The Role of Muramyl Depeptide in Glucagon-Like Peptide-1 Regulation

by

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Abstract

Type 2 diabetes (T2D) accounts for 90% of all cases of diabetes, and it is one of the world's most threatening chronic and metabolic diseases in the world. Researchers have been investigating the possible links between the glucagon-like peptide1 (GLP-1) incretin hormone and bacterial metabolites in the gut in order to improve T2D treatments. In this study, I hypothesized that muramyl dipeptide (MDP) enhances the release of GLP-1 to improve hyperglycemia. Various muropeptides were tested in GLP-1 secreting cell lines (NCI-H716 and GLUTag) for their ability to regulate hormone secretion. The intracellular mechanism of lipid-modified L18MDP was examined via western blot and pharmacological inhibition. Pilot studies using siRNA for the MDP receptor NOD2 were completed. GLP-1 secretion was significantly induced by different concentrations of L18MDP in the two cell lines, (5, 10 µg/ml) in GLUTag and (10 µg/ml) in NCI-H716. Treatment did not affect cell lines viability. The p38 MAPK signaling pathway was slightly stimulated at the 10-minute L18MDP treatment as was demonstrated by western blot. Pharmacological NOD2 inhibition with GSK717 at different doses (5, 10 µM) failed to block the L18MDP stimulation of GLP-1 secretion in GLUTag and NCI-H716 cell lines. This work demonstrates that L18MDP can significantly stimulate GLP-1 release; however, future work will be required to resolve the precise mechanism.

Keywords: Glucagon-like peptide 1, Muramyl dipeptide, L18MDP, p38 MAPK

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Introduction

Type 2 Diabetes:

Diabetes is a primary cause of mortality and cardiometabolic morbidity, as the worldwide incidence of diabetes has witnessed rapid growth in the past few decades. Globally, around 415 million people have diabetes, and it is estimated that the prevalence will increase up to 642 million people by 2040[1]. Type 2 diabetes (T2D) is characterized by both insulin resistance and beta cell dysfunction, which lead to persistent hyperglycemia. Beta cells are found in the islets of Langerhans and are responsible for the production of insulin, a peptide hormone. In beta cell dysfunction, insufficient insulin is produced or released, leading to reduced cellular uptake of glucose in the body[2]. Diabetes can lead to serious long-term complications that increase the risk of death such as microvascular (nephropathy, neuropathy, and retinopathy) or macrovascular (cardiovascular disease, peripheral artery disease, stroke) complications[3–5]. Moreover, it is important to know that some risk factors for T2D include obesity, overweight, poor diet, and physical inactivity [6].

Initial T2D treatment often involves non-pharmacological interventions including diet and exercise. These are often supplemented with pharmacological approaches including antihyperglycaemic agents such as metformin and hypoglycemia agents such as insulin injections[7,8]. However, studies have shown that these approaches are not always satisfactory, as Type 2 diabetic patients are becoming resistant to these kinds of treatments[9]. Also, insulin has to be injected, and it is associated with many risk factors such as colorectal cancer, hypoglycaemia and weight gain[8]. An emerging area of antihyperglycaemic treatments is incretin-based therapies that mimic the function of glucagon-like peptide-1 (GLP-1) [8]. Indeed, GLP-1 therapies are becoming a widely used antidiabetic in Canada[10].

Glucagon-Like peptide 1 (GLP-1):

Incretin hormones secretion in humans is mediated by two peptide hormones. They are secreted from gut endocrine cells known as enteroendocrine cells (EECs) in response to nutrient intake. Additionally, they are able to enhance the release of insulin in what is known as the "incretin effect"[11]. The first hormone, which was discovered in 1973, is called glucose-dependent insulinotropic polypeptide (GIP), and it is produced from K-cells in the proximal duodenum[9]. GLP-1 was the second incretin hormone discovered, and it is produced in L cells located in the distal small and large intestines. Specifically, GLP-1 is expressed in a low density in the duodenum and in a higher density in the ileum, colon, and rectum[12]. This study will focus on GLP-1 due to its importance in treating T2D as an insulin-stimulating glucoregulatory hormone. It is also known that glucose-stimulated insulin secretion can be potentiated by GLP-1. GLP-1 has multiple functions such as delaying gastric emptying, suppressing glucagon secretion, promoting satiety. GLP-1 has triggered the interest of experts because of the different GLP-1 receptor agonists that have been developed for treating T2D[13].

GLP-1 Production:

GLP-1 is a powerful incretin hormone produced in the L-cells by the tissue-specific posttranslational processing of the proglucagon gene[14]. The bioactive GLP-1 has two equally potent forms while circulating in the bloodstream: GLP-1 $^{7\text{-}36NH2}$ and GLP-1$^{7\text{-}37}$, the former of which is more abundant[15]. The physiological effects of GLP-1 produced in the gut cells are widespread. From gut motility, decreasing gastric emptying to inhibiting glucagon secretion and increasing diuresis, the roles of GLP-1 are varied and have piqued the interest of researchers[14,16]. Many nutrients, ranging from dietary fiber to fatty acids, play a crucial part in up-regulating the transcription of the gene that encodes GLP-1, thereby catalyzing the

production of GLP-1[14]. Although the majority of L-cells are located in the distal ileum and colon, the levels of GLP-1 increase whenever food ingestion occurs. Following its release, GLP-1 affects multiple target tissues throughout the body, the actions of which are supposed to be mediated by a single G-protein-coupled receptor isoform[14].

GLP-1 is a member of the glucagon peptide family and is obtained from the glucagon gene (GCG) located on chromosome 17[17]. The GCG is found in the alpha cells of the pancreas and in the enteroendocrine L-cells in the gut, predominantly in the distal ileum and colon regions. It is also expressed in a specific population of neurons present in the nucleus tractus solitarii region of the brainstem[16]. The resultant gene product is translated to preproglucagon, which is post-translationally cleaved by specific convertase enzymes known for cleaving proprotein and propeptide substrates at the C-terminus to trigger the generation of the active peptides. In particular, propeptide convertase 1/3 (PC1/3) is actively involved in the generation of GLP-1 and GLP-2 in the gut[17]. These cleavage sites within the proglucagon molecule along with the presence of particular prohormone convertase enzymes are responsible for determining the nature of the peptide molecules that are formed. For instance, the N-terminal cleavage of proglucagon is responsible for the formation of hormones such as glicentin, glicentin-related polypeptide (GRPP) and oxyntomodulin (OXM)[16]. Moreover, GLP-1 is also formed due to the cleavage of proglucagon molecules and protease enzymes in the L-cells. In other words, the proglucagon molecules undergo a tissue-specific differential process that leads to the formation of OXM in the gut and glucagon alongside the creation of the N-terminal fragment of glicentin in the pancreas[16].

As GLP-1 is comprised of thirty amino acids, it undergoes amidation at the carboxyl terminus. This amidation process together with the histidine of GLP-1 is fundamental in the insulinotropic and glucagon-inhibiting activities of GLP-1[17]. Additionally, amidation also determines the lifespan of circulating GLP-1 in the bloodstream. The processing of the proglucagon gene is explained in Figure 1[18].

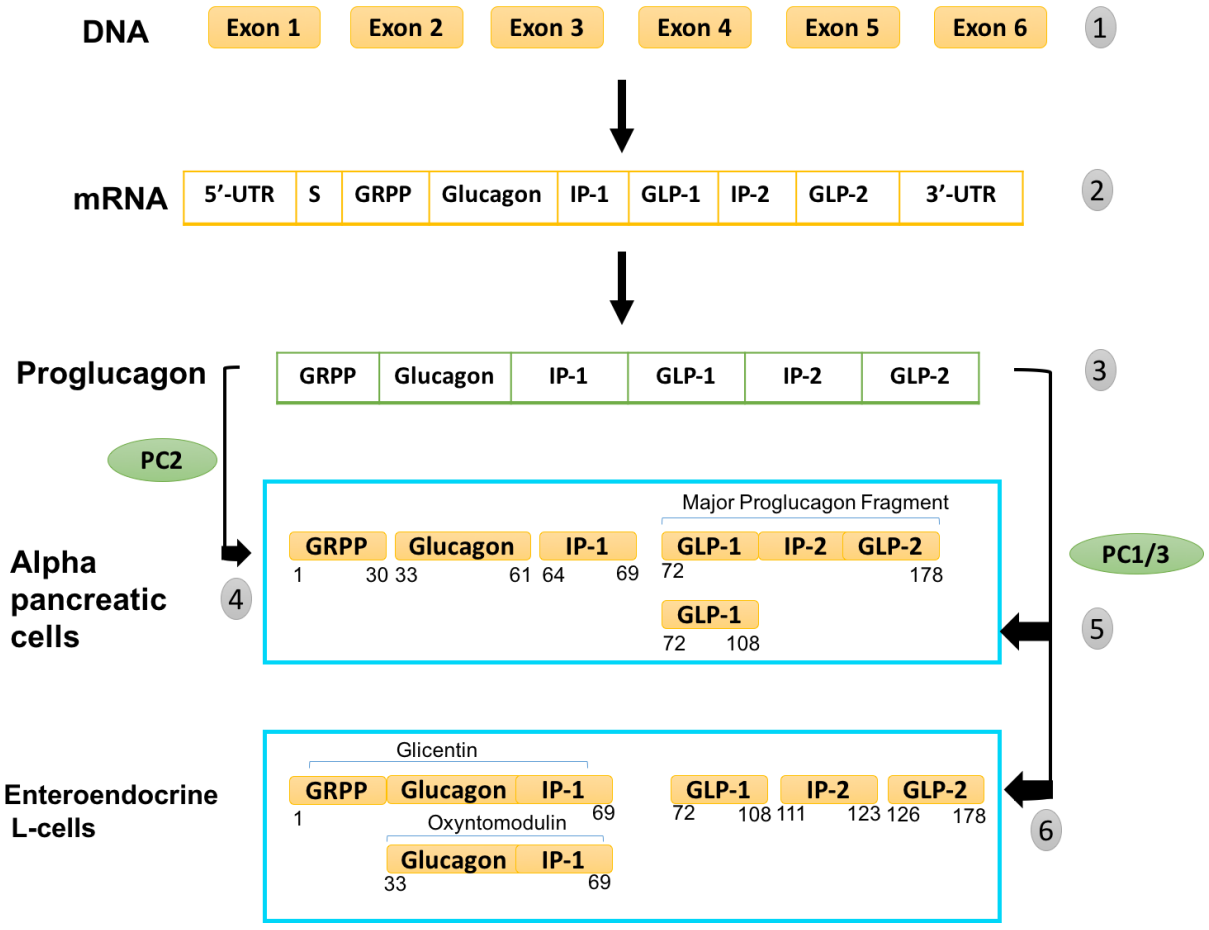


Figure 1: The formation of proglucagon-based peptides from the processing of the proglucagon gene.

Adapted from reference 18.

Intestinal GLP-1 Secretion:

As mentioned above, L-cells are located in the distal ileum and colon regions of the small intestine in both humans and rodents[19]. GLP-1 has two biphasic patterns once released after meal intake. The early phase occurs after 10 to 15 minutes, and then the longer phase occurs after 30 to 60 minutes[20]. Therefore, the initial rapid release of GLP-1 likely originates from neural stimulation, and its secretion occurs from the circulating or luminal nutrients (such as glucose, bile acids and fatty acids) that bind directly to the surface receptors on the cell membrane[19].

Glucose sensing:

Many studies have shown that luminal glucose is a potent trigger of GLP-1 release in both animals and humans. However, the most extensively studied glucose-sensing cells are the pancreatic beta cells, which increases their metabolic rates as direct concentrations of ATP and ADP occur, leading to the closure of K_{ATP} channels on the surface of cell membranes[21]. Though many early studies conducted on this topic dismissed the possibility that pancreatic β-cells and L-cell glucose sensors may be identical, recent studies have proved that glucose-dependent responses are mediated by sodium ions and are mimicked by a wide range of monosaccharides, some of which are galactose, α-methylglucopyranoside,and 3-O-methylglucose[22]. These results demonstrate the sensitive nature of sodium-glucose cotransporter (SGLT) and support the conclusion that SGLT is linked with the release of GLP-1. However, in the GLUTag cell line, it was found that glucose application led to depolarisation of the cell membrane, an increase in intracellular calcium ions along with an increase in the firing of the action potentials. Further, the release of GLP-1 that is mediated by the cells suggests that these cells contain the essential glucose-sensing machinery responsible

for creating the in vivo release of GLP-1[21].

Subsequently, GLP-1 secretion from the GLUTag cells is triggered by SUR1 receptors, namely glibenclamide and tolbutamide, which catalyse the process of insulin secretion in the pancreatic beta cells. These phenomena take place due to the presence of a K_{ATP} channel-dependent pathway in the GLUTag cells[21]. However, the presence of a K_{ATP} channel-dependent pathway is not the sole reason for the glucose-sensing mechanism. For instance, non-glucose analogues such as α-methylglucopyranoside can also create a range of excitation activities and trigger the release of GLP-1 secretion in GLUTag when applied at high concentration levels[22]. Likewise, SGLTs are utilised by the border cells of the intestine region to absorb sugars from the gut lumen. This inward influx of Na^+ ions at higher concentration levels along with substrate intakes create a potential inward current to neutralise the balance created by the cell membranes in place of the depolarisation process[21].

Fatty Acids:

In addition to glucose, fat ingestion is also known to be a potential trigger for GLP-1 release. The affinity of G-protein-coupled receptors towards fatty acids and fats with a particular chain length and degree of saturation has piqued the interests of the researchers in studying the role of lipids in catalysing GLP-1 release. In this regard, two receptors have gained more attention[19]: GPR40 and GPR120. Both receptors respond positively to unsaturated, longer-chain fatty acids. While GPR40 is abundantly expressed in insulinoma cells, GPR120 is found in the gut and STC-1 cells. The toning down of GPR120 by siRNA creates a decrease in GLP-1 secretion, which emphasises the importance of both these receptors in triggering the release of GLP-1.

Bile Acids:

Bile acids (BAs) can be defined as cholesterol-based metabolites that trigger the intestinal absorption and movement of edible, dietary lipids[22]. BAs also play a major role as signalling molecules in controlling energy, lipid and glucose metabolisms by binding to the nuclear receptor farnesoid X receptor (FXR) and the cell surface receptor Takeda G protein receptor 5 (TGR5) in various organs. By doing so, BAs regulate the intestinal secretion of incretin hormones, inflammation levels, gluconeogenesis, glycogen synthesis and determine the composition of gut microbiomes[22]. FXR, which is abundantly expressed in entero-hepatic organs such as the intestines and the liver, plays a crucial role in energy homeostasis[23]. The BAs produced by cholesterol in the liver get transformed into secondary BAs in the intestine by gut microbiota. These microbiota also modulate the GLP-1 secretion in the L-cells by bonding to the transmembrane receptor TGR5 and the nuclear receptor FXR[22]. FXR is expressed mainly in the enteroendocrine L-cells, where it modulates the production of GLP-1 in response to glucose stimulation. That is, the FXR activation in L-cells decreases the expression of the glucose-induced proglucagon gene along with the secretion of GLP-1 by inhibiting glycolysis and the transcription factor ChREBP[23]. Within 15 minutes, the level of GLP-1 in the circulation increases after a meal and reaches a peak at 40 and 90 minutes[24]. GLP-1 has a short half-life (1 to 2 minutes). It is degraded by two mechanisms: the dipeptidylpeptidase-4 (DPP-4) enzyme action and renal elimination. At the N-terminal dipeptide, DPP-4 cleaves both GLP-1^{7-36} and GLP-1^{7-37} to form GLP-1^{9-36} or GLP-1^{9-37}. The formed GLP-1 is cleared from the circulation through the kidneys.

GLP-1 Receptor Signaling:

GLP-1 signalling begins by binding to its receptor 'GLP-1R' and triggering a downstream signalling cascade[21]. This phenomenon induces a powerful stimulation of glucose-stimulated insulin secretion (GSIS) in β-cells along with the inhibition of glucagon release in the pancreatic α-cells. Analogues of GLP-1, namely Exendin-4 and liraglutide, cannot be drastically reduced by DPP-4, unlike GLP-1, inducing continuous therapeutic actions[21]. This action cannot be executed by circulating GLP-1, as it has a brief life span. GLP-1R is a B-class G-protein-coupled receptor abundantly expressed in the cental nervous system (CNS), lower levels of the gut region, liver, kidneys, lungs, heart, muscles, peripheral nervous system, and other tissues. Therefore, upon binding to the receptor, GLP-1 along with its analogues, Exendin-4 and Liraglutide, initiate a wide-range of anti-diabetic effects, such as an increase in satiety levels, the replenishment of insulin levels, a reduction in gastric emptying as well as anti-inflammatory and cytoprotective actions on beta cells[21].

Upon the completion of the ligand-binding process, GLP-1R initiates a cascade through which membrane-bound Adenylyl Cyclase (AC) and the continuous production of cyclic adenosine monophosphate (cAMP) occurs[21]. Through downstream cAMP formation, several signal transduction pathways begin, which commonly requires the activation of cAMP effectors, namely protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC)[25]. The mediated effects of GLP-1R can impact both calcium flux and insulin secretion in a posttranslational manner. In β-cells, GLP-1-stimulated pathways can act quickly to initiate glucose-dependent insulin release[21]. This process will be followed by a rapid increase in the cAMP levels, which leads to an increase in vesicles containing insulin[26]. Fundamentally, receptor agonists work by inducing insulin secretion and inhibiting glucagon secretion in a

glucose-dependent way, which explains why agonist drugs have a zero risk of hypoglycemia. More specifically, the GLP-1RAs can effectively improve the glucose sensitivity of β-cells. They can improve the glycemic profile of a patient by suppressing appetite, providing satiety, and reducing gastric emptying, thereby providing weight loss[27].

GLP-1 Therapeutics:

The constant increase in the number of drugs available to treat T2D and the increasing complexity of patients with this condition present a considerable challenge when it comes to identifying the right set of treatment measures for patients with T2D. GLP-1 receptor agonists (GLP-1RAs) are regarded as safe, non-insulin injectable options for the management of T2D[27]. Also known as aGLP-1, these agonist therapeutic drugs have a wide range of effects including a prominent cardio-protective effect among patients with T2D[28]. Known as an effective method for improving glycemic control with almost zero risks of hypoglycemia and providing effective weight loss, the agonist drugs are a vital addition to the existing armamentarium[27].

Many GLP-1RAs, such as dulaglutide, liraglutide, lixisenatide, exenatide BD, exenatide QW, and semaglutide are currently administered in countries such as Canada[28]. Among these drugs, liraglutide is the only receptor agonist drug that does not have complete resistance to DPP-4 inactivation[29]. A one-year administration of liraglutide to patients who have already achieved weight loss through a hypocaloric diet triggered greater weight loss in those patients[28]. Dipeptidyl peptidase IV can quickly degrade GLP-1, which explains its extremely short life span (approximately two minutes)[15]. Therefore, aGLP-1 drugs need to avoid DPP-4 degradation[29]. There are also oral anti-hyperglycemic agents known as DPP-4 inhibitors. Some of the available inhibitors are saxagliptin, sitagliptin, and linagliptin[30]. These

inhibitors have been used to treat T2D for more than a decade by blocking the action of the DPP-4 enzyme. While these GLP-1-based therapies are a vital part of anti-diabetic therapies, new approaches that enhance the endogenous levels of GLP-1 are of interest.

Bacterial Metabolites in T2D:

The gastrointestinal tract (GI tract) is a highly complicated organ that consists of the intestinal microbiota, intestinal epithelium and local immune system[31]. The gut microbiota have a mutually symbiotic relationship with the host (i.e.) the intestine. The gut microbiome plays a significant role in energy homeostasis, insulin sensitivity and glucose metabolism[32]. However, in a recent research, it was found that Bacteroides and Bifidobacterium are the two most frequently found bacterial genera that offer people significant protection against T2D[32]. However, other bacterial genera such as Blautia, Ruminococcus and Fusobacterium were most commonly found in higher levels in patients with T2D. There were also some discordant results from Lactobacillus genus, where species such as *L.gasseri*, *L.acidophilus* and *L.salivarius* were profoundly elevated in T2D patients. In contrast, other species such as *L.amylovorus* were noted as having significantly lower levels[32].

The introduction of prebiotics, probiotics and postbiotics into the human diet is highly favorable to the development of gut microbiota-based anti-diabetics. Prebiotics are defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health"[33]. Probiotics are live microorganisms like bacteria and yeast that help benefiting the alteration of the host's intestinal microbiome[34]. Prebiotics are used as an alternative to probiotics or as their supplement. However, different prebiotics can stimulate the growth of different types of gut microbiomes. Likewise, prebiotics and probiotics can be

combined to form synbiotics, in which a prebiotic component will favour the development of a probiotic microorganism[31]. Similarly, the 'postbiotics' is new in the "biotics" family. Postbiotics are beneficial bioactive chemicals that are generated in the gut by probiotic bacteria that feed on prebiotics to promote health. Postbiotics are made up of different constituents such as metabolites, enzymes, peptides, and muropeptides[35]. Like prebiotics and probiotics, postbiotics also have direct immunomodulatory effects on a person's overall health and development, but their impact on glucose regulation is largely unknown[36]. Some postbiotics include bacterial endotoxins like lipopolysaccharides that can promote insulin resistance during metabolic endotoxemia[37]. Conversely, others, including the bacterial cell wall-derived muramyl dipeptide (MDP), are known to enhance insulin sensitivity. This insulin-sensitising postbiotic requires the presence of the nucleotide-binding oligomerisation domain containing 2 (NOD2)[37]. NOD2 is an innate pattern recognition receptor that senses the release of muramylpeptides from bacterial peptidoglycan, especially MDP[37].

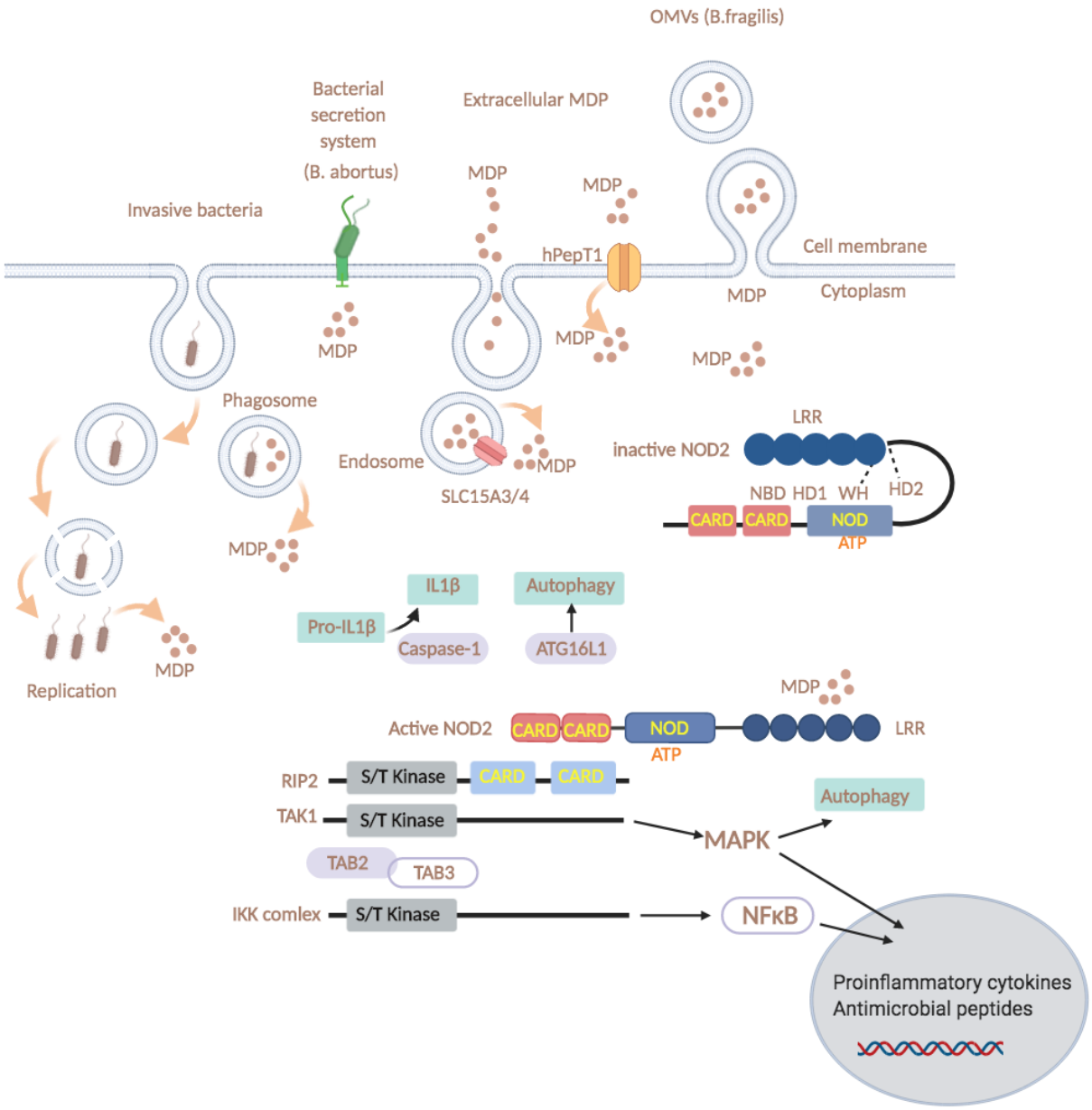
It is important to understand the potential relationship between MDP and GLP-1 in the gut to identify how it can be used in treating T2D. In the context of recognising bacterial patterns and component releases, it is remarkable that PEPT1, a receptor that mediates peptide-induced GLP-1 secretion, can transport small bacterial peptides such as MDP[38]. MDP can be recognized by intracellular receptors like NOD or NOD-like receptors that belong to the innate immune system and can identify pathogen-related molecular patterns[38].

Muramyl Dipeptide MDP signaling:

NOD-like receptors, as mentioned, are cytosolic innate immune receptors that can identify bacterial peptidoglycans. More specifically, NOD1 and NOD2 can act as immune sensors for peptidoglycan-derived molecules like MDP and y-D-glutamyl-Meso-

diaminopimelic acid (iE-DAP)[39]. The human intestinal tract is a host to trillions of microorganisms, ranging from archaea to viruses that are collectively represented by the term 'gut microbiome'. Therefore, a mutual relationship between the host and the gut microbiome is necessary for preserving immune homeostasis[40]. In the abdominal region, NOD2 is expressed by a variety of cell types that include hematopoietic cells, such as dendritic cells, mast cells, and T-cells, along with an array of non-hematopoietic cells like paneth cells, enterocytes, stem cells and goblet cells[40].

NOD2 senses the arrival of MDP via the partial degradation of bacterial peptidoglycan (PGN). MDP starts binding to the nucleotide domain of NOD2 (from amino acids 216-821 and with an optimal pH level of 5.0 to 6.5)[40]. While NOD2 can detect various types of PGN, its degree and level of activation depends on the origin of the PGN. Following the activation of MDP, NOD2 triggers the activation of MAPK and NF-kB signalling processes (Figure 2). This activation process provides a sizeable contribution to the enhancement of the host defence through the continuous production of mucins, anti-inflammatory cytokines and antimicrobial molecules[40]. Both NOD1 and NOD2 are vital for both myeloid and epithelial cell signalling mechanisms in lieu of the release of microbial products from a range of bacterial species. However, the mechanisms through which MDP and, most importantly, PGNs enter the cells and trigger the activation of NOD2 receptors is poorly understood even today. Host intestinal cells absorb or internalise MDP in three ways such as phagocytosis of microbes and endocytosis, uptake of fragments of PGN from the outer membrane vesicles (OMVs), or using transmembrane channels such as hPepT1 for NOD2 signaling[40].



In addition to the processes used to trigger NOD2 activation, the NOD2 receptor should remain in the vicinity of MDP release for the activation process to take place. Then, two peptide transporters: SLC15A3 and SLC15A4 can translocate MDP towards the cytosolic

Figure 2: The mechanism through which MDP enters the host cells to activate NOD2 signaling. MDP enters the host cells through transmembrane channels such as hPepT1, phagocytosis of whole bacteria, or uptaking of PGN fragments from OMVs. Once NOD2 is activated, it induces the activation of both NF-kB and MAPKs pathways. Adapted from reference 40.

compartment of the NOD2 receptor[40]. There are three protein domains that are important to a functional NOD2 receptor: the leucine-rich repeat (LRR) domain, nucleotide-binding oligomerisation domain (NOD) and caspase activation and recruitment domains (CARDSs)[40]. Each NOD molecule has a NOD domain along with a nucleotide-binding domain (NBD) and a winged-helix (WH) domain along with two helices (HD1 and HD2). HD1 and HD2 are two helix domains. The interactions between WH and NBD are essential to maintain the stability of NOD2 molecules in an inactive form. These interactions are mediated by adenosine diphosphate (ADP). Upon the confirmation of ligand binding, HD2 mediates the conformational changes of NBD molecules and WH. This process serves as a precursor for the ADP-ATP exchange, downstream signalling, leading to a self-oligomerisation process[40]. Here, CARDs, effectively mediate all forms of the aftermath interactions of LRR domains and MDP. However, here, the oligomerisation process of NOD2 signaling creates new signaling complexes known as nodosomes. Nodosomes are seen in plasma membranes where gut microbiota play a significant role. The NOD2 receptors attract a new form of protein kinase called receptor-interacting serine/threonine-protein kinase 2 (RIP2) through a homotypic CARD-CARD interaction. This process is closely followed by transforming growth factor beta-activated kinase 1 (TAK1), and, consequently, the formation of TAK1 binding proteins 2 and 3 (TAB2 and TAB3) will take place. The kinases activity of TAK1 will trigger the activation of MAPK and NF-kB signaling pathways. Additionally, the interaction of NOD2 molecules with others such as Caspase-1 and ATG16L1 results in autophagy. Therefore, the NOD2 receptor functions as an intracellular pattern recognition receptor for the MDP of both gram-negative and gram-positive bacteria[40]. This led me to develop the hypothesis described below.

Hypothesis and Predictions

GLP-1 is an incretin hormone that has metabolic functions and many potential pharmacological applications. It is produced from the L-cells in the intestine in response to

nutrients ingestion. It also plays several key roles in promoting normal glucose levels. Microbial metabolites such as MDP have been shown to have a role in regulating GLP-1. MDP is known as a bacterial cell wall component that acts via NOD2 receptors to improve insulin resistance, metabolic tissue inflammation and glucose tolerance in obese mice. The aim of this study is to understand the way MDP regulates GLP-1 secretion to improve T2D treatments. Therefore, I hypothesized that bacterial products such as MDP play a role in enhancing GLP-1 seretion.

This hypothesis was examined via testing the effect of MDP and its derivative, the fatty acid-modified L18MDP, in two GLP-1-secreting cell models called GLUTag and NCI-H716. Second, I explored the role of NOD2 signalling and its downstream cascade in the two cell lines. It is predicted that MDP will enhance GLP-1 secretion through binding to the NOD2 receptor and enhancing P38 MAPK signaling. Finally, I explored if the pharmacological inhibition of NOD2 using GSK717 and NOD2 genetic knockdown will prevent MDP-mediated GLP-1 secretion from cells.

Materials and Methods

Cell Culture:

Two secreting GLP-1 cell lines were used in my research: GLUTag and NCI H716. GLUTag cells are derived from colonic tumors of transgenic mice (passage 10-30; a generous contribution from Dr. Drucker, Lunenfeld-Tanenbum Research Institute, Toronto, ON). Cells were cultured in Dulbecco's Modified Eagle's Medium-low glucose DMEM (Cat NO. SH30021.01, GE Healthcare Life Sciences, Utah) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (10,000 I.U/ml). GLUTag cells were trypsinized every 48 hours and reseeded at 1:3-dilution. NCI H716 cells were derived from the human tumor cells of a 33-year-caucasian male; the cell line was deposited by Dr. Adi F. Gazdar and purchased from (ATCC,UK). They are normally grown in a suspension culture. They were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Cat NO.SH30027.01, GE Healthcare Life Sciences, Utah) supplemented with 10% FBS and 1% penicillin. At least 500,000 cells/ml for NCI H716 cells was required, as lower amounts cause stalled growth. The number of cells doubled every three days and needed to split once a week. Both types of cells were kept at 37˚C, 5% CO_2 until 70-85% confluent, then split and transferred to a fresh complete medium.

Glucagon-Like Peptide-1 Secretion:

For GLP-1-stimulatory activity, GLUTag cells were seeded into 6-well plates (1,000,000 cells/well). In addition, 24-well plates were seeded with GLUTag (250,000 cells/well) and NCI H716 (200,000 cells/well) to be able to handle different treatments at once. Human NCI H716 cells require a basement membrane matrix coating to allow the cells attach to plates. The Matrigel (NO.354234, Corning®, Life Sciences, USA) was thawed on ice at 4˚C. The wells were coated with 200 µl of 0.5 g/ml Matrigel diluted in a cold Hanks Buffered Saline Solution (HBSS).

The wells with Matrigel were allowed to polymerize for 1 hour at room temperature. When finished, the wells were rinsed with room temperature HBSS and then allowed to dry for 30 minutes. Plates were then ready to be plated with NCI H716 cells. In all conditions, plates were incubated at 37˚C, 5% CO_2 for 48 hours to ensure the cell growth reached approximately 80% confluency before receiving treatments using the hemocytometer. On the day of treatment, wells were rinsed with secretion media (low-glucose DMEM, 0.5% FBS, 1% penicillin) for GLUTag and RPMI 1640 secretion media (0.5% FBS, 1% penicillin) for NCI H716 cells. For baseline secretion, the control was only the secretion media without any treatments. 10 µM Forskolin was added to secretion media and used as a positive control for stimulated secretion. In some of the GLUTag GLP-1 secretion treatments, I tested C12-iE-DAP (InvivoGen, San Diego, CA, USA) at different concentrations 2,5,10 µg/ml. Moreover, in both cell lines, I tested L18MDP at concentrations of 2,5,10 µg/ml (InvivoGen, San Diego, CA, USA) and 0.5, 2, 5, 8, and 10 µM of NOD2 inhibitor (GSK717) (Sigma-Aldrich). All plates were co-incubated for 2 hours at 37˚C, 5% CO_2. Then, I collected the media into 2-ml screw cap tubes that already had been prepared with 20 µl of 0.1% trifluoroacetic acid (TFA). All acidified collected media were stored at -20˚C to be used for GLP-1 quantification.

GLP-1 Hormone ELISA:

To quantify GLP-1 amounts in the cell culture media, I used Enzyme-Linked immunosorbent assay ELISA (BMS2194, Invitrogen, Thermo Fisher Scientific, Whitby, Ontario) following the manufacturer's guidelines. Briefly, GLP-1 standard was reconstituted by adding distilled water and then was allowed to reconstitute for 20 minutes at room temperature. Then, it was mixed well before making dilutions. I prepared 1:2 serial dilutions for the standard curve by pipetting 250 µl of sample diluent into all the labeled 1.5-ml tubes for making the standard. Then, I pipetted 250 µl of reconstituted standard (concentration = 100 pg/ml) into the first tube and mixed it. The concentration of standard 1= 50 pg/ml.

After that, I pipetted 250 µl of standard 1 into the second tube and mixed it before the next transfer. Then, I repeated the serial dilutions five more times to make the standard curve points range from 50 pg/ml to 0.8 pg/ml. For GLUTag culture media, 20 µl of media was added to assay wells along with 50 µl of sample diluent and 30 µl of assay buffer. In contrast, because of the high amount of GLP-1 present in the NCI H716 media, I diluted 10 µl of collected media into 90 µl of assay buffer, then added 10 µl of the diluted mixture to 50 µl of sample diluent and 40 µl of assay buffer. To this, 50 µl of biotin-GLP-1 conjugate was added to all wells and incubated for 2 hours. When incubation ended, I washed the wells five times with wash buffer and proceeded to the next step before the wells dried. Then, I added 100 µl of streptavidin-HRP to all wells and incubated for 1 hour on a shaker at room temperature. The wells were washed five times with wash buffer and 50 µl of a tetramethyle-benzidine TMB-substrate solution was added to all wells. The plate was covered with foil and incubated in the dark for 30 minutes. After the incubation ended, I added 50 µl of 1M Phosphoric acid (stop solution) to all wells to terminate the reaction and measure the absorbance of GLP-1 at 450 nm using SoftMax Pro software. GLP-1 concentrations were calculated using 4 parameter log curve fitting. This ELISA's limit of detection is 0.32 pg/ml. Also, the calculated overall intra-assay and inter-assay coefficient of variation were 3.8%.

Neutral Red cell viability:

Under similar conditions, I determined the effect of drug cytotoxicity and cell viability by performing a quantitative test known as a neutral red uptake assay as explained in reference 41[41]. Cells were incubated with various concentrations of L18MDP for 2 hours at 37˚C, 5% CO_2 with media containing a neutral red dye (N129-25, Fisher Chemical), or 30% hydrogen peroxide as a positive control. The dye enters and is trapped in the lysosomes via non-ionic passive diffusion, and with the use of an acidified ethanol solution, the dye is extracted from the viable cells. A spectrophotometer was used to measure the absorbance of the solubilized dye at 540 nm[42].

Western Blot:

GLUTag cells were seeded into 6-well plates (1,000,000/well) for 48 hours at 37˚C, 5% CO_2. On the day of treatment, I rinsed the wells with secretion media (low-glucose DMEM, 0.5% FBS, 1% penicillin). Then, I treated the cells with secretion media for controls and secretion media containing 5 µg L18MDP for the treatment's wells. These treatments lasted 0, 5, and 10 minutes. Media were removed. Then, I immediately rinsed the wells once with 1 ml of ice-cold 1x phosphate-buffered saline (PBS) to remove any remaining media and quickly lysed with lysis buffer. To lyse the cells, I added 100 µl of lysis buffer (Cell Signaling Technologies, Danvers, MA Technologies) containing a phosphatase inhibitor (Cat NO. A32957, PhosphoSTOP, ThermoFisher Scientific), and a protease inhibitor (EDTA-Free cOmplete Mini by Roche). Cells were collected, sonicated on ice (10 seconds at power 3), and centrifuged (5 minutes, 13,000 xg, 4 ˚C). Protein concentration was measured using Bradford protein assay, and the expression levels of phospho-P38 MAPK and total P38 MAPK were analyzed by Western Blotting. 35 µg of protein was loaded per lane on TruPAGE Precast Gels 4-12% (Sigma-Aldrich, Oakville, Ontario) and run at 180 V. The protein was then transferred for 1.5 h at 200 V onto a polyvinylidene fluoride (PVDF) membrane, blocked in 5% skim milk powder and 0.1% Tween 20 TRIS buffered saline (TBST) for 30 minutes, then incubated in primary antibodies (see table) in TBST containing 5% BSA on a shaker at room temperature for 4 hours. After washing in TBS (3 x 5 minute), the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody at a concentration of 1:1000 for 1 hour on a shaker at room temperature. The signal was developed by exposing the membrane to Luminata Forte Western HRP Substrate (Cat NO. WBLUF0100, Millipore Corporation, Billerica, Ma) for 1 minute. A BioRad Chemidoc XRS documentation apparatus was used to record a chemiluminescence signal and analyzed using the program Quantity One. To analyze the band's intensity, Densitometry was used to compare the protein ratio between P-P38 MAPK and total P38 MAPK.

To detect NOD2 in the NCI H716 cell line, a 10-cm plate was treated after 48 hours with RPMI 1640 secretion media for 2 hours. Then, following all the described protocol above, the NOD2 primary antibody was diluted as 1:1000 and incubated at room temperature for 4 hours on a shaker. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody at a concentration of 1:1000 for 1 hour at room temperature on a shaker.

Table 1: List of Primary Antibodies						
Peptide/Protein Target	Antigen Sequence	Name of Antibody	Manufacturer	Species Raised in	Dilution used	Antibody Type
NOD2	2D9	Anti-NOD2 clone 2D9	Sigma-Aldrich	Mouse	1:1000	Monoclonal
P38 MAPK		P38 MAPK	Cell Signaling Technologies	Rabbit IgG	1:1000/ 1:2000	Monoclonal
Phospho-P38 MAPK	Thr180/ Tyr182	*Phospho*-P38 MAPK	Cell Signaling Technologies	Rabbit IgG	1:1000	Monoclonal

Table 2: List of Secondary Antibodies				
Peptide/Protein Target	Name of Antibody	Manufacturer	Dilution	Antibody Type
NOD2	Goat Anti-Mouse IgG H&L (HRP) (ab6789)	Abcam	1:1000	Polyclonal
P38 MAPK	Anti-rabbit IgG, HRP linked antibody	Cell Signaling Technologies	1:1000	Polyclonal/ Monoclonal
P-P38 MAPK	Anti-rabbit IgG, HRP linked antibody	Cell Signaling Technologies	1:1000	Polyclonal/ Monoclonal

Gene Knockdown:

To investigate if NOD2 is required for the MDP-based stimulation of GLP-1 secretion, I conducted NOD2 siRNA knockdown experiments. First, the MISSION esiRNA (negative control, mouse NOD2 esiRNA, and transfection reagent) (Cat NO. S1452) needed for NOD2 knockdown was purchased from Sigma-Aldrich, Oakville, Ontario. Second, I started to knock down the NOD2 gene in GLUTag cells. I seeded the cells into two 6-well plates (500,000 cells/well) for 24 or 48 hours at 37˚C, 5% CO_2 until 30-50% confluent as recommended by the transfection protocol used. The day of treatment, I prepared two 1.5 ml-tube for NOD2 siRNA and Control siRNA. Then, I added 1.4 ml of serum-free DMEM into each 1.5 ml-tube plus 3.85 µl of (NOD2 siRNA into its tube/Control siRNA into its tube) for the final concentration of 5 nM. However, during the troubleshooting, I increased the concentration of siRNA transfection reagent to 25 nM, and I added 19.25 µl of (NOD2 siRNA into its tube/Control siRNA into its tube). The prepared siRNA tubes were incubated at room temperature for 15 minutes. Then, media were replaced with a 2 ml/well fresh complete media once the 15-minute incubation was about to end, then 200 µl of each siRNA tube was added to its well containing 2 ml complete DMEM. The plates were incubated at 37˚C, 5% CO_2 for 24 or 48 h. The next day, I treated the plates for 2-h GLP-1 secretion treatment as mentioned on page 17 with 5 µg/ml L18MDP or control media.

RNA Extraction Analysis:

The protein collection and RNA isolation were performed using an illustra RNAspin Mini RNA Isolation Kit from GE Healthcare Life Sciences. Once the 2-h secretion treatment ended, I added the 350 µl lysis solution and 3.5 µl of ß-mercaptoethanol to each well, and the cells were scraped off the surface of the wells into the lysis solution. Homogenization was done using a 0.5 mm needle fitted to a 1-ml syringe for 1 minute or until no clumps were visible. The homogenized lysate applied to the RNAspin mini filter and centrifuged at 11,000 x g for 1 min. Then, I transferred the filtrate without any formed pellet to a new 1.5-ml RNase-free microcentrifuge tubes.

350 µl of 70% ethanol was added to each 1.5 ml-tube where a stringy precipitate may have become visible, and each tube was vortexed twice for 5 seconds. The lysate was pipetted up and down for 2-3 times before loading it onto the RNAspin mini column. It was then centrifuged for 30 s at 8000 x g. The column was then transferred to new collection tubes. To desalt the lysate to make the subsequent DNase digestion more effective, I added 350 µl of desalting buffer onto each column, then centrifuged at 11,000 x g for 1 min to dry the membrane. The flowthrough was discarded, and the RNAspin mini columns were returned to the collection tubes. Then, applied 95 µl of a mixture of (10 µl of reconstituted DNase 1 and 90 µl of DNase reaction buffer) was applied onto the center of the silica membrane of the column. Tubes were incubated for 15 minutes at room temperature on the bench top. When the incubation period ended, I added 200 µl of wash buffer 1 onto the RNAspin mini column to inactivate the DNase and centrifuge for 1 min at 11,000 x g. The flowthrough was discarded, and 600 µl of wash buffer 2 was added to each column and centrifuged for 1 min at 11,000 x g. Then, 250 µl of wash buffer 2 again was added to the same columns and centrifuged for 2 min at 11,000 x g to dry the membrane completely. RNA was eluted by adding 40 µl of RNase free water to each column and centrifuged for 1 min at 11,000 x g. After that, 2 µl off the total RNA extraction was resuspended in 398 µl of RNase-free water in a 0.5-ml quartz cuvette and quantified using the UV Spectrophotometer. The absorbance at 260 nm provided a quantitative measure of the concentration of the total RNA. At this step, RNA samples can be stored at -80˚C.

cDNA and End Point PCR Analysis:

Total RNA (1µg) was reverse transcribed into cDNA using a SensiFAST cDNA synthesis kit (BIOLINE) by adding 1 µl of reverse transcriptase, 4 µl of trans amp buffer, and an amount of RNase/DNase-free water required to make a final volume of 20 µl. The cDNA was placed in the thermocycler, set to 25˚C for 10 minutes, 42˚C for 15 minutes, and 85˚C for 5 minutes, and then held at 4˚C.

The cDNA was used to prepare polymerase chain reactions (PCRs) by using specific designed primers for NOD2 and a housekeeping reference gene known as RPL13a (Integrated DNA Technologies, Ontario, CA). The primers sequences are as follows:

Items	Forward (5’ to 3’)	Reverse (5’ to 3’)
NOD2	5’-TGTCCAACAATGGCATCACCT-3’	5’-TGTGTTCCCTCGAAGCCAAA-3’
RPL13a	5’-GAAGCAGATCTTGAGGTTACGGA-3’	5’AGGCATGAGGCAAACAGTCT-3’.

The annealing temperature of both primers was determined using an end point gradient PCR. 50 µl reactions were prepared by adding 22 µl of RNase/DNase-free water, 25 µl of Taq 2X Master Mix,1 µl of cDNA, 1 µl of forward primer, and 1 µl of reverse primer for each sample. The samples were then placed in the thermocycler set as a gradient annealing temperatures between 60 and 65 ˚C. For NOD2 and RPL13a, the thermocycler had an initial denaturation step of 95°C for 30 seconds, followed by 40 cycles of 95°C for 15 seconds, 63°C for 30 seconds and 68°C for 20 seconds. The optimal annealing temperature found for NOD2 and RPL13a was 63°C. The amplified fragments were analyzed by electrophoresis on a 2% agarose gel in 1x TBE buffer, then stained with a 1:20000 RedSafe (Froggabio, Oakville, ON) solution for 30 minutes on a shaker . After destaining the gel with 1x TBE buffer for another 30 minutes on a shaker, I visualized the gel using the BioRad Chemidoc XRS system transilluminator and Quantity One software. Predicted band size for PCR products is 98bp for NOD2 and 129bp for RPL13a.

Statistical Analysis

All data is presented as the mean ± standard error of the mean (SEM). Experiments with multiple doses of a single treatment were analyzed by a one way ANOVA. Experimetns with an inhibitor and treatment were analyzed by two way ANOVA. Tukeys post hoc test was used for multiple comparisons between groups. Significance was interpreted as a p value <0.05.

Results

GLP-1 secretion treatments and cell viability:

Different treatments were tested to stimulate GLP-1 secretion in both GLUTag and NCI-H716 cell lines. Either GLUTag or NCI-H716 cells were treated with one of the products or control media for 2 hours to perform all secretion experiments. In Fig. 3A, GLUTag cells were treated with regular MDP between 0.001-100 µg/ml. No significant effect on GLP-1 secretion was observed. The lipid- modified NOD1 agonist (C12-IE-DAP) Fig. 3B was also tested on GLUTag cells at 2,5, and 10 µg/ml and did not have any effect on GLP-1 secretion. I therefore opted to test the lipid-modified L18MDP on GLUTag to enhance cell uptake. Different concentrations (0.01, 0.1, 1, 2, 5, 10 µg/ml) of L18MDP were examined on GLUTag cells, and Fig. 3C shows a significant increase in GLP-1 secretion at 5 and 10 µg/ml ($P<0.001$ one-way ANOVA). Next, I examined the effect of L18 MDP on NCI-H716 (Fig. 3D), and it significantly increased GLP-1 secretion at the highest concentration of 10 µg/ml ($P<0.001$ one-way ANOVA).

To ensure that treatments had no effect on cell viability I used the neutral red uptake assay. The neutral red assay demonstrated that the cell viability in GLUTag and NCI-H716 cells (Fig. 3E and 3F respectively) were not affected by L18MDP.

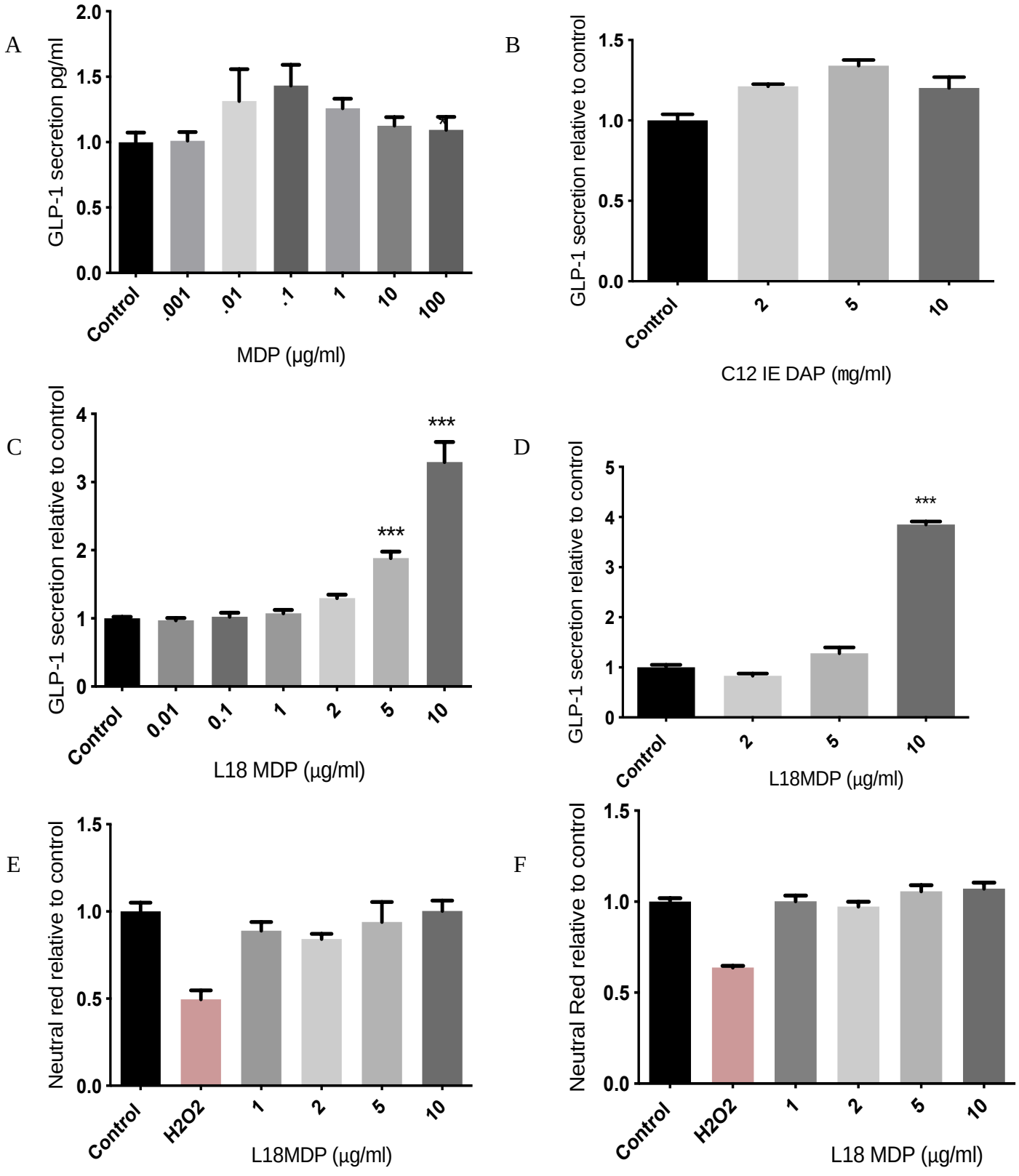


Figure 3: GLP-1 secretion and cell viability.

GLP-1 secretion (A, B, C, D) was examined in GLUTag after a 2-hour treatment with MDP (A), C12-IE-DAP (B), L18 MDP (C) and in NCI-H716 with L18MDP (D). Also, the cell viability was examined using L18MDP in GLUTag (E) and NCI-H716 (F). N=8 from different passages, *** = $p<0.001$.

P38 MAPK Signaling Pathway Optimization:

To determine a potential intracellular mechanism involved in the enhanced GLP-1 secretion by L18 MDP, I examined cytosolic protein from GLUTag cells treated with L18MDP. While a modest effect for P38MAPK activation was observed in the GLUTag cells (Fig. 5A), many additional experiments and optimization were attempted for the cell signaling study. At the beginning, I used an expired P38 MAPK antibody, and I incubated the GLUtag cells membranes with a primary antibody for overnight at 4 °C. However, no signals were observed. Then, I tried to strip and re-probe the same membranes on a shaker for different room temperature time periods (1, 2, 3, 4 hours). Once again, no signal was observed. With fresh cell lysates, and a 4-hour room temperature primary incubation, I was able to generate a signal on the Western Blot (Fig. 4); however, these blots produced many non-specific bands for both GLUTag and NCI cells (Fig. 4). With the purchase of fresh antibodies for phospho P38 and total P38, the blots improved significantly.

P38 MAPK signaling pathway:

In Fig. 5A, GLUTag cells were treated with 5 µg/ml for 10 and 20 minutes and the phosphorylation of P38 MAPK was examined by western blot. Phospho P38 MAPK/total was slightly elevated compared to the control (0.359±.011 compared to 0.325±0.007, p=0.032). However, the difference was not observed at the longer 20-minute incubation (Fig. 5B). A similar treatment was performed on the human NCI-H716 cells; however, I was not able to successfully get a clean band detection due to bubbles in the western blot. Therefore, this experiment needs to be repeated (Fig. 6).

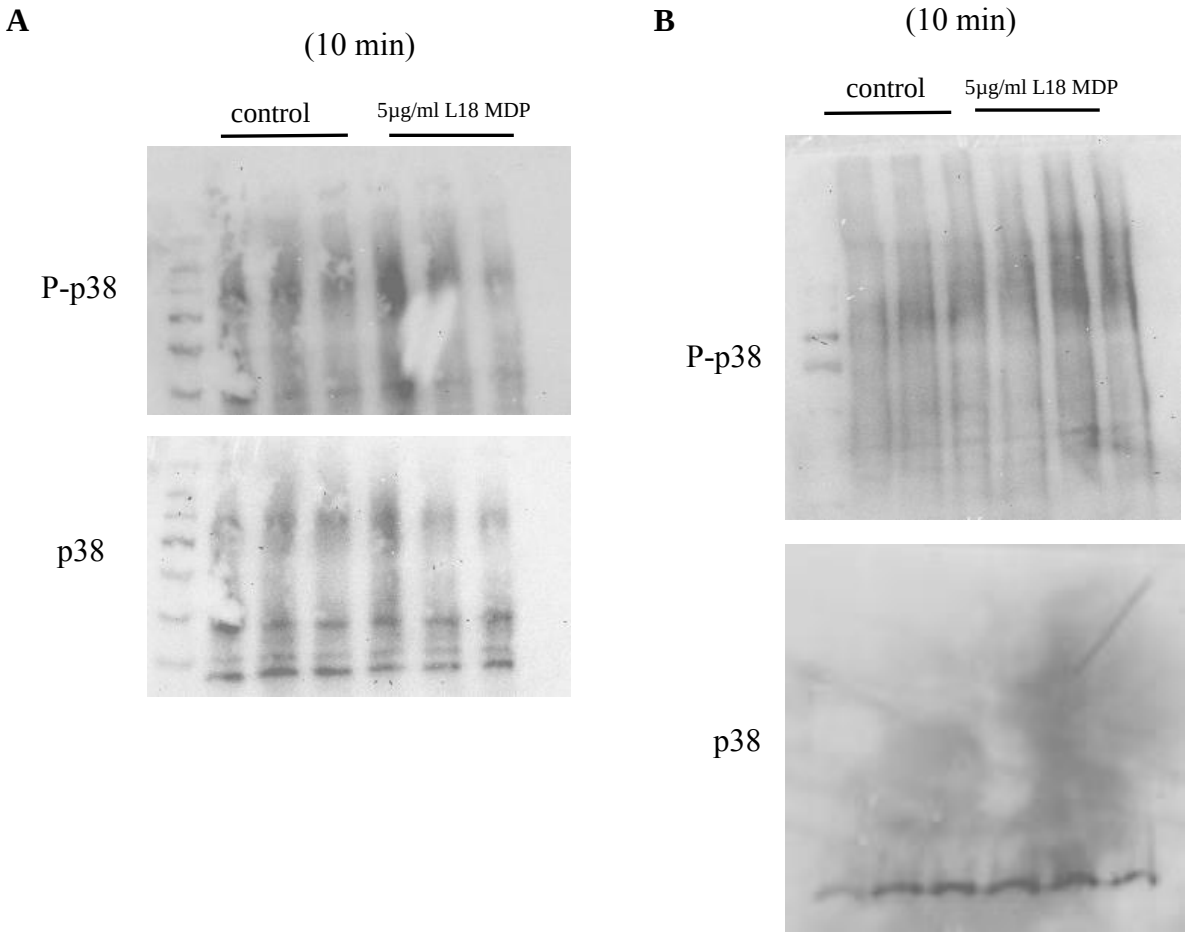


Figure 4: P38 MAPK Western Blot Optimization in GLUTag and NCI-H716 cells.

P38 phosphorylation and total P38 (43 kDa) were examined in GLUTag cells treated with 5 µg/ml L18MDP for 10 minutes by Western Blot. The expired P38 MAPK antibody was used, showing many signals for different proteins in GLUTag cell lysate (A) and in NCI-H716 cell lysate (B). N=3

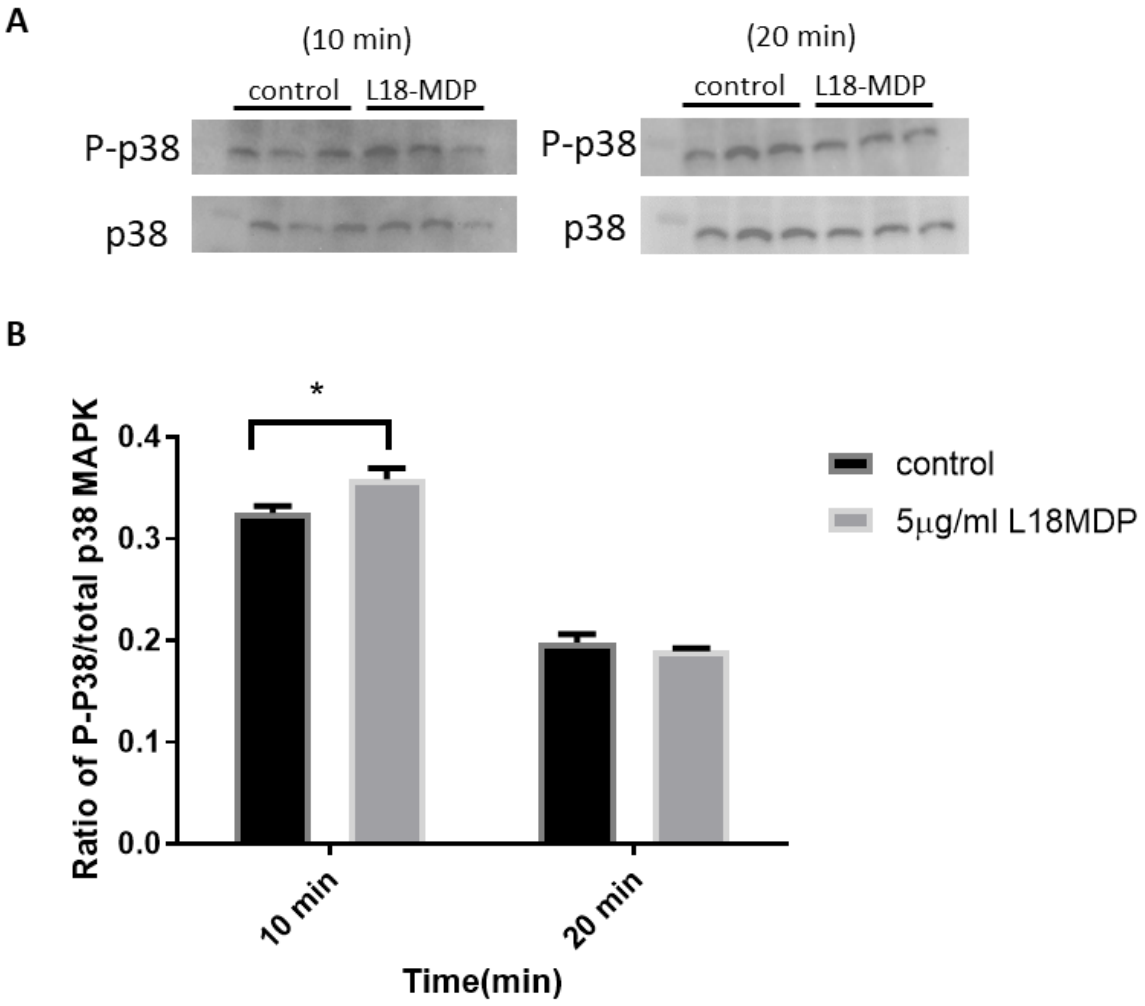


Figure 5: L18MDP and P38 MAPK signaling in GLUTag cells.

P38 phosphorylation and total P38 was examined by Western Blot in GLUTag cells treated with 5μg/ml L18MDP for 10 and 20 minutes(A). The P38 phosphorylation/total P38 ratio was determined via densitometry. N=3, *=P<0.05.

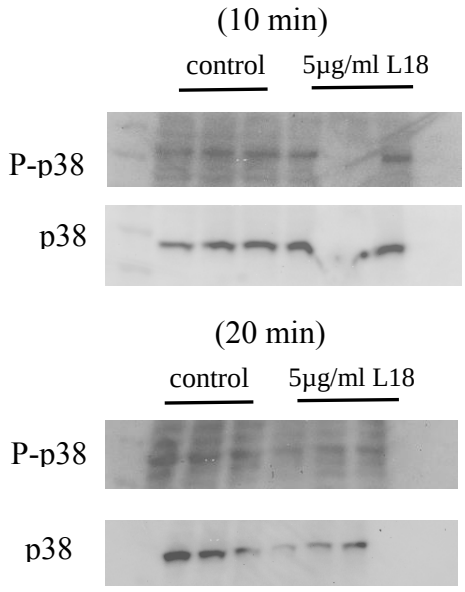


Figure 6: L18MDP and P38 MAPK signaling in Human NCI-H716 cells.

P38 phosphorylation/total P38 was examined in NCI-H716 cells treated with 5 µg/ml L18MDP for 10 and 20 minutes by Western Blot. N=3

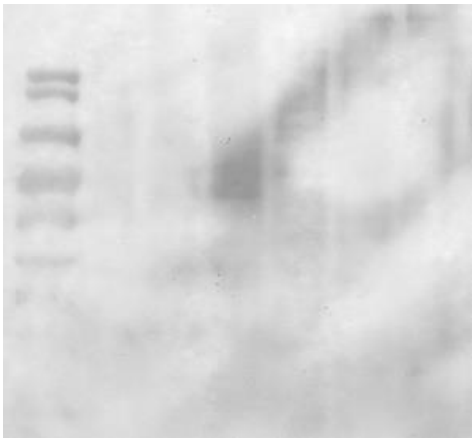
NOD2 receptor expressed in GLP-1-secreting cells Optimization:

As NOD2 is a documented cytosolic receptor for MDP, I started to explore the presence of NOD2 receptor in GLUTag and NCI-H716 cells. I used anti-mouse NOD2 (2D9) antibody. I tested both an overnight 4°C and room temperature incubation, but no signals were observed for the GLUTag models. Then, I tried to use a fresh protein lysate of mouse colon tissue and GLUTag cells and loaded different amounts of protein (35, 45, and 55 µg, corresponding to lanes 2,3, and 4, respectively, in Figure 7A). With a 4-hour room temperature primary antibody incubation, no clear signal was observed either for the colon tissue or GLUTag (Fig. 7A). Fig. 7B shows a photo of a western blot in NCI-H716 using the NOD2 (2D9) antibody at 1:2000 as recommended and incubated for 4 hours at room temperature, but the signal was weak. I then completed a 1:1000 dilution of the NOD2 primary antibody, which produced the signal expressed in Fig. 8B.

NOD2 receptor expressed in GLP-1-secreting cells:

Figure 8A shows that the NOD2 receptor is expressed in GLUTag cells by endpoint PCR, and it is also expressed in the human NCI-H716 cells by Western Blot (Fig. 8B). Unfortunately, I was unable to detect a NOD2 band of the appropriate size in the mouse GLUTag cells.

A



B

Figure 7: GLP-1 secreting cells express the NOD2 receptor (the optimization).

It shows no expression of the NOD2 receptor in both mouse colon tissue and untreated GLUTag cells at different protein loadings (A). However, it shows the NOD2 signal in NCI-H716 cells at room temperature using 1:2000 NOD2 (2D9) antibody dilution (B).

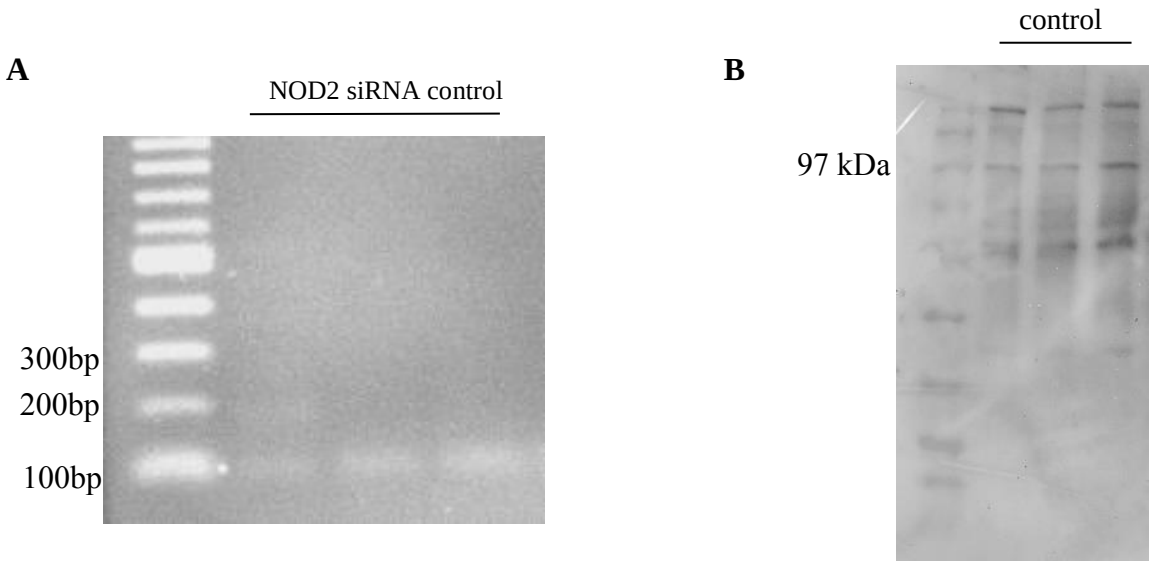


Figure 8: GLP-1 cells express the NOD2 receptor.

Agarose gel of endpoint PCR using NOD2 specific primers (A) showing the 98 base pair product of the NOD2 gene. (B) The NOD2 protein was detected in NCI-H716 cells at the size of 97 kDa.

The Effect of NOD2 inhibition on the regulation of GLP-1 Secretion Optimization:

The NOD2 inhibitor GSK717 was used to examine the role of NOD2 in the regulation of GLP-1 secretion in GLUTag and NCI-H716 cells. As I was unsuccessful in blocking MDP's effect, I tested low doses including 2 µg/ml (Fig. 9A, 9C). Despite having less MDP in the treatment, GSK717 was unable to block GLP-1 secretion (Fig. 9A, 9C). Several doses of the GSK717 inhibitor (0.5, 5, 8, 10 µM) in GLUTag were also tested but failed to block MDP's effects (Fig. 9). In addition, I did 30 minutes of pre-incubation for the GSK717 inhibitor before spiking in the L18MDP treatment; however, this pre-incubation did not block MDP's effect on GLP-1 (Fig. 9B).

The Effect of NOD2 inhibition on the regulation of GLP-1 Secretion:

Fig. 9 shows that 5 µg/ml of L18MDP was used in my experiments, as it causes a modest increase but ideally could be blocked by the NOD2 inhibitor. Cells were treated for 2 hours with two different doses of GSK717 (5, 10 µM), with or without co-incubation of 5 µg/ml of L18 MDP. While GSK717 caused a modest reduction (not significant) in basal GLP-1, the inhibitor was not able to block the L18MDP-stimulated secretion in GLUTag cells (Fig. 10A) and in NCI-H716 cells (Fig. 10B).

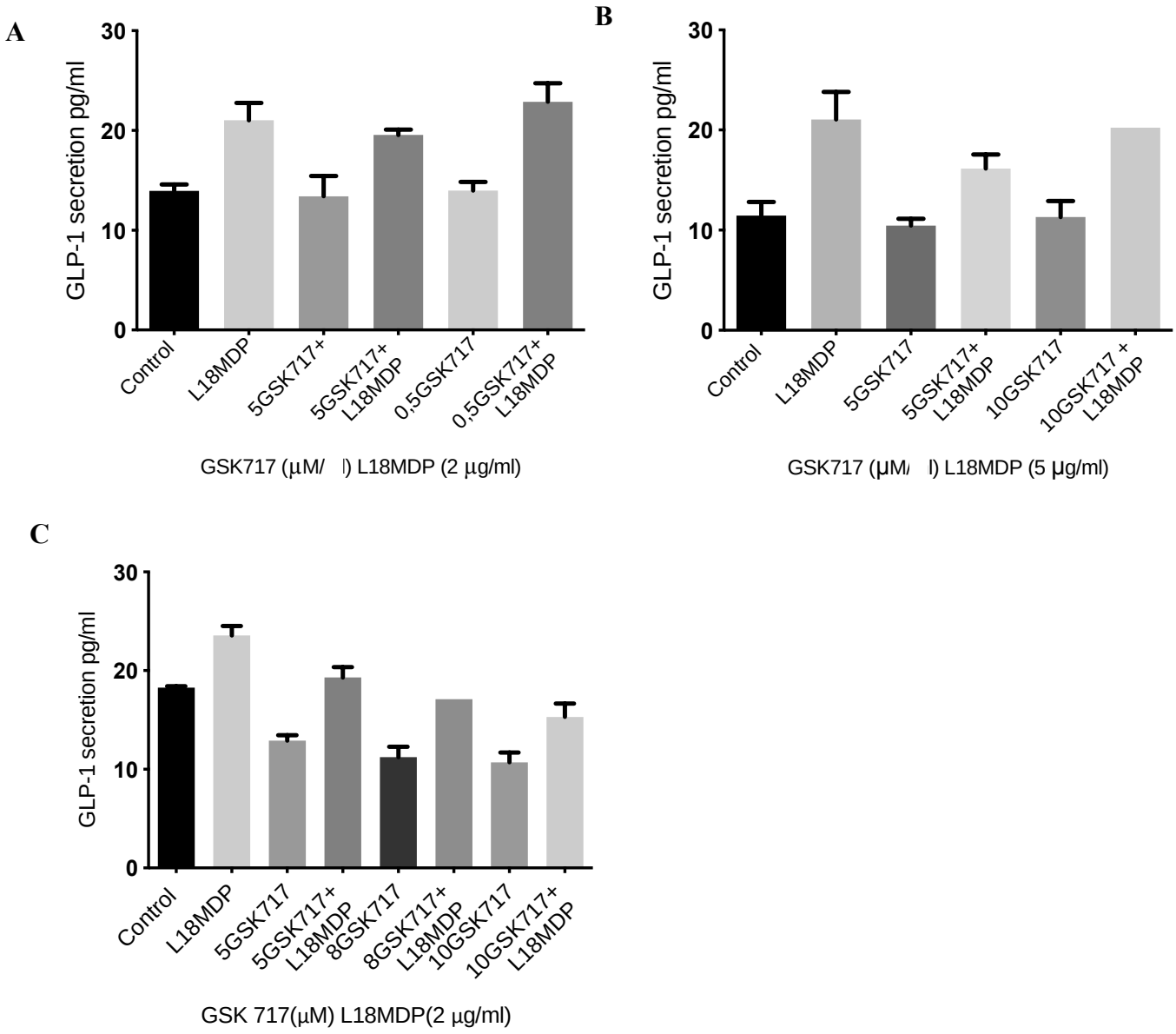


Figure 9: NOD2 inhibitor GSK 717 did not block GLP-1 secretion (optimization).

GLUTag cells were treated for two hours with two different doses of GSK717 and L18MDP. The inhibitor did not show any inhibition on GLP-1 secretion. N=4

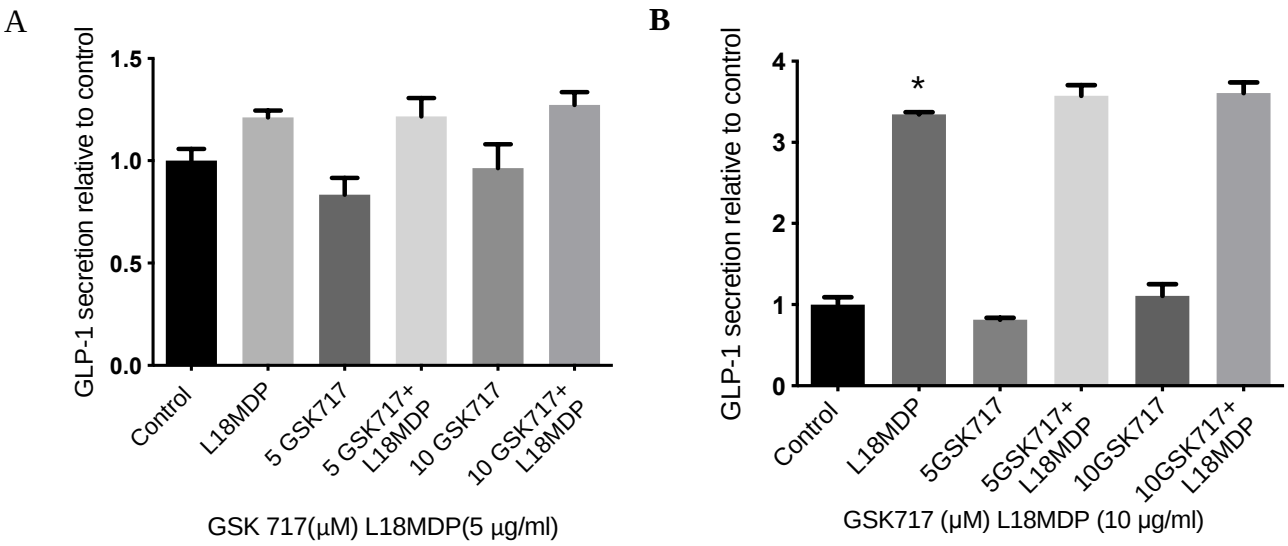


Figure 10: NOD2 inhibitor GSK 717 did not block GLP-1 secretion.

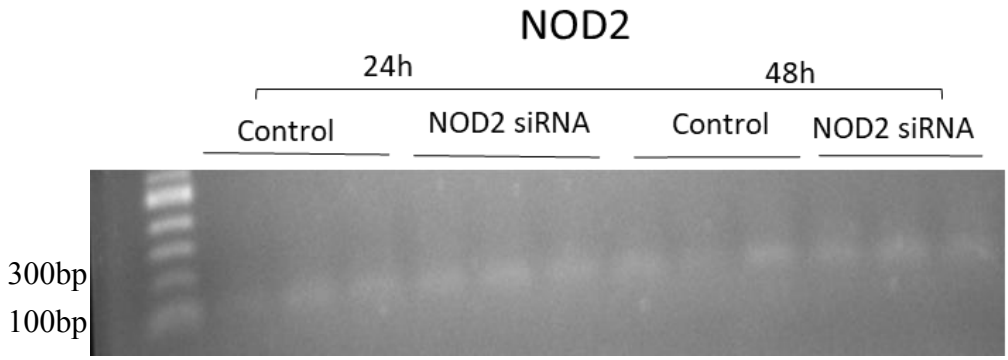
Two-hour GLP-1 secretion was examined in GLUTag cells (A) and NCI-H716 cells (B). Both cell lines were treated with GSK717 (5 or 10μM) with L18MDP. N=8, normalized from two different passages.

NOD2 small interfering RNA (siRNA) knockdown:

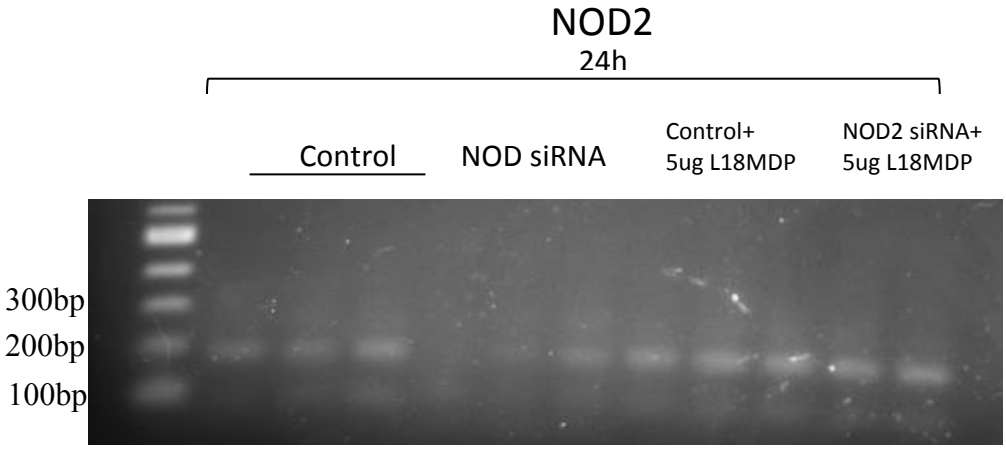
To confirm the importance of the NOD2 receptor in regulating GLP-1 secretion, I attempted to knock down the NOD2 gene expression in GLUTag cells. In Figure 11 (A), I examined the NOD2 siRNA knockdown at two time durations (24 and 48 h) and treated the cells

with control siRNA and NOD2 siRNA (5 µM). A clear NOD2 siRNA knockdown was not shown, and that led me to increase the amount of siRNA in my subsequent experiment. In Figure 11 (B), 25 µM of siRNA was tested with a 5 µg/ml L18MDP treatment to examine a 2-hour GLP-1 secretion experiment (C). However, my PCR product showed a 200 base pair band, which did not correspond with the 98 base pair band in (A). The PCR reaction in (B) was amplifying another PCR product, which might be due to a contamination. New NOD2 primers are required to continue knocking down NOD2.

A



B



C

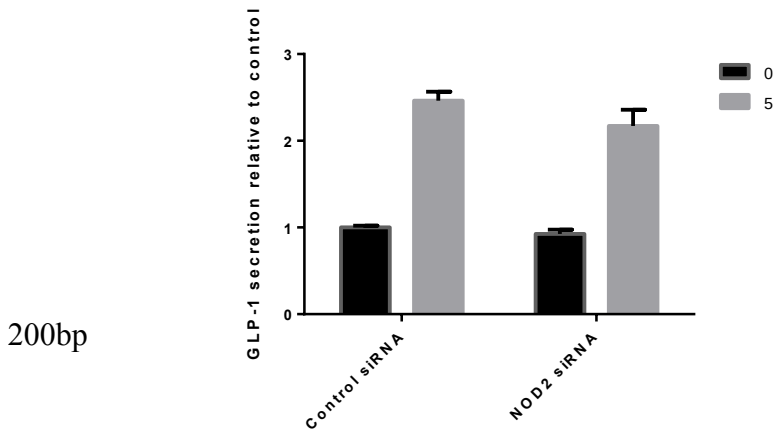


Figure 11: NOD2 siRNA knockdown and GLP-1 secretion.

Electrophoresis of RNA samples in 2% agarose gel, stained with a RedSafe solution showing the knockdown siRNA of NOD2 in GLUTag cells (A). A different size band is generated (B) 2-hour GLP-1 secretion ELISA is not showing any difference with or without the 5 µg/ml L18MDP treatment on the NOD2 knockdown experiment (C). The Controls in A and B present siRNA controls.

Discussion

Intestinal microbiota studies have gained widespread attention for their correlation with human pathologies. Also, there is a complex interaction between diet, microbiota, metabolic

makeup and host genetics. Conditions such as obesity and T2D are associated with microbial changes in terms of diversity and richness. Using probiotics, postbiotics, and prebiotics have been shown to be a potentially effective therapeutic approach to treating patients with T2D and inflammatory bowel disease[43]. Research from our group has demonstrated that postbiotics and prebiotics can increase GLP-1 secretion[37]. Moreover, T2D treatments using GLP-1-based strategies have become a major therapeutic approach for dealing with this disease[43]. In this thesis, I investigated the impact of the bacterial cell wall postbiotic (modified L18MDP) in the regulation of GLP-1 from several GLP-1 producing cell lines.

Surprisingly, the initial work with standard MDP had no effect on GLP-1 secretion. This lack of an effect may have been due to the difficulty of this muropeptide to enter the cell and have an effect in the 2-hour time period of this experiment. In the literature, standard MDP has been shown to affect human colonic Caco2/bbe cells in shorter periods[44]; however, the GLP-1 cells may differ in terms of their responses, and may require longer incubations[37]. While extended incubation duration could have been done, our group was informed that a lipid-modified version of MDP could be tested in place of standard MDP[37] (Dr J Schertzer, personal communication). The modified lipid L18MDP would allow for rapid diffusion into the cytoplasm. Therefore, I continued my experiments with the modified L18MDP. I have shown in my thesis that L18MDP at 5 and 10 µg/ml stimulated GLP-1 secretion in both mouse (GLUTag) and human (NCI-H716) cell lines after a 2-hour time period.

In Schertzer's et al.'s 2017 study, they demonstrated that the MDP postbiotic acted through the NOD2 receptor and prevented insulin resistance and metabolic tissue inflammation in obese mice. Liver insulin resistance and fat inflammation were reduced as well by MDP via NOD2[37].

It is also important to explain that, in this study, L18MDP was shown to be well-tolerated by GLUTag and NCI-H716 cells since they were devoid of cytotoxicity in Neutral Red assay. In

support of our lack of toxicity, another study on human foreskin fibroblasts treated with MDP showed no toxicity in an MTT assay at any of their concentrations used (1, 10, and 100 µg/ml)[45].

Based on different studies, NOD2 is expressed in many cells in the intestine, including-non immune cells (such as stem cells[46], paneth cells[47], and globlet cells[48]) and immune cells (such as B cells and T cells)[49]. Studies demonstrated that NOD2 is expressed in small bowel Paneth cells, and a recent study explained that NOD2 is highly expressed in intestinal stem cells[50]. Moreover, a study generated a western blot analysis of NOD2 expression in different intestinal epithelial cell modules as well as in HEK293 and COS7. On the other hand, little or no NOD2 protein was expressed in T84 and Caco-2. The aspects of their study I am considering in relation to my work are the transfection reagent (LipofectAMINE 2000) they used as well as the rabbit NOD2 antiserum HM2559[51]. In my study, I have shown the expression of the NOD2 receptor in GLUTag cells by performing endpoint RT-PCR, and in NCI-H716 cells by a Western blot. However, the NOD2 receptor failed to be detected in GLUTag cells with Western blot. In regard to this, a study performed a western blot using GLUTag cells to detect Epac2 and FLAG-GPR119. They had successful results, which may be related to their short time incubation, as they blocked the PVDF membranes at room temperature for 1 hour in TBST and dry fat milk; the second incubation was done for 1 hour at room temperature. They diluted the primary monoclonal antibody to (1:100-fold) and the secondary antiserum to (1:5000- fold)[52]. In the future, more troubleshooting needs to be done to test if NOD2 can be detected in GLUTag cells.

In order to confirm the role of NOD2 in MDP-induced GLP-1 secretion, I tested the only commercially available NOD2 inhibitor (GSK717). In my experiments, I was unable to block MDP-induced GLP-1 secretion with this inhibitor. It is possible that longer periods of pre-incubation of the inhibitor may be required to block the effect of MDP. In support of this, another study that used HEK293T cells with a NOD2 reporter system pre-incubated the cells with the inhibitor for 1 h before a 16-h MDP treatment[53]. It should be noted that this study used a different NOD2 inhibitor, benzimidazole diamides, to achieve their inhibition. More commercially-

available NOD2 inhibitors are required to support the mechanistic studies in this field[53].

It has been established that the MAPK family is involved in cell differentiation, proliferation, and apoptosis[54]. In addition, GLP-1 secretion is known to be regulated in part by the p38 MAPK pathway[55,56]. I next examined if this pathway was implicated in L18MDP-mediated stimulation. After testing multiple doses and multiple time points in L18MDP-treated cells, I had a modest P38 MAPK increase in one of the time points (10 min) over the other tested time points in GLUTag cells. This was surprising, as a recent study in grass crap (Ctenopharyngodon idella) demonstrated that p38 gene expression was elevated by MDP. Furthermore, the p38MAPK inhibitor SB203580 can inhibit MDP-induced effects, and this explains that p38MAPK has a key role in bacterial-induced intestinal inflammation[57]. On the other hand, the stimulation of NOD2 results in activating the MAPK: p38, extracellular signal- regulated kinase (ERK), and c-Jun N-terminal kinase (JNK), which are involved in cell growth, migration, proliferation, inflammation, cellular processes, and survival (MAPK: JNK, ERK, P38)[58]. Further studies are needed on the enteroendrine cells in this field to investigate which other pathways could be involved in GLP-1 release.

In the NOD2 siRNA experiments, I was not successful in achieving NOD2 knockdown. Additionally, I observed some inconsistencies with the PCR product size for NOD2. There are many possible explanations for the lack of knockdown in the GLUTag cells. The additional optimization of NOD2 siRNA concentration, transfection reagent, and incubation time may be required. Indeed, a study that performed successful siRNA knockdown of NOD2 in PK-15 cells did so with a 36-hour transfection incubation. Our longest incubation was 48 hours. Also, Lipofectamine 3000 was used as their transfection reagent[59]. It is also important to note that the NOD2 knockdown PCR validation experiments showed a band that did not correspond to the predicted 98 bp PCR product. In light of this, I cannot properly optimize the siRNA conditions. Additional NOD2 PCR validation experiments using a suite of controls will be required first. This includes a no RT negative control which will ensure contamination is not present and generating a

false positive band. Additionally, a positive control template cDNA that is known to express NOD2 would ensure that our experiment allows for detection of the appropriate NOD2 band. Once these controls are in place, NOD2 knockdown experirments will be better equipped to be done in the future.

Limitations and alternative approaches:

Several of the experiments had limitations and future work with alternative approaches are required. To confirm the inhibition of NOD2 with GSK717, a validated positive control experiment should be done. Here I would use human monocytes as a positive control cell model since in human monocytes cytokine serection was successfully inhibited using GSK717[60]. For NOD2 protein expression Western Blot expirments, a postive control cell lystate should be used. Cell lysates known to show expression of NOD2 by Western Blot include RAW 264.7 and HEK293T [61,62]. Since some of the end point PCR from GLUTag cells provided NOD2 products of an inorrect size, an additioanl cDNA preparation from mouse immune cells could be used as a positive control

Conclusion

In this study, I have provided evidence that the modified L18MDP enhanced the secretion of GLP-1 hormone in GLUTag and NCI-H716 cell lines *in vitro*. The L18 MDP was well-tolerated by both cell lines when testing the cell toxicity. A goal of this research was to examine the cellular mechanism of L18MDP on GLP-1 cells. Since it is well-known that MDP is recognized by NOD2 in immune cells and it activites the MAPK signaling pathway, I chose to examine these in the L-cell models. In my study, I was unable to confirm the role of NOD2 and the P38 MAPK in MDP-induced GLP-1 secretion. However, I was only able to detect a modest increase of P38 MAPK in the 10-min time point. For future directions, I would examine other different signaling pathways that could be involved in GLP-1 release such as JNK and ERK when performing western blot. Also, I would optimize the NOD2 PCR experiments with the appropriate controls so that siRNA knockdown experiments for NOD2 could be properly interpreted. All in all, a greater understanding of the possible links between GLP-1 and bacterial metabolites like MDP may produce novel approaches in the treatment of T2D.

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