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Single-Cell Transcriptomics of Intestinal Epithelial Cells: Insights into the Prediabetic condition

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Table of Contents

List of abbreviations

Acknowledgements

Abstract

Single-Cell Transcriptomics of Intestinal Epithelial Cells: Insights into the Prediabetic condition
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Background: Obesity is a chronic condition characterised by excess adiposity that may be accompanied by different structural and functional abnormalities and with increased comorbidity and premature mortality risk thus reducing quality of life (Jastreboff et al., 2019; Lustig et al., 2022).

The main cause of obesity is the energetic imbalance due to increased caloric intake and little expenditure. This induces metabolic and hormonal changes e.g. increase in blood sugar levels that induces a prediabetic status with increased risk of developing type 2 diabetes mellitus (T2DM), heart disease, and stroke.

T2DM is a major non-communicable disease and one of the world's fastest growing health problems, with a projected increase in the number of diabetic patients to 700 million by 2045 (Saeedi et al., 2019). T2DM is associated with significant morbidity, including increased risk of cardiovascular diseases (CVD) and stroke, hypertension, etc. These place an enormous burden on individuals, society, and the healthcare system (Brorsson & Pociot, 2015). T2DM is a non-reversible but preventable condition with overweight and obesity being major risk factors. The onset of T2DM is gradual, with most individuals progressing from normoglycemia through a pre-diabetic state.

There is substantial evidence for the role of gut microbiota and impaired barrier in metabolic diseases including T2DM (Brunkwall & Orho-Melander, 2017). Recent clinical trials using Glucagon-like peptide-1 (GLP-1) receptor agonists (an incretin hormone produced by the enteroendocrine L cells in the distal intestine) have shown benefits to patients with these conditions. However, a role for other intestinal epithelial cell subsets in obesity and diabetes is yet to be determined. Thus, the aim of this project is to:

1) Identify alterations in intestinal epithelial cell subsets
2) Identify alterations in pathways contributing to obesity and/or diabetes pathophysiology.

For this purpose, the student will be performing a bioinformatics analysis on in house and publicly available bulk and single cells RNASeq data sets. Findings from this project, will provide a better understanding on how gut epithelial cells, which are in close contact to the microbiota, might support and/or sustain the development of these chronic conditions.

Aim

1) Identify alterations in intestinal epithelial cell subsets
2) Identify alterations in pathways contributing obesity and/or diabetes pathophysiology.

Introduction

Type 2 Diabetes Mellitus (T2DM) has emerged as a global health crisis, with its prevalence quadrupling over the past three decades. Characterized by insulin resistance and inadequate insulin secretion leading to hyperglycemia, T2DM is often preceded by a condition known as prediabetes. This precursor state is marked by impaired fasting glucose, impaired glucose tolerance, or raised HbA1c levels, and is associated with obesity, sedentary lifestyle, and genetic factors (American Diabetes Association, 2021).

While the systemic effects of T2DM and prediabetes are well-documented, recent evidence has highlighted the crucial role of the intestinal epithelium in the development and progression of these conditions. The intestinal epithelium, serving as the primary interface between the external environment and the body's internal milieu, performs critical functions in nutrient absorption, barrier protection, and hormone secretion. Alterations in the structure and function of this epithelium have been observed in prediabetic individuals, potentially contributing to the dysregulation of glucose homeostasis and overall metabolic health (Aliluev et al., 2021; Xie et al., 2020).

Key areas of interest in studying the intestinal epithelium in the context of prediabetes include changes in the gut microbiota, intestinal permeability, inflammation, macronutrient metabolism alterations, and intestinal stem cells (ISCs). These changes can significantly impact nutrient absorption and metabolism, potentially contributing to systemic inflammation and insulin resistance (Filippello et al., 2022; Paone et al., 2022). Understanding these intricate relationships between the intestinal epithelium and metabolic dysfunction requires advanced research techniques that can capture the complexity of cellular responses at a high resolution.

Single-cell RNA sequencing (scRNA-seq) has emerged as a powerful tool to address this need. This technology allows for the characterisation of gene expression profiles at the individual cell level, enabling researchers to uncover cellular heterogeneity, identify rare cell populations, and elucidate cell-specific responses to physiological changes. The scRNA-seq workflow, typically

involving single-cell isolation, cell lysis, reverse transcription of RNA to cDNA, amplification, library preparation, and sequencing, has revolutionized our ability to study complex biological systems.

In light of these technological advancements and the growing recognition of the intestinal epithelium's role in metabolic health, this research aims to employ scRNA-seq analysis to identify genes and pathways within the intestinal epithelium that are characteristic of the prediabetic state. Using a high-fat, high-sugar diet (HFHSD) mouse model to study diet-induced prediabetes, this study seeks to characterise the transcriptional profiles of individual cell types within the intestinal epithelium in both normal and prediabetic states. By identifying differentially expressed genes and altered signaling pathways associated with prediabetes, we aim to investigate the link between prediabetes-induced changes in the intestinal epithelium and alterations in metabolic function.

The significance of this research lies in its potential to advance our understanding of the molecular basis of prediabetes, particularly in relation to the intestinal epithelium. By providing a high-resolution map of transcriptional changes in individual cell types, this study aims to reveal novel mechanisms contributing to prediabetes progression in the intestinal epithelium and potentially identify new targets for therapeutic interventions. Ultimately, this research aims to elucidate the molecular mechanisms underlying the link between diet, intestinal epithelial function, and systemic metabolism.

Chapter 1: The intimate link between the intestinal epithelium and prediabetes.

1.1 Intesintal Barrier Structure and Function

The intestinal barrier is a dynamic system of several specialised components regulating the absorpotion of nutrients while preventing the entry of harmful substances and microorganisms. Understanding the structure and function of the intestinal barrier is essential for comprehending its role in health and disease, particularly in the context of metabolic disorders such as prediabetes. This section explores the key components of the intestinal barrier, including the

gut microbiota, mucus layer, intestinal epithelium, immune barrier as well as factors that can modify barrier function. Furthermore, dysfunctions of the intestinal epithelium in the prediabetic model are discussed in detail.

1.1.1 Microbiota

The intestinal microbiome forms the initial line of defense in the gut barrier consisting of four main phyla Proteobacteria, Bacteroidetesm Actinobacteria, Firmicutes, with Bacteroides and firmicutes totalling to approximately 90% of the total gut microbiota (Rajilić-Stojanović et al., 2007)(Vos et al., 2022). The density of these microorganisms increase along the gastrointestnial tract reaching its peak in the colon (McGhee and Fujihashi, 2012). The gut microbiota also plays a crucial role in nutrient metabolism. The microbiota derives its nutrients mostly through carbohydrates in the diet but also lipids, proteins, vitamins and various phenolic compounds. Carbohydrates are primarily used as an energy source by the microbiota and are primarily metabolised by members of the genus Bacteroides by expressing various carbohydrate digesting enzymes (Jandhyala et al., 2015). Nutrients which are not digestible by the host such as fibers make their way to the colon and are metabolised by the microbiota into a wide array of metabolites such as short-chain fatty acids (SCFAs) which play an important role in maintaining health and disease displaying roles in inducing reactive oxygen species, altering cell proliferation and function, antinflammatory, antitumorigenic and antimicrobial effect. SCFAs are also used directly as an energy source by enterocytes in the colon or are transported across the epithelial layer into the blood (Tan et al., 2014). Interactions between the host and the microbiome significantly impacts the maturation of the immune system. Literature indicates that different bacterial species can trigger distinct immune responses, suggesting that microbiota composition significantly influences immunity. The microbiota's impact extends beyond the gut, affecting systemic immune function and influencing disease processes in various organs. Depending on the bacterial species involved, these effects can range from disease promotion to protection (Kosiewicz et al., 2011).

1.1.2 Mucus Layer

The mucus layer forms the second component of the intestinal barrier, facilitated by a layer mucins which are highly O-glycosylated proteins with gel-like properties secreted by goblet cells. This layer is respnsible for a unique role in maintaining the intestinal lining from pathogens and mechanical damage (Johansson and Hansson, 2016). The mucus layer of the small intestine is penetrable to microbes however the microbiota are kept at a distance from the intestinal epithelium through antimicrobial metabolites. Lysozyme, secretory IgA, and defensins produced by Paneth cells are found in the inner mucus layer, providing a mechanism that helps keep bacteria away from the brush border of enterocytes (Gibbins et al., 2015). Additional antimicrobial peptides and proteins, such as REG3G, lypd8, and ZG16, further contribute to bacterial exclusion from the mucosa (Bergström et al., 2016). On the other hand in the large intestine, the mucus layer is stratified into two main layers. The inner layer is impenetrable to the microbiota composed largely of Muc2 multimers whereas the outer layer provides a comfortable penetrable environment for the microorganisms (Johansson and Hansson, 2016). The microbiota influences mucin composition and structure through various mechanisms, including the ratio of Bacteroides and Firmicutes (Wrzosek et al., 2013). Dietary factors, such as fiber content, also impact mucus thickness and the abundance of mucin-degrading bacteria (Desai et al., 2016). Certain bacteria, like Akkermansia muciniphila, play a crucial role in mucus degradation and modulation of inflammatory changes and crosstalk between them and the intestinal epithelium has been shown to modulate obesity (Everard et al., 2013).

1.1.3 Intestinal Epithelium

The intestinal epithelium forms a critical component of the intestinal barrier, consisting of a single layer of cells that provide both physical and functional protection [ref]. This layer comprises various cell types, each contributing to barrier integrity and function. Enterocytes, the most abundant cell type, produce antimicrobial peptides and form the main absorptive surface [ref]. Goblet cells,

responsible for mucus production, contribute to the mucus layer discussed earlier [ref]. Tuft cells possess chemosensory functions and secrete interleukins involved in innate immunity [ref]. Enteroendocrine cells produce hormones such as CCK, VIP, GLP1, GLP2, and PYY [ref]. Paneth cells, located in crypt regions, produce antimicrobial peptides and contribute to the immune barrier [ref]. M cells, found in the small intestine, are involved in antigen uptake and mucosal immune responses [ref].

The epithelial cells are interconnected by junctional complexes, with tight junctions (TJs) being the most critical for barrier function. TJs comprise over 40 proteins, including claudins, occludin, and zonula occludens (ZO) proteins [ref]. These structures regulate the paracellular passage of molecules and ions, acting as a selective barrier based on size and charge [ref].

The epithelial barrier allows for both transcellular and paracellular transport of molecules. The transcellular route permits the passage of lipids, small hydrophilic compounds, and ions, while the paracellular route is controlled by TJs [ref]. The permeability of this barrier varies along the intestinal tract, with the colonic epithelium being less permeable than the small intestinal epithelium [ref].

Various factors can influence TJ function, including cytokines (e.g., IL-13, TNF-α, IFN-γ) and signaling kinases [ref]. Impaired intestinal barrier function has been observed in animal models of obesity, diabetes mellitus, and inflammatory bowel diseases [ref].

1.1.4 Immune Barrier

The immune barrier comprises various immune-competent cells and molecules that work in concert with the physical barriers to maintain intestinal homeostasis. This system includes immune cells such as Paneth cells, dendritic cells, B and T lymphocytes, and phagocytes [ref]. Antimicrobial peptides, including defensins, cathelicidines, and lectins, accumulate within the intestinal lumen in the inner layer of the host mucin [ref]. Intraepithelial lymphocytes play

a role in host defense against pathogens, wound repair, and homeostatic interactions with the microbiota and nutrients [ref].

The gut-associated lymphoid tissues (GALT), including Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles, form part of the mucosa-associated lymphoid tissue (MALT) [ref]. This system facilitates the migration of immune cells from mucosal inductive tissues to effector tissues via the lymphatic system [ref].

Several distinct subsets of immune cells contribute to intestinal barrier function. Innate lymphoid cells (ILCs) interact with both innate and adaptive immune cells and are involved in tissue repair and metabolic homeostasis [ref]. CD4+Foxp3+ regulatory T-cells (Tregs) regulate peripheral tolerance and control autoreactive T cells [ref]. Th17 cells, a subset of CD4 T cells, produce IL17-A, IL17-F, and IL-22, contributing to tight junction efficiency and epithelial cell regeneration [ref].

Pattern-recognition receptors, including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors, recognize microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs). These receptors facilitate gut-microbiota interactions and recruit dendritic cells in case of intestinal barrier damage [ref].

The relationship between these immune components and the gut microbiota plays a critical role in the maturation of immune cells and the maintenance of intestinal barrier integrity [ref]. This sophisticated system ensures a balanced response to environmental stimuli while maintaining the barrier function of the intestinal epithelium.

1.1.5 Modifiers of the Intestinal Barrier

The intestinal barrier's integrity and function can be significantly influenced by various factors. These modifiers play crucial roles in maintaining or altering barrier function, which may have implications for metabolic disorders such as prediabetes.

Alcohol consumption, both acute and chronic, can disrupt the intestinal barrier through direct damage to enterocytes and disruption of tight junctions [ref]. In animal models, chronic alcohol administration induces intestinal dysbiosis, bacterial overgrowth, and translocation [ref]. Clinical studies have shown that excessive alcohol intake can lead to alterations in duodenal histology and increased intestinal permeability [ref].

Dietary components significantly impact barrier function. High-fat diets may increase gut permeability by altering the expression of tight junction proteins and promoting inflammatory responses [ref]. In rodent models, high-fat diets have been associated with decreased mRNA or protein expression of tight junction components such as claudins-1-3 and ZO-1 [ref].

Conversely, certain dietary elements can enhance barrier function. Fibers, particularly microbiota-accessible carbohydrates (MACs), contribute to intestinal barrier health by increasing the production of short-chain fatty acids (SCFAs) [ref]. SCFAs, including butyrate, propionate, and acetate, enhance barrier function by regulating tight junction proteins and reducing inflammation [ref].

Polyphenols found in fruits and vegetables can improve intestinal barrier function and inhibit intestinal dysbiosis [ref]. Specific compounds like resveratrol have shown protective effects on the intestinal barrier in animal models of high-fat diet-induced metabolic disorders [ref]. Quercetin has been found to inhibit the decrease of tight junction proteins caused by inflammatory stimuli in cell culture models [ref].

Certain nutrients have been associated with improved intestinal permeability. Glutamine supplementation has shown protective effects on tissue integrity and intestinal permeability in conditions such as irritable bowel syndrome and Crohn's disease [ref]. Vitamin D levels have been linked to improved intestinal permeability in some clinical studies [ref]. The food supplement zinc carnosine appears to protect the intestinal barrier via a proliferative response, as demonstrated in both cell culture and clinical studies [ref].

Emulsifiers, commonly found in processed foods, can potentially damage the intestinal barrier by decreasing microbial diversity and inducing mucosal

inflammation [ref]. In vitro studies have shown that certain emulsifiers can alter the thickness of the mucus layer and increase bacterial translocation [ref].

Probiotics, prebiotics, and synbiotics may enhance intestinal barrier function, although their clinical efficacy requires further investigation [ref]. These agents have shown promise in responding to stressor or disease states, but more research is needed to establish their role in maintaining barrier integrity [ref].

The relationship between diet, microbiota, and the intestinal barrier are of great importance in the study of metabolic diseases. Future research should focus on understanding the mechanisms by which these modifiers affect barrier function and their potential role in the development and progression of prediabetic conditions.

1.2 Intestinal Epithelium Alterations in prediabetes

1.2.1 Microbiota Alterations

Prediabetes is associated with significant alterations in the gut microbiome, which may contribute to the progression towards type 2 diabetes mellitus (T2DM). These changes in microbial composition and diversity are increasingly recognized as potential factors in the development of metabolic disorders [ref]. Studies have consistently reported a reduction in microbial diversity and richness in individuals with prediabetes, mirroring observations in patients with established diabetes [ref]. This decreased diversity may compromise the beneficial functions of the gut microbiome, potentially contributing to metabolic dysregulation.

Several bacterial genera have been found to be differentially abundant in prediabetic individuals compared to those with normal glucose metabolism. Notably, studies have reported lower abundances of Blautia, Faecalibacterium, Bifidobacterium, Clostridium, Anaerostipes, Mediterraneibacter, and Butyricicoccus in prediabetic fecal samples [ref]. These bacteria are known to play important roles in maintaining intestinal health, including the production of short-chain fatty acids (SCFAs) and the maintenance of gut barrier integrity.

Particularly noteworthy is the reduced abundance of Akkermansia muciniphila in individuals with prediabetes [ref]. A. muciniphila has been associated with improved metabolic health, and its depletion may contribute to increased intestinal permeability and metabolic disturbances. Experimental studies have shown that oral administration of A. muciniphila can improve glucose intolerance and insulin resistance in animal models, possibly through Toll-like receptor 2 signaling [ref].

Conversely, some bacterial genera have been found to be more abundant in prediabetic individuals. These include Dorea, Ruminococcus, Sutterella, and Streptococcus [ref]. Additionally, increased abundances of Bacteroides, Phascolarctobacterium, Parabacteroides, and Paraprevotella have been observed in prediabetic fecal samples [ref]. The functional implications of these increases are not fully understood and require further investigation.

Interestingly, the advancement of diabetes in animal models has been associated with an increase in facultative anaerobes [ref]. This shift in microbial composition may reflect adaptations to changing intestinal conditions as the disease progresses. The alterations in gut microbiota composition are thought to contribute to the pathogenesis of prediabetes through various mechanisms. These include changes in intestinal barrier function, modulation of the immune response, and alterations in metabolite production. For instance, the decreased abundance of butyrate-producing bacteria may lead to reduced production of this important SCFA, which plays a crucial role in maintaining gut barrier integrity and regulating inflammation [ref].

Moreover, the gut microbiota changes observed in prediabetes may influence several physiological metabolic pathways. These include alterations in glucose-phosphate isomerase metabolism, defects in insulin transmembrane signaling, and overexpression of retinoic acid-inducible gene I (RIG-I), potentially contributing to immune system dysregulation and impaired glucose control [ref].

It's important to note that while these microbial alterations are consistently observed in prediabetic individuals, the causal relationship between gut dysbiosis and prediabetes development remains to be fully elucidated. Factors such as diet, which accounts for approximately 57% of gut microbiota composition, play a significant role in shaping the microbial community [ref]. This underscores the potential for dietary interventions in modulating the gut microbiome and, potentially, in preventing or managing prediabetes.

Future research should focus on understanding the functional consequences of these microbial alterations and their specific contributions to the development and progression of prediabetes. Additionally, investigating the potential of microbiome-based interventions, such as targeted probiotic therapies or dietary modifications, may offer new avenues for preventing or managing prediabetes and its progression to T2DM.

1.2.2 Intestinal Permeability

Alterations in intestinal permeability play a crucial role in the pathogenesis of prediabetes and its progression to type 2 diabetes mellitus (T2DM). The intestinal barrier, comprising various elements including the epithelium, tight junctions, and mucus layer, regulates the passage of substances between the intestinal lumen and the circulation [ref]. In prediabetic conditions, several studies have reported increased intestinal permeability, often referred to as "leaky gut." This increased permeability is associated with alterations in the structure and function of tight junctions, which are critical components of the paracellular barrier [ref]. Tight junctions consist of various proteins, including claudins, occludin, and zonula occludens (ZO) proteins, which work together to regulate the selective passage of molecules between intestinal epithelial cells [ref].

Research has shown that high-fat diet (HFD) intake, often associated with prediabetes, can lead to significant changes in intestinal permeability. In vitro studies have demonstrated that exposure to intestinal content, particularly from

the small intestine of mice fed a HFD, can disrupt the tight junction-mediated epithelial barrier in cell culture models [ref]. This suggests that components of the intestinal lumen in HFD conditions may directly impact barrier integrity. In animal models of prediabetes, structural changes in tight junctions have been observed in various segments of the intestine. Notably, these alterations occur early in the development of prediabetes, often preceding major metabolic changes. The duodenum and jejunum appear to be particularly affected, with significant reductions in the junctional content of tight junction proteins [ref]. These changes in the upper small intestine are especially relevant given its role in nutrient absorption and metabolic signaling.

Interestingly, the increase in intestinal permeability observed in prediabetic animal models has been reported to occur without significant changes in systemic and intestinal zonulin, tumor necrosis factor-α (TNF-α), and lipopolysaccharide (LPS) concentrations [ref]. This suggests that the mechanisms underlying increased permeability in prediabetes may be distinct from those observed in other inflammatory conditions and may involve alternative pathways or mediators.

The consequences of increased intestinal permeability in prediabetes are multifaceted. Enhanced paracellular passage of luminal contents may lead to the absorption of dietary antigens, bacterial products, and other potentially harmful substances. This, in turn, could contribute to low-grade systemic inflammation and metabolic disturbances characteristic of prediabetes [ref]. Furthermore, the disruption of the intestinal barrier may alter the intricate relationship between the gut microbiota and the host. Changes in permeability could allow for increased translocation of bacterial products, potentially triggering immune responses and further contributing to metabolic dysfunction [ref]. It's important to note that while increased intestinal permeability is consistently observed in prediabetic conditions, the exact mechanisms linking this phenomenon to the development and progression of metabolic disorders are still being elucidated. Factors such as diet composition, microbiota

alterations, and host genetic susceptibility likely interact in complex ways to influence barrier function and metabolic health [ref].

Future research should focus on further characterizing the molecular and cellular changes in the intestinal barrier during the progression from normal glucose tolerance to prediabetes and T2DM. Additionally, investigating potential therapeutic interventions targeting intestinal permeability may offer new strategies for preventing or managing prediabetes and its associated complications.

1.2.3 Inflammation

Inflammation plays a crucial role in the pathogenesis of prediabetes and its progression to type 2 diabetes mellitus (T2DM). Systemic inflammation in prediabetes is characterized by elevated levels of inflammatory markers and alterations in immune function. Studies have reported increased levels of inflammatory proteins in prediabetic patients, including resistin, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and C-reactive protein (CRP) [ref]. The ratio of CRP to albumin is also elevated, indicating a shift towards a pro-inflammatory state [ref].

Prediabetes is associated with systemic immune dysregulation, affecting multiple tissues involved in glucose homeostasis, such as pancreatic islets and the liver [ref]. Impaired immune responses include heightened activation of the complement cascade and alterations in hemostasis [ref]. Changes in T cell function have been observed, with regulatory T cells (Tregs) promoting T helper 17 differentiation and cytokine production in prediabetes, but not in established diabetes [ref]. Environmental factors contribute to inflammation in prediabetes. Long-term exposure to air pollutants has been linked to inflammatory pathway activation and glycemic disorders [ref]. Endocrine-disrupting compounds like bisphenol A can trigger hypothalamic inflammation through toll-like receptor 4

(TLR4)-dependent mechanisms, promoting prediabetic metabolic dysfunction [ref].

In the context of intestinal inflammation in prediabetes, current hypotheses focus on the relationship between diet, gut microbiota, and intestinal barrier function. One prevalent hypothesis suggests that high-fat diet intake leads to intestinal microbiota dysbiosis, resulting in increased luminal lipopolysaccharide (LPS) and zonulin secretion by the intestine [ref]. These alterations are thought to increase paracellular permeability and LPS absorption, leading to metabolic endotoxemia and low-grade chronic systemic inflammation, which may trigger or exacerbate peripheral insulin resistance [ref].

However, conflicting evidence exists in the literature regarding the relationship between intestinal permeability, inflammation, and prediabetes development. Some studies report significant increases in intestinal permeability to large molecules, associated with endotoxemia and systemic inflammation [ref]. In contrast, other research has found that increased intestinal tight junction (TJ) permeability in prediabetic mice occurs without significant changes in systemic and intestinal levels of zonulin, TNF-α, and LPS [ref]. These discrepancies may be partially explained by differences in experimental models, including variations in animal strains and diet composition. For instance, studies using diets with very high fat content (e.g., 72% of energy from lipids) have observed significant metabolic and intestinal changes, including metabolic endotoxemia and increased cecal LPS levels, after relatively short exposure periods [ref]. In contrast, studies using more moderate high-fat diets (e.g., 40% of energy from lipids) found that animals became prediabetic after longer periods without significant changes in microbiota composition or luminal LPS levels [ref].

Another area of conflict in the literature concerns the timing and localization of intestinal barrier disruption in prediabetes. Some research indicates that TJ-mediated epithelial barrier disruption occurs early in prediabetes development,

particularly in the duodenum and jejunum, and precedes major metabolic changes [ref]. However, the extent of these changes and their relationship to systemic inflammation remain debated. These conflicting findings highlight the complex and potentially nuanced relationship between intestinal barrier function, inflammation, and prediabetes development. The disruption of the TJ-mediated paracellular barrier, particularly in the upper small intestine, appears to be an early event in prediabetes development. However, this disruption can occur in the absence of detectable endotoxemia or systemic inflammation, suggesting that the progression from intestinal alterations to systemic metabolic dysfunction may involve additional factors or mechanisms not yet fully understood.

Further research is needed to reconcile these conflicting observations and elucidate the specific mechanisms linking intestinal barrier dysfunction to systemic inflammation and metabolic disturbances in prediabetes. Future studies should focus on characterizing the temporal relationship between intestinal epithelial alterations, local and systemic inflammatory responses, and metabolic changes in the context of prediabetes development. Additionally, investigating the role of specific intestinal luminal components and intracellular signaling pathways in regulating TJ structure and function may provide valuable insights into the pathogenesis of prediabetes and potential therapeutic targets.

1.2.4 Macronutrient Metabolism Alterations

The intestinal epithelium undergoes significant changes in macronutrient metabolism during the development of prediabetes, particularly in response to high-fat diets (HFDs) and high-fat high-sugar diets (HFHSDs). These alterations affect lipid, carbohydrate, and protein metabolism, contributing to the progression of metabolic dysfunction.

HFDs significantly impact the expression of intestinal genes involved in fatty acid metabolism. A notable example is the Scd1 gene, which converts saturated fatty acids to monounsaturated fatty acids and is upregulated more than tenfold in the jejunum by coconut oil [ref]. Other affected genes include those involved

in linoleic acid and arachidonic acid metabolism (e.g., Cyp2c, Cyp2j, Cyp4a, Ephx2), which are associated with pro-inflammatory processes [ref]. In prediabetic conditions, lipid metabolism pathways are vastly altered. Proteins involved in mitochondrial β-oxidation (HADHA, ACADVL, ACADL, ECH1, ECHS1) and peroxisome β-oxidation (ECH1, ACOX1) are upregulated in gut-derived extracellular vesicles (GDEs) from HFD-fed animals [ref]. This suggests a shift towards fatty acids as a preferred energy source over glucose. A family of acyl-CoA thioesterases (ACOTs) is particularly upregulated, acting as intermediaries in directing fatty acids to either the TCA cycle or storage [ref]. The activation of ACOTs in obesity may direct fatty acids towards triglyceride synthesis for incorporation into chylomicrons or VLDL particles [ref]. ATP-citrate lyase, responsible for converting citrate into acetyl-CoA, is upregulated in intestinal cells under prediabetic conditions, suggesting an increased capacity for de novo lipogenesis [ref].

Carbohydrate metabolism in the intestinal epithelium is significantly altered in prediabetes. Key glycolytic enzymes, including hexokinase (HK) and phosphofructokinase (PFKL), show reduced abundance in GDEs from HFD-fed animals [ref]. This decrease suggests changes in sucrose utilization and lactate production. Pyruvate dehydrogenase A1 (PDHA1), part of the pyruvate dehydrogenase complex, is upregulated in HFD conditions. This change may represent a compensatory mechanism to maintain acetyl-CoA production for the TCA cycle and other biochemical reactions [ref]. The intestine's role in fructose metabolism gains importance in prediabetic states. Recent studies have demonstrated the capacity for intestinal fructose metabolism coupled to intestinal gluconeogenesis [ref]. In conditions of high dietary fructose, as often seen in prediabetic diets, the intestine may play a crucial role in regulating fructose metabolism and its systemic effects. Enterocytes show increased expression of genes linked to carbohydrate uptake and intracellular fat accumulation in response to HFHSD [ref]. This metabolic rewiring is reminiscent of hepatic steatosis and may contribute to increased calorie intake and fat accumulation.

Protein and amino acid metabolism also undergo changes in the prediabetic intestinal epithelium. HFDs affect the expression of genes involved in amino acid metabolism, despite diets containing the same amount of protein [ref]. These changes could play a role in select signaling pathways. Lysine metabolism pathway alterations have been observed in GDEs from HFD-fed animals [ref]. Lysine plays a role in carnitine synthesis, a key factor in fatty acid metabolism. Carnitine deficiency due to poor lysine intake can induce triglyceride accumulation [ref]. L-arginine metabolism is notably altered in prediabetic conditions. Nine out of ten regulated proteins identified in the arginine and proline metabolism pathway are upregulated in HFD conditions [ref]. This upregulation aligns with arginine's role as an insulin secretagogue, stimulating the release of glucagon-like peptide 1 in the intestine, which may contribute to characteristic prediabetic hyperinsulinemia [ref].

These alterations in macronutrient metabolism within the intestinal epithelium reflect the complex metabolic changes occurring in prediabetes. The shift in lipid metabolism towards increased fatty acid oxidation, changes in carbohydrate utilization, and alterations in amino acid metabolism all contribute to the dysregulation of energy homeostasis. Understanding these changes provides insight into the role of the intestinal epithelium in the progression of prediabetes and may offer potential targets for therapeutic interventions.

1.2.5 Intestinal Stem Cell Function Alterations

Prediabetes induces significant changes in intestinal stem cell (ISC) function, contributing to altered intestinal homeostasis and potentially increasing the risk of gastrointestinal complications. These alterations are primarily driven by dietary factors associated with prediabetes, particularly high-fat high-sugar diets (HFHSDs).

Research has shown that HFHSDs lead to hyperproliferation and altered differentiation of ISCs and progenitor cells [ref]. This increase in proliferative activity is accompanied by accelerated differentiation and cell turnover, which

can disrupt the normal balance of cell types within the intestinal epithelium [ref]. The effects of HFHSD on intestinal structure are notable. Studies have observed enlargement of the small intestine, elongation of villi, and decreased crypt density [ref]. These morphological changes are associated with alterations in the cellular composition of both crypts and villi, as confirmed by lineage-tracing studies [ref].

At the molecular level, the hyperproliferative state induced by HFHSD is not driven by the previously implicated Pparδ-mediated activation of Wnt/β-catenin signaling [ref]. Instead, it involves the upregulation of several key pathways. Pro-proliferative Pparγ signaling, Srebp1-mediated lipogenesis, and InsrIgf1r–Akt signaling are all elevated in response to HFHSD [ref]. The activation of these pathways, particularly Pparγ and Srebp1-mediated de novo lipogenesis, has been linked to inflammation, increased proliferation, and tumor progression in various cancer types [ref]. This connection suggests a potential mechanism by which HFHSD-induced metabolic changes might increase the risk of gastrointestinal cancer in prediabetic conditions [ref].

The rapid turnover of ISCs in HFHSD-fed mice presents a challenge in studying these changes. While single-cell RNA sequencing (scRNA-seq) data shows a decrease in ISCs, Lgr5-reporter mice show no change in ISC numbers [ref]. This discrepancy is explained by the accelerated division and differentiation of ISCs, where newly formed cells still express high levels of Lgr5 but also show markers of differentiated cells [ref].

The altered ISC function in prediabetes also affects the composition of enteroendocrine cells. There is a decrease in serotonergic enteroendocrine cytotypes and an increase in peptidergic types [ref]. These changes in the enteroendocrine system may contribute to the dysregulation of intestinal function and systemic metabolism observed in prediabetes. The chronic activation of pro-proliferative and lipogenic pathways in ISCs, as observed with prolonged HFHSD exposure, may lower the threshold for oncogenic transformation [ref]. This metabolic reprogramming of ISCs could explain, in part, the increased risk of gastrointestinal cancers associated with obesity and prediabetes.

Understanding these alterations in ISC function provides insight into the mechanisms underlying intestinal maladaptation in prediabetes. It also highlights potential targets for interventions aimed at maintaining intestinal homeostasis and reducing the risk of gastrointestinal complications in individuals with prediabetes.

Chapter 2: scRNA-seq and modelling approaches for revealing alterations in the prediabetic disease state.

2.1 single cell RNA-sequencing

Multiomics technologies have advanced with major breakthroughs in the last two decades, driven by developments in bioinformatics, computational biology, and multi-omics technologies. The ability to capture large amounts of molecular data through high-throughput technologies provides a new landscape of information in which systems biology can be studied. These approaches collectively enable the comprehensive characterization of biological systems at multiple levels, including the transcriptome, proteome, metabolome, epigenome, and genome. Multi-omics techniques integrate several methodologies to provide a holistic view of biological processes. The intersections of each of these disciplines are revealing new understandings and mechanisms by which organisms operate, on the multicellular, larger perspective, but also focussed perspectives at the single-cell resolution. Genomics focuses on the complete set of genetic material within an organism, while transcriptomics examines the complete set of RNA transcripts produced by the genome under specific conditions. Proteomics, complementing genomics and transcriptomics, centers on the identification and quantification of proteins within a cell, tissue, or organism, providing insights into protein identity, structure, and function. Metabolomics analyzes the complete set of small-molecule metabolites within a biological sample, and epigenomics investigates modifications to the genome that do not involve changes to the underlying DNA sequence.

In the context of prediabetes and Type 2 Diabetes Mellitus (T2DM) research, these multi-omics approaches enable researchers to elucidate alterations at multiple biological levels, from genetic predisposition to changes in gene expression, protein function, and metabolic pathways. The integration of these data sets allows for a more hollistic understanding of the complex biological processes underlying these metabolic disorders.

Among these techniques, single-cell transcriptomics, particularly single-cell RNA sequencing (scRNA-seq), has emerged as a leading approach due to its ability to provide high-resolution insights into cellular heterogeneity and gene expression dynamics at the individual cell level. This technology has proven particularly valuable in studying the diverse cell populations within the intestinal epithelium and their roles in metabolic health and disease (Aliluev et al., 2021; Xie et al., 2020).

The application of multi-omics approaches in prediabetes and T2DM research has the potential to reveal novel mechanisms contributing to disease progression, identify new biomarkers for early detection, and uncover potential therapeutic targets. As these technologies continue to evolve, they promise to provide increasingly detailed and nuanced understanding of the molecular basis of metabolic disorders.

Single cell RNA sequncing technologies are employed in this research project in tandem with various bioinformatics and computational biology methods in an attempt to understand the role of the individual cell types of the intestinal epithelium in prediabetes.

2.1.1 Upstream scRNA-seq analysis pipelines

The term ‘pipeline’ can take various meanings in different contexts, particularly in the field of bioinformatics. In the context of this research project, it means a series of automated and sequential steps for processing and analysing biological data, ensuring efficient and reproducible workflows. In this scRNA-seq analysis, the upstream pipeline is focussed on taking a matrix of raw gene counts for individual cells and outputting a Uniform Manifold Approximation and Projection (UMAP) reducing the extremely large and complex dataset to two

dimensions by which varation of the transcriptomes of each cell can be compared. From here an unsupervised leiden clustering algorithm can be employed to separate the cells into different groups, where each group represents a cluster of cells of similar transcriptomic value. With this clustered UMAP, annotation labels are applied which represent the type of cells present in the cluster.

2.1.2 Downstream scRNA-seq analysis pipelines

2.2 Mouse Models in Prediabetes Research

As discussed in sections 1 and 2, there are many risk factors which lead to prediabetes and subsequent T2DM with the primary risk factors being diet, activity, and weight. As a result, when one is divising a method for modelling these systems, one must choose sensible risk factors accordingly to induce these metabolic changes. Currently, the primary means of inducing a diabetic system is through diet interventions, chemical actions, and genetic modifications.

2.2.1 Diet-induced models

Diet-induced models are commonly used to study prediabetes and T2DM pathophysiology. These models primarily utilize high-carbohydrate, high-sucrose, high-fructose, or high-fat diets (HFD). HFDs, with fat concentrations ranging from 20% to 60%, are frequently employed and lead to increased triglyceride formation [ref]. Fructose consumption induces increased energy intake, body weight, adiposity, and various metabolic disturbances [ref]. Sucrose-enriched diets, ranging from 20% to 77%, are used to induce obesity and insulin resistance, although they are generally less effective than equivalent amounts of fructose [ref].

C57BL/6J mice fed a 60% HFD for 11-16 weeks typically develop obesity, hyperglycemia, insulin resistance, and hypertension [ref]. Extended HFD feeding (36 weeks) can lead to additional abnormalities in the renin-angiotensin system and cardiac remodeling [ref]. Combinations of diet and drug interventions, such as HFD with nitric oxide synthase inhibitors, have also been employed to induce more comprehensive cardiometabolic syndrome phenotypes [ref].

It's important to note that diet-induced phenotypes can vary based on genetic background, gut microbiota, diet composition, duration of intervention, age, and sex of the mice [ref]. These factors should be considered when interpreting results from diet-induced models. Additionally, different diets may trigger varying metabolic signatures, simulating different aspects of cardiometabolic complications rather than the complete syndrome [ref].

Not all high-fat diets produce equivalent metabolic effects. A study comparing isocaloric diets with different fat sources and fructose content in C57/BL6 male mice revealed distinct metabolic outcomes. A diet high in soybean oil (rich in polyunsaturated fatty acids) proved to be more obesogenic and diabetogenic than diets high in coconut oil or fructose. It induced more significant weight gain, adiposity, diabetes, glucose intolerance, and insulin resistance compared to a diet primarily composed of coconut oil [ref]. The soybean oil diet also caused more severe hepatic steatosis and alterations in gene expression related to obesity, diabetes, and inflammation. In contrast, a high-fructose diet, while less impactful on overall weight gain and diabetes markers, led to its own set of metabolic disturbances. These findings underscore the importance of considering specific dietary components, rather than just total fat content, when designing and interpreting diet-induced metabolic studies [ref].

2.2.2 Chemical and drug-induced models

Chemical and drug-induced models offer alternative approaches to studying prediabetes and T2DM. Streptozotocin administration is a widely used method to induce diabetes in mice. It acts by destroying pancreatic β-cells, accumulating in these cells via the GLUT2 glucose transporter, and primarily leads to a Type 1 diabetes-like state [ref]. Another approach involves glucocorticoid-induced metabolic syndrome. Glucocorticoids regulate glucose homeostasis by promoting gluconeogenesis in the liver and decreasing glucose uptake and utilization in skeletal muscle and white adipose tissue. Mice treated with glucocorticoids exhibit glucose intolerance, reduced insulin sensitivity, weight gain, dyslipidemia, central and peripheral fat accumulation, and hypertension [ref]. These chemical-induced models provide valuable tools for studying specific aspects of diabetes and metabolic syndrome, although they may not fully recapitulate the complex etiology of human T2DM.

2.2.3 Genetic-induced Models

Genetic engineering strategies for mouse models of prediabetes and T2DM focus on altering lipid metabolism, weight regulation, glucose homeostasis, and blood pressure. Two well-characterized dyslipidemic models are the low-density

lipoprotein receptor (Ldlr) and apolipoprotein E (Apoe) deficient mice. Ldlr-/- mice develop moderate hypercholesterolemia on a normal diet and are responsive to atherogenic diets, developing obesity, insulin resistance, and impaired glucose tolerance [ref]. Apoe-/- mice exhibit severe hyperlipidemia and spontaneous atherosclerosis, but typically do not become obese or insulin resistant without specific dietary interventions [ref].

Obesity models include leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice. These models display increased food intake, extreme obesity, and reduced energy expenditure, with strain-dependent effects on glucose metabolism [ref]. The agouti yellow obese (Ay/a) mouse and melanocortin 4 receptor (MC4-R) knockout mouse are additional models exhibiting adult-onset obesity, hyperinsulinemia, and glucose intolerance [ref].

Nonobese lipodystrophic models, such as A-ZIP F-1 and aP2-SREBP-1c mice, feature restricted white adipose tissue capacity. These models demonstrate that fat ablation can lead to liver steatosis, diabetes, and elevated blood pressure [ref].

Together, these three model classes form a solid foundation for interrogating the prediabetic / T2DM metabolic system. There still however remain limitations and questions. Do these models truly capture the prediabetic / T2DM system exactly how it pertains to human populations? As discussed, there are several risk factors which are attributed to these metabolic disturbances, some of which are not captured by these models. Smoking, ethnicity, human genetics, varying human diets, alcohol consumption are not captured within these models and resultingly may not display the intricacies of the true human diabetic system. Some alterations of the human system may not be seen in these models, similarly some alterations may be seen which are not typically seen in the human system. Perhaps for this reason it is worth aggregating studies from multiple different diabetic models as well as human studies when investigating the landscape of disease systems.

Chapter 3: The potential for synthetic and natural agents in reversing prediabetic disturbances in the gut.

3.1 Current Pharmacological Interventions for Prediabetes and T2DM

Currently, the best options for preventing T2DM is through the screening of prediabetes in populations and subsequent lifestyle changes in reducing the usual risk factors of T2DM. There do however exist preventative/management options regarding pharmacological interventions. These usually involve drugs promoting weight loss and/or the reduction of glucose levels. Typical pharmacological interventions include metformin, a-glucosidase inhibitors, thiazolidinodiones, SCLT2 inhibitors, and GLP-1 agonists.

Metformin

GLP-1 agonists

a-glucosidase inhibitors

thiazolidinodiones

SCLT2 inhibitors

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10033948/

3.2 Emerging Therapies: Focus on Brown Seaweed Extract

Brown seaweed contains the highest quantity of antioxidants, polyphenols and other bioactive compounds out of the three seaweed classes (brown, red and green). Evidence is emerging attributing a plethora of pharmacological benefits through supplementation of extracts of these marine organisms. More specifically, numerous studies, in vitro, in vivo and clinical studies have been carried out investigating the potential for brown algae to ameliorate symptoms of the prediabetic and diabetic system although with mixed results. Algae strains which have been shown to demonstrate the largest benefits are *A. nod* and *F. ves*.

One in vitro study involved the extraction of inhibitors from 3 species …

Another study reviewed the potential of species1 and species 2 in manageing MetS

Another study investigated the effect of brown seaweed extract on weight-loss, blood glucose homeostasis in association with the metabolic and inflammatory response.

One other study investigated the ability for borwn seaweed algae to control salmonella infections in pig populations, a common seasonal issue in the agricultural industry during the stressful weaning period of young pigs. This is of particular relevance to diabetics who are more susceptible to Salmonella infections.

Materials and Methods

Mouse models

The dataset used throughout this study consists of scRNA-seq (10X) data from the small intestinal crypts of control diet and HFHSD obese FVF-enriched mice (Foxa2-FVF/FVF) as well as villus samples from the small intestine of control diet and HFHSD C57BL/6N mice.

All mice subjects in the dataset are male. Mice living conditions consisted of 2 to 4 mice per group, 23C 45-65% humidity with a 12 hr light/dark cycle.

Mice diets are reported to have begun at 10-12 weeks old and were randomised into different test groups matched for body weight with similar variance and given ad libitum access to either the control diet or HFHS diet for 11-13 weeks.

Glucose tolerance and insulin secretion tests.

To assess glucose tolerance, an oral glucose tolerance test (oGTT) was performed on FVF mice after 12 weeks on either a control diet (CD) or a high-fat high-sugar diet (HFHSD). Following a 6-hour fast, each mouse received an oral dose of glucose (1.5 mg/g body weight of 20% (wt/v) d-(+)-glucose solution in PBS). Blood glucose levels were measured at 0, 15, 30, 60, and 120 minutes post-glucose administration using a handheld glucometer (Abbott). For insulin secretion analysis, blood samples were collected from the tail vein at 0, 15, and 30 minutes during the oGTT. Plasma was isolated by centrifuging the blood samples (3,500 rpm, 15 minutes, 4°C), and insulin levels were quantified using the Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, 90080) following the manufacturer's protocol.

Insulin resistance and beta-cell function were evaluated using the homeostasis model assessment of insulin resistance (HOMA-IR) and HOMA-β, respectively. These indices were calculated 12 weeks after diet initiation, using fasting blood

glucose and plasma insulin levels obtained after a 6-hour fast. The conventional formulas applied were HOMA-IR = fasting blood glucose (mg/dL) × fasting insulin (μU/mL) / 405 and HOMA-β = fasting insulin (μU/mL) × 360 / (fasting glucose (mg/dL) - 63).

Crypt and Villus Isolation and Single-Cell Preparation

The isolation of small intestinal crypts was performed following established protocols. Briefly, small intestines (SIs) were excised and rinsed with cold PBS. The villi were carefully scraped away using a glass slide. The remaining tissue was then cut into 2-cm sections, repeatedly washed with cold PBS, and incubated in 2 mM EDTA/PBS for 35 minutes at 4°C with gentle agitation. Crypts were released by vigorous shaking and filtered through a 70-μm mesh to remove any villous debris. For the preparation of single cells, the isolated crypts were treated with TrypLE (Life Technologies, no. 12605) first on ice for 5 minutes, followed by 5 minutes at 37°C, and subsequently incubated with 10 μg/mL DNase in crypt complete medium (DMEM/F-12 with 10% FCS) for 5 minutes at 37°C. The resulting single-cell suspension was achieved by gentle, repeated pipetting. Cells were then washed twice with 2% FCS in PBS and pelleted by centrifugation at 300g for 5 minutes at 4°C. For flow cytometry analysis, cells were resuspended in 1-2 mL of FACS buffer (2% FCS, 2 mM EDTA in PBS; Sigma-Aldrich, no. Y0503) and passed through 40-μm cell strainer caps attached to FACS tubes.

For the isolation of villus cells, the villi were scraped and processed into a single-cell suspension using the same TrypLE treatment protocol as described for crypt cells.

Flow Cytometry

For gene expression analyses, including microarray, single-cell transcriptomics, and western blotting, small intestinal crypt cells were sorted using a FACS-Aria III (BD Bioscience) with FACSDiva software v.6.1.3 and a 100-µm nozzle. In all experiments, cells were gated based on their forward scatter area (FSC-A) and side scatter area (SSC-A). Singlets were identified using forward scatter width (FSC-W) and forward scatter height (FSC-H), and dead cells were excluded using 7-AAD (eBioscience, no. 00-6993-50). For quantitative PCR with reverse transcription (qRT-PCR), cells were directly sorted into Qiazol lysis reagent (QIAGEN, no. 79306). To enrich FVF-positive small intestinal crypt cells for scRNA-seq, 30,000 FVF+ (FVFlow and FVFhigh) cells were sorted along with 30,000 live crypt cells per sample. The sorted cells were collected in modified FACS buffer (2% FCS, 0.02 mM EDTA in PBS).

scRNA-seq

Crypt and villus samples were prepared as described above. Dead cells were excluded via flow cytometry after 7AAD labelling. Dead cell exclusion was controlled by trypan blue staining and sorted cells were counted. Single-cell libraries were generated using the Chromium Single cell 3′ library and gel bead kit v2 (10X Genomics, no. 120237) according to the manufacturer’s instructions. Libraries were sequenced on a HiSeq4000 (Illumina) with 150-bp paired-end sequencing of read 2.

Upstream scRNA-seq pipeline

Quality control was performed using the Scanpy package to ensure the integrity and accuracy of the single-cell RNA sequencing (scRNA-seq) data. The quality control steps focused on identifying and mitigating potential confounding factors, such as the presence of mitochondrial and ribosomal, which can indicate cellular stress or contamination. Identification of Mitochondrial and Ribosomal genes: Mitochondrial genes were identified using the prefix "mt-" for mouse genes. Ribosomal genes were identified using the prefixes "Rps" and

"Rpl". The `calculate_qc_metrics()` function from Scanpy was used to compute common QC metrics, including the percentage of counts attributed to mitochondrial and ribosomal. The metrics were calculated and stored in the AnnData object for subsequent analysis.

Cells were removed with greater than 10% mitochondrial gene content, less than 200 genes by counts as well as cells with less than 500 total counts.

To identify potential doublets, the scrublet tool was used utilising a nearest neighbour classifier of observed transcriptomes and simulated doublets. Predicted doublets were subsequently removed from the dataset.

Counts were normalised per cell by total counts over all genes so that every cell has the same total count after normalisation. Each cell is normalised to a total count equal to the median of total counts for cells before normalisation. The data are then logarithmised using log1p where each count is transformed to the natural log of 1 plus the original count value. This method allows for the data to be transformed as well as zero values.

PCA Nearest neighbour graph and UMAP visualisation.

Filtering lymphocytes and ambient genes.

Annotating the data with relevant cell type labels

Downstream scRNA-seq Pipeline

DEG analysis
KEGG pathway analysis
GO Term Analysis

Results

Discussion

Mitochondrial dysfunction

Abnormal nutrient intake represents a critical determinant in obesity, associated with a condition defined as “nutri-stress” where metabolic alterations induce an impaired heat shock proteins (HSPs) response, leading to mitochondrial damage, dysfunctional energy metabolism, high glucose levels, and IR [42]. Upon metabolic alterations, intestinal cells of prediabetic patients secrete higher levels of exosomal vesicles related to lipid metabolism and oxidative stress, compared to non-prediabetic subjects [43]. In obese patients, dysfunctional adipose tissue releases reactive oxygen species (ROS), inflammatory cytokines, and free fatty acids (FFAs), whose elevated plasmatic levels deter-

Alternative splicing

Proteasome Dysregulation

Peroxisome

Intestinal Permeability

Endoplasmic Reticulum Stress

RNA transport

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Appendix