Novel clinico-molecular insights into the role of bisphenol-A (BPA) in the etiology of diabetes

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Novel clinico-molecular insights into the role of bisphenol-A (BPA) in the etiology of diabetes

by

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Abbreviations

2,4-BPS	-	2,4-Bisphenol S
AR	-	Androgen Receptor
AHR	-	Aryl Hydrogen Receptor
AO	-	Acridine Orange
β-GAL	-	β-galactosidase
BDP	-	Bisphenol A bis (diphenyl phosphate)
BMI	-	Body Mass Index
BPA	-	Bisphenol A
BPAF	-	4,4'-(hexafluoroisopropylidene) diphenol
BPAP	-	4,4'-(1-phenylethylidene)bisphenol
BPA-DGE	-	Bisphenol A diglycidyl ether
BPA-G	-	BPA-glucuronide
BPA-S	-	BPA sulphate
BPB	-	2,2-bis(4-hydroxyphenyl)butane
BPBP	-	4,4′-(Diphenylmethylene) diphenol
BPC	-	Bis(4-hydroxyphenyl)-2,2-dichloroethylene
BPE	-	1,1-Bis(4-hydroxyphenyl)ethane
BPF	-	4,4'-dihydroxydiphenylmethane
BPFL	-	4,4′-(9-Fluorenylidene) diphenol
BPG	-	2,2-Bis(4-hydroxy-3-isopropylphenyl) propan
BPM	-	4,4′-(1,3-Phenylenediisopropylidene) bisphenol
BPP	-	4,4'-(1,4-phenylenediisopropylidene) bisphenol
BPPH	-	2,2-Bis(2-hydroxy-5-biphenylyl) propane
BP-TMC	-	4,4'-(3,3,5-trimethyl cyclohexane-1,1-diyl) diphenol
BPS	-	Bisphenol S
BPZ	-	4,4'-cyclohexylidenebisphenol

BSA	-	Bovine serum albumin
BTUM	-	4,4'-bis (Ncarbamoyl-4- methylbenzene sulfomide) diphenylmethane
C/EBPβ	-	CCAAT/enhancerbinding protein-β
CDKs	-	Cyclin-Dependent Kinases
d-8	-	4- Hydroxyphenyl 4-isoprooxy phenylsulfone
d-90	-	Phenol, 4,4'- sulfonylbis-, polymer with 1,1'-oxybis[2- chloroethane]
DCR2	-	Decoy receptor 2
DD-70	-	1,7-bis(4- Hydroxyphenylth io)-3,5- dioxaheptane
DDR	-	DNA-damage response
DDT	-	Dichlorodiphenyltrichloroethane
DEC1	-	Differentiated embryo chondrocyte expressed gene 1
DEHP	-	Di(2-ethylhexyl) phthalate
DES	-	Diethylstilbestrol
DM	-	Diabetes Mellitus
EDCs	-	Endocrine Disruptor Chemicals
ER	-	Endoplasmic reticulum
ERR	-	Estrogen related receptors
ERs	-	Estrogen receptors
GADA	-	Glutamic acid decarboxylase antibodies
Gck	-	Glucokinase
GDM	-	Gestational diabetes mellitus
GPR30	-	G-protein coupled receptor 30
HbA1c	-	Glycated hemoglobin A1c
HBCDD	-	Hexabromocyclododecane
HDL	-	High-density Lipoprotein
HMEC	-	Human mammary epithelial cells
HOMA-IR	-	Homeostasis assessment model-Insulin Resistance

IDF	-	International Diabetes Federation
IGF1	-	Insulin-like growth factor 1
IRS	-	Insulin receptor substrate
IVF	-	*In vitro* fertilization
LADA	-	Latent autoimmune diabetes in adults
LC -96	-	Light cycler 96
LDL	-	Low-density lipoprotein
L-Pk	-	Pyruvate kinase
MBHA	-	Methyl bis (4-hydroxyphenyl) acetate
MetS	-	Metabolic syndrome
MIDD	-	Maternally Inherited Diabetes and Deafness
MIDY	-	Mutant INS-Gene Induced Diabetes of Youth
MO	-	Morpholino
MODY	-	Maturity-onset diabetes of the young
MRC	-	mitochondrial respiratory chain
NDM	-	Neonatal diabetes mellitus
NEFA	-	Non-esterified fatty acids
NF-κB	-	Nuclear factor-κB
NGT	-	Normal glucose tolerance
NHANES	-	National Health and Nutrition Examination Survey
NOAEL	-	No-observed-adverse-effect-level
PAI1	-	Plasminogen activator inhibitor 1
PBDEs	-	Polybrominated diphenyl ethers
PBMC	-	Peripheral blood mononuclear cells
PCBs	-	Polychlorinated biphenyls
PCOS	-	Polycystic Ovarian Syndrome
PFOA	-	Perfluorooctanoic acid

PFOS	-	Perfluorooctanesulfonate
PTU	-	Phenylthiourea
POPs	-	Persistent organic pollutants
PVDF	-	Polyvinylidene fluoride
Rb1	-	Retinoblastoma-1
RITA	-	Reactivation of p53 and induction of tumour cell apoptosis
SAHF	-	Senescence-associated heterochromatin foci
SASP	-	Senescence-associated secretory phenotype
SA-β-GAL	-	Senescence-associated β-galactosidase
SIPS	-	Stress induced pre-mature senescence
SPSS	-	Statistical Package for Social Science
T1DM	-	Type 1 diabetes
T2DM	-	Type 2 diabetes Mellitus
T/S	-	Telomere (T) over single gene copy number
TBT	-	Tributyltin
TGSA	-	4,4′-Sulfonylbis[2-(2-propen-1-yl)phenol
TL	-	Telomere Length
TMBPA	-	Tetramethyl Bisphenol A
UGTs	-	UDP-glucuronosyltransferases
UU	-	Urea urethane compound
VLDL	-	Very low density Lipoprotein
WHO	-	World Health Organization

Chapter 1

Introduction and Review of Literature

1.1 Diabetes

Diabetes Mellitus (DM) was first recognized around 3000 years ago as a disease by the ancient Indians and Egyptians, illustrating some clinical characteristics that are now seen in diabetes (Frank, 1957). The word diabetes is adopted from the Greek word, '*siphon*' meaning to pass through coined by Araetus of Cappodocia (81-133AD). Later, the Latin word 'Mellitus', meaning '*honey sweet*' was introduced by Thomas Willis in 1675. By 1776, excess sugar in blood and urine was confirmed for the first time by Dobson of Great Britain (Ahmed, 2002). With the development of science and technology, the knowledge of diabetes and its etiology and pathogenesis became widespread.

According to the World Health Organization (WHO), "*Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads over time to serious damage to the heart, blood vessels, eyes, kidneys, and nerves.*"
For our body to function properly, cells require constant supply of energy which is derived from the D-glucose obtained from food with the help of a hormone called insulin (Wang & Lee, 2015). Insulin is a peptide hormone, synthesized by β-cells of islets of Langerhans present in pancreas (Bosco et al., 2010). The size of an insulin molecule is 5.8 kDa approximately comprising 51 amino acids and contains two polypeptide chains (A & B) which are inter-linked by two disulphide bonds (Mo et al., 2014). Cells cannot absorb glucose from blood directly. For this purpose, insulin is synthesized and released from the pancreatic β-cells that enables insulin-dependent glucose uptake in our body and hence lowers the blood glucose levels and maintains glucose homeostasis (Röder et al., 2016). In those people affected by diabetes, pancreas produce insufficient amount of insulin and/or have diminished tissue responses to insulin action (American Diabetes Association, 2014; Arnold et al., 2018).

1.1.1 Types of diabetes

Diabetes Mellitus is classified into two major types: type 1 diabetes and type 2 diabetes (Canivell & Gomis, 2014). Type 1 diabetes (T1DM) is an auto-immune disorder that is characterized by the absolute deficiency of insulin due to the destruction of insulin secreting β-cells (Wu et al., 2015). Type 2 diabetes (T2DM) is highly heterogeneous and is characterized by insulin resistance, which precedes the onset of the disease condition. Insulin resistance followed by progressive decline in insulin secretion is the hallmark of T2DM pathogenesis (Tangvarasittichai, 2015). About 75-85% of patients with diabetes are classified

as affected by T2DM (Ahlqvist et al., 2018). In addition to T1DM and T2DM there are other forms of diabetes: Gestational diabetes mellitus (GDM), is defined as glucose intolerance that occurs during second or third trimester of pregnancy (Donazar-Ezcurra et al., 2017); Latent autoimmune diabetes in adults (LADA), a hybrid form of diabetes is defined by the presence of autoimmune marker glutamic acid decarboxylase antibodies (GADA) similar to T1DM patients along with insulin resistance similar to type 2 diabetes (Löfvenborg et al., 2016).

In addition to these, other atypical monogenic forms of diabetes are as follows: 1) Maturity-onset diabetes of the young (MODY), is an autosomal dominant inherited monogenic form of diabetes (Majidi et al., 2018). Currently, 13 types of MODY are classified (Brunerova et al., 2018). 2) Neonatal diabetes mellitus (NDM) is a monogenic form of diabetes that is defined as diabetes mellitus with onset before six months of age (Thomas & Philipson 2015). 3) Maternally Inherited Diabetes and Deafness (MIDD) is a monogenic form of diabetes caused due to mitochondrial disorder that is characterized by the onset of sensorineural hearing loss and diabetes (Naing et al., 2014). 4) Mutant INS-Gene Induced Diabetes of Youth (MIDY) (Liu et al., 2010) results from the expression of a mis-folded mutant pro-insulin protein in pancreatic β-cells.

1.1.2 Prevalence of Type 2 diabetes and the Indian context

Type 2 diabetes has now become a major public concern and its increasing prevalence has drawn a major public outcry. It is considered to be one of the most commonly diagnosed chronic conditions worldwide following hypertension, arthritis and dyslipidemia (Hsiao et al., 2010). As per the International Diabetes Federation Atlas 2017 report, around 425 million people are diagnosed with diabetes and it is expected to reach 693 million by 2045. A total of approximately 72.9 million cases of diabetes were reported in India. India ranks second in the world in terms of harbouring a huge population of people with diabetes (IDF, 2017). In an ICMR-INDIAB study by Anjana et al. (2017), the cumulative data from 15 Indian states suggests the prevalence of diabetes in India to be 7.3% and the prevalence of pre-diabetes to be 10.3% of the population.

1.1.3 Determining criteria for type 2 diabetes

According to the International Diabetes Federation (IDF), diabetes is determined by the following diagnostic criteria: Fasting plasma glucose concentration >=7mmol/L (126 mg/dL) or >=11.1mmol/L (200mg/dL) in 2 hours following a 75g of glucose load.

Glycated hemoglobin A1c (HbA1c) is a standard marker for measuring long term glucose control (Lee, 2015). HbA1c is a measure of an individual's average blood glucose levels of previous two or three months (Sherwani et al., 2016). Hemoglobin is glycated at one or both valines in N-terminal of beta chains (Nasir et al., 2010). HbA1c is a measure of beta-N-1-deoxy fructosyl component of haemoglobin (Miedema, 2005). IDF categorizes diabetes as HbA1c ≥ 48 mmol/mol (equivalent to 6.5%).

Apart from HbA1c, other alternative biomarkers that are in use today for glycemic control monitoring are Fructosamine, Glycated Albumin, 1,5-Anhydroglucitol and continuous glucose monitoring (Lee, 2015).

1.1.4 Comorbidities of Insulin resistance

Diabetes is a complex heterogeneous disease characterized by insulin resistance (Karalliedde & Gnudi, 2016). In addition to insulin resistance, the other comorbidities found in T2DM patients are hyperglycaemia, inflammation, dyslipidaemia, hypertension, atherosclerosis, microvascular complications and renal failure (Arnold et al., 2018).

1.1.5 Risk factors for type 2 diabetes

Non-modifiable risk factors

Age

Age is a primary risk factor and thus risk of occurrence of T2DM increases with age (Zou et al., 2016). However currently, T2DM is also affecting young adults and children (Wu et al., 2014).

Race

People of certain races are more likely to develop type 2 diabetes than others. For example, Asians are more susceptible to metabolic diseases at lower BMI when compared to other population across the globe (Wen et al., 2009). Body fat composition is higher, and lesser

muscle mass is seen in Asian-Indians. These are characteristics of the Asian-Indian phenotype.

Genetic factors

Inherited genetic susceptibility remains an independent major risk factor for T2DM (Scott et al., 2013). Subjects with family history of diabetes i.e., either or both parents or siblings affected with the disease, have more chances of acquiring the disease (Murea et al., 2012).

Modifiable risk factors

Overweight/Obesity

There are many modifiable risk factors for T2DM. Being overweight or obese is associated with an increased risk for developing insulin resistance and type 2 diabetes (Grover et al., 2015; Hsu et al., 2015). In obese individuals, adipose tissue undergoes extensive hypertrophy and the expansion of the visceral adipose tissue is a strong predictor of the development of insulin resistance (Gustafson et al., 2015). In addition to increased fat accumulation, adipose tissue releases excessive amount of pro-inflammatory cytokines (Pirola & Ferraz., 2017), non-esterified fatty acids (NEFA) (Nemati et al., 2017), and other factors that are involved in the genesis of insulin resistance followed by subsequent decline in β-cell function that results in the development of type 2 diabetes (Kahn, 2003). According to the WHO, Body Mass Index (BMI, calculated as weight in kilograms divided by height in square meter (kg/m^2)) criteria that determines global obesity is: BMI greater than or equal to 25 is considered to be overweight and BMI greater than or equal to 30 is considered to be obese. The BMI range for Asian population differs from that of the world population (Gujral et al., 2013). For Asian-Indian population overweight is defined as a BMI of 23 or higher and BMI of 27 or higher falls under obesity (Misra, 2015). Waist Circumference and Waist Hip Ratio are some of the important parameters associated with diabetes in Asians (Lear et al., 2007).

Sedentary life style

Physical activity helps in utilizing glucose as an energy source more efficiently (Stanford & Goodyear., 2014). Endurance exercise training has been shown to enhance the GLUT-4 expression in skeletal muscles (Higashida et al., 2011; Richter & Hargreaves, 2013). This increase in GLUT-4 expression increases the glucose uptake in skeletal muscle (Hussey et al., 2012; Richter & Hargreaves, 2013).

Carbohydrates are the preferred source of energy during intense or moderate exercise. When the high intake of carbohydrates is not utilized properly due to physical inactivity, it can lead to metabolic diseases like diabetes (Mul et al., 2015). A study by Anjana et al. (2014) in the Indian population reported about the prevalence of physical inactivity among large portion of the population. This may be one of the reasons for the high prevalence of metabolic disorders in the Asian-Indian population (Wells et al., 2016). Association of sedentary behaviour with the increased odds of occurrence of T2DM was well documented (Hayes et al., 2002; Van der Berg et al., 2016; Engelen et al., 2017). A study by Balkau et al. (2008) which included 801 healthy individuals, demonstrated that the sedentary time is inversely associated with insulin sensitivity. A meta-analysis by Wilmot et al. (2012) examined 10 studies and suggested that larger duration of sedentary behaviour has 112% greater relative risk for type 2 diabetes.

Gestational diabetes

Several follow up studies have shown that the occurrence of gestational diabetes mellitus (GDM) during pregnancy increases the risk of developing T2DM in both mother and child later in life (Krishnaveni et al., 2007; Herath et al., 2017). Risk of developing diabetes was reported to be 9.6 fold higher in women who had history of GDM (Lee et al., 2007). Metabolic alterations such as increase in insulin secretion, increased postprandial free fatty acids (FFA), increased hepatic glucose production are witnessed in the women affected by GDM which later on results in insulin resistance leading to T2DM (Barbour et al., 2007). Hormonal imbalance during the GDM phase in women may also be responsible for development of T2DM. For example, 30-fold increase in human placental lactogen (hPL) is seen throughout pregnancy of women affected by GDM that induces insulin secretion from the pancreas (Brelje et al., 1993). Studies have shown that hPL can cause peripheral insulin resistance (Barbour et al., 2007). Epigenetic changes such as histone methylation are recorded in women with GDM that can be a potential predictor for occurrence of T2DM in the future (Michalczyk et al., 2016). Maternal hyperglycaemic condition tends to bring in epigenetic modifications in the offspring genes, resulting in obesity and diabetes in the child (Michalczyk et al., 2016).

Prediabetes

Prediabetes is a predecessor condition of diabetes where blood sugar level is higher compared to normal subjects, but not high enough to be classified as diabetes. If left untreated, a large proportion of the individuals with prediabetes may progress to T2DM (Tabák et al., 2012).

IDF classifies prediabetes as those having fasting plasma glucose 100–126 mg/dL and/or 2 hours plasma glucose 140–199 mg/dL after glucose load.

Metabolic syndrome

Metabolic syndrome is a complex condition characterized by the presence of a group of metabolic risk factors like reduced high-density lipoprotein (HDL), hypertriglyceridemia, insulin resistance, dyslipidemia, abdominal obesity, hyperglycemia and hypertension in the same individual which cumulatively increases the risk for developing T2DM, cardiovascular disease, atherosclerosis, stroke, vascular inflammation, liver, kidney and heart diseases (Bonomini et al., 2015; Heindel et al., 2017).

1.1.6 Type 2 diabetes is a reflection of gene(s) and environment interactions

Type 2 diabetes is a multifactorial metabolic disease (Nowotny et al., 2015), where interactions between genes and environmental factors contribute toward causation of the disease (Kaul & Ali, 2016). Though important, genetic factors alone could contribute only around 10% for predisposition of diabetes (Herder & Roden, 2011; Billings & Florez, 2011). More of environmental influence is seen in the occurrence of the disease. Major hallmarks of T2DM like mitochondrial dysfunction, aging, dyslipidemia are the result of combined effects of genes and environment (Ling et al., 2009).

T2DM can be heritable. Evidence support that inherited genetic susceptibility as a risk factor for developing T2DM (Murea et al., 2012). Genome-wide association studies (GWAS) have identified and reported around 116 genes and 161 SNPs that are linked with T2DM (Cheng et al., 2017). Epidemiological evidences from the past suggest that environmental factors play a key role in development of diabetes. Individuals who were exposed to famine prenatally in The Dutch Hunger Winter Families Study showed impaired glucose tolerance in comparison to their unexposed siblings of the same sex (Ravelli et al., 1998; Lumey et al., 2007; Heijmans et al., 2008; Tobi et al., 2009). Military personnel who got exposed to dioxins during the Vietnam War have shown to be associated with a higher risk of developing diabetes (Henriksen et al., 1997). Increased incidence of diabetes was also reported in women who were exposed to dioxins during Seveso industrial explosion in Italy (Bertazzi et al., 2001).

There are also reports suggesting the association between prenatal environment with the incidence of insulin resistance and risk of developing T2DM at later age (Ravelli et al., 1998;

Jensen et al., 2002). Rats fed with high-fat diet during pregnancy were shown to deliver offspring that developed impaired glucose homeostasis and mitochondrial dysfunctions later in life (Armitage et al., 2003; Taylor et al., 2005).

These studies attest that along with genes, environmental factors play a significant role in the genesis of diabetes and its complications.

1.2 Endocrine Disrupting Chemicals

The incidence of metabolic complications related to T2DM is highly associated with an unhealthy lifestyle such as high calorie diet intake and lack of physical activity (Sami et al., 2017). In addition to this, a particular group of environmental pollutants defined as Endocrine Disruptor Chemicals (EDCs) appear to play a pathophysiological role in the genesis of metabolic disorders (Alonso-Magdalena et al., 2011; Fénichel & Chevalier, 2017).

According to the Endocrine Society**,** EDCs are defined as "*an exogenous [non-natural] chemical, or mixture of chemicals, that interferes with any aspect of hormone action.*"
Studies on endocrine disruption began during the famous meeting at the Wingspread Conference Center, Racine, Wisconsin in July 1991, organized by Theo Colborn and his coworkers (Kabir et al., 2015).

EDCs are highly heterogeneous exogenous agents (Diamanti-Kandarakis et al., 2009; Polyzos et al., 2012) that include synthetic chemicals interfering with synthesis, secretion, transport, storage, binding action and elimination of hormones in our body (Mangochi, 2010).

Plausible intervention of more than 800 compounds with the endocrine system is known but only few are well studied (Xin et al., 2015). Some of the widely known EDCs include: Polychlorinated biphenyls (PCBs), polybrominated biphenyls, bisphenols, o-phenyl phenols, tributyltin (TBT), diethylstilbestrol (DES), dioxins, chlorophenols, phthalates, atrazine, triazines, triazoles, triclosan, vinclozin, fluoxetine, alkylbenzene, alkylphenol, persistent organic-pollutants (POPs), polybrominated diphenylethers(PBDEs), perfluorooctanesulfonate (PFOS), levonorgestrel, hexabromocyclododecane (HBCDD), cyclic methyl siloxanes, perfluorooctanoic acid (PFOA), Di(2-ethylhexyl) phthalate (DEHP), Benzo[a]pyrene (BaP), 4-Nonylphenol, 4-tert-butylphenols, parabens, methyl-mercury and pesticides like Dichlorodiphenyltrichloroethane (DDT) derivatives and metabolites (Kabir et al., 2015; Maqbool et al., 2016; Darbre, 2017).

1.2.1 Sources of EDC

As shown in **Figure 1**, most of the EDCs are man-made which has their source from pesticides, industrial effluents, plastic industries, pharmaceuticals, rocket propellants, food additives, etc. (Neel & Sargis, 2011).

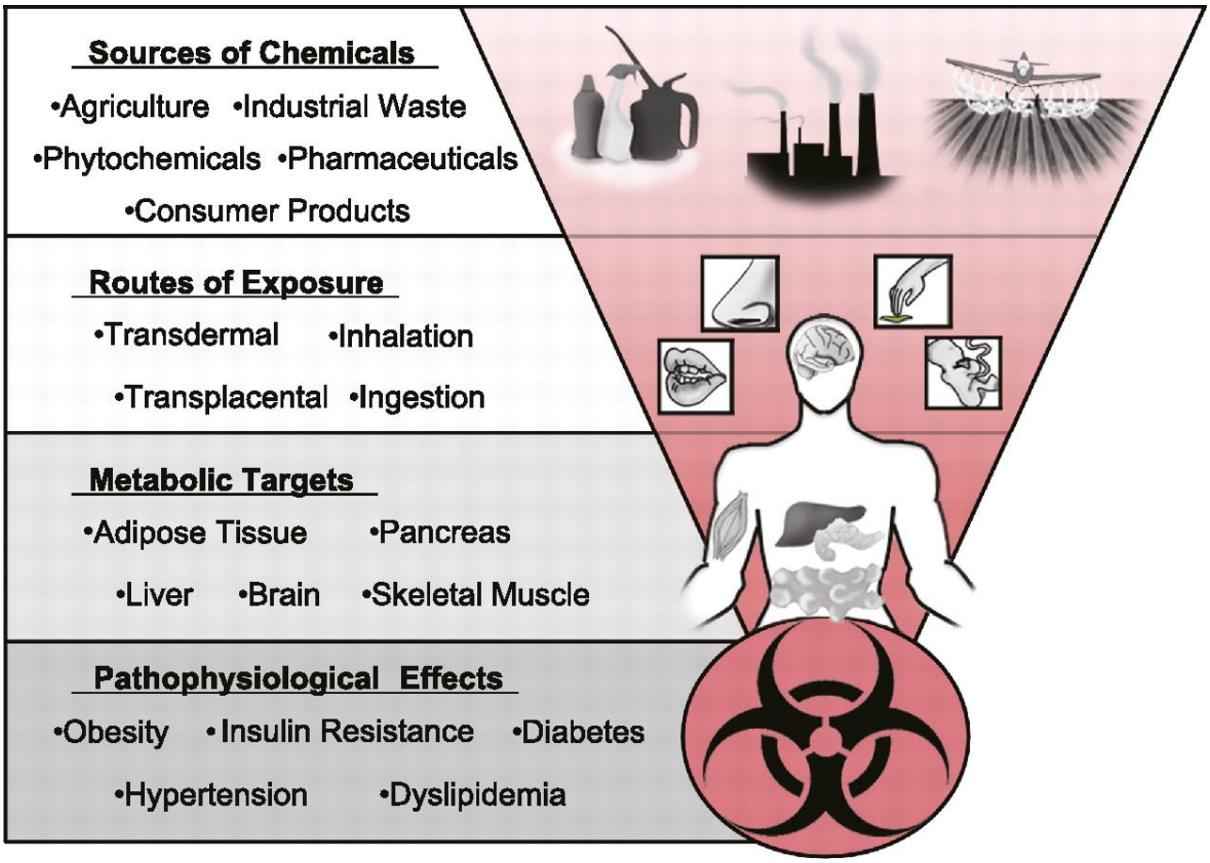


Figure 1: Adopted from Neel and Sargis (2011). Pictorial illustration showing the sources and targets of EDCs causing metabolic disorders. Sources of EDC exposure include Agriculture wastes to consumer products. Through various routes of exposure, EDC enters into the human body and affects metabolic tissues causing metabolic disorders with pathophysiological effects such as obesity, insulin resistance, diabetes, hypertension and dyslipidemia.

1.2.2 Endocrine disruptors and metabolic disease

Several studies across the globe have provided evidences for the links between EDC and reproductive and developmental failures (Diamanti-Kandarakis et al., 2009; Main et al., 2010; Knez, 2013; Costa et al., 2014), cancer (Byford et al., 2002; López-Carrillo et al., 2010; Hsieh et al., 2011; Khanna et al., 2014), and metabolic diseases like diabetes, obesity and metabolic syndrome (Neel & Sargis, 2011; Trasande et al., 2012; Goodman et al., 2014;

Lee et al., 2014; Dirinck et al., 2015; Desai et al., 2015; Le Magueresse-Battistoni et al., 2017). The metabolic target tissues for EDCs include adipose, liver, pancreas, skeletal muscle and brain (Neel & Sargis, 2011).

In the current study, our focus is mainly on bisphenol A which is a major EDC, and its effects on pathogenesis of T2DM.

1.2.3 Bisphenol A and its sources

Though Bisphenol A (BPA), (2,2-bis(4-hydroxyphenyl)propane) was first synthesized in 1891 by A.P. Dianin, only during the late 1940s and 1950s, BPA found its usage in plastic industry (Rubin, 2011). BPA is a synthetic compound that is used as a raw material in the manufacture of polycarbonate plastics and epoxy resins (Brede et al., 2003) and is widely applied in daily consumer products (Huang et al., 2018) such as baby toys, infant feeding bottles, water pipes, eye glass lens, compact discs, dental sealants, consumer electronics food and beverage packaging containers, thermal paper, among many others (Vandenberg et al., 2007; Fu and Kawamura, 2010; Bernier & Vandenberg, 2017). **Figure 2** illustrates possible sources of BPA exposure.

1.2.4 Routes of BPA entry

Bisphenol A enters the body by oral ingestion, by dermal exposure, or through inhalation. Ingestion is considered to be the major route of exposure to BPA in humans (Vandenberg et al., 2010). BPA leaches out from polycarbonate plastics, epoxy resins or from other storage materials which are in contact with food and water, can enter by oral ingestion through food intake or drinking water (Schecter et al., 2010). Non-food source of entry is by dermal contact and inhalation. BPA absorption by skin can be through handling of thermal papers or products of thermal paper (Zalko et al., 2011; Hormann et al., 2014). Mielke et al. (2011) had predicted that dermal exposure of BPA can have a significant contribution to the overall BPA exposure in an individual.

Figure 2: Representative figure illustrating various source materials for the exposure of bisphenol A. The source ranges from cosmetics to medical equipment. Plastic grade numbers 3 and 7 in the centre indicate PVC plastic and mixture of plastics, respectively, which majorly utilize BPA for the manufacturing.

There is a possibility of BPA getting released from epoxy-based floorings, adhesives, etc. into indoor air and dust. However, exposure through air and dust inhalation is considered to be minimal and not an important source of exposure (Dekant and Völkel, 2008; Geens et al., 2012).

1.2.5 Bisphenol A analogues

Chemicals with structural or functional similarity to bisphenol A (BPA) are often referred to as BPA analogues or derivatives. BPA analogues have been detected in foodstuff (Cacho et al., 2012; Liao and Kannan, 2013), thermal paper (Becerra and Odermatt, 2013), river and lake sediments near industrialized areas (Liao et al., 2012), and chlorinated and brominated

analogues in flame retardants (Voordeckers et al., 2002). Analogues of BPA are also seen in human biological specimens (Cobellis et al., 2009; Cobellis et al., 2010; Liao et al., 2012).

There are totally 31 BPA analogues identified to date (Chen et al., 2016; Pelch et al., 2017; Owczarek et al., 2018). They are: 4,4'-(hexafluoroisopropylidene) diphenol (BPAF), 4,4'-sulfonyldiphenol (BPS), 4,4'-(1-phenylethylidene)bisphenol (BPAP), 2,2-bis(4-hydroxyphenyl)butane (BPB), 4,4'-(1,4-phenylenediisopropylidene) bisphenol (BPP), 4,4'-cyclohexylidenebisphenol(BPZ), Bis(4-hydroxyphenyl)-2,2-dichloroethylene (BPC), BPC-dichloride,4,4′-Ethylidene bisphenol, 1,1-Bis(4-hydroxyphenyl)ethane (BPE), 4,4'-dihydroxydiphenylmethane (BPF), 2,2-Bis(4-hydroxy-3-isopropylphenyl) propan (BPG), 4,4′-(1,3-Phenylenediisopropylidene) bisphenol (BPM), 2,2-Bis(2-hydroxy-5-biphenylyl) propane (BPPH), 4,4'-(3,3,5-trimethyl cyclohexane-1,1-diyl) diphenol (BP-TMC), Bisphenol A diglycidyl ether (BPA-DGE), 4,4′-(Diphenylmethylene) diphenol (BPBP), 4,4′-Sulfonylbis[2-(2-propen-1-yl)phenol (TGSA), 4- Hydroxyphenyl 4-isoprooxy phenylsulfone (d-8), Phenol, 4,4'-sulfonylbis-polymer with 1,1'-oxybis [2- chloroethane] (d-90), 1,7-bis (4-Hydroxyphenylth io)-3,5- dioxaheptane (DD-70), 2,4-Bisphenol S (2,4-BPS), Bisphenol S-MAE, Bisphenol S-MPE, Methyl bis (4-hydroxyphenyl) acetate (MBHA), 4,4'-bis (Ncarbamoyl-4-methylbenzene sulfomide) diphenylmethane (BTUM), Tetramethyl Bisphenol A (TMBPA), Bisphenol A bis (diphenyl phosphate) (BDP), 2,4' Bisphenol F, Pergafast 201, Urea urethane compound (UU) and BPA bis (diphenyl phosphate) (BDP), 4,4′-(9-Fluorenylidene) diphenol (BPFL).

1.2.6 Structure of BPA and its receptor-binding abilities

Bisphenol A with chemical formula $(CH_3)_2C(C_6H_4OH)_2$, belongs to a group of diphenylmethane derivatives with two hydroxyl groups. The structure of BPA is similar to endogenous hormone estradiol (E2) **(Figure 3)**, thus possessing estrogenic activity (Maruyama et al., 2013). Hence, BPA is known as xenoestrogen (Wang et al., 2017) that mimics estradiol activity. BPA has the potential to induce the expression of estrogen responsive genes and can promote proliferation of MCF-7 cells similar to estradiol (Wang et al., 2018). Previous studies have reported that BPA can act as either agonist or antagonist depending on the Estrogen Receptor (ER) subtype and cell type (Kurosawa et al., 2002). BPA has a low binding affinity for nuclear estrogen receptors (ERs) α and β, compared to estradiol (Kuiper et al., 1998; Wolstenholme et al., 2011; Baker & Chandsawangbhuwana, 2012). Interestingly, studies have reported that BPA can act as either agonist or antagonist

depending on the Estrogen Receptor (ER) subtype and cell type (Kurosawa et al., 2002; Acconcia et al., 2015).

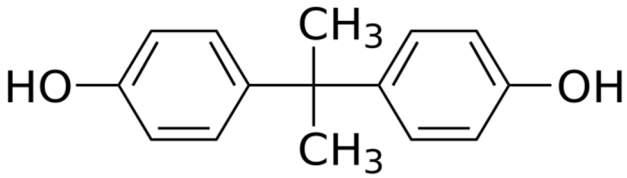


Figure 3A: **Structure of Bisphenol A (BPA; 2, 2-Bis (4-hydroxyphenyl) propane)**

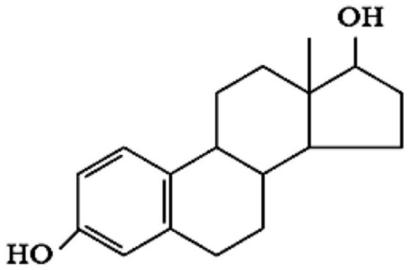


Figure 3B: Structure of estradiol [E2]

Figure 3: Illustration showing the structure of Bisphenol A and Estradiol. The chemical structures of (A) Bisphenol A (B) Estradiol are presented. The two molecules are structurally similar enabling BPA to interact with the estrogen receptors and other estrogen-like receptors similar to estrogen action.

Estrogen related receptors (ERR-α, ERRβ and ERR-γ), which belong to orphan nuclear receptor family have significant homology with ERs (Giguère, 2002; Horard & Vanacker, 2003). Studies have demonstrated that there was a positive association of higher concentration of BPA with higher expression levels of ERβ and ERR-α in peripheral blood leukocytes (Melzer et al., 2011; Cipelli et al., 2014). Recent studies had revealed that one of the major *in vivo* receptors for BPA is ERR-γ (Takayanagi et al 2006; Okada et al 2008; Tohme et al 2014). Reports have suggested that BPA can bind to thyroid hormone receptor and act as an antagonist disrupting thyroid hormone action (Moriyama et al., 2002; Sun et al., 2009; Delfosse et al., 2014). Molina et al. (2013) through *in vitro* bioassays demonstrated that BPA acts as a strong Androgen Receptor (AR) antagonist. Pereira-Fernandes et al. (2013) stated that BPA potentially induces adipogenesis by activating PPARγ. Association of BPA with PPARγ is also supported by studies conducted by Riu et al. (2011) demonstrating that BPA and its halogenated forms possess the capability of binding to PPARγ. By binding to PPARγ, BPA and its halogenated forms are capable of altering early adipogenesis (Somm et

al., 2009) and the dysregulation of PPARγ is shown to cause the onset of diabetes and obesity (Swedenborg et al., 2009). Studies have also suggested that BPA at low concentrations is capable of triggering the effects of G-protein coupled receptor 30 (GPR30) promoting cellular proliferation (Bouskine et al., 2009; Chevalier et al., 2012).

1.2.7 Metabolism and toxicokinetics of BPA

Acute exposure to BPA has been reported to cause adverse health outcomes in experimental animals (Richter et al., 2007); similar findings have also been reported in observational epidemiological studies, where BPA exposure was linked to adverse health outcomes (Vandenberg et al., 2007). BPA exposure during fetal development or during postnatal life is of utmost concern as the potential of BPA to disrupt normal development is higher because the process of metabolizing BPA through conjugation in liver may not be fully developed at the early part of life (Gerona et al., 2013; Divakaran et al., 2014). Liver is the major organ involved in metabolism of toxic chemicals and substances like BPA (Malaguarnera et al., 2012).

Once BPA gets ingested into the body as a free active unconjugated form, the majority of it gets quickly conjugated to glucuronide by enzyme Uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferases) and gets converted to BPA-glucuronide (BPA-G) (Street et al., 2017). This process is called glucuronidation which is a major form of xenobiotic metabolism that occurs in the liver and gut to detoxify and eliminate both endogenous compounds and drugs (Dutton, 1980). This conjugated form of BPA is more hydrophilic and excreted in urine (Midoro-Horiuti & Terumi, 2017). In humans, UGT2B15 is the major isoform of UGT that is responsible for glucuronidation of BPA (Hanioka et al 2008). However, other UGTs including UGT1A1, UGT1A3, UGT1A9, UGT2B4 and UGT2B7 showed significant levels of glucuronidation activities (Street et al., 2017). Though a major part of BPA is conjugated to BPA-G, to a lesser extent, unconjugated free BPA gets converts into other metabolites, mainly BPA sulphate (BPA-S) through the help of cytosolic enzyme sulfotransferase (Gerona et al., 2013; Thayer et al., 2015). The conjugated forms of BPA are considered to be biologically inactive as they cannot bind to the estrogen receptors. However, recent studies have reported that BPA even in conjugated form is capable of evoking cellular response in prolactinoma cells through interaction with ERα (Vinas et al., 2013).

The half-life of BPA in human body is about 5.4 hours (Ye et al., 2006) and will be completely eliminated in 24 hours by renal clearance (Volkel et al., 2002). In the primates, the BPA-G is directly eliminated through renal clearance, whereas in rat and mouse, entero-hepatic circulation of BPA glucuronide results in slow excretory rate (Tominaga et al., 2006). **Figure 4** illustrates the process of BPA conjugation and excretion.

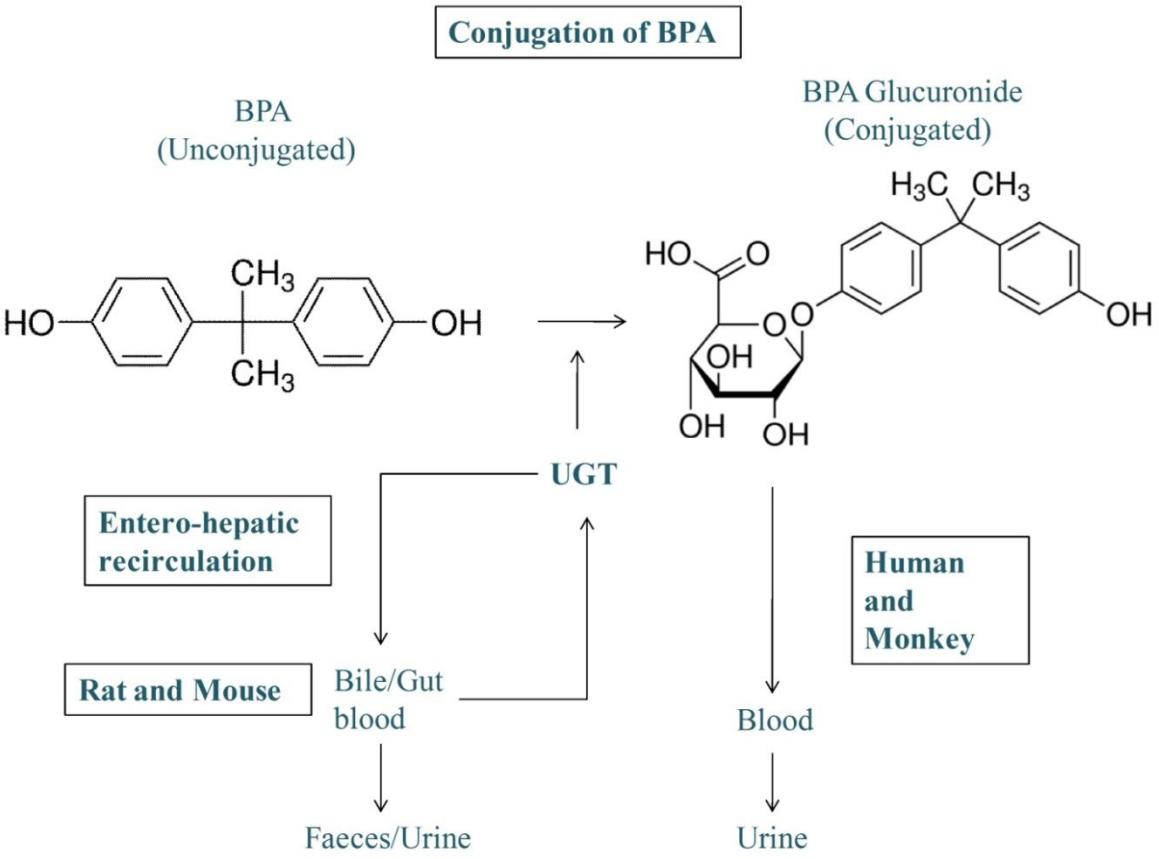


Figure 4: Illustration showing the mechanism of BPA conjugation, metabolism and its elimination. Unconjugated free active form of BPA is detoxified by conversion into BPA Glucuronide (BPA-G) with the help of enzyme Uridine 5'-diphospho-glucuronyl transferase (UGT). Clearance of BPA in primates is in rapid phase and through urine. However, in rodents, BPA Glucuronide enters into entero-hepatic circulation that result in slow excretion through faeces.

The interspecies difference in the BPA pharmacokinetics was studied by Collet et al. (2015). The study identified that plasma clearance of BPA was high and of same order of magnitude as hepatic blood flow across species. BPA clearance in humans is through highly efficient hepatic metabolism. However, BPA absorbed through skin and sublingual mucosal

membrane can directly enter into systemic circulation surpassing liver metabolism (Mielke et al., 2011; Patel et al., 2011; Gayrard et al., 2013).

1.2.8 Bisphenol A- a cause for concern?

Bisphenol A ingested orally undergoes rapid metabolization in liver followed by excretion (Volkel et al., 2002; Thayer et al 2015). However, BPA that enters through skin and oral mucosal membrane escapes liver metabolism (Gayrard et al., 2013) and is metabolised with lower efficiency compared to liver metabolism (Gundert-Remy et al., 2014; Toner et al., 2018). This increases the overall bioavailability of BPA in circulation. BPA even in conjugated form is reported to cause adverse effects. Boucher et al. (2015) demonstrated that conjugated form of BPA i.e., BPA-G, when treated to primary human pre-adipocytes, induced adipogenesis, which indicates that BPA-G is not an inactive metabolite as previously thought. Further research in this direction is needed. In addition, deconjugation of BPA from BPA-G and BPA-S to free form was also reported in foetus (Corbel et al., 2015; Gauderat et al., 2016). Possibility of this process in adults has to be researched further.

Toxicology studies have established no-observed-adverse-effect-level (NOAEL) dose for BPA ranging from 5 to 50 mg/kg/day (Draganov et al., 2015). Continuous exposure to BPA through various sources is inevitable (Vandenberg et al., 2009). Effect of BPA depends on many factors such as gender, dose and timing of exposure. Effects due to the exposure of BPA during gestation or on foetus is considered to be the most severe, compared to other timing of exposure (Liu et al., 2013).

1.2.9 Bisphenol A exposure levels across population

Bisphenol A was a subject of concern as early as 1960. Gaul (1960), first reported a case where a young man developed allergic reaction to epoxy resin containing BPA. However, there was not much concrete evidence to report that BPA causing such allergic reactions. Currently, large body of evidences point towards the links between BPA and adverse health outcomes detected in humans, animals and in *in vitro* models (Vom Saal et al., 2007; Richter et al., 2007). Several studies have determined the levels of BPA in human serum and urine across the globe confirming that BPA is ubiquitous, and humans are subjected to wide spread exposure of BPA (Vandenberg et al., 2010; Rochester, 2013).

Oral intake of BPA was reported to be the main source of BPA entry into humans (Kang et al., 2006). Lorber et al. (2015) determined the daily BPA intake through canned food to be 12.6 ng/kg-day in adult U.S. population that was then compared with National Health and Nutrition Examination Survey (NHANES) reports from 2005 to 2010 that determined the BPA intake to be between 30 ng/kg-day and 70 ng/kg-day. The difference in estimation was explained as due to the fact that possibilities of exposure to BPA from non-canned food and also from other non-dietary sources such as dermal entry from thermal papers, dust and air borne exposures (Lorber et al., 2015).

The European Food Safety Authority, based on previous studies, has reported the exposure levels of BPA according to age group of the population. Average dietary exposure of BPA in infants aged 0-6 months was 30 ng/kg/day; in toddlers aged 1-3 years was 375 ng/kg/day; in children aged 3-10 years was 290 ng/kg/day; in adolescents aged 10-18 years was 159 ng/kg/day; in adults aged 18-45 year was 132 ng/kg/day; in adults aged 45-65 year was 126 ng/kg/day; and in elderly aged 65 above was 116 ng/kg/day. Among non-dietary exposure category, thermal paper exposure through skin had the highest average of 94 ng/kg/day in adolescents (aged 10-18 years). Other major source and routes of exposure estimated include cosmetics (dermal), dust (oral/ingestion), toys (oral/ingestion) and air (inhalation) (Ćwiek-Ludwicka, 2015).

Teeguarden et al. (2013) determined human serum BPA concentration using various BPA exposure estimates and human biomonitoring data. The result estimated the serum BPA concentrations to be between 0.003 pM and 290 pM. Apart from urine and serum, BPA was also detected in different body fluids, such as milk (Sun et al., 2004; Migeot et al., 2013; Mendonca et al., 2014; Altamirano et al., 2015; Niu et al., 2017; Lee et al., 2018; Mercogliano & Santonicola, 2018), maternal urine (Callan et al., 2013; Ferguson et al., 2015), amniotic and placental fluids (Ikezuki et al., 2002; Schönfelder et al., 2002; Shekhar et al., 2017; Lee et al., 2018) and saliva (Sasaki et al., 2005; Zimmerman-Downs et al., 2010; Berge et al., 2017).

In Indian population, very few studies have analyzed the levels of BPA. Zhang et al. (2011) determined the total urinary BPA concentration from samples collected from seven Asian countries: China, India, Japan, Korea, Kuwait, Malaysia, and Vietnam. The geometric mean concentration of BPA levels was 2.7 pM, and India accounted for 3.84 pM. Xue et al. (2015) measured the urinary levels of endocrine disruptor chemicals including BPA in obese and

non-obese Indian children. The study reported geometric mean and SD of BPA levels as 11.41 pM and concluded that there was no association between BPA and obesity. Apart from these studies, there was not much of detailed analysis of BPA levels in the Indian population; and especially no data on BPA levels in the diabetes clinical setting.

Evidences from various studies have associated BPA with developmental disorders, reproductive failure and cancer. A brief account on the link between BPA and these disorders are summarized below.

1.2.10 Effects of BPA on development

The concept of "the fragile foetus" was proposed by an epidemiologist, Howard Burn, in the early 1970s and confirmed by a series of experiments exposing mouse embryos to BPA. It has been shown that active unconjugated form of BPA can surpass placental barriers and reach the foetus (Balakrishnan et al., 2010; Mørck et al., 2010), which has the potential to affect the unborn. Epidemiological studies have shown the detrimental effects of BPA exposure during gestation and development. Recurrent miscarriages (Sugiura et al., 2005) and premature delivery (Cantonwine et al., 2010) were associated with exposure to BPA during gestation. Snijder et al. (2013) suggested that BPA exposure during gestation may impair the foetal development. *In utero* exposure to BPA can affect birth weight of the offspring (Miao et al., 2011; Chou et al., 2011; Philippat et al., 2012). Recent findings from Pinney et al. (2017) also reported that BPA concentration in amniotic fluid was associated with reduced birth weight in infants. Conversely, a recent meta-analysis by Hu et al. (2018) did not find any association between BPA exposure during gestation and lower birth weight.

In vivo experiments in animal models have demonstrated the effects of BPA on embryogenesis. Pastva et al. (2001) reported transient embryonic deformities in medaka (Japanese rice fish) when it was exposed to environmentally relevant concentrations of BPA. Experiments of Ramos et al. (2003) demonstrated that male rats when prenatally exposed to environmental doses of BPA can induce both transient and permanent changes in male reproductive axis. Duan et al. (2008) reported that high dose exposure of BPA during embryogenesis of zebrafish resulted in mortality while sub-lethal dose resulted in cardiac edema and tail malformation. Tse et al. (2013) showed that exposure to BPA during early stages of embryogenesis, affected segmentation, dorsoventral patterning and brain development in zebrafish. Gibert et al. (2011) demonstrated that exposure to BPA during

early embryogenesis of zebrafish resulted in malformation of otolith. Head malformation was also noted in *Xenopus laevis* embryos when they were exposed to BPA (Imaoka et al., 2007).

1.2.11 Effects of BPA on reproduction

Several epidemiological studies have linked BPA with infertility both in men and women. Studies have observed that the increase in urinary BPA levels was significantly associated with decreased libido and sexual desire, difficulty in erection and lower ejaculation rate (Li et al., 2010), low sperm count, sperm concentration, sperm vitality, sperm motility, increased DNA damage in sperms (Meeker et al., 2010; Li et al., 2011) and may influence human steroidogenesis (Vitku et al., 2016). A cross-sectional study by Liu et al. (2015) on Chinese men associated the increased BPA exposure to significantly increased estradiol, prolactin, and sex hormone binding globulin levels in male and concluded that it may contribute to male infertility. Studies have also reported that BPA exposure may increase the risk for developing cryptorchidism (Komarowska et al., 2015). Studies have found significant association between higher levels of BPA exposure in women with Polycystic Ovarian Syndrome (PCOS) (Takeuchi et al., 2004; Kandaraki et al., 2011; Tarantino et al., 2013). Some studies have stated that BPA exposure may influence the oocyte quality and fertility rates during *in vitro* fertilization (IVF) (Fujimoto et al., 2010; Ehrlich et al., 2012).

Experiments in *in vivo* model organisms have also given enormous evidence for adverse effects of BPA on reproduction. BPA exposure during gestational period is reported to develop adverse effects on reproductive capacity in both male and female animals. A study by Salian et al. (2009) on rats demonstrated that the mothers who were exposed to BPA during their gestation and lactation, gave birth to infertile male offspring. *In utero* exposure to BPA was shown to affect reproductive function for several generations in female mice (Ziv-Gal et al., 2015). Perinatal exposure to BPA was reported to affect folliculogenesis and steroidogenesis in female rats (Santamaría et al., 2016). An experiment on rhesus monkey by Hunt et al. (2012) demonstrated that BPA alters oogenesis and follicle formation. Reports have suggested that neonatal exposure to BPA may affect the reproductive potential of animals later in life (Aikawa et al., 2004; Adewale et al., 2009) and may also lead to poly cystic ovarian syndrome in female animals (Fernández et al., 2010). Gurmeet et al. (2014) reported that BPA is harmful to male reproductive system even at doses that are lower than NOAEL (no observed adverse effect levels) dose. D'Cruz et al. (2012) found that BPA exposure even at low doses could decrease GLUT-8 expression in spermatocytes of rat and

can impair testicular function by disturbing glucose homeostasis. Experiments in male rats by Tiwari and Vanage (2013) reported that BPA may act as weak germ cell mutagen and can cause DNA damage in sperm. Male Sprague-Dawley rats which were exposed to BPA developed lesions in Sertoli and Leydig cells (Jiang et al., 2016). Studies performed with rat and goat sertoli cells demonstrate that BPA may decrease the vitality of sertoli cells and thus induce apoptosis (Iida et al., 2003; Qi et al., 2014; Wang et al., 2015; Zhag et al., 2017). Wang et al. (2017) reported that BPA induces excess generation of reactive oxygen species (ROS) and mitochondrial dysfunction in sertoli cells and thereby leads to apoptosis.

1.2.12 BPA and carcinogenesis

Occurrence and prevalence of cancer of endocrine target organs such as breast, prostate and testis are increasing. Endocrine disruptors like BPA have been reported to contribute to this increasing trend of cancers (Seachrist et al., 2015). Exposure to BPA showed the enhanced susceptibility for occurrence of cancer, as it is capable of initiating pro-inflammatory markers (Savastano et al., 2015) and other genes associated with cancer (Hussain et al., 2015) and capable of altering epigenomics during development and promotes the cancer development with aging (Ho et al., 2006). BPA is reported to induce transition and stimulates migration of cancer cells (Recchia et al., 2004; Kim et al., 2015). The first clinical evidence for association of BPA with prostate cancer was reported by Tarapore et al. (2014). The study reported that the urinary BPA levels were higher in patients with prostate cancer than those without. BPA is reported to act as mammary gland carcinogen. Sprague et al. (2013) reported that increased serum BPA levels in postmenopausal women correlated with mammographic breast tissue density. Reports have also indicated that occupational exposure to BPA may increase the incidence of breast cancer (DeMatteo et al., 2012). An animal study suggests that, rat exposed to BPA during developmental stages may induce mammary gland neoplasms (Jenkins et al., 2009; Acevedo et al., 2013) and can increase the secretion of progesterone and cell number, making it susceptible to develop breast cancer (Ayyanan et al., 2011).

1.2.13 BPA and metabolic disorders

Incidence and progression of metabolic anomalies like metabolic syndrome, obesity and diabetes are steadily increasing worldwide. Lifestyle factors such as lack of physical activity, high calorie diets, and genetic predisposition are the causes for the raise of these metabolic disorders. Recent evidences suggest that along with other factors, endocrine disruptor

chemicals (EDC) may play a key role in initiation and progression of metabolic disorders (Newbold et al., 2009; Chevalier & Fénichel, 2016). Among them, BPA is one of the major EDCs that is increasingly studied worldwide for its role in metabolic abnormalities. The potential role of BPA in initiation and progression of metabolic disorders had been investigated in some epidemiological and animal studies (Lang et al., 2008; Alonso-Magdalena et al., 2010). Several studies have suggested an association of urinary BPA levels with insulin resistance and obesity (Carwile & Michels, 2011; Wang et al., 2012; Trasande et al., 2012; Oppeneer & Robien, 2015).

1.3 BPA and type 2 diabetes

Findings of various studies that reported an association between BPA and T2DM, and tissue specific effects of BPA are discussed in the following sections.

1.3.1 Epidemiological studies in Humans

BPA has been shown to be associated with T2DM in many epidemiological studies. However, some studies have also reported no association.

A number of cross-sectional studies on BPA had used dataset from National Health and Nutrition Examination Survey (NHANES). The first among them was conducted by Lang et al. (2008) who used NHANES data from 2003-04 and reported an association between higher urinary BPA levels and diabetes. Subsequent study by Melzer et al. (2010) that used data from 2005-06 did not find any association and reported lower levels of BPA compared to 2003-04 data. However, a pooled data from 2003-08 by Shankar and Teppala (2011) reported an association between BPA and T2DM. Association between BPA and metabolic syndrome (Teppala et al., 2012), and association between BPA and prediabetes (Sabanayagam et al., 2012) was also reported by the same group. Beydoun et al. (2014) also used NHANES data to report an association between BPA levels with increased insulin resistance in men. A similar kind of study from Canada showed that urinary BPA levels were associated with diabetes and increased glycemic parameters such as HbA1c and blood glucose levels (Tai & Chen, 2016). A study from Thai population reported higher levels of BPA in patients with diabetes (Aekplakorn et al., 2009). The study concluded by stating that, the association found was stronger in men compared to women. A recent study from the same Thai population, noted an association between BPA and impaired fasting glucose (Chailurkit et al., 2017). However, the study did not find any significant association between BPA and diabetes. A longitudinal

study by Sun et al. (2014), reported the association between urinary levels of BPA and the risk of developing T2DM in middle aged women. However, the study could not find any relation between BPA levels and risk of developing T2DM in older women.

Similar studies across the globe have reported significant association between BPA and diabetes (Wang et al., 2012; Mouneimne et al., 2017) or risk of developing diabetes (Ahmadkhaniha et al., 2014), and also BPA levels linked to insulin resistance (Lee et al., 2013; Khalil et al., 2014; Menale et al., 2017) and other comorbidities of diabetes such as high glucose level and inflammation (Savastano et al., 2015; Choi et al., 2017). Conversely, a few recent studies did not find any association between BPA levels and T2DM. A recent follow-up study from China reported no association between BPA level and T2DM (Shu et al., 2018). A Danish study in children reported opposite associations as well. A Study by Carlsson et al. (2018) reported an association between higher BPA levels and lower insulin resistance. Despite the fact that Indians are highly prone to insulin resistance, diabetes and cardiovascular diseases (Shah & Mohan, 2015), there is lack of data on BPA in the clinical setting.

1.3.2 Animal studies

Most of the studies involving gestational exposure of BPA have reported to have adverse effects on male offspring. Gestational exposure of mice to low doses of BPA resulted in male offspring with glucose intolerance and mild disruption to β-cell function (Alonso-Magdalena et al., 2010). Angle et al. (2013) have reported that BPA exposure during gestation and lactation led to increased postnatal body weight in male mice which was accompanied by increased liver weight, changes in adipocyte number and volume of abdominal fat and glucose intolerance. Liu et al. (2013) also demonstrated that perinatal exposure to BPA led to development of glucose intolerance in male mice. García-Arevalo et al. (2014) demonstrated that exposure to BPA during pregnancy disrupted glucose homeostasis and β-cell function in male mice which mimic T2DM in humans. Studies have additionally shown that BPA when combined with metabolic stressor such as high-fat diet (HFD), aggravated the underlying metabolic disorder. A study by Wei et al. (2011) exposed rats to BPA during gestation and lactation, with the pups later placed on either normal or HFD. The results showed that BPA was able to induce glucose intolerance, increase body weight and insulin levels in male offspring. These effects were aggravated in male offspring that were given a HFD which resulted in enhanced obesity, dyslipidemia, glucose intolerance, and structural damages to

pancreatic β-cells. Interestingly, another study by Susiarjo et al. (2015) have reported that early life exposure to BPA can disrupt metabolic health across multiple generations – particularly in the male offspring – through stable inheritance of DNA methylation changes resulting in higher body fat and perturbed glucose homeostasis.

Exposure to low doses of BPA for 4 days, increased insulin resistance and glucose intolerance and reduced β-cell function in adult mice (Alonso-Magdalena et al., 2006; Ropero et al., 2008). Studies with 8 days of exposure to BPA have reported the occurrence of insulin resistance (Batista et al., 2012). Long-term exposure experiment in mice was reported to develop hyperglycaemia and hypercholesterolemia (Marmugi et al., 2014; Moghaddam et al., 2015). A recent study by Veissi et al. (2018) on male mice showed that BPA exposure disrupts glucose homeostasis and elevates glucose levels by reducing insulin secretion and β-cell function. Several other experiments on different animals such as mice (Moon et al., 2015), rat (Mullainadhan et al., 2017; Song et al., 2014), rabbit (Fang et al., 2015) and zebrafish (Zhao et al., 2018) have demonstrated that BPA exposure may disrupt glucose homeostasis and induce hyperglycaemia and insulin resistance.

1.4 Effect of BPA on metabolically active target tissues

Several studies have demonstrated the potential of BPA to impair glucose metabolism by targeting metabolic tissues viz., liver, pancreas, adipose tissue, skeletal muscles and central nervous system.

1.4.1 Potential effects of Bisphenol A on Liver

The energy demand in our body is met by glucose homeostasis that is controlled by the liver through various metabolic pathways that includes glycogenolysis (break down of glycogen), glycogenesis (formation of glycogen), glycolysis (break down of glucose) and gluconeogenesis (generation of glucose from non-carbohydrate substrates) (Han et al., 2016).

In addition to these, liver plays a major role in detoxifying chemical/toxic substances, drugs and environmental contaminants. BPA as an environmental contaminant gets metabolized in the liver (Malaguarnera et al., 2012). Several studies have reported that BPA exposure may induce liver damage in animals (Yıldız & Barlas, 2013; Avci et al., 2016; Thoene et al., 2017). Experiments of Hassan et al. (2012) with rat model demonstrated that BPA induces hepatotoxicity by creating oxidative stress. Jayashree et al. (2013) reported that, adult male

albino rats when exposed to BPA showed reduction in hepatic glucose oxidation together with reduced insulin receptor expression and reduced glycogen production. This shows that BPA has the potential to disrupt hepatic glucose oxidation and reduces glycogenesis. Naville et al. (2013) demonstrated that, even at low doses, BPA can induce hepatic metabolic dysfunction. A study conducted by Xia et al. (2014) in rat offspring demonstrated that perinatal exposure of BPA leads to mitochondria mediated apoptosis in liver. The study also found that mitochondrial activities like ATP synthesis, MRC (mitochondrial respiratory chain) activity and mitochondrial membrane potential were progressively reduced in offspring exposed to BPA leading to the development of hepatic steatosis later in life. A study by Marmugi et al. (2014) analyzed expression levels of genes that are involved in hepatic carbohydrate metabolism in adult male CD1 mice after exposing it to BPA with doses below No Observed Adverse Effect Level (NOAEL). The results showed a marked increase in the expression of glucokinase (Gck) and pyruvate kinase (L-Pk) genes that are involved in glycolysis. Also, the study showed over expression of genes involved in biogenesis of cholesterol. This shows that BPA by altering gene expression signature affects glycolysis and cholesterol biogenesis leading to hyperglycaemia and hypercholesterolemia even at low doses. Several studies have also reported the potential effect of BPA to induce epigenetic alteration in liver resulting in metabolic dysfunction. Ma et al. (2013) reported abnormal DNA methylation in rats exposed to BPA during gestation. The study reported an increase in promotor hypermethylation and reduction in gene expression of hepatic glucokinase resulting in insulin resistance and impaired glucose tolerance in adulthood. Another study showed that long-term exposure of mice to BPA can induce hepatic lipid accumulation through epigenetic reprogramming of genes involved in lipid metabolism (Ke et al., 2016).

1.4.2 Potential effects of Bisphenol A on Pancreatic β-cells

Pancreas produces two major hormones- insulin and glucagon, which play a pivotal role in the regulation of glucose homeostasis (Jouvet & Estall, 2017). While glucagon is produced in α-cells, insulin is produced in β-cells of pancreatic islets. Any dysregulation to pancreatic function can affect production of insulin and glucagon that further affects glucose homeostasis leading to metabolic disorders like insulin resistance and T2DM (McGarry, 2001). Estrogen is known to regulate pancreatic function through activating estrogen receptors that are expressed in pancreatic cells that aids in insulin secretion and sensitivity and proper β-cell function (Nadal et al., 2009). BPA as a xenoestrogen, can mimic estradiol

(a form of estrogen) in pancreas and lead to over-production of insulin through ERα by activating ERK1/2 pathway (Alonso-Magdalena et al., 2006, 2008). *In vitro* studies by Adachi et al. (2005), showed the potential of BPA to release insulin in response to glucose when rat islets were treated with BPA for 24 hours 10 g/L. In another *in vitro* study. Makaji et al. (2011) examined the effect of six environmental contaminants on pancreatic β-cell function. Among them, only BPA at concentration of 100 ng/mL, affected the insulin secretion and long-term exposure to BPA resulted in endoplasmic reticulum (ER) stress in the β-cells. ER stress is one of the important contributors to the pathogenesis of T2DM (Back et al., 2012). Lin et al. (2013) studied the effect of BPA on insulin secretion *in vitro* using INS-1 cells. The study showed that exposure to higher BPA concentration of 0.20 or 2.0 M resulted in reduction in Glut 2 and GCK gene expression. BPA exposure also resulted in apoptosis, which leads to mitochondrial defects that, in turn, resulted in the decline of glucose-stimulated insulin secretion. *In vivo* studies on mice have also revealed that BPA even at low doses starting from 10μg/kg/day, has the capacity of disrupting pancreatic β-cell function and resulting in insulin resistance in male mice (Alonso-Magdalena et al., 2006).

1.4.3 Potential effects of Bisphenol A on Adipose tissue

White adipose tissue is an endocrine tissue that secrets adiponectin and leptin which are involved in energy metabolism and homeostasis (Coelho et al., 2013). Leptin mainly acts on the central nervous system, specifically hypothalamus and helps in regulating energy intake and fat deposition uptake (Gao & Horvath, 2008). Adiponectin plays a major role in regulating glucose (Ben-Jonathan et al., 2009). Xenoestrogens like BPA can control adipogenesis and its function (Newbold et al., 2005; Hugo et al., 2008). Using 3T3-L1 adipocytes, Ariemma et al. (2016) reported that BPA exposure at low doses during adipogenesis can lead to metabolic dysfunction. Several *in vivo* and *in vitro* studies have showed that BPA is capable of modulating expression patterns of several genes related to adipocyte differentiation and function, which may result in metabolic dysfunction and obesity (Somm et al., 2009; Linehan et al., 2012; Ohlstein et al., 2014; Song et al., 2014). Valentino et al. (2013) through their *in vitro* study in human adipocytes observed that BPA at low doses diminishes insulin dependent glucose utilization and up-regulates inflammatory pathways. Sakurai et al. (2004) reported that BPA is capable of altering the expression of glucose transporter (GLUT4) and thereby affecting the glucose uptake by adipocytes. Angle et al. (2013) investigated the perinatal effects of BPA exposure in male mice. The study found an

increase in body weight and abdominal fat. In addition, the study also found higher levels of insulin and leptin in the blood with an associated reduction in serum adiponectin levels. These results indicate that BPA exposure can disrupt adipogenesis and cause metabolic dysfunction in adipose tissues.

1.4.4 Potential effects of Bisphenol A on Neuroendocrine system

Only in recent years, effects of BPA exposure on the central nervous system got attention, as studies are reporting that BPA can accumulate in several regions of brain. An *in vivo* study by Mita et al. (2012) on Balb-C mice offspring revealed that, based on gender and dose, BPA can accumulate in the brain. The study failed to report any subsequent phenotypic changes associated with it. A study by Elsworth et al. (2013) in non-human primates revealed that prenatal BPA exposure may decrease synaptogenesis and hippocampal spine synapses and impact the formation of mid brain dopamine neurons.

The central nervous system regulates energy expenditure and feeding behaviour through hypothalamus, and studies have revealed the impact of BPA exposure on hypothalamus. Experiments in Zebrafish embryos by Kinch et al. (2015) showed that low dose exposure to both BPA and its analogue BPS (Bisphenol S) induces premature hypothalamic neurogenesis.

Hypothalamic estrogen receptors: estrogen receptor α (ERα/ESR1) expression is crucial in regulating food intake, body fat distribution, energy expenditure (Frank et al., 2014); and postnatal sex-specific expression of estrogen receptor (ERβ/ESR2) is vital in brain for socio-sexual behaviour (Cao et al., 2014). Rebuli et al. (2014) studied the effects of low dose oral exposure to BPA on estrogen receptors expression in hypothalamus of juvenile and adult female rats. The result suggested that BPA reduces ESR1 expression levels in juvenile female rats and reduced ESR2 expression levels in adult female rats.

1.4.5 Potential effects of Bisphenol A on skeletal muscle

Skeletal muscles are an important metabolic tissue that plays a role in maintaining proper glucose metabolism. It is the primary insulin-sensitive tissue in the body that induces insulin-stimulated glucose uptake (Stump et al., 2006). This process is carried out with the help of glucose transporter-4 (GLUT-4), which translocates into the plasma membrane through the intracellular signalling mechanism involving insulin receptors and Akt (Protein kinase B) that facilitates the glucose uptake (Saltiel & Kahn, 2001; Satoh, 2014). For maintaining proper

blood glucose levels in circulation, insulin promotes glucose utilization in muscle cells, thus inhibiting hepatic gluconeogenesis (Fritsche et al., 2008).

A study by Indumathi et al. (2013) in male rats reported that BPA exposure resulted in the reduction in the levels of insulin receptors, Akt, Akt phosphorylation and GLUT-4. Mullainadhan et al. (2017) have also reported similar results. The study demonstrated that BPA exposure in male rats, reduced protein expression levels of Insulin receptor (IR), Insulin receptor substrate (IRS-1) and reduced Akt phosphorylation along with dysregulation of GLUT-4 translocation that was achieved by altering SNARE protein that is required for GLUT-4 translocation. These data suggest that BPA by disrupting glucose homeostasis in skeletal muscle can lead to insulin resistance.

1.5 Cellular Senescence

Cellular senescence is an irreversible process where the damaged cells are permanently arrested from replication in G1/G0 phase of cell cycle as a result of different types of damaging stimuli (Muñoz-Espín & Serrano, 2014) like external stress signals, DNA damage or oncogene activity (Lee et al., 2015; Ben-Porath et al., 2005; Lloyd, 2002; Serrano et al., 2001). This phenomenon for the first time was described by Hayflick as he observed senescent cells in serially passaged human fibroblast cell culture (Hayflick and Moorhead, 1961). **Figure 5** illustrates cell cycle check points and effect of senescence stimuli leading to cellular senescence. Senescence majorly occurs in aged organisms where it contributes to the decline of tissue function, promoting inflammation and cell cycle arrest (López-Otín et al., 2013); it is also seen during embryogenesis where it helps in remodelling tissues (Hernandez-Segura et al., 2018).

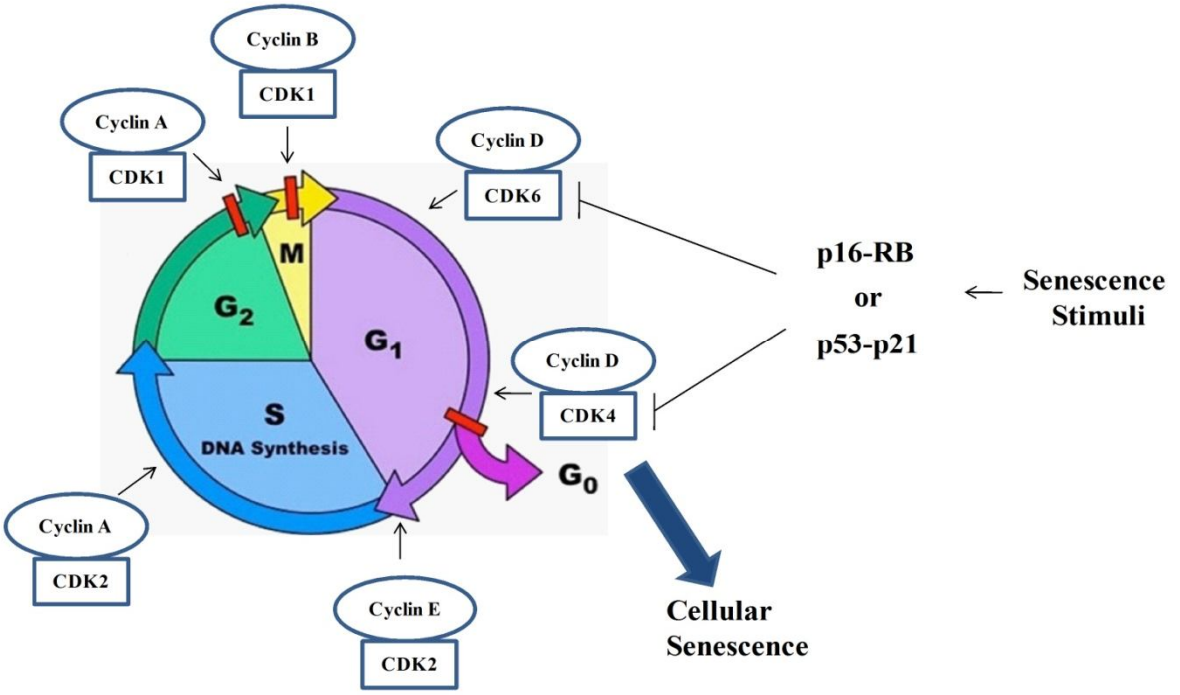


Figure 5: Illustration of cell cycle check points and effect of senescence stimuli. External stimuli such as stress, oncogene activation, DNA damage are capable of inducing p16 and/or p53 pathways leading to inhibition of cyclin dependent kinases resulting in cell cycle arrest and cellular senescence.

1.5.1 Types of cellular senescence

Cellular senescence is of different types based on the sources of induction: Oncogene-induced senescence, Replicative senescence, Stress-induced premature senescence, DNA damage-induced senescence, mitochondrial dysfunction-associated senescence, epigenetically induced senescence, Chemotherapy-induced senescence and Paracrine senescence (Hernandez-Segura et al., 2018).

Oncogene-induced senescence

Activation of oncogenes such as BRAF or RAS and/or inactivation of tumor suppressor genes like PTEN can lead to oncogene-induced senescence (Muñoz-Espín & Serrano, 2014; Sharpless & Sherr, 2015).

Replicative senescence

Replicative senescence refers to the arrest of proliferative potential of the cells after multiple cell cycle divisions (Druelle et al., 2016). Telomere shortening is observed and noted as a cause for this type of senescence (Sharpless & Sherr, 2015).

Telomere shortening and DNA-damage-induced senescence

Irrepairable damage to the DNA or telomere attrition may trigger DNA-damage response (DDR). DDR involves secretion of DNA damage kinases such as ATR, ATM, CHK1 and CHK2 which further induces the phosphorylation of p53 which in turn transactivates the expression of p21, which inhibits CDK–cyclin complexes leading to senescence (Muñoz-Espín & Serrano, 2014).

Mitochondrial dysfunction-associated senescence (MiDAS)

Wiley et al. (2016) have reported that mitochondrial dysfunction can induce senescence, which has a characteristic of distinct MiDAS associated secretary phenotype.

Stress Induced Premature Senescence (SIPS)

Senescence that occurs as a result of induction by exogenous chemicals, oxidizing agents (also known as oxidative stress-induced senescence), ionizing radiation or by DNA damaging agents. SIPS show similarities in mechanism with Replicative senescence (Rajarajacholan & Riabowol, 2015; Hernandez-Segura et al., 2018).

Epigenetically induced senescence

Histone deacetylase such as sodium butyrate and suberoylanilide hydroxamic or DNA methylase inhibitors such as 5-azacytidine and 5-aza-2'-deoxycytidine are known to induce senescence (Venturelli et al., 2013; Petrova et al., 2016; Sidler et al., 2017).

Chemotherapy-induced senescence

Anticancer drugs such as doxorubicin, bleomycin, abemaciclib or palbociclib are capable of inducing senescence mechanism either through DNA damage or by cyclin dependent kinase inhibition (Petrova et al., 2016).

Paracrine senescence

Paracrine senescence is the induction of senescence in neighbouring cells by a primary senescent cell in a paracrine manner that is achieved by producing senescence-associated secretory phenotype (SASP) (Acosta et al., 2013).

1.5.2 Biomarkers to identify cellular senescence

While cellular senescence can be triggered by a wide range of conditions, the senescent cells display a number of morphological characteristics that allow their identification. Cells undergoing senescence tend to display morphological changes like enlargement and extension, vacuolizing, and becoming multinucleated (Kuilman et al., 2010).

Some of the biomarkers, separately or in combination, are widely accepted to define senescence in both cell culture and in tissues (Collado & Serrano, 2006). Expression of markers that are mediators of senescence or cell cycle arrest include: p15, p16, $p14^{ARF}$, p53, p21, p27 and retinoblastoma (RB) (hypophosphorylated) (Muñoz-Espín & Serrano, 2014). Absence of proliferative markers, nuclear aberrations and activation of tumor suppressor networks are also considered to be biomarkers that are indicative of cellular senescence (Görg et al., 2015). The most widely accepted and used biomarker for detection of cells undergoing senescence is β-galactosidase assay. It is the histochemical detection of β-galactosidase activity at pH 6.0 and this assay is also known as senescence-associated β-galactosidase (SA-β-GAL) (Dimri et al., 1995). Senescent cells show increased lysosomal content, which enables the detection of enzyme lysosomal β-galactosidase at pH 6.0 (Kurz et al., 2000). The increased lysosomal content also reflects on autophagy that occurs in senescent cells. The major limitation of this assay is that it needs fresh samples to detect senescence. In addition to this, Sudan Black B is also used to stain senescent cells positively and can also be used in paraffin-embedded tissues. It detects the lysosomal aggregate known as lipofuscin in the cells (Georgakopoulou et al., 2013).

In addition to these biomarkers, senescent cells secrete a number of extracellular factors such as insulin-like growth factor 1 (IGF1) -binding proteins, plasminogen activator inhibitor 1 (PAi-1), transforming growth factor-β (TGFβ), inflammatory cytokines and chemokines that help in pathogenesis of senescence process in an autocrine and paracrine manner (Kuilman & Peeper, 2009; Acosta et al., 2013). Other markers present in cells that undergo senescence are decoy receptor 2 (DCR2), differentiated embryo chondrocyte expressed gene 1 (DEC1) and senescence-associated heterochromatin foci (SAHF) (Xu et al., 2012).

1.5.3 Cellular Senescence-signalling pathways

Cyclin-Dependent Kinases (CDKs) regulate multiple proteins involved in cell cycle progression (Suryadinata et al., 2010). Senescent cells undergo cell-cycle arrest through CDK inhibitors which is encoded in the CDKN2A ($p16^{INK4a}$/p16 and $p14^{ARF}$/p14), CDKN2B (p15) and CDKN1A (p21) loci.

Senescence-inducing signals, such as telomere attrition, DNA damage, oxidative stress, oncogenes, excessive mitogenic signalling and many others, may trigger DNA-damage response (DDR) as well as many other stresses that further engage either in p53 dependent (p53-p21) or p53 independent (p16-RB) pathway inducing cellular senescence (Campisi & d'Adda di Fagagna, 2007; Gorgoulis & Halazonetis, 2010; Lämmermann et al., 2018). **Figure 6** illustrates the two major pathways through which senescence happens.

p16 is an inhibitor of Cyclin dependent kinase 4 and 6 (CDK4/6), that prevents phosphorylation of retinoblastoma protein. Hypophosphorylated RB halts cell proliferation by suppressing the E2F activity, a transcription factor that induces the expression of genes necessary for cell-cycle progression (Narita et al., 2003). Studies have correlated the levels of p16 with the chronological aging of tissues that are analyzed in both mice and humans which highlights the importance of p16 as a biomarker to detect senescent cells (Krishnamurthy et al., 2004; Ressler et al., 2006; Baker et al., 2011; Burd et al., 2013). Alternate reading frame (ARF) protein has been shown to inactivate p53 degrading E3 ubiquitin-protein ligase HDM2 (MDM2 in mice) in such a way that it maintains p53 stability (Kim & Sharpless, 2006; Gil & Peters, 2006). p53 transactivates p21 (cyclin-dependent kinase (CDK) inhibitor) expression (Hernandez-Segura et al., 2018), which suppresses the phosphorylation of RB leading to cell cycle arrest resulting in senescence.

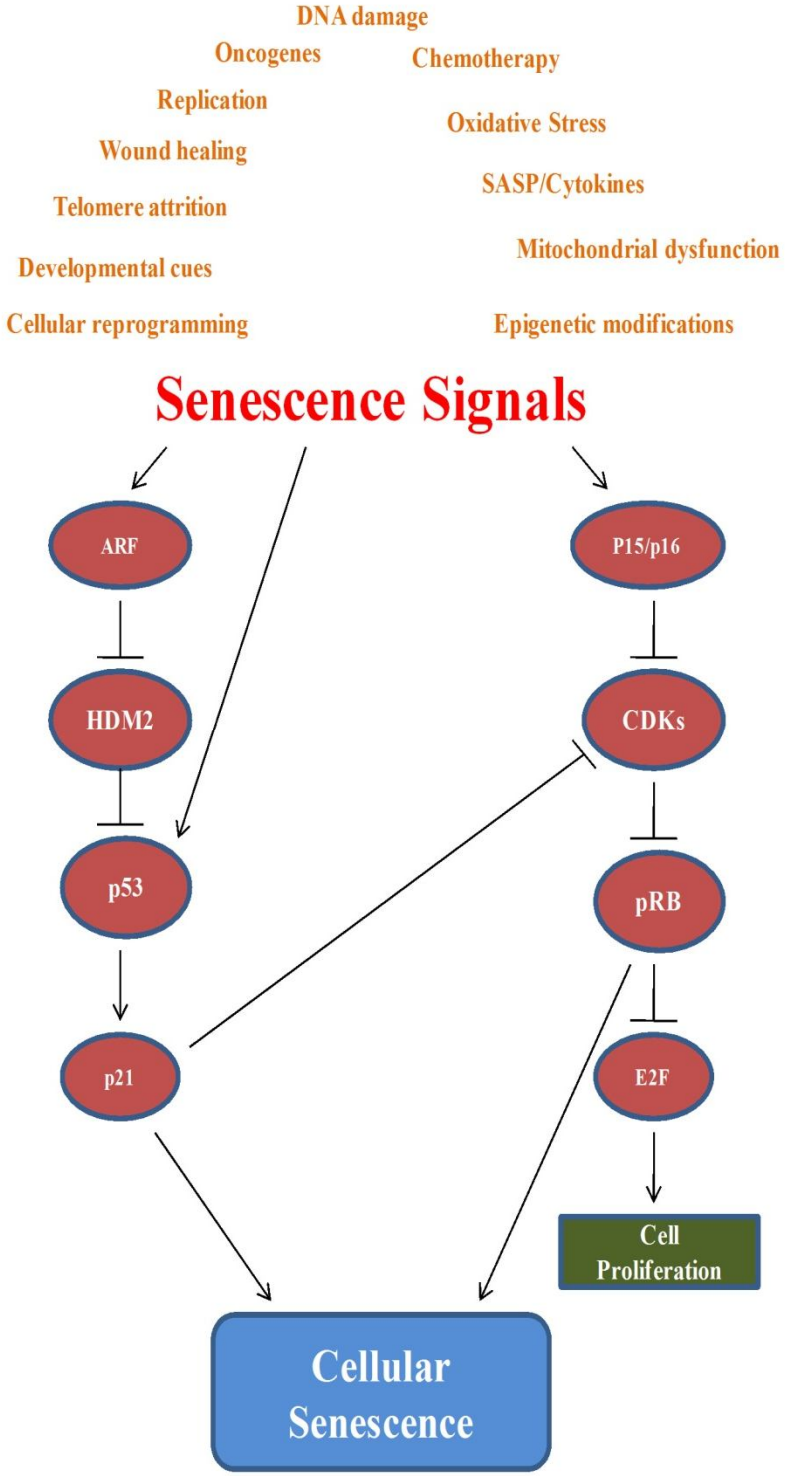


Figure 6: Illustration of senescence pathways. Various senescence-inducing signals trigger cells to undergo senescence through either p53-p21 axis and/or p16-Rb axis, which are two major pathways for senescence.

p15 (CDKN2B) has similar structure, function and biochemical properties like p16; however not much attention has been given to p15 in the context of senescence mechanism (Li et al., 2011) as p16 is recognized widely as an important marker for senescence.

1.5.4 Senescence-Associated Secretory Phenotype (SASP)

In a landmark study by Coppé et al. (2008), the authors demonstrated that senescent cells when induced by genotoxic stress secrets a complex number of factors that are associated with inflammation. This was termed as Senescence-Associated Secretory Phenotype (SASP). Though senescent cells stop dividing, they still remain metabolically active and implement a complex pro-inflammatory response secreting cytokines, chemokines, interleukins and proteinases that are collectively known as SASP (Kuilman & Peeper, 2009; Evan & d'Adda di Fagagna, 2009; Coppé et al., 2010; Campisi, 2013). The SASP is mediated by the transcription factors such as nuclear factor-κB (NF-κB), GAT4A and CCAAT/enhancerbinding protein-β (C/EBPβ) (Acosta et al., 2008; Rovillain et al., 2011; Kang et al., 2015). NFkB mainly induces the expression of inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Apart from this, Jak2/ Stat3 pathway is another pathway that upregulates immunosuppressive cytokines such as, IL-10, IL-13, M-CSF, GM-CSF, CXCL1/CXCL2 (Hayakawa et al., 2015). SASP components such as TGFβ can induce senescence on its own in neighbouring cells in a paracrine manner (Hubackova et al., 2012; Nelson et al., 2012; Ritschka et al., 2017).

1.5.5 Cellular Senescence and Diabetes

Senescence is considered to play a prominent role in the pathogenesis of age-associated diseases like diabetes (Palmer et al., 2015). The association between type 2 diabetes and senescence is complex. Whether senescence is the cause for disease or the senescence is a consequence of pathology of the disease is still under debate (Childs et al., 2015).

Several *in vitro* studies have shown that high glucose exposure which mimics hyperglycaemic condition, is capable of inducing cellular senescence in various tissue models such as fibroblasts, endothelial cells, mesenchymal cells, and adipose-derived stem cells (Blazer et al., 2002; Yokoi et al., 2006; Cramer et al., 2010; Legzdina et al., 2016; Zhang et al., 2017). Ksiazek et al. (2008) predicted mitochondrial dysfunction, over production of reactive oxygen species, or DNA damage as mechanism through which high glucose induces

accelerated senescence. Cellular senescence may accompany mitochondrial dysfunction that causes oxidative stress related to the development of insulin resistance in ageing muscle tissues (Petersen et al., 2003; Passos et al., 2010). Studies have reported age-associated reduction in the proliferative potential, through senescence in pancreatic β-cells, as the cause for the pathogenesis and increased prevalence of T2DM among elderly population (Sone & Kagawa, 2005; Helman et al., 2016).

Starting from the study by Adaikalakoteswari et al. (2005) which demonstrated an association of increased telomere shortening in patients with T2DM, several studies attested that there exists a link between accelerated senescence (ageing) and T2DM (Adaikalakoteswari et al., 2007; Balasubramanyam et al., 2007; Monickaraj et al., 2012; Monickaraj et al., 2013).

Several GWAS studies have revealed that polymorphisms in close proximity to CDKN2A ($p16^{INK4a}$/$p14^{ARF}$) and CDKN2B (p15) locus, which are a major locus for senescence associated genes, are genetically linked to several age-associated pathologies that includes type 2 diabetes cardiovascular complications, glaucoma and Alzheimer's (Doria et al., 2008; Jeck et al., 2012). Baker et al. (2011) demonstrated in a mouse model that by eliminating p16-positive senescent cells, age-related pathologies can be delayed.

Senescence-associated secretory phenotypes, such as MCP-1, IL-6 and IL-8, are notably increased in obese individuals and contribute to the pro-inflammatory state (Kim et al., 2006; Freund et al., 2010). This inflammation is reported to be a major contributing factor in the development of insulin resistance (Xu et al., 2003; Esser et al., 2014). These results attest that there is a strong interlink between diabetes and cellular senescence.

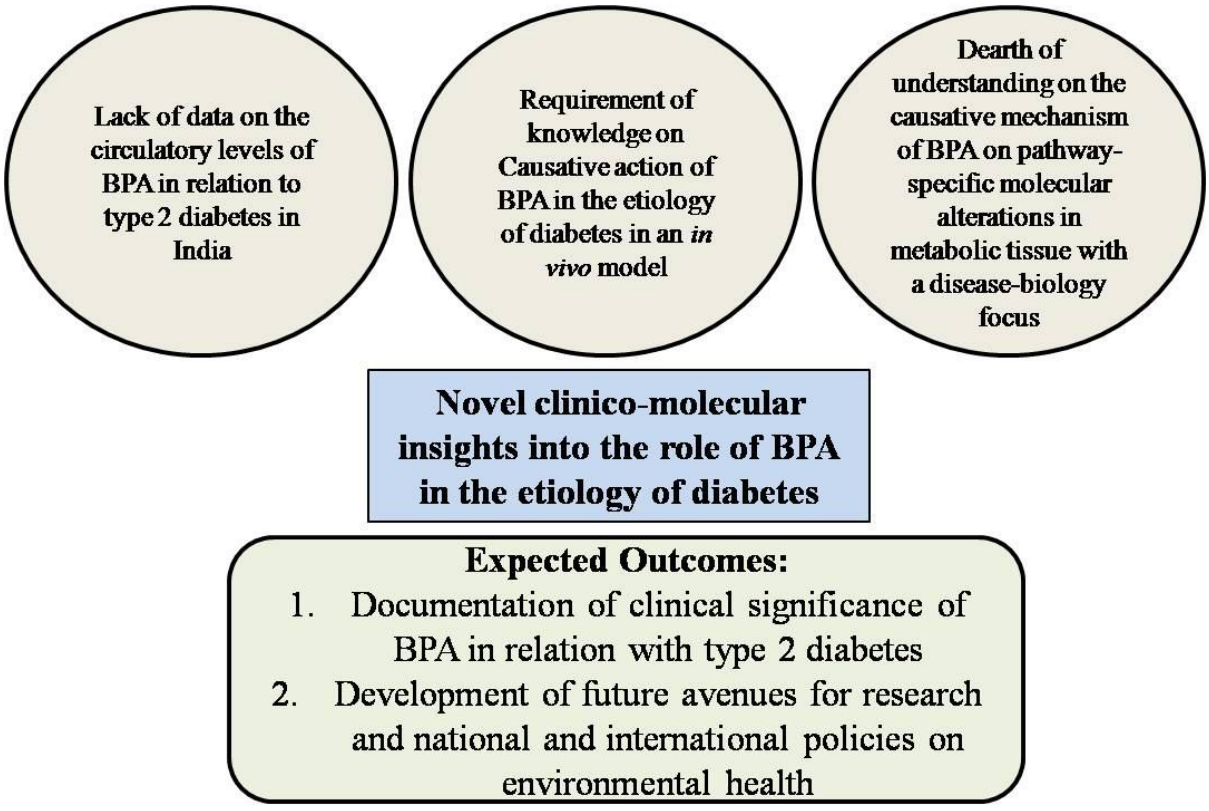
The detailed literature on the possible role of BPA in the pathogenesis of diabetes warrant more clinically relevant studies backed up by studies in animal model and metabolic tissues and this thesis has been holistically focused on this directionality.

1.6 Research Gaps

- Despite the worldwide concern on the BPA and environmental health, there is a lack of data on the circulatory levels of BPA in relation to type 2 diabetes and obesity in the clinical context in India.
- Several studies that involved in vivo models, were only focused on consequence of BPA exposure on metabolic disorders rather than focusing on the context of pathophysiology of the disease.
- While the review of literature accounts for the varied tissue-specific effects of BPA on metabolism, there is lack of research that systematically studies the effects of BPA on pathway-specific molecular alterations with a disease-biology focus and using the state-of-the-art molecular tools.

Our study tried to address these research gaps and create a knowledge pool for future studies of translational research.

1.7 Rationale behind this study



1.8 Study Objectives

- To investigate the circulating levels of BPA in subjects with type 2 diabetes compared to control subjects (normal glucose tolerant individuals) and to derive an association with glycemic control, insulin resistance, and senescence.
- To investigate the causative mechanism of BPA in the pathogenesis of metabolic disease like hyperglycemia using zebrafish as an *in vivo* animal model.
- To study the tissue-specific (skeletal muscle) effects of BPA on senescence mechanisms and glucose metabolism.

Chapter 2

Elevated systemic levels of bisphenol-A (BPA) are linked to poor glycemic control, cellular senescence and insulin resistance in patients with Type 2 Diabetes

2.1 Abstract

Evidences are accumulating to point out an interaction of genes and environment in the etiology of type 2 diabetes (T2DM). Recently, endocrine disrupting chemicals (EDCs) like bisphenol-A (BPA) have received special attention for their mechanistic role in metabolic disruption. Despite the increasing awareness on the role of BPA in the development of insulin resistance and T2DM, there is a lack of clinically relevant data regarding Asian Indians, a population which is more susceptible to T2DM and other metabolic disorders. Therefore, the objective of this study is to measure systemic levels of BPA in patients with T2DM (n=30) compared to control individuals with normal glucose tolerance (n=30) and to assess its status for correlation with other risk variables. Serum BPA levels were estimated using ELISA kit and biochemical determinations were done by standard protocols. In addition, we have also utilized peripheral blood mononuclear cells (PBMCs) to profile the gene expression alterations with special reference to inflammation, estrogen receptors and cellular senescence in these subjects. Serum levels of BPA was significantly higher in patients with T2DM compared to control individuals. BPA levels in T2DM subjects were also positively correlated to poor glycemic control and insulin resistance. Gene expression analysis in PBMCs revealed that patients with T2DM exhibited significantly elevated mRNA levels of senescence (GLB1, p16, p21 and p53) and inflammatory (IL6 and TNF-α) markers. The chronic burden of senescence and inflammation was also reflected by significantly reduced and shortened telomeres in patients with T2DM compared to control subjects. Interestingly, PBMCs from patients with T2DM also exhibited significantly elevated mRNA expression of ERRγ, a recently identified receptor for BPA. This study is the first to demonstrate an association of increased BPA levels with cellular senescence, pro-inflammation, poor glycemic control, insulin resistance and shortened telomeres in patients with T2DM. Studying the biological consequences of cellular alterations of BPA is expected to unravel novel drug targets and development of newer therapeutic measures.

2.2 Introduction:

The emergence of type 2 diabetes mellitus (T2DM) as a global epidemic as well as pandemic is one of the major challenges to human health in the 21st century (Jaacks et al., 2016). According to the latest International Diabetes Federation (IDF) Atlas, around 425 million people worldwide are diagnosed with diabetes and India alone harbours more than 73 million of individuals with diabetes (IDF, 2017). T2DM is conceived as a metabolic (Marin et al., 2018) and vascular disease (Hayward et al., 2015), who's pathogenesis originates from multiple cellular alterations (Geraldes & King, 2010) and gene-environment interactions (Kaul & Ali, 2016). Peripheral insulin resistance in combination with impaired pancreatic β-cell function leads to the clinical manifestation of T2DM (Defronzo, 2009). Recent genome wide association studies (GWAS) have identified ~120 genetic variants linked to diabetes; however, these genetic variants could explain only 10-15% of disease risk, while the remaining >80% of risk is not yet clearly understood and often suggested to be product of gene-environment interactions (Imamura et al., 2016). In this context, environmental contaminants such as endocrine disrupting chemicals (EDCs) received special attention in causing the metabolic disruption in relation to obesity, insulin resistance, T2DM and other metabolic diseases (Chevalier & Fenichel, 2016; Mimoto et al., 2017; Heindel et al., 2017). Among EDCs, bisphenol A (BPA), which mimics natural endogenous estrogen, has been reported to be involved in the pathogenesis of several diseases and, over the last decade, it has been claimed to have potential diabetogenic and obesogenic actions (Fenichel et al., 2013; Provvisiero et al., 2016). While several epidemiological studies have suggested that BPA exposure is positively associated with an increased risk of T2DM (Sowlat et al., 2016; Provvisiero et al., 2016; Chailurkit et al., 2017), there is lack of data on this in Asian Indians, a population highly prone to the epidemic of diabetes and its vascular complications (Gujral et al., 2015).

T2DM among Asian Indians is characterized by the onset at a younger age, greater abdominal obesity despite of having relatively lower BMI, greater insulin resistance, and early decline in beta cell function (Shah & Mohan, 2015). Tissue dysfunction occurs with aging (McHugh & Gil, 2018) and has systemic effects, including peripheral insulin resistance, inflammation, ectopic lipid accumulation and accelerated senescence, may have a causal role in the pathogenesis of T2DM (Le Lay & Dugail, 2009; Palmer et al., 2015).

Senescent cells may play a role in the pathogenesis of T2DM by impacting pancreatic β-cell function, senescence-associated secretory phenotype (SASP) mediated tissue damage, and involvement in multiple tissue defects (Regulski, 2018). One of the well-established features of senescence is the up regulation of p16-RB and/or p53-p21 proteins which typically involves in the inhibition of cyclin dependent kinase activity resulting in cell cycle arrest (López-Otín et al., 2013; Althubiti et al., 2014; Caliò et al., 2015). Hence, it becomes important for our study to analyze the expression levels of these genes as an indicator of senescence. Increased levels of lysosomal enzyme and its activity is a hallmark of senescence (Wagner et al., 2015). Thus, increased GLB1 gene (β-gal) expression which encodes lysosomal enzyme was used as one of the markers of senescence in this study.

SASP, which is produced in senescent cell comprises of pro-inflammatory cytokines, growth factors that alters tissue microenvironments and induce malignant phenotypes in nearby cells (Watanabe et al., 2017). TNF- α and IL-6 are among the major secreted pro-inflammatory cytokines during senescence (Tchkonia et al., 2013; Prattichizzo et al., 2016; Meyer et al., 2017) that are also shown to be elevated during the pathogenesis of T2DM (Mirza et al., 2012). Our study analysed the expression patterns of these inflammatory and associated markers like suppressor of cytokine signalling 3 (SOCS3), which is a major regulator of inflammatory cytokines among SOCS family of proteins (Carow & Rottenberg, 2014; Liu et al., 2015).

In one of our pioneering works (Adaikalakoteswari et al., 2005), we have reported increased shortening of telomeres in patients with T2DM. Telomere shortening is also a validated biomarker for cellular senescence (Bernadotte et al., 2016). In addition to the comparison of telomere length between diabetic subjects and normal subjects, our study also examined the correlation between BPA levels found in the study participants with the telomere length, which we believe will show the impact of BPA exposure on telomere length.

Our study also focused on analysing the expression levels of several other important genes such as estrogen receptors, estrogen related receptors, AR, AHR, NOD-1, NOD-2 and metabolic receptors that are involved in the BPA mechanism of action in the pathogenesis of metabolic diseases.

While BPA exposure has been linked to DNA damage and senescence (Qin et al., 2012; Herz et al., 2017), there is lack of data on the cellular and molecular alterations in the clinical setting. With this background, the present study has determined the systemic levels of BPA in

patients with T2DM compared to healthy individuals and have assessed the biochemical and molecular correlations with special reference to senescence and inflammatory markers.

2.3 Methodology

Most of the methods followed in this chapter was adopted from Sathish et al. (2016) and other collegues from Madras Diabetes Research Foundation.

2.3.1 Ethical Statement

Human research ethics approval was obtained from the Institutional Ethics Committe (IEC) of Deakin University as well as Madras Diabetes Research Foundation according to national guidelines: DU-HREC:2015-169. The study was conducted adhering to the principles of Declaration of Helsinki.

2.3.2 Recruitment of study participants

The study participants were recruited from the on-going epidemiology studies at the Madras Diabetes Research Foundation and from Dr. Mohan's Diabetes Specialities Centre, a tertiary diabetes centre at Chennai, South India. The study groups comprised of participants (age between 35 and 55 years) with normal glucose tolerance subjects (NGT, n=30) and patients with Type 2 Diabetes mellitus (T2DM, n=30) clinically well-characterized based on World Health Organization (WHO) criteria for the diagnosis of diabetes. According to WHO, subjects with fasting plasma glucose $\geq$ 126 mg/dL (7.0 mmol/L) and/or 2 hours plasma glucose concentration $\geq$ 200 mg/dL (11.1 mmol/L) after orally consuming 75 grams of anhydrous glucose in an oral glucose tolerance test (OGTT) are classified as diabetes. Subjects with fasting plasma glucose <100 mg/dL and a 2-hour post load plasma glucose of <140 mg/dL are classified as normal glucose tolerant subjects.

Fasting blood samples were collected in the presence and absence of anticoagulant tubes and samples were centrifuged at 4,000 rpm (rotations per minute) for 10 minutes, followed by 12,000 rpm for 15 minutes to completely remove cell debris and serum was stored at -80°C in BPA-free polypropylene cryo-tubes (Catalog#FC430488, Corning) and was handled separately for BPA estimation.

2.3.3 Anthropometric measurements

Anthropometric measurements that includes height, weight and waist circumference were measured using standardized techniques. Height was noted down using a tape measured to the nearest centimeter. Weight was measured in kilograms using electronic weighing machine that was kept on a firm horizontal surface. Waist circumference was measured by a non-

stretchable fiber measuring tape. Body Mass Index (BMI) which is a simple index commonly used for determining underweight, overweight and obesity in adults (WHO) was calculated as the weight in kilograms divided by height in metre square (kg/m^2). Blood pressure was recorded from the right arm in a sitting position to the nearest 2 mmHg using mercury sphygmomanometer (Diamond Deluxe BP apparatus, Pune, India). Readings were recorded twice in the interval of 5 minutes and the mean of those was taken as blood pressure.

2.3.4 Biochemical estimation

Biochemical estimation of fasting plasma glucose (glucose oxidase-peroxidase method), serum triglycerides (glycerol phosphate oxidase-peroxidase-amidopyrine method), serum cholesterol (cholesterol oxidase-peroxidase-amidopyrine method), and HDL cholesterol (by direct method-polyethylene glycol-pre-treated enzymes) were measured using Hitachi-912 Autoanalyser (Hitachi, Mannheim, Germany). The inter assay and intra assay coefficient of variation for these biochemical assays were lesser than 5%. Low-density lipoprotein (LDL) cholesterol was estimated using the Friedewald formula (Friedewald et al., 1972). Glycated haemoglobin (HbA1c) was measured using high-pressure liquid chromatography (HPLC) with the variant machine (Bio-Rad, Hercules, Calif., USA). Serum Insulin level was estimated by enzyme-linked immunosorbent assay (Catalog#IS130D, Calbiotech, CA). The inter-assay and intra-assay coefficients of variation for the insulin assay were lesser than 9%. Insulin resistance was calculated using the homeostasis assessment model (HOMA-IR) formula: (Fasting insulin (μIU/mL) **x** Fasting glucose (mmol/L)) / 22.5.

2.3.5 Quantification of serum Bisphenol-A

To estimate total BPA, serum samples (100 μL) was hydrolyzed by adding 215 μL of serum β-glucuronidase enzyme (Catalog#G0751-100 KU, Sigma-Aldrich) to serum samples and this mixture was incubated at 37°C for 16 hours. After incubation, samples clean-up was performed using Strata^{TM} X 33 μm Polymeric Reversed Phase 60 mg/3 mL, tubes (Catalog#8B-S100-UBJ, Phenomenex, India). Strata X column was pre-conditioned with absolute methanol and distilled water, followed by addition of hydrolyzed serum sample mixtures into the column. Final elute was collected in glass tube and samples were air dried using nitrogen gas and the dried product was reconstituted in 10% methanol and it was used for investigation. Total BPA (Free and conjugated) from serum was measured in the 100 μL of serum sample using Super sensitive ELISA kit (Catalog#92893, Ecologiena,

EnviroChemical, Ltd.) as per manufacturer instruction. The intra-assay and the inter-assay coefficient of variation for the assay were <12% and <8% respectively.

2.3.6 Preparation of peripheral blood monolayer cells and RNA isolation

Whole blood (10 ml) was collected in EDTA tube from all the study subjects**.** Peripheral blood monolayer cells (PBMC) were isolated using Histopaque (Catalog#1077, Sigma-Aldrich) following the standard protocol. Blood was overlaid on the density gradient solution and centrifuged at 1500-1800 rpm for 30 minutes. The buffy coat layer that contained PBMCs were collected and washed with phosphate buffered saline (PBS, pH 7.2-7.4). Isolated PBMCs were used immediately for total RNA extraction.

2.3.7 Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from PBMCs using Trizol reagent (Catalog#9109, RNAiso Plus, Takara) as per manufacturer's instruction and RNA was eluted using 100 µl of nuclease free water. The total RNA quality and the quantity were measured using NanoDrop 2000 (Thermo Scientific) and RNA samples were stored at -80°C. First strand cDNA was synthesized using 1 µg of total RNA, 40 µM Oligo-dT18 primer (New England Biolabs), 10x RT buffer and 2.5 mM each of dNTPS and 100 units reverse transcriptase enzyme. The reaction condition includes 42°C for 1 hour which is followed by 95°C for 5 minutes to heat inactivate the enzyme.

2.3.8 Gene expression by Real-time PCR

Quantitative real-time PCR was performed for specific genes by preparing reaction mixture with SYBR green master mix (Catalog#F416S Dynamo Color flash Sybr Green QPCR Kit). PCR amplification was carried out using LC -96 (Light cycler 96, Roche cycle) with the following cycle conditions: Initial cycle at 50°C for 2 minutes, Initial denaturation at 95°C for 15 minutes, 45 cycles of denaturation at 95°C for 15 seconds and Annealing/Extension at 60°C for 1 minute). The relative quantification was calculated using 2^{-ddct} method and normalized by β-actin. List of genes analyzed in this study are as follows: β-actin, GLB1 (β-Gal), p16, p21, p53, ER-α, ER-β, ERR-α, ERR-β, ERR-γ, Socs-3, IL-6, TNF-α, CYP2C9, CYP2C18, AHR, AR, NOD-1, NOD-2, GPER-1 and PPAR-γ. List of primers used were given in **Table 1**.

2.3.9 Telomere length measurement by Real-time PCR

For the measurement of telomere length, DNA was isolated using back extraction method (Triant & Whitehead, 2009). Real-time PCR was used to determine the length of telomere as previously described (Monickaraj et al., 2012). 30 ng of DNA was used for the experiment. Amplification was carried out using LC-96 (Light cycler 96, Roche cycle) with cycle conditions (45 cycles of 95°C for 15 seconds, 60°C for 1 minute). Telomere length (TL) was expressed as a ratio of number of repeated copies of the telomere (T) over single gene copy number (T/S). The beta-hemoglobin gene (36B4) was used as a reference single-copy gene. Primers used were given in **Table 1**.

Gene	Forward Primer (5' - 3')	Reverse primer (5' - 3')
β-ACTIN	ACCTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG
GLB1	ACGGTGGACTTTGGAACAG	TGATTGTGGAGTGAGGTTGG
p16	GATGTCGCACGGTACCTG	TCTCTGGTTCTTTCAATCGGG
p21	TGTCACTGTCTTGTACCCTTG	GGCGTTTGGAGTGGTAGAA
p53	GCCATCTACAAGCAGTCACAG	TCATCCAAATACTCCACACGC
IL-6	TGGATTCAATGAGGAGACTTG	TGTTCTGGAGGTACTCTAGG
TNF-α	GGACGAACATCCAACCTTCCC	GTGGTCTTGTTGCTTAAAGTTCTAAG
SOCS-3	TCGATTCGGGACCAGC	GCGGGAAACTTGCTGT
ER-α	CGACTATATGTGTCCAGCCAC	CCTCTTCGGTCTTTTCGTATCC
ER-β	GGTCGTGTGAAGGATGTAAGG	TTCCCACTTCGTAACACTTCC
ERR-α	TGCCAATTCAGACTCTGTGC	CCAGCTTCACCCCATAGAAA
ERR-β	GCAAAGTGCCCATGCACAAA	TGATCCCTGCTTGTGAAGGC
ERR-γ	GAAATCACAAAGCGCAGACG	CTGTTCTCCGCATCTATCCTG
NOD-1	ACAATCTCAACGACTACGGC	CTTAGCACCTTTACCCCACC
NOD-2	AACTCTGTGGGTGACATTGG	GATGCCTCGGTCTGAGATATTG
GPER-1	CATTCCAGACAGCACCGAG	AGTTTAGAGACATGACGTGGC
PPAR-γ	TCTCTCCGTAATGGAAGACC	GCATTATGAGACATCCCCAC
AHR	CCAACATCACCTACGCCAG	CCAAACGGTCCAACTCTGTAT
AR	AATCCCACATCCTGCTCAAG	AAGTCCACGCTCACCATG
CYP2C9	CACGAGGTCCAGAGATACATTG	CATGTAGCACAGAAGTCAGGG
CYP2C18	AACATCCCTGACTTCTGTGC	TCTCCCATACACATCCGTTTTC
36B4	CAGCAAGTGGGAAGGTGTAATCC	CCCATTCTATCATCAACGGGTACAA
TELOMERE	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT

Table 1: Shows the list of primers used in this study for real time PCR.

2.3.10 Statistical analysis

All the data were expressed as Mean ± SEM or Mean ± SD. Comparisons between groups were performed using unpaired student's t-test. Pearson correlation analysis was performed to determine the relation between BPA levels with other variables such as metabolic risk variables and gene expression. The correlation is done between BPA levels and other variables irrespective of the disease status of an individual. Binary logistic regression analysis was used to determine the association of independent molecular signatures with BPA. p-value <0.05 was considered statistically significant. All the statistical analyses were carried out using Windows based Statistical Package for Social Science (SPSS Version 20, Chicago, IL).

2.4 RESULTS

2.4.1 Clinical & Biochemical characteristics of the study groups

Clinical and biochemical characteristics of the study subjects were given in **Table 2**. BMI was significantly higher in T2DM patients compared to control participants. Patients with T2DM exhibited significant increase ($p<0.05$) in fasting plasma glucose levels, 2 hours post glucose levels and HbA1c compared to the control subjects. Patients with T2DM were also insulin-resistant as characterized by the significant elevation in HOMA-IR values. Compared to control subjects, there were also significant higher levels of lipids (total cholesterol, triglyceride and VLDL cholesterol) in patients with T2DM.

	Normal Glucose Tolerance (NGT)	Type 2 Diabetes Mellitus (T2DM)
Male/Female	(15/15)	(16/14)
Age (years)	43.5 ± 6.1	48.5 ± 6.7*
Body Mass Index (kg/m2)	25.5 ± 3.9	27.7 ± 3.7*
Waist/Hip	0.92 ± 0.1	0.95 ± 0.1
Fasting plasma glucose (mg/dL)	91.2 ± 8.3	132.3 ± 35.1*
2 hours plasma glucose (mg/dL)	103.8 ± 16.9	271.7 ± 62.9*
Fasting Insulin (uIU/mL)	9.43 ± 5.6	10.9 ± 6.1
HOMA IR	2.1 ± 1.4	3.5 ± 1.9*
HbA1c (%)	5.5 ± 0.4	7.2 ± 1.2*
Total Cholesterol (mg/dL)	175.1 ± 33.2	193.7 ± 30.4*
Serum Triglyceride (mg/dL)	116.3 ± 58.7	168.4 ± 78.2*
LDL Cholesterol (mg/dL)	108.4 ± 29.1	117.6 ± 26.4
HDL Cholesterol (mg/dL)	43.33 ± 11.1	41.3 ± 8.5
VLDL Cholesterol (mg/dL)	23.4 ± 11.7	32.4 ± 13.0*
Systolic Blood Pressure (mm/Hg)	122.4 ± 18.6	127.5 ± 9.7
Diastolic Blood Pressure (mm/Hg)	79.1 ± 11	81.6 ± 7.1

Table 2 shows the **clinical characterization of the study subjects**. Comparison is between type 2 diabetes subjects (n=30) with normal glucose tolerant subjects (n=30). Data represented as mean ± SD. *$p<0.01$ compared to NGT subjects.

2.4.2 Systemic BPA levels in the study participants

Serum BPA levels (Mean ± SEM) in patients with T2DM (52.4±3.8 µg/L) were found to be significantly higher ($p<0.05$) compared to NGT individuals (41.7±3.0 µg/L) (**Fig 1**). T2DM subjects showed approximately, a 0.3 fold increase in BPA levels over NGT subjects, which was visible between the groups.

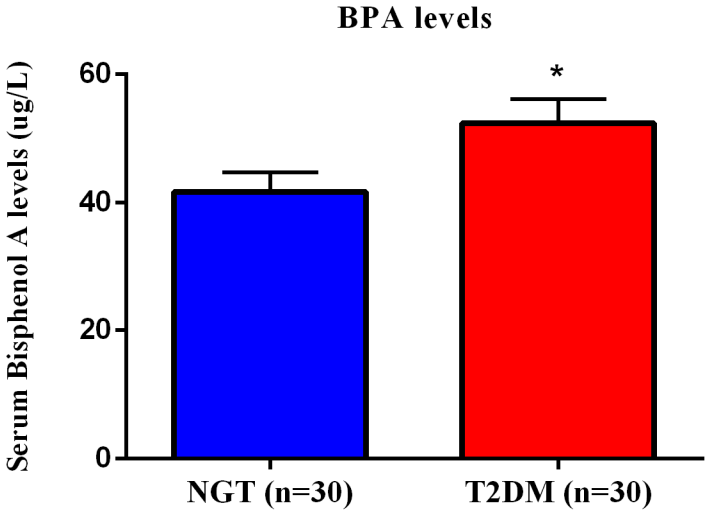


Figure 1: Elevated BPA levels in the patients with type 2 diabetes: Serum BPA concentration quantified from patients with type 2 diabetes (n=30) and normal glucose tolerance (n=30). Bars represent the mean ± standard mean error. *$p<0.01$ compared to control subjects.

2.4.3 Transcriptional levels of senescence, inflammatory, estrogen and metabolic receptor markers:

Gene expression amplified by Real-time PCR and sequences of the primers used for their amplification are depicted in **Table 2**. **Figure 2** shows PBMCs gene expression levels of senescence markers (GLB1, p16, p21 and p53) in T2DM patients compared to NGT individuals. All the senescence markers were found significantly elevated in PBMCs from patients with T2DM compared to NGT. GLB1 expression showed an increase in expression levels in T2DM by ~0.4 fold of the control and p16 expression levels showed an increase in

T2DM by ~0.8 fold of the control. Whereas, gene expression levels of p21 and p53 in T2DM subjects showed significant increase by ~0.6 fold of the NGT. The process of senescence is not limited to cell cycle arrest. Senescent cells are metabolically active and acquire the state of Senescence Associated Secretary Phenotype (SASP) (Wiley & Campisi, 2016). Therefore, we analyzed the gene expression state of pro-inflammatory signatures in these cells. As shown in **Figure 3**, transcriptional levels of inflammation and associated markers (IL-6, TNF-α and SOCS-3) were found significantly elevated in patients with T2DM compared to control subjects. IL-6 and TNF-α showed significant increase in T2DM by ~0.6 and ~0.7 fold of the NGT respectively, while SOCS-3 had ~1.1 fold significant increase in T2DM over NGT. This is indicative of presence of increased SASP in T2DM subjects which is a component of senescence process.

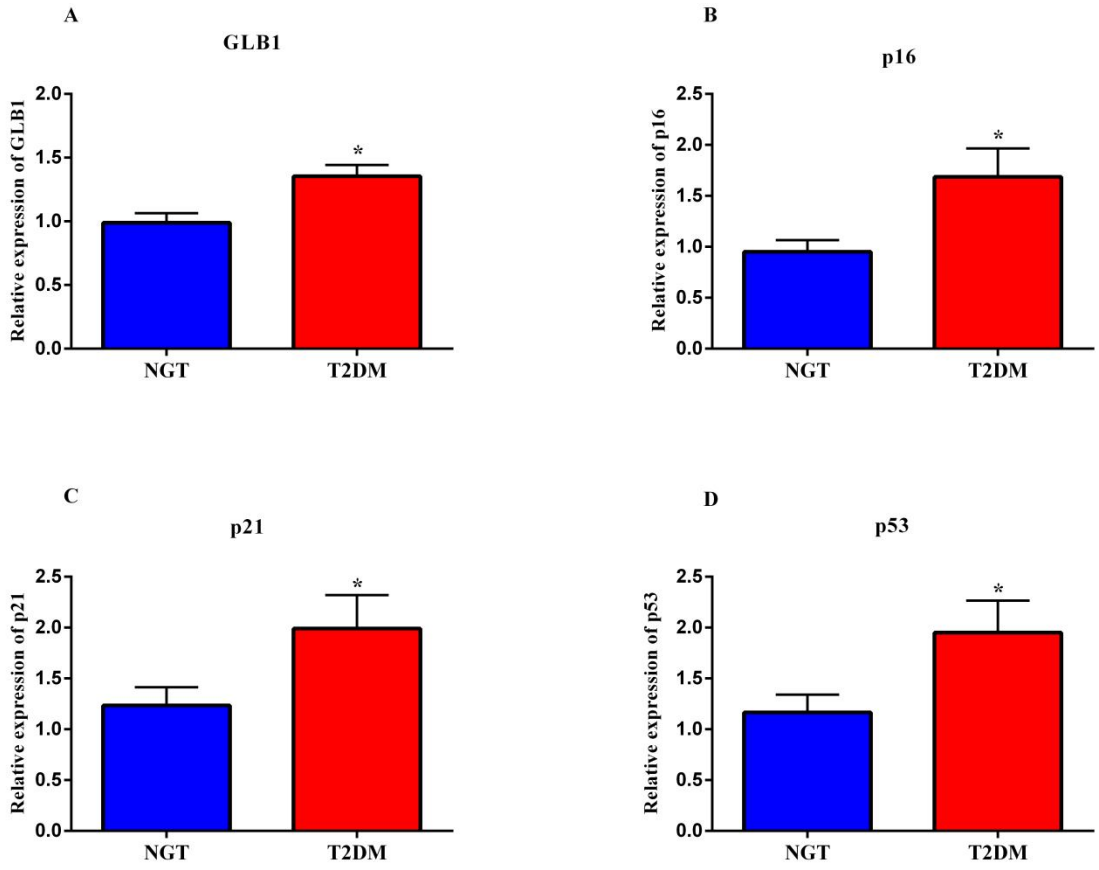


Figure 2 (A-D): **Gene expression analysis of senescence markers:** Real-time quantitative RT-PCR analysis of mRNA expression levels of Senescence markers- (A) GLB1 (β-GAL), (B) p16, (C) p21, (D) p53. All the senescence markers are seen significantly elevated in T2DM subjects (n=30) compared to NGT subjects (n=30). Bars represent the mean ± standard mean error. *p<0.05 vs NGT.

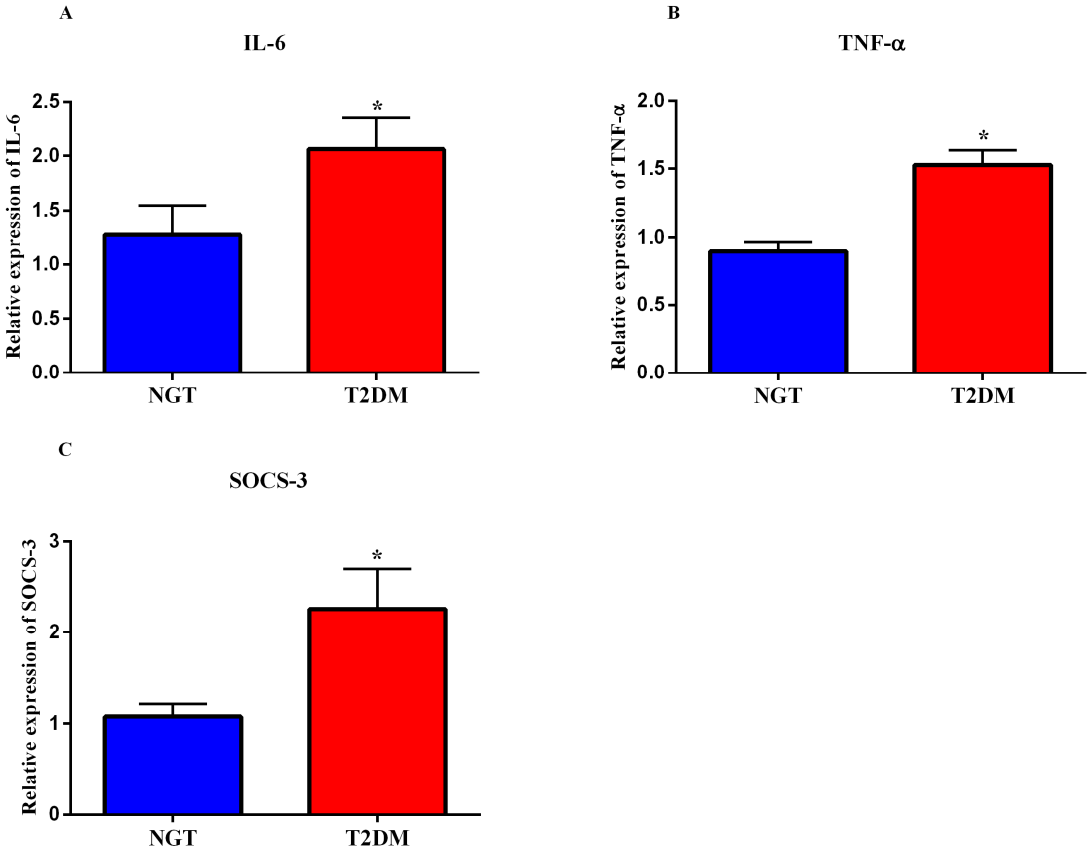


Figure 3 (A-C): **Gene expression analysis of Inflammatory markers:** Real-time quantitative RT-PCR analysis of mRNA expression levels of inflammatory markers- (A) IL-6, (B) TNF-α, (C) Socs-3. All the three inflammatory markers are seen significantly elevated in T2DM subjects (n=30) compared to NGT subjects (n=30). Bars represent the mean ± standard mean error. *p<0.05 vs NGT.

Among the profiled gene expression levels of various estrogen receptors (ER-α, ER-β) and estrogen related receptors (ERR-α, ERR-β, ERRγ), expression levels of ERRγ were found significantly elevated in T2DM patients which is ~0.6 fold of the NGT (**Figure 4**). Among the other transcriptional signatures tested, gene expression levels of PPARγ and NOD-2 showed ~1.5 fold and ~0.4 fold respectively, significant increase in patients with T2DM over NGT (**Figure 5**), whereas receptors like Aryl Hydrogen receptor (AHR), Androgen receptor (AR), GPER-1 and NOD-1 did not show differential expression with statistical significance. Interestingly, BPA metabolism related enzyme like CYP2C9 showed an increase in expression levels in T2DM by ~1.2 fold of the NGT (**Figure 5)**.

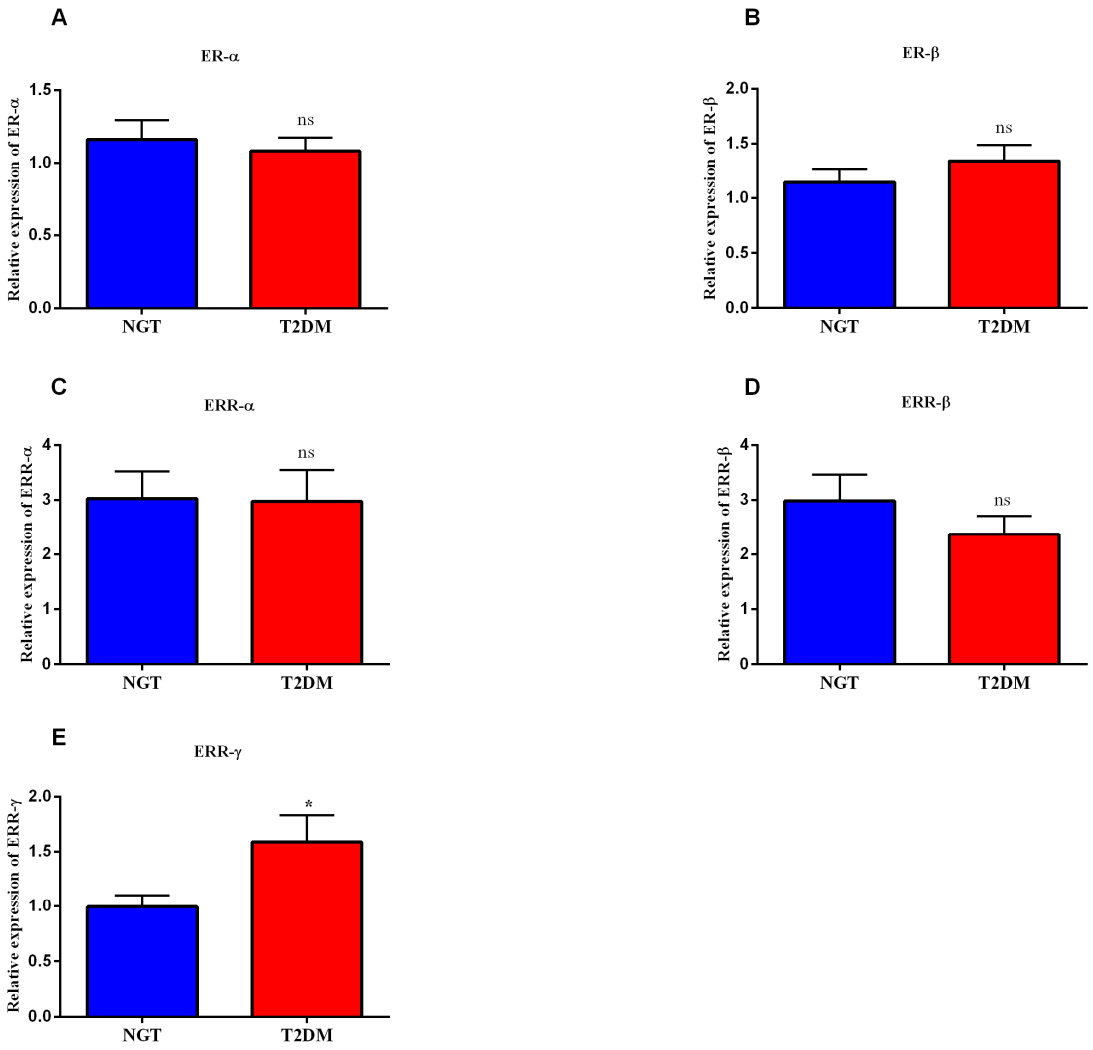


Figure 4 (A-E): **Gene expression analysis of estrogen and estrogen related receptors:** Real-time quantitative RT-PCR analysis of mRNA expression levels of estrogen receptors- (A) ER-α and (B) ER-β; and estrogen related receptors- (C) ERR-α, (D) ERR-β, (E) ERR-γ. No significant difference is seen in any of the receptor mRNA levels apart from ERRγ, which was found to be significantly increased in T2DM subjects (n=30) compared to NGT subjects (n=30). Bars represent the mean ± standard mean error. *p<0.05 vs NGT.

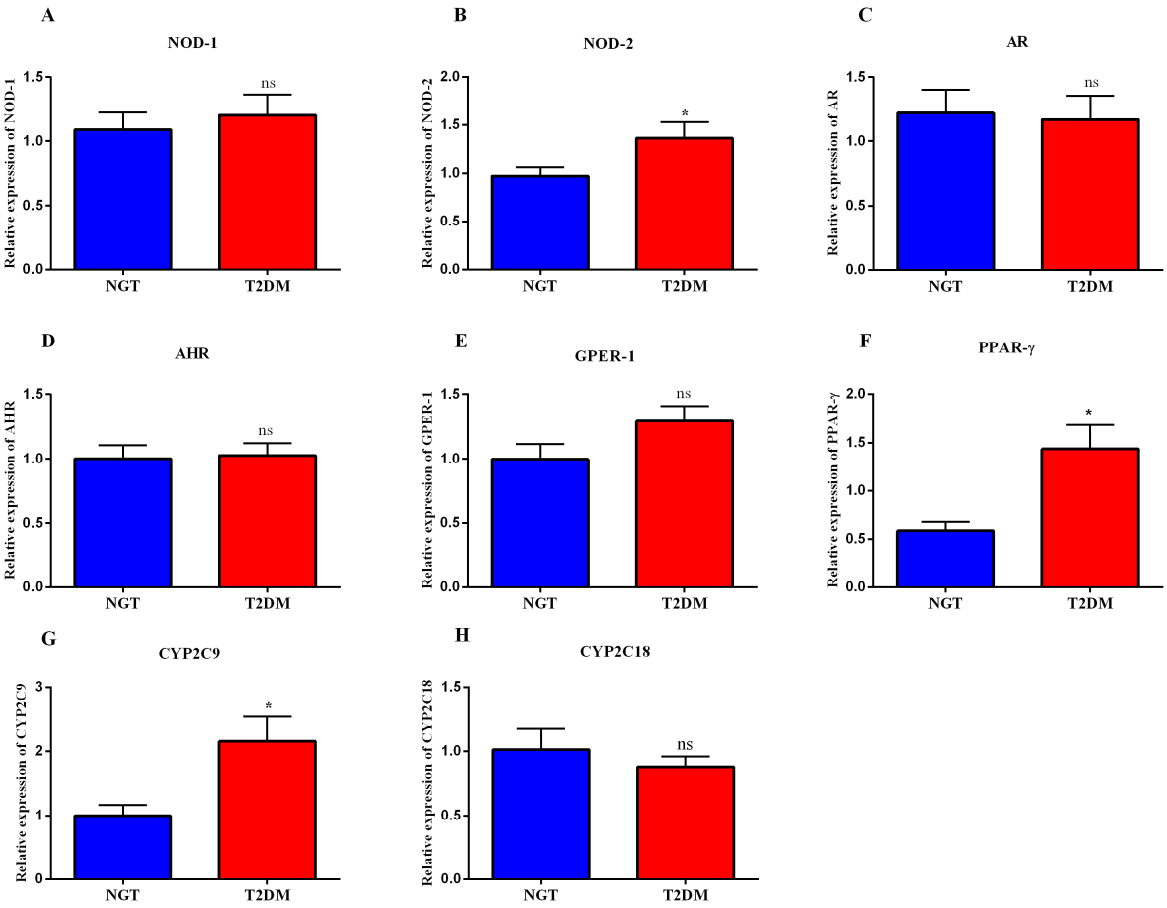


Figure 5 (A-H): **Gene expression analysis of metabolic receptors:** Real-time quantitative RT-PCR analysis of mRNA expression levels of (A) NOD-1, (B) NOD-2, (C) AR, (D) AHR, (E) GPER-1, (F) PPARγ, (G) CYP2C9, (H) CYP2C18. No significant difference was seen in any mRNA levels of NOD-1, AR, AHR, GPER-1 and CYP2C18. Significant increase in mRNA expression of NOD-2, PPARγ and CYP2C9 are seen in T2DM subjects (n=30) compared to NGT subjects (n=30). Bars represent the mean ± standard mean error. *p<0.05 vs NGT.

2.4.4 Shortened telomeres in patients with T2DM

Consistent with the accelerated senescent markers at the transcriptional level, patients with T2DM exhibited significantly shorter telomeres compared to NGT subjects (**Figure 6**). Mean ± SD of telomere length (T/R ratio) of T2DM and NGT subjects were (1.42 ± 0.04) and (1.44 ± 0.04) respectively.

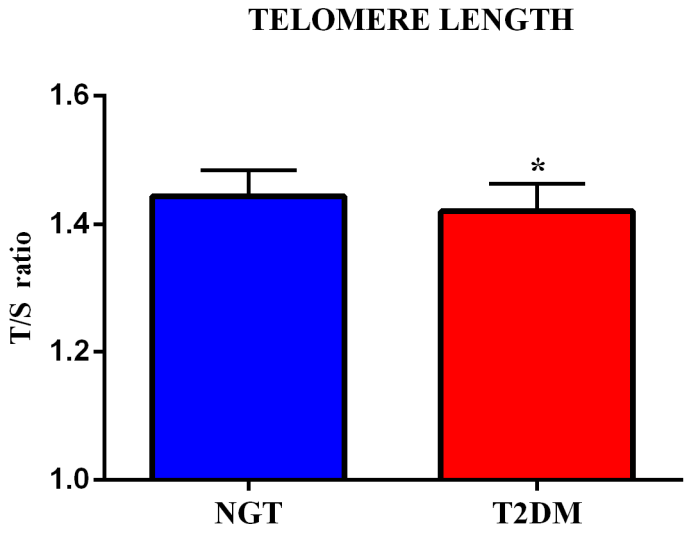


Figure 6: **Telomere length** as measured by Real-time PCR. Telomere length in T2DM subjects (n=30) were significantly lesser when compared to NGT subjects (n=30). Bar diagram represent T/S ratio in the patient with T2DM and NGT with ± SD. *p<0.05 vs. NGT.

2.4.5 Correlation between BPA levels and Biochemical parameters/Molecular signatures

Biochemical parameters like plasma glucose (Correlation coefficient (r) =0.400, p=0.002), HbA1c (r=0.395, p=0.002), triglyceride (r=0.417, p=0.001), and VLDL (r=0.381, p=0.003) were significantly and positively correlated with BPA levels while there was a negative correlation between HDL and BPA levels (r=-0.352, p=0.006). Insulin resistance HOMA-IR showed positive correlation with BPA levels (r=0.356, p=0.006). Senescence indicators: GLB1 (r=0.414, p=0.001), p16 (r=0.353, p=0.006), p21 (r=0.375, p=0.003), p53 (r=0.279, p=0.032), inflammatory markers: TNF-α (r=0.309, p=0.017) and IL-6 (r=0.294, p=0.024) and receptors like ERRγ (r=0.284, p=0.029), GPER-1 (r=0.256, p=0.05) and AHR (r=0.304, p=0.019) were significantly and positively correlated with BPA levels. Telomere length showed negative correlation with BPA levels (r=-0.256, p=0.049) (**Table 3**). This indicates that as the level of BPA increases the telomere length reduces. This is the first study to report a negative correlation between BPA levels and telomere length in a clinical setup.

Clinical parameter and mRNA levels	BPA levels	
	r-value	*p*-value
Fasting plasma glucose (mg/dL)	0.400*	0.002
2 hours plasma glucose (mg/dL)	0.395*	0.002
HOMA-IR	0.356*	0.006
HbA1c (%)	0.338*	0.009
Body Mass Index (BMI) (kg/m2)	-0.103	0.438
Weight/Hip Ratio	0.226	0.086
Total Cholesterol (mg/dL)	0.092	0.488
Triglyceride (mg/dL)	0.417*	0.001
HDL Cholesterol (mg/dL)	-0.352*	0.006
LDL Cholesterol (mg/dL)	-0.034	0.798
VLDL Cholesterol (mg/dL)	0.381*	0.003
Systolic Blood Pressure (mm/Hg)	0.013	0.925
Diastolic Blood Pressure (mm/Hg)	0.187	0.155
GLB1	0.414*	0.001
p16	0.353*	0.006
p21	0.375*	0.003
p53	0.279*	0.032
SOCS-3	0.166	0.208
IL-6	0.294*	0.024
TNF-α	0.309*	0.017
ER-α	0.140	0.29
ER- β	0.148	0.264
ERR-α	0.095	0.473
ERR- β	-0.011	0.936
ERRγ	0.284*	0.029
GPER-1	0.256*	0.05
AHR	0.304*	0.019
AR	-0.063	0.633
PPAR γ	0.112	0.397
NOD-1	0.074	0.575
NOD-2	0.176	0.183
CYP2C9	0.134	0.311
CYP2C18	-0.159	0.228
Telomere Length	-0.258*	0.049

Table 3: Pearson correlation analysis of serum BPA levels with biochemical parameters and gene expression in the study subjects. Adjusted for age. *$p<0.005$ indicates significance.

2.4.6 Association analysis

Binary logistic regression analysis was performed to identify the association between BPA and diabetes. Unadjusted BPA showed significant association with diabetes (β-value 1.032, CI- 1.002-1.063, $p<0.05$). When adjusted for confounding factors like age, gender and BMI, BPA was still significant (β-value 1.052, CI- 1.011-1.096, $p<0.05$). Whereas, when adjusted with HOMA-IR, senescence markers (GLB1, p16, p21, p53), and inflammatory markers (Socs-3, IL-6 and TNF-alpha), ERRγ, and telomere length, it lost its significance indicating that BPA was associated with above markers (**Table 4**).

Parameters	β-value	95% C.I. for EXP(B)	p-value
Dependent Variable: T2DM			
BPA unadjusted	1.032	1.002-1.063	0.036
BPA adjusted For:			
Age, Gender, BMI	1.052	1.011-1.096	0.014
Age, Gender, BMI, HOMA IR	1.033	0.990-1.078	0.134
Senescence Markers (GLB1, p16, p21, p53)	1.017	0.984-1.052	0.311
Inflammatory Markers (SOCS-3, IL-6, TNF-α)	1.016	0.979-1.054	0.411
ERRγ	1.024	0.993-1.056	0.126
Telomere Length	1.027	0.996-1.059	0.091

Table 4: Binary logistic regression analysis of serum BPA levels with biochemical parameters and gene expression in the study subjects. *$p<0.005$ indicates significance.

2.5 Discussion

Increasing exposure to EDCs is suspected as a contributive factor to T2DM along with known causes such as reduced insulin sensitivity, obesity and defective β-cell mass and function (Ashley-Martin et al., 2014; Garcia-Arevalo et al., 2015). Several epidemiological studies found higher BPA and chlorinated BPA levels to be associated with T2DM (Shankar & Teppala, 2011; Sun et al., 2014; Aekplakorn et al., 2015; Andra et al., 2015). In the absence of data on Indians, our study not only reports an association of increased systemic levels of BPA in patients with T2DM but also provides preliminary data on association among elevated BPA levels, poor glycemic control, insulin resistance, accelerated senescence and telomere shortening in patients with T2DM.

Cellular senescence is characterized by irreversible replicative arrest, apoptosis resistance, and frequent acquisition of a pro-inflammatory, tissue-destructive senescence-associated secretory phenotype (SASP) (Kirkland & Tchkonia, 2017). In our study, several senescence markers (GLB1, p16, p21, p53) at their transcriptional state were seen increased and correlated positively to the elevated BPA levels in patients with T2DM. The fact that cells from patients with T2DM also exhibited increased gene expression of inflammatory markers (TNF-α and IL-6) reflects that these cells acquired SASP status. As previous studies have demonstrated an induction of DNA damage, senescence and epigenetic changes by BPA exposure (Qin et al., 2012; Ribeiro-Varandas et al., 2014; Jalal et al., 2017), our study suggests that studying the link between BPA and SASP should be a priority in the prevention as well as management aspects of metabolic disorders including T2DM.

It has been reported from *in vivo* and *in vitro* studies that BPA exerts its disrupting effects on the classical nuclear receptors as well as non-classical types so as to mediate metabolic dysfunction (Alonso-Magdalena et al., 2012). Among the gene expression patterns of ERs and ERRs profiled in our study, most strikingly there was a significant increase in the gene expression of ERR-γ in cells from T2DM subjects and it was positively correlated to the systemic levels of BPA. This is an important finding in that ERRγ has been identified as an *in vivo* receptor for BPA (Tohmé et al., 2014) and it has been shown to promote hepatic gluconeogenesis (Misra et al., 2016) – one of the hallmark pathogenesis of T2DM. While our study reports increased gene expression of PPARγ in patients with T2DM, it is important to realize that several environmental pollutants including BPA exert their adverse impact on adipogenesis and metabolic dysfunction through disturbing the PPAR signalling pathway

(Huang & Chen, 2017). Increased gene expression of nucleotide-binding oligomerization domain-2 (NOD2) in patients with T2DM seen in our study is also consistent with our previous findings (Shiny et al., 2013) and its emerging role in proinflammation (Negroni et al., 2018).

Increased gene expression of CYP2C9 in patients with T2DM compared to NGT individuals is an important observation in our study. CYP2C9 is an important enzyme in the liver metabolism which is responsible for metabolizing a variety of clinically prescribed drugs including anti-diabetic medication (Goldstein & de Morais, 1994). Findings by Xu et al. (2017) suggested that BPA exposure has a potential risk of developing adverse health effects in human liver metabolism by up regulation of CYP2C9 expression. While diabetic patients with variant CYP2C9 genotypes were shown more likely to encounter hypoglycemic adverse effects (Holstein et al., 2005), recent findings suggest that CYP2C9*3 genetic variant independently contributes to good glycaemic control of patients with T2DM treated with glibenclamide (Castelan-Martinez et al., 2018). As variable distribution of CYP2C9 amino acid substitution alleles has also been reported in South Indian diabetes patients (Rao et al., 2017), further studies are needed to unravel the association of increased BPA levels and increased CYP2C9 gene expression.

Our study is of its first-kind to report an association among elevated BPA levels, poor glycemic control, insulin resistance, accelerated senescence and telomere shortening in patients with T2DM. The decrease in mean telomere length from 1.44 to 1.42 is relatively small, but it is statistically significant. Moreover, telomere attrition is a slow senescence/ageing related factor and even with the current limitations of the gold-standard qPCR evaluation of telomere length, such small differences have been demonstrated to reflect biological significance as demonstrated in several studies (Monickaraj et al., 2013; Piplani et al., 2018; Sathishkumar et al., 2018). Our pioneering works have unravelled shortened telomeres linked to the etiology of T2DM (Adaikalakoteswari et al., 2005; Monickaraj et al., 2012). Although genetic variants could influence shortening of telomeres in T2DM (Zhou et al., 2016), recent studies imply a predominant role by environmental factors. Environmental and occupational exposures that include traffic-related air pollution and pesticides were reported to induce telomere shortening (Zang et al., 2013; Ziegler et al., 2017). Exposure to Persistent Organic Pollutants (POPs) has been earlier shown to predict shortened telomeres in the Helsinki Birth Cohort Study (Guzzardi et al., 2016). Very recently, Yuan et al. (2018) also observed that the POPs exposure was highly associated with the occurrence of the

T2DM, shortened and dysfunctional telomeres with senescence-associated secretary phenotype characteristics.

While there are also reports on the exposure to BPA and gender differences (Caporossi & Papaleo, 2015), a recent study reported an association of higher urinary BPA concentrations with shortened telomeres in females (Awada et al., 2018). Since the epidemic of T2DM in India is also linked to higher prevalence of gestational diabetes mellitus (GDM) (Bhavadharini et al., 2016) and the endocrine disruptors have been considered as potential risk factors for GDM (Ehrlich et al., 2016), we caution that future studies are warranted to expose the BPA-associated gender-specific health burden in women.

One of the limitations of our study is of its cross-sectional nature. As such we could not extrapolate causal link between elevated systemic levels of BPA and T2DM and it needs a prospective follow-up study. Moreover, BPA concentrations were only determined in single spot serum samples, which may not reflect chronic exposure levels of bisphenols. However, some studies have reported that the single spot-sampling approach may adequately reflect an average body burden of bisphenols (Ye et al., 2011; Chen et al., 2016). It is suggested that future studies should also examine BPA levels in individuals with prediabetes (ie. individuals with impaired glucose tolerance, IGT) in order to infer and determine whether elevated BPA levels could predate and play a role in the genesis of diabetes. The strength of the study is that for the first-time in a clinical diabetes setting, our study provides a directionality of elevated BPA levels and accelerated senescence (ageing) in patients with T2DM. BPA exposure has been linked to accelerated aging process in the nematode *Caenorhabditis elegans* (Tan et al., 2015) and several cellular stress signalling pathways including increased senescence has been demonstrated in human fetal lung fibroblasts on exposure to BPA (Mahemuti et al., 2018). As one of the characteristics of Asian Indian Phenotype is early onset T2DM (Shah & Mohan, 2015), future studies should focus on the role of BPA as an important environmental contributor to the accelerated aging mechanisms linked to the genesis of diabetes and its complications.

To conclude, our study is first of its kind to report an association among elevated BPA levels, poor glycemic control, insulin resistance, accelerated senescence and telomere shortening in patients with type 2 diabetes.

Chapter 3

Bisphenol A exposure under metabolic stress induces cellular senescence *in vivo* in a p53 independent manner

3.1 Abstract

Senescence or accelerated ageing is an irreversible process that is a characteristic of age-associated diseases like Type 2 diabetes (T2DM). This process of accelerated ageing can be initiated or enhanced by other external stimuli such as stress or environmental contaminants like Bisphenol-A (BPA). BPA, one of the most common endocrine disruptor chemicals, received special attention in the development of insulin resistance and T2DM. However, the causal mechanisms are not clearly understood. For better understanding of the causal mechanisms of BPA, zebrafish embryos were treated with BPA in the absence and presence of metabolic stress (hyperglycaemia) condition. Interestingly, the increase in glucose levels seen in zebrafish embryos under hyperglycaemia was significantly augmented in the presence of BPA. Transcriptional levels of senescence markers such as p15, p53, Rb1 and β-galactosidase (β-gal) were increased in combination of metabolic stress and BPA exposure, and this was also supported by β-gal enzyme expression patterns. In addition, embryos exposed to hyperglycaemia and BPA also exhibited increased apoptosis as evident from acridine orange and cleaved caspase-3 antibody staining. The fact that senescent markers were elevated even in p53 knockdown conditions suggests that senescence might majorly involve p15-Rb1 pathway when embryos were treated with metabolic stress coupled to BPA exposure. Our work exposes a novel and unique converging role of senescence and apoptosis axis contributing to glucose dyshomeostasis. Altogether, we conclude that BPA adds up to the existing metabolic stress which induces increased senescence that leads to aggravation of disease phenotype in age-associated diseases like T2DM.

3.2 Introduction

In the previous chapters, we discussed about the environmental disruptor chemicals and their role in incidence and progression of metabolic diseases such as diabetes. Among them Bisphenol A (BPA), a known xenoestrogen has been reported to be associated with the pathophysiology of metabolic disorders, especially diabetes (Fang et al., 2015; Provvisiero et al., 2016). Diabetes is an age-associated disorder in which cellular senescence plays a prominent role in disease progression (Palmer et al., 2015). Studies have shown that metabolic stress such as high glucose treatment is capable of inducing stress induced pre-mature senescence (SIPS) (Park et al., 2014; Kong et al., 2015; Maeda et al., 2015).

Epidemiological evidences have implied a positive association of BPA with insulin resistance (Savastano et al., 2015; Menale et al., 2017; Hong et al., 2017) and *in vivo* studies have shown that BPA is capable of disrupting β-cell function, insulin signalling and cause insulin resistance (Alonso-Magdalena et al., 2006; Batista et al., 2012).

On the other hand, few studies have linked BPA with cellular senescence. $p16^{INK4a}$ or p16 is an inhibitor of Cyclin dependent kinase 4 and 6 (CDK4/6) resulting in cell cycle arrest (Narita et al., 2003) leading to senescence. In a study by Qin et al. (2012), human mammary epithelial cells (HMEC) that were exposed to BPA showed increased levels of p16 protein which induced cellular senescence. Gestational BPA exposure in animal studies has been linked to pancreatic β-cell senescence in mothers post-delivery as evident from increased expression of p16 and p53 along with enhanced apoptosis, which were associated with pancreatic β-cell dysfunction (Alonso-Magdalenaet al., 2015). Further, the study also showed that BPA can induce epigenetic alterations leading to senescence. Tan et al. (2015) studied the effects of BPA on *C. elegans* and suggested that the animal on exposure to BPA generated ROS and superoxide anions creating oxidative stress condition, which resulted in accelerated aging.

Apart from these limited works, there was no proper scientific evidence for the effect of BPA on accelerated senescence and its causative mechanism under the influence of metabolic stress, especially in an *in vivo* model organism.

Therefore, the aim of this chapter is to study the effect of high glucose-induced senescence changes to see whether that could be augmented by external environmental contaminant like BPA. The study utilized zebrafish as an *in vivo* model.

Zebrafish is an established model organism to study development and to model human diseases (Kari et al., 2007; Veldman & Lin, 2008). In recent years, it is increasingly becoming a suitable model organism to study metabolic anomalies. In fact, zebrafish is a good model organism to study metabolism as it possesses all the key organs involved in energy homeostasis similar to human in addition to the advantages like easy handling, being cost effective, high fecundity rate and lesser life span (Elo et al., 2007; Seth et al., 2013).

This study holds importance in this context, as this is the first unique study highlighting the impact of BPA on the process of accelerated ageing under the influence of metabolic stress in zebrafish (*Danio rerio*) as an *in vivo* model.

3.3 Methodology

3.3.1 Ethical Statement

Animal ethics approval was obtained from Deakin University animal welfare committee in accordance with national guidelines: AWC: G17-2015.

3.3.2 Chemical Treatment

Zebrafish embryos were collected and maintained in E3 media at 28°C. Embryos were treated with 100 nM BPA (Catalog#239658, Sigma), 2.5% of high glucose condition, combination of 100 nM BPA and high glucose and DMSO only as a control. 500 nM reactivation of p53 and induction of tumour cell apoptosis (RITA) (Catalog#213261-59-7, Cayman chemicals) was used as a positive control. All the treatments were done from 3 days post fertilization (3dpf) to 6 days post fertilization (6dpf) **(Figure 1)**. At the end of day 6, experiments were carried out. For those experiments involving staining, de-pigmentation was carried out by treatment by phenylthiourea (PTU), used at concentration of 1:100 or combination of 1% Potassium hydroxide & 3% Hydrogen peroxide for an hour at room temperature.

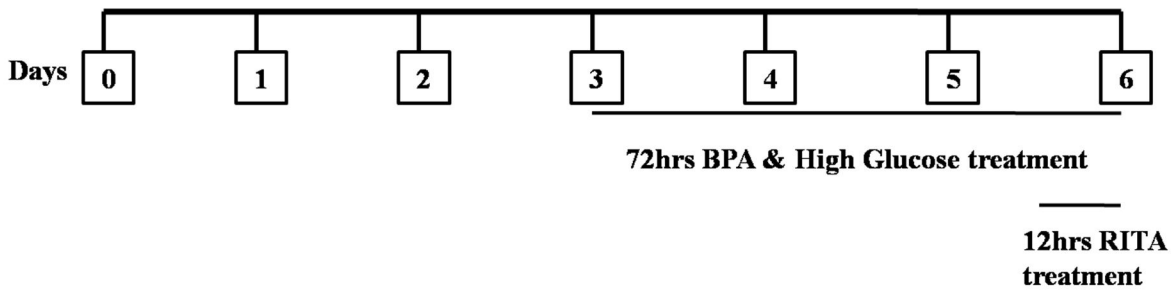


Figure 1: **Illustration depicting treatment timings:** 100 nM BPA and 2.5% high glucose treatments were carried out from day 3 to day 6 of post fertilization. RITA was treated in the last 12 hours of day 6.

3.3.3 Glucose Measurement

At the end of treatment conditions, embryos were pooled together and E3 media was removed, followed by PBS washes. The embryos were then homogenised with electrical homogenizer. Resulting suspension was centrifuged and the supernatant was taken for

glucose measurement. Whole body glucose measurements were recorded with Accu-Check Performa glucose meter.

3.3.4 RNA isolation and qRT-PCR analysis

Real-time PCR was used to examine senescence-related mRNA expression. At the end of the treatment period, embryos were collected and pooled into groups of 30 and processed for RNA isolation using RNeasy Mini Kit (Catalog#74106, QIAGEN). cDNA was synthesised from RNA using Maxima H Minus First Strand cDNA synthesis kit (Catalog#K1652, Thermo Scientific). This was followed by quantitative real-time qRT-PCR assay performed using Power SYBR green PCR master mix (Catalog#4367659, Applied Biosystems). Detection and analysis were performed in PikoReal 96 Real time PCR machine (Thermo Scientific). The amount of single strand DNA (ssDNA) or cDNA concentration was determined in the RT samples using a Quant-iTOliGreen ssDNA Assay Kit (Catalog#O11492, Invitrogen) as previously described (Lundby et al., 2005). For each sample, the amount of a given target cDNA was normalized to the ssDNA content in the sample. Gene expression of senescence markers (viz. β-gal, p15, p21, p53, Rb1) were analysed.

3.3.5 Senescence Associated β-galactosidase assay (SA-β-gal)

Measuring the senescence associated β-galactosidase enzyme (SA-β-gal) activity is recognized as a standard procedure for detecting cellular senescence (Dimri et al., 1995). SA-β-gal assay was performed after the treatment period to analyse the intensity of SA-β-gal expression in the zebrafish embryos using Senescence β-galactosidase Staining Kit (Catalog#9860, Cell Signaling Technology). Embryos were washed and fixed for at least 4 hours. It is then followed by addition of β-galactosidase staining solution (pH 6.0) and kept at 37°C overnight. On the subsequent day, the embryos were stored in 70% glycerol and used for imaging under light microscope.

3.3.6 Acridine Orange staining (Live Imaging)

Acridine Orange (AO), a nucleic acid-sensitive dye was used to stain and visualize the presence of apoptotic cells (Plemel et al., 2017). The cell undergoing apoptosis will produce numerous lysosomes (Mrschtik & Ryan, 2015). AO has the tendency to penetrate cell membrane and bind to the lysosomes and can also intercalate with the nucleic acid (Mpoke et al., 1997; Pierzyńska-Mach et al., 2014). At the end of treatment, embryos were added with

2.5 g/mL of AO staining solution in E3 media. This was followed by incubation at room temperature for 30 minutes. After incubation, embryos were washed with E3 media. Prior to imaging, the embryos were anesthetised with 1X Tricaine, followed by imaging using ZOE fluorescent cell imager (BioRad, CA, USA).

3.3.7 Immunohistochemistry analysis using Anti-Cleaved Caspade-3 antibody staining

At the end of treatment, embryos were fixed in 4% PFA overnight. Fixing solution was removed and the embryos were added with 100% ice-cold methanol and incubated at -20°C for 2 hours or overnight. It is then followed by subsequent washes with 1x PDT (1x PBST, 0.3% Triton-X100, 1% DMSO). Blocking buffer (10% FBS, 2% BSA, 1x PBSTw) was added and incubated at room temperature with gentle rocking for an hour. 1 l of rabbit anti-cleaved (anti-activated) caspase-3 antibody (1:500 dilution) was added. It is then incubated at room temperature with gentle rocking for 2 hours or incubated overnight at 4°C. Next, the blocking buffer was removed, and tubes are washed with 1xPDT. Blocking buffer step was repeated as before but this time with anti-rabbit fluorophore conjugated secondary antibody at a concentration of 10 g/mL. After incubating for 2 hours, the embryos were washed with 1x PDT and were ready for imaging. ZOE fluorescent cell imager was used for capturing fluorescent images.

3.3.8 Morpholino Injection

Antisense morpholino oligonucleotides, obtained from Gene Tools, Inc. (Philomath), for p53 were injected into the embryos at 1 cell stage of post fertilization. Oligo sequence is given in **Table 1**. 4 nl of 1 mM p53 MO stock was injected with phenol red at 4:1 concentration. Treatments were carried out as mentioned before. After appropriate treatment conditions, these p53MO+ embryos were used for determining the expression levels of senescence markers, which is followed by SA-β-gal assay to determine β-gal enzyme levels following the methodology described previously.

3.3.9 Statistical Analysis

All bar graphs are drawn using GraphPad Prism 6. Data represent mean ± SEM. The statistical significance ($*P \leq 0.05$) was determined by One-way ANOVA using GraphPad Prism 6.

3.4 Results

3.4.1 Glucose measurement shows the disruption of glucose homeostasis

One of the criteria to design a study related to high glucose is to ensure that levels of glucose are elevated after the high glucose treatment. Initially, we determined the whole-body glucose level of zebrafish embryos after treatment with 100 nM BPA. Environmentally relevant dose of BPA was determined to be 10-100 nM (Pfeifer et al., 2015). Based on dose response treatment for toxicity, with concentrations varying from 10-100 nM BPA, we determined 100 nM BPA as the optimum for our experiment; as best results in terms of no mortality rate, as well as optimal gene expression of senescence markers were achieved in that concentration. BPA was combined with and without metabolic stress condition for 3 to 6dpf (days post fertilization). Metabolic stress was achieved by treating embryos with 2.5% high glucose. After dose response treatment, 2.5% high glucose was chosen for the treatment. Beyond that concentration mortality rate was seen increased and there was changes in morphogenesis of the embryos. Media with DMSO was used as a vehicle control. 500 nM RITA (reactivation of p53 and induction of tumour cell apoptosis) was used as a positive control. Based on previous publications (Yang et al., 2009; Ahmed et al., 2011) and dose response treatment to check toxicity, 500 nM concentration of RITA was chosen for treatment. 500 nM treatment of RITA for 12 hours showed no mortality and no changes in morphogenesis was observed. At the end of day 6, glucose measurement was carried out with Accu-Check Performa glucose meter. **Figure 2A** shows the levels of whole-body glucose measurement. Presence of metabolic stress was confirmed by the higher levels of whole-body glucose induced by high glucose treatment. The combination of high glucose with BPA had a 2.5-fold increase in the glucose level compared to high glucose condition. Negligible levels of glucose were present in control and BPA and RITA, which was assigned an arbitrary value of 0.6 mmol/L according to manufacturer's protocol. Four-fold increase in the glucose levels of embryos treated with combination of BPA with high glucose treatment in comparison with control indicates the disruption of glucose metabolism.

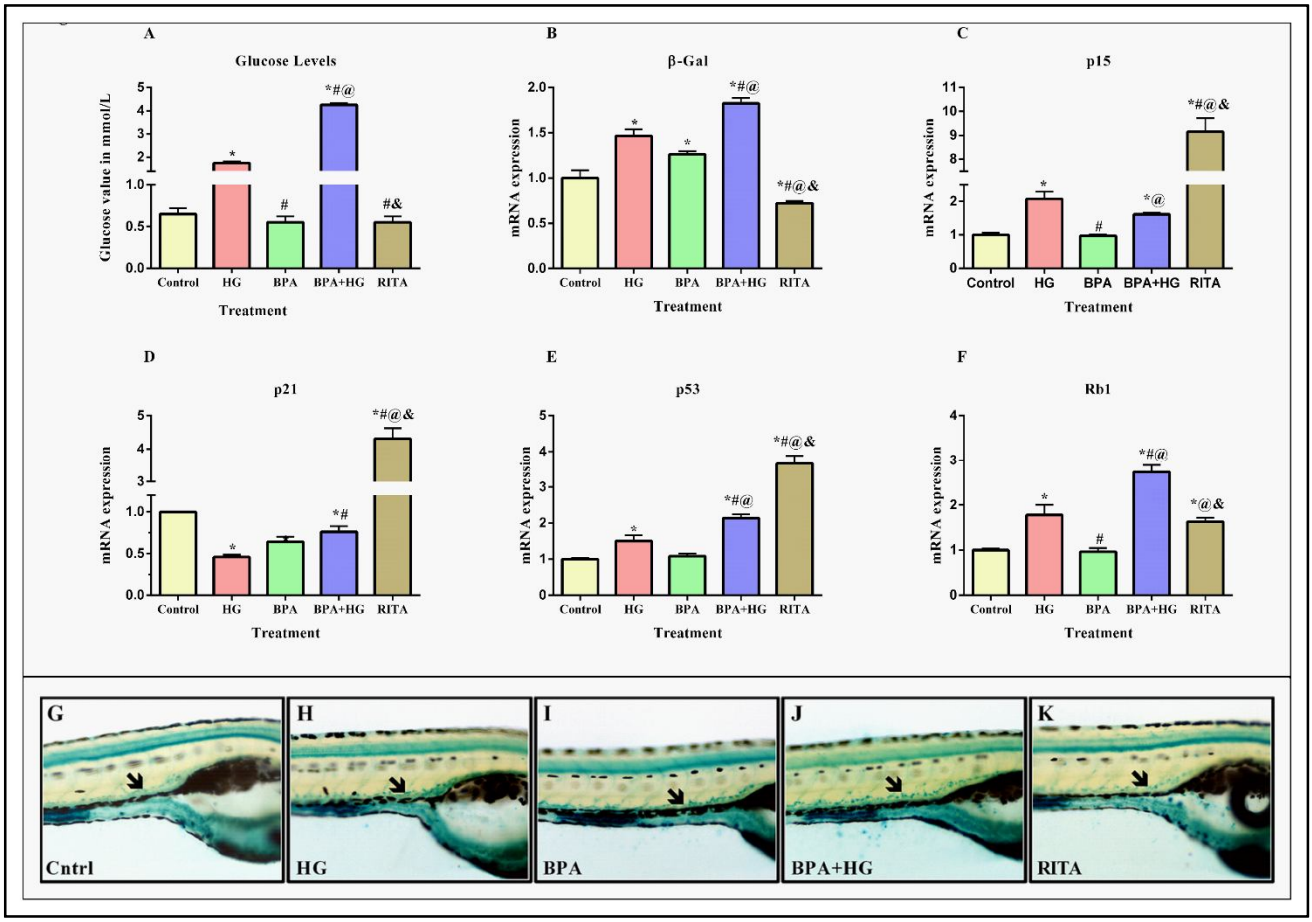


Figure 2: (A) **Whole body glucose measurement** done in 6dpf zebrafish embryos treated from 3dpf to 6dpf, with DMSO (vehicle control), 2.5% glucose (HG), 100 nM BPA, combination treatment of high glucose and BPA (HG+BPA) for 3dpf to 6dpf. 500 nM RITA (reactivation of p53 and induction of tumour cell apoptosis) treated for 12 hours at the end of treatment period was used as a positive control. Accu-Check Performa glucose meter was used to measure glucose levels. Data represented as Mean ± SEM from n=3 independent measurement with ≥30 embryos per group. L0 value is taken as 0.6 mmol/L as per glucose meter indications. *p<0.05 vs. Control, #p<0.05 vs. High Glucose, @p<0.05 vs. BPA, &p<0.05 vs. BPA + High Glucose.

(B)-(F) **mRNA expression of senescence markers**: (B) β-Gal, (C) p15, (D) p21, (E) p53, (F) Rb1 in 6dpf zebrafish embryos after appropriate treatment (as mentioned above). Data represented as Mean ± SEM from n=3 independent measurement with ≥30 embryos per group. *p<0.05 vs. Control, #p<0.05 vs. High Glucose, @p<0.05 vs. BPA, &p<0.05 vs. BPA + High Glucose.

(G)-(K) **Representative images of β-galactosidase enzyme expression pattern** in 6dpf zebrafish embryos after appropriate treatment (as mentioned above). (G) Control, (H) High glucose, (I) BPA, (J) BPA+ High glucose, (K) RITA. Black arrow head indicates the β-gal enzyme expression pattern showing the cells undergoing senescence (green marks) localized near the ventral blood vessels of the trunk. Scale bar: 50 µm.

3.4.2 Transcriptional levels of senescence markers indicate the higher rate of accelerated ageing

Accelerated cellular senescence is observed in people with diabetes (Palmer et al., 2015). Senescence-associated genetic signals are shown to be higher in individuals with hyperglycaemic condition (Maeda et al., 2015; Stefano et al., 2016). As our study involves metabolic stress condition (hyperglycaemia) induced by high glucose, it was necessary for us to check the expression of senescence markers such as β-gal (Beta-Galactosidase/glb1), p15, p21, p53 and Rb1 (Retinoblastoma-1). Our focus here was on the gene expression pattern of senescence markers when BPA was combined with metabolic stress. Embryos after appropriate treatment conditions **(Figure-1)** were utilized to extract RNA, which was then converted to cDNA by the enzyme reverse transcriptase. Followed by this qRT-PCR analysis was done to determine the gene expression pattern of senescence markers. **Figure 2(B-F)** shows various levels of senescence markers. All the markers associated with senescence are seen elevated. β-gal expression in all treatment conditions was significantly higher than in control. BPA with high glucose condition was significantly elevated compared to all other conditions. As the expression of β-gal is the most important indicator of senescence, our results indicate that high glucose and BPA have the potential to cause senescence on their own or when combined.

Similar to β-gal, p53 showed increased expression in all conditions. Interestingly p21, which is a downstream signalling molecule of p53 dependent pathway for senescence, is significantly lower in BPA with and without high glucose. This may indicate that the senescence occurrence is through a p53 independent pathway. The other pathway for senescence is p15-Rb pathway. In the p15 expression alone, BPA condition did not show any significant difference from control. However, when high glucose is added to the combination, it elevates the p15 expression significantly compared to the control. Rb1 gene expression pattern was similar to β-gal gene expression where combination of BPA and high glucose shows few folds higher expression than control. These results indicate that BPA in combination with metabolic stress can possibly increase the intensity of senescence through p53 independent, p15-Rb1 associated pathway. All primers used in this study are listed in **Table 1.**

Zebrafish Primers Sequence	
β-gal forward primer	5'-ATGGGCAGAATCAACTACGG-3'
β-gal reverse primer	5'-CGACACTCAGGGAATACACAG-3'
p15 forward primer	5'-CGAGGATGAACTGACCACAGC-3'
p15 reverse primer	5'-CAAGAGCCAAAGGTGCGTTAC-3'
p21 forward primer	5'-CAAGCCAAGAAGCGTCTAGTG-3'
p21 reverse primer	5'-AACGGTGTCGTCTCTGGTTC-3'
p53 forward primer	5'-GCGATCATGGATTTAGGCTC-3'
p53 reverse primer	5'-CTTATAGATGGCAGTGGCTCG-3'
Rb1 forward primer	5'-CTGCTCACCTCACATCCAGA-3'
Rb1 reverse primer	5'-ATCCACGTTTTTCACCTTGC-3'
p53 MO	5'-GAATTGATTTTGCCGACCTCCTCT-3'

Table 1 shows the list of primers used for qPCR analysis and p53 morpholino sequence used to knockdown p53 expression in zebrafish embryos.

3.4.3 Visualization of senescence associated β-galactosidase *in vivo*

Gene expression analysis gave us a hint on the possible mode and intensity of senescence. To further confirm this, we performed Senescence Associated β-gal assay (SA-β-gal). β-gal is a hydrolytic enzyme that hydrolysis β-galactosides, which is only present in cells undergoing senescence (Dimri et al., 1995). Post treatment, embryos were subjected to β-gal assay. **Figure 2(G-K)** shows the expression of β-gal that indicates the intensity of senescence in that particular region. Liver (the hotspot of metabolism), the ventral part of the trunk where blood vessels are located (arrow) and other regions near the tail showed higher intensity of β-gal expression in BPA with high glucose-treated embryos. Treatment with BPA alone did not have much difference from control, whereas high glucose treatment alone or in combination with BPA showed an intense stain. This assay confirms the presence of senescence and also shows the intensity at which senescence is occurring in various parts of the embryo.

3.4.4 Possibility of dual mechanism involving apoptosis:

Our gene expression results indicated that senescence is happening in a p53-independent pathway. However, we had also shown increase in p53 expression, which indicated the possibility of presence of apoptosis. Apoptosis is a factor that occurs in individuals with diabetes, especially in the pancreatic β-islet cells, which results in the reduction in number of the cells (Anuradha et al., 2014). It is not a surprise that metabolic stress on its own can cause

apoptosis, along with senescence. However, in the current study, we have shown that BPA along with metabolic stress can also lead to apoptosis simultaneously with senescence.

Acridine Orange (AO) staining was performed to visualize the presence of apoptotic cells in live embryos with using fluorescent microscopy. AO is a fluorescent dye that traverses cell membrane and intercalates with nucleic acids and also binds to lysosomes that are present abundantly in apoptotic cells (Mpoke et al., 1997). Embryos were treated with the dye prior to imaging. This was followed by imaging which was done in ZOE fluorescent cell imager. **Figure 3(A-E)** shows the presence of apoptotic cells in the embryos. BPA in combination with high glucose and high glucose alone showed the presence of more apoptotic cells compared to the control embryos. BPA alone did not show much change from the control.

For further re-confirmation on apoptotic process, immunohistochemical analysis using Cleaved caspase-3 Antibody staining was performed. Caspases are a family of protease enzyme that are involved in apoptosis (Li & Yuan, 2008). Among other caspases, caspase-3, -6, -7, -8, and -9 are the ones involved in apoptosis (McIlwain et al., 2013). Among these, caspase-3 was identified to be the major caspase involved in apoptosis, as it is the primary regulator of DNA fragmentation and Poly (ADP-ribose) polymerase (PARP) cleavage which are hallmarks of apoptosis (Kiraz et al., 2016). Hence it was important for us to analyze the presence of activated caspase-3 which further re-confirms the occurrence of apoptosis. Presence of apoptotic cells was imaged with ZOE fluorescent cell imager. **Figure 3(F-J)** shows the inverted image of Cleaved caspase-3 Antibody staining. The results were similar to acridine orange staining, where BPA along with high glucose was shown to increase apoptosis in comparison to control. These results expose a novel and unique converging role of senescence and apoptosis axis contributing to glucose dyshomeostasis.

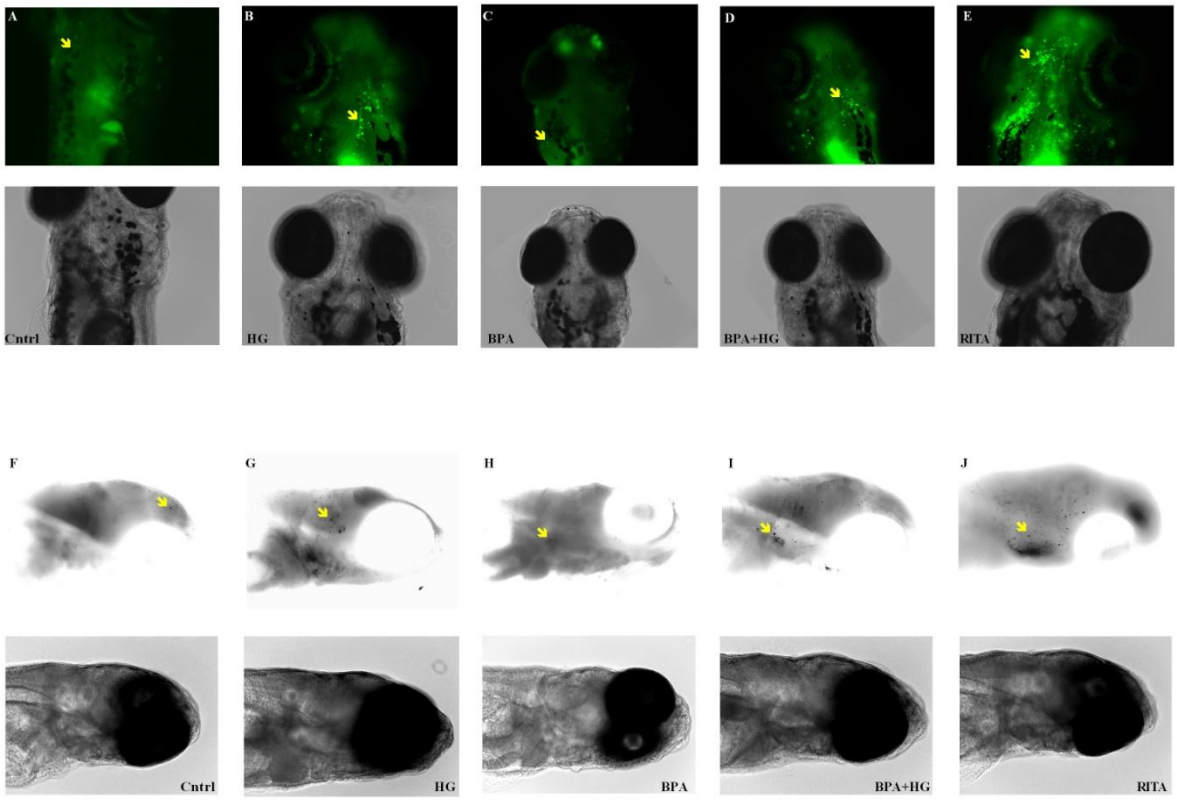


Figure 3: **(A)-(E) Representative images of acridine orange staining** done in 6dpf zebrafish embryos treated from 3dpf to 6dpf, with DMSO (vehicle control), 2.5% glucose (HG), 100 nM BPA, combination treatment of high glucose and BPA (HG+BPA) for 3dpf to 6dpf. 500 nM RITA (reactivation of p53 and induction of tumour cell apoptosis) treated for 12 hours at the end of treatment period was used as a positive control. Live fluorescent imaging of the Zebrafish embryos was done using ZOE live cell imager. (A) Control, (B) High glucose, (C) BPA, (D) BPA+ High glucose, (E) RITA. In the images at top, yellow arrow head indicates the presence of apoptotic cells (green marks). The images at the bottom are bright field images of the embryos in representation figure (above). Scale bar: 50 µm.

(F)-(J) **Representative images of cleaved caspase-3 antibody staining** done in 6dpf zebrafish embryos after appropriate treatment (as mentioned above). Fluorescent imaging of the zebrafish embryos was taken using ZOE live cell imager. (F) Control, (G) High glucose, (H) BPA, (I) BPA+ High glucose, (J) RITA. Yellow arrow heads in the inverted images above indicate the presence of apoptotic bodies (black marks). The images at the bottom are bright field images of the embryos in representation figure (above). Scale bar: 50 µm.

3.4.5 Expression of Senescence markers even in the absence of p53

The purpose of knockdown of p53 is to confirm whether senescence runs unaffected or lowered in BPA treated conditions; even in the absence of p53. Knockdown of p53 was achieved by targeting morpholino (MO) to p53 mRNA in zebrafish embryos which effectively blocks the translation of p53, resulting in lowering p53 protein levels with no visible phenotype produced (Duffy & Wickstrom, 2007). Embryos were microinjected with p53 MO at single-cell stage that resulted in knockdown of p53 mRNA expression. Embryos were then cultured as regularly and treated with appropriate treatment conditions as in **Figure-1**. The embryos were then pooled for RNA extraction and cDNA conversion followed by qRT-PCR analysis to determine the expression levels of senescence markers: β-gal, p15 and Rb1. **(Figure 4A-C)**. Both β-gal and Rb1 gene expression patterns were similar, and this indicates that senescence runs uncontrolled even in the absence of p53. High glucose and combination of BPA with high glucose have shown significant increase in senescence markers compared to the control. Though the expression levels of p15 in combination of BPA and high glucose condition had slight increase compared to the control, it was not significant.

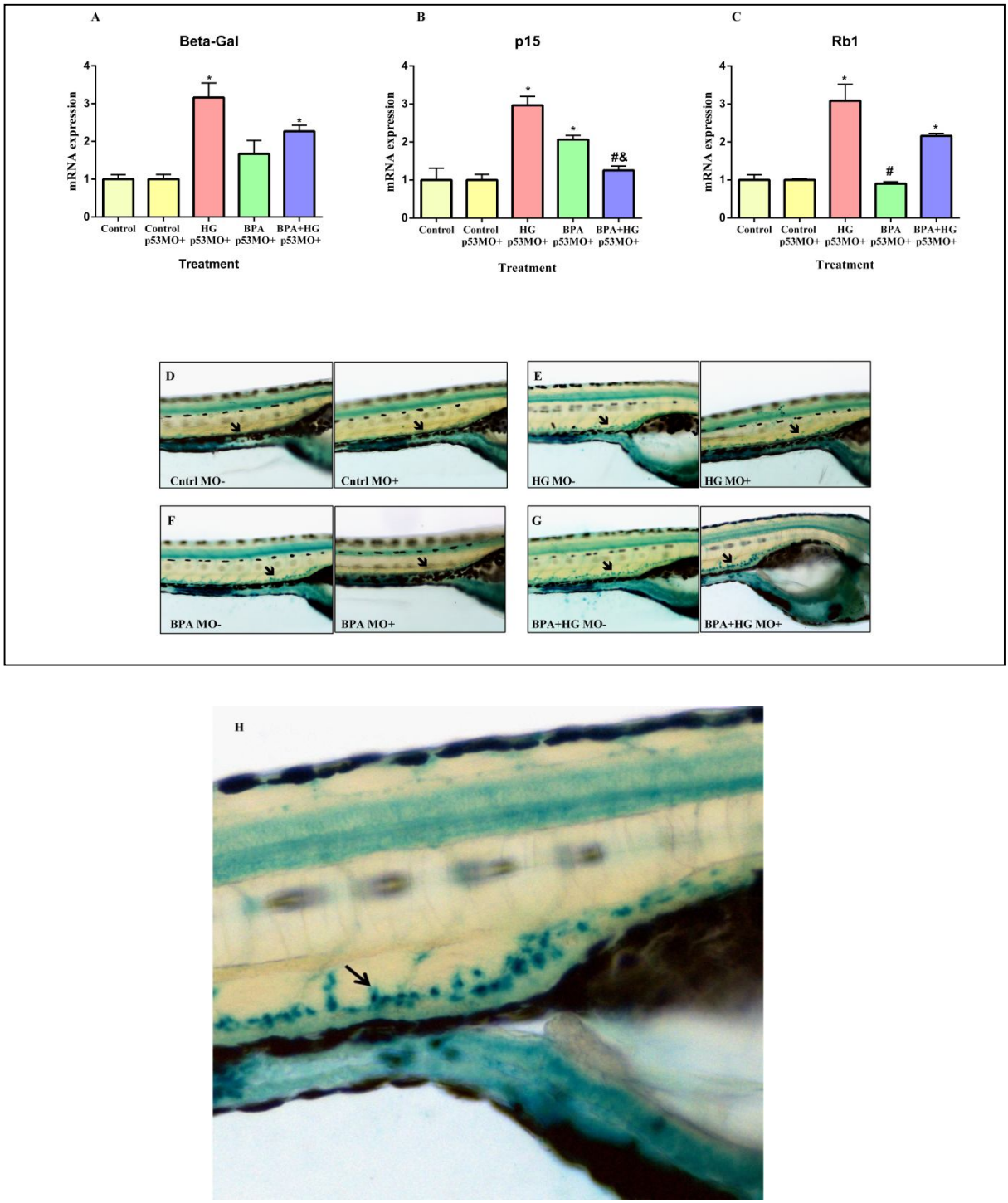


Figure 4: (A)-(C) **mRNA expression of senescence markers**: (A) β-Gal, (B) p15, (C) Rb1, in zebrafish embryos that are p53 knockdown, achieved by injecting p53 morpholino (p53 MO+). qRT-PCR was performed after zebrafish embryos were treated from 3dpf to 6dpf, with DMSO (vehicle control), 2.5% glucose (HG), 100 nM BPA and combination treatment of high glucose and BPA (HG+BPA). Data represented as Mean ± SEM from n=3 independent measurement with ≥30 embryos per group. *p<0.05 vs. Control, #p<0.05 vs. High Glucose, @p<0.05 vs. BPA, &p<0.05 vs. BPA + High Glucose.

(D)-(G) **Representative images of β-galactosidase enzyme expression pattern** in p53 MO+ zebrafish embryos (right side images) after appropriate treatment (as mentioned above). (D) Control, (E) High glucose, (F) BPA, (G) BPA+ High glucose. The p53 MO+ representation images are compared with wild type (p53 MO-) representation image (left side images). Black arrow head indicates the β-gal enzyme expression pattern showing the cells undergoing senescence (green marks) localized near the ventral blood vessels of the trunk. Scale bar: 50 μm.

(H) **Magnification of a portion** from figure 4 (G). Black arrow head indicates the β-gal enzyme expression pattern showing the cells undergoing senescence (green marks) localized near the ventral blood vessels of the trunk.

Senescence Associated β-gal assay was also performed to further confirm the results. All the p53 MO+ embryos were treated appropriately. SA-β-gal assay was performed following the treatment. **Figure (4D-G)** shows the intensity of β-gal expression in various treatment conditions in p53MO+ embryos and was compared with the expression of β-gal in p53 MO- embryos**.** In accordance with gene expression analysis, the result suggests that senescence is still occurring even in the absence of p53, though slightly altered. The results suggest that senescence happens uncontrolled, though altered in levels, in a p53- independent manner but mainly through p15-Rb1 pathway.

3.5 Discussion:

In this study, we have demonstrated that the direct treatment of zebrafish embryos with BPA did not alter the senescence levels, which suggests that BPA has no direct effect to increase cellular senescence in zebrafish embryos. However, we observed an increase in senescence following BPA treatment along with high glucose. This study for the first time showed that the combination of BPA and metabolic stress can accelerate senescence process. Few *in vitro* studies have reported an occurrence of cellular senescence in BPA treated cells.

Initially, the whole-body glucose measurement from 6dpf embryos after appropriate treatment conditions was measured. The rise in glucose levels compared to control confirmed the establishment of a metabolic stress model. The increased glucose levels in combination of BPA and high glucose condition is indicative of the fact that BPA under metabolic stress may have the potential to elevate the glucose levels or is capable of causing dyshomeostasis in glucose metabolism.

Studies have shown that metabolic stress like high glucose treatment is capable of inducing senescence (Park et al., 2014; Kong et al., 2015; Maeda et al., 2015). On the other hand, studies have shown that exposure to BPA can affect glucose homeostasis and lead to glucose intolerance and insulin resistance (Moon et al., 2015; Fang et al., 2015). Moghaddam et al. (2015) demonstrated in mice model that BPA has the potential to induce hyperglycaemic conditions and complications associated with that. In addition to that, Qin et al. (2012) have demonstrated that human mammary epithelial cells when exposed to BPA showed a remarkable increase in the levels of p16 indicating the occurrence of accelerated cellular senescence. However, there is no proper report on the combined effect of BPA and metabolic stress on senescence and signalling pathway associated with that. The current study tried to address the same.

SA-β-gal assay measuring the activity of a lysosomal β-galactosidase is a prominent marker for detecting senescence (Severino et al., 2000; Wagner et al., 2015; Piechota et al., 2016). In our study, both the β-gal gene expression and SA-β-gal enzyme activity are shown to increase in high glucose and combination of BPA and high glucose conditions compared to vehicle control. Interestingly, BPA on its own showed significant increase over control but lesser than high glucose and combination treatment. All together, BPA when combined with metabolic stress significantly increased senescence compared to other individual treatments.

Now that, we had established the presence of senescence, the molecular pathway underlying it has to be established.

The molecular mechanism of cellular senescence involves p16 and p53 mediated pathways (Jacobs & de Lange., 2004; Lee at al., 2014). p15 (cdkn2a/b) in fish, which is similar in function to p16 (cdkn2a) in higher organisms, is one of the major proteins involved in cell cycle regulation. It binds and inhibits the action of cyclin-dependant kinases 4/6, thereby preventing the phosphorylation of Retinoblastoma (Rb1). Rb1 remains associated with transcriptional factor E2F1 preventing the transcription of genes necessary for G1 to S phase progression of cell cycle. This leads to cell cycle arrest and senescence (Rayess et al., 2011).

Our *in vivo* results show that p15 levels significantly increased when BPA combined with high glucose compared to control. Higher p15 expression in high glucose compared to control was in accordance with previous studies (Maeda et al., 2015; Zhang et al., 2015). Rb1 levels were significantly elevated in combination treatment compared to either BPA or high glucose alone. This shows that BPA along with metabolic stress may induce senescence through p15-Rb1 pathway in zebrafish.

The other major pathway for senescence is p53 dependent. p53, as a transcription factor activates downstream effector genes, which includes p21 (Sullivan et al., 2018). *p21* inhibits activity of various cyclin-dependent kinase complexes (CDK1, CDK2, and CDK4/6), mediating p53 dependant G1 growth arrest (Deng et al., 1995; Abbas & Dutta., 2009). A study by Kitada et al. (2014) in mice model, reported that during early stages of diabetic nephropathy, hyperglycaemia causes cellular senescence by p21-dependent pathway. In our study, though significant increase in the level of p53 was seen in combination treatment, whereas p21 showed significantly reduced levels compared to control. This suggests that BPA when combined with metabolic stress aggravates senescence in a p53 independent pathway.

Rodriguez and Meuth. (2006) through their experiment with human colon cancer cell lines demonstrated that, during unfavourable conditions, cells undergo p21-mediated cell cycle arrest, preventing it from entering into S-phase. It is transient and once the damage is successfully repaired, the cells may resume the cell cycle. However, prolonged cell cycle arrest leads to up-regulation of p16 gene expression that further leads to permanent cell cycle arrest (Childs et al., 2014). Result from our gene expression analysis suggests that, p15 gene expression was up-regulated in the treatment condition involving BPA with high glucose and

high glucose alone condition, which indicates that the embryonic cells were under permanent cell cycle arrest followed by accelerated senescence.

The increase in p53 and reduction in p21 expression suggested that there may be a possibility of apoptotic pathway simultaneously happening along with senescence. To validate this, our study utilized acridine orange (AO) staining of the embryos. AO is a cell-permeant fluorescence dye used for staining acidic vesicles such as lysosomes and can bind to nucleic acids. It emits green fluorescence if intercalated with double stranded DNA and with single stranded RNA it emits red fluorescence (Osman et al., 2018). Normal cells produce light green fluorescence, whereas early apoptotic cells produce bright green fluorescence due to the presence of fragmented chromatin and late apoptotic cells produce orange fluorescence (Ribble et al., 2005). Our results from live imaging showed green fluorescent cells implying the presence of early apoptotic cells. The apoptotic cell number was seen increased in both high glucose and combination of BPA with high glucose conditions.

Immunohistochemical analysis by cleaved caspase-3 Antibody staining of zebrafish embryos was carried out to re-confirm the presence of apoptotic cells. The results were similar to acridine orange staining assay where BPA in combination with high glucose increased apoptotic bodies. These results suggest that BPA under the influence of metabolic stress like high glucose is capable of aggravating both senescence and apoptosis simultaneously. Chen and Liu. (2000) through their experiments, subjected normal human fibroblast cells to external stress factor and showed that the cells that undergo apoptosis were distributed mainly in S-phase of the cell cycle, whereas cells undergoing senescence were predominantly seen in G1/G2 phase of the cell cycle. Our result suggests that the apoptotic cells were majorly seen in dorsal side of the head region of the embryos **(Figure 3)**. Whereas, β-gal assay reveals that, cells that undergo senescence were majorly localized near the ventral blood vessels of the trunk region **(Figure 2)**. It is quite possible that ventral blood vessel cells were in G1 phase, whereas the cells near the dorsal head region were in S-phase of the cell cycle. Gene expression analysis may not reveal the exact detail of the cellular status under stress conditions, tissue region wise, as we utilized whole embryo for gene expression analysis. In future study, it is necessary for us to analyze the gene expression in various tissue regions in the embryo which will help us to understand the tissue specific effect of BPA.

The last part of our study focused on re-establishing our finding that accelerated senescence happens in embryos treated with BPA under metabolic stress condition through p15-Rb1,

independent of p53 pathway. As there was increased p53 expression in combination of BPA and high glucose and high glucose alone condition **(Figure 2)**, it was necessary for us to determine whether p53 knockdown affects senescence induced by BPA and metabolic stress conditions. To achieve this, p53 morpholino was used to silence the p53 gene expression. Even after p53 knockdown, the expression levels of senescence markers like β-Gal and Rb1 significantly increased in combination of high glucose and BPA or high glucose alone condition compared to control. SA-β-gal assay re-confirms the presence of senescence, which remains unaltered even in the absence of p53. This experiment proves that silencing p53 does not affect senescence mechanism and further goes on to show that BPA along with metabolic stress aggravates senescence in p53 independent manner. As p53 inhibition did not block the senescence-induction effects of BPA, it was suggested that p15 pathway could be a central mechanism contributing to the accelerated senescence effects of BPA. However, the correct interpretation of the senescence data would require functional analysis of p15.

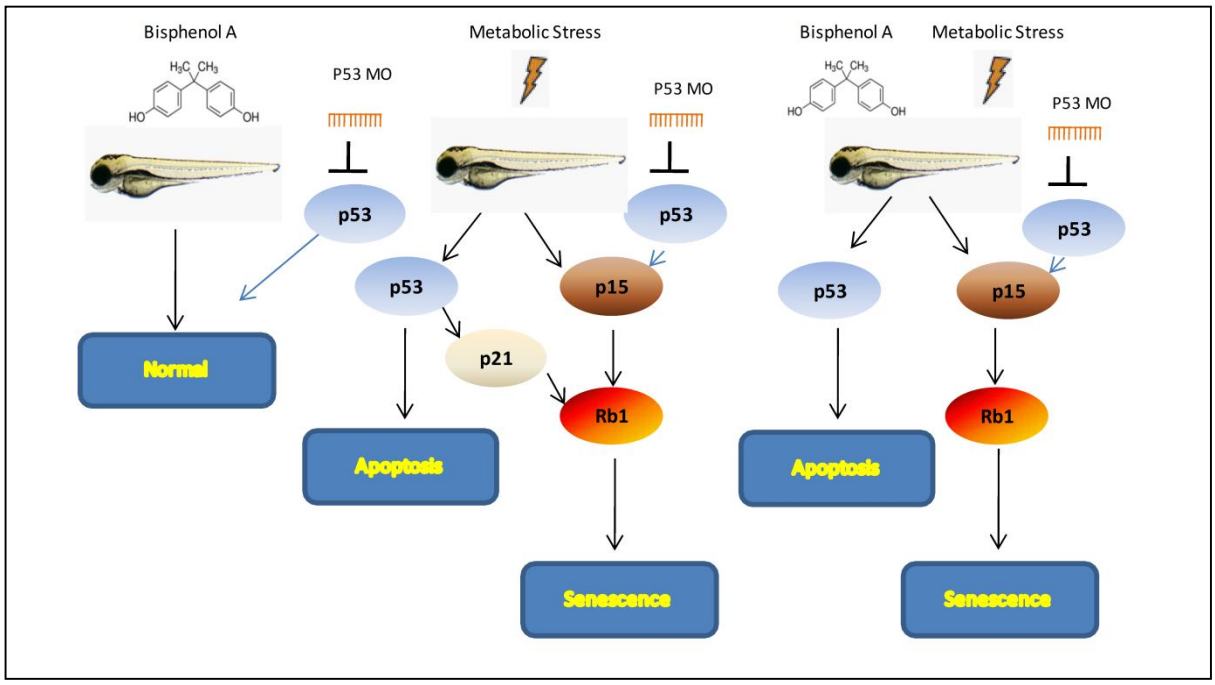


Figure 5: Schematic representation of major outcomes of the study. BPA along with metabolic stress induces senescence in p53-independent pathway through p15-Rb1. This was confirmed when senescence occurs unaffected even in the absence of p53 expression.

Major outcomes of our study are highlighted in **figure 5**. It precisely describes the action of BPA with and without metabolic stress condition. Cells under the metabolic stress conditions

may either lead to senescence through p15-Rb1/p53-p21 pathway or may lead to apoptosis through p53 pathway. But when metabolic stress combines with BPA, both apoptosis and senescence happen simultaneously, in which senescence happens through p15-Rb1 pathway. Also, p53 knockdown experiment shows that senescence runs unaffected even in the absence of p53.

In conclusion, this study for the first time has shown in a vertebrate model that BPA in combination with metabolic stress like high glucose has the potential to aggravate the senescence in p53 independent manner and majorly through p15-Rb1 pathway. The study also reveals that BPA along with metabolic stress may lead to apoptosis coupled to senescence simultaneously and the mechanisms or signals that lies as a thin line between these two processes has to be well studied in future.

Chapter 4

Bisphenol A exposure under metabolic stress induces cellular senescence and impairs glucose uptake in skeletal muscle cells

4.1 Abstract

Cellular senescence is an irreversible process that is commonly seen in age-associated diseases like Type 2 diabetes (T2DM). Bisphenol A (BPA), an endocrine disruptor, is known to be associated with metabolic anomalies like diabetes. The mechanism by which BPA impacts the diabetes pathogenesis is still unclear. While there is lack of studies to demonstrate the potential of BPA to cause cellular senescence, hardly any studies have focused on the impact of BPA on insulin-target tissues under metabolic stress condition. The mechanism by which BPA aggravates the pathogenesis of diabetes is still unknown. Through our clinical and *in vivo* studies, we deciphered that BPA under metabolic stress induces accelerated senescence through p16 pathway, which may further lead to aggravation of disease pathogenesis. However, this mechanism has to be demonstrated in metabolically active target cell models, which are responsible for glucose metabolism and homeostasis. Our study utilized skeletal muscle cells for this purpose. Skeletal muscle comprises of metabolically active cells which are essential for glucose regulation and maintenance in our body. The results obtained were consistent with our *in vivo* study, wherein senescence occurred through p16 pathway, independent of p53. In addition to this, deterioration of insulin stimulated glucose uptake and reduction in GLUT-4 protein expression were witnessed. Altogether, we conclude that BPA adds up to the existing metabolic stress, which induces increased senescence along with reduction in insulin-stimulated glucose uptake, further leading to the aggravation of the pathogenesis of diabetes.

4.2 Introduction

In previous chapters, we reported that BPA was associated with increased senescence markers in diabetic patients. *In vivo* results also demonstrated that BPA induces accelerated senescence under metabolic stress conditions through p15/p16-Rb1 axis. The causative mechanism has to be confirmed in a metabolically active insulin target tissue and this form the premise for this chapter.

Skeletal muscles are primary insulin-sensitive metabolic tissues that play an important role in glucose regulation and maintenance (Stump et al., 2006). Contracting skeletal muscles requires more glucose and hence it uptakes glucose through insulin stimulation, which helps in maintaining the glucose levels in circulation (Fritsche et al., 2008). This insulin-stimulated glucose uptake happens through glucose transporter-4 (GLUT-4), which translocates into the plasma membrane with the help of intracellular signalling mechanism involving insulin receptors and Akt-phosphorylation (Saltiel & Kahn, 2001; Satoh, 2014).

Defective insulin-stimulated glucose uptake in skeletal muscle is the hallmark pathogenesis in patients with T2DM (Abdul-Ghani & DeFronzo, 2010). Insulin resistance in skeletal muscles is considered to be the primary defect in T2DM patients (DeFronzo & Tripathy, 2009). Hence, it becomes important for us to confirm our previous findings in skeletal muscle cells.

Only few studies have reported that BPA induces cellular senescence. Two separate studies by Indumathi et al. (2013) and Mullainadhan et al. (2017) showed that BPA exposure in animal model can affect GLUT-4 translocation and leads to deregulation of insulin stimulated glucose uptake process. However, the effect of BPA in metabolic tissues under the influence of metabolic stress has not been studied in the background of senescence mechanism.

Therefore, the aim of this chapter is to confirm our clinical and *in vivo* animal model findings in an *in vitro* skeletal muscle cell model with a focus on the effect of BPA on senescence and insulin-stimulated glucose uptake.

4.3 Methodology

4.3.1 Cell culture treatment

C2C12, an immortalized mouse myoblast cell line, with passage number 14-19 was used for the experiments. Cells were maintained in low glucose (5 mM) DMEM containing 10% FBS. The cells were treated with 100 nM BPA. The concentration was selected based on the concentration used in zebrafish experiment. Moreover, environmentally relevant dose of BPA was determined to be 10-100 nM (Pfeifer et al., 2015). Based on previous studies on C2C12, 25 mM glucose condition was determined to be high glucose condition (Hanke et al., 2011; Leontieva et al., 2014) and the same was used in our treatment condition. Along with separate BPA and high glucose conditions, a combination of 100 nM BPA with high glucose was used as one of the conditions. 1 μM reactivation of p53 and induction of tumour cell apoptosis (RITA) was used as a positive control. Based on MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay treatment concentration of RITA was fixed; where the concentration beyond 1 μM decreased the cell viability. The MTT assay determines mitochondrial activity in the cell. MTT gets converted into formazan crystals in the living cells which determine the total number of viable cells present in the culture dish (van Meerloo et al., 2011). High glucose used in the treatment conditions was provided as a glucose excursion, wherein alternate high and low glucose was supplied, mimicking the glycemic excursions seen in diabetes patients (Hezarkhani et al., 2013; Fujimoto et al., 2018). All the treatments were carried out for 48 hours and, at the end of treatment, cells were harvested and processed for further experiments.

4.3.2 RNA isolation and real-time PCR analysis

After different experimental manoeuvre, cells were harvested and processed for real-time PCR. Total RNA from tissues were isolated as described previously (Balasubramanyam et al., 2011). The quality of the RNA and its concentration was measured using NanoDrop 2000 (Thermo Scientific). First strand cDNA was synthesized using 1 μg of total RNA, 40 μM Oligo-d(T)18 primer (Catalog#S13168, New England Biolabs), 2.5 mM dNTPs, 10xRT buffer, 20 U RNase inhibitor and 100 units reverse transcriptase enzyme. The resultant mix was subjected to 1 hour of incubation at 42˚C, followed by 95˚C for 5 minutes. Real-time PCR was performed for senescence-associated genes: β-gal (glb1), p16, p21, p53 and retinoblastoma (Rb1) using SYBR green master mix (Cat #F416S Dynamo Color flash Sybr

Green QPCR Kit). Amplification was carried out using LC-96 (Light cycler 96, Roche cycle) and normalized using endogenous β-actin.

4.3.3 Quantitative assay of β-Gal activity

β-galactosidase activity in skeletal muscle cells was measured by performing a fluorometric assay following a modified version of the methodology previously described (Spazzafumo et al., 2017). Protein was extracted and quantified using BCA kit. 1 mM 4-Methyl-umbelliferyl-β-D-galactopyranoside in citrate-phosphate buffer (pH 4.0) was used as a substrate. 20 µg of protein was incubated with 200 µl substrate for 1 hour at 37°C. The reaction was stopped by 1 ml of glycine-carbonate buffer. The fluorescence liberated by 4-Methylumbelliferone was captured on EnSpire Multimode Plate Reader (PerkinElmer) at excitation wavelength of 360 nm and at emission wavelength of 446 nm. The enzymatic activity of β-gal was expressed in relative frequency units (RFU).

4.3.4 Western blot protein expression

Protein lysates from C2C12 cells were prepared using RIPA Lysis and Extraction Buffer (Catalog#89900, Thermo Scientific) containing 1X protease inhibitor cocktail (Catalog#P8340, SIGMA). Cells were sonicated and incubated for at least an hour in ice, followed by centrifugation at 13000 rpm for 30 mins at 4°C. The supernatant was used to quantify the protein content using Pierce™ BCA Protein Assay Kit (Catalog#23225, Thermo Scientific). 30 µg of protein was resolved on 10% and 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. After an hour of blocking in 5% bovine serum albumin (BSA), the membrane was probed with the following antibodies in 1% BSA in Phospho-buffered saline with 0.5% Tween 20 (PBS-T): anti-p16 (Catalog#sc-81156, Santa Cruz), anti-p21 (Catalog#sc-6246, Santa Cruz) and anti-p53 (Catalog#ab31333, Abcam). A mouse anti-β-actin antibody (Catalog#sc-47778, Santa Cruz) was used as an internal control for loading. It was then followed by exposure to HRP-conjugated secondary antibodies: anti-mouse IgG HRP Conjugate (Catalog#A4416, SIGMA) and goat anti-rabbit IgG HRP Conjugate (Catalog#A6154, SIGMA). Detection was performed using enhanced chemiluminescence kit (GE Healthcare) and blots were quantified using Image-J software. Protein of interest was normalized using β-actin.

4.3.5 Glucose uptake assay

2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) was used as a marker for detecting insulin stimulated glucose uptake (Chen et al., 2015). Myogenic differentiation of C2C12 cells was induced by changing the growth medium to differentiation medium, which was supplemented with 2% FBS for 4 days. Treatment protocol as mentioned earlier was carried out in the last 48 hours of differentiation period. Myotubes were then washed with PBS and kept for starving in DMEM containing no glucose for 3 hours at 37°C. Cells were then shifted to Hepes buffer containing 0.5% BSA with no glucose. The cells were then incubated with 100 nM insulin for 30 mins followed by treatment with 60 µM 2-NBDG (Catalog#N13195, Thermo Scietific) for an hour at 37°C. Total amount of 2-NBDG incorporated into the cells was measured in cell lysates by fluorescence. The fluorescence read-out was measured using EnSpire Multimode Plate Reader (PerkinElmer) at excitation wavelength of 485 nm and emission wavelength of 535 nm. Final fluorescence readings were represented as percentage (%) insulin stimulated glucose uptake.

4.3.6 Statistical Analysis

All bar graphs are drawn using GraphPad Prism 6. Data represent mean ± SEM. The statistical significance (*$P \leq 0.05$) was determined by One-way ANOVA using GraphPad Prism 6.

4.4 Results

4.4.1 Elevation of senescence markers in skeletal muscle cells under treatment conditions

Transcriptional levels of senescence markers were quantified using real-time PCR. mRNA expression of senescence markers- β-gal, p16, p21, p53 and Rb1 was analysed (**Figure 1A-E).** β-gal and Rb1 expressions in all treatment conditions were higher than control, and significant elevation was seen in combination of BPA and high glucose conditions compared to control. Both β-gal and Rb1 showed approximately (~) 1.25 fold increase compared to control. p16 was equally elevated in both high glucose and combination treatment. p16 expression was ~1.6 fold higher in high glucose condition compared to control. Combination of BPA and high glucose treatment showed ~2 fold increase compared to the control. p53 and p21 mRNA expressions in individual treatments of high glucose and BPA showed slight increase compared to control. However, in combination treatment, no significant increase was observed. This shows that, BPA when combined with metabolic stress follows p16 pathway to cause senescence, which is independent of p53 pathway. These results were consistent with our findings obtained in *in vivo* model (Chapter 3). List of primers used in this study was given in **Table 1**.

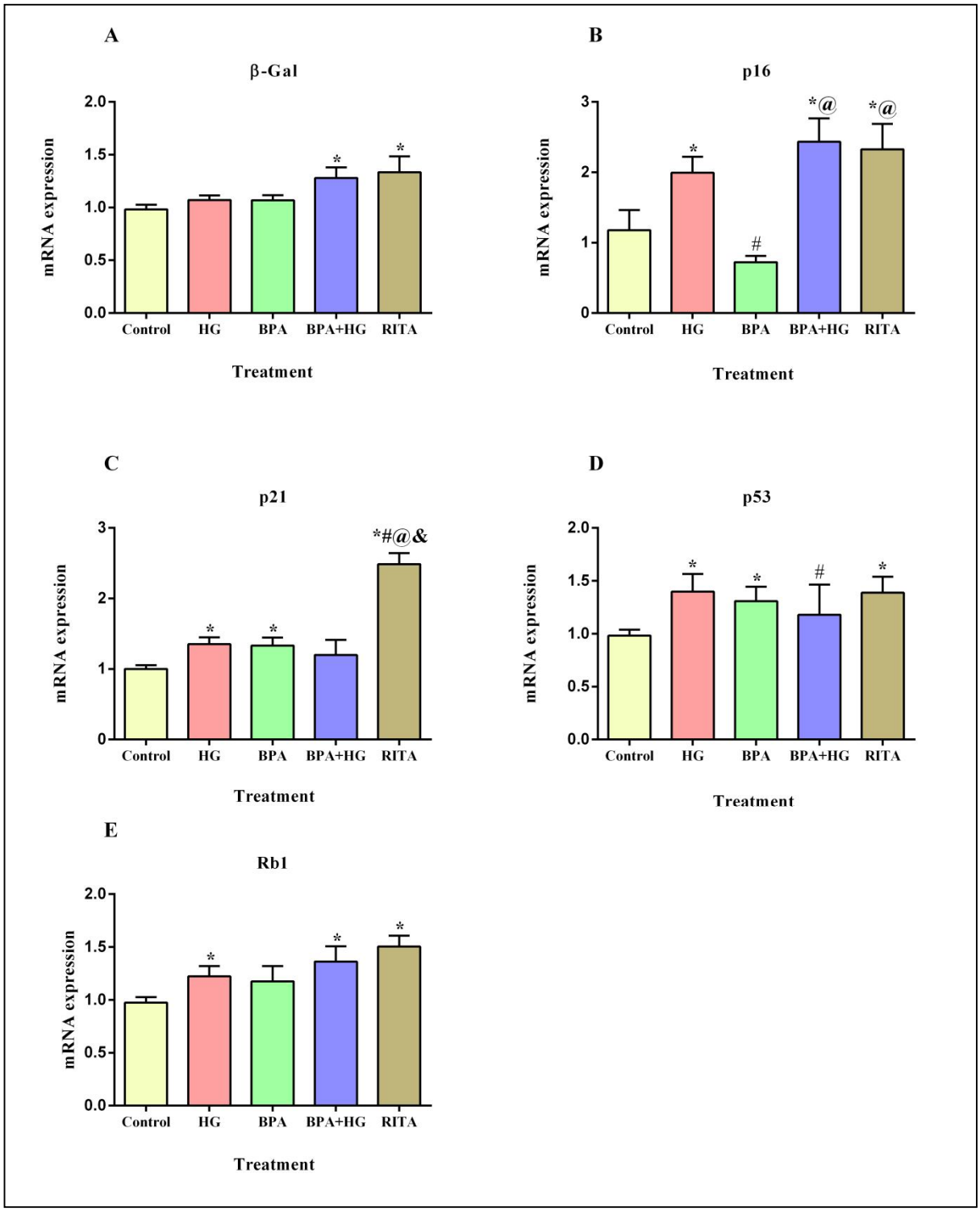


Figure 1: (A)-(E) mRNA expression of senescence markers: (A) β-gal, (B) p16, (C) p21, (D) p53, (E) Rb1 in C2C12 myoblast cells. Treatment condition includes control, 25 mM glucose (HG), 100 nM BPA and combination treatment of high glucose and BPA (HG+BPA). High glucose was provided as glucose excursion, wherein alternate high and low glucose was provided every 12 hours. 1 µM RITA (reactivation of p53 and induction of tumour cell apoptosis) was used as a positive control. All the experiments are repeated individually at least thrice. Data represented as Mean ± SEM. *p<0.05 vs. Control, #p<0.05 vs. High Glucose, @p<0.05 vs. BPA, &p<0.05 vs. BPA+ High Glucose.

Mouse Primers	
β-gal forward primer	5'-GTGAAAAGCCTCTATCCCCTG-3'
β-gal reverse primer	5'-ATCACGGACACCATTGAAGG-3'
p16 forward primer	5'-CGCCTTTTTCTTCTTAGCTTCA-3'
p16 reverse primer	5'-AGTTTCTCATGCCATTCCTTTC-3'
p21 forward primer	5'-CTTGCACTCTGGTGTCTGAG-3'
p21 reverse primer	5'-GCACTTCAGGGTTTTCTCTTG-3'
p53 forward primer	5'-ATGTTCCGGGAGCTGAATG-3'
p53 reverse primer	5'-CCCCACTTTCTTGACCATTG-3'
Rb1 forward primer	5'-CAGGCTTGAGTTTGAAGAAATTG-3'
Rb1 reverse primer	5'-ATGCCCCAGAGTTCCTTCTTC-3'

Table 1 showing the list of primers used in this study for real-time PCR.

4.4.2 β-Galactosidase activity assay shows significant increase in BPA-treated and combination treatment

Quantitative assay of β-gal activity showed significant increase in β-gal activity in all three conditions compared to control **(Figure 2)**. BPA and BPA in combination with high glucose were also significantly elevated compared to high glucose alone condition. This suggests that BPA individually has the potential to raise the senescence level; in addition, when combined with high glucose induces even more senescence in the skeletal muscle cells.

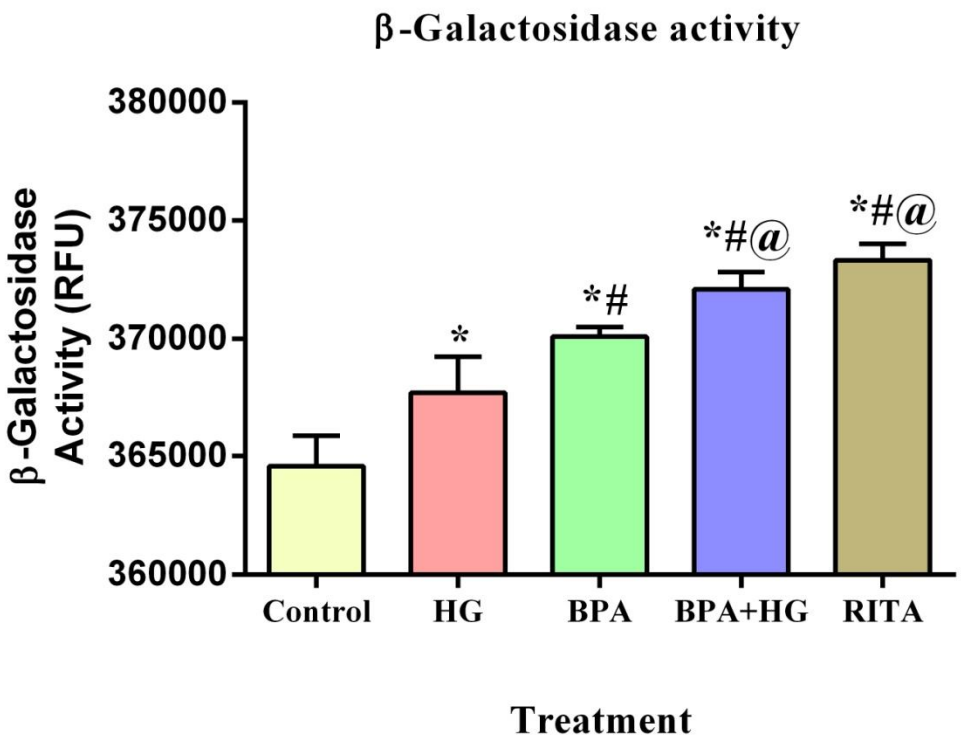


Figure 2: Quantitative assay of β-gal activity: β-galactosidase activity evaluated in C2C12 myoblast cells after appropriate treatment (as mentioned above). β-galactosidase activity was reported as Relative Frequency Units (RFU). Data represented as Mean ± SD. All the experiments are repeated individually at least thrice. *p<0.05 vs. Control, #p<0.05 vs. High Glucose, @p<0.05 vs. BPA.

4.4.3 Western blot analysis confirms the elevated levels of senescence markers

Western blot analysis for senescence markers showed significantly higher protein levels of p16 and p53 in BPA treated condition compared to control **(Figure 3).** Apart from BPA treatment, other treatments showed no significant increase in p53 protein levels compared to control. The increase in p53 protein expression in BPA treated was similar to the gene expression result. However, unlike in gene expression, p21 protein expression in BPA treated cells did not show any significant difference, that in turn indicates the presence of apoptosis. Previously, in our *in vivo study* (Chapter 3), we had already showed that BPA might be capable of inducing both senescence and apoptosis simultaneously. There was a significant increase in p16 expression in all the other treatments compared to control. C2C12 cells treated with BPA showed a significant increase in p16 protein expression, although it was not similarly reflected in p16 gene expression. The difference could be due to BPA impacting on

post-transcriptional regulation, such as translation or protein stability, allowing greater p16 levels despite no change in p16 mRNA (Li et al., 2011). Alternatively, it could be related to protein expression related feed-back mechanism potentially involving miRNA(s) that might return a transient increase in p16 mRNA back to normal levels (Overhoff et al., 2014). Only high glucose was shown to significantly increase p21 expression.

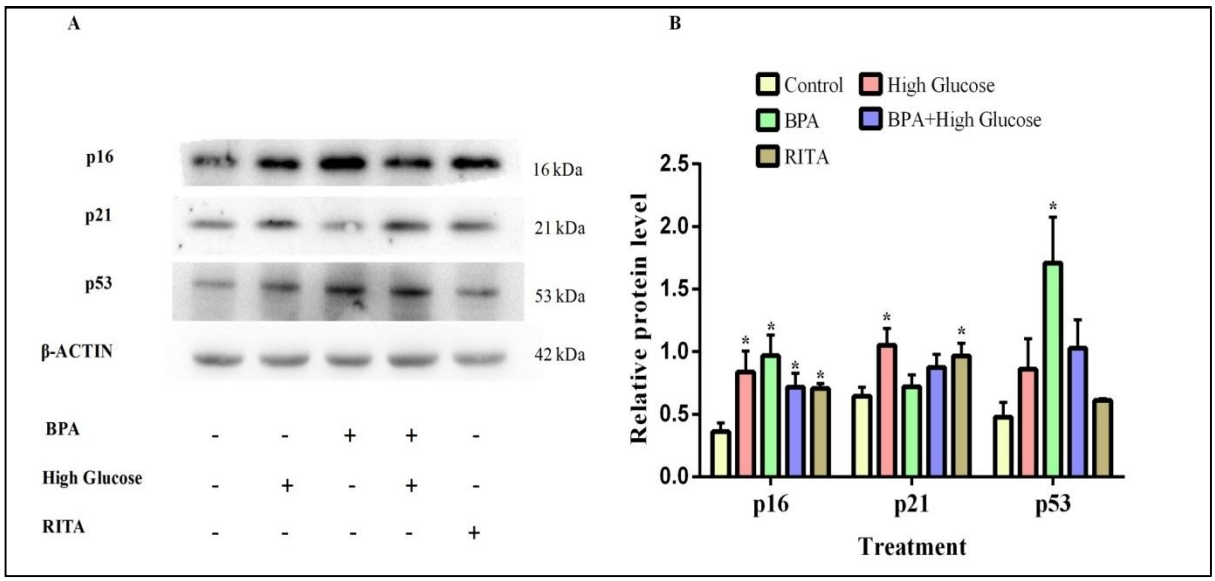


Figure 3: (A) Representative images of western blot analysis of p16, p21 and p53 in C2C12 myoblast cells. Treatment condition includes control, 25 mM glucose (HG), 100 nM BPA and combination treatment of high glucose and BPA (HG+BPA). High glucose was provided as glucose excursion, wherein alternate high and low glucose was provided every 12 hours. 1 μM RITA was used as a positive control. β-ACTIN was used as internal control.

(B) Relative protein levels of p16, p21 and p53. All the experiments are repeated individually at least thrice. Data represented as Mean ± SEM. *$p<0.05$ vs. Control.

4.4.4 2-NBDG assay reveals the impact of BPA on insulin stimulated glucose uptake

2-NBDG glucose uptake assay shows difference in insulin stimulated glucose uptake in cells subjected to different treatments (**Figure 4**). All the treatment groups showed significant reduction in glucose uptake compared to control. Comparatively, BPA treated condition showed marked decrease in glucose uptake compared to both control and high glucose. These results imply that BPA in combination with high glucose or BPA alone has the potential to disrupt glucose uptake potential of the cells.

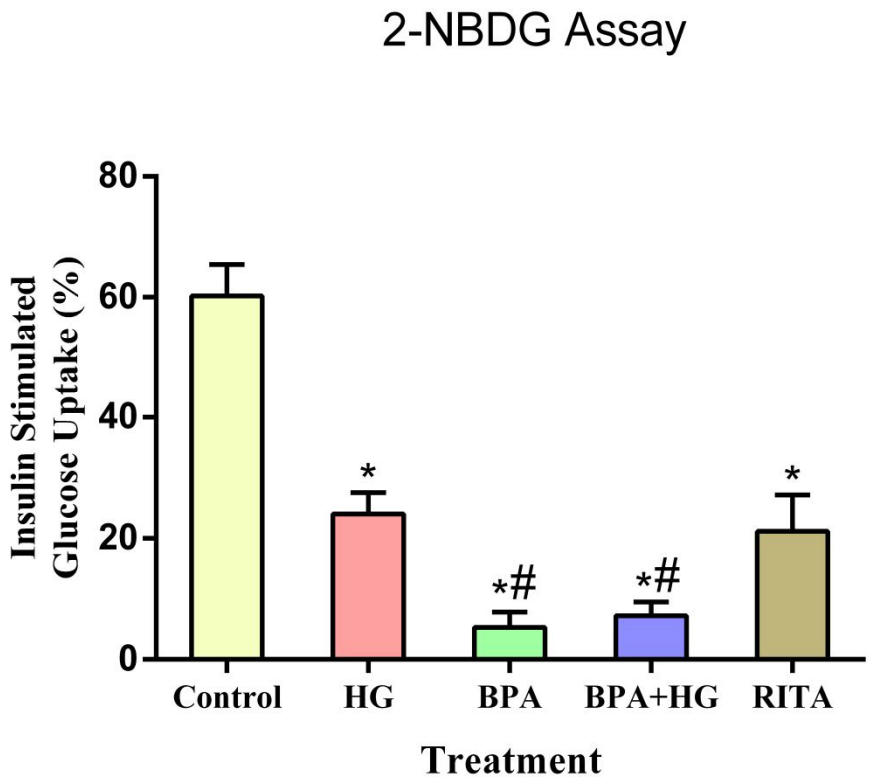


Figure 4: 2-NBDG glucose uptake assay showing percentage change in insulin-stimulated glucose uptake between treatment groups: Control, HG-High Glucose, BPA, combination treatment of (BPA+HG) and RITA. All the experiments are repeated individually at least thrice. Data represented as Mean ± SEM. *$p<0.05$ vs. Control and #$p<0.05$ vs. High glucose.

4.4.5 GLUT-4 protein expression supports the impact of BPA on reduced glucose uptake

Translational analysis of GLUT-4 protein showed significant decrease in the combination treatment of BPA and high glucose **(Figure 5)**. Decreasing trend was observed in the individual treatments though it was not significant. This confirms that BPA along with high glucose may impair glucose transporter, subsequently leading to poor glucose uptake in skeletal muscle cells.

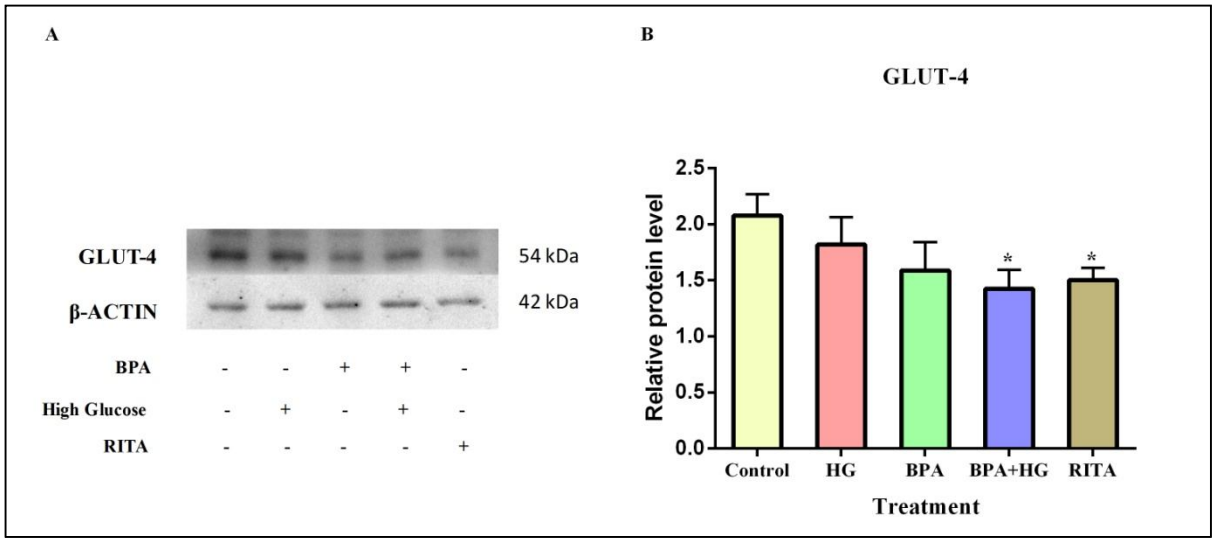


Figure 5: (A) Representative images of western blot analysis of GLUT-4 in C2C12 myoblast cells. Treatment condition includes control, 25 mM glucose (HG), 100 nM BPA and combination treatment of high glucose and BPA (HG+BPA). High glucose was provided as glucose excursion, wherein alternate high and low glucose was provided every 12 hours. 1 μM RITA was used as a positive control. β-ACTIN was used as internal control. **(B) Relative protein levels** of GLUT-4. All the experiments are repeated individually at least thrice. Data represented as Mean ± SEM. *$p<0.05$ vs. Control.

4.5 Discussion

Cellular senescence was reported as a cause and consequence of T2DM (Testa & Ceriell., 2007). This was also inferred from our clinical and zebrafish animal model studies. Hence it was necessary for us to replicate our *in vivo* results in a metabolic tissue model like skeletal muscle cells. Skeletal muscle cells are metabolically active (Slentz et al., 2009). Transcriptional regulation of genes involved in glucose metabolism was reported in muscle tissues (Baskin et al., 2015). It is the major insulin-sensitive tissue in our body that induces insulin-stimulated glucose uptake (Stump et al., 2006). In order to maintain proper blood glucose levels in circulation, insulin promotes glucose utilization in muscle cells, thus inhibiting hepatic gluconeogenesis (Fritsche et al., 2008). Our study utilized C2C12, an immortalized mouse myoblast cell line.

The results from cell culture study were consistent with animal model aberrations, where β-gal gene expression was significantly increased in the combination of BPA and high glucose condition. Accumulation of β-gal expression is an indicator of cells undergoing senescence (Wagner et al., 2015). In our study, β-gal gene expression was significantly higher in the combination treatment condition of BPA and high glucose compared to control. This shows that the level of senescence is highly influenced by BPA especially when it combines with metabolic stress. In addition to this, β-galactosidase activity assay **(Figure 2)**, in which the activity of the β-gal protein is measured, also confirmed the presence of increased senescence in the combination treatment.

In the *in vivo* study in zebrafish, we observed that senescence happened through the p15-Rb1 axis. Here we analysed the levels of p16 which is the mammalian ortholog of p15, which are similar in biochemical properties and function (Li et al., 2011), as p16 is considered to be a robust biomarker for prediction of cellular ageing (Ressler et al., 2006; Dimri 2004, Sharpless & DePinho 2007). Our study found a significant increase in the p16 and Rb1 gene expression levels.

Cyclin dependent kinase 4 and 6 (CDK4/6) negatively regulates Rb1 (Tang et al., 2012) and these kinases are inhibited by cyclin-dependent kinase inhibitors such as p16 and p21 which initiates senescence (Leontieva & Blagosklonny, 2013).

A study by Bontempo et al. (2009) in human acute promyelocytic leukemia (NB4) cells had reported an increase in p16 and p21 protein expression levels when cells were treated with

BPA. The study noted unchanged p53 protein levels. On the same lines, a study by Qin et al. (2012) in human mammary epithelial cells (HMEC) had also reported an increase in p16 protein levels when cells were exposed to BPA inducing cellular senescence. However, the study showed no significant increase in p53 protein levels which was contrary to our result, wherein p53 protein levels showed marked increase compared to the control. The difference in the results might be due to the difference in dose and cell models utilized in the studies. Increased p16 protein levels in BPA compared to control was similar to the study by Qin et al. (2012). But there was no change observed in p21 protein levels in the presence of BPA **(Figure 3)**. This confirms that BPA along with metabolic stress may induce cellular senescence through p15/p16-Rb1 axis independent of p53 pathway. In addition to this, there was a possibility of apoptosis happening when tissues get exposed to BPA. This was inferred from the significant increase in p53 protein levels and unaltered p21 protein levels in BPA treated. This result was consistent with our *in vivo* experiment **(Chapter 3)**. Our study is the first to show the presence of senescence and its pathway, when BPA combines with metabolic stress condition.

In addition to these, our study measured the levels of glucose uptake in skeletal muscle cells to understand the impact of BPA on glucose metabolism. Skeletal muscle is the major site for insulin-mediated glucose uptake (DeFronzo & Tripathy, 2009). GLUT-4 is the major glucose transporter expressed in skeletal muscle cells which aids in glucose uptake (Zorzano et al., 2005). Reduction in GLUT-4 expression in the skeletal muscle cells is a characteristic feature of patients with type 2 diabetes, resulting in the reduced capability to process glucose (Maier & Gould, 2000).

Previous studies have reported a detrimental effect of BPA on glucose uptake and GLUT-4 expression in animal models. A study by Indumathi et al. (2013) in wister rats had reported a significant reduction in the GLUT-4 expression levels in skeletal muscle cells when rats were administered with BPA. The study concluded that BPA has drastic effect on GLUT-4 translocation. A recent study by Mullainadhan et al. (2017) had also demonstrated a significant decrease in GLUT-4 expression and insulin-stimulated glucose uptake in male albino rats after the animals were exposed to BPA. However, there is a dearth of understanding on the extent to which BPA affects the GLUT-4 translocation when it adds up to the metabolic stress condition. Our study focused on this aspect.

Insulin-stimulated glucose uptake was measured in skeletal muscles by 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) assay. 2-NBDG is a reliable marker to identify the levels of insulin-stimulated glucose uptake (Chen et al.,2015). The results obtained in our study were in accordance with the results reported by Indumathi et al. (2013) and Mullainadhan et al. (2017). Both the treatments involving BPA (BPA alone and combination treatment) showed significant reduction in the levels of insulin-stimulated glucose uptake in skeletal muscle cells **(Figure 4)**. However, there was no significant change between BPA alone and combination of BPA and high glucose condition. This indicates that BPA on its own or in combination with metabolic stress produces the same level of effectiveness on the process of glucose uptake. Further studies are required on this direction on the effectiveness of BPA under the influence of metabolic stress on glucose uptake with different concentrations of BPA.

Protein analysis through western blot indicated a decreasing trend in GLUT-4 protein in BPA treated and a significant decrease in BPA and high glucose combination treatment compared to control **(Figure 5)**. C2C12 cells treated with BPA showed significantly reduced glucose uptake, albeit with only a decreased trend of GLUT-4 protein expression. This could be due to post-translational modifications (PTMs) linked to biological activity such as glucose uptake (Sadlier et al., 2013). Deterioration of GLUT-4 expression will result in impaired insulin-stimulated glucose uptake in the skeletal muscle cells, which is commonly seen in T2DM patients (Stanford & Goodyear, 2014). Our study for the first time showed that BPA under the influence of metabolic stress condition will diminish the GLUT-4 expression. Future studies must focus on the effect of BPA on glucose metabolism under the influence of metabolic stress conditions in animal models.

In conclusion, our study demonstrated that BPA under the influence of metabolic stress can dysregulate glucose metabolism affecting the glucose uptake in a metabolic tissue such as skeletal muscle through mechanism of increased senescence.

Chapter 5

Nut-shell Findings and Future Directions

While several epidemiological studies have suggested that BPA exposure is positively associated with an increased risk of T2DM (Sowlat et al., 2016; Provvisiero et al., 2016; Chailurkit et al., 2017), there is lack of data on this in Asian Indians, a population highly prone to the epidemic of diabetes and its vascular complications (Gujral et al., 2015). To fulfil this important and contemporary research gap, this study for the first-time, unravelled the link between BPA and T2DM in a holistic way utilizing the clinical samples, *in vivo* animal model and *in vitro* skeletal muscle cell model based biochemical and molecular investigations.

Our clinical study revealed that serum levels of BPA were significantly higher in patients with T2DM compared to control individuals. BPA levels in T2DM subjects were also positively correlated to poor glycemic control and insulin resistance. Gene expression analysis in PBMCs revealed that patients with T2DM exhibited significantly elevated mRNA levels of senescence (GLB1, p16, p21 and p53) and inflammatory (IL6 and TNF-α) markers. The chronic burden of senescence and inflammation was also reflected by significantly reduced and shortened telomeres in patients with T2DM compared to control subjects. Interestingly, PBMCs from patients with T2DM also exhibited significantly elevated mRNA expression of ERRγ, a recently identified receptor for BPA. This study is the first to demonstrate an association of increased BPA levels with cellular senescence, pro-inflammation, poor glycemic control, insulin resistance and shortened telomeres in patients with T2DM.

Our clinical study sets the tone for rest of the studies where in causative mechanism of BPA action in the pathogenesis of T2DM was studied in detail through *in vivo* (zebrafish) and *in vitro* (skeletal muscle cell) studies.

In order to better understand the causal mechanisms of BPA, zebrafish embryos were treated with BPA in the absence and presence of metabolic stress (hyperglycaemia) condition. Interestingly, the increase in glucose levels seen in zebrafish embryos under hyperglycaemia was significantly augmented in the presence of BPA. Transcriptional levels of senescence markers such as p15, p53, Rb1 and β-galactosidase (β-gal) were increased in combination of metabolic stress and BPA exposure, and this was also supported by β-gal enzyme expression patterns. In addition, embryos exposed to hyperglycaemia and BPA also exhibited increased apoptosis as evident from acridine orange and cleaved caspase-3 antibody staining. The fact that senescent markers were elevated even in p53 knockdown conditions suggests that

senescence might majorly involve p15-Rb1 pathway when embryos were treated with metabolic stress coupled to BPA exposure. Our work exposes a novel and unique converging role of senescence and apoptosis axis contributing to glucose dyshomeostasis.

Interestingly, our study revealed that senescence happens simultaneously with apoptosis. In general, senescent cell activates several pro-survival genes thus becoming resistant to apoptosis (Zhu et al., 2015). However, we showed that both senescence and apoptosis occur simultaneously. It has to be seen further whether BPA induces ectopic expression of p16 induced p53 mediated apoptosis, as this was reported to be happening in different cancer cells (Katsuda et al., 2002; Tamm et al., 2002).

When analysed the effects of BPA under the influence of metabolic stress (hyperglycemia) in a metabolic tissue like skeletal muscle, our results reconfirmed the presence of accelerated senescence that is mediated through p16-Rb1 axis. Skeletal muscle cells exposed to glycemic excursions showed impaired glucose transporter expression as well as decreased glucose uptake and this was significantly augmented in the presence of BPA.

One of the limitations of our study is of its cross-sectional nature and the causal link between elevated systemic levels of BPA and T2DM needs to be further investigated in a prospective follow-up study.

While our clinical study supports the acquisition of Senescence Associated Secretary phenotype (SASP) nature of cells and tissues in patients with T2DM, the underlying molecular signatures and mechanisms needs to be studied in model organisms and in vitro studies with much more focus.

While Asian Indians are suffering from T2DM at an early age compared to others, the concept of accelerated aging and related pathways has become a thrust area of research for both prevention as well as management of metabolic disorders including T2DM. In this direction, the present study is considered as a significant research contribution.

While the prevalence of gestational diabetes is increasing worldwide contributing to the escalating epidemic of T2DM in women, recent studies have already imposed a ‘cause for concern’ on the genesis of gestational diabetes that could originate from the exposure to

EDCs like BPA. Therefore, future in-depth studies are warranted to dissect out the mechanisms and prevention strategies.

With important findings and futuristic avenues, this study helped us to understand the impact of environmental contaminants like BPA on the pathogenesis of diabetes. By acquiring in-depth knowledge on this we hope in future, targeted therapies can be improved upon by avoiding these confounding external factors that may form an impediment in treatment.

The Strategic Approach to International Chemicals Management (SAICM) considers EDCs as an "emerging policy issue". While many countries take actions to mitigate their dangers, the issue is not in the public domain in India, and there is very little information and data available. Thus, with better knowledge and understanding of these environmental pollutants and toxicants, we can look forward for appropriate national and international policies and laws to regulate the usage of endocrine disruptor chemicals like BPA.

Future is full of hopes!

Plastic free….. BPA-free….. and Free of Diabetes and other Metabolic Disorders…..

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