CHAPTER 2

REVIEW OF LITERATURE

2.1 DISEASE BURDEN

2.1 .1 Disease Burden: Global

Diabetes is a chronic disease impacting world population and leads to serious threat for morbidity and mortality across the populations. 451 million individuals acquire Diabetes and projected that the incidence may increase upto 693 million by 2045 (Cho, N., *et al*. 2018). Impaired glucose tolerance (IGT) or pre-diabetic is a major challenge where in 2017; 374 million people were living with the same. International Diabetic Federation (IDF) estimated

that, about 145.7 and 279.2 million population with diabetes is living in rural and urban area respectively and about 4 million deaths worldwide were attributable to diabetes in 2017 (Table 2.1) (Cho, N., *et al*. 2018). USD 727 billion in 2017 was estimated for the global healthcare expenditure on people with diabetes (Cho, N., *et al*. 2018). Direct and indirect losses in gross domestic product (GDP) due to diabetes worldwide from 2011 to 2030, will total US$ 1.7 trillion, US$ 900 billion for high-income countries and US$ 800 billion for low- and middle-income countries respectively (Table 2.1) (Cho, N., *et al*. 2018).

Table 2.1: Global estimation of Diabetes: Estimated from 2017 and predicted for 2045 (Cho, N., *et al*. 2018).

	2017	**2045**
Prevalence (20-79 years)	8.8per cent (7.2-11.3per cent)	9.9per cent (7.5-12.7per cent)
Number of people with diabetes (20-79 years)	425 million	629 million
Number of people with diabetes (20-79 years), Rural	145.7 million	156.0 million
Number of people with diabetes (20-79 years) , Urban	279.2 million	472.6 million
Number of deaths due to diabetes (20-79 years)	4.0 (3.2-5.0) million	-
Total Healthcare Expenditures for Diabetes (20-79 years)	USD 727 billion	USD 776 billion
Global prevalence of Impaired glucose tolerance (IGT)	7.3per cent (4.8-11.9per cent)	8.3per cent (5.6percent-13.9per cent)
Number of people with IGT (20-79 years)	352.1 million	531.6 million

Economic progression and altered lifestyle along with genomic susceptibility provides a comprehensive threat for Diabetes associated morbidity and mortality among Indian population. According to International Diabetes Federation (IDF) the prevalence of diabetes in adults is diverse in South East Asia (SEA) and the range is 4.0 per cent in Nepal to 8.8 per cent in India (Figure 2.1) (Hills, A. P., *et al.*2018).

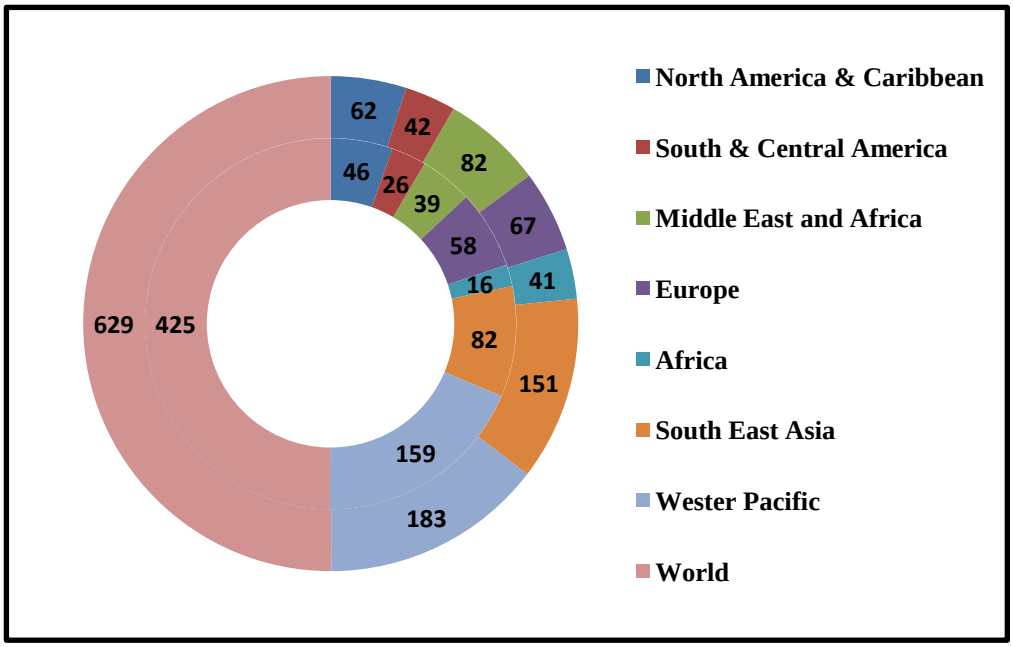


Figure 2.1: Global prevalence of diabetes estimated in 2017 inner circle and predicted for 2045 in outer circle (Hills, A. P., *et al.*2018).

2.1.2 Disease Burden: India

In recent years, incidence of diabetes and its associated micro and macro vascular complications are significantly increased in India (Rajadhyaksha 2018). It is reported that, diabetics in India is steadily increased to about 72.0 million in 2017, 65.0 million in 2016 from 26.0 million in 1990 (Tandon, N., *et al*. 2018, IDF Diabetes Atlas, 9th edition, 2019). The National Health Policy of India aims to increase screening and treatment of 80 per cent of people with diabetes and 25 per cent reduction in mortality from diabetes by 2025(Tandon, N., *et al*. 2018). Diabetes contributed to 3·1per cent of all deaths in India from 1990 to 2016. It is predicted that approximately 98 million people in India will suffer from type 2 diabetes by 2030.

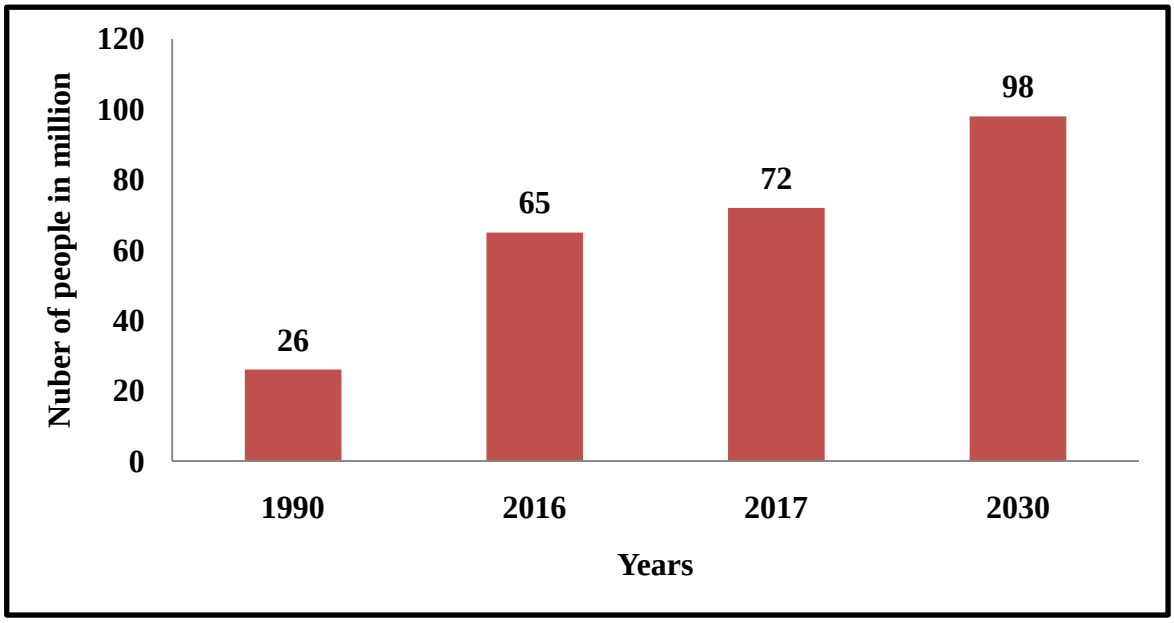


Figure 2.2: Burden of diabetes in India (Tandon, N., *et al*. 2018, IDF Diabetes Atlas, 9th edition, 2019)

Rapid urbanization and economic progression associated with altered lifestyle contributed the increased health care burden due to diabetes in India. A decline in nutrition quality, reduced physical activity, and increased sedentary behaviours are reflected in the increasing prevalence of the same and its associated risk factors in the region.

ICMR-INDIAB study estimated the prevalence of diabetes and pre diabetes in India to be 7.3per cent and 10.3per cent (Anjana, R. M., *et al*. 2017). There was vast difference of prevalence for diabetes among Indian states, where 4·3per cent in Bihar to 10·0per cent in Punjab and was higher in urban areas (11·2 per cent) than in rural areas (5·2 per cent) (Anjana, R. M., *et al*. 2017). Among north-eastern states Meghalaya has lowest prevalence (4.5 per cent) and Tripura accounting for highest i.e; 9.4 per cent (Anjana, R. M., *et al*. 2017). Assam possesses of 5.5 per cent diabetic according to the study (Anjana, R. M., *et al*. 2017). ICMR-INDIABstudy also revealed that prediabetes was found to be highest in the north-eastern states of Tripura (14.7 per cent) and lowest in Mizoram (6.0 per cent). Development of socioeconomic status directly proportionate to prevalence of diabetes in all over India and is a threat for health care (Anjana, R. M., *et al*. 2017).

2.2 THE DISEASE DIABETES:

Hesy-Ra, an Egyptian physician described for the first time in 1552 B.C. the symptom of frequent urination of a mysterious disease (Krisha 2009). The term diabetes was used for first time to denote an excessive passage of urine and also ascribed as the aetiology to the kidney by an Egyptian physician named Apollonius Memphites around 230 BC (Papaspyros 1964). In 150 AD, the Greek physician Arateus described the diabetes as "the melting down of flesh and limbs into urine." In ancient time people used to test urine of people suspected to have it weather sweet or not. The word “Diabetes” is a Greek word meaning to siphon or pass through and the “mellitus” is a Latin word which means honey or sweet (Diabetes: Past treatments, new discoveries, Krisha 2009). In 1675, the word "mellitus" was added to the name "diabetes" (Krisha 2009). The Indian synonym of diabetes was “madhumeha” (‘honey urine’) because urine of patients is attracted by ants. Sushruta and the surgeon Charaka (400–500 A.D.) identified the two types of diabetes, i.e; Type I and Type II diabetes (Frank 1957, Tipton 2008 and Lakhtakia 2013). The major driving factors of the global type2 diabetes mellitus (T2DM) epidemic include overweight and obesity, sedentary lifestyle and increased consumption of unhealthy diets containing high levels of red meat and processed meat, refined grains and sugar-sweetened beverages (Zheng, Y., *et al.* 2018). Since then, researches were going on with the advancement of technologies to understand the mechanism, progression and prevention of diabetes but it remained elusive (Polonsky 2012). Various breakthrough have been achieved across the centuries by scientific community for the understanding the disease and starting from F.G. Banting and J.J.R. Macleod for the discovery of Insulin in 1923 (Polonsky 2012). Seven Nobel Prizes were awarded to scientists for commendable works in this field (Table 2.2) (Polonsky 2012).

Table 2.2: Nobel Prize in the field of diabetes related work (Polonsky 2012).

Year	Recipients	Description
1923	F.G. Banting and J.J. R. Macleod	Discovery of Insulin
1947	C.F.Cori and G.T	Discovery of the course of the catalytic conversion of

	Cori	glycogen
1947	B.A. Houssay	Discovery of the role of hormones released by the anterior pituitary lobe in the metabolism of sugar
1958	F. Sanger	Work on the structure of protein, especially insulin
1971	E.W. Sutherland	Discoveries concerning the mechanism of action of hormones
1977	R. Yalow	Development of radioimmunoassay for peptide hormones
1992	E.H. Fischer and E.G. Krebs	Discoveries concerning reversible protein phosphorylation as a biologic regulatory mechanism

The universal symbol for diabetes i.e.; The Blue Circle was taken to give a common identity to diabetes by International Diabetes Federation (IDF) on 17 March 2006 (Figure 2.3) (IDF 2006). Glucose metabolism plays a central role for the incidence and development of diabetes. In 19th century, Claude Bernard showed that liver play a major role on regulation of blood glucose levels especially from non glucose precursors (Robin 1979).

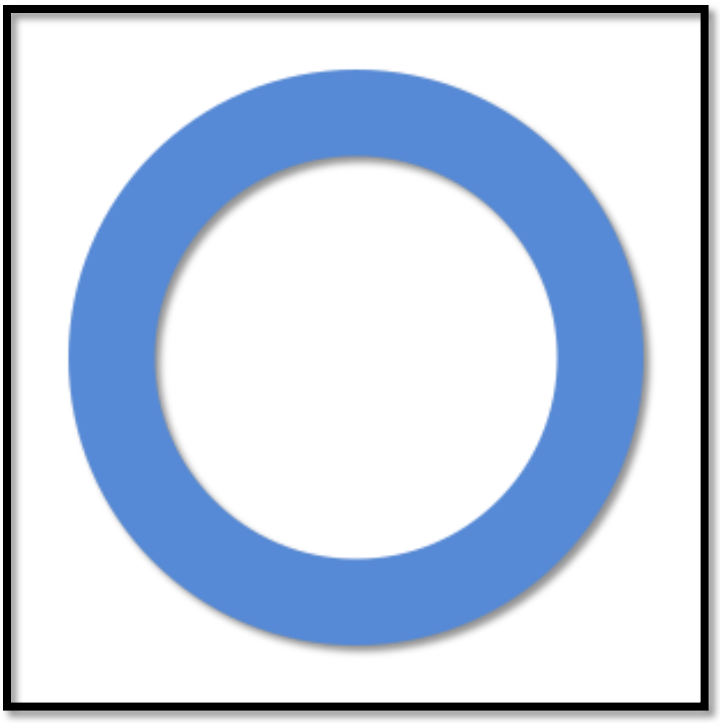


Figure 2.3: The Blue circle: universal symbol for diabetes (IDF 2006)

Joseph von Mering and Oskar Minkowski in 1889 discovered that pancreas is the regulator of blood glucose concentration (Rolles 1987, Brogard, J. M. *et al.* 1992). Series of investigation was done by various investigators during early 19th century including, Edward Albert Sharpey-Schafer's (1910) hypothesis about the deficiency of chemical from pancreas is responsible for diabetes and further named it as "Insulin" from Latin word "*insula*" meaning island and referring to the pancreatic islet cells of Langerhans (Polonsky 2012). After the various breakthrough experiments during 1910 to onwards, James Collip and John Macleod purified the hormone insulin from bovine pancreases and use it to treat a patient with diabetes. The productionof insulin and its therapeutic use quickly spread around the world (Polonsky 2012). Over past two centuries it has been investigated and established that diabetes is a complex, heterogeneous disorder (Polonsky 2012). Insulin resistance was first proposed by Harold Himsworth in 1936 and successive studies postulated that insulin resistance is essential in the pathogenesis of type2 diabetes (Himsworth 1936, Cavaghan, M. K. *et al.* 2000). Insulin resistance, upper-body obesity, hypertension, hypertriglyceridemia and low levels of high-density lipoprotein cholesterolare high risk factors for glucose intolerance and diabetes and clinically termed as metabolic syndrome (Reaven 2005, Fajans, S. S. *et al.* 2001). Weight loss, polyuria (increase urination), polydipsia (increase thirst) and polyphagia (increase hunger) are

the major sign and symptom of untreated diabetes (Figure 2.4) (Cooke, D. W., and Plotnick, L. 2008). Incase of type 1 diabetes symptoms may develop rapidly (weeks or months) but much more slowly and may be subtle or absent in type 2 DM Cooke, D. W., and Plotnick, L. 2008). According to American Diabetic Association (ADA), symptom such as Blurred vision, headache, fatigue, slow healing of cuts and itchy skin are also associated to diabetes.

Diabetes is also considered as polygenic disorder because multiple genes and environmental factor contribute to the development of the same (Polonsky 2012). Previous studies identified over 40 genetic variants that increase the risk of type 2 diabetes and postulates that the same increased risk of developing diabetes 10-15per cent (Stolerman, E. S., and Florez, J. C. 2009, Ahlqvist, E. *et al.* 2011).

Clinical presentation of diabetes is insulin deficiency and vast majority of diabetic patients are overweight and have a combination of insulin resistance and impaired insulin secretion.Though efforts have been made continuously since past 200 years and positive results were reported regarding diabetes but prevalence of diabetes rose consistently to become epidemic (Polonsky 2012).

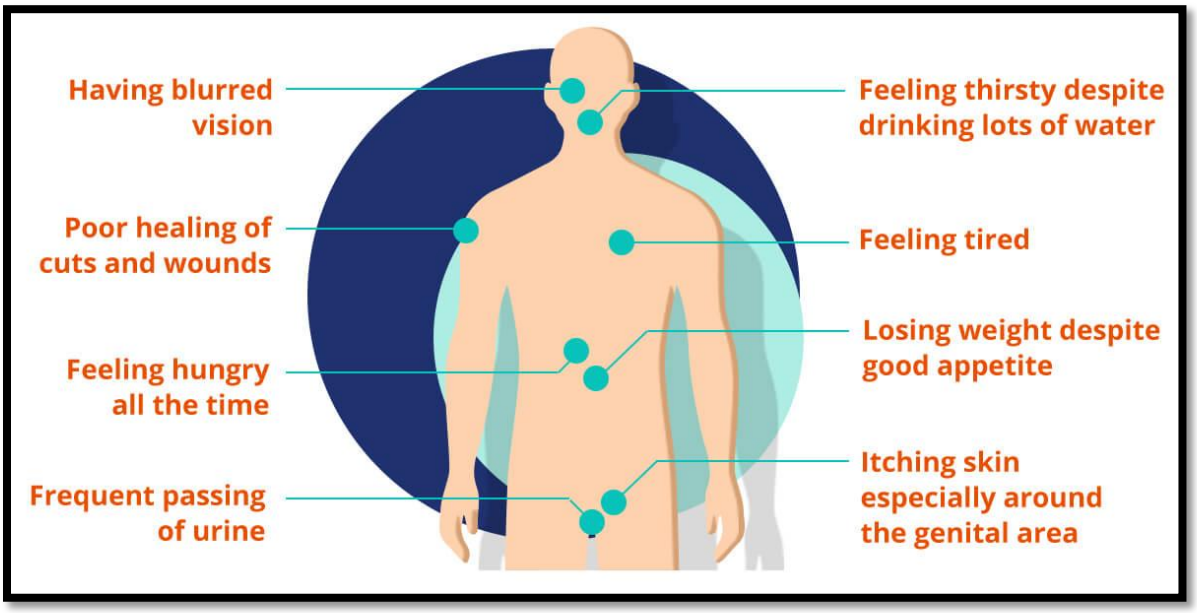


Figure 2.4: Sign and symptom of diabetes [www.healthhub.sg]

2.3 PATHOPHYSIOLOGY OF DIABETES:

Diabetes Mellitus (DM) is a chronic metabolic disorder characterized by the presence of persistent hyperglycemia either immune-mediated (Type 1 diabetes), insulin resistance (Type 2), gestational or others (environment, genetic defects, infections, and certain drugs).

2.3.1 Type 1 Diabetes

Type I diabetes is an autoimmune disorder where insufficient insulin production occurred due to destruction of insulin-producing beta cells in the islets of the pancreas gland by host's immune system. (IDF2017) There was a 21per cent increase in the number of youth with type1 diabetes in the U.S. between 2001 and 2009. Though diagnosis of type 1diabetes frequently occurs in childhood, 84per cent of people living with type1 diabetes are adults (Skyler, J. S., *et al.* 2017). Type 1 diabetes is mostly due to genetic risk factors. The genetic basis of type 1 DM is still remains unclear but a number of major genetic determinants such as alleles of the major histocompatibility locus (HLA) are associated with Type 1 diabetes (Forbes, J. M., and Cooper, M. E. 2013). The incidence of type 1 diabetes is increasing worldwide, but there is huge variation across the countries (Skyler, J. S., *et al.* 2017).

2.3.2. Type 2 Diabetes:

Type 2 diabetes is more common than type I DM and account for more than 90per cent of all cases of diabetes. Type 2 diabetes develops due to relative insulin deficiency or insulin resistance. Previously it has been referred as non insulin dependent diabetes mellitus also. Defective insulin secretion is prime factor to the pathophysiology of type 2 diabetes. Insulin resistance leads to condition termed as hyperinsulimaemia, further increased insulin destroy the beta cells function and leads to hyperglycemia (Hackett, E., and Jacques, N. 2009). Untreated diabetes is the major risk factor for patients with all forms of diabetes for developing the chronic complications, although rates of progression may differ. Overweight, adult age, having family history of diabetes, high blood pressure, have a history of gestational diabetes and sedentary lifestyle identified as crucial risk factors for developing type 2 diabetes (Skyler, J. S., *et al.* 2017).

2.3.3. Gestational Diabetes (GDM):

GDM Defined high blood glucose levels during pregnancy and usually disappears after given birth. Second or third trimester of pregnency is more common stage to develop GDM. It develops in one in 25 pregnancies worldwide and is associated with complications to both mother and baby. Usually GDM disappears after pregnancy but women with GDM and their children are prone to develop type2 diabetes in later life (Melchior, H. *et al*. 2017). It is estimated that 4 million women are affected in India at any point of time and prevalence of GDM has been reported to range from 3.8per cent in Kashmir to 17.9per cent in Tamil Nadu (Mithal, A., *et al*. 2015).

2.3.4. Maturity Onset Diabetes of the Young (MODY):

MODY is a rare form of diabetes associated with genomic variation of certain genes that actively involved insulin catabolic and metabolic pathway (ADA 2017). Several reports postulate that autosomal dominant genomic variation may be critical determinant for the same that established at adolescence or early adulthood (Rubio-Cabezas, O. 2014). MODY accounts for up to 2 per cent of all cases of diabetes in the United States among younger people aged upto 25 (Pihoker 2013). Study estimated that, 1-2per cent of all diabetic cases are MODY (Gaal Z., and Balogh I. 2019). Comprehensive genetic studies across the world identified about 13 genes on different chromosomes are critical determinant for MODY (ADA 2018). MODY further subcategorized in with the aid of genomic variation that includes GCK-MODY (MODY2), HNF1A-MODY (MODY3), and HNF4A-MODY (MODY1) (Fernandez 2006, Hattersley, A. T., & Patel, K. A. 2017, ADA 2018).

2.4. DIAGNOSIS OF DIABETES

Criteria to diagnose Diabetes is mainly based on plasma glucose either fasting plasma glucose test (FPG) or 2 hours oral glucose tolerance test (OGTT). ADA emphasizes about HbA1c (glycated heamoglobin) test for diagnosis of diabetes (ADA. 2018). HbA1c are measures by various methods such as immunoassay, ion-exchange high-performance liquid chromatography (HPLC), boronate affinity HPLC, and enzymatic assays to separate the glycated and nonglycated forms of hemoglobin (Kilpatrick 2004). HbA1c measurement

provides the more reliable results about the glucose control over periods of 3 months. It is recommended that HbA1c should be under 7 per cent to consider good glycemic control (Kilpatrick 2004). According to ADA diagnostic criteria for diabetes are described in table 2. 3. Patients with symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose >200 mg/dL (11.1 mmol/L) should go for repeated diagnosis by following criteria (Table 2.3). ***(ADA2017)***

Table 2.3: Criteria for the diagnosis of diabetes (ADA 2017)

	HbA1c (per cent)	**Fasting Plasma Gucose *(mg/dL)**	**Oral Glucose Tolerance Test# (mg/dL)**
Diabetes	≥6.5	≥126	≥200
Prediabetes	5.7-6.4	100-125	140-199
Normal	~5.7	≤99	≤139

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

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

2.5. Complication of Type 2 Diabetes:

Epidemiological studies reveal that persistent hyperglycaemia frequently leads to several microvascular and macrovascular complications in different organs. Microvascular complications (due to damage to small blood vessels) include diabetic retinopathy (DR) diabetic nephropathy (DN) and diabetic neuropathy (DN) (Figure 2.5) (Forbes, J. M., and Cooper, M. E. 2013, Papatheodorou, K. *et al*. 2018).

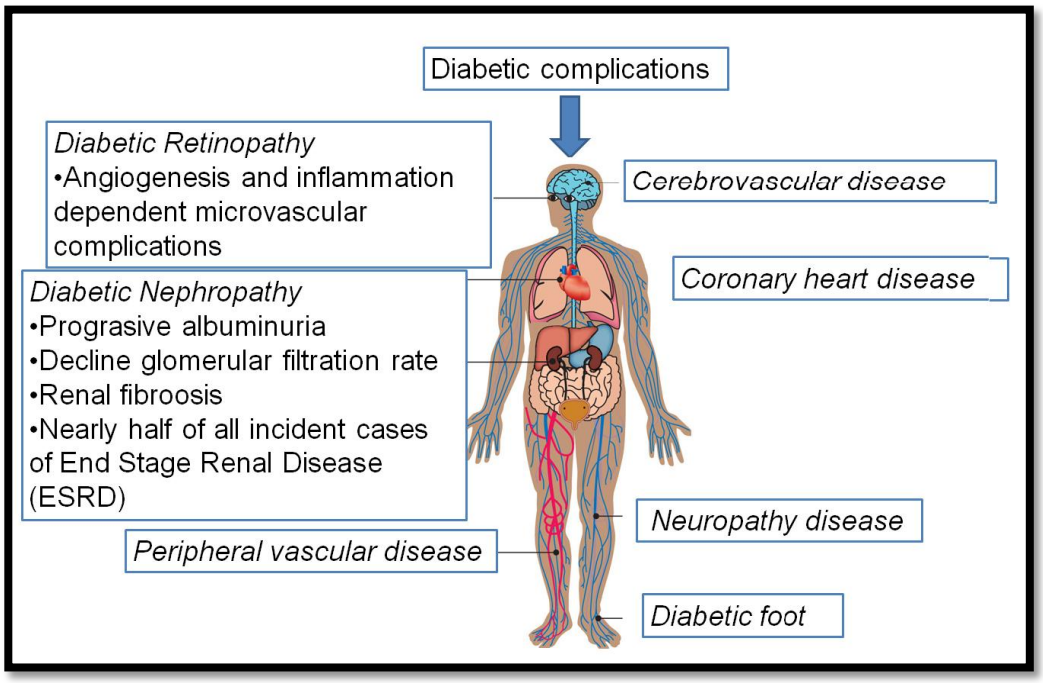


Figure 2.5: Complications of Type 2 diabetes (Forbes, J. M., and Cooper, M. E. 2013)

The major macrovascular complications (due to damage to the arteries) include cardiovascular disease, stroke, and peripheral artery disease. (Forbes, J. M., and Cooper, M. E. 2013, Papatheodorou, K. *et al*. 2018).

Studies estimated that about 47 per cent of retinopathy, 17 per cent for nephropathy, and 14 per cent for cardiovascular disease associated with T1DM (Nathan, D. M. *et al.* 2009). Data for T2DM is relatively enigmatic across the population but it has been documented that about 20per cent of T2DM cases develop several micro and macro vascular complications after 10 years of establishing the T2DM (Fowler 2008). Asians have higher prevalence of nephropathy but a lower incidence of cardiovascular disease than Caucasians among the T2DM subjects (Chan 2004, Moore, D. J. *et al*. 2009).

2.5.1. Diabetic Nephropathy:

Diabetic Nephropathy (DN) is characterized by the development of proteinuria with a subsequent decline in glomerular filtration rate (GFR), which progresses over a long period of time, often over 10–20 years with diabetes. Hypertension and poor glycemic control are responsible to accelerate the progression of severity of diabetic nephropathy (DCCTRG

1993,UKPDS34 1998, UKPDS38 1998). Elevated concentrations of glucose induce specific cellular pathways, which affect various kidney cells including endothelial cells, smooth muscle cells, mesangial cells, podocytes, immune cells and myofibroblasts for the pathogenesis of kidney disease. Proteinuria, a condition where urine contains an abnormal amount of protein often reflects changes in renal hemodynamics and is linked to changes in the glomerular filtration rate, etc. (Mauer, S. M., *et al.* 1984, Diabetes - A Major Risk Factor for Kidney Disease, National Kidney Foundation).

2.5.2. Diabetic Retinopathy:

Diabetic retinopathy (DR) is microvascular complication due to prolonged hyperglycemia affecting the retina and is the leading cause of blindness among adults aged 20–74 years (Frank 2004, Hirai, F. E., *et al*. 2011). Diabetic retinopathy develops almost all patients with type1 diabetes and type2 diabetes (Roy, M. S., *et al*. 2004, Kempen, J. H., *et al*. 2004, Hirai, F. E., *et al*. 2011). Changes in vascular permeability, capillary microaneurysms, capillary degeneration, and excessive formation of new blood vessels (neovascularization) are the major clinical manifestation of DR. DR is categorized into nonproliferative and proliferative disease stages. Thickening of the basement membrane due to hyperglycemia contribute changes in the integrity of blood vessels within the retina, which alters the blood-retinal barrier and vascular permeability (Frank 2004). Proliferative retinopathy is common in type 1 diabetes and macular edema in type 2 diabetes (Kohner 2003).

2.5.3. Diabetic Neuropathy

Diabetic neuropathy is defined as a microvascular complication leading to nerve damage due to prolonged diabetes. More than half of all individuals with diabetes eventually develop neuropathy (Abbott, C. A., *et al.* 2011).Vascular abnormalities, such as capillary basement membrane thickening, endothelial hyperplasia and hypoxia are traditional clinical characteristics of diabetic neuropathy (DN). Prolonged diabetes or uncontrolled hyperglycemia affects the neurons and longer nerve fibers that losses nerve conduction velocity. Therefore, tingling, loss of sensation and reflexes are occurred in the feet and other areas. This syndrome is commonly termed a “glove and stocking” distribution. Consistent with

other complications, the duration of diabetes and lack of glycemic control are the major risk factors for neuropathy in both major forms of diabetes (UKPDS34 1998, Nathan, D. M., *et al.* 1993).

2.5.4. Macrovascular Complications of Diabetes: Cardiovascular Disease

Cardiovascular diseases are one of the major contributors for more than half of the mortality in diabetes (Haffner SM, *et al.* 1998, Laing, S. P., *et al.* 2003). Diabetes increases the risk of myocardial infarction compared with the non diabetic subjects (Domanski, M., *et al.*2002). Cardiovascular disorders in diabetes include premature atherosclerosis, myocardial infarction and stroke as well as impaired cardiac function. Diabetic cardiomyopathy is the condition where myocardium is damaged (Boudina, S., & Abel, E. D. 2007). Long-term glycemic control, measures of HbA1C, remains the best predictor of CVD risk in both type 1 (Borg, R., *et al.* 2010) and type 2 diabetic individuals (Cederberg, H., *et al.* 2010). Oxidative stress and chronic inflammation contribute to the altered gene expression within the vasculature and there is also a failure of vascular repair in diabetes further enhancing complications in multiple organs (Tepper, O. M., *et al.* 2002, Feng, L., *et al.* 2005).

2.5.5. Cerebrovascular Disease:

Diabetes is a risk factor for cerebrovascular diseases (CVD). About 20per cent-40per cent patients with type 2 diabetes suffer from cerebral blood vessel diseases. The major clinical manifestations are asymptomatic cerebral atherosclerosis, stroke, cerebral small vessel disease and acute CVD. Type 1DM and Type 2DM Patients are at markedly increased risk of death due to CVD (Ergul, A., *et al.* 2012). Thomas Almdal *et al*. 2004 reported an increased relative risk for developing stroke in men by 1.5 to 2 fold and 2 to 6.5 fold in women having type 2DM compared to healthy control and the risk of death increased by 2-fold (Almdal, T., *et al*. 2004).

2.6. DIABETIC THERAPY:

According to American Diabetic Association, screening, diagnostic, and therapeutic actions are known or believed to favourably affect health outcomes of patients with diabetes. Metformin is the preferred pharmacological agent for type 2 diabete (George, M. M., and Copeland, K. C. 2013, Nasri, H. and Rafieian-Kopaei, M. 2014). Metformin work on hyperglycemia by inhibiting hepatic glucose production via AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms (Rena, G., *et al.* 2017). Upon resistance to metformin monotherapy a combination of metformin with sulfonylureas, thiazolidinediones, dipeptidyl peptidase-4 inhibitors, sodium–glucose cotransporter 2 (SGLT2) inhibitors, glucagon-like peptide-1 (GLP-1) agonists or insulin therapy are used for management of diabetes (George, M. M. and Copeland, K. C. 2013, Nasri, H. and Rafieian-Kopaei, M. 2014, Inzucchi, S. E. *et al* 2015). Recently, pre-therapeutic marker against different angiogenic disorder provides the new opportunity to personalize the therapeutic strategy. Major clinical trials have demonstrated that medication along with diet and lifestyle modifications are also effective in preventing T2DM in high-risk individuals (Zheng, Y., *et al.* 2018).

2.7. DIABETIC NEPHROPATHY:

2.7.1 Disease Burden and Epidemiology:

Diabetic Nephropathy (DN) seems the leading cause of morbidity and mortality among the hyperglycemic subjects both type1 and type2 in the age group of 20 to 75 years across the globe (Persson, F., & Rossing, P. 2018). Third National Health and Nutrition Examination Survey (NHANES III) estimated that 42.3 per cent individuals were having diabetic nephropathy and the mortality rate was 31.1per cent (95 per cent CI, 24.7–37.5) (Afkarian, M., *et al.* 2013). Table 2.4 describes about the epidemiology and economic impact DN (Vijayan, M., *et al*. 2016).

Table 2.4: Epidemiology of diabetic nephropathy (Vijayan, M., *et al*. 2016).

	India	Globe
Prevalence of Diabetic Nephropathy	31.3per cent of total CKD (2.2% overt nephropathy and 26.9% microalbuminuria)	25per cent of diabetic population

Poor glycemic control seems the crucial accelerator for end-stage renal disease (ESRD) and it increases the risk of same by 10 folds (Bash, L. D., *et al*. 2008). Parving, H. H. *et al.* in 2006 revealed the prevalence of normo, micro and macroalbuminuria was 51, 39, and 10per cent respectively among T2DM individuals (Parving, H. H. *et al.* 2006). Study reported that, after being diagnose with T2DM 2.0, 2.8 and 2.3percent persons per year progress to microalbuminuria, microalbuminuria to macroalbuminuria and from macroalbuminuria to overt nephropathy respectively (Adler, A.I. *et al*.2003). DN is highest (30.3per cent) among chronic renal failure patients followed by chronic interstitial nephritis (23per cent) and chronic Glomerulonephritis (17.7per cent) (Dabla 2010). It was estimated that the prevalence of overt nephropathy and microalbuminuria was 2.2 and 26.9per cent, respectively among urban Asian (Unnikrishnan, R. *et al.* 2007). The prevalence of CKD estimated by the International Society of Nephrology's Kidney Disease Data Center was 17per cent and the range from 1per cent to 13per cent in different region of India (Varughese, S. and Abraham, G. 2018). Epidemiological studies postulates that, rate of development of DN is comparatively less among Caucasians than the population like African Americans, American Indians and Hispanics or Latinos (Dabla 2010). DN rarely develops more frequently among T2DM patients than type1 diabetes before the age of 10 years (Gheith, O. *et al*. 2015). Population based study revealed that Diabetic kidney disease is the single most common cause of End Stage Renal Disease (ESRD) in many parts of the world including Europe, Japan, USA, India along with other south east Asian countries (Persson, F., and Rossing, P. 2018).

2.7.2. Pathology and Diagnosis of DN:

DN is characterized by progressive kidney damage with presence of increasing albuminuria, impairment in renal function leading to decline in glomerular filtration rate (GFR) (Dabla 2010, Persson, F., and Rossing, P. 2018). Micro and macroalbuminuria is the main clinical phenotype of DN which is based on the amount of urinary albumin excreation (UAE) on timed, 24-hrs, and spot urine samples (Persson, F., & Rossing, P. 2018, Care 2004). According to ADA the cutoff value for diagnosis of micro and macroalbuminuria is based on timed, 24-h, and spot urine collection for estimation of urinary protein, presented in table Table 2.5 (Inzucchi, S. E., *et al*. 2015).

Table 2.5: Diagnosis and main clinical characteristics of Diabetic nephropathy based on urine albumin excretion

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

* Albumin to Creatinine Ratio (ACR)

Previous studies documented that, the risk of diabetic nephropathy was about 30 fold high in patients with type2 diabetes (Murussi M, *et al.* 2002). Microalbuminuria is considered as a risk factor for macroalbuminuria, although not all patients progress to this stage and rather some may regress to normoalbuminuria (Caramori, M. L., *et al.* 2000). Studies during 1980s demonstrated that approximately 80per cent of microalbuminuric patients progressed to proteinuria over a period of 6 –14 years especially for type1 DM (T1DM) (Mogensen, C. E., & Christensen, C. K. 1984**,** Parving, H. H., *et al* 1982, Viberti, G. C., *et al* 1982). Recent studies predicted that over 10 years of duration, 30–45per cent of microalbuminuric patients have been progressed to proteinuria stage; more intensive glycemic and blood pressure control strategies may be the reason (Caramori, M. L., *et al.* 2000). It is recommended that the

screening for diabetic nephropathy must be initiated at the time of diagnosis in patients with type 2 diabetes because it was estimated that 7per cent of them already have microalbuminuria at time of diagnosis of type 2DM (T2DM) (Adler, A. I., *et al.* 2003). In case of T1DM the same is recommended after 1 year of diabetes diagnosis, because 18per cent patients develop microalbuminuria within 5 years of diabetes. (Stephenson, J. M., *et al.*1994, Care 2004). Some diabetic patients possessed decreased glomerular filtration rates (GFR) though albuminuria status is normal. **(**Caramori, M. L., *et al.* 2003**,** MacIsaac, R. J., *et al.* 2004) Third National Health and Nutrition Examination Survey (NHANES III) study observed that low GFR (<60 ml min^{-1} 1.73 m^{-2}) was present in 30per cent of patients in the absence of micro or macroalbuminuria and retinopathy (Garg, A. X. *et al.* 2002, Kramer, H. J., *et al.* 2003). Various techniques are used to measure the GFR such as Insulin clearance Cr-EDTA, I-iothalamate and iohexoletc are the major (Gaspari, F., *et al.* 1997). In clinical practice, GFR can be estimated based on serum creatinine, age and weight by Cockroft and Goult formula [CCr={((l40–age) x weight)/(72xSCr)}x 0.85 (if female)] (Cockcroft, D.W. and M.H. Gault. 1976). Estimated GFR (eGFR) from serum creatinine, age and weight based on gender can be calculated using an online formula available at http:/www.kidney.org/ klsprofessionals/gfr_calculator.com. ADA and National Institute of Health (NIH) have recommended that estimated GFR should be calculated from serum creatinine at least once a year in all patients of diabetes for diagnosis of kidney dysfunction (Dabla 2010). Adjustment for conventional risk factors of DN including age, sex, duration of diabetes, smoking, obesity, blood pressure, glycemic and lipid control and presence of diabetic retinopathy and eGFR remains an independent and significant predictor for diagnosis of DN (Garg, A. X. *et al.* 2002, Dabla 2010). The reference range of GFR values in young individuals is presented in table 2.6. Annual monitoring of urinary albumin-to-creatinine ratio, estimated GFR (eGFR), and blood pressure is also recommended. Several new biomarkers or profiles of biomarkers have been investigated to improve prognostic and diagnostic precision, but none have yet been implemented in routine clinical care.

Table 2.6: Severity and diagnosis of DN according to Glomerular Filtration Rate and the clinical renal manifestation. (Haneda, M., *et al* 2015, Gheith, O., *et al.* 2016**)**

Stage of DN (severity)	**Glomerular Filtration rate (mL/min/1.73 m^2)**	**Duration of diabetes (years)**	**Renal manifestation**
G1	≥90	0-5	Size of the kidneys is increased by nearly 20per cent Renal plasma flow is increased by 10per cent-15per cent, but without albuminuria or hypertension
G2	60 to 89	2-5	Thickening of basement membrane and mesangial proliferation
G3	30 to 59	5-15	Glomerular damage and microalbuminuria (albumin 30-300 mg/day)
G4	15 to 29	15-25	CKD with irreversible proteinuria (>300 mg/day), Hypertension
G5	<15	25-30	ESKD

Approximately 40 per cent of DN patients if untreated reach the stage G3. Nearly 50per cent of patients of stage 5 may require renal replacement therapy (peritoneal dialysis, hemodialysis or kidney transplantation (Haneda, M., *et al* 2015, Gheith, O., *et al.* 2016). Though other techniques are available for diagnosis of DN in early stage but proteinuria and GFR are the best indicators of the degree of the kidney damage (Figure 2.6) (Mogensen, C. E. 2000, Gheith, O., *et al.* 2016).

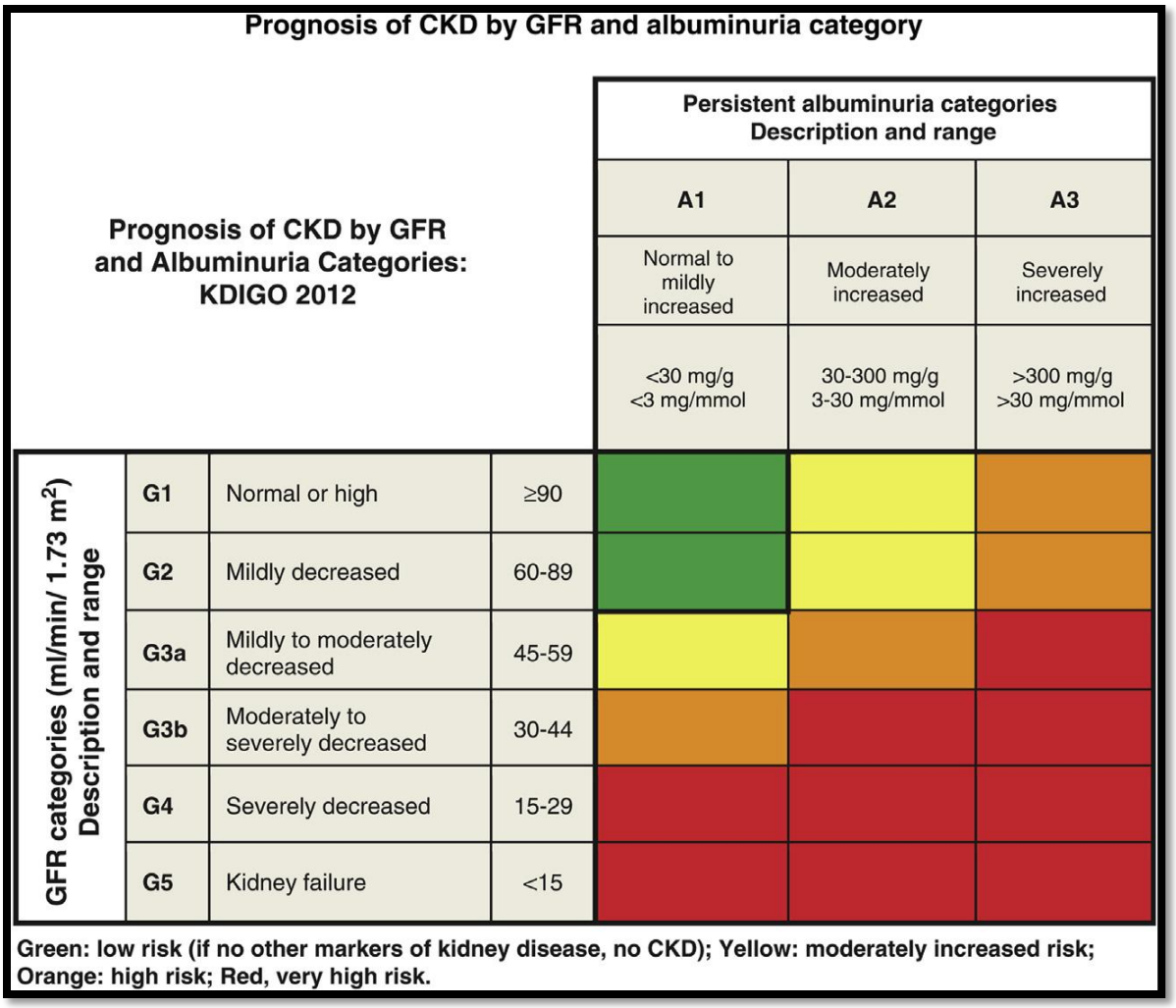


Figure 2.6: Staging of chronic kidney disease (Persson, F., and Rossing, P. 2018).

2.8: ANATOMICAL AND STRUCTURAL CHANGES OF GLOMERULI IN DIABETES

Anatomical changes in glomeruli due to diabetes can be seen using various techniques like staining with hematoxylin and eosin, periodic acid–Schiff (PAS), Masson trichrome, and periodic acid methenamine silver stains for light microscopy (Movat, H. Z., and Mcgregor 1959, Jain 2012). Immunofluorescence requires the use of antibodies against IgA, IgG, IgM, C3, C1q, and kappa and lambda light chains to rule out other renal diseases (Tervaert, T. W. C *et al*. 2010). Glomerulosclerosis due to diabetes is characterized by increased glomerular basement membrane width, diffuse mesangial sclerosis, hyalinosis, microaneurysm, and

hyalinearteriosclerosis (Mauer S.M. *et al.*1981, Brito, P. L., *et al.*, 1998, Katz, A., *et al.*, 2002). Kimmelstiel-Wilson nodules or nodular mesangial expansion characterized by expansion of extreme mesangial areas are observed in 40–50per cent of patients developing proteinuria (Kimmelstiel, P., and Wilson, C. 1936). It is well documented that type 2 diabetes has more structural heterogeneity than patients with type 1 diabetes consisting of Micro and macroalbuminuria (Osterby, R. *et al*. 1993, Fioretto, P. *et al.*1996). It is essential to evaluate renal tissue using appropriate standards for renal biopsy (Figure 2.7).

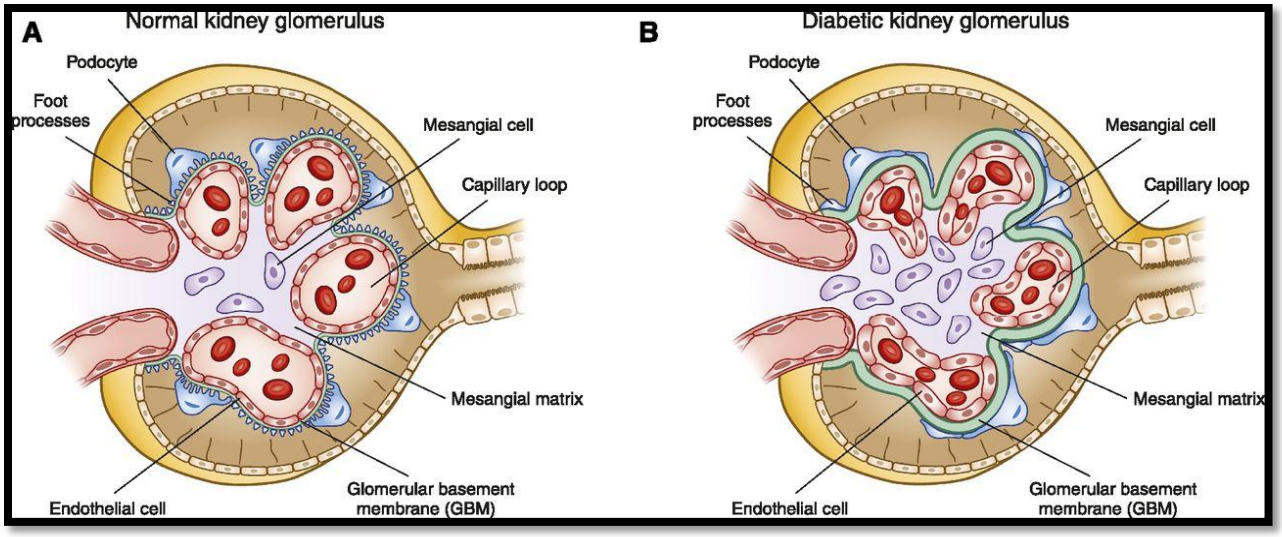


Figure 2.7: Morphology and structural changes in normal and diabetic kidney glomerulus. (Alicic, R. Z., *et al*. 2017).

2.9 CLASSIFICATION OF DIABETIC NEPHROPATHY:

Pathologic classification of glomerular changes in diabetic kidney disease is described below (Figure 2.8 and 2.9).

2.9.1 Class I: Glomerular Basement Membrane Thickening.

Glomerular Basement Membrane (GBM) thickening i.e; class I referred as Microscopic examination of biopsy tissue imaged with absence of mesangial expansion, nodular increases in mesangial matrix (Kimmelstiel–Wilson lesions) and global glomerulosclerosis of more than 50per cent of glomeruli. Direct measurements with Electron

microscopy (EM) shows that glomerular basement membrane (GBM) on average is 430 nm thicker in males and 395 nm thicker in females (Haas 2009). Previous studies described that thickrning of GBM increases with the duration of diabetes and the characteristic of early change in both type 1and type 2 DN (White, K. E., & Bilous, R. W. 2000, Mauer, S. M.,*et al*. 1984, Drummond, K., & Mauer, M. 2002). GBM thickening resulted with increased deposition of normal extracellular matrix components such as collagen types IV and VI, laminin, and fibronectin (Falk, R. J., *et al.* 1983**,** Kim, Y., *et al.* 1991).

2.9.2 Class II: Mesangial Expansion, Mild (IIa) or Severe (IIb).

Class II encompasses those patients classified with mild or severe mesangial expansion. Class II pathological conditions arise with the increase of extracellular material in mesangium. Mild and severe mesangial expansion is based on whether the expanded mesangial area is smaller or larger than the mean area of a capillary lumen (Mauer, S. M., *et al*. 1984, White, K. E., & Bilous, R. W. 2000).

2.9.3 Class III: Nodular Sclerosis (Kimmelstiel– Wilson lesions)

Kimmelstiel–Wilson lesion was name after Paul Kimmelstiel and Clifford Wilson in 1936 as they first described nodular lesions in glomeruli from eight maturity onset diabetes patients (Tervaert, T. W. C., *et al*. 2010). It is also stated that nodular sclerotic lesions may also occur in hypertension, smoking, hypercholesterolemia and extra-renal vascular disease in absence of DN (Markowitz, G. S., *et al.* 2002). Lytic changes in the mesangial area called mesangiolysis and detachment of endothelial cells from the GBM are observed during initial stage of developing nodular sclerotic lesions in DN. (Nishi, S., *et al.* 2000). Fragmented red blood cells were first detected by Paueksakon*et et al.* 2002 in Kimmelstiel– Wilson lesions (Tervaert, T. W. C., *et al*. 2010). Presence of fragmented red blood cells weighted the postulation that, microvascular injury contributes to the pathogenesis of Kimmelstiel– Wilson lesions. Aaccumulation of mesangial matrix with collagen fibrils, small lipid particles and cellular debris are associated with Kimmelstiel– Wilson lesions (Glick, A. D., *et al*. 1992).

Kimmelstiel–Wilson lesion destroys the normal structure of glomerular tuft with a decrease in mesangial cells. Study postulated that longer duration of diabetes is associated with Kimmelstiel– Wilson lesion along with more severe retinopathy and higher serum creatinine level (Schwartz, M. M., *et al.* 1998 Hong, D., *et al.* 2007). This lesion is considered as transitional phases from early or moderately advanced stage to a progressively more advanced stage of DN (Hong, D., *et al.* 2007**,** Mason, R. M., & Wahab, N. A. 2003).

2.9.4 Class IV: Advanced Diabetic Glomerulosclerosis.

Class IV or advance diabetic glomerulosclerosis (ADG) is the end point of multifactorial mechanisms that lead to excessive accumulation of extracellular matrixproteins such as collagen types I, III and IV and fibronectin in the mesangial space. (Horlyck, A., *et al*. 1986). Figure 2.8 and figure 2.9 describe the morphologic lesions in DN and classification of DN respectively.

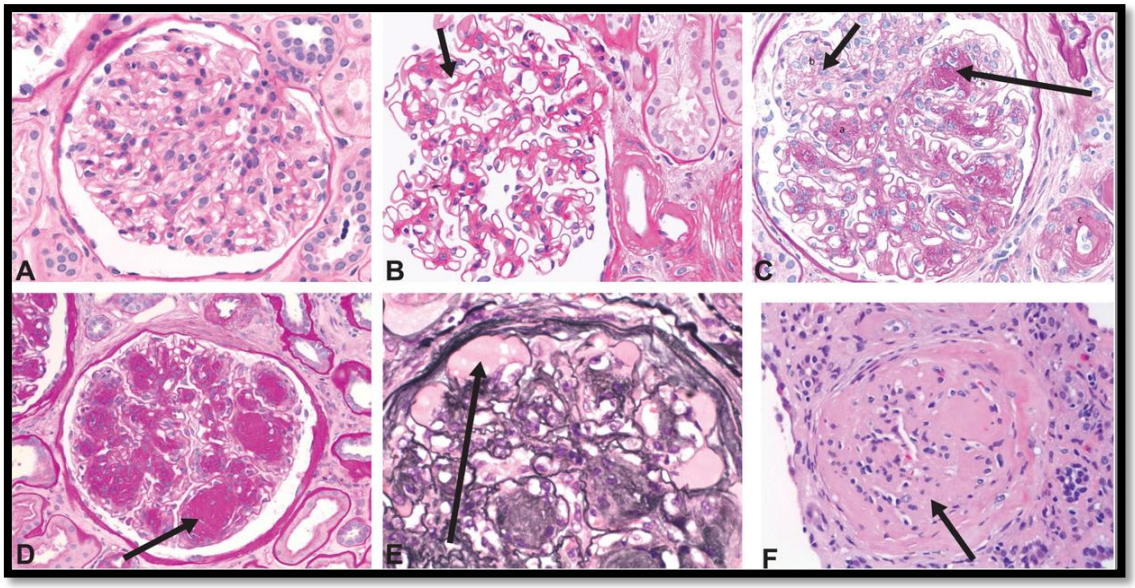


Figure 2.8: Morphological lesions in DN. Changes in glomerular histology in diabetic glomerulopathy (A) Normal glomerulus. (B) Diffuse mesangial expansion with mesangial cell proliferation. (C) Prominent mesangial expansion with early nodularity and mesangiolysis. (D) Accumulation of mesangial matrix forming Kimmelstiel–Wilson nodules. (E) Dilation of capillaries forming microaneurysms, with subintimal hyaline. (F) Obsolescent glomerulus. (Alicic, R. Z., *et al.* 2017).

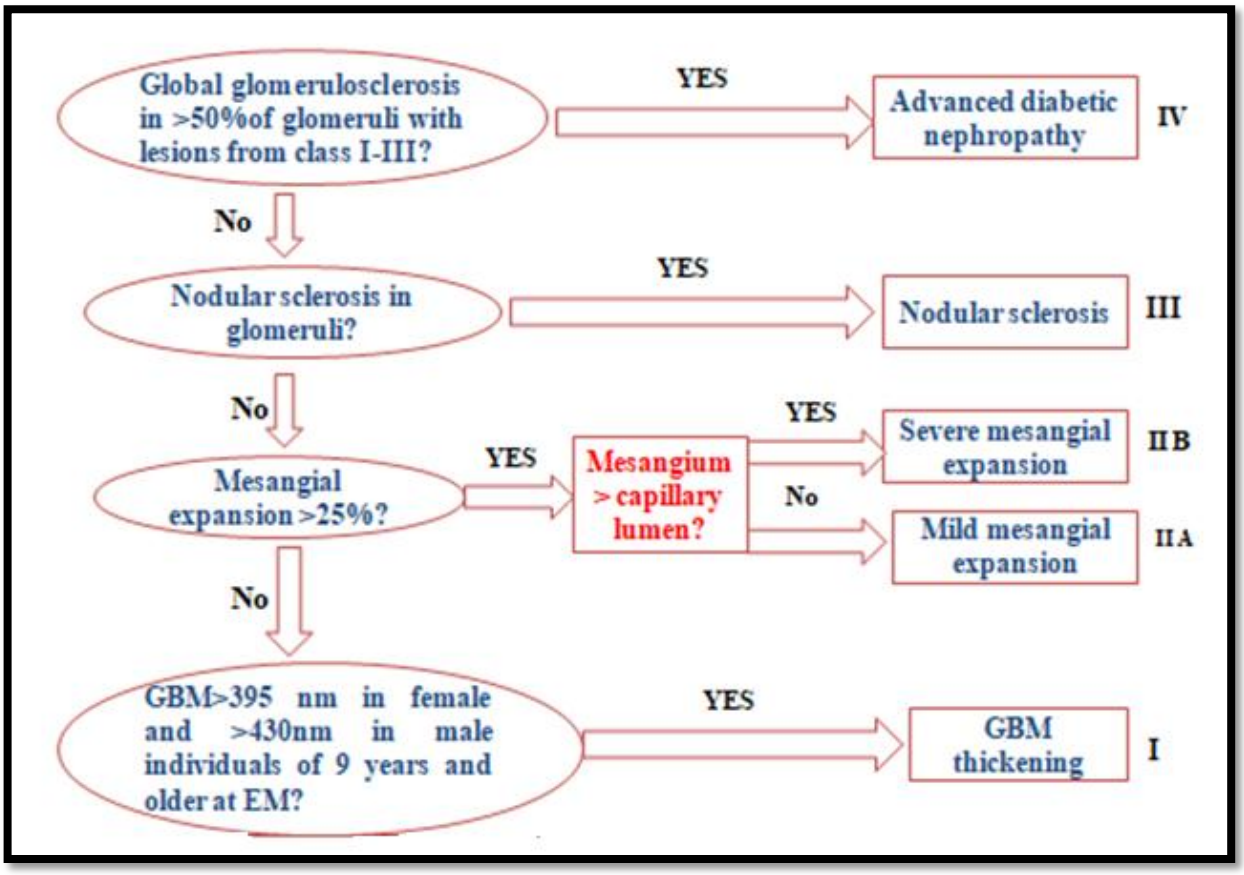


Figure 2.9: Pathologic classification of DN based on glomerular changes (Tervaert, T. W. C., *et al*. 2010)

2.9.5 Tubulointerstitial and Vascular Lesions in DN:

Glomerular and interstitial lesions have a significant impact on DN prognosis and could be used as an independent risk factor for the same. Study with 396 type 2 diabetes patients revealed that the severity of DN is due to the presence of Glomerular and interstitial lesions (Table 2.7) (An, Y., *et al.* 2014). According to the pathological changes under light microscope pathological classification of DN was divided into 3 categorises, C1: normal/near normal, C2: typical diabetic nephropathy with predominantly glomerular changes and C3: atypical patterns of injury which is associated only mild diabetic glomerular changes (Fioretto, P., *et al.* 1996, Qi, C., *et al.* 2017).

Table 2.7: Scoring system of interstitial and vascular lesions of DN (Qi, C., *et al.* 2017).

Lesion	Criteria	Score
Tubulointerstitial lesions	No IFTA*	0
	IFTA < 25per cent	1
	25per cent < IFTA < 50per cent	2
	IFTA > 50per cent	3
Interstitial inflammation	Absent	0
	Relate to IFTA	1
	In areas without IFTA	2
Arteriolar hyalinosis	Absent	0
	One hyaline arteriole	1
	More than one hyaline arteriole	2
Arteriosclerosis	No intimal thickening is observed	0
(most severely affected artery)	Intimal thickening is less than the thickness of the media	1
	Intimal thickening is more than the thickness of the media	2

*****IFTA: tubulointerstitial fibrosis and tubular atrophy.

2.10. THERAPY FOR DN:

Therapeutic option for DN includes targeting its modifiable initiators and promoter (Kim 2017)**.** Control of blood glucose and blood pressure by inhibiting the renin-angiotensin system is the conventional treatments option for DKD (Kim 2017). Renoprotective effect of two oral hypoglycemic agents, dipeptidyl peptidase 4 (DPP-4) inhibitors and sodium-glucose cotransporter 2 (SGLT2) inhibitors have been suggested through various clinical trial (Gilbert 2014, Panchapakesan, U., and Pollock, C. 2015, Penno, G., *et al.* 2016, Solini 2016). Various prospective studies including UKPDS and ADVANCE studies suggested that BP control is associated with reduced levels of diabetic microvascular complications, including nephropathy (Adler, A. I., *et al.* 2000, Patel 2007, Kim 2017). Angiotensin II receptor blockers (ARBs) or angiotensin-converting enzyme (ACE) inhibitors are recommended to control BP (Kim 2017). Glycemic control and BP control are not sufficient to prevent DN progression in many patients hence additional preventive strategies are being searched. Several clinical trials using novel drug have been completed and some are going on to prevent ESRD (Figure 2.10).

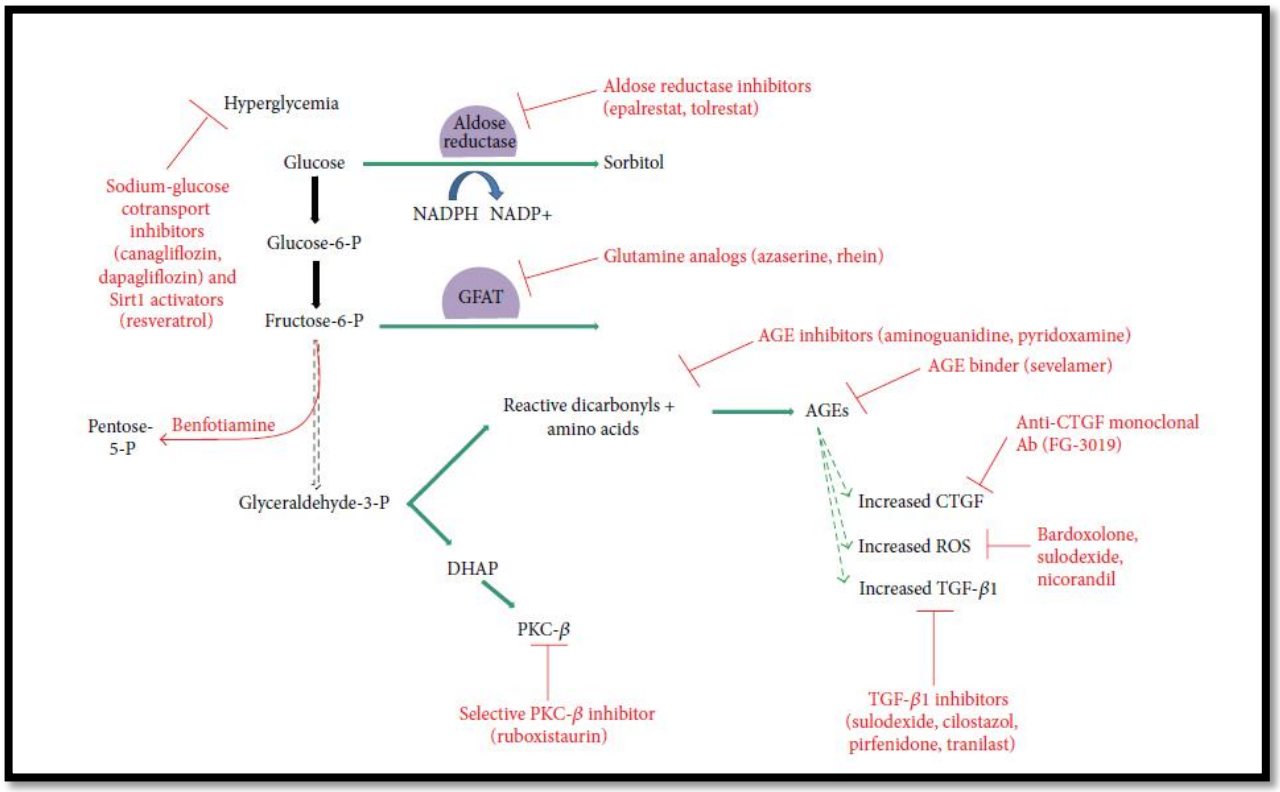


Figure 2.10: Various therapeutic agents identified in the metabolic and alternative pathways of DN (Toth-Manikowski, S., & Atta, M. G. 2015).

2.11 RISK FACTOR FOR DIABETIC NEPHROPATHY:

Risk factor for the progression of DN can be distinguished as susceptibility factors, initiation factors and progression factors (Gross, J. L. *et al*. 2005, Staples, A., and Wong, C. 2010). Susceptibility factors includes, age, sex, race or ethnicity and family history i.e; genetic susceptibility. The initiation factors include hyperglycemia and acute kidney injury (AKI). Hypertension, dietary factors and obesity fall under the progression factors for DN incidence and pathogenesis (Alicic, R. Z. *et al.* 2017). Hyperglycemia is the principal factor responsible for occurrence of DN through structural alterations and renal damage directly or through hemodynamic modifications (Kanwar, Y. S. *et al*. 2011). Intensive blood glucose control in early course of the disease reduces the progression of DN, a fact that has been well established through various trials (Tonna, S. *et al.* 2010, Alicic, R. Z. *et al.* 2017). The beneficial effects of intensive glycemic control on diabetic complications to revert the usual metabolic control (often poorer) has been described as ‘metabolic memory’ by the Diabetes Control and

Complications Trial (DCCT)/Epidemiology of Diabetes Intervention and Complications (EDIC) study (Nathan, D. M., *et al.* 2014). The term 'legacy effect' was given for the same phenomenon by the United Kingdom Prospective Diabetes Study (UKPDS) group (Tonna, S. *et al.* 2010, Alicic, R. Z. *et al.* 2017, Testa, R., *et al.* 2017). Previous studies documented that, increasing systolic blood pressure by 10mmHg was associated with a 15per cent increases of both micro and macroalbuminuria and impaired kidney function (Retnakaran, R. *et al.* 2006). Among the genetic factors, Uromodulin, APOL1 and renin–angiotensin system genes are associated with DN progression (Kazancioglu 2013).

2.12 PATHWAYS INVOLVED IN THE PATHOGENESIS OF DN:

DN pathology involves histological changes in kidney attributed primarily through metabolic and hemodynamic alteration. Hemodynamic alteration referring to the hyperfiltration occurs due to activated renin-angiotensin-aldosterone system (RAAS) mediated arteriolar vasoconstriction (Toth-Manikowski, S., & Atta, M. G. 2015). Increased level of Angiotensin II produced from the activated RAAS causes the arteriolar vasoconstriction which is associated with increased albuminuria and nephropathy in both humans and mice (Toth-Manikowski, S., & Atta, M. G. 2015). M.Brownlee in 2001 first described hyperglycemia as metabolic pathway for DN (Brownlee 2001, Toth-Manikowski, S., and Atta, M. G. 2015). Persistent hyperglycemia induced several independent biochemical pathways such as, glucose-induced activation of protein kinase C (PKC) isoforms; increased formation of glucose-derived advanced glycation end products; and increased glucose flux through the aldose reductase pathway, polyol pathway, hexosamine pathway, oxidative stress and accumulation of proinflamatory molecules (Figure 2.11) (Shiju, T. M., and Pragasam, V. 2012, Sharma, V., and Sharma, P. L. 2013). Reactive oxygen species (ROS) is a potent activator for aldose reductase, induce diacylglycerol, activate PKC, induce advanced glycation end product formation and activate the pleiotropic transcription factor nuclear factor-kappa B (NF-κ;B) (Giacco, F., and Brownlee, M. 2010, Sharma, V., and Sharma, P. L. 2013). A number of studies have demonstrated that hyperglycemia induced the development of DN by inducing endothelial dysfunction, excessive extracellular matrix (ECM) production, podocyte

abnormality and tubulointerstitial fibrosis (Lee, H. B. et al. 2003, Kawanami, D., Matoba, K., and Utsunomiya, K. 2016). Key factors that are involved in diabetic kidney damage are oxidative stress, overproduction of advanced glycation end products (AGE), apoptosis and inflammation due to the local release of proinflammatory cytokines (Sharma, V., and Sharma, P. L. 2013 , Behl, T. *et al*.2014,). Hyperglycemia mediated activation of various pathways for the pathogenesis of DN is described below (Figure 2.11).

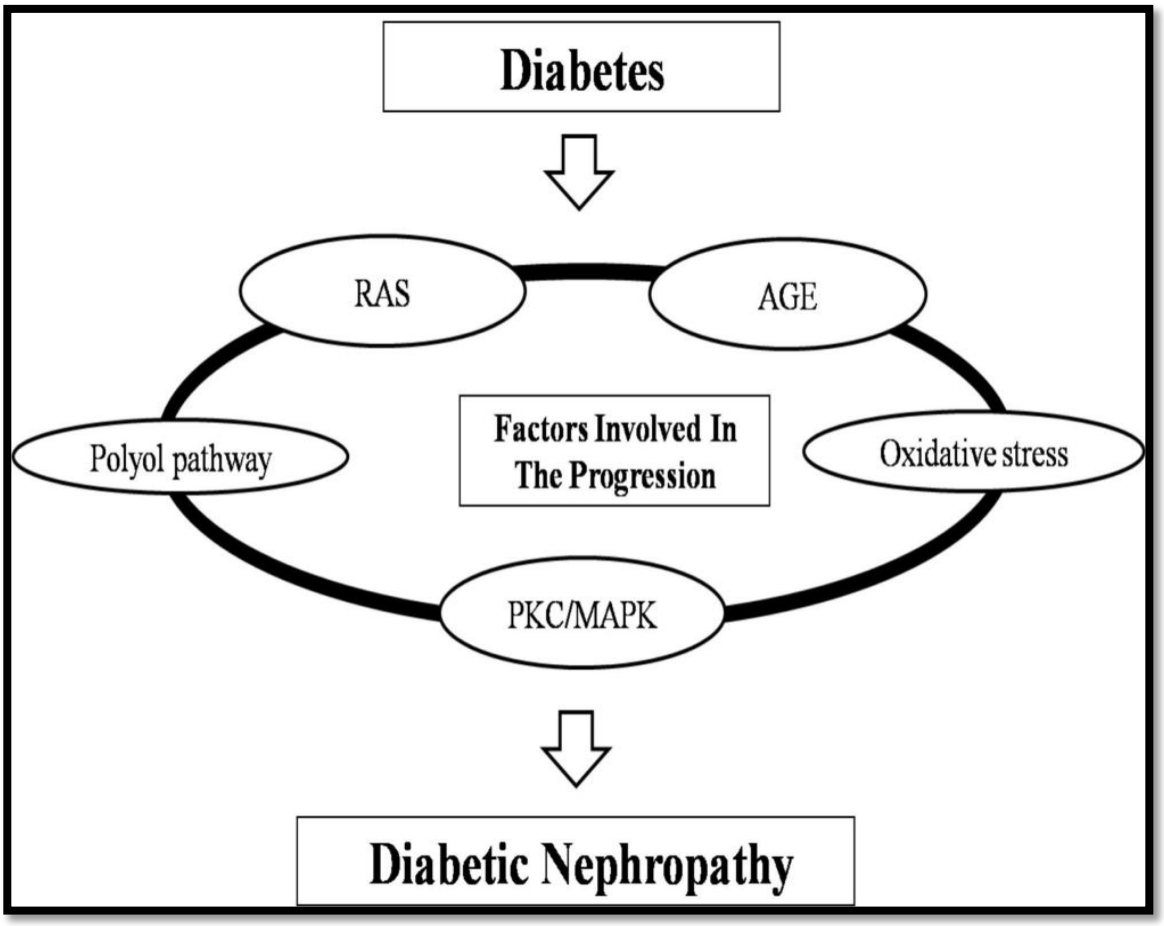


Figure 2.11: Various pathways involve in the pathogenesis of DN

2.12.1 Hyperglycemia Increase Superoxide Production and Role of Oxidative Stress in DN:

Hyperglycemia is a single unifying mechanism to increased production of reactive oxygen species (ROS), serves as a causal link between elevated glucose and each of the three major pathways responsible for diabetic damage (Nishikawa, T., *et al*. 2000). In

hyperglycemic condition, normal glucose metabolism in TCA cycle has been disturbed and as a result more glucose has to oxidize (Brownlee 2005). Mitochondrial electron transport chain has to bear more electron donors (NADH and $FADH_2$) hence electron transport is blocked in complex III and generating superoxide (Brownlee 2005). Approximate 30 mmol/L glucose increased ROS formation in aortic endothelial cells, by 250per cent within 24 hours, and resultant lipid peroxidation by 330per cent by 168 hours (Nishikawa, T., *et al*. 2000). Thus, hyperglycemia rapidly increases intracellular ROS production in cells affected by diabetic complications. Hyperglycemia-induced ROS can trigger DN pathology via early loss of podocytes by inducing a variety of pathological events which include apoptosis, detachment of podocytes, etc (Fakhruddin, S., *et al.* 2017). Hyperglycemia mediated oxidative stress occurs due to an imbalance between Reactive Oxygen Species (ROS) and intracellular antioxidants (Sharma, V., and Sharma, P. L. 2013). It has been documented that, ROS is responsible for the activation of various signaling pathways including PKC, MAPk, JAK/STAT and transcription factors (NF-κ β) tumor growth factor-β1 (TGF-β1) which is leads to accumulation of extra cellular matrix (ECM) in kidney (Brownlee 2005, Palacios, H, H., et al. 2011, Sharma, V., and Sharma, P. L. 2013).

2.12.2 Polyol Pathway:

The polyol pathway which consists of two enzymes namely, Aldose Reductase (AR), with its cofactor NADPH reduces glucose to sorbitol and the second enzyme sorbitol dehydrogenase (SDH), with its co-factor NAD^+ converts sorbitol to fructose (Sharma, V., and Sharma, P. L. 2013, Toth-Manikowski, S., and Atta, M. G. 2015). Excess glucose in cell reduced by aldose reductase to sorbitol later oxidized to fructose results in higher consumption of cofactor NADPH. NADPH is responsible for the regeneration of antioxidant reduced glutathione. Hence hyperglycemia induced polyol pathway increases susceptibility to intracellular oxidative stress, increased level of Advance Glycation End Products (AGE) formation and further signaling cascade for the pathogenesis of DN (Sharma, V., and Sharma, P. L. 2013, Toth-Manikowski, S., and Atta, M. G. 2015).

2.12.3 Advanced Glycation End Products (AEG) Pathway:

Advance glycation end products (AEG) are the products of irreversible glycation of proteins in hyperglycemic condition (Peppa M., *et al*. 2005, Huebschmann, A. G., *et al*. 2006). Production of AEG precursors occurs via oxidation of glucose to make glyoxal, degradation of amadori products and aberrant glycolysis which pushes glyceraldehyde-3-phosphate into forming methylglyoxal (Peppa M., *et al*. 2005, Huebschmann, A. G., *et al*. 2006, Sharma, V., and Sharma, P. L. 2013, Toth-Manikowski, S., and Atta, M. G. 2015). Previous animal model studies confirmed AEG as mediator of tissue injury in DN (Sharma, V., and Sharma, P. L. 2013). Receptors for AEG (RAGE) presents on proximal tubular cells, mesangial cells and podocytes of renal cell type. Interaction of AGE and RAGE promotes activation and expression of proinflamatory cytokine, growth factor and adhesion molecule such as VEGF andCTGF, TGF-β1, IGF-1, PDGF, TNF-α, IL-1β, and IL-6 (Serpillon, S., *et al*. 2009, Bonventre 2012, Sharma, V., and Sharma, P. L. 2013).

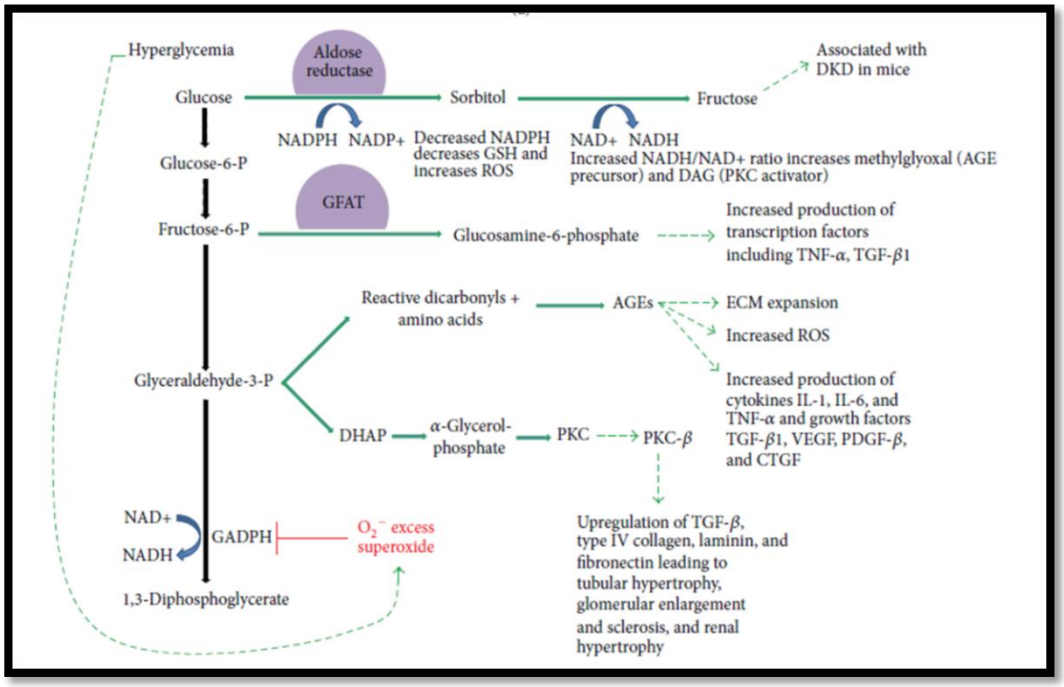


Figure 2.12: Hyperglycemia mediated activation of various pathways for the progression of DN (Toth-Manikowski, S., and Atta, M. G. 2015).

2.12.4 Protein Kinase C Pathway:

Hyperglycemia induces the conversion of glyceraldehyde-3-phosphate into dihydroxyacetone phosphate (DHAP) and finaly diacylglycerol (DAG) (Sharma, V., and Sharma, P. L. 2013). DAG is potent activator of protein kinase-C (PKC) family. PKC activation contributes to pathogenesis of DN through various ways including the changes in mesangial expansion, production of extracellular matrix to enhance contractility, vascular permeability and vascular cell proliferation along with increase production of pri-inflammatory cytokines (Figure 2.12) (Koya, D., and King, G. L. 1998, Sharma, V., and Sharma, P. L. 2013, Teng, B., *et al.* 2014).

2.12.5 Hexosamine Pathway:

High glucose concentration in cell activates an enzyme called glutamine fructose-6 phosphate amidotransferase (GFAT) which convert fructose-6-phosphate to glucosamine-6-phosphate finally to uridine diphosphate (UDP) N-acetylglucosamine. Glucosamine is used to increase the expression of tumor necrosis factor α (TNF-α) and TGF-β1. Increased TGF-β1 is well known factor to promote renal cell hypertrophy and increased matrix component in mesangial in DN (Koya, D., and King, G. L. 1998, Sharma, V., and Sharma, P. L. 2013, Teng, B., *et al.* 2014, Toth-Manikowski, S., and Atta, M. G. 2015).

2.12.6 Inflammatory Pathways of DN:

Hyperglycemia leads to the activation of many inflammatory pathways supports the postulation of low grade inflammation in DN may involve in pathology of the same. It is well documented that innate immune system are activated through various signaling cascade in DN patients. Hyperglycemic condition increases the expression of NF-*κ*B and it is associated with proteinuria and interstitial cell infiltration in cyclic fashion (Toth-Manikowski, S., and Atta, M. G. 2015). NF-*κ*B also regulates multiple gene expression related to inflammation, apoptosis and localizes to glomerular and tubular epithelial cells in human kidney (Figure 3.13) (Toth-Manikowski, S., and Atta, M. G. 2015).

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway is activated through ROS in hyperglycemic condition associated with hypertrophy of mesangial cells through interaction with various inflammatory molecules (Garcia-Garcia, P. M., *et al.* 2014, Toth-Manikowski, S., and Atta, M. G. 2015). Inflammatory cytokines such as IL-1, IL-6, IL-18 and TNF-α are highly expressed in DN patients as well as animal model (Sekizuka, K., *et al.* 1994, Navarro, J. F., *et al.* 2006). Increased serum IL-6, IL-18 and TNF-α has been found to be associated with increased kidney weight and urine albumin excretion diabetic model (Navarro, J. F., *et al.* 2006, Donate-Correa, J. *et al.* 2015). Cytokines contributes in DN pathology through increase vascular endothelial cell permeability, glomerular hyper-cellularity, GBM thickening, induce apoptosis of endothelial cells and can be directly toxic to renal cells (Toth-Manikowski, S., and Atta, M. G. 2015). Macrophages infiltrate damaged tissues and play an important role in the production of inflammatory cytokines and profibrotic cytokines. Differential macrophage activation has been documented in DN. M1 macrophage exacerbate the renal cells and M2 macrophage promotes epithelial and vascular repair (Zheng, Z., and Zheng, F. 2016). Accumulating evidence suggest that innate immunity play a significant role on DN development and progression via activation of various immune cells including monocytes, macrophages, as well as molecules like monocyte chemoattractant protein-1 (MCP-1), adhesion molecules, nitric oxide synthase and vascular endothelial growth factor (VEGF) (Zheng, Z., and Zheng, F. 2016, Tesch 2017). Activation of Inflammation leads to pyroptosis, a distinct form of programmed cell death characterized by cellular lysis, release of intracellular components and an inflammatory response (Kroemer, G., *et al*. 2009, Bergsbaken, T., *et al*.2009, Labbe, K., & Saleh, M. 2011, Shi, J., *et al.* 2017). Caspase-induced pyroptosis has been demonstrated in macrophages, dendritic cells, enterocytes and hematopoietic progenitors (Labbe, K., & Saleh, M. 2011). Pyroptosis is therefore a critical feature of inflammasome activation in a wide variety of cells leads to adverse prognosis of disease including DN (Bergsbaken, T., *et al*.2009, Sharma, D., & Kanneganti, T. D. 2016).

Inflammation in DN is considered as sterile, chronic and initiated through Damage or danger signals known as Damage associated molecular pattern (DAMP) sensing by TLRs and NLRs (Wada, J., & Makino, H. 2016, Zheng, Z., and Zheng, F. 2016, Tesch 2017). Hyperglycemia mediated activation of TLRs and NLRP3 inflammasome result in the production of various proinflammatory cytokines that can induce inflammation in renal cell and further progression of DN pathogenesis (Wada, J., & Makino, H. 2016, Zheng, Z., and Zheng, F. 2016, Tesch 2017)(Figure 2.13).

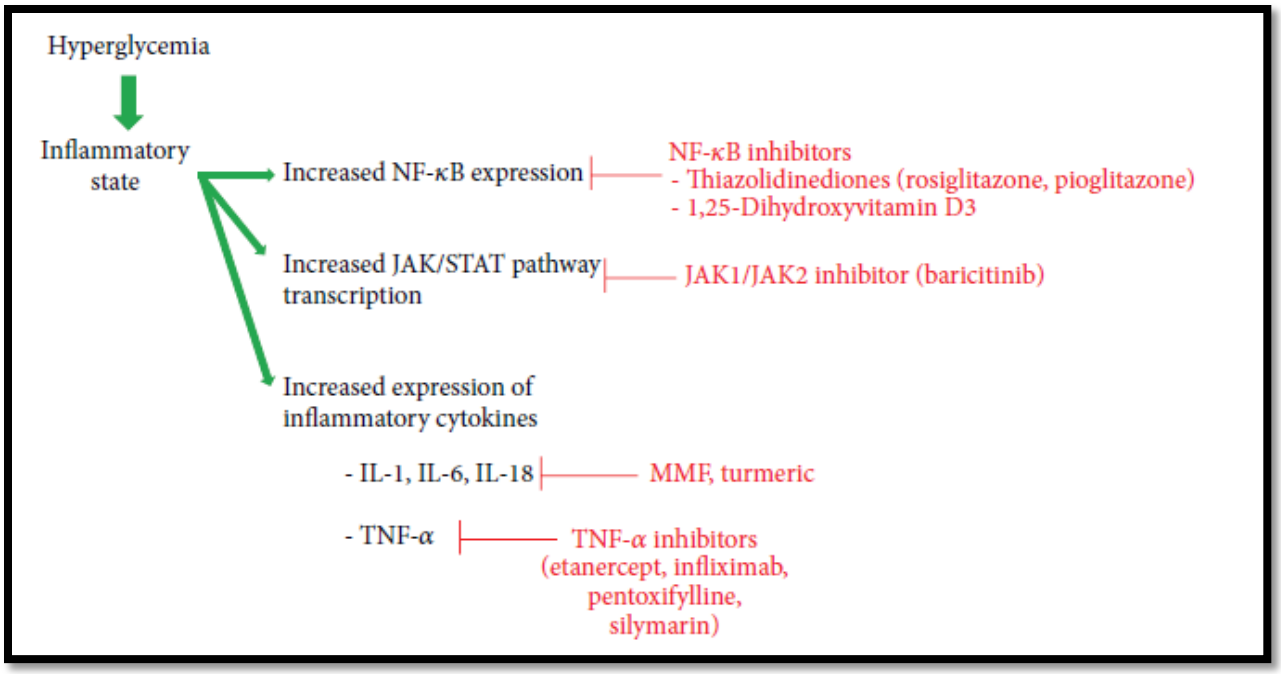


Figure 2.13: Inflammatory pathway in DN and schematic summary of the therapeutic targets. (Toth-Manikowski, S., and Atta, M. G. 2015)

2.13 INFLAMMASOME COMPLEX

The innate immune system is regulated by activation of Pattern Recognition Receptors (PRRs) by Pathogen Associated Molecular Patterns or Damage Associated Molecular Patterns (DAMPs) in response to stress tissue injury or cell death (Schroder and Tschopp, 2010). DAMPs trigger innate immune molecules by activating Inflammasome (Anders and Muruve, 2011; Anders and Schaefer, 2014; Harijith, Ebenezer, and Natarajan, 2014; Rosin and Okusa, 2011). The Intracellular multiprotein inflammatory machinery, the Inflammasome comprising

PYCARD, NLRP3 and Caspase 1 crucially modulates the inflammatory process (Schroder and Tschopp, 2010). Activation of Inflammasome complex is a necessary and critical defense mechanism for the clearance of pathogens or damaged cells. It has been also postulated that overt inflammasome activation is also a major driver of several autoimmune and metabolic disorders (Sharma, D., and Kanneganti, T. D. 2016). Inflammasome formation or activation requires a pattern recognition receptor (PRR) as the sensor (He, Y. *et al.* 2016). PRRs are expressed by macrophages, monocytes, dendritic cells, neutrophils and epithelial cells, as well as cells of the adaptive immune system. In humans four types of PRRs are recognized various stimuli. These include the transmembrane protein such as Toll like receptor (TLRs) and C-type lectin receptor (CLRs), and two types of cytoplasmic proteins including Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi, O., & Akira, S. 2010). The PRRs recognized microbial conserved structure which is called Pathogen associated molecular patter (PAMPs) and also recognized endogenous molecules released from damaged cells, termed damage associated molecular patterns (DAMPs) (Takeuchi, O., & Akira, S. 2010). Recognition of PAMPs and DAMPs by PPRs are depends upon the nature of both the responding cells and inducer. The output signal from these responses of PPRs leads to a common molecular signaling moldules of activation of the NF-kB transcription factors that drive proinflammatory cytokine/chemokine production (Takeuchi, O., & Akira, S. 2010). The inflammatory response due to signaling from PPRs response orchestrated by proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6,IL18 etc. These cytokines are involved in the regulation of cell death of inflammatory tissues, modify vascular endothelial permeability, recruit blood cells to inflamed tissues, and induce the production of acute-phase proteins (Takeuchi, O., & Akira, S. 2010).

2. 13. 1 The NLR Family:

The NLRs are comprised of 22 human genes. The NLR genes are composed of a central nucleotide-binding and oligomerization (NACHT) domain which is common to all members, a C-terminal leucine-rich repeats (LRRs) and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains (Schroder, K., & Tschopp, J. 2010). The functions of LRRs are ligand sensing and autoregulation, whereas CARD and PYD domains mediate

homotypic protein-protein interactions for downstream signaling (Schroder, K., & Tschopp, J. 2010). The central NACHT domain is enables to activation of the signaling complex via ATP-dependent oligomerization (Schroder, K., & Tschopp, J. 2010). The phylogenetic analysis of NLR family reveals 3 distinct subfamilies that includes, (i) NODs (NOD1-2, NOD3/ NLRC3, NOD4/NLRC5, NOD5/NLRX1, CIITA), (ii) the NLRPs (NLRP1-14, also called NALPs) and (iii) the IPAF subfamily, consisting of IPAF (NLRC4) and NAIP (Ting, J. P. Y., *et al.*, 2008, Schroder, K., & Tschopp, J. 2010) (Figure 2.14).

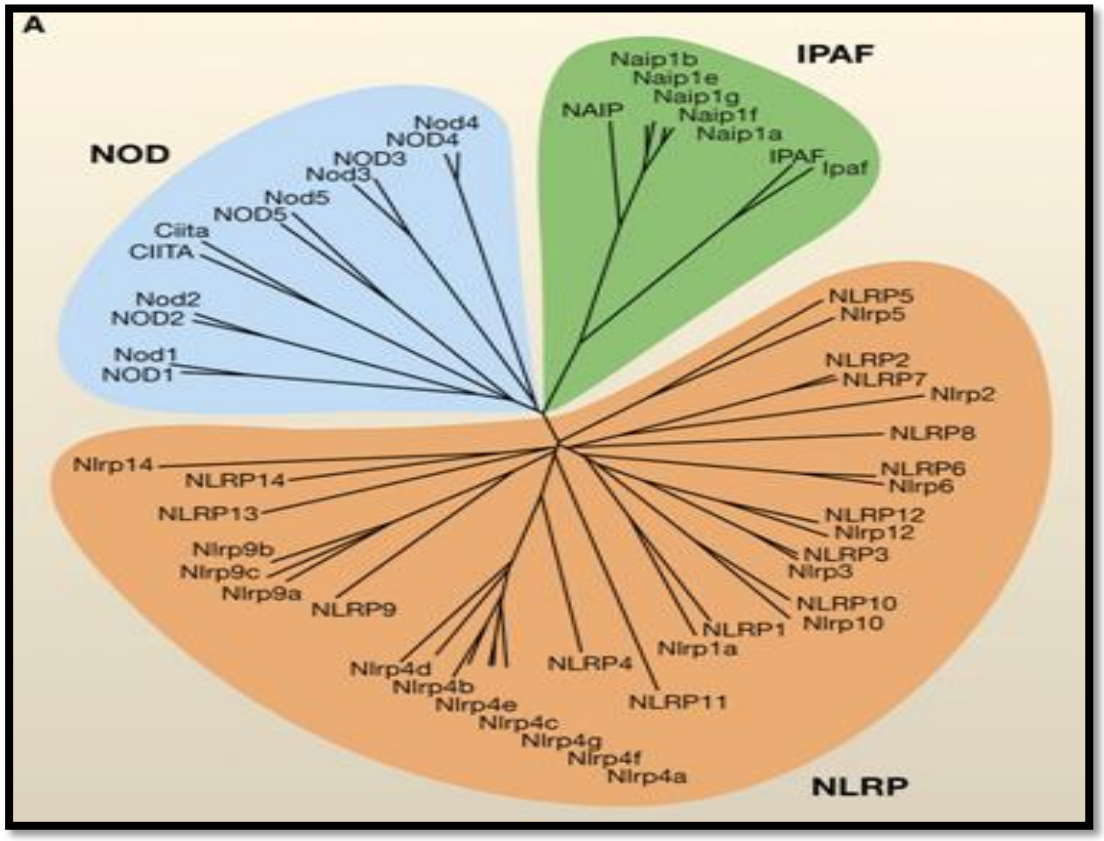


Figure 2.14. Represents the Human and Mouse NLR Family Members (Schroder, K., & Tschopp, J. 2010).

2.13.2 NLR Family Pyrin Domain Containing 3 (NLRP3):

NLRP3 is the best studied NLR family protein and associated with diseases like cancer, cardiovascular disease, HCV and other communicable disease. The NLRP3 Inflammasome is a protein coding gene located in chromosome 1q44 position (Figure 2.15)

and named abbreviates as NLR family, pyrin domain containing 3(NLRP3), where NLR refers to nucleotide-binding domain, leucine-rich repeat. External ID for NLRP3 genes is HGNC: 16400 Entrez Gene: 114548 Ensembl: ENSG00000162711 OMIM: 606416 UniProtKB: Q96P20 (National Center for Biotechnology Information (NCBI), Wheeler, D. L., *et al.* 2007). The NLRP3 gene encodes a pyrin-like protein expressed predominantly in peripheral blood leukocytes (Hoffman *et al.*, 2001). Alternative names of NLRP3 genes are CIAS1 GENE; CIAS1, CRYOPYRIN, NACHT DOMAIN-LEUCINE-RICH REPEAT- AND PYD-CONTAINING PROTEIN 3; NALP3, PYRIN DOMAIN-CONTAINING APAF1-LIKE PROTEIN 1; PYPAF1AII/AVP RECEPTOR-LIKE (NCBI Gene database).

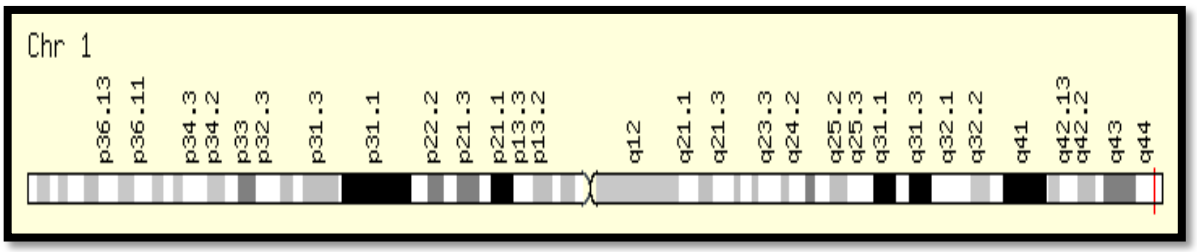


Figure 2.15: Chromosomal position of NLRP3 protein (1q44). (GeneCards – the human gene database)

NLRP3 gene is 34,270 base pair long and located on chromosome 1 (1q44) (Figure 2.15). This gene comprises of 9 exon and the protein is of 1036 amino acid long with the molecular weight of 118173 Da. (Gene Card, Human Gene database and NCBI Gene Database, ENSEBLE). NLRP3, protein sequence contains several distinct motifs including a pyrin domain in the amino terminus (amino acids 13 through 83), a central nucleotide-binding site (NBS; NACHT subfamily) domain in exon 3 (amino acids 217 to 533), and a C-terminal leucine-rich repeat (LRR) domain containing 7 leucine-rich repeats (amino acids 697 through 920) (NCBI OMIM). Gene tree showed the cluster of human NLRP3 with the species of Orangutan (Figure 2.16). Differential expression of NLRP3 among different tissues are presented below figure 2.17 and showed that NLRP3 are highly expressed in blood cells (whole blood) (Figure 2.17)

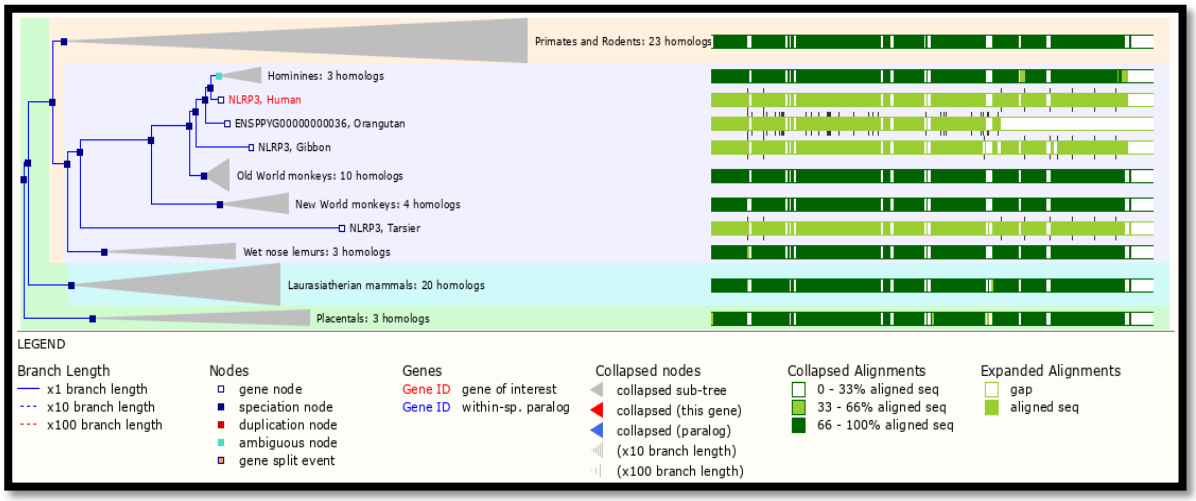


Figure 2.16: Gene tree of NLRP3 (Ensembl)

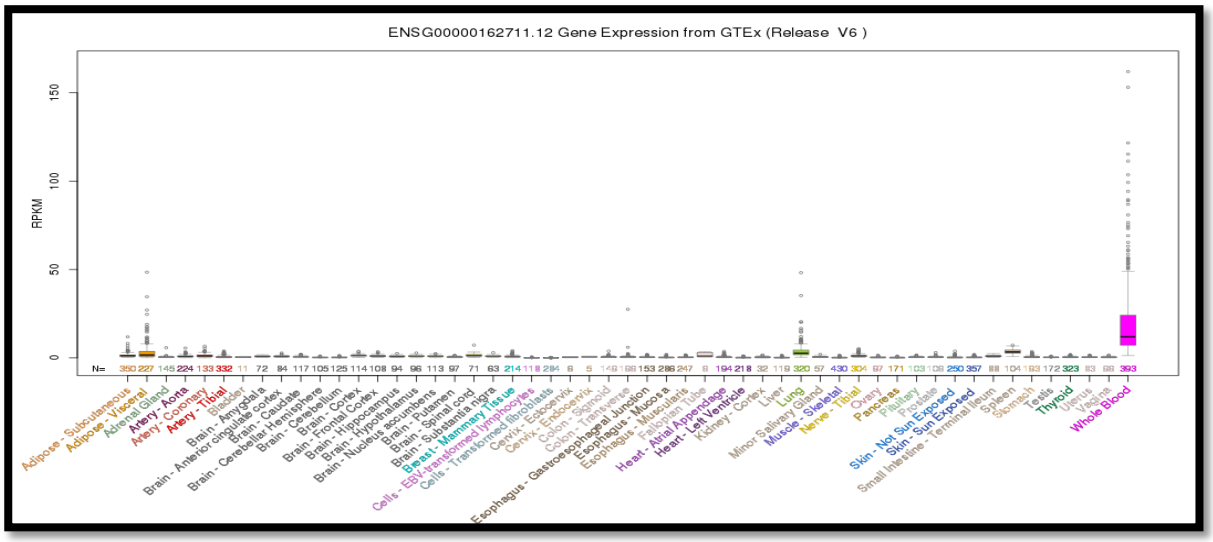


Figure 2.17: Differential expression of NLRP3 (UCSC Genome Browser)

2.13.3 NLRP3 gene function: NLRP3 was first identified and predicted the protein sequence by Mao *et al*. in 1998 (Mao, M., *et al*.1998). The protein found mainly in cytosol and the endoplasmic reticulum of macrophages. NLRP3 interacts with the apoptosis-associated speck-like protein PYCARD/ASC, which contains a caspase recruitment domain and is a member of the NALP3 inflammasome complex (Stutz, A., *et al.* 2017). This colocalization allows the activation of NLRP3 inflammasome assembly.

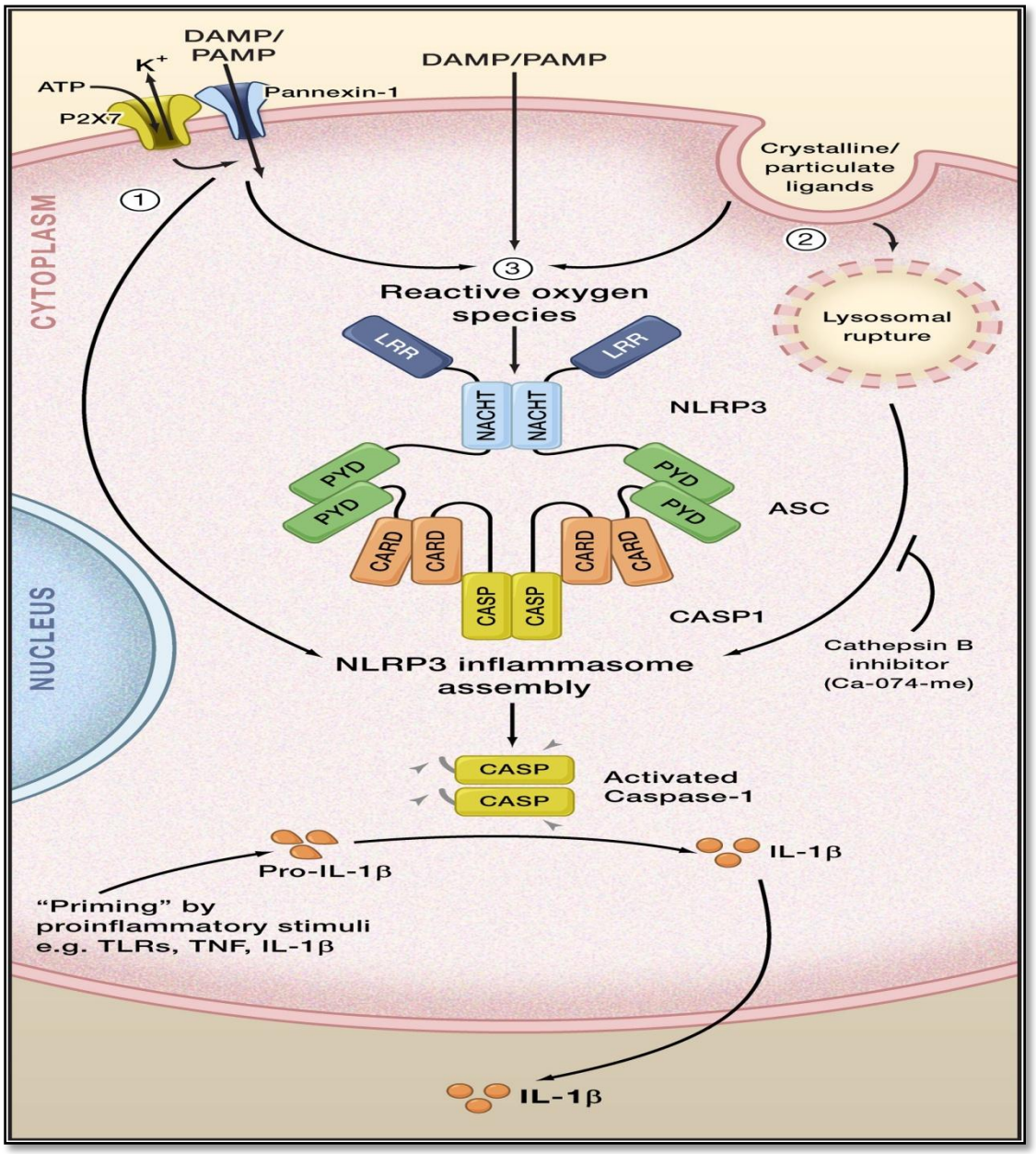


Figure 2.18: Assemble and activation of NLRP3 Inflammasome (Schroder, K., & Tschopp, J. 2010).

Upon activation, NLRP3 oligomerizes with PYCARD and procaspase1 and leads to autocatalytic cleavage and activation of caspase1 (Agostini, L., *et al.*2004, Schroder, K., & Tschopp, J. 2010, Lu, A., and Wu, H. 2015). Active Caspase-1 catalyzed IL1B and IL18 maturation and secretion in the extracellular milieu (Figure 2.18). Activated Inflammasomes can also induce pyroptosis, an inflammatory form of programmed cell death. Phagocytosis induces lysosomal damage and inflammasome activation in the recipient cells (Baroja-Mazo,

A., *et al.* 2014). NLRP3 inflammasome complex functions as an upstream activator of NF-kappaB signaling, and it plays a role in the regulation of inflammation, the immune response, and apoptosis. Various study demonstrated the association of mutation in NLRP3 gene and diseases like familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), chronic infantile neurological cutaneous and articular (CINCA) syndrome, and neonatal-onset multi system inflammatory disease (NOMID) (Arostegui, J. I., *et al.* 2010, Cantarini, L., *et al.* 2011, Kuemmerle 2015). NLRP3 activation stimuli include extracellular ATP, reactive oxygen species, K(+) efflux, crystals of monosodium urate or cholesterol, amyloid-beta fibers, environmental or industrial particles and nanoparticles, cytosolic dsRNA, etc (Schroder, K., *et al.* 2010, He, Y., *et al.* 2016). Dostert, C., *et al*. in 2008 demonstrated that asbestos and silica are capable of NLRP3 inflammasome activation, whose subsequent activation leads to IL1β secretion (Dostert, C., *et al*. 2008). Perivious study demonstrated that mice lacking PYCARD, CASP1 or NLRP3 significantly increased mortality in response to influenza virus infection (Allen, I. C., *et.al;* 2009). Activation of the NLRP3 inflammasome in response to virus or to RNA and reactive oxygen species production in human cells. NLRP3 also essential in host defense against influenza infection through the sensing of viral RNA (Allen, I. C., *et.al;* 2009).

2. 13. 4 CASPASE1: Caspase1 is an evolutionarily conserved protein which is a member of the cysteine-aspartic acid protease (caspase) family, which proteolytically cleaves other proteins, such as the precursors of the inflammatory cytokines interleukin 1β and interleukin 18. The chromosomal location of the gene Caspase 1 (CASP1) is 11q22.3 (Figure.2. 19 and 2. 20) and the Entrez Gene ID for Caspse1 is 834 (NCBI, Wheeler, D. L., *et al.* 2007). This gene comprises of 12 exon. CASP1 gene also known as ICE; P45; IL1BC. The gene CASP1 encodes the protein Caspase1 which is 404 amino acids long and Molecular mass is 45159 Da. The protein symbol is P29466-CASP1_HUMAN (GeneCards – the human gene database, Stelzer G, *et al.* 2016).

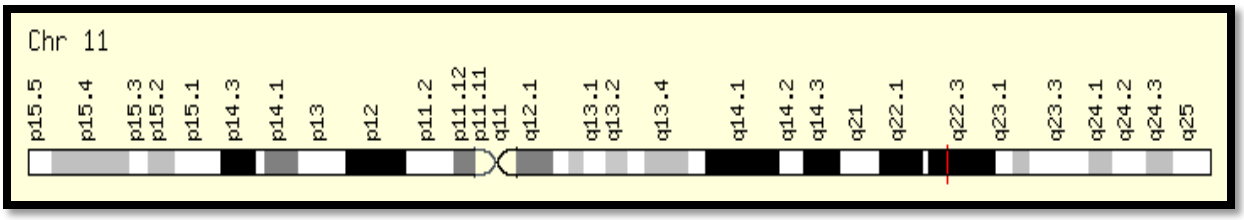


Figure 2.19: Chromosomal location of CASP1 gene (GeneCards – the human gene database)

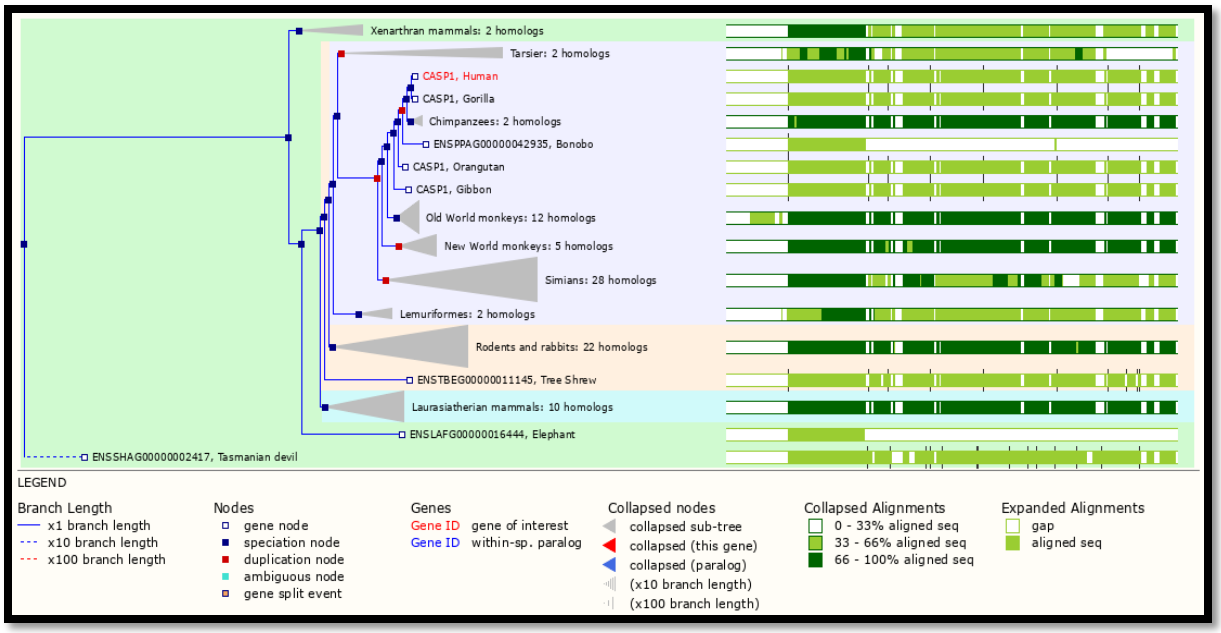


Figure 2.20: Gene tree of CASP1 (Ensembl)

The protein Caspae1 is found in cytosol of human cells and the broad expression in small intestine (RPKM 39.5), duodenum (RPKM 37.3) and 21 other tissues. [NCBI] Autoproteolysis of pro-Caspase-1 generate p20 and p10 subunits the catalytically active Caspase1, and also removed N-terminal CARD (Caspase Activation Recruitment Domain) (Broz, P., *et al.* 2010). Caspase1 interacts with Apoptosis-Associated Speck-like Protein Containing a CARD (ASC/PYCARD) and NLRP3 through CARD-CARD interactions in the formation of inflammasomes (Broz, P., *et al.* 2010, Hornung, V., & Latz, E. 2010). The crystallographic structure of caspase1 reported in 1994 (Mariathasan, S., *et al.* 2004, Scheer 2013, Lu, A., *et al*. 2016).

2. 13. 5 General Functions and Association with Disease:

In 1989 Caspase1 (previously named as interleukin 1-beta-converting enzyme (ICE) was described as a cysteine protease, involved in cleaving of pro IL1β on Asp and Ala, and IL18 releasing the mature IL1β and IL18 which is involved in a variety of inflammatory processes (Hornung, V., & Latz, E. 2010, Wang, Y., *et al.* 2017). Caspase1 also promotes apoptosis and inducing inflammation in various condition upon activation during pathogen associated molecular pattern (PAMP) and DAMP (Wang, Y., *et al.* 2017). In 1996, the specific nomenclature was introduced to describe this proteolytic enzyme "caspase" where 'c' reflects a cysteine proteinase mechanism, and 'aspase' relates to their ability to cleave after Asp, the most distinguishing catalytic property of this family (Scheer 2013).

2. 13. 6 PYCARD: PYCARD also referred to as ASC (Apoptosis-associated speck-like protein containing a CARD) is an adaptor protein that is composed of two protein-protein interaction domains: N-terminal PYRIN-PAAD-DAPIN domain (PYD) and C-terminal caspase-recruitment domain (CARD) in humans is encoded by the PYCARD gene (Stehlik, C., *et al.* 2003, NCBI Entrez Gene). It is localized primarily to the nucleus in resting monocytes or macrophages and rapidly redistributed to the cytoplasm, perinuclear space, endoplasmic reticulum and mitochondria upon pathogen infection and it is a key adaptor protein in activation of the NLRP3 Inflammasome complex (Stehlik, C., *et al.* 2003, Dunn, J. H., and Fujita, M. 2015). The gene PYCARD is located on chromosome 16 (p11.2) (Figure. 2. 21 and 2. 22). PYCARD is broadly expression in spleen (RPKM 25.5), colon (RPKM 25.4) and 22 other tissue (NCBI Entrz Gene). The protein is 195 amino acids long and the Molecular mass is 21627 Da.

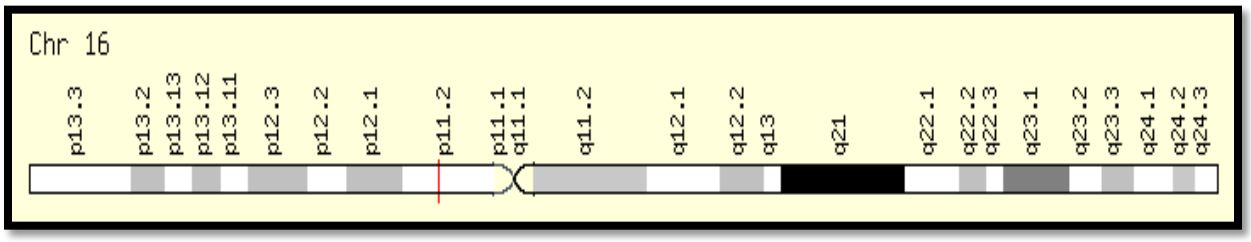


Figure 2.21: Chromosomal location of PYCARD. (GeneCards – the human gene database)

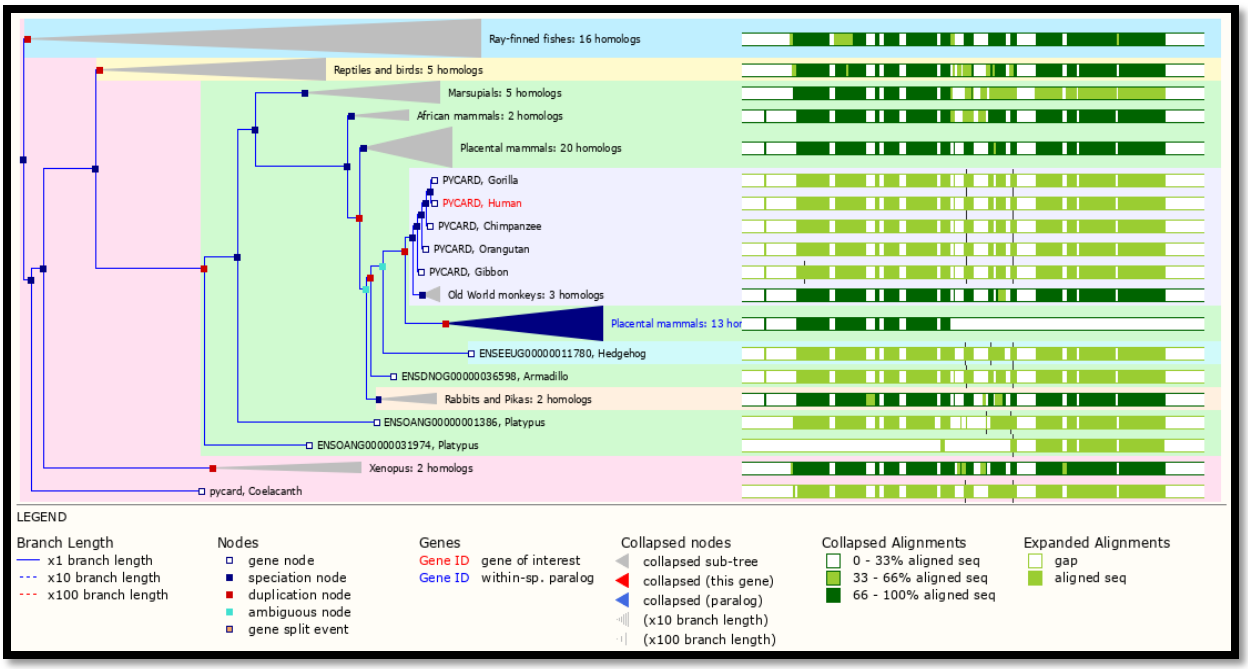


Figure 2.22: Gene tree of PYCARD (Ensembl)

2. 13.7 Functions of PYCARD and Association with Disease:

PYCARD or ASC act as key mediator in apoptosis and inflammation (Stehlik, C., *et al.* 2003, Dunn, J. H., and Fujita, M. 2015. This protein involved in macrophage pyroptosis, a caspase1 dependent inflammatory form of cell death. ASC is belived to be the integral part of Inflammasome assembly and activation to induced IL1β and IL18 mediated inflammation of Innate Immune responses (Agostini, L., *et al*. 2004, Mariathasan, S., *et al*. 2004, Stutz, A., *et al*. 2017).This is the major constituent of the ASC pyroptosome which forms upon potassium depletion and rapidly recruits and activates caspase1 (Fernandes-Alnemri, T., *et al.* 2007). PYCARD is also a crucial regulator of adaptive immunity. It involved in maturation of dendritic cells to stimulate T-cell immunity, transcriptional activation of cytokines and chemokines independent of the Inflammasome, regulation of NF-kappa-B activating and inhibiting etc (Fernandes-Alnemri, T., *et al.* 2007, Wang, Y., *et al.* 2017).

2.14: TOLL LIKE RECEPTORS AND INFLAMMATION

2. 14. 1 Toll Like Receptors (TLRs):

Toll-like receptors (TLRs) are a class of proteins that play important roll role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors usually expressed in immune cells such as macrophages and dendritic cells that recognize structurally conserved molecules derived from microbes (Uematsu, S., & Akira, S. 2008). Toll-like receptors (TLRs) were the first PRRs to be identified. TLRs are well characterized and recognize a widerange of PAMPs (Akira 2006, Kono, D. H., *et al.* 2009, Medzhitov 2007). TLR genes were first indentified by German scientists and named it “Toll” which transIates to "Great" in English (Figure 2. 23) (Christmas 2010). Discovery of TLRs was breakthrough event in the field of immunology especially in innate immunity research and awarded Nobel Prize in Physiology or Medicine to Jules Hoffmann and Bruce Beutler in 2011(O'neill, L. A., *et al.* 2013). There are 10 different functional TLRs in human (12 in mouse) respond to a variety of PAMPs, including lipopolysaccharide (TLR4), lipopeptides (TLR2 associated with TLR1 or TLR6), bacterial flagellin (TLR5), viral dsRNA (TLR3), viral or bacterial ssRNA (TLRs 7 and 8), and CpG-rich unmethylated DNA (TLR9) (Figure 2. 24) (Kumar, H., *et al*. 2009, Botos, I., *et al*. 2011, Kawai, T., and Akira, S. 2011).

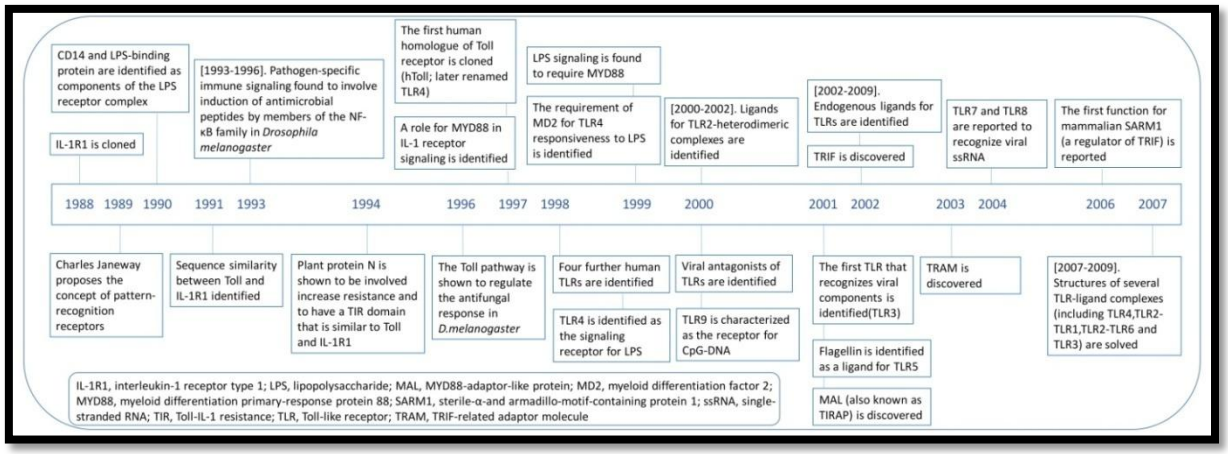


Figure 2.23: History of Toll Like Receptors related significant acheivments (Panneerselvam, Suresh (2016): The history of Toll like receptors. figshare. Dataset)

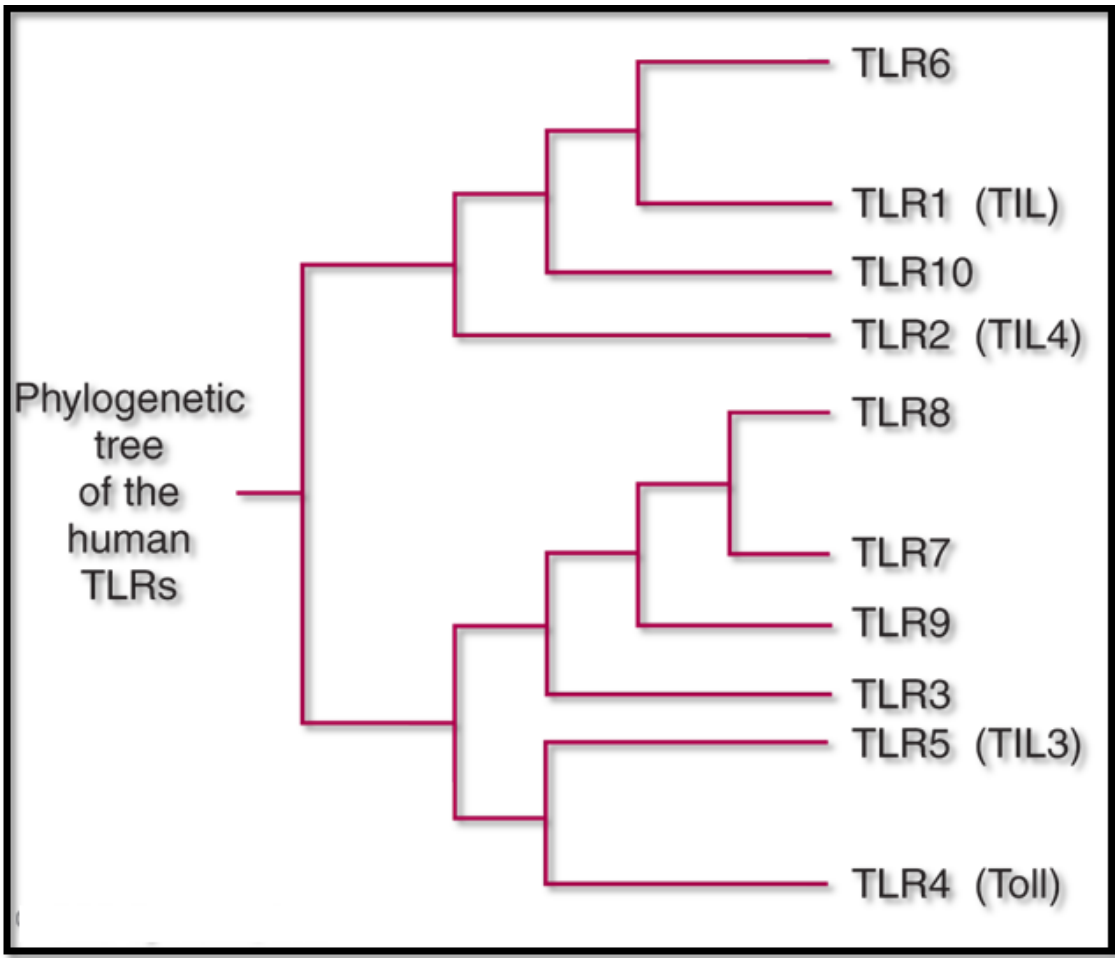


Figure 2.24: Evolutionary relationships of all 10 human TLRs. (Chuang, T. H., & Ulevitch, R. J. 2001)

Human TLRs are basically transmembrane protein and comprises of 700-1000 amino acid long. TLRs consist of extracellular N-terminal ligand recognition domain, a single transmembrane helix, and a C-terminal cytoplasmic signaling domain (Bell, J. K., *et al.,* 2003**,** Botos, I., *et al*. 2011). The n-terminal ectodomains (ECDs) are glycoprotein and contsists of 550-800 amino-acid residues and responsible for the recognition of DAMPs or PAMPs (Figure 2. 25 and 2. 26) (Bell, J. K., *et al.,* 2003**,** Botos, I., *et al*. 2011). The unique TLR ECDs are tandem copies of typically 22–29 residues long motif known as the leucin rich repeat (LRR) (Bell, J. K., *et al.,* 2003).

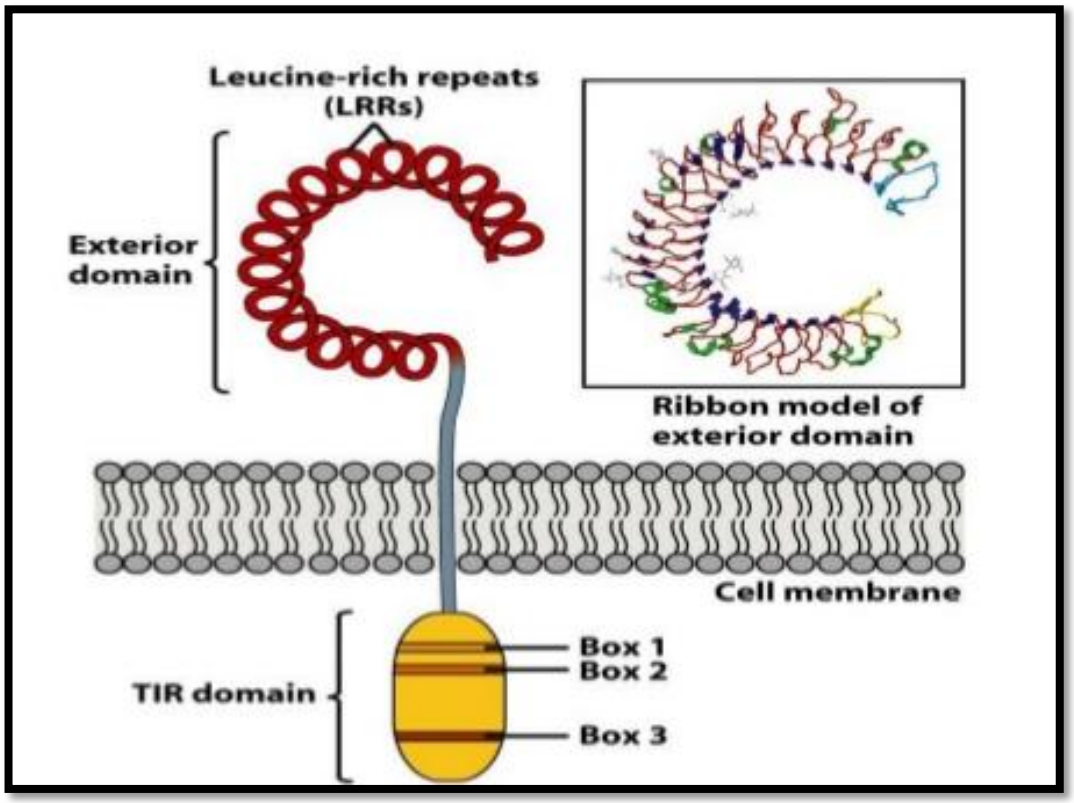
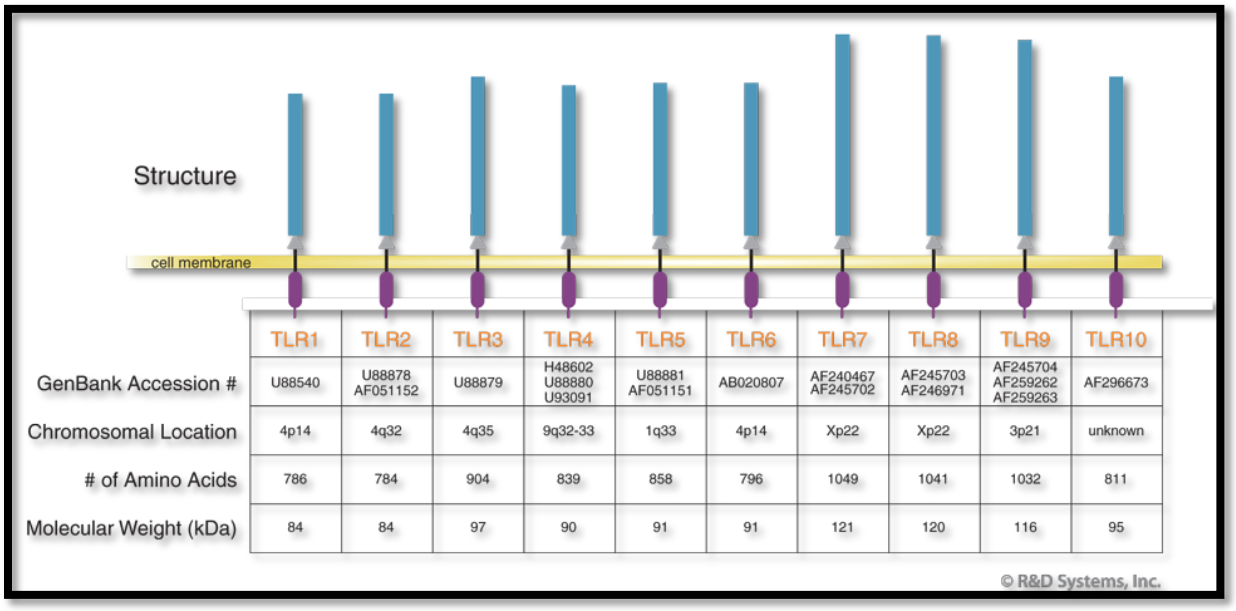


Figure 2.25: Structure of TLRs (Toll like Receptors, microbionotes.com).



	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10
GenBank Accession #	U88540	U88878 AF051152	U88879	H48602 U88880 U93091	U88881 AF051151	AB020807	AF240467 AF245702	AF245703 AF246971	AF245704 AF259262 AF259263	AF296673
Chromosomal Location	4p14	4q32	4q35	9q32-33	1q33	4p14	Xp22	Xp22	3p21	unknown
# of Amino Acids	786	784	904	839	858	796	1049	1041	1032	811
Molecular Weight (kDa)	84	84	97	90	91	91	121	120	116	95

Figure 2.26: Toll-like receptor (TLR) protein structure, location and amino acid variations with Gene Bank Accession numbers (The Toll-like Receptor Family, R&D Systems).

2. 14.2 Cellular Localization of TLRs and Cell-Specific Expression

TLRs 1, 2, 4, 5, and 6 are located in the plasma membrane, where they recognize and interact with microbial membrane components. TLRs 3, 7, 8 and 9 are expressed within intracellular vesicles such as endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes of innate immune cells and recognize nucleic acids (Blasius, A. L., & Beutler, B., 2010). The intracellular localization of TLRs 3, 7, 8 enables to recognize nucleic acid of viruses, other pathogens and infected cells in intracellular compartment. This intracellular localization also important to avoid the contact with “self” nucleic acid present in the extracellular environment which may risk the initiation of autoimmune disease (Kawai, T., and Akira, S. 2011).

TLRs have complex pattern of expression in different cell types. Different types of white blood cells: mast cells, macrophages, and dendritic cells have the significant TLRs expression. Innate immune responses are initiated by mast cells and macrophages, whereas adaptive immune responses are primarily initiated by dendritic cells (Christmas 2010, Chuang, T. H., and Ulevitch, R. J. 2001).

2. 14. 3 TLR Signaling Pathways:

TLR signaling is broadly divided in two distinct pathways; the Myeloid differentiation primary response gene 88 (MyD88) dependent and Toll-interleukin 1 receptor domain containing adaptor protein inducing IFNβ (TRIF) dependent pathways (Figure 2. 27) (Barton, G. M., & Kagan, J. C. 2009, Kawai, T., and Akira, S. 2011). Except TLR3, all other TLRs use MyD88 pathway for signal transduction and only TLR4 utilized both pathways (Figure 2.27) (Barton, G. M., & Kagan, J. C. 2009, Kawai, T., and Akira, S. 2011). MyD88 dependent pathway for TLRs signaling initiates with the complex formation of MyD88 with IRAK kinase family members. IRAK1 is activated from IRAK4 and autophosphorylated to release from MyD88 (Kawasaki, T., and Kawai, T. 2014). IRAK associated with TRAF6 and promotes K63-linked polyubiquitination to activate TAK1 (Chen 2012, Ajibade, A. A., *et al.* 2013). Activation of TAK1 leads to activation of IKK complex-NF-kB pathway and-Mitogen-activated protein kinase (MAPK) pathway. Activation of IKK complex-NF-kB pathway initiates the binding of TAK1 with IKK complex through ubiquitin chains which allows translocation of NF-kB into the nucleus to induce pro-inflammatory gene expression (Kawasaki, T., and Kawai, T. 2014). MAPK family members such as ERK1/2, p38 and JNK also activates through the activation of TAK1, which mediates stabilization of mRNA to regulate inflammatory responses through activation of AP-1 family transcription factors (Akira, S., *et al.* 2006, Kawai, T., and Akira, S. 2010). TRIF dependent pathway also leads to the activation of NF-kB and MAPKs and induction of inflammatory cytokines. Here TRIF interacts with TRAF6 and recruits the RIP-1 kinase, which in turn interacts with and activates the TAK1 complex (Akira, S., *et al.* 2006, Kawai, T., and Akira, S. 2010). TLR4 among all TLRs activates both the MyD88 and TRIF dependent pathways and it is controlled by several molecules to induce appropriate responses. Balanced production of inflammatory cytokines and type I IFN through TLR mediated MyD88 and TRIF dependent pathways may be important for controlling tumor cell growth and autoimmune diseases (Kawasaki, T., and Kawai, T. 2014).

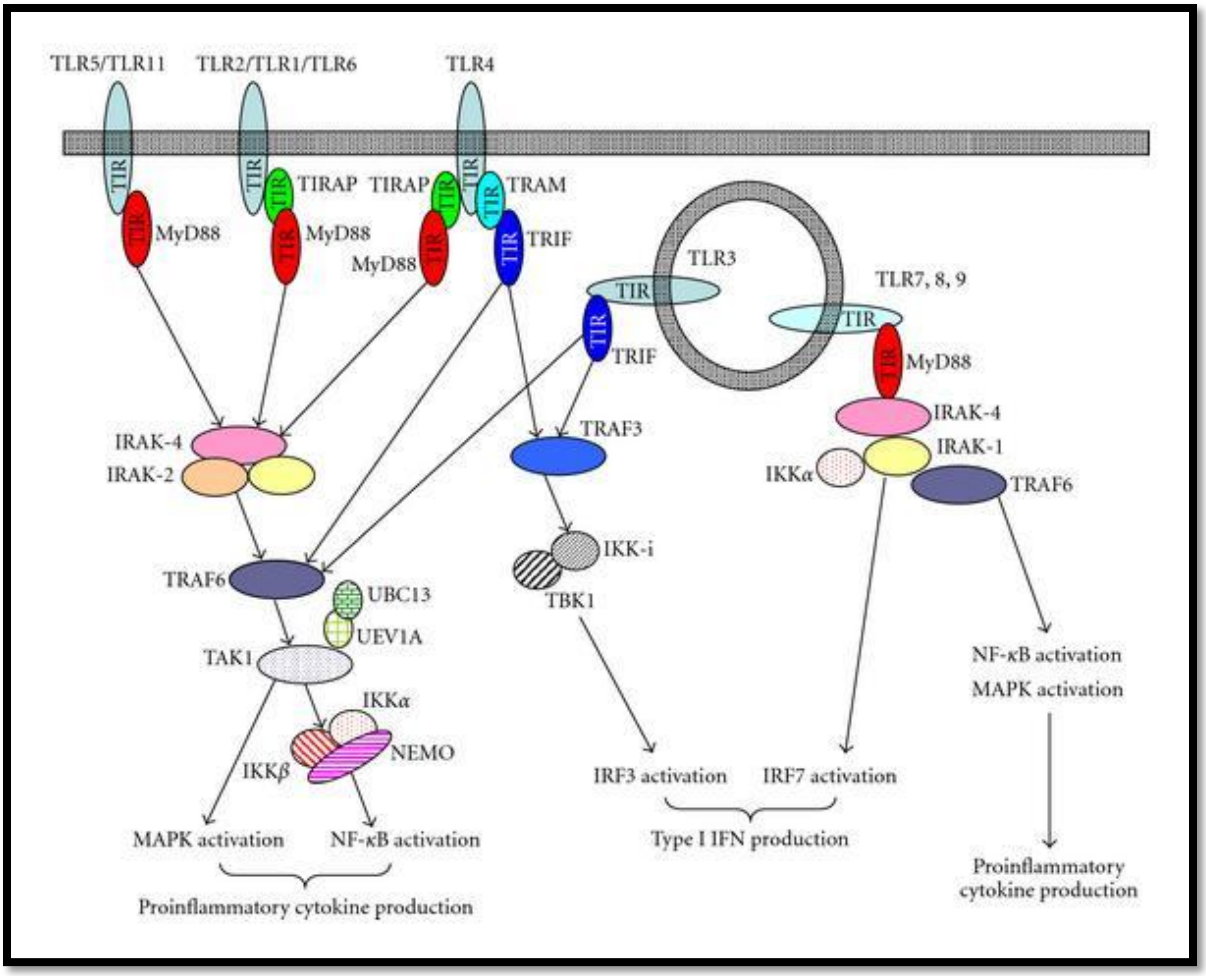


Figure 2.27: TLR signaling pathways (Yamamoto, M., and Takeda, K. (2010).

2. 15 INFLAMMATORY CYTOKINES: INTERLEUKIN 1B (IL1β), INTERLEUKIN 18 (IL18) AND TUMOR NACROSIS FACTOR A (TNFα):

Cytokines are signalling molecules of immune system that regulates wide range of biological function through extracellular signalling cascade secreted from immune cells. Cytokines are primarily involved in inflammatory responses of host immune function. Depending upon the presumed function, cell of secretion, or target of action, cytokines are named as lymphokines or interleukins (ILs), monokines and chemokines. Cytokines and their receptor exhibits in close proximity and high affinity for each other therefore picomolar concentration of the same can initiat biological function (What are Cytokines, Sino Biological, Zhang, J. M., and An, J. 2007, O'Shea, J. J., *et al.*2013). Interleukin 1beta (IL1β), Interleukin

18 (IL18) and TumorNacrosis Factor α (TNFα) are the downstream signalling molecules of innate immune response mediated through NLR and TLR pathway.

2. 15. 1 Interleukin 1Beta (IL1β):

The protein IL1β is coded by the IL1β gene (Gene ID: 3553) and produced by the macrophages. The mature and active form of this protein is generated by the proteolytical processed of pro IL1β by caspase1. IL1β is an important mediator for the activation of inflammation, cellular activities and apoptosis (NCBI Entrz Gene). This gene located on chromosome no 2 (2q14.1) along with other 8 interleukin 1 family genes bearing 7 exon count (Figure 2.28). [***provided by RefSeq, Jul 2008***] The other names of IL1β are Interferon-Gamma-Inducing Factor, Interleukin-1 Gamma (GeneCards – the human gene database).

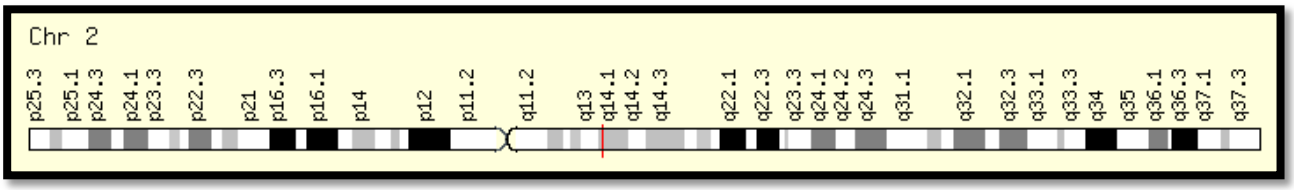


Figure 2.28: Chromosomal location of IL1β (GeneCards – the human gene database).

It is documented that, highest expression of IL1β was in bone marrow of human. The mature IL1β production occurs in 2 steps, first, inflammatory signals, such as Lipopolysaccharides (LPS), stimulate the synthesis and promote the accumulation of cytosolic stores of pro-IL1β (priming). The second steps is the Caspase1 mediated pro-IL1β processing and eventually secretion of the active cytokine after inflammasome assembly (GeneCards – the human gene database). The IL1β is 269 amino acids long and 30748 Da in molecular mass. This is localized in Cytoplasm, cytosol, lysosome, secreted exosomeetc; of cells (GeneCards – the human gene database).

2. 15. 2 Interleukin 18 (IL18):

Interleukin 18 (IL18) is an inflammatory cytokine encoded by the gene IL18 (Gene ID: 3606) and located onchromosome 11 (11q23.1) comprises of 6 exon count (Figure 2. 29). The

protein IL18 is 193 amino acids long and 22326 Da in molecular mass. IL18 mainly localize to the nucleus and the Golgi apparatus. In addition IL18 also localized to the cytosol and extracellular region (The human protein atlas).

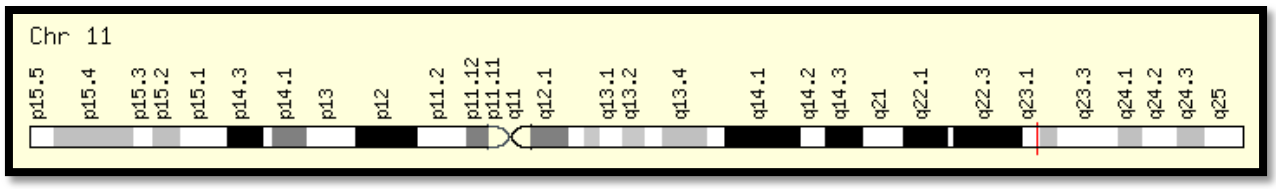


Figure 2.29: Chromosomal location of IL18 (GeneCards – the human gene database).

The protein IL18 encoded by IL18 gene is a proinflammatory cytokine that increases the activity of natural killer cell in spleen cells and stimulates interferon gamma production in T-helper type I cells (Kato, Z., *et al.* 2003, Tsutsumi, N., *et al.* 2014). It is documented that, highest expression of IL18 in human was in skin (NCBI). Mature IL18 first binds to IL18R1 forming a low affinity binary complex, which then interacts with IL18RAP to form a high affinity ternary complex that signals inside the cell (Kato, Z., *et al.* 2003, Tsutsumi, N., *et al.* 2014, Wei, H., *et al.* 2014). IL18 play major role in host innate immunity against infections and tumors. Along with macrophages, a wide variety of cells including mononuclear cells, keratinocytes, osteoblasts, intestine, renal epithelial cells and dendritic cells express IL18. Various mouse model study postulates that blockage of IL18 has potential therapeutic effect (Edelstein, C. L. (Ed.). 2016).

2. 15. 3 Tumour Necrosis Factor Alpha (TNFα): Tumour Necrosis Factor alpha (TNFα) is an proinflammatory cytokines responsible for signalling cascade leading to the necrosis or apoptosis TNFα is produced during inflammation by macrophages from its encoded gene belongs to tumour necrosis (TNF) superfamily located on chromosome 6 (6p21.33) with 4 exon (Figure 2. 30) (Idriss, H. T., and Naismith, J. H. 2000, NCBI). TNFα is synthesized as a 26-kD and 233 amino acids long transmembrane protein found on the surface or processed to release the 17-kD soluble form (Idriss, H. T., and Naismith, J. H. 2000, GeneCards – the human gene database). Lloyd J. Old from Memorial Sloan-Kettering Cancer Center, New York, named the tumor necrosis factor (TNF) in 1975 (Bodnar, R. J., *et al.* 1989). DIF, TNF-alpha, TNFA, TNFSF2 and TNLG1F are the other names of TNFα (NCBI Entrz Gene). TNFα

mainly localize to the plasmamembrane, nucleus, cytosol and extracellular region (The human protein atlas).

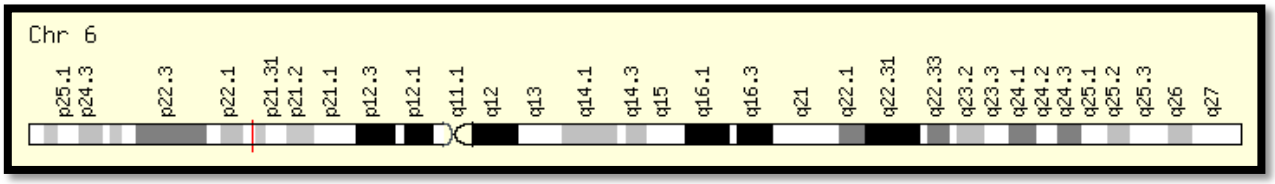


Figure 2.30: Chromosomal location of TNFα (GeneCards – the human gene database).

TNFα has the ability to induce tumour cell apoptosis and considered as central mediator of inflammation to play a protective role in immune response of body (Pfeffer 2003). This cytokines is associated with various disease including autoimmune diseases, insulin resistance, and cancer. Knockout studies in mice also suggested the neuroprotective function of this cytokine (Idriss, H. T., & Naismith, J. H. 2000, Pfeffer 2003). TNFα binds to either a 55 kDa cell membrane receptor termed TNFR-1 or a 75 kDa cell membrane receptor termed TNFR-2 for performing its activity (Idriss, H. T., & Naismith, J. H. 2000).

TNFα signal transduction starts with binding of TNFα with the receptor TNFR1 and TNFR2. The inhibitory protein, silencer of domains (SODD) is released from the intracellular domain of TNFR1 by binding the TNFα trimer to the extracellular domain of TNFR1. This free intracellular domain of TNFR1 is then bound to acoplex of protein comprises of TNF receptor-associated death domain (TRADD), receptor interacting protein-1 (RIP-1), a serine/threonine kinase and TNFR-associated factor 2 (TRAF2), an E3 ubiquitin ligase. This complex is then internalized and the TRADD-RIP-1-TRAF2 complex is released from TNFR1 and involved in activating key signaling pathways (Takeuchi, M., *et al.* 1996, Parameswaran, N., and Patial, S. 2010). TRADD-RIP-1-TRAF2 complex can initiated three pathways, such as Activation of NF-κB, Activation of the MAPK pathways and Induction of death signaling (Parameswaran, N., and Patial, S. 2010). Activation of NF-κB initiates with the recruitment of TRAF2 and RIP by TRADD. The inhibitory protein IκBα that normally binds to NF-κB and inhibits its translocation, phosphorylated by IKK which is recruited by TRAF2 degraded and releasing the NF-κB. This NF-κB translocates to the nucleus and mediates the transcription of

a vast array of proteins involved in cell survival and proliferation, inflammatory response, and anti-apoptotic factors (Parameswaran, N., and Patial, S. 2010).

TNFα induce the activation of MAPK pathways by stress related c-Jun N-terminal kinases (JNK) pathways via phosphorylation of various intermediate proteins. Activated JNK translocates to the nucleus and activates transcription factors such as c-Jun and ATF2. The JNK pathway of MAPK cascade is involved in cell differentiation, proliferation and is generally pro-apoptotic (Micheau, O., and Tschopp, J. 2003, Parameswaran, N., and Patial, S. 2010). TNFα induces TNFR1 activation to initiate pro-apoptotic signaling via the Fas-associated death domain (FADD). Micheau *et al.*2000 explained that TNFR1-induced pro-apoptotic signaling is mediated by two complexes. Complex-I is consists of TNFR1, TRADD, RIP, TRAF2 and c-IAP1at the plasma membrane and this complex-I triggers NFκB response without affecting apoptosis (Micheau, O., and Tschopp, J. 2003, Parameswaran, N., and Patial, S. 2010). The second complex is consists of FADD and procaspases-8 and -10 but lakes of TNFR1. The complex FADD and procasepae-8 and -10 formed in the cytoplasm and initiates apoptosis (Parameswaran, N., and Patial, S. 2010). TNFα is considered to be a "master regulator" of pro-inflammatory cytokine production (Maini, R. N., *et al.* 1996). TNFα activates macrophage by priming with interferon gamma (IFNγ) to stronger activation of NFκB (Parameswaran, N., and Patial, S. 2010). Activated macrophages can recruits to the inflammation site and lyse the pathogens (Parameswaran, N., and Patial, S. 2010). This activation leads to the production of toxic oxygen species and nitric oxide (NO). Magez*et al*. 2007 revealed the soluble TNFα dependent control of *Trypanosoma congolense* infection (Magez, S., *et al.* 2007).

2. 16 DAMAGE-ASSOCIATED MOLECULAR PATTERNS (DAMPs):

Damage associated molecular patterns (DAMPs) are molecules released upon cellular stress or tissue injury and are regarded as endogenous danger signals and activate the innate immune system by interacting with pattern recognition receptors (PRRs) (Roh, J. S., and Sohn, D. H. 2018). Several DAMPs have been identified since the introduction of danger model by Polly Matzinger in 1994 (Matzinger 1994). DAMPs released after cellular injury or death in disease condition from the extracellular or intracellular space (Figure 2. 31). These DAMPs

are recognized by PRRs and initiats inflammatory pathways via TLRs and Inflammasome (Figure 2.32) (Roh, J. S., and Sohn, D. H. 2018). In DN condition various DAMPs have released due to hyperglycemia induced cell death or renal cell necroptosis (Anders, H.J., and Schaefer, L. 2014). It is well known that DAMPs play a critical role in disease progression including DN through initiating inflammation. Inhibition of DAMP release can be promising therapeutic strategy in inflammatory disease as well as DN.

Origin		Major DAMPs	Receptors
Extracellular matrix		Biglycan	TLR2, TLR4, NLRP3
		Decorin	TLR2, TLR4
		Versican	TLR2, TLR6, CD14
		LMW hyaluronan	TLR2, TLR4, NLRP3
		Heparan sulfate	TLR4
		Fibronectin (EDA domain)	TLR4
		Fibrinogen	TLR4
		Tenascin C	TLR4
Intracellular compartments	Cytosol	Uric acid	NLRP3, P2X7
		S100 proteins	TLR2, TLR4, RAGE
		Heat shock proteins	TLR2, TLR4, CD91
		ATP	P2X7, P2Y2
		F-actin	DNGR-1
		Cyclophilin A	CD147
		Aβ	TLR2, NLRP1, NLRP3, CD36, RAGE
	Nuclear	Histones	TLR2, TLR4
		HMGB1	TLR2, TLR4, RAGE
		HMGN1	TLR4
		IL-1α	IL-1R
		IL-33	ST2
		SAP130	Mincle
		DNA	TLR9, AIM2
		RNA	TLR3, TLR7, TLR8, RIG-I, MDA5
	Mitochondria	mtDNA	TLR9
		TFAM	RAGE
		Formyl peptide	FPR1
		mROS	NLRP3
	ER	Calreticulin	CD91
	Granule	Defensins	TLR4
		Cathelicidin (LL37)	P2X7, FPR2
		EDN	TLR2
		Granulysin	TLR4
	Plasma membrane	Syndecans	TLR4
		Glypicans	TLR4

Figure 2.31: List of DAMPs and their receptors (Roh, J. S., and Sohn, D. H. 2018).

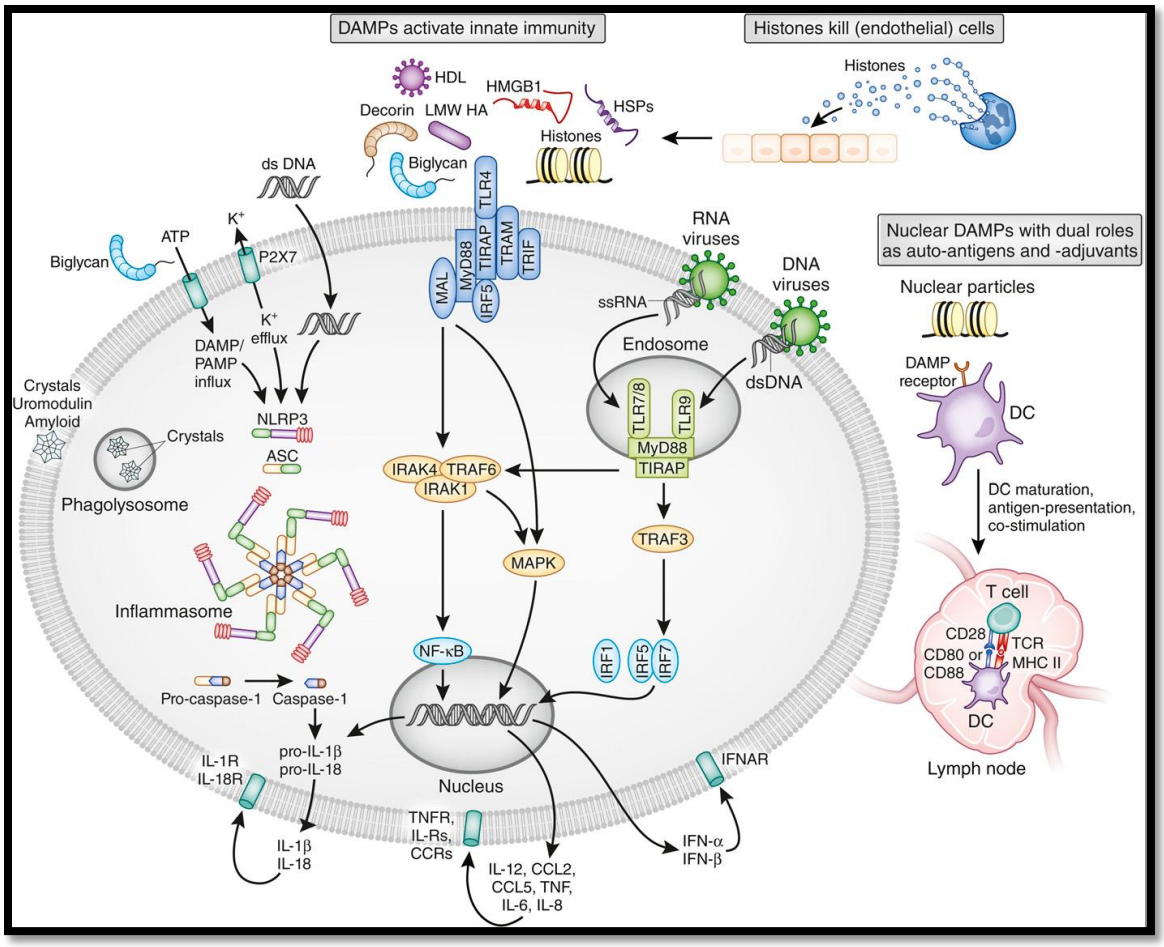


Figure 2.32: DAMP associated activation of immune response (Anders, H.J., and Schaefer, L. 2014).

2. 16.1 DAMP Mediated NLRP3 Inflammasome Activation :

Activation of potent inflammatory molecule viz. TNFα, IL6, IL1β and IL18 has been documented in-vivo and in-vitro (Donate-Correa, J. *et al.* 2015). Nod Like Receptors (NLRs) family containing 3 (NLRP3) inflammasome has been postulated for the pathogenesis of various diseases. NLRP3 functions as a pattern recognition receptor for ATP, cholesterol crystals, β-amyloid and monosodium urate crystals (Schroder, K., and Tschopp, J. 2010, Baroja-Mazo, A., *et al.* 2014). Stimulation with danger signals triggers the formation of a large multimolecular complex, namely, NLRP3 inflammasome and process inflammatory cytokine IL-1β and IL18 to its bioactive form (Latz, E., *et al.* 2013, Baroja-Mazo, A., *et al.* 2014). NLRP3 inflammasome is a potent contributor to the occurrence of damage renal cells by activating inflammatory cascade involving of macrophage (Vilaysane, A., *et al.* 2010,

Skeldon, A. M., *et al.* 2014). Hyperglycemia seems the potent activator for Inflammasome by mitochondrial reactive oxygen species (ROS) but yet to establish. On the other hand Damage Associated Molecular Patterns (DAMPs) in response to stress, tissue injury or cell death[23,24] trigger innate immune molecules by activating Inflammasome (Schroder, K., and Tschopp, J. 2010, Anders, H.J., and Muruve, D. A. 2011, Anders, H.J., and Schaefer, L. 2014), Harijith, A., *et al.* 2014). Receptor for Advance Glycation End product (RAGE) is one of the best-studied DAMP receptors in various metabolic conditions including DM (Schaefer 2014). Studies have revealed that AGE-RAGE axis play an important role in the pathogenesis of DN (Yan, S. F., *et al.* 2010). NLRP3 inflammasome activation can occur as a response to a number of distinct DAMPs (Schaefer 2014). Uric acid act also an inducer of NLRP3 inflammasome, a process that requires the LRR domain of NLRP3 (Riteau, N., *et al.* 2010). Biglycan a abarent damage associated molecules act as DAMP to activate the NLRP3 inflammasome by clustering P2X7 with TLR2/4 (Babelova, A., *et al.* 2009). Histones release from necrotic cells was recently shown to independently activate the NLRP3 inflammasome (Pisetsky 2013, Allam, R., *et al.* 2013). The action of inflammation in the kidney among diabetic patients has captured scientific interest very recently. Importantly, small but seminal human studies have also provided evidences that anti-inflammatorytherapy can improve glycemia and β cell 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 (Fu, Y., *et al.* 2017).

Several in-vitro and in-vivo studies suggested that NLRP3 Inflammasome activation is a key mechanism to induce inflammation and insulin resistance in diabetic complications including DN. Reduce level of NLRP3 Inflammasome in diabetic patients was observed after weight loss through lifestyle intervention suggesting its clinical relevance (Dixit 2013). NLRP3 mediated inflammation via activation of IL1β and IL18 contributes to pathophysiology of various microvascular diseases (Figure 2.33). Inflammasome effectors (eg: IL1 receptor antagonists and IL1β) inhibiting therapeutics has been approved but no NLRP3 antagonist therapy have been approve till now through various clinical trials are going on (Papademetriou, V., *et al.* 2015, Mulay 2019).

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.

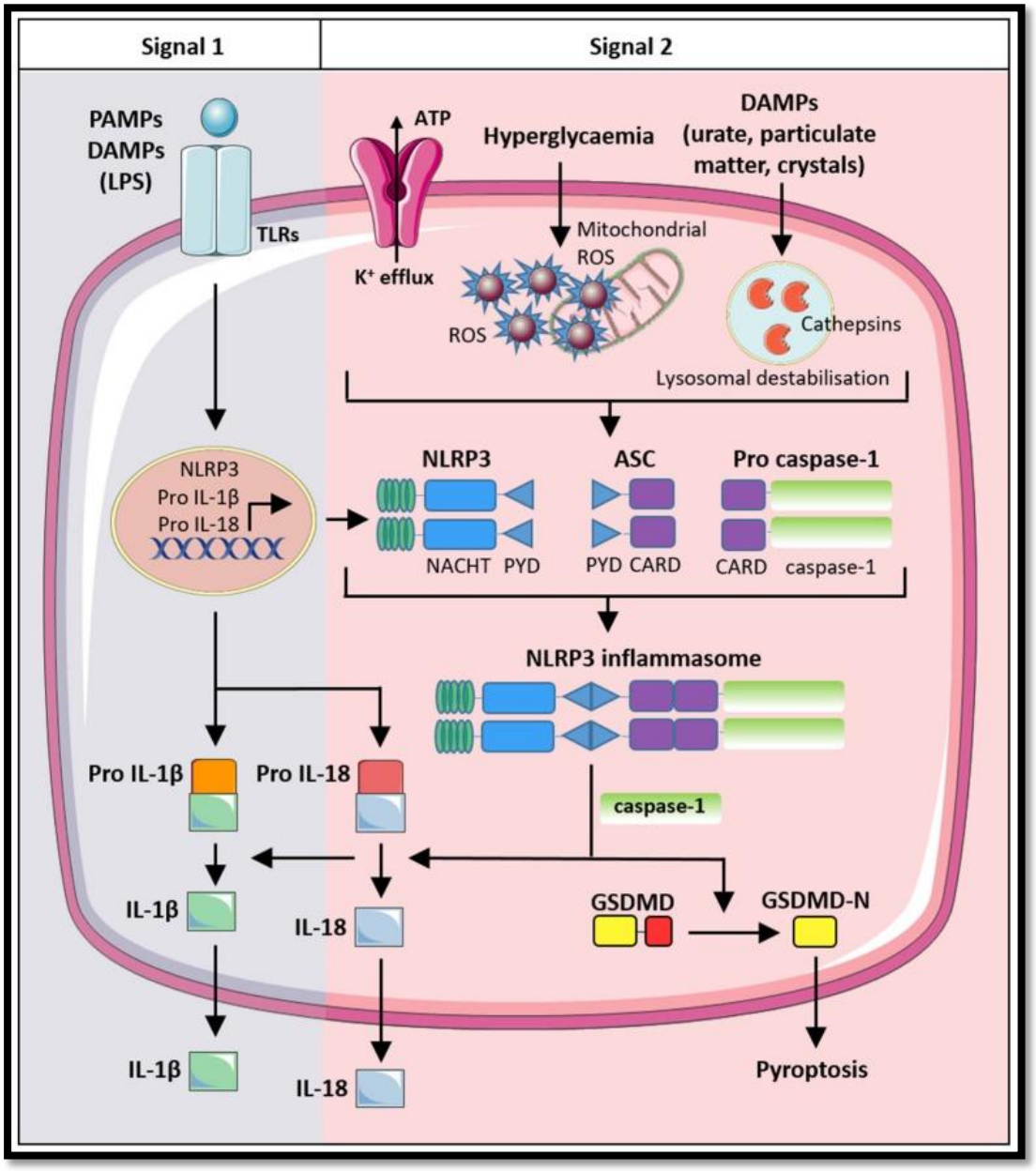


Figure 2.33: The NLRP3 inflammasome activation through mitochondrial reactive oxygen species (Schroder, K., and Tschopp, J. 2010).

2. 16. 2 DAMP Mediated TLRs Activation

Comprehensive knowledge has been generated on growth factor viz. VEGF, TGF mediated patho-mechanism but it is still enigmatic to explain the disease completely. Cellular damage, endothelial dysfunction and matrix degradation due to persistent hyperglycemia have been postulated. Elevated expression of cell adhesion molecules, growth factors, chemokines and pro-inflammatory cytokines are associated with various diseases (Donate-Correa, J., *et al*. 2015). It is well documented that TLRs also recognize endogenous (self) components released by dead/ dying cells or damaged tissues (Toll like Receptors, microbionotes.com). Recently, Toll Like Receptor 4 (TLR4) are shown as a putative factor for activation of inflammation in noncommunicable diseases (Ma, J., *et al.* 2014). Though TLR has been well established for viral and bacterial disease as Pattern Recognition Receptor (PPR) but recent knowledge with limited studies (animal model) postulates that TLRs may crucial to understand the disease pathology of Diabetic Nephropathy.

2. 17 NON CODING RNAs: OVERVIEW:

Non coding RNAs are functional RNA segment transcribed from DNA but does not translate to protein. Since 2001, after human genome project it was revealed that only 1.2per cent gene of total genome encodes proteins and rest was termed as non coding RNA (Jarroux, J., *et al.* 2017). Various studies proposed and characterized long non coding RNAs (ncRNAs) containing >200 nucleotides in many species and were shown to be involved in regulatory processes, revealing a new layer of regulation in eukaryotic cells (Jarroux, J., *et al.* 2017). The discovery of noncoding RNA was described under the knowledge of eukaryotic gene repression or silencing. Complex system of gene expression are controlled by various post-transcriptional mechanisms and scientists characterized these mechanisms are the result of small noncoding pieces of RNA called small inhibitory RNA or interference RNA (siRNA), and microRNA (miRNA) or antisense RNA (Phillips 2008). Lee *et al*. 1993 first observed the control of gene expression by these small noncoding RNA i.e. miRNA in *Caenorhabditis elegans* larvae (Lee *et al*., 1993). miRNA complemented to the sense strand of mRNA and

bound to its 3' untranslated region and inhibiting translation (Lee *et al*., 1993). Gene expression and regulatory mechanisms through involvement of noncoding small RNA are collectively known as RNA silencing or RNA interference (Carthew, R. W., and Sontheimer, E. J. 2009). Small RNAs are classified on three main categories in eukaryotes: short interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs) (Figure 2.34) (Carthew, R. W., and Sontheimer, E. J. 2009, Losko, M., *et al.* 2016). Craig C. Mello and Andrew Fire received 2006 Nobel Prize in Physiology or Medicine for the discovery of the RNAi mechanism (Fire, A, *et al.* 1998, Fire, A. Z., and Mello, C. C. 2006). Some other noncoding small RNAs are, small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), extracellular RNA (exRNAs), small cajal body-specific RNAs (scaRNAs) are the recent addition (Collins, L. J., *et al*. 2011).

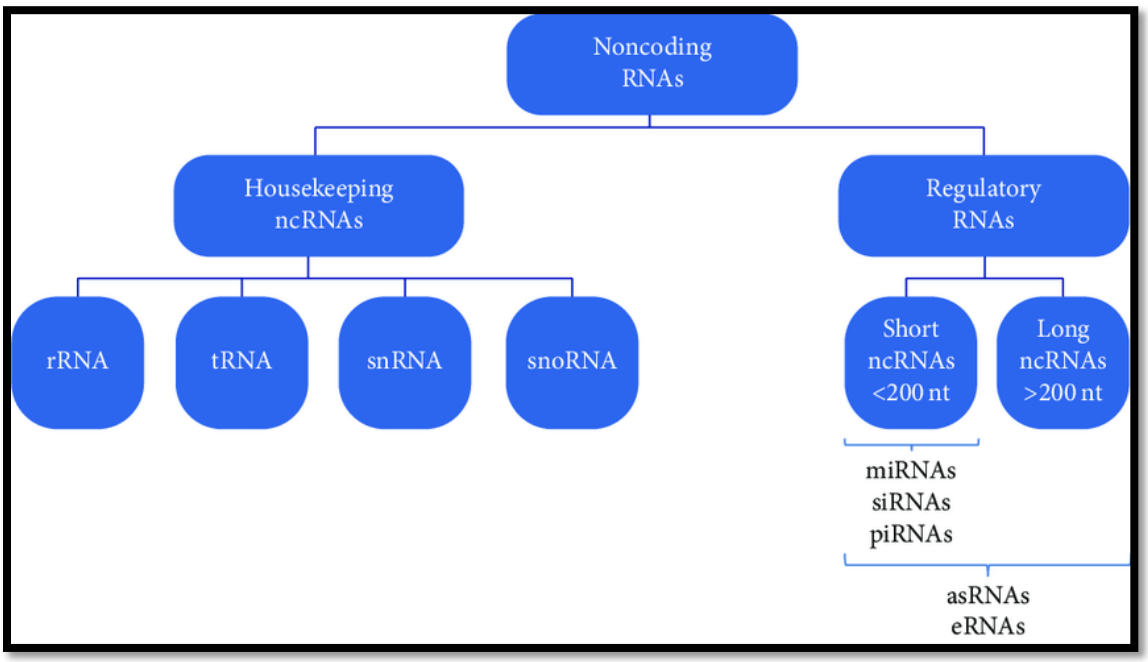


Figure 2.34: Types of non coding RNAs (Losko, M., *et al.* 2016)

2. 17. 1 Micro RNAs:

MicroRNAs (miRNAs) are non coding smallRNA molecules approximately 20-22 nucleotides long that regulate other genes in the human genome (Boyd 2008, Machnicka, M. A., *et al.* 2012). 1000 different kinds of miRNAs are discovered and the numbers increasing with time. The first miRNA was lin-4 discovered in *C. elegans* about 15 years ago by Lee *et*

al; in 1993 which is responsible to inhibit translation of the messenger RNA of a different gene by binding to its 3' untranslated region of lin-14 gene (Lee *et al;* 1993, Bartel 2004). miRNAs that regulate gene expression during posttranscriptional repression through mRNA degradation in a sequence-specific manner and repressing translation (Figure 2.35) (Carthew 2006). The roles of miRNAs are just beginning to be understood, but with the development of recent high-throughput sequencing technologies and bioinformatics predictions has enhancing the regulatory and target predictions research of miRNA (Lai, E. C., *et al.* 2003, Nam, J.W., *et al.* 2005, Li, S.C., *et al.* 2006, Huang,T.H., *et al.* 2007). miRNAs are also involved in diverse function including cell proliferation, cell death, hematopoietic differentiation, immunity and the same can be described as "multivalent," as one miRNA able to target multiple genes (Wahid, F., *et al.* 2010). Recent development of miRNA studies revealed two distinct types of miRNA according to their origin of generation. Canonical miRNAs are generated from protein-coding transcriptional units produced by RNA polymerase II and noncanonical miRNAs are generated from noncoding regions between genes transcribed by RNA polymerase III (Bracht, J., *et al.* 2004, Lee, Y., *et al.* 2004, Borchert, G. M., *et al.* 2006, Lin, S. L., *et al.* 2006, Tran, T. H., and Montano, M. A. 2017). Most of human miRNA genes are present within the introns of protein coding region (Boyd 2008). The abundance of highly expressed miRNA in a cell may upto 10^4 copies which is much higher than that of a mRNA (Lim *et al.*, 2003, Zeng 2006). The sequence of miRNA may be evolutionarily conserved and the expression patterns are tissue and developmental stage specific (Zeng 2006). Approximately 1000 miRNAs (3per cent of the total numbers of gene) has been predicted to encode from human genome (Bentwich, I., *et al.* 2005, Berezikov, E., *et al.*, 2006). Though all predicted miRNAs are not experimentally verified but it is clear that miRNAs are a large family of RNA molecules that can be expressed at vastly different levels (Zeng 2006).

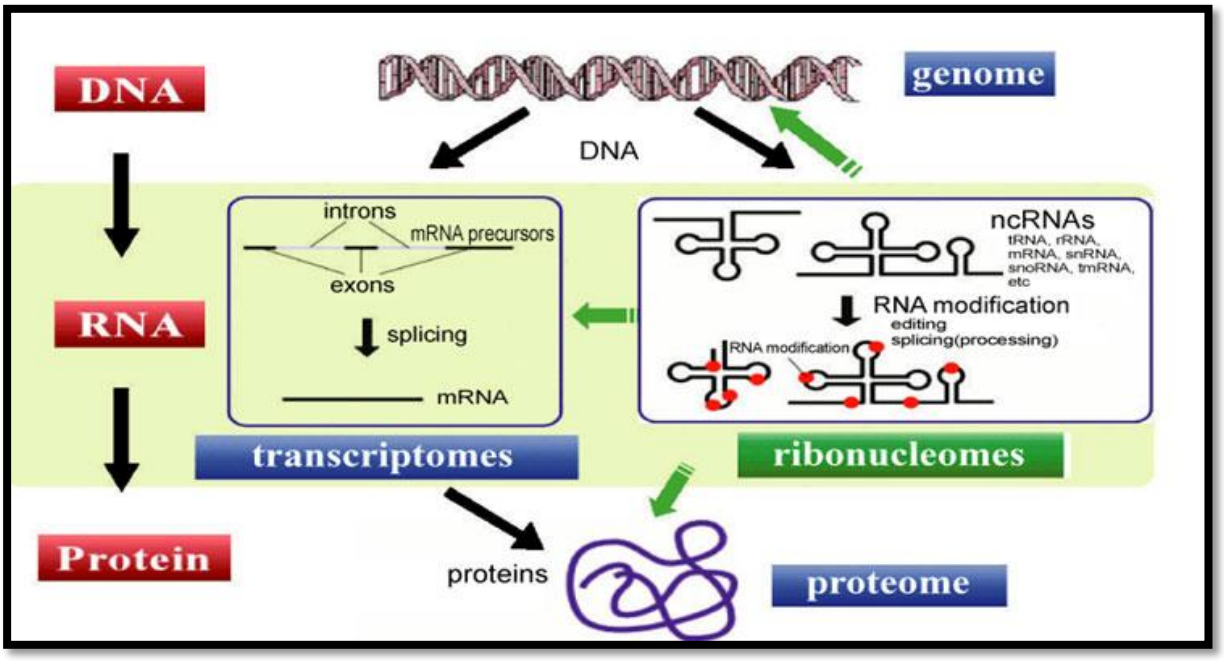


Figure 2.35: Generation of miRNA (In vivo function of NcRNA in living organisms. (Retrieved from:https://www.whatisepigenetics.com/non-coding-rna/#fn-174-2)

2. 17. 1. 1 Biogenesis, Structure and Functions of miRNA:

Majority of miRNAs are located in intergenic regions and found in close proximity to other miRNAs. These clustered miRNAs are expressed as poly-cistronic primary transcripts. miRNA genes can be classified in four different group based on their genomic location. (i) intronic miRNAs in coding transcription units (TUs), (ii) intronic miRNAs in noncoding TU, (iii)exonicmiRNAs in coding TU, and (iv) exonic miRNAs in noncoding TU (Figure 2. 36) (Wahid, F., *et al.* 2010).

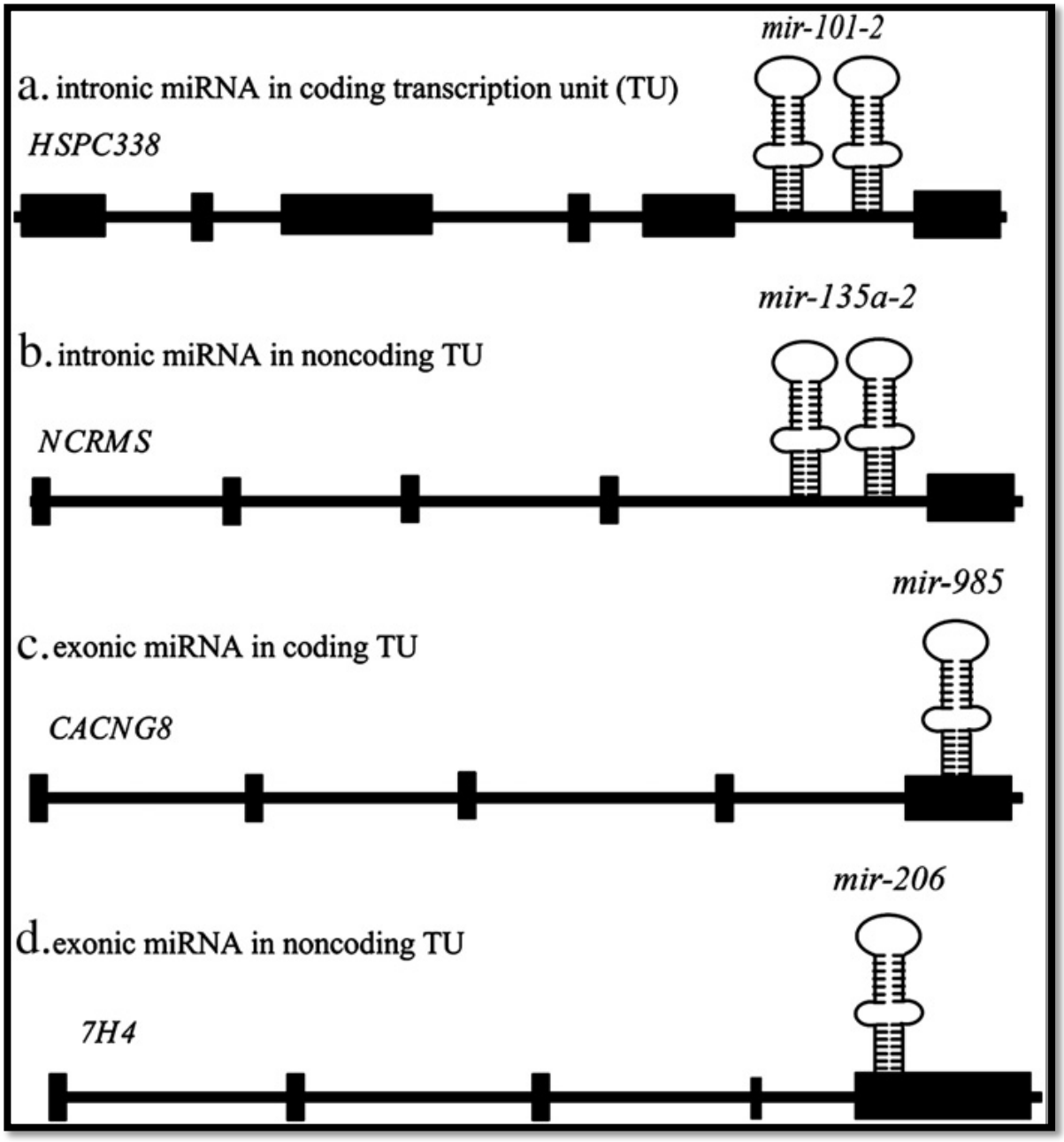


Figure 2.36: Structure of miRNA genes. (a) intronic miRNAs in coding transcription units (TUs), for example, the mir-101-2 cluster (b) Intronic miRNAs in noncoding TU, such as the mir-135a-2 cluster (c) Exonic miRNAs in coding TU (d) Exonic miRNAs in noncoding TU(Wahid, F., *et al.* 2010).

In human single stranded mature miRNAs are derived from longer primary transcripts (pri-miRNA) (Lee, Y., *et al.* 2002, Kim 2005). The pri-miRNA is transcribed by the action of RNA polymerase II, RNA polymerase III also involved in some different species (Figure 2.37)

(Zeng 2006). Various series of enzymatic process has to be undergone to produce a 22 nt mature miRNA from a much longer (>1 kb) pri-miRNA (Bartel 2004, Zeng 2006, Boyd 2008). The first step of pri-miRNA processing is to produce approximately 60nt long hairpin RNA termed as precursor miRNA (pre-miRNA) in nucleus. Synthesis of pre-miRNA from pri-miRNA transcript is an enzymetic process and it involve two enzyme namely, Drosha and DiGeorge syndrome critical region in gene 8 (DGCR8) in humans or Pasha in flies (Figure 2.37) (Cullen 2004, Denli, A. M., *et al.* 2004, Han, M. H., *et al*., 2004, Meister, G., Landthaler, *et al.*, 2004, Lee, Y., *et al.*, 2006, Winter, J., *et al.* 2009, Wahid, F., *et al.* 2010). Drosha the RNaseIII type endonucleases cleaves RNA duplexes about 11 bp away from the ssRNA-stem loop junction and produce pre-miRNA containing a 5' phosphate and a 3'-OH, with usually a 2 nucleotide overhang at the 3' end. Drosha, which requires the DGCR8 as cofactor to help the catalytic acticvity of Drosha subunit to recognize the correct substrates (Wahid, F., *et al.* 2010).

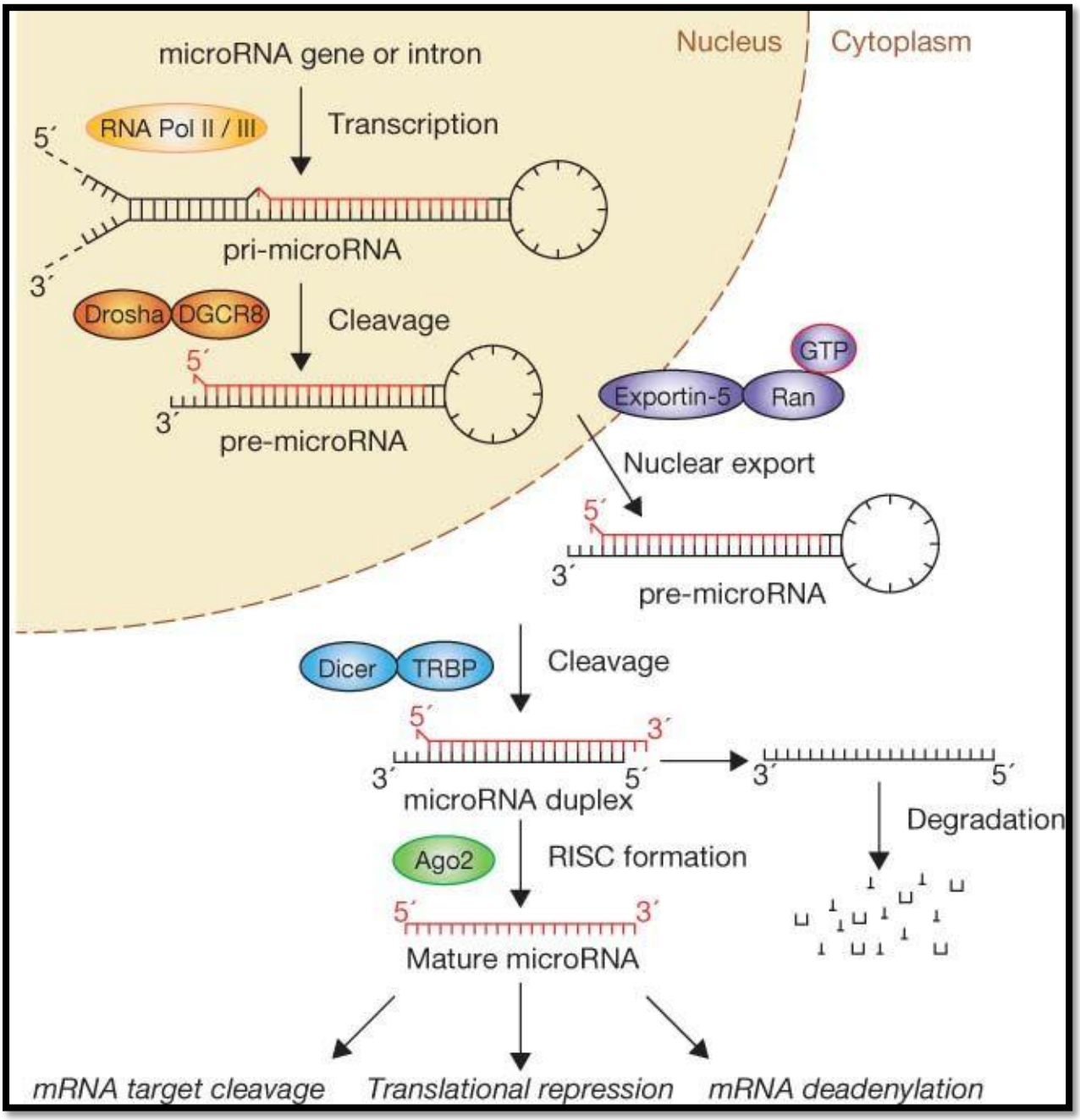


Figure 2.37: Biogenesis and function of miRNA (Winter, J., *et al.* 2009)

The newly formed pre-miRNA, approximately 60nt long are exported from nucleus to cytoplasm by Exportin5 (Exp5) and its Ran-guanosine triphosphate (GTP) cofactor through nuclear pore complex (Zeng, Y., *et al*., 2005, Wahid, F., *et al.* 2010 Bohnsack, M. T., *et al.*, 2004*;* Lund, E., *et al.*, 2004). Exp5, a member of the karyopherin family is specialized at

binding to minihelix containing RNAs with a 3' overhang (Gwizdek, C., *et al.,* 2003). The pre-miRNA complex with Exp5 exports to cytoplasm, where hydrolysis of the GTP results in the release of the pre-miRNA (Wahid, F., *et al.* 2010). The processing of pri-miRNA to pre-miRNA in cytoplasm involved another RNaseIII type enzyme called Dicer. Dicer is a highly conserved protein that exists in almost all eukaryotic organisms with a N-terminal ATPase/Helicase domain, DUF283 (domain of unknown function), PAZ (Piwi/Argonaute/Zwilli) domain, and two tandem RNase III nuclease domains (RNase IIIa and RNase IIIb) located at the C-terminal followed by a dsRNA-binding domain (dsRBD) (MacFarlane, L. A., & R Murphy, P. 2010). Dicer cleaves pre-miRNA at 3' overhang approximately 20 nt away via its PAZ domain (Billy, E., *et al.*, 2001, Grishok, A., *et al*., 2001; Hutvagner, G., *et al*. 2001, Ketting, R. F., *et al.*, 2001; Provost, P., *et al.*, 2002; Han, J., *et al.*, 2004). The product is a miRNA: miRNA* duplex intermediate containing approximately 2 nt 3' overhangs at both ends. The miRNA duplex produced by Dicer yet to process to formation of active single stranded miRNA complex. In the process miRNA duplex enter into the RNA induced silencing complex (RISC) for unwinding, stable association of only one of the two strands with the Argonaute protein 2 (Ago2) effector protein (Meister, G., and Tuschl, T. 2004, Tomari, Y., and Zamore, P. D. 2005). The selection of miRNA strand from the duplex is based on the thermodynamic stability of the two strands (Zeng 2006, Carthew, R. W., and Sontheimer, E. J. 2009, Wahid, F., *et al.* 2010). The miRNA strand (guide strand or miRNA) with relatively lower stability of basepairing at its 5` end is incorporated into RISC, whereas the other miRNA strand (passenger strand or miRNA*) is typically degraded due to endo-nucleolytic enzyme activity of the Ago protein (Zeng 2006, Carthew, R. W., and Sontheimer, E. J. 2009, Wahid, F., *et al.* 2010). An RNA helicase is responsible for unwinding and removal of the unselected strand of the miRNA duplex. The processed miRNA guides the RISC to its target mRNA, for silencing through degradation or translation repression by base-pairing interactions (Figure 2. 38) (Zeng 2006, Carthew, R. W., and Sontheimer, E. J. 2009, Wahid, F., *et al.* 2010, Berezikov 2011).

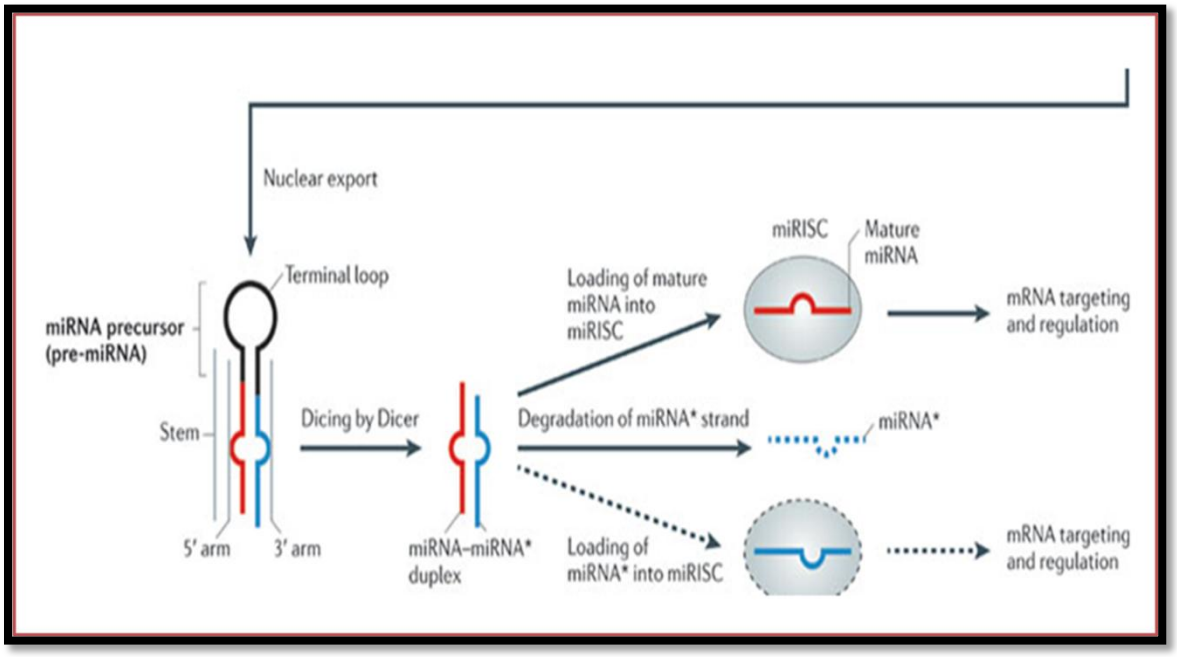


Figure 2.38: Mechanism of action of miRNA (Berezikov 2011)

2.17.1.2 miRNA-Mediated Silencing:

miRNA mediated mRNA silencing is depends on the sequence complementary between mRNA and miRNA. Silencing occurs through the interaction of miRNA to the 3` UTR of mRNA (Figure 2.39) (Eulalio, A., *et al.* 2008). miRNAs guide miRISC to specifically recognize messenger RNA(mRNA) and downregulate gene expression by one of the two posttranscriptional mechanisms: (i) translational repression and (ii) mRNA cleavage. In mammal, key feature of recognition of target mRNA involves Watson-Crick base pairing of seed region of miRNA which is 2-8 nucleotides long (Zeng 2006, Carthew, R. W., and Sontheimer, E. J. 2009, Wahid, F., *et al.* 2010). Silencing of mRNA occurs upon perfect complementarity and catalytic cleavage of the mRNA strand (Carthew, R. W., and Sontheimer, E. J. 2009). Translational repression is the default mechanism of miRNA mediated mRNA silencing both in animals and plants through central mismatches mechanism. The process of mRNA degradation is better understood than translational repression by miRNA. The mRNA degradation requires Ago, GW182, and the cellular decapping and deadenylation machinery (Eulalio, A., *et al.* 2008). RISC complex containing Argonaute protein family (Ago 1-4) along with the GW repeat containing protein GW182 (TNRC6A-C in

humans) is provided the platform to interact with other proteins requires for the mRNA degradation (Wilczynska, A., and Bushell, M. 2015). First, steps of degradation starts with the interaction of poly(A) binding protein (PABP), CCR4-NOT and PAN2-PAN3 deadenylase complexes to induces target deadenylation of thepoly(A) tail. Following the deadenylation of poly (A) tail, DCP1-DCP2 decapping complexes remove the 5` terminal cap and the target mRNA is degraded by Xrn1 5`-3` exonuclease (Fabian, M. R., and Sonenberg, N. 2012, Inada, T., and Makino, S. 2014).

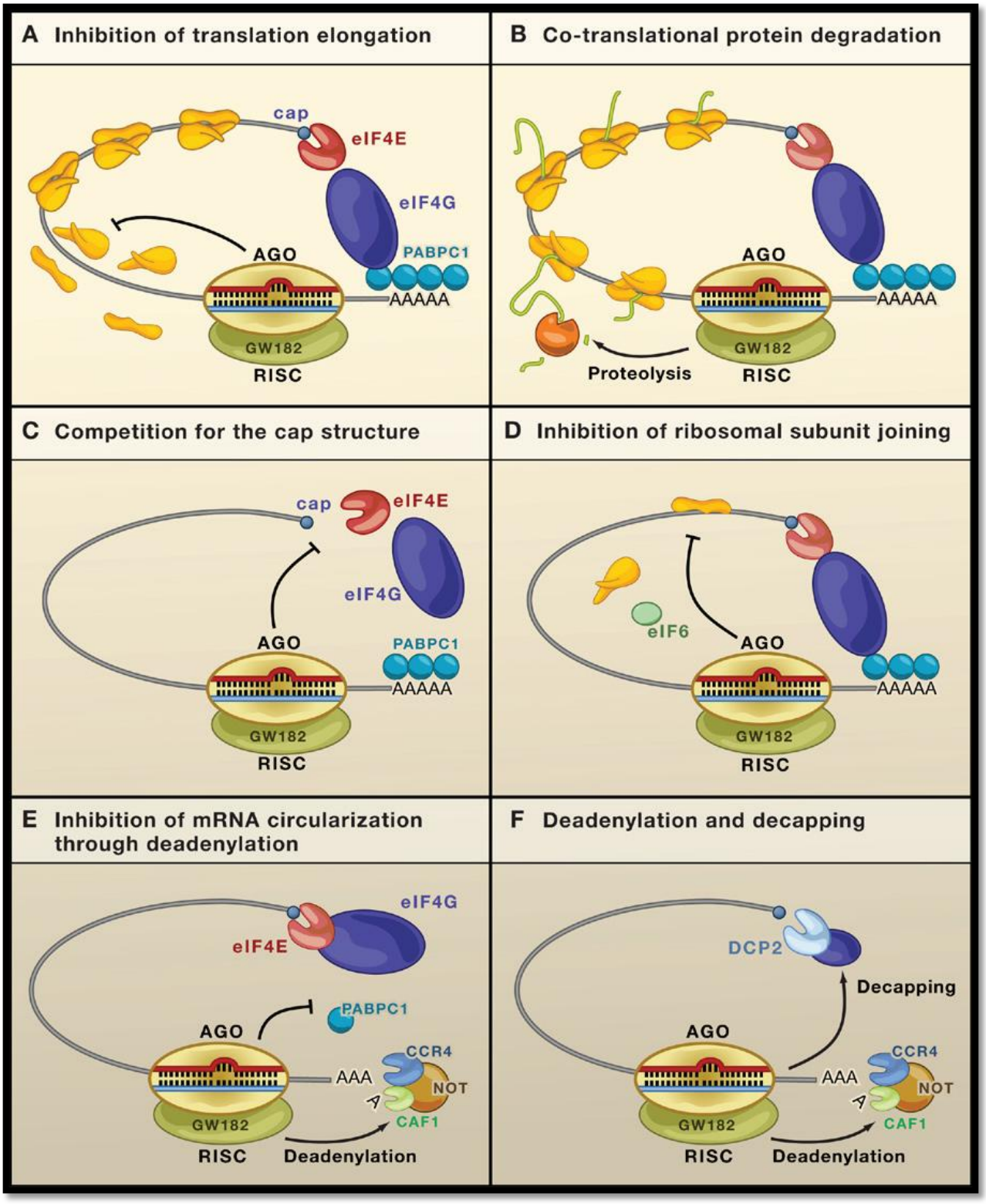


Figure 2.39: Mechanisms of miRNA-mediated Gene Silencing (Eulalio, A., *et al.* 2008)

The mechanism of translational repression by miRNAs remains elusive but studies documented that miRNAs block translation initiation or blockin elongation (Bushati, N., and Cohen, S. M. 2007). Previous studies postulated that, miRISCs compete with eIF4E for binding to the mRNA 5′ cap structure, resulted in failure of the translation initiation process (Mathonnet, G., *et al.* 2007, Thermann, R., and Hentze, M. W. 2007). miRISC induce translation inhibition has been taken place by preventing the mRNA from circularizing. (Behm-Ansmant, I., *et al.* 2006, Wakiyama, M., *et al*. 2007). The prevention of 60S and 40S ribosomal assembly by RISC complex results the inhibition of translation initiation is the another model of translational repression. According to this model, 40S ribosomes are attached to the targeted mRNA, but the 60S ribosomal subunit fails to attach (Chendrimada, T. P., *et al.* 2007, Wang, Y., *et al.* 2008).

miRNA play a distinct role in the gene regulation in mammal and thus a potential novel class of therapeutic targets as well as biomarker (Wahid, F., *et al.* 2010)**.** miRNA mediated gene silencing mainly based on the complementarities between the mRNA and seed sequence and thermodynamic stability to ensure the binding with the 3′ end of miRNA (Wilczynska, A., and Bushell, M. 2015). Theoretically there are hundreds of potential mRNA targets for each miRNA hence the actual principles of target recognition remain obscure (Wilczynska, A., and Bushell, M. 2015). A single mRNA might be regulated by more than one miRNA and a single miRNA can regulates more than one mRNA and it is depend upon the abundence of complementarities between miRNA and mRNA sequence (Wu, S., *et al.* 2010, Wilczynska, A., and Bushell, M. 2015). Development with the hightroughput technology, various methods has been developed to predict the miRNA targets and provided broader view of the nature of this regulation. Extensive target repertoires for most miRNAs have been suggested from the large datasets. The knowledge of miRNA functions proved that, a single miRNA may function predominantly as a regulator of one target mRNA under certain conditions (Wilczynska, A., and Bushell, M. 2015).

The miRNA functions have been studying since last two decades and established the strong association of the same in developmental and pathological processes (Quevillon Huberdeau, M., and Simard, M. J. 2019). It has been estimated that over 60per cent of human protein-coding genes have regulated through pairing to miRNA sequences (Friedman, R. C., *et*

al. 2009). miRNA has multiple mRNA target and vice versa, therefore it is difficult to characterize the action of miRNAs on their mRNA targets. Computational prediction and subsequent experimental validation of these miRNA:mRNA interactions is necessary to address the better functional characterization of miRNAs in biological processes and predict their effects (Riffo-Campos, A., *et al.* 2016, Quevillon Huberdeau, M., and Simard, M. J. 2019) The miRNA sequence, the 3` UTR of mRNA, the Seed region, free energy and accessible energy, miRNA 3` site, conservation status and other target sites are the principal biological fundamentals of the miRNA:mRNA interactions and to develop the algorithms use for sequence-based prediction (Riffo-Campos, A., *et al.* 2016). More robust computational tools have been developed to predict miRNA targets are miRDB, miRBase, TargetScan, miRanda and DIANA microT etc (Riffo-Campos, A., *et al.* 2016). Bioinformatic tools have been developed to predict miRNA:mRNA interactions to identify potential cellular role and functional characterization (Riffo-Campos, A., *et al.* 2016) (Table 2.8). Functional validation is necessary to monitor the biological outcome of computationally predicted miRNA:mRNA interaction.

Table 2.8: The most relevant web-based tools for miRNA sequence-based prediction (Riffo-Campos, A., *et al.* 2016).

Name of database	URL
miRDB	http://mirdb.org/
miRBase	http://www.mirbase.org/
TargetScan	http://www.targetscan.org/
Diana Tools	http://diana.imis.athena-innovation.gr/DianaTools/index.php
miRanda	http://www.microrna.org/microrna/getGeneForm.do
PITA	http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html
PicTar	http://pictar.mdc-berlin.de/
RNA22	https://cm.jefferson.edu/rna22/

RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/
miRTar	mirtar.mbc.nctu.edu.tw/
TargetS	http://liubioinfolab.org/targetS/mirna.html
miRU	http://plantgrn.noble.org/psRNATarget/
EIMMo	http://www.mirz.unibas.ch/

2.17.1.3 Location of Mature miRNA:

miRNA basically found in extracellular compartment. Extracellular or circulating miRNAs found in biological fluids, such as plasma and serum (Chen, X., *et al.* 2008, Arroyo, J. D., *et al.* 2011), cerebrospinal fluid (Cogswell, J. P., *et al.* 2008), saliva (Gallo, A., *et al.* 2012), breast milk (Zhou, Q., *et al.* 2012), urine, tears, colostrum, peritoneal fluid, bronchial lavage, seminal fluid (Weber, J. A., *et al.* 2010), and ovarian follicular fluid (da Silveira, J. C., *et al.* 2012). Extracellular miRNAs are highly stable and also resist from degradation at room temperature, extreme deleterious conditions such as boiling, multiple freeze-thaw cycles and wide range of pH (Chen, X., *et al.* 2008, Mitchell, P. S., *et al.* 2008) compare to the cellular RNA species. Group of extracellular circulation miRNA are found in vesicles such as exosomes, microvesicles, and apoptotic bodies (Gallo, A., *et al.* 2012) while the other is associated with proteins, especially AGO2 (Turchinovich, A., *et al.*2011, Gallo, A., *et al.* 2012).

2.17.1.4 Association of miRNA with Disease:

miRNA mediated regulation of gene expression represents a novel epigenetic mechanism which may alter the homeostatic process and pathological condition within the cells (Riffo-Campos, A., *et al.* 2016, Shi, C., *et al.* 2016). Dysfunction and poor interaction of miRNA has been associated with large number of diseases. Sekar *et al* 2016 described the role of miR-21 in different types of diabetes, the miRNAs of the hsa-let-7 family and others are

associated with obesity and related metabolic diseases are documented (Shi, C., *et al.* 2016). Recent studies suggested the role of miRNA in the pathogenesis or association of arthritic diseases, kidney disease, cardiovascular diseases, etc (Denby, L., and Baker, A. H. 2016, Hackfort, B. T., and Mishra, P. K. 2016). The role of miRNA in cancer has been extensively studied over the recent years and regarded as either tumor suppressors or inducers. Oncogenic miRNAs collectively known as "oncomiRs" which act directly on mRNAs of pro-apoptotic or anti-proliferative pathways (Schickel, R., *et al*. 2008, Voorhoeve 2010). The role of miRNA in diabetic microvascular complication such as retinopathy, nephropathy, wound healing and myocardial injury and listed various miRNA as involved in insulin relevant tissue has been reviewed in earlier studies (Table 2.9) (Zhang, Y., *et a*l. 2017). Previous studies postulates that, miRNA may offer new targets for early detection and therapeutic intervention of various diseases (Schickel, R., et al. 2008, Voorhoeve 2010, Zhang, Y., *et a*l. 2017).

Table 2.9: List of miRNAs involved in Vascular complications (Zhang, Y., *et a*l. 2017).

miRNA	Target	Function
Macrovascular complications		
let-7a/b		Prevents atherosclerosis
miR-1		Prevents endothelial dysfunction
miR-125b	Suv39h1	Promotes the inflammatory reaction
miR-126	Spred-1	Promotes EPC proliferation and migration
miR-143/145		Increases VSMC cell area and reduces cell proliferation
miR-144-3p	ABCA1	Promotes THP-1 macrophage differentiation into foam cells
miR-146a	IRAK1, IL-6	Alters insulin sensitivity
miR-155	p65	Contributes to the progression of atherosclerosis Promotes macrophage-derived foam cell formation
miR-181b	Importin-a3 PHLPP2 Card10	Confers anti-inflammatory effects in the macro- and microvasculature by regulating NF-kB and insulin signalling
miR-195	SIRT1	Increases expression of FN
miR-200	Zeb1	Induces MCP-1 and COX-2 expression and promotes

		monocyte binding
miR-206		Enhances cholesterol efflux
miR-21	SPI	Stimulates VSMC proliferation and reduces VSMC differentiation marker gene expression
miR-24		Reduces intraplaque macrophage proliferation
miR-492	resistin	Reduces insulin resistance and endothelial dysfunction
miR-9	ACAT1	Decreases THP-1 macrophage-derived foam cell formation
miR-92a	MKK4 and JNK	Inhibits oxidative stress–induced VSMC apoptosis
Microvascular complications		
miR-130a	Runx3	Maintains the normal EPC function
miR-135a	TRPC1	Improves renal function and ameliorates progression
miR-146	CARD10 HuR	Inhibits NF-kB activation
miR-146a	IRAK1	Reduces IL-1b–induced ICAM-1 expression
miR-146a	FN	Inhibits thrombosis
miR-146a	IRAK1 TRAF6	Promotes the complications of diabetic sensory neuropathy
miR-195		Prevents SIRT1-mediated tissue damage
miR-200b	VEGF	Prevents the glucose-induced increased vascular permeability and angiogenesis
miR-21	DAXX	Prevents endothelial apoptosis
miR-29b		Abolishes TGF-b/Smad3 pathway activation
miR-29c	Spry1	Inhibition of miR-29c reduces mesangial matrix accumulation and albuminuria
miR-320		Inhibits EC migration and tube formation
miR-34a		Alleviates glomerular hypertrophy
miR-93	Msk2/VEGF-A	MiR-93 overexpression reduces features of diabetic nephropahy

In a breakthrough study it is claimed that, “mRX34” a liposomal miR-34 mimic is the first-in-human trial of microRNA cancer therapy in patients with advance solid tumor. Down-regulation of targeted oncogenes, miRNA levels and other potential pharmacodynamic markers were being evaluated through qRT-PCR and next-generation sequencing (NGS) analyses from white blood cells, tumor and tissue biopsies of patiens (Beg, M. S., *et al.* 2016). This tumor suppressor miRNA is capable to down-regulate expression of >30 oncogenes (eg, MET, MEK1, PDGFR-α, CDK4/6, BCL2, WNT 1/3, NOTCH1, CD44) as well as genes involved in tumor immune evasion (eg, PD-L1, DGKζ) (Beg, M. S., *et al.* 2016). In-vivo and in-vitro studies are going on to overcome the obstacle in RNA-based therapeutics and will soon enter into the clinic as next generation drugs. miRNA based therapeutics definitely has the potential to contribute significantly to the future of medicine as targeted therapy. miRNAs are the best-studied among small noncoding RNAs and there is an increasing awareness towards their role in disease pathophysiology, uses as biomarkers and as potential therapeutics (Voskarides, K., and Felekkis, K. 2015). Transmitting role of miRNA for m-RNA regulation may yield novel insights into molecular mechanisms of various complex disease pathogenesis.

2. 18: HUMAN GUT MICROBIOME

Human Gut microbiome (GM) composition is recently evolved as a promising area of research as it consists of 200 prevalent bacteria and 1000 uncommon species approximately (Ley, R. E., *et al.* 2008). Several factors including diet, host immune response genetic background affect the composition of GM (Mandal, R. S., *et al.* 2015). GM architect of an organism have a large impact on biological homeostasis of the same by mutual benefit of symbiosis and often pathogenic (Backhed, F., *et al.* 2005, Dubey, A. K., *et al.* 2018). Pioneering studies from early 2000s and recent advancements of technology insists researcher to investigate the interaction of GM with host physiology and biochemistry in details. Recent development of next generation sequencing technology using 16S rRNA gene or whole genome sequencing (WGS), being used to study the descriptive metagenomics and functional metagenomics (Mandal, R. S., *et al.* 2015, NIH Human Microbiome Portfolio Analysis Team

lita. proctor@ nih. gov 2019). Descriptive metagenomics reveals the structure, relative abundance and variation of microbiome based on different physiological and environmental conditions (Garmendia, L., *et al*. 2012, Sun, F., and Xia, L. C. 2015). Functional metagenomics can predict the host-microbes and microbe-microbe interaction associated with certain condition (Chistoserdova 2009, Faust, K., and Raes, J. 2012). Functional as well as descriptive GM studies in human and other different host revealed that gut microbiota play beneficial role in metabolism and immunity of the host (Mandal, R. S., *et al.* 2015). Metagenomic sequencing revealed the inter-individual conservation of bacteria constitutethe overwhelming majority of gut microbiota at the phylum level (Fernandes, J., *et al.* 2014). *Bacteroides* and *Firmicutes* are the two major phyla represents in more than 90 per cent of healthy individuals (Fernandes, J., *et al.* 2014).

2. 18.1: Human Gut Microbiome and Disease

Gut microbiota play important role on an organism's health by providing nutrients, digesting complex polysaccharides, metabolizing drugs and environmental toxins and harvesting energy (Jia, W., *et al.* 2008, Shanahan 2009 and 2012, Clemente, J. C., *et al*. 2012, Blumberg, R., and Powrie, F. 2012). Microbial dysbiosis interms of diversity and abundance of individual phylotype may occurs due to certain conditions including dietary and environmental alteration (Clemente, J. C., *et al*. 2012, Blumberg, R., and Powrie, F. 2012). GM dysbiosis has been associated with various diseases such as inflammatory inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), non-alcoholic hepatitis, obesity, metabolic diseases, neurological disorders like Alzheimer's disease, asthma, cancer and Crohn's disease (Wang, T., *et al*. 2012, Karlsson, F. H., *et al*. 2012, Chen, X., *et al*. 2013, Greenhill 2013, Azad, M. B., *et al*. 2013, Schippa, S., and Conte, M. 2014, Kennedy, P. J., *et al*. 2014, Moreno-Indias, I., *et al*. 2014, Z Alam, M., *et al*. 2014). The increased abundance of Bacterial species that were reported in previous studies is described in Table 2.10 (Mandal, R. S., *et al.* 2015).

Table 2.10: Highly-abundant bacterial species in different disease conditions (Mandal, R. S., *et al.* 2015).

Disease	**Name of prevalent bacteria**
Symptomatic atherosclerosis	*Escherichia coli, Eubacterium rectal, Eubacteriumsiraeum Faecalibacteriumprausnitzii, Ruminococcusbromii, Ruminococcus sp. 5_1_39BFAA*
Type 2 diabetes	*Akkermansiamuciniphila, Bacteroides intestinalis Bacteroides sp. 20_3, Clostridium bolteae, Clostridium ramosum Clostridium sp. HGF2, Clostridium symbiosum, Colstridiumhathewayi, Desulfovibrio sp. 3_1_syn3, Eggerthellalenta Escherichia coli*
Obesity/IBD/Crohn's disease.	*Acidimicrobidaeellin 7143, Actinobacterium GWS-BW-H99 Actinomyces oxydans, Bacillus licheniformis, Drinking water bacterium Y7, Gamma proteobacterium DD103, Nocardioides sp. NS/27, Novosphingobium sp. K39, Pseudomonas straminea Sphingomonas sp. AO1*
Colorectal cancer	*Acinetobacter johnsonii, Anaerococcusmurdochii, Bacteroides fragilis, Bacteroides vulgates, Butyrate-producing bacterium A2-166, Dialisterpneumosintes, Enterococcus faecalis Fusobacterium nucleatum E9_12, Fusobacterium periodonticum Gemellamorbillorum, Lachnospirapectinoschiza, Parvimonasmicra ATCC 33270, Peptostreptococcusstomatis, Shigella sonnei*

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 health (Parekh, P. J., *et al*. 2014). Various microbial metabolites such as folate, indoles, secondary bile acids, trimethylamine-N-oxide (TMAO), neurotransmitters (eg, serotonin, gamma amino butyric acid), and eventually short-chain fatty acids (SCFAs) influence host metabolism by binding specific host membrane or receptors (Cani 2018). SCFAs are recognising by G-protein-coupled receptors and stimulates to secretion of intestinal peptides which involved in glucose metabolism or food intake (Brown, A. J., *et al.* 2003, Kimura, I., *et al.* 2014, McKenzie, C., *et al.* 2017). Microbial metabolites such as propionate act as an immune regulator by regulating immune cells to produce antimicrobial factors (Cani 2018). Various in-vitro or in-vivo studies postulates that, a distinct microbial metabolite may play different roles in host metabolism, ranging from the regulation of glucose levels to immunomodulatory effects (Cani 2018).

2. 18. 2: Human Gut Microbiome and Host Immunity

GM and host immune system are mutually maintain the host's homeostasis. Normal gut flora regarded as Commensal microorganisms are required to maturation of the immune system (Lazar, V., *et al*. 2018). Pattern recognition receptors (PPRs) such as TLRs and NLRs of innate immune system are responsible for recognition and monitoring of microbes. TLRs and NLRs together recognize pathogen associated molecular pattern (PAMPs) derived from microorganisms such as lipopolysaccharides, peptidoglycans, lipoteichoic acid, flagellin and muramyldipeptide (Lazar, V., *et al*. 2018, Cani 2018). Inspite of close proximity of gut microbiota and innate immune system, perfect symbiosis are maintained until any dysbiosis of gut microbiota arises (Figure 2. 40) (Cani 2018). Functional studies proposed that, dysbiosis of gut microbiota leads to develop low grade inflammation and further associated with pathogenesis of disease (Mezouar, S., *et al.* 2018, Lazar, V., *et al*. 2018, Cani 2018). Gram negative bacteria constitute lipopolysaccharides (LPS) which are the key factors triggering low-grade inflammation and insulin resistance as a result of interaction between gut microbes and TLR-4 (Cani, P. D., et al. 2007). Mice model studies revealed the causal role of other PAMPs such as peptidoglycans or flagellin derived from gram negative bacteria in metabolic pathways (Vijay-Kumar, M., *et al*. 2010, Chassaing, B., *et al*. 2014, Denou, E., *et al*. 2015). Gut microbes such as *Bacteroides fragilis, Bacteroides vulgatus, Escherichia coli, Citrobacter*

rodentium, Citrobacter freundii were found to be linked with cancer (Mbulaiteye, S. M., *et al*. 2009). GM dysbiosis with reduced abundance of certain bacteria such as *Sutterella (Proteobacteria)* and *Prevotella (Bacteroidetes)* were found in multiple sclerosis (MS) patients but restored after treatment (Colpitts, S. L., and Kasper, L. H. (2017). Lower prevalence of *Akkermansia, Faecalibacterium* and *Bifidobacterium* along with higher abundance of fungi such as *Rhodotorula* and *Candida* in neonates which may involve in modulation of T-cell differentiation further lead to allergy susceptibility was reported in previous study (Van Den Elsen, L. W., *et al*. 2017). Accumulating data suggested that gut barrier is controlled by fine maintenance of cross talk between gut microbes and host immune system (Cani 2018).

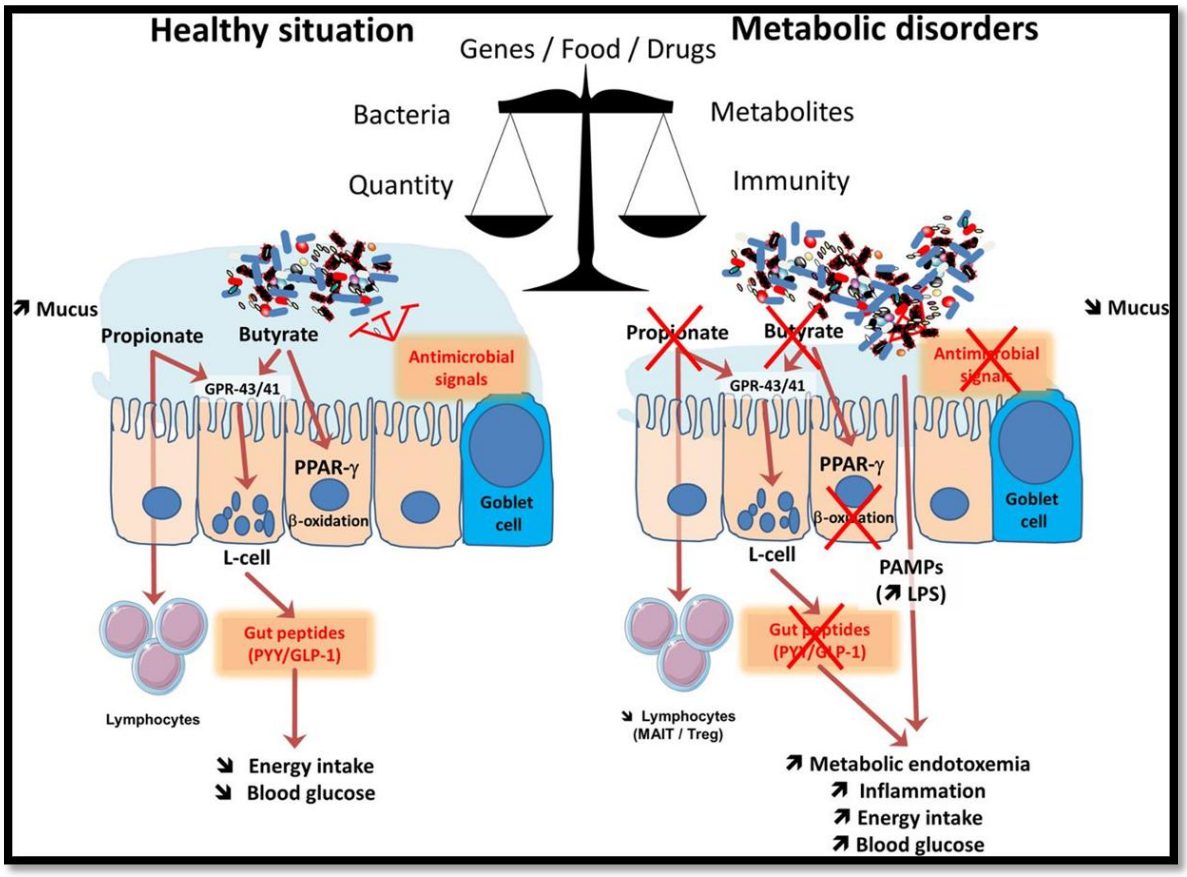


Figure 2.40: Figure represents the crosstalk between microbes and host (Cani 2018).

Dysbiosis of microbe–microbe or host –microbe cross-talk may influence the disease outcome. Identification of microbial colonization profiles and characterization associated with health and disease state still remains a challenge due its complexity and inter-individual

differences (Lazar, V., *et al*. 2018). GM interacts with the immune system and alteration in cross talk associated with various diseases including inflammatory and autoimmune diseases, allergies, cancer, and metabolic disorders. This phenomenon could lead to develop potential biomerkers for screening or therapeutics by altering of GM architecture as probiotic treatments are exercise in various diseases (Cani, P. D., *et al*. 2008, Larsen, N. *et al.* 2010, Lazar, V., *et al*. 2018).