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

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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.

Introduction

Type 2 Diabetes Mellitus (T2DM) has emerged as a global health crisis, with its prevalence quadrupling over the past three decades (Zheng et al., 2018). 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 (IFG), impaired glucose tolerance (IGT), or raised HbA1c levels (5.7 – 6.4%), and is closely associated with obesity, diet, 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 role of the intestinal epithelium in the development and progression of prediabetes. The intestinal epithelium, serving as the primary interface between the diet and internal biological systems, performs important functions in nutrient absorption, barrier protection, and hormone secretion. Alterations in the structure and function of this epithelium have been observed in prediabetic individuals, with evidence suggesting a dysregulation of the intestinal barrier and metabolism (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 composition, intestinal permeability, inflammation, macronutrient metabolism alterations, and intestinal stem cell (ISC) functions and are reviewed here in detail. Understanding these intricate relationships between the intestinal epithelium and metabolic dysfunction requires advanced research techniques which 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 reveal cell-specific, transcriptional responses to physiological changes.

In light of these technological advancements and the growing recognition of the intestinal epithelium's role in metabolic health, this research project aims to

employ scRNA-seq analysis to identify genes and signalling 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 reveal 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, a significantly underresearched area. 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.

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 component on the gut barrier consisting of four main phyla Proteobacteria, Bacteroidetes, 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), a key metabolite which plays 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 (Tan et al., 2014). 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 alterations in the microbiota composition can range from disease promotion to protection and is implicated in the progression of prediabetes and T2DM discussed in more detail later (Kosiewicz et al., 2011).

1.1.2 Mucus Layer

The mucus layer forms the second element of the intestinal barrier, facilitated by a layer of mucins. These are highly O-glycosylated proteins with gel-like properties secreted by goblet cells (Kim and Ho, 2010). This layer is responsible 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 (Johansson and Hansson, 2016). Lysozyme, secretory IgA, and defensins are secreted by Paneth cells are found in the inner mucus layer, providing a mechanism that helps keep bacteria away from the surface of enterocytes (Portincasa et al., 2021; Gibbins et al., 2015). Other antimicrobial molecules, such as REG3G, lypd8, and ZG16, further contribute to bacterial exclusion from the mucosa (Portincasa et al., 2021; Bergström et al., 2016). Contrastingly, 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, a key risk factor of prediabetes (Everard et al., 2013).

1.1.3 Intestinal Epithelium

The intestinal epithelium forms the main component of the intestinal barrier, consisting of a single layer of cells having roles in nutrient acquisition and thus roles in protection from the external environment. The structure of the epithelium differs between the small and large intestine. The small intestine contains structural protrusions, increasing the surface area for nutrient

absorption while the large intestine is flat, reducing the potential for damage from more solid material (Allaire et al., 2018). Furthermore, both the small and large intestine contain invaginations called ‘crypts’, harbouring proliferative intestinal stem cells (ISCs) important in the constant turnover of new intestinal epithelial cells (Allaire et al., 2018). The epithelial layer comprises six primary cell types, with each contributing to these roles. Enterocytes, the most abundant cell type, form the main absorptive surface and play a direct role in the immune response (Snoeck et al., 2005). ISCs as mentioned are involved in regenerating and producing new cells. Goblet cells, responsible for mucus production, contribute to the mucus layer as discussed previously. Tuft cells remain elusive in their functions although exhibit chemosensory functions and secrete effector molecules involved in innate immunity and play an important role in barrier maintenance (Silverman et al., 2024). Enteroendocrine cells are diverse secretory cells which produce hormones regulating nutrient absorption, intestinal barrier function and ISC homeostasis (Nwako and McCauley, 2024). Paneth cells, located in the intestinal crypts produce antimicrobial peptides and proteins to mediate host-microbe interactions and innate immunity, as well as factors that help sustain and modulate the ISCs (Clevers and Bevins, 2013).

The epithelial cells are interconnected by junctional complexes, including tight junctions (Tjs), adherens junctions (Ajs), and desmosomes. TJs are found at the top of the junction, establishes polarity and regulates permeability (Lessey et al., 2022). They comprise over 40 proteins, including claudins, occludin, and zonula occludens proteins (Portincasa et al., 2021). Directly below the TJs are the AJs playing a crucial role in cell-cell adhesion. Below the AJs are the desmosomes which strengthen the adhesion while withstanding mechanical stress (Lessey et al., 2022).

The epithelial barrier allows for both transcellular and paracellular transport of molecules. The paracellular and transcellular routes permit the passage of water, macromolecules, small hydrophilic compounds, lipids, and ions (Lessey et al., 2022). The permeability of this barrier varies along the intestinal tract, with the colonic epithelium being less permeable than the small intestinal epithelium

(Portincasa et al., 2021). Various factors can influence junction integrity, including diet, microbiota composition and inflammation which are discussed in detail later. Impaired intestinal barrier function has been observed in animal models of obesity, prediabetes and T2DM, and inflammatory bowel diseases.

The intestinal barrier's integrity and function can be significantly influenced by many factors, most notably, diet, microbiota, physical acitvity, and medication. These modifiers play crucial roles in maintaining or altering the intestinal barrier, potentially having roles in intestinal diseases and dysfunctions such as prediabetes.

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 recognised as potential factors in the development of metabolic disorders. Studies have consistently reported a reduction in microbial diversity and richness in individuals with prediabetes, mirroring observations in patients with established diabetes (Chang et al., 2024). 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 Bifidobacterium, Blautia, Clostridium, Faecalibacterium, Mediterraneibacter, Anaerostipes, and Butyricicoccus in prediabetic stool samples (Chang et al., 2024). 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 (Rathi et al., 2023). 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 (Everard et al., 2013; Shin et al., 2014; Plovier et al., 2017; Rathi et al., 2023).

Conversely, some bacterial genera have been found to be more abundant in prediabetic individuals. These include Ruminococcus, Dorea, Streptococcus, Sutterella as well as facultative anaerobes (Rathi et al., 2023; Piccolo et al., 2024). Additionally, increased abundances of Bacteroides, Parabacteroides, Phascolarctobacterium, and Paraprevotella have been observed in prediabetic fecal samples (Chang et al., 2024). The functional implications of these increases are not fully understood however and require further investigation.

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 understood. Factors such as diet, which accounts for nearly 60% of gut microbiota composition, play a significant role in shaping the microbial community (Zhang et al., 2010). This underscores the potential for dietary interventions or supplementation 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 thus its progression to T2DM.

1.2.2 Intestinal Barrier Permeability

Alterations in intestinal permeability play a crucial role in the pathogenesis of prediabetes and its progression to type 2 diabetes mellitus (T2DM). As previously discussed, the intestinal barrier and its various components are integral in regulating the passage of substances into the body. 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, critical components of the paracellular barrier (Nascimento et al., 2021).

Olivera et al. have shown that high-fat diet (HFD) intake, often associated with prediabetes, can lead to significant changes in intestinal permeability. In vitro models using Caco-2 cell lines have demonstrated that exposure to intestinal content from the small intestine of mice fed a HFD, can disrupt the tight junction-mediated epithelial barrier in cell culture models (Oliveira et al., 2019). This suggests that an element 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 (Nascimento et al., 2021). These findings highlight the potential importance of intestinal barrier dysfunction as an early event in the pathogenesis of prediabetes and metabolic disorders associated with high-fat diets. The prevailing theory suggests that alterations in the microbiota via a high-fat diet induces this increase in intestinal permeability and potentially leads to the translocation of bacteria and antigens leading to diabetic like disturbances such as insulin resistance (de Kort et al., 2011; Matheus et al., 2017). 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 clarified. Factors such as diet composition,

microbiota alterations, lifestyle factors and genetic susceptibility likely interact in complex ways to influence and progress the metabolic condition.

Future research should focus on further characterising 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 T2DM in the larger systemic context. Systemic inflammation in prediabetes is characterised by elevated levels of inflammatory markers and alterations in immune function (Weaver et al., 2021). Studies have reported increased levels of inflammatory proteins in prediabetic patients, including interleukin-6, interleukin-1β, tumor necrosis factor-α, monocyte chemoattractant protein-1, resistin, and C-reactive protein (CRP). The ratio of CRP to albumin is also elevated, indicating a shift towards a pro-inflammatory state (Colloca et al., 2024). Hypoteheses based on protein and gene analysis of pancreatic tissues and isolated islets suggest that inflammation in prediabetes may be initiated by a decrease in CD163+ cells leading to reduced anti-inflammatory protection and thus increased production of pro-inflammatory cytokines and resistin (Weaver et al., 2021). There are significant implications for inflammation in prediabetes. Inflammation during the prediabetic state seems to be a driving force behind pancreatic beta cell dysfunction and insulin resistance, dyslipidemia, and cardiovascular diseases and is a risk factor for peripheral cascular diseases (Saghir et al., 2023). Initiation of inflammation in the prediabetic subject is not fully understood, although research is suggesting that it may begin in the intestines.

In the context of intestinal inflammation in prediabetes, recent hypotheses are focussed on the relationship between diet, gut microbiota, and intestinal barrier function. One prevalent hypothesis suggests that high-fat diet intake leads to alterations in intestinal microbiota composition. These alterations are thought to increase paracellular permeability and absorption of LPS, dietary antigens, and translocation of bacteria leading to metabolic endotoxemia and low-grade chronic systemic inflammation, which may trigger or exacerbate peripheral insulin resistance (Geurts et al., 2014; Gomes et al., 2017; Nascimento et al., 2021).

However, conflicting evidence exists in the literature regarding the relationship between intestinal permeability, inflammation, and prediabetes development. While some studies report significant increases in intestinal permeability to large molecules, associated with endotoxemia and systemic inflammation, other research has found that increased intestinal TJ permeability in prediabetic mice occurs without significant changes in systemic and intestinal levels of zonulin, TNF-α, and LPS (Nascimento et al., 2021). These discrepancies may be partially explained by differences in prediaebetic 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 (Cani et al., 2008; Nascimento et al., 2021). Consistent with this are reports demonstrating that isocaloric diets can have varying diabetogenic and obesogenic effects based on their macronutrient composition, particularly the type of fat (polyunsaturated vs. saturated) and the presence of fructose, rather than just total calorie content (Deol et al., 2015).

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 characterising 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 Ppary 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 characterisation 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 reveal alterations at multiple biological levels, from genetic predisposition to changes in gene expression, protein function, and metabolic pathways.

Among these techniques, 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 Analysis Techniques

The term ‘pipeline’ can take on 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 variation of the transcriptomes of each cell can be compared. From here an unsupervised leiden clustering algorithm is employed to separate the cells into different groups, where each group represents a cluster of cells of similar transcriptomic features. With this clustered UMAP, annotations are applied to each cluster representing the cell type of that cluster.

2.1.2 Quality Control

Quality control (QC) is a critical initial step in scRNA-seq analysis, aimed at identifying and removing low-quality cells and genes that could skew downstream analyses. The process typically involves evaluating three main aspects of the data: the number of counts per cell (count depth), the number of

genes detected per cell, and the fraction of counts from mitochondrial genes per cell. Cells with exceptionally low count depths, few detected genes, or high fractions of mitochondrial counts are often considered damaged or dead and are removed from the dataset.

However, it's important to note that these metrics should be considered jointly, as deviations in one parameter may reflect specific biological conditions rather than technical artifacts. For instance, high mitochondrial counts could indicate cells with heavy respiratory activity, while low counts might represent quiescent cells. Therefore, QC thresholds should be set carefully to avoid unintentionally filtering out viable cell populations.

Advanced methods for doublet detection, such as scrublet, have been developed to identify and remove cell multiplets, which can confound downstream analyses. These methods can distinguish between "embedded" doublets (same cell type) and "neotypic" doublets (different cell types), improving the accuracy of cell type identification.

2.1.3 Normalisation

Normalisation is essential for making gene expression levels comparable between cells and addressing technical biases introduced during library preparation and sequencing. The most common approach is linear normalisation, such as counts per million (CPM), which scales the count data to obtain relative gene expression abundances. This method assumes that all cells initially contained an equal number of mRNA molecules, and differences in count depth arise solely due to sampling.

For datasets with strong batch effects, non-linear normalisation methods may be more appropriate, particularly for plate-based scRNA-seq data. These methods often employ parametric modeling to correlate technical and biological sources of variability and correct for both simultaneously.

In full-length sequencing protocols, methods that consider gene length, such as transcripts per million (TPM), are often preferred. Additionally, the use of unique molecular identifiers (UMIs) during library preparation can help correct for

amplification bias, providing a more accurate representation of absolute molecular counts.

2.1.4 Dimensionality Reduction

High-dimensional scRNA-seq data is often reduced to lower dimensions to facilitate visualisation and downstream analyses. Principal Component Analysis (PCA) is commonly used as an initial step to capture the main sources of variation in the data. However, PCA assumes linear relationships between features and may not fully capture the complex structure of scRNA-seq data.

Non-linear dimensionality reduction techniques, such as t-distributed Stochastic Neighbor Embedding (t-SNE) and Uniform Manifold Approximation and Projection (UMAP), are frequently employed for visualisation and clustering. These methods aim to preserve local similarities between cells in the high-dimensional space while projecting them into two or three dimensions. UMAP has gained popularity due to its ability to preserve both local and global structure, faster runtime, and higher reproducibility compared to t-SNE.

2.1.5 Clustering

Clustering is a crucial step in scRNA-seq analysis, aiming to group cells with similar gene expression profiles. Graph-based clustering methods, such as the Louvain and Leiden algorithms, have become popular due to their efficiency and ability to handle large datasets. These methods typically operate on a K-nearest neighbor (KNN) graph, where cells are represented as nodes connected to their most similar neighbors based on their gene expression profiles.

The Leiden algorithm, an improvement over the Louvain algorithm, guarantees that the identified communities are connected, addressing a major limitation of its predecessor. Clustering can be performed at multiple resolutions to explore the data at different levels of granularity, allowing for the identification of both broad cell types and finer subpopulations.

2.1.6 Annotation

Once clusters have been identified, the next step is to assign biological identities to these cell groups. This process, known as annotation, typically

involves identifying marker genes that characterise each cluster. Differential expression analysis between clusters is often used to identify these marker genes, with statistical tests such as the Wilcoxon rank-sum test or t-test employed to rank genes by their difference in expression.

Automated annotation methods, which compare cluster-specific marker genes to reference datasets or known cell type signatures, can expedite this process. However, manual curation by domain experts is often necessary to ensure accurate annotation, particularly for novel or rare cell types.

2.1.7 Differential Gene Expression Analysis

Differential gene expression (DGE) analysis in scRNA-seq aims to identify genes that are differentially expressed between conditions or cell types. This analysis must account for the unique characteristics of scRNA-seq data, including high sparsity, technical noise, and complex experimental designs involving multiple subjects and conditions.

Various methods have been developed for scRNA-seq DGE analysis, including adaptations of bulk RNA-seq methods (e.g., edgeR, DESeq2) and specialized single-cell methods (e.g., MAST, NEBULA). These methods differ in their underlying statistical models and how they handle the sparsity and overdispersion typical of scRNA-seq data.

2.1.8 Gene Set Enrichment and Enrichment Map

Gene set enrichment analysis helps interpret large gene lists by identifying functionally coherent gene sets (e.g., KEGG pathways, biological processes) that are statistically overrepresented. However, as gene set collections grow larger and more complex, enrichment results can become difficult to interpret due to redundancy between sets.

Enrichment Map addresses this challenge by organising enriched gene sets into a network visualisation. In this network, nodes represent gene sets, and edges represent the overlap between sets. This approach clusters related gene sets, making it easier to identify major functional themes in the data. Enrichment Map can be particularly useful for comparing enrichment results across multiple

conditions or experiments, providing a more intuitive and comprehensive view of the biological processes involved.

In conclusion, the scRNA-seq analysis workflow comprises several interconnected steps, each addressing specific challenges posed by single-cell data. As the field continues to evolve, new methods and tools are being developed to improve the accuracy and interpretability of scRNA-seq analyses, furthering our understanding of cellular heterogeneity and function.

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 use high-carbohydrate, high-sucrose, high-fructose, or high-fat diets (HFD). HFDs, with fat concentrations ranging from 20% to 60%, are frequently uesd 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 remodelling [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 utilisation 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 prediabetic 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.

The intestine is a heavily understudied area of prediabetes lacking in an in depth understanding of the specific dysfunctions experienced during prediabetes as well as a lack of in vitro models of the prediabetic gut. Understanding these dysfunctions are critical in producing pharmacological agents, new dietary advice, as well as discovering effective natural supplements which aid in ameliorating the prediabetic gut. Currently there are a selection of drugs and supplements which aid in the prevention of prediabetes. Here we review some of the major players and how they may aid in the dysfunctions of the prediabetic gut as discussed previously, as well as potential gaps or dysfunctions which may present themselves as targets for new drugs/supplements.

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, a biguanide compound, is the first-line pharmacological intervention for prediabetes and type 2 diabetes mellitus (T2DM) management. Its primary mechanism involves reducing hepatic glucose production and improving insulin

sensitivity. Recent research has revealed that metformin's effects extend beyond glucose metabolism, particularly in relation to the intestinal environment.

Metformin significantly alters the gut microbiota composition, with studies reporting increased abundance of Akkermansia muciniphila and various Lactobacillus species. These changes are associated with improved metabolic parameters, suggesting that microbiome modulation may contribute to metformin's therapeutic effects. The drug's impact on microbial communities varies along the gastrointestinal tract, with more pronounced effects observed in the upper small intestine.

Regarding intestinal permeability, metformin has demonstrated protective effects on the intestinal barrier. It enhances the expression of tight junction proteins such as zonulin-1 and occludin, thereby reducing paracellular permeability. This action may be partly mediated through increased mucin production stimulated by the drug's effects on goblet cells and A. muciniphila abundance.

Metformin exhibits anti-inflammatory properties in the intestinal environment. It suppresses the TNF-α-induced NF-κB pathway and attenuates both acute and chronic colitis in animal models. These effects may contribute to the overall improvement in metabolic health by reducing low-grade systemic inflammation associated with prediabetes.

In terms of macronutrient metabolism, metformin influences several pathways in intestinal epithelial cells. It promotes glucose utilization through glycolysis and increases lactate production, creating a futile intestinal-liver cycle that may contribute to its glucose-lowering effects. Additionally, metformin enhances fatty acid oxidation in intestinal cells at pharmacological concentrations, potentially altering lipid metabolism and absorption.

Emerging evidence suggests that metformin affects intestinal stem cell (ISC) function, although this area requires further investigation. Preliminary studies indicate that metformin may suppress Wnt signaling, a critical pathway in ISC differentiation. This effect could influence the balance of cell types within the

intestinal epithelium, potentially impacting nutrient absorption and barrier function.

These diverse effects of metformin on the intestinal environment highlight its multifaceted role in prediabetes management, extending beyond its classical action on hepatic glucose production. Further research into these mechanisms may reveal new therapeutic targets and strategies for metabolic disease prevention and treatment.

3.2 Emerging Therapies: Focus on Brown Seaweed Extract

Brown seaweed, classified under the phylum Phaeophyta, represents a rich source of bioactive compounds, including polysaccharides, polyphenols, proteins, and polyunsaturated fatty acids. These marine organisms have garnered significant attention in recent years due to their potential therapeutic applications, particularly in the context of metabolic disorders such as prediabetes and type 2 diabetes mellitus (T2DM). Among the various seaweed species, Ascophyllum nodosum and Fucus vesiculosus have demonstrated notable benefits in metabolic health studies.

The impact of brown seaweed extract on gut microbiota has been a focus of recent research. Studies have shown that brown seaweed polysaccharides (BSPs) can modulate the composition of intestinal microbial communities. Specifically, dietary supplementation with fucoidan, a sulfated polysaccharide from brown seaweed, has been associated with increased relative abundances of beneficial bacteria such as Akkermansia muciniphila, Alloprevotella, Blautia, and Bacteroides in animal models. A. muciniphila, in particular, has gained attention as a novel probiotic species linked to improved metabolic health. The prebiotic effects of BSPs are attributed to their ability to reach the large intestine largely undigested, where they can selectively stimulate the growth of beneficial microorganisms.

Regarding intestinal permeability, certain brown seaweed extracts have demonstrated the ability to enhance barrier function. In inflammation-induced Caco-2 cell models, extracts from species such as Saccharina japonica and Hizikia fusiforme significantly increased transepithelial electrical resistance (TEER) values and decreased permeability. These effects were associated with reduced expression of pro-inflammatory markers and increased expression of tight junction proteins. Similar beneficial effects on colon health indices were observed in dextran sulfate sodium (DSS)-induced colitis mouse models, suggesting potential applications in managing inflammatory bowel conditions and associated intestinal barrier dysfunction.

The anti-inflammatory properties of brown seaweed extracts have been documented in various studies, although the mechanisms are not fully elucidated. Some species have shown the ability to reduce the expression of pro-inflammatory cytokines such as TNF-α and the inducible nitric oxide synthase (iNOS) in intestinal epithelial cells. These anti-inflammatory effects may contribute to the overall improvement in intestinal barrier function and metabolic health.

In terms of macronutrient metabolism, brown seaweed extracts have demonstrated significant inhibitory effects on carbohydrate digestive enzymes, particularly α-glucosidases. Extracts from species like A. nodosum have shown potent inhibition of both maltase and sucrase activities, suggesting a potential role in reducing postprandial glucose excursions. The inhibitory potency and specificity vary among different seaweed species and are influenced by factors such as extraction methods and molecular weight distribution of the bioactive compounds.

While the effects of brown seaweed extract on intestinal stem cell (ISC) function have not been extensively studied, the observed impacts on intestinal barrier function, inflammation, and microbiota composition suggest potential indirect

effects on the intestinal epithelium renewal and homeostasis. However, further research is needed to elucidate any direct effects on ISC function.

The diverse effects of brown seaweed extracts on intestinal health parameters highlight their potential as a natural intervention for prediabetes management. However, it is important to note that the efficacy can vary significantly between species and extraction methods. Further research, particularly well-designed clinical trials, is needed to fully elucidate the mechanisms of action and establish optimal formulations for therapeutic use in prediabetic individuals.

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