Chapter 2

Review of literature

2.1 Obesity and type 2 diabetes (T2D): Epidemiological prevalence

Obesity is characterized by the accumulation of uncontrolled masses of fat at various parts of the body most specifically abdomen which is measured by an individual's body mass index (BMI) or the index of weight for height of an individual. Accordingly, BMI of $\geq$ 25 kg/m^2 is overweight; BMI of $\geq$ 35kg/m^2 is severely obese and BMI of $\geq$ 40kg/m^2 is morbidly obese [1]. People with high BMI's develop high levels of blood glucose, cholesterol and blood pressure which eventually progress with diabetes, coronary heart disease, chronic kidney disease (CKD), musculoskeletal problems respectively [2]. Entitled as a global non-communicable disease (NCD) by the World Obesity Federation (WOF), obesity targeted a population size of nearly 520 million with the fastened spike in the frequency of obesity cases from 2.2% to over 5.1% in the year span of 1998-2015 [3]. The most obese and overweight cases were recorded with a portion of ~38.2 million acquired mainly by children under the age of five years thus indicating hazardous future [4]. As estimated based on the fastened pace of affected people, nearly 60% of the world population by 2030 will be obese, if remained unchecked [5]. Overweight and obesity is posing the potential threat to the human race by becoming the underlying cause for the progression of many metabolic disorders such as type 2 diabetes (T2D), atheroschelorsis, hypertension, hypertriglyceridemia, arthritis, hyperuricemia and ischaemic heart disease [6]. According to the 2019 figures published by the International Diabetes Federation (IDF), 463 million adults around the world between the ages of 18 and 79 suffer from type 2 diabetes (T2D). Ranked as ninth leading cause of death, diabetes resulted in estimated deaths of 1.5million with 48% of the deaths belonging to patients before 70years of age. More than 95% of people diagnosed with diabetes posses T2D. T2D is characterized as an epidemic disease, it is anticipated that the mass epidemic effect of T2D of 400 million people worldwide will get doubled in the next 20 years [7]. The premature mortality rate caused by diabetes is mostly reported in lower-middle-income countries thus posing a great need for research in this area in order to curb this menace.

2.2 Obesity mediated type 2 diabetes: Interrelation

Obesity, ageing and physical inactivity are the causes for the progression of T2D that results in the more serious and permanent disorders in the body that includes macro vascular complications (including atherosclerosis and amputations) and micro vascular

complications (including retinopathy, nephropathy and neuropathy). The major reason underlying the prevalence of obesity is the imbalance in the energy homeostasis where in the co-ordinated homeostatic regulation of energy inflow and energy outflow is alarmingly disturbed [8]. The increased intake of fat-rich, sugar-rich food supplements that satisfies the sedentary and modern life style of the urban society has put the individuals at the risk line alongside the lack of physical activity to process out the harmful calories and sugar from the imbalanced system. Thus, the fastened intake of over the counter processed food items is the main bait to the modern sector followed by physical inactivity that roots the harmful processed fats to the core of multiple organs of the body causing diseases listed as cardiovascular diseases, type 2 diabetes mellitus, osteoarthritis, breast, liver, colon and kidney cancer, hypertension, respiratory diseases, dyslipidaemia and hyperuricaemia [9]. Ford et. al suggested that an overweight individual possess the 4.5% to 9% risk of developing a diabetic state with per kilogram gain of body weight with a seven fold risk for obese subject and three fold risk for the over weights [10]. Around 60% to 90% diabetic cases belong to obese patients with 10-fold to 11-fold relative risk rate of diabetes progression in male and female obese individuals, respectively [11].

2.3 Obesity and adipose tissue inflammation: The interrelation

Over the last decade, mounting evidence has emerged demonstrating a close link between a state of chronic low-level inflammation in adipose tissue and obesity induced insulin resistance and T2D [12]. Increasing accumulation of intra-abdominal adipose tissue in obese subjects is frequently associated with the enhanced rate of free fatty acids (FFAs) mobilization and higher levels of circulating FFAs which trigger inflammatory pathways that compromise insulin sensitivity [13]. Anatomically, obese body constitutes large masses of fat-filled mature adipocytes or adipose tissues (AT). Adipose tissues are class of connective tissues that constitutes heterogeneous mass of preadipocytes, stromal cells, histiocytes, endothelial cells, fibroblasts and macrophages that are classified as white adipose tissue (WAT), brown adipose tissue (BAT) and beige or brite/inducible BAT. On the basis of localisation and functionality, WAT is further classified as visceral WAT (vWAT) that line the peritoneum and internal organs, subcutaneous WAT (sWAT) that is present beneath the skin, inter-(itMAT) and intra muscular adipose tissue (iMAT) [14]. In case of obesity, vWAT constituting almost

20% of body's fat ought to be more accumulated and is responsible in onset of various metabolic disorders [15]. WAT act as energy depot and has a central role in governing insulin sensitivity and whole body glucose homeostasis by maintaining a balanced sequestration of lipid inside the adipocytes and secretion of appropriate levels of adipokines. Adipose tissue undergoes two major growth phenomenon namely adipocyte hyperplasia (adipocyte proliferation) and adipocyte hypertrophy (sequestration of triglycerides inside the existing adipocytes leading to adipocyte enlargement) [16]. High fat diet or overnutrition results in excessive deposition of FFAs resulting in hypertrophic adipocytes. These fat filled adipocytes further result in alarmingly high level of adipokines secretion leading to a low grade pro-inflammatory state that eventually prevents the insulin sensitivity effect of the adipose tissue [17]. The various discovered adipokines with their cellular origins are enlisted in Table 2.3.

Type/class	Adipokine	Secretion location	Effects
Adipocytokines	Leptin(16-kDa)	Mature adipocytes	Pleiotropic effect
	TNF-α (17-kDa)	Mature adipocytes and stromal cells	Pro-inflammatory, Promotes insulin resistance, obesity-induced T2D
	IL-6 (21–28 kDa)	Mature adipocytes and stromal cells	Pro-inflammatory, promotes obesity-induced T2D
	MCP-1(11-13 kDa)	Mature adipocytes and stromal cells	Monocyte/macrophage recruitment
	IL-10 (18-kDa)	Stromal cells	Anti-inflammatory
Cysteine-rich resistin-like molecules	Resistin (12-kDa)	Mature adipocytes	Promotes insulin resistance
Collagen and complement factor structure like proteins	Adiponectin (28-kDa)	Mature adipocytes	Anti-inflammatory, antiatherogenic, stimulates angiogenesis
Pre-B cell colony-enhancing factor	Visfatin (52-kDa)	Mature adipocytes and macrophages	Differentiation of preadipocytes to adipocytes, pro-inflammatory
Biologically active peptides	Omentin	Mature adipocytes	Modulate insulin action
	Chemerin	Mature adipocytes	Modulate insulin action
	RBP4	Mature adipocytes and macrophages	Systemic insulin resistance
	Lipocalin 2	Mature adipocytes and macrophages	Promotes insulin resistance and inflammation
	Macrophage migration inhibitor factor	Stromal cells	Monocyte/macrophage recruitment

Table 2.3: Secretion source and effects of some key adipokines

As supported by various reports WAT of the lean or underweight subjects maintain the energy homeostasis in the body with a regulated safe level of adipokine secretion whereas in case of obese subjects there is a dysregulation of adiposity and the imbalanced secretion of adipokines resulting into an excessive and riskful increase in the secretion levels of pro-inflammatory adipokines namely leptin, tumour necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin 6 (IL-6), resistin and interleukin 1 beta (IL-1β) in the plasma that finally cause insulin resistance (IR) [18]. This chronic low grade inflammation of the adipose tissue finally results in the adipocyte dysfunction characterized by the presence of inflamed hypertrophic adipocytes and increased accumulation of macrophages, which leads to the development of insulin resistance.

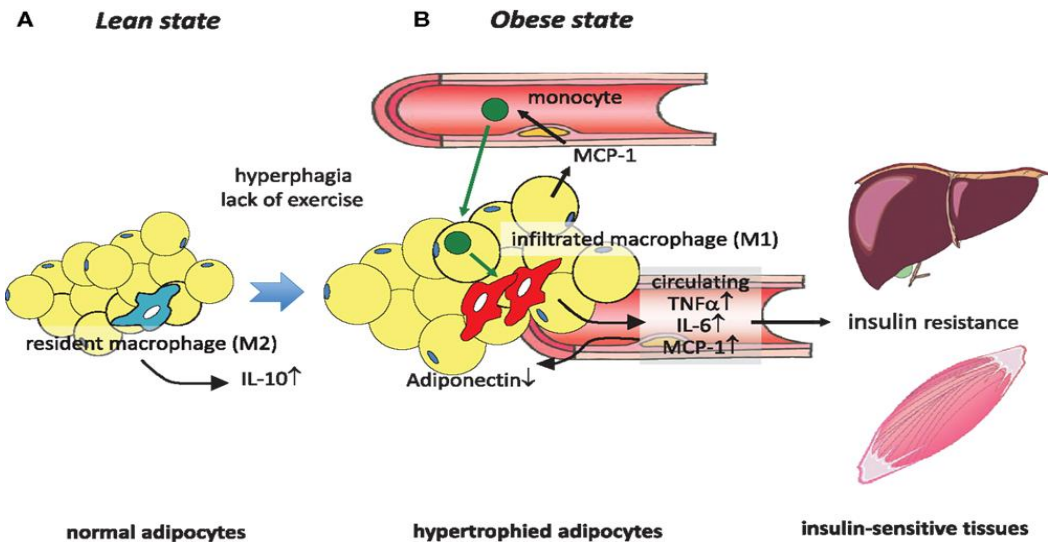


Fig 2.3 A schematic representation of obesity mediated adipokine disregulation and related health disorders.

2.4 Adipose tissue inflammation leading to insulin resistance: Mechanism

Chronic low grade inflammation of the adipose tissue results in the adipocyte dysfunction characterized by the presence of inflamed hypertrophic adipocytes and increased accumulation of macrophages, which leads to the development of insulin resistance (IR) which further progresses to type 2 diabetes (T2D). Insulin resistance (IR) is an impairment of insulin stimulated glucose disposal in insulin responsive cells which is a major defect and early sign for future development and progression of type 2 diabetes (T2D) pathogenesis [19]. Heavy FFA influx demands adipocyte expansion

occuring via adipocyte hypertrophy and lead to low grade adipose tissue inflammation marked by amalgamation of deregulated adipocytokine secretion from the hypertrophic adipocytes which activates the pro-inflammatory signalling cascades. The adipose tissue inflammatory status is further augmented by macrophage infiltration which initiates when the adipose tissue is undergoing fat accumulation and transforming into a hypertrophic state. At this stage, obese adipocytes recruit monocytes via adipocyte secreted MCP-1 mediated chemotaxis. The monocytes differentiate into macrophage after interacting with adipose tissues and are called adipose tissue macrophages (ATMs) [20-21]. The increase in the number of resident ATMs is elevated to 50% in obese subjects than in the lean state [22]. The main trigger for macrophage infiltration is adipocyte death. The acute protein factor CRP/MCP-1 cause the chained recruitment of more ATMs into the hyper inflamed adipose tissue to form a crown like structure (CLS) which helps in degrading the necrotic adipocytes associated debris [23]. Macrophage polarization is the adaptation of diverse phenotypes by macrophages in order to assist the body for maintaining homeostasis, tissue repair, remodelling as well as vasculogenesis [24]. There are two response phenotypes mentioned as M1 (classically activated macrophages) and M2 (alternatively activated macrophages) and metabolic stress such as high calorific diet drives the phenotypic switching of obese adipocytes from the anti inflammatory M2 macrophages marked by the down regulated gene expression of YM-1, Arginase-1, IL-10 and IL-4 to pro inflammatory M1 macrophages which is marked by the up-regulated gene expression of TNF-α, iNOS, IFN-γ, IL-6 and MCP1 [25]. Macrophage infiltration and polarisation recruited M1 macrophages and obese adipocytes via paracrine interaction intensify the inflammatory status of the adipose tissue by secreting excessive levels of pro-inflammatory chemokines (MCP-1, CCL2, CCL3) and cytokines (TNF-α, iNOS, IFN-γ, IL-6) which augment the AT inflammation in obese subject finally resulting in glucose intolerance and insulin resistance [26]. The high levels of TNF-α further activates the inflammatory signalling molecules IKK, p38 MAPK, JNK, and PKC which inactivates insulin receptor substrate (IRS) by impairing serine and tyrosine phosphorylation thus compromising the insulin sensitivity of adipose tissue, muscle cells and liver [27].

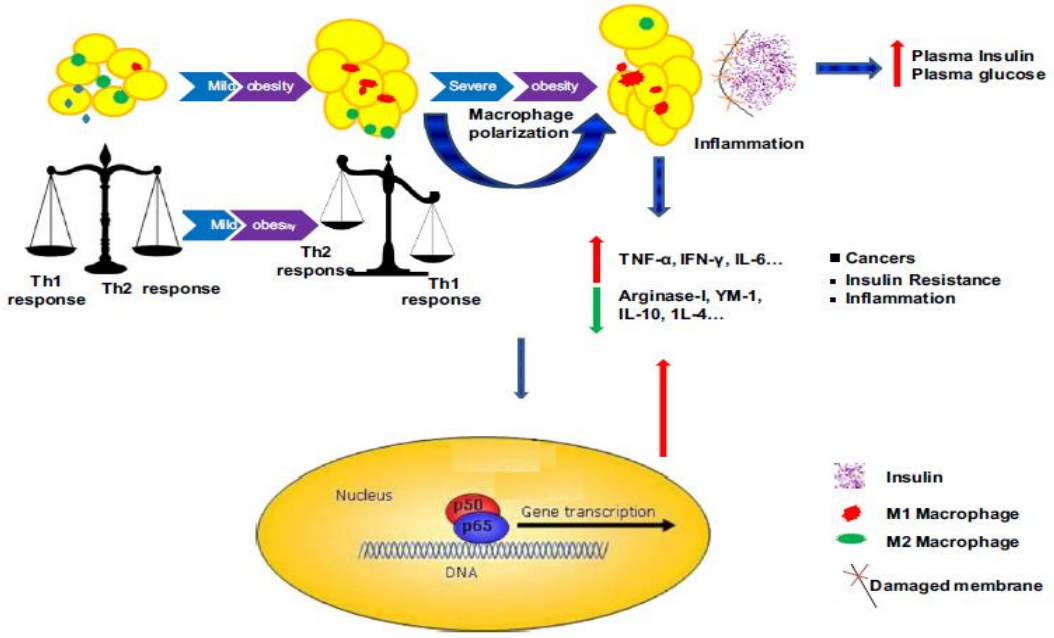


Fig 2.4: A schematic representation of mechanism of macrophage polarization and inflammation.

2.5 Inflammation and T2D: A brief introduction

The typical underlying factor which drives the pathology and progression of the metabolically associated diseases such as type 2 diabetes, cardiovascular diseases and arthritis is inflammation [28]. Inflammation is the innate body response towards eliciting a protection against pathogenic and harmful invasions into the body namely bacteria, viruses, allergens, pathogens etc. The first line of defence is flagged by the migration of neutrophils to the attacked area which initiates the cascade of the inflammatory responses that trigger the rescue of the affected area from the inducers. At first, the microbe and pathogen specific pathogen-associated molecular patterns (PAMPs) are recognised by the cell surface receptors Toll-like receptors (TLRs) or the NOD-like receptors (NLRs) located on the affected local cells that trigger downstream inflammatory cascades for the pathogen invasion resolution [29]. Upon PAMP-TLR/NOR binding, a set of cytokines are released by various transcription factors such as nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 3 (STAT3) that facilitate the availability of various adhesion molecules on the inflamed cells allowing the healing leukocytes to enter the affected tissue [30]. The inflammatory resolution is further amplified by the recruitment and activation of additional macrophages by various pro-inflammatory cytokines at the inflamed site which mediate

phagocytosis of the affected cells by vascular dilation, release of the mediators i.e. cytokines and resolution of the antigen, removal of the debris of the dead phagocytosed cells and finally repair the tissue damage at the area [31]. The successful occurrence of inflammation at the affected site is marked by the display of the five parameters which are heat (calor), pain (dolor), redness (rubor), swelling (tumor) and loss of function (functiolaesa). Acute (short termed) and chronic (long termed) inflammation are the two branches of inflammation. With the acute inflammatory response most of the infections are resolved by the resident immune cells namely macrophages, dendritic cells, kupffer cells and mast cells. These first line innate immune cells lead to the adaptive immune responses [32]. In case of chronic inflammation the helping role of inflammation advances to the harmful aspect as the chronic trail becomes uncontrolled and the inflammatory responses result in the unstoppable recruitment of leukocytes at the injury site. This results in the tissue damage and consequently to various disorders underlying this. As chronic inflammation lasts for weeks to months it prolongs its tissue damaging attribute until it causes a disorder. The diseases underlying uncontrolled chronic inflammation are namely crohn's disease, psoriasis, type 2 diabetes, atherosclerosis, asthma, rheumatoid arthritis, and cancer [33]

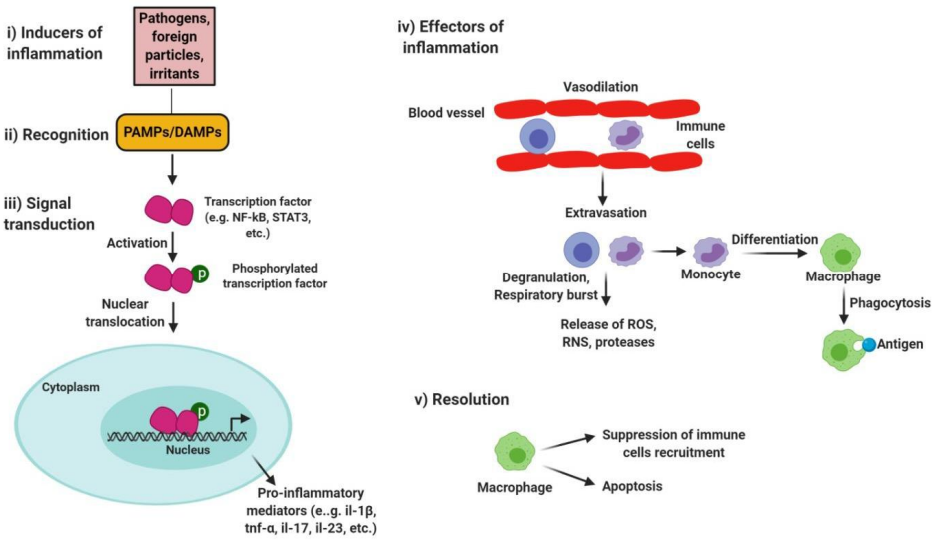


Fig 2.5 A schematic representation depicting the process of inflammation.

Increasing accumulation of intra-abdominal adipose tissue in obese subjects is frequently associated with the enhanced rate of free fatty acids (FFAs) mobilization and higher levels of circulating FFAs which trigger inflammatory pathways that compromise insulin sensitivity [34,35]. Consequently, targeting adipose tissue

inflammation by genetic knockdown of inflammatory receptors or mediators has a beneficial effect on insulin sensitivity and glucose homeostasis. Toll-Like Receptor 4 signalling pathway and Adenosine Receptor signalling pathway play pivotal role in regulating adipose tissue inflammation, while former promotes and later resolve the inflammation. Hence, targeting these signalling pathways could improve the adipose tissue dysfunction in order to resolve the insulin resistance.

2.6 Adenosine signalling pathway: Role and target of treatment

Adenosine is a ubiquitous purinergic substrate that belongs to the group of heterocyclic aromatic purines [36]. Adenosine remain in 20-200 nanomolar (nM) concentration during normal condition but during stress or hypoxic conditions as observed in tumours the nanomolar amount of adenosine spikes up to micromolar (μM) concentrations [37]. Thus, suggesting that adenosine act as a stress rescue marker molecule that resolve the inflammation and promotes tissue wound healing as developed on the affected tissue due to the disease state ischemia, acute inflammation, or fibrosis trigger the breakdown of intracellular adenosine triphosphate (ATP) into adenosine monophosphate (AMP) by the action of ectoenzyme CD39 (ectonucleoside triphosphate diphosphohydrolase1) which is then dephosphorylated to adenosine via ecto-5′-nucleotidase CD73. The extracellular Adenosine transport of adenosine is mediated and controlled by equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs) [38].

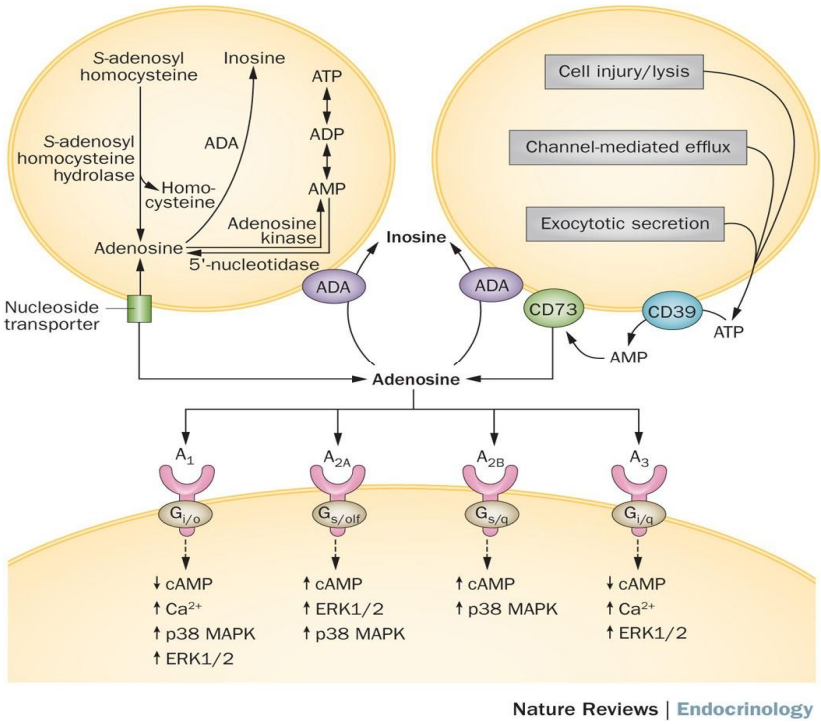


Fig 2.6 Detailed representation of Adenosine signalling pathway activation and dowmstream signalling

Extracellular relocated adenosine then mediate its effects by binding to specific G-protein–coupled adenosine receptors (ARs) which are widely distributed in metabolically active sites such as adipose tissue, liver, pancreas and various immune cells. Adenosine signals by binding to any of the following four G-protein-coupled receptors listed as Adora1 (A_1), Adora2a (A_{2A}), Adora2b (A_{2B}) and Adora3 (A_3), respectively [39].

Adenosine receptor	GPCR type	Effect	Physiological effect
Adora1 (A_1AR)	Gi/Go family	Inhibition of adenylyl cyclase (AC) activity, decreases cyclic AMP (cAMP) concentration	Improvement of insulin sensitivity and glucose homeostasis
Adora3 (A_3AR)	Gi/Go family	Inhibition of adenylyl cyclase (AC) activity, decreases cyclic AMP (cAMP) concentration	Glucose metabolism
Adora2a (A_{2A}AR)	G_s family	Activation of adenylyl cyclise (AC), increases cyclic AMP (cAMP) concentration	Increases gluconeogenesis and glucose release
Adora2b (A_{2B}AR)	G_s family	Activation of adenylyl cyclise (AC), increases cyclic AMP (cAMP) concentration	Glucose homeostasis, protective effect against HFD-induced tissue inflammation and insulin resistance in mice

Table 2.6 Types of adenosine receptors and their physiological effects

A valuable set of research has been done till date throwing light on the anti-inflammatory aspect of $A_{2A}AR$ mediated adenosine signalling. Adenosine signalling pathway functions as an immunosuppressive agent by regulating inflammation via $A_{2A}AR$ by inhibiting inflammatory cytokines IL-2 and TNF-α in both T-cell, Tc1 and Tc2 cell population [40]. $A_{2A}AR$ agonist which positively activates the signalling cascade was reported to impart rescue to the kidneys in diabetic nephropathy [41]. Thus supporting the fact that among the four different subtypes of adenosine receptor, A_1, A_{2A}, A_{2B}, and A_3, adenosine orchestrates its anti-inflammatory effect through the activation of $A_{2A}AR$ and $A_{2B}AR$. Accumulating evidence highlights a critical role of adenosine signalling in the regulation of insulin synthesis from the pancreatic beta cells and also modulates the insulin responsiveness in adipose tissue, muscle, and liver that governs glucose homeostasis [42-47]. Adenosine signalling was also reported to promote the pancreatic β-cells regeneration process by adenosine agonist 5'-N-ethylcarboxamidoadenosine (NECA) via $A_{2A}AR$ signalling activation in zebra fish model thus restoring the β cell compromised diabetic state [48]. Therefore the signalling pathway of these receptors subtypes are more intensely studied to counter the pathophysiology of various inflammatory diseases including T2D.

2.7 Toll-like receptor signalling pathway: Introduction and role in resolving obesity underlying inflammation

2.7.1 TLRS: Structure and classification

Toll-like receptors (TLRs) are one of the specifically identified pattern recognition receptors (PRRs) among the other receptor families that include AIM2-like receptors (ALRs), nucleotide-binding oligomerization domain (NOD) - Leucin Rich Repeats (LRR)- containing receptors (NLRs), retinoic acid-inducible gene 1 (RIG-1)- like receptors (RLRs) and C-type lectin receptors (CLRs). TLRs belong to toll-IL-1 receptor (TIR) super family of proteins and are characterized by single-pass membrane spanning domain [48]. Human possess 10 TLRS (TLR1-10) and mice possess 12 TLRs (TLR1-9, TLR11-13), respectively that protect the host body from a wide range of pathogenic and auto-immune ligands. TLRs are localized on immune cells that include macrophages, neutrophils, mast cells, dendritic cells (DC), natural killer (NK) cells and on non-immune cells namely endothelial cells and fibroblasts, respectively [49-50]. Structurally, TLRs belong to type I integral transmembrane proteins which consist of a

membrane bound amino-terminal domain (NTD) rich in repetitive leucine-rich repeat (LRR) which imparts the specificity to the TLR for binding of foreign ligands, a single-pass transmembrane domain and a cytoplasmic carboxyl-terminal TIR domain that propagates ligand induced inflammatory response signaing inside the affected cell [51]. The principal function of TLRs is to recognize the microbial and pathogenic invasion in the host blood stream by identifying and binding specific pathogen-associated molecular patterns (PAMPs) and damage associated molecules patterns (DAMPs) [52]. The TLRs bind specific fungal, bacterial, viral specific ligands in order to mediate innate immune response to eliminate the microbial lipids, polysaccharides and proteins from the host body. Depending on the subcellular localization of the receptors on the cells, TLRs are broadly grouped as cell surface receptors listed as TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 and intracellular receptors present on the endoplasmic reticulum (ER) or endolysosome that include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13. The various TLRs listed above bind specific ligands derived from viruses, fungi, bacteria and pathogens that include TLR1 specific lipopeptides and glycoproteins, TLR2 specific lipotechoic acid, heat shock protein70 and zymosan, TLR3 specific double-stranded viral RNA (poly I:C), TLR4 specific lipopolysaccharide (LPS), TLR5 specific bacterial flagellin and parasitic profilin, TLR6 specific diacyl lipopeptides, TLR7 and TLR8 specific single-stranded viral RNA, TLR9 specific unmethylated CpG-DNA motifs; TLR11 specific flagellin, TLR13 specific bacterial 23S rRNA, respectively[53].

2.7.2 TLR4-LPS mediated signaling pathway: Introduction and mechanism

Toll-like receptor 4 (TLR4) is a cell surface PRR which specifically recognizes and binds the Gram negative bacteria specific endotoxin LPS (lipopolysaccharide) which is characterized by a outermost repetitive glycan polymer termed as O-antigen, connecting core composed of heptose and 3-Deoxy-D-manno-oct-2-ulosonic acid that link to lipid A hydrophobic domain. Lipid A is the conserved and prime toxic component of LPS which induce inflammatory responses in the host blood circulation and is recruited by TLR4-MD-2 complex as its specific ligand [54]. Alongside MD-2 as co-receptor, TLR4 mediated LPS recognition is accompanied by two more accessory proteins namely LPS binding protein (LBP) and myeloid-lineage cells located cluster of differentiation 14

(CD14), respectively. In order to initiate the immune response against the LPS populating in the cell surface vicinity, LBP at first bind LPS aggregates and LBP-LPS present LPS to membrane associated-glycosylphosphatidylinositol (GPI)-linked CD14 homodimer. CD14 then disintegrate the LPS aggregates into LPS monomers which gets translocated from CD14 to MD-2 in TLR4-MD-2 complex that triggers the BB loop mediated TIR dimerization that causes the juxtapositioning of the TIR domains for the recruitment of intracellular adaptors. The conformational changes in the TIR domains facilitate the binding of adaptor molecules namely myeloid differentiation factor 88 (MyD88), TIR-domain containing adaptor molecule (TRIF), TIR domain containing adaptor molecule/MyD88-adaptor-like (TIRAP/MAL), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif-containing protein (SARM) to the C-terminal region of the respective TLR [54]. LPS polymers bind to TLR4/MD-2 complex to trigger the innate immune response against bacterial endotoxin. MyD88-dependent and/or MyD88-independent pathways are activated upon LPS binding that lead to the production pro-inflammatory cytokines; chemokines, and type I interferons (IFN α and IFN-β), respectively [55].

2.7.3 MyD88-dependent pathway

LPS-TLR4 binding complex activates the MyD88 dependent pathway where in TIRAP/MAL, a sorting adaptor molecule via its phosphatidylinositol (4,5) bisphosphate (PIP2) binding domain senses the dimerized TLR4 molecule and facilitates the attachment of MyD88 to the cell surface membrane which is followed by the formation of the supramolecular organizing center (SMOC) called myddosome which is filamentous in nature and constitutes of adaptor molecule MyD88 and IL-1R-associated kinase (IRAK) members IRAK1, IRAK2, IRAK4 wherein the death domain (DD) of serine/threonine IRAK members is recognized by the N-terminal DD of MyD88 [56]. Upon MyD88 recruitment, at first IRAK4 is activated and autophosphorylated at its central kinase domain (KD) leading to activation of both IRAK1 and IRAK2 via autophosphorylation which release IRAK1 from the myddosome complex. IRAK1 then bind and activates the E3 ubiquitin (Ub) ligase TNF receptor-associated factor 6 (TRAF6). TRAF6 then activates TGF-β activated kinase 1 (TAK1) complex via recruitment of the regulatory TGFβ-activated kinase 1 binding protein (TAB2) and TAB3 to the complex facilitated via the K63-linked polyubiquitination and

oligomerization of TRAF6 [57]. Activated TAK1 results in the successful activation of IkB kinase (IKK) complex comprised of catalytic IKK-α, IKK-β, and regulatory IKK-γ (also known as NF-κB essential modulator, NEMO) pathway or canonical NF-κB pathway. Ubiquitin chain linked TAK1 phosphorylate IKK- β protein which cause the phosphorylation and subsequent proteasomal degradation of IκBα, protein that inhibits the transcription factor NF-κB and restricts its movement to the nucleus. Once IκBα degradation takes place, NF-κB, is now free to translocate from cytoplasm to nucleus [58]. Alongside NF-κB activation, TAK1 complex simultaneously activate the transcription factors activator protein-1 (AP-1) via activation of ERK1/2 and JNK as well as cAMP response element-binding protein (CREB) via p38 activation in order to induce the gene expression of inducible nitric oxide synthase (iNOS), inducible cyclo-oxygenase (COX-2), pro-inflammatory cytokines (TNF-α, IL-6, IL-1, COX-2), interferon IFN-γ and chemokines (MCP-1, IL-8), respectively. Alongside NF-κB activation, TRAF6 also activates another transcription factor activator protein 1 (AP-1) which via the phosphorylation of mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK 1/2) and p38 results in the up-regulation of the pro-inflammatory genes [59].

2.7.4 MyD88-independent/TRIF-dependent pathway

LPS binding to TLR4 also mediate the endocytosis of the LPS-TLR4 complex to cytoplasmic endosomes where TLR4 recruits the sorting adaptor molecule TRAM and TRIF, adaptor molecule meant for downstream signaling. TRIF then facilitates its interaction with TRAF6 and TRAF3. TRAF3 then activates IKKi and TBK1 which further lead to the phosphorylation of the transcription factor IRF3. The nuclear translocation of IRF3 result in the transcriptional activation of type I interferons (IFN-α and IFN-β) and IFN stimulated genes (ISGs), respectively [60]. Obesity mediated elevated plasma levels of long-chain fatty acids, free fatty acids (FFA), diacylglycerols (DAG), hypoxia, endoplasmic reticulum (ER) stress, contribute to the activation of TLR4 signaling pathway leading to lipid-induced insulin resistance and T2D [61].

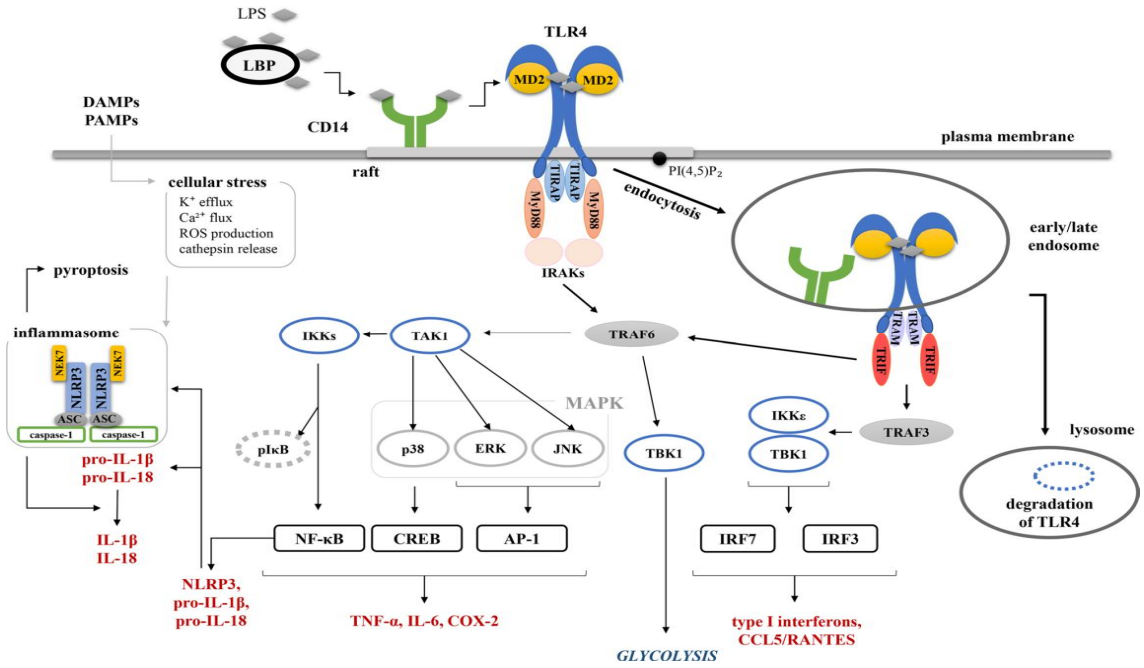


Fig 2.7.4 Schematic representation of LPS-TLR4 signaling pathway

2.8 TLR4 signaling pathway, chronic inflammation and T2D

Obesity mediated chronic inflammation in metabolically active tissues listed as liver, skeletal muscle, intestine and pancreatic islets is the prime cause for the development of insulin resistance and T2D [62]. TLR4 is the prime causative factor to develop obesity-induced adipose tissue inflammation and insulin resistance and is activated by a high level of plasma free fatty acid (FFA) uptake in insulin sensitive mature adipocytes, macrophages, $CD8^+$ effector memory T cells and $CD4^+$ Th1 cells, respectively [63]. High FFA mediated TLR4 activation contribute to the AT inflammation and insulin resistance via the activation of NF-κB and MAPK pathways that eventually induce excessive level of various pro-inflammatory cytokines (IL-1β, IFN-γ, TNF-α), chemokines, reactive oxygen species (ROS) and eicosanoids [64]. The cytokines and chemokines affect the target insulin sensitive cells in an endocrine or paracrine manner [65]. This was supported with the observation where hypertrophic adipose tissue expressed high levels of pro-inflammatory cytokine TNF-α, MCP-1, macrophage inflammatory protein 2 alpha (MIP-2α) [66]. LPS mediated TLR4 signaling results in the induction of inflammatory cytokines TNF-α and IL-6 in peripheral blood mononuclear cells (PBMNC) isolated from the patients suffering from T2D as compared to healthy controls was also reported [67]. TLR4 and TLR2 mRNA is highly expressed in T2D state as suggested by the real time PCR analysis of three experimental

groups with two groups belonging to diabetic and impaired glucose tolerance patients and one healthy control. The highly expressed TLR4 mRNA levels further induces the excessive secretion of TNF-α, IL-6, 1L-1β, MCP-1 and 1L-1 by activating MyD88-dependent pathway [68]. Saturated fatty acid (SFA) was unable to induce insulin resistance with reduced activation of NF-κB pathway in TLR4 deleted L6 myotubes and TLR4 deficient mice model [69]. In high fat diet (HFD) fed mice, increased number of adipose tissue macrophage (ATM) contribute to elevated TNF-α and MCP-1 levels which induce insulin resistant state both in mice and human [70]. In addition to the concrete data that suggests obesity induces high TLR4 mRNA expression which result in the inflammation of the adipocytes, researchers were able to show that TLR4 enhances the lipid-induced insulin resistance via its endogenous ligand, fetuin A (FetA or Ahsg) [71]. Alongside adipocytes and macrophages, other insulin sensitive organs such as pancreatic islets were also shown to secrete chemokines MCP-1 and CXCL1 via the activation of FFA induced TLR4/MD-2-MyD88 pathway [72].

2.9 Protein kinase C (PKC) mediated impairment of insulin signaling pathway

In addition to FFA induced TLR4 signalling mediated insulin resistance in adipocytes and macrophages as stated in the above sections, diacylglycerol (DAG) contribute to FFA-induced insulin resistance in liver cells via protein kinase C (PKC) activation. DAG possesses strongest affinity towards PKCϵ than other PKC isoforms [73]. HFD mediated increased hepatic DAG activates PKCϵ activation that inactivate insulin receptor kinase that further compromise the tyrosine phosphorylation of insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) resulting in the development of lipid-induced insulin resistance in liver [74].

2.10 Small molecules and their roles in combating health complications to metabolic disorders

Small molecules are low molecular weight chemical compounds with a size range 0.1-1 kDa. They contribute to almost 85-90% of available therapeutic drugs [75]. Characterized by a smaller size range than the size of native biological molecules namely nucleic acids, peptides, enzymes and polysaccharides, small molecules have the advantage to diffuse through the cell membrane and bind to the 5% biological targets

that include extracellular cell-membrane receptors such as G protein–coupled receptors and intracellular target proteins such as protein kinases [76-77]. Small molecules include sugars, lipids, phenols, alkaloids and glycosides. These chemically synthesized molecules are developed with good bioavailability with adsorption (A), easy distribution (D), metabolized (M) and then excreted (E) from the body satisfying the Lipinski's rule of five [78]. Small molecules are extracted from various fungi and plant species. Plant extracts are the source of a large portion of drug candidates that include fruits, vegetables, root, and leaves as well as flower extracts and secreted plant secondary metabolites. Plant extracts play a very pronounced role as anti obesity, anti inflammatory, anti diabetic activities [79].

2.11 Natural products and their derivatives: their role as drug molecules

Natural products and derived natural products have been explored for their invaluable role as potential drug candidates in the cure of a wide range of human health complications [80-81]. The United States Food and Drug Administration (FDA) approved 75% fraction of the naturally derived drugs showing majorly anticancer and antimicrobial activities [82]. The different categories of sources from where the small molecules or drug molecules are extracted and synthesized are as follows Biological source (B); Natural product (N); Natural product "Botanical" (NB); semi synthetic modification of natural product or derived natural product (ND); synthetic molecule (S) and vaccine (V). From 1981 to 2019, out of the aforementioned drug sources a fair portion of natural products (N) and derived natural products (ND) are reported to curb medical disorders listed below in Table 2.11.1 [83].

Serial No.	Medical disorder	Number of approved natural products (N)	Number of approved derived natural products (ND)
1.	Alzheimer's disease	1	1
2.	Analgesic	1	2
3.	Anti allergic	1	4
4.	Anti arthritic	1	4
5.	Anti bacterial	11	78
6.	Anti cancer	18	43
7.	Anti diabetic	1	8
8.	Anti parasitic	2	7

9.	Anti thrombotic	1	5
10.	Anti ulcer	1	12
11.	Hypocholesterolemic	4	1
12.	Immunostimulant	3	2
13.	Immunosuppressant	5	3

Table 2.11.1: Natural products (N) to derived natural products (ND) ratio for treating various medical disorders

A close analysis on the numbers depicted in table 2.11.1 draws an insight that the ratio of drug molecules from derived natural products (ND) to natural products (N) is high which is more followed when the drugs are categorised according to their proposed and proved beneficial activities. From the last two decades, combinatorial chemistry is a fruitful approach that utilises the structural backbone of the natural products and chemical modifications are introduced in order to derive compounds with improved pharmacokinetics attributes, solubility, and bioavailability with reduced toxicity as compared to the parent molecule [84]. The percentage of drug molecules from N and ND sources over the year 1981 to 2019 also supports the observation that the modified structure of parent natural products results in the improved effectiveness of the drug molecule as depicted in Table 2.11.2 as follows.

Serial No.	Drugs by source	Percentage of drugs from natural product (N)	Percentage of drugs from derived natural product (ND)
1.	Anti bacterial	4	78
2.	Anti parasitic	2	7
3.	Anti cancer	43	18
4.	Small anti cancer	43	18
5.	Anti diabetic	8	1

Table 2.11.2 Percentage of natural products (N) and derived natural products (ND) and their effects

Thus, the synthesis of derivatives from the parent chemical backbone results in the development of manifold effective and less toxic analogues that could be successfully employed as potent drug candidates. Many natural products are active components of our daily dietary consumption and thus could be judiciously imbibed onto our body in a harmless and effective manner.

2.12 Indirubin and Indirubin derivatives

Indirubin is a member of indigoids family and it is chemically characterised as a 3,2'-bisindole, an alkaloid. This group of compounds possess their utilisation mostly in the field of dye and medicine [85]. Indirubin is an active component of a traditional Chinese medicinal formulation named Danggui Longhui Wan which is used as an anti proliferative agent to cure chronic myelocytic leukemia and it is also known as 3, 2'-bisindole which is an active red isomer of indigo [86]. The various plant species from where indirubin is extracted are namely *Indigofera tinctoria L., Isatis tinctoria L., Cnidii fructus, Isatis indigotica, Strobilanthes cusia, and Polygonum tinctorium* [87]. Parent indirubin possess very low solubility and absorption with gastrointestinal toxicity which is compensated by various indirubin analogues or derivatives with improved pharmacokinetic attributes listed as bioavailability, solubility, and lowered toxicity with better pharmacokinetics efficacy. By utilizing the basic bicyclic heterocycle indole skeleton structure, various derivatives of indirubin is synthesized chemically [88]. Indirubin derived molecules are classified as i) Halogenated and alkylated derivatives; ii) substituted indirubins as N-substituted and O-substituted derivatives. Some examples are N-ethyl-indirubin, 5-halogen-indirubins, N-methylisoindigotin (meisoindigo), indirubin-3'-monoxime, indirubin-5-sulfonic acid, 5-iodoindirubin-3'-monoxime, 3'-substituted 7-halogenoindirubins, et cetera [89].

2.12.1 Indirubin and its derivatives: Role as anti cancer and anti inflammatory agent

With the knowledge of curing chronic myelogenous leukemia (CML) as reported in 1980s, indirubin and its derivatives was explored to have multifaceted medicinal aspects by various researchers both in vitro and in vivo. The underlying healing action of indirubin on CML patients was triggered by the inhibition of cell cycle regulator cyclin-dependent kinases (CDKs)/cyclin complexes such as CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E and inhibition of an important tumour growth marker glycogen synthase kinase-3β (GSK-3β) [90]. Indirubin and its derivatives act as potent inhibitor of serine/threonine kinases CDK and GSK-3β by competitively inhibiting for the ATP binding to the kinase active site by forming van der waals and hydrogen bonding eventually leading to G2/M arrest and apoptosis [91]. It was evidenced by indirubin-5-sulphonate (E226) mediated active and competitive inhibition of CDK2 and pCDK2-

cyclinA complex with an IC_{50} (50% inhibitory concentrations) value of 35nM [92]. Also, indirubin analogues namely 6-bromoindirubin, 5-bromoindirubins, 5,5'-bisubstituted analogs 6-bromoindirubin-3'-oxime exhibited CDK inhibition with very attractive IC_{50} values ranging from 0.91 to 0.46 μM in leukemic cell lines K562 cells, KCL22 cells, mutant leukemia cell lines as well as various solid tumors [93]. 6-bromoindirubin acetoxime inhibited GSK-3β which caused the inhibition of migratory pediatric and adult gliomas [94]. With IC_{50} values ranging between 5-50nM, indirubins competitively bind to the ATP bindng active pocket of GSK-3β kinase thus contribute in treating the GSK-3β triggered abnormal tau phosphorylation in alzheimer's disease (AD) [95]. Aurora kinases are serine/threonine kinases classified as Aurora A, B and C which are responsible for chromatid segregation which regulate mitosis whose over expression cause various cancers thus making it a potential target to treat breast, ovarian, gastrointestinal cancers and tumours [96]. A bromine substitution from 6C to 7C position turned 6-bromoindirubin-3'-oxime (6BIO), a GSK-3β inhibitor into 7-bromoindirubin-3'-oxime (7BIO), a potent Aurora B and C kinases inhibitor [97]. A novel indirubin derivative LDD-1819 alongside acting as a GSK-3β inhibitor was reported to act as potential inhibitor for Aurora A kinase which is responsible to regulate carcinogenesis [98]. FMS-like tyrosine kinase-3 (FLT3) regulates cell growth and proliferation of hematopoietic progenitor cells and its mutation result in development of acute myeloid leukemia (AML) marked by abnormal number of leukemia cells [99]. 5-fluoro-indirubin-3'-oxime (IC_{50} = 15nM) and indirubin-3â²-oxime was explored as an active FLT3 inhibitor in MV-4-11 cells [100]. 5- and 6-bromoindirubin derivatives act as c-Src or Aurora A kinase, and JAK/STAT molecules inhibitor in prostate DU145, melanoma A2058, pancreatic MIA-PaCa2, lung A549, breast MDA-MB-231 and ovarian SKOV3 marked by decrease in viable cell numbers [101-102]. Further indirubin was also successful in inhibiting cell viability in human ovarian and its derivative IDR-E804 caused cytotoxicity in human breast and prostate cancer cells by down regulating the phosphorylation status of signal transducer and activator of transcription (STAT)-3 [103-104]. Another indirubin derivative, 5-methoxyindirubin-3'-oxime claimed to reduce cell viability in pancreatic ductal adenocarcinoma (PDAC) [105]. The treatment of cancer by oxime-mediated kinase inhibition also display potential anti-inflammatory effect as the CDKs and GSK-3β are responsible for the inflammatory responses underlying the secretion of pro-inflammatory cytokines via the activation of the transcription factors NF-κB, STAT3

and AP-1 as tested and observed in various cell lines and animal models [106]. Indirubin proved to be a potent anti-inflammatory molecule as it improved the pro-inflammatory state in LPS-induced mastisis state in mouse model [107]. Indirubin derivative IDR-E804 was also reported to impart anti-angiogenic action in human umbilical vein endothelial cells (HUVECs) [108]. Indirubin as an active molecule of Indigo naturalis was further explored to have a preventive role against psoriasis by inhibiting active cell division and differentiation of human epidermal keratinocytes [109]. A very recent report displays the role of indirubin to have a preventive role in attenuating obesity by encouraging the browning of WAT and the up-regulated activity of brown adipose tissue (BAT) with an enhanced uncoupling protein-1 (UCP-1) expression in adipocytes [110]. By inducing potent PPARγ expression, Indirubin significantly improved the adipogenesis process in 3T3-L1 pre-adipocytes thus marking itself as a potential curative for T2D [111]. The above stated various research finding suggest that indirubin and its analogues act as potent inhibitors of a wide range of kinases whose over expression or mutation result in uncontrolled cell growth and division leading to a cancerous state which confer their potential therapeutic efficacies as anti-cancer, anti-inflammatory, anti-angiogenic, anti-psoriatic and anti-diabetic functions.

2.12.2 Indirubin-3'-monoxime (I3M)

Indirubin-3'-monoxime (I3M) stands out to be one of the most studied stable indirubin derivative with many synonyms such as indirubin-3'-oxime, indirubin-3-oxime, indirubin-3-monoxime, indirubin oxime, and indirubin-3'-monoxime [112]. I3M, a member of biindoles class was synthesized by Li et al in the year 1996 and it consists of oxindoles, a bisindole, a ring assembly, a ketoxime and an alkaloid [113].

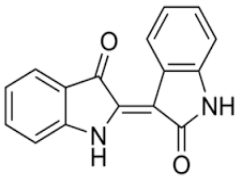
Chemical formula	$C_{16}H_{11}N_3O_2$
Chemical structure	
Molecular weight	277.3
Physical appearance	Dark red crystalline solid
Solvent solubility	DMSO (6 -10 mg/ml) Ethanol (2-4 mg/ml)

Table 2.12.2 Physicochemical characteristics of indirubin-3'-monoxime

I3M has enhanced solubility and improved drug scaffold than and thus the carbon skeleton of I3M is explored by many researchers for its various medicinal attributes all around the globe. Scientists worldwide have reported that I3M possess anti leukemic efficacy and anti cancer activity to treat renal cancer, and laryngeal carcinoma [114]. I3M also efficiently inhibited STAT-3 signaling in activated vascular smooth muscle cells (VSMCs) as well as Notch1 signaling. I3M and its derivatives exhibit potent CDK2/cyclin E1 and CDK9/Cyclin T1 inhibition as supported by molecular docking analysis [115-117]. Literature review supports that I3M has anti cancer activity against breast, prostate, lung, and kidney cancers [118]. I3M in hybrid with thymoquinone (Tq) improved Lung cancer (LC) in A549 cells and A549 mouse xenograft model via Akt/mTOR/NF-κB signalling pathway [119]. Evident data support that the I3M is a cell permeable GSK-3β inhibitor which triggers the inhibition of cytokine production and neurotoxicity in SH-SY5Y neuroblastoma cells. I3M also efficiently inhibited cancer cell proliferation in human osteosarcoma (OS) with induced apoptosis [120-121]. I3M was also successful in showcasing anti-inflammatory activity in LPS-induced murine macrophages RAW264.7 marked by a notable down regulation in iNOS and COX-2 expression followed by the reduced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production and a significant decrease in the secretion of pro-inflammatory cytokines, IL-6 and 1L-1β via the inhibition of the major inflammatory molecules NF-κB and JNK [122]. Impaired GSK-3β activity is reported to be one of the causal factors behind the high fat diet induced impairment of insulin signalling or insulin resistance which has a crucial interrelation with the pathogenesis Alzheimer's diseases (AD). Thus, I3M which is a proven GSK-3β inhibitor resulted in a neuroprotective effect in HFD mice with cognitive impairment in a dose-dependent manner [123].

A fair amount of research observation are in hand which document the potential medicinal aspects of indirubin and its analogue indirubin-3'-monoxime in combating various health and metabolic disorders including tumours, cancers, obesity, inflammation, T2D, AD, etc. However, the underlying mechanisms of many of these effects remain largely unexplained and that considerably delays their integration into the modern healthcare system.

2.13 Vanillin (VNL): A brief introduction and its anti-cancer, anti-microbial and anti-inflammatory attribute

Vanillin (2-hydroxy-3-methoxy benzaldehyde) is a widely used flavouring agent in food industry, confectioneries, as an aromatic component in perfume industry and as a chemical intermediate in many drug industries [124]. Vanillin is isolated from the seed pods of the *Vanilla planifolia*, *Vanilla tahitensis* and *Vanilla pompona* [125]. It is a phenolic aldehyde and its structure is composed of aldehyde, methoxy and ether as functional groups.

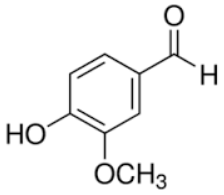
Chemical formula	$C_8H_8O_3$
Chemical structure	
Molecular weight	152.149
Physical appearance	Pleasant smelling white monoclinic crystals
Solvent solubility	Water (10mg/ml) Glycerol (50mg/ml)

Table 2.13 Physicochemical characteristics of Vanillin

Due to its multifaceted uses as flavouring agent, in food industry, as a health supplement due to its very low calorific value and as a rich source of antioxidants, vanillin is positioned as a valuable industrial attribute which drives its worldwide mass production marked by a yield of 16000 tons in the year 2015 [126]. Vanillin at a concentration of 300mg/kg had non-toxic effect on the vitals such as blood cells, liver and kidney when administered on rats orally exhibiting a neuroprotective activity thus

making it a safe and potential candidate to be explored for the various beneficial attributes [127].

2.13.1: Vanillin as anti-cancer and anti-mutagenic agent

Dating back to the year 1998, vanillin was reported to show anti mutagenic effect on a primary cell culture where in it prevented X-ray and UV-induced chromosomal damage at four different concentration of 0,5,50 and 100 µg/ml [128]. Vanillin acted as a DNA-PKc inhibitor as it selectively interrupted the process of non-homologous end joining (NHEJ) which resulted in the failure of the successful NHEJ mediated repair step completion and thus indirectly prevented cancer development mediated by chromosomal abnormalities [129]. Vanillin displayed anti-metastatic activity in breast cancer cell (spontaneous mammary adenocarcinoma cell line 4T1) [130]. It also showcased anti-proliferative property by preventing the uncontrolled cell division in HepG2 and SH-SY5Y cell lines [131] and by disturbing the stability of mitochondrial membrane and enhancing DNA fragmentation in leukemic (Jurkat) cells [132].

2.13.2: Vanillin as anti-microbial agent

Vanillin also exhibited potent anti-microbial effect as it provided intriguing data suggesting a species specific bacteriostatic action of vanillin on three different bacterial species namely *Escherichia coli*, *Listeria innocua* and *Lactobacillus plantarum* at a concentration range of 10-100 mM via respiration inhibition and proton gradient dissipation machanism [133]. Vanillin efficiently displayed anti microbial activity towards bacterial (minimum inhibitory concentration, MIC = 10 to 13.3 mM), fungal (MIC = 12.5 to 13.3 mM) and yeast (MIC = 5 to 6.7 mM) strains which are the causal microbes for the food spoilage in fresh cut slices of mango. [134]. Vanillin reduced the plasma ROS levels in oral administered mice as supported by various antioxidant assays [135]. The fungistatic activity of vanillin was also tested where it was able to inhibit the growth of the strain *Alternaria alternate* at a concentration of 250 mg/L [136].

2.13.3: Vanillin as anti-inflammatory agent

In-vitro experiments also displayed very promising results supporting the anti inflammatory action of vanillin. As demonstrated in a study on LPS induced COX2 expression as well as inactivation of NF-κB expression in RAW 264.7 macrophages. As

a member of the phenolic group of molecules, vanillin imparts anti inflammatory action as proved early in 19th century in 1978 by RW Egan et.al where they developed the link between the phenols and their non-steroidal anti-inflammatory effect [137]. Vanillin alongside its other members from the phenolic group namely guaiacol, phenol, eugenol were proved to show inhibition to prostaglandins cyclooxygenase enzyme which catalyzes the production of inflammatory prostaglandins and thromboxane from the precursor arachidonic acid [138]. Vanillin has the property to imbibe LPS-activated macrophages with anti-inflammatory effect by inhibition of iNOS that eventually leads to the decreased production of nitric oxide (NO) and thus reducing the inflammation response in the LPS-induced inflamed RAW macrophages [139]. Another group observed that vanillin treatment led to a significant reduction in the volume of paw edema at 100mg/kg and 200mg/kg vanillin concentration on carrageenan induced paw edema rat models [140]. Vanillin's anti-inflammatory facet was further proven as it successfully blocked the expression of pro-inflammatory cytokines IL-8, IL-1β, IL-6 and TNF-α, notably suppressed the expression of the inflammatory molecules iNOS, PGE_2, NO and COX2 in LPS-activated human THP-1 macrophages [141]. Vanillin's anti-inflammatory mode of action became the healing and repairing wing towards various health complications underlying inflammation. Mice model with LPS-induced acute lung injury (ALI) was notably prevented by vanillin treatment via the inhibition of inflammatory molecules ERK1/2, p38, NF-κB and pro-inflammatory cytokines [142-144]. Vanillin treatment also lead to the rescue of the mammary glands from LPS-induced mastitis via the vanillin mediated blockage of LPS-induced inflammatory responses as marked by the reduction in TNF-α, IL-6, IL-1β, iNOS, COX-2 levels [145-146]. A chitosan-vanillin hydrogel formulation was developed and subjected to wound healing on rat skin samples which alongside wound healing also showcased itself as anti-inflammatory marked by the lowering of pro-inflammatory TNF-α and IL-1β with an enhanced anti-inflammatory IL-10 and TGF-β gene expression, respectively [147-148]. Again, a very recent report suggested that vanillin portrayed a gastroprotective effect by downregulating the expression levels of pro-inflammatory genes IL-6, IL-1β, TNF-α and IFN-γ thus improving the ethanol triggered inflammatory status of gastric tissue [149].

2.13.4: Vanillin bioavailability

Vanillin as a drug when administered into the body could be stored in fat tissue with a slow release with time owing to the lipophilic nature of the drug. Also, it has a fair bioavailability percentage of 7.6 with a probable enterohepatic circulation (EHC) after administration [150]. Recently, nanoparticles (NP) have been used as a delivery carrier for vanillin that has proved an enhanced bioavailability with good solubility and improved pharmacokinetic properties [151-152]. Vanillin was also reported to accelerate the rate of passive transportation and absorption of moderately bio-available drugs wherein vanillin interfered with the lipid bilayer assembly to facilitate the entry of the accompanied drugs inside the cell cytoplasm in Caco-2 bidirectional transport set up [153]. With the gathered knowledge of proven data supporting the potent anti-inflammatory activity of various small molecules and phytochemicals have provided the ground to further work on the exploration of the bioactive attributes of vanillin (VNL) and indirubin-3'-monoxime (I3M).

Objectives of the study

Thus, in order to address these loopholes, the following objectives were framed for the present study:

1. To study the efficacy of different small molecules on the induction of insulin sensitivity through A_{2A}AR signalling pathway.
2. To investigate the role of indirubin 3'-monoxime (I3M)-induced A_{2A}AR signalling in alleviating lipid-induced adipocyte insulin resistance.
3. To explore the molecular target(s) of vanillin (VNL) in the impairment of LPS-induced macrophage inflammation.

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