Jejunal control of glucose homeostasis in the human body

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Immunofluorescence staining on the cover shows ketogenesis rate-limiting enzyme HMGCS2 (green) and nuclei (blue) in jejunal *villus* of an obese individual subjected to RYGB surgery.

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“You should always go to other people’s funerals.
Otherwise, they won’t come to yours.”

Yogi Berra (1925-2015), catcher New York Yankees

To Cornelia, Kasper, Fanny, Melvin and Leo

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ABSTRACT

Background: The prevalence of obesity and type-2 diabetes mellitus (T2DM) have risen dramatically over the last decades, and are major threats to the human health. Obesity, and also T2DM, is treated with lifestyle modifications, medications or by bariatric surgery, with varying success. Recent findings demonstrate that the improved glucose homeostasis following bariatric surgery is partially weight-loss independent. Improvements in glucose homeostasis and incretin secretion profiles are seen already before the patients leave the operating hospital after Roux-en-Y gastric bypass (RYGB) surgery. Therefore, it has been speculated that this improvement is induced by the anatomical reconfiguration of the gut. The exact mechanism for this has been elusive. The overall aim of this thesis was to investigate how jejunum, and the expression of different proteins in the jejunum, helps regulate glucose homeostasis in the human body.

Methods: In Paper I, global proteomics was used on jejunal mucosa biopsies in patients undergoing RYGB surgery, and biopsies retrieved from the Roux limb 6-8 months after surgery to search for major regulations in the proteome. The biological functions of the proteomics findings were further studied *in vivo* in mice and *in vitro* in murine primary jejunal enteroendocrine cells (EECs). In Paper II and III, the effect of two weeks of iso-caloric high-fat diet (HFD) and high-carbohydrate diet (HCD) were assessed in healthy and normal weight volunteers in a cross-over design. For Paper II, a mixed meal test (MMT) with sequential blood sampling was performed at end of each dietary period to examine the glucose homeostasis. Metabolomics was also used to explore the effect of each dietary period on metabolite profiles. For Paper III, jejunal mucosa biopsies were retrieved following each dietary period, and the protein expression was assessed with western blot while functional characteristics of glucose transport were evaluated with Ussing chambers. The findings from the biopsy material were further studied *in vitro* in Caco-2 cells. In Paper IV, *in vitro* cultures of murine GLUTag cells and differentiated human jejunal

enteroid monolayers were used to study the effect of the ketone body β-hydroxybutyrate (βHB) on glucose-induced GLP-1 secretion.

Results: From global proteomics analysis, it was shown that the ketogenesis rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) is drastically down-regulated in jejunum after RYGB. Furthermore, prolonged HFD in mice increased the jejunal expression of the same enzyme. In the diet study, MMT resulted in similar glucose and insulin profiles, while the secreted levels of GLP-1 were higher after HFD. The HFD also increased the levels of several metabolites, *i.e.* valine, leucine and creatine, that has previously been shown to be indicators of early insulin resistance. Evaluation of the jejunal mucosa revealed that HFD decreased the expression of sodium-glucose linked transporter 1 (SGLT1) and the acetylation of histone 3 at lysine 9, while the expression of HMGCS2 was increased. *In vitro* studies in Caco-2 cells stipulate a sirtuin dependent regulation of SGLT1 expression, induced by jejunal ketogenesis. The ketone body βHB had significant inhibitory effect on glucose-induced GLP-1 secretion in murine primary jejunal EECs, in GLUTag cells and in differentiated human jejunal enteroid monolayers. Protein expression analysis displayed an increased phosphorylation of kinase Akt as well as expression of kinase ERK1/2 after addition of βHB to GLUTag cells.

Conclusions: The results from this doctoral thesis display an interesting role of intestinal ketogenesis. The rate-limiting enzyme of ketogenesis HMGCS2, and therefore intestinal ketogenesis, is induced by a prolonged fat-dominated diet, and is almost completely abolished following RYGB surgery. The results also display a capacity of the jejunal ketogenesis to influence glucose homeostasis both by inhibition of GLP-1 secretion from EECs and by regulation of jejunal glucose transporters.

Keywords: glucose homeostasis, ketogenesis, jejunum

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SAMMANFATTNING PÅ SVENSKA

I mag-tarmkanalen sker kroppens första kontakt med nyintagen föda. Under sin väg genom mag-tarmkanalen utsätts födan för både mekaniska och kemiska krafter som bryter ned den till mindre komponenter. Dessa kan absorberas och senare användas till olika biologiska processer i kroppen. De makromolekyler kroppen främst utnyttjar är proteiner, kolhydrater och fetter. Den största delen av denna absorption sker i tunntarmen som utgör över 90% av den totala slemhinneytan i mag-tarmkanalen. Den del av mag-tarmkanalen där det mesta av födoämnesupptaget sker är i jejunum, d.v.s. den mellersta delen av tunntarmen.

Fetma, och även typ-2 diabetes som innebär försämrad blodsockerreglering, har ökat dramatiskt världen över de senaste decennierna. Dessa sjukdomar behandlas med livsstilsförändringar och mediciner, eller genom fetma-reducerande kirurgi. Nya fynd visar att den förbättrade blodsockerregleringen som uppnås genom fetma-reducerande kirurgi är delvis viktoberoende och sker redan innan patienterna lämnat sjukhuset efter operation. Det har spekulerats i att denna förbättring beror på den omkoppling av mag-tarmkanalen som det kirurgiska ingreppet innebär. Jejunum utgör den del av mag-tarmkanalen som påverkas mest vid den fetma-reducerande kirurgiska operationen Roux-en-Y gastrisk bypass. Vid denna operation kopplas den största delen av magsäcken och den första delen av tunntarmen bort från födans väg och maten går i stället direkt från matstrupen till jejunum via en liten kvarlämnad magsäcksficka.

I detta avhandlingsarbete var vi intresserade av hur jejunum, och uttrycket av olika proteiner i jejunum, hjälper till att reglera glukosnivåerna i kroppen, dels beroende av mängden kolhydrater och fett i dieten hos friska frivilliga, dels vid kirurgi-inducerade förändringar hos överviktiga. De viktigaste frågeställningarna var:

- Hur förändras proteinuttrycket i jejunum med Roux-en-Y gastrisk bypass? (delarbete I)
- Vilka ämnesomsättningsförändringar sker vid fett- respektive kolhydratdominerad diet hos friska frivilliga? (delarbete II)
- Hur förändras uttrycket av sockertransportörer i jejunal-slemhinnan vid fett- respektive kolhydratdominerad diet hos friska frivilliga? (delarbete III)
- Kan slemhinneproducerade ketonkroppar i jejunum reglera utsöndringen av mättnadshormoner från tarmens hormonproducerande celler? (delarbete IV)

För att besvara dessa frågor användes biopsimaterial från jejunum hos patienter som opererades med Roux-en-Y gastrisk bypass, samt 6 till 8 månader efter operationen. Dessutom utfördes en studie på friska frivilliga som under två veckor fick en individanpassad diet med, i slumpmässig ordning, högt innehåll av kolhydrater respektive fett. I slutet av varje dietperiod utfördes ett måltidstest med ett standardiserat mål mat följt av kontinuerlig blodprovsinsamling. Efter ytterligare en dag genomfördes en enteroskopi med provtagning från jejunum. Förutom dessa humana vävnadsprover användes också musmodeller samt cellodlingsmodeller. Det insamlade materialet analyserades med proteomik, metabolomik, western blot, immunofluorescence samt ELISA-liknande analyser.

Arbetena visar att det enzym som är hastighetsbegränsande för ketonkroppsbildningen ökar i jejunum av fet kost, samt kraftigt minskar i jejunum efter en Roux-en-Y gastrisk bypassoperation. Vi såg också att en fettdominerad kost hos friska frivilliga gav upphov till en ökad utsöndring av mättnadshormoner. Samtidigt ökade nivåerna av metaboliter som anses vara tidiga markörer för insulinresistens. Uttrycket av sockertransportörer, främst SGLT1, nedreglerades vid fettdominerad diet. Genom att studera sockertransportören SGLT1 i cellmodeller kunde vi se att SGLT1 minskar genom en epigenetisk process som är beroende av ketonkroppsbildningen i jejunum. Studier i cellmodeller visade också att ketonkroppar direkt hämmar GLP-1-utsöndring från tunntarmens hormonproducerande celler.

Sammanfattningsvis visar våra studier att jejunum anpassar sig till förändringar i dieten och likaså efter Roux-en-Y gastrisk bypasskirurgi. Detta kan i sin tur ge viktiga insikter om uppkomst och behandling av till exempel typ-2 diabetes. Hämmad ketonkroppsbildning i jejunum efter Roux-en-Y gastrisk bypassoperation kan vara en ny mekanism som genom ökad GLP-1-produktion kan bidra till operationens viktoberoende effekt mot typ-2 diabetes. Ketonkroppsbildningen i jejunum kan också genom epigenetiska förändringar styra uttrycket av sockertransportörer i jejunum, vilket skulle kunna vara en delförklaring till uppkomsten av typ-2 diabetes.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. Wallenius V, Elias E, **Elebring E**, Haisma B, Casselbrant A, Larraufie P, Spak E, Reimann F, le Roux CW, Docherty NG, Gribble FM, Fändriks L. *Suppression of enteroendocrine cell glucagon-like peptide (GLP)-1 release by fat-induced small intestinal ketogenesis: a mechanism targeted by Roux-en-Y gastric bypass surgery but not by preoperative very-low-calorie diet.*
Gut 2020; 69(8): 1423-1431.

II. Wallenius V[#], **Elebring E**[#], Casselbrant A[#], Laurenius A, le Roux CW, Docherty NG, Biörserud C, Björnfot N, Engström M, Marschall HU, Fändriks L. *Glycemic control and metabolic adaptation in response to high-fat versus high-carbohydrate diets – data from a randomized cross-over study in healthy subjects.*
Nutrients 2021; 13(10): 3322.
[#]Shared first authorship.

III. **Elebring E**[#], Wallenius V[#], Casselbrant A[#], Docherty NG, le Roux CW, Marschall HU, Fändriks L. *A fatty diet induces a jejunal ketogenesis which inhibits local SGLT1-based glucose transport – results from a randomized cross-over study between iso-caloric high-fat versus high-carbohydrate diets in healthy volunteers.*
Manuscript.
[#]Shared first authorship.

IV. **Elebring E**, Casselbrant A, Fändriks L, Wallenius V. *The ketone body β-hydroxybutyrate inhibits glucagon-like peptide-1 (GLP-1) secretion through a G-protein coupled receptor mediated mechanism in GLUTag and human enteroid cells.*
Manuscript.

CONTENT

ABBREVIATIONS

Akt	Protein kinase B
ALI	Air-liquid interface
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
βHB	β-hydroxybutyrate
BMI	Body mass index
CPT1A	Carnitine palmitoyltransferase 1 A
DAPI	4′,6-diamidino-2-phenylindole
DGAT1	Diglyceride acyltransferase 1
DMEM	Dulbecco's modified eagle medium
DPP-4	Dipeptidyl peptidase-4
ECLIA	Electrochemiluminescence immunoassay
EEC	Enteroendocrine cell
ELISA	Enzyme-linked immunosorbent assay
ERKs	Extracellular signal-regulated kinases
FATP4	Fatty acid transport protein 4
FBS	Fetal bovine serum
FFAR	Free fatty acid receptor
fsk	Forskolin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GPCR	G-protein coupled receptor
GPR	G-protein coupled receptor
GTP	Guanosine triphosphate

H3K9	Lysine 9 at histone 3
H3K9ac	Acetylation of lysine 9 at histone 3
HCD	High-carbohydrate diet
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High-fat diet
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
IBMX	3-isobutyl-1-methylxanthine
ITS	Insulin-transferrin-selenium
LFD	Low-fat diet
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
MMT	Mixed meal test
NEAA	Non-essential amino acids
NAD	Nicotinamide adenine dinucleotide
PEST	Penicillin/streptomycin
PPAR	Peroxisome proliferator-activated receptor
PTX	Pertussis toxin
RYGB	Roux-en-Y gastric bypass
SCOT	Succinyl-CoA-3-oxaloacid CoA transferase
SF	Serum free
SG	Sleeve gastrectomy
SGLT	Sodium-glucose linked transporter
T1DM	Type-1 diabetes mellitus
T2DM	Type-2 diabetes mellitus
VLCD	Very low calorie diet
WB	Western blot
WHO	World Health Organization
Wnt	Wingless/integrated

DEFINITIONS IN SHORT

Caco-2	Immortalized cell line of human colorectal adenocarcinoma cells used as a model for small intestinal epithelial cells
GLUTag	Immortalized cell line of differentiated murine enteroendocrine cells.
Human jejunal enteroids	Cell culture established from stem cells isolated from crypts of human jejunum. When cultured in Wnt3a free media, cells will differentiate spontaneously into the different cell types of the jejunal mucosa.

1 INTRODUCTION

This thesis investigates the role of the jejunum in the control of glucose homeostasis in the human body. Obesity and its associated metabolic diseases, foremost type-2 diabetes mellitus (T2DM), are major threats to the human health. These have been treated with lifestyle changes, medications or by bariatric surgery with varying success. Recent findings demonstrate that the improved glucose homeostasis achieved by bariatric surgery is partially weight-loss independent. An improvement in glucose homeostasis is seen already before the patients leave the hospital after surgery. Therefore, it has been speculated that this improvement is induced by the anatomical reconfiguration of the gut by the surgical procedure *per se*. The exact mechanism for this has been elusive. The aim of the present thesis was to explore how the jejunum, and functional aspects of the jejunum, control glucose homeostasis in the human body.

1.1 BASIC GLUCOSE HOMEOSTASIS

In order for the body to function properly, control of blood glucose level is essential. Glucose homeostasis is achieved through an interplay between the pancreas, brain, gut, liver, muscle tissue and adipose tissue [1, 2]. In this interplay, the pancreas is pivotal. The endocrine cells, α- and β-cells, of the pancreatic islets secrete the hormones glucagon and insulin, respectively [3]. Insulin lowers blood glucose levels, while glucagon has the opposite effect [1]. Insulin secretion from the pancreas is induced by increasing levels of blood glucose [4]. Insulin then stimulate glucose uptake in muscle and adipose tissue, lowering blood glucose levels [5]. Insulin also stimulates glycogenesis and inhibits gluconeogenesis in the liver [1]. Glucagon, on the other hand, is secreted from pancreas when blood glucose levels are low to promote glycogenolysis and gluconeogenesis in the liver. A schematic overview of the basic interplay to achieve glucose homeostasis can be seen in Figure 1.

As hormones secreted from the pancreas influence functions of other tissues, several hormones, neurotransmitters and cytokines secreted from various tissues also influence pancreatic function [1]. For example leptin, a hormone secreted by adipocytes, has been shown to reduce insulin secretion from the pancreas [6]. Hormones secreted from the gut that have the ability to influence insulin and glucagon secretion are called incretins [1]. As discussed more in detail later in this introduction, incretins are secreted from enteroendocrine

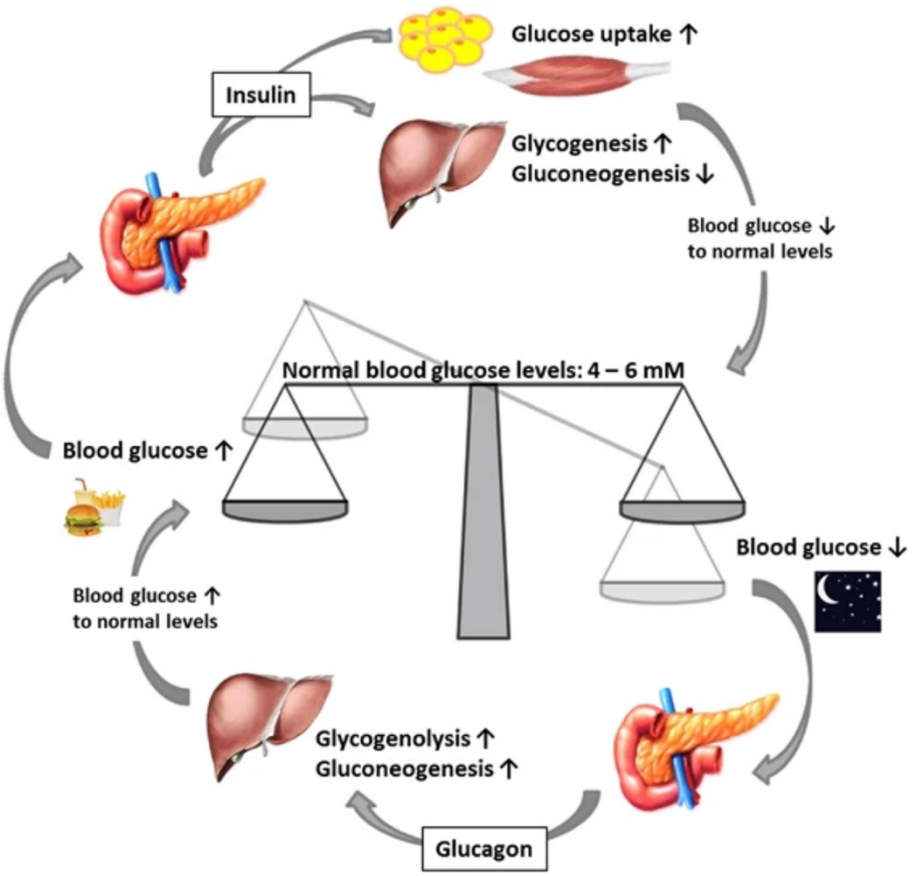

Figure 1. *Glucose homeostasis interplay. At increased levels of blood glucose, insulin is secreted from the pancreas to increase uptake of blood glucose by adipose and muscular tissue and to increase storage of carbohydrates in the liver. At decreased levels of blood glucose, glucagon is secreted from the pancreas to increase release of stored carbohydrates in the liver. Image reprinted under CC-BY-NC-ND license from Röder, et al., Pancreatic regulation of glucose homeostasis. Exp Mol Med 48(3), 2016.*

cells (EECs) in the gut upon stimulation by nutrients like carbohydrates and free fatty acids.

1.2 METABOLIC DISEASES

The metabolic syndrome, a term first introduced in second part of the 20th century, is the clustering of medical conditions obesity, dyslipidemia, hyperglycemia and hypertension together [7]. Metabolic diseases, mainly obesity and T2DM, are increasing health problems worldwide. Already at the

last millennial shift, obesity was specifically recognized by the World Health Organization (WHO) as an escalating disease [8]. Since then, during the past decades, obesity has still more than doubled in many countries and is now considered a global pandemic [9, 10]. In 2019, the prevalence of diabetes mellitus was estimated to be almost 10% of the adult population in the world and the numbers are projected to continue to increase [11]. T2DM is estimated to account for almost 90% of the total cases of diabetes mellitus.

1.2.1 OBESITY AND OVERWEIGHT

According to convention used by WHO, an individual with body mass index (BMI) of 30 kg/m^2 is defined as obese [12]. Overweight is defined as a BMI between 25.0 and 29.9 kg/ m^2 [13]. The dogma of obesity is an imbalance between ingested energy and expended energy [9]. Through evolution, humans have had to live through long periods of undernutrition and therefore individuals with good ability to store energy and with low energy expenditure had favorable characteristics. Over the last centuries, periods of undernutrition have become rare and instead overnutrition has become a problem. With overnutrition, the genotypes favored by evolution in times of famine are instead at risk of developing obesity and obesity associated comorbidities [14, 15].

Obesity increases the risk of several other metabolic diseases besides T2DM, *i.e.* hypertension and cardiovascular disease, non-alcoholic fatty liver disease and different kinds of cancers [16, 17].

1.2.2 DIABETES AND IMPAIRED GLUCOSE TOLERANCE

There are three different main types of diabetes mellitus: type-1 diabetes mellitus (T1DM), T2DM and gestational diabetes mellitus [11]. Diabetes mellitus is the result of a disturbance in the glucose homeostasis [1]. T1DM is characterized by the disrupted synthesis of insulin, while T2DM is characterized by inefficient response to insulin [18]. Gestational diabetes is elevated levels of blood glucose during pregnancy without a history of T1DM or T2DM. Only T2DM is clearly associated with obesity, and will therefore be the main focus of this thesis. The relative risk of developing T2DM for an obese subject was reported to be seven times higher compared to a normal weight subject [19]. For overweight subjects the same relative risk increased almost three times.

Impaired glucose tolerance is defined as an intermediate state between normal glucose homeostasis and diabetes mellitus [20]. With time, and without any

intervention, the majority of subjects with impaired glucose tolerance will develop T2DM.

1.2.3 OBESITY AND DIABETES MELLITUS IN THE WORLD

Metabolic diseases are a great burden in the world today. In 2014, the annual economic burden of obesity was estimated to $2.0 trillion [21]. In the same year, the annual direct cost of diabetes in the world was estimated to $850 billion [22]. These numbers are not estimated to decrease within the near future. Instead, the prevalence of both obesity and diabetes are projected to increase [9-11]. From 1975 to 2016, the mean BMI increased worldwide for both adults and children [23]. During this time period, the prevalence of obesity also increased globally for both adults and children. Today, almost half a billion adults are estimated to live with diabetes mellitus, and the numbers have increased with more than 60% the last 10 years [11]. Apart from almost 10% having T2DM, another 7.5% of the adults in the world were 2019 estimated to have impaired glucose tolerance. Also these numbers are estimated to increase over the following decades. Until 2045, the number of adults with diabetes mellitus is projected to increase to 700 million.

1.3 CURRENT THERAPIES FOR OBESITY AND T2DM

The current therapies for obesity and T2DM include lifestyle modifications, pharmacotherapies and obesity surgery.

1.3.1 LIFESTYLE MODIFICATIONS

Lifestyle modifications for treatment of obesity and T2DM include dietary adaptions and an increased level of physical activity [24]. Lifestyle modification programs generally result in, at best, a 10% initial weight-loss the first 16-26 weeks of treatment [25]. The dietary modifications can include meal replacement with restricted calorie content, portion-controlled servings of normal food, low-carbohydrate high-fat diets and diets with low glycemic index. Dietary changes have been reported to result in improvements of diabetes-related parameters [26, 27]. Physical activity interventions include increased planned activity or increased lifestyle activity [24]. The moderate physical activity of walking has been reported to, in combination with dietary change, result in both greater weight reduction and better glucose homeostasis, compared to dietary changes alone [28]. Alone, increased physical activity has

been reported to result in less weight-loss compared to increased physical activity in combination with dietary adaptions [29].

1.3.2 PHARMACOTHERAPIES

The pharmacotherapies for obesity and T2DM are represented with a broad range of drugs with different mechanisms of action.

For obesity drugs, the most common target is either neural or hormonal pathways controlling food intake and satiety in the brain [30]. These include drugs increasing anorexigenic signaling, and also incretin receptor agonists. Incretin receptor agonists have been shown to not only have glucose lowering effects, but also for other obesity related comorbidities [31]. Further, analogous of the adipocyte-produced satiety-inducing hormone leptin have been tried for the treatment of obesity but have proven to not have much impact on weight in obese subjects since that seems to have developed a leptin resistance [32]. Apart from neural and hormonal drugs, obesity pharmacotherapies also include drugs that inhibit fat absorption. Orlistat, an inhibitor of gastric and pancreatic lipases, has also been shown to have positive effects on patients with T2DM via an unknown mechanism [33].

Pharmacotherapies for T2DM include drugs that target hepatic glucose production, insulin secretion from β-cells, intestinal absorption of carbohydrates, insulin sensitivity and renal reabsorption of glucose [34, 35]. Also more modern drugs, like incretin analogous and DPP-4 (dipeptidyl peptidase-4) inhibitors are used for treatment of T2DM [36]. Metformin is the most commonly prescribed drug against T2DM and is recommended as the optimal first-line drug [37].

1.3.3 BARIATRIC SURGERY

Bariatric surgery, or obesity surgery, is the most successful long-term therapy for obesity and is considered when other therapies have failed [38]. The idea of surgery for the treatment of obesity emerged in the middle of the last century [39]. The initial surgical procedures were designed to induce nutrient malabsorption [40]. Later these procedures were combined with surgical techniques that also resulted in volumetric restriction of the stomach capacity. As of now, it is believed that today's dominating bariatric surgery procedures, Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG), induce weight loss through mechanisms that are not mainly dependent on malabsorption or restriction [40, 41]. Bariatric surgery results in greater weight loss compared to non-surgical procedures, and is the most effective treatment

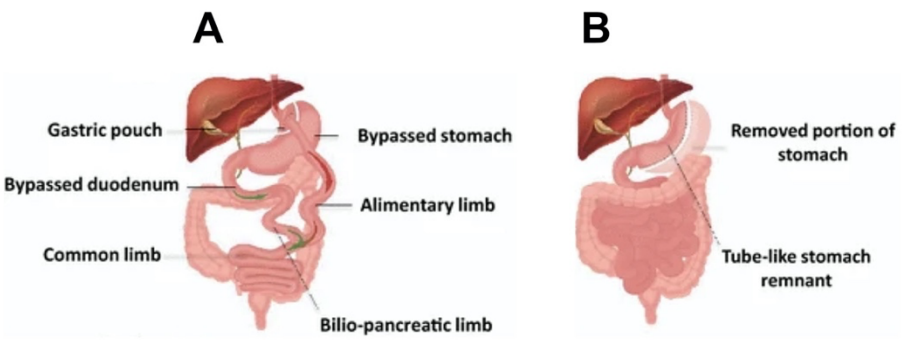

Figure 2. *Schematic representation of RYGB (A) and SG (B). In RYGB, a small gastric pouch is created and connected to the Roux limb constructed of the original jejunum. Ingested food bypass the stomach and the duodenum. Bile acids and pancreatic secretions mix with nutrients in the common limb. In SG, the stomach is removed along the greater curvature, creating a tube-like new stomach. Image adopted under CC-BY license from Pucci et al., Mechanisms underlying the weight loss effects of RYGB and SG: similar, yet different. J Endocrinol Invest 42(2), 2019.*

for individuals with severe obesity, also in the long-term [42, 43]. Schematic representations RYGB and SG are presented in Figure 2.

With RYGB, the stomach is divided into a small gastric pouch of about 20-30 ml, which is connected to the mid-jejunum, creating the so-called Roux limb or alimentary limb [40]. Ingested nutrients pass through the Roux limb and therefore completely bypass a major part of the stomach and the duodenum. Bile acids and pancreatic juices are mixed with the ingested food in the so-called common limb where the Roux limb is connected back to the jejunum just distal to the ligament of Treitz. In SG, a large portion (about 2/3) of the stomach is removed along the greater curvature, resulting in a tube-like stomach remnant that does not function as a food reservoir and thus ingested food reaches the duodenum faster.

The complete mechanism for body weight reduction with these bariatric procedures is still not clear, but much evidence points towards the beneficial effects on weight loss not being mainly through malabsorption or volumetric restriction alone [44, 45]. Instead, it is believed that the reduced energy intake after bariatric surgery is due to reduced hunger and reduced desire for food because of an increased satiety-signaling from the gut in response to food ingestion [40]. Since food intake is decided by the brain, it is believed that the different changes induced by bariatric surgery are ultimately affecting hypothalamic satiety signaling [46]. It has also been reported that RYGB results in increased postprandial energy expenditure [47, 48].

The weight reducing of effect of RYGB and SG have been reported to be fairly similar, with a slight edge for the former [49]. RYGB has been reported to result in a subtly higher degree of resolution of obesity-related comorbidities, while SG may be somewhat safer with fewer complications [50].

Apart from reducing body weight, bariatric surgery procedures have demonstrated effects on hyperglycemia and remission of T2DM [51]. The long-term effect on T2DM was greater with bariatric surgery compared to medical treatment. The effects of RYGB on glucose homeostasis will be discussed more in detail later in this introduction.

1.4 SMALL INTESTINE AND ITS ROLE IN GLUCOSE HOMEOSTASIS

The small intestine facilitates digestion and absorption of nutrients from food, and is essential for the digestive system [52-55]. The area of the small intestine is large, allowing for much of the nutrient absorption to occur [56].

1.4.1 ANATOMY AND PHYSIOLOGY OF SMALL INTESTINE

The small intestine constitutes of three distinct parts: duodenum, jejunum and ileum [52, 53]. The inside surface of the small intestine has *villi*, which increase the luminal surface area and therefore also the absorption potential. The enterocytes also have *microvilli* making the surface area even greater. In duodenum, digestive enzymes from pancreas and bile acids are entered. These factors aid in digestion of ingested nutrients. The main function of the jejunum is to absorb carbohydrates, lipids and proteins. In the ileum, much of the remaining nutrients are absorbed, particularly vitamin B12 and bile acids.

Carbohydrates are ingested in the form of monosaccharides, disaccharides or longer poly-saccharides [54, 55]. Carbohydrates can only be absorbed in the form of monosaccharides and more complex carbohydrates must therefore be enzymatically digested before absorption can occur. Digestion of complex carbohydrates is carried out by amylase secreted in the mouth and with the pancreatic juice. Absorption is carried out by enterocytes of the epithelial layer through specialized transporters. Glucose and galactose are both absorbed in cotransport with sodium by the sodium-glucose linked transporter 1 (SGLT1). This transporter utilizes a sodium gradient generated by an energy dependent ion channel. Fructose, on the other hand, is absorbed through glucose transporter 5 (GLUT5) via diffusion, and can therefore not be transferred against the concentration gradient. All these monosaccharides are further

transported into the blood circulation through GLUT2 on the basolateral side. Also lipids and proteins are absorbed by enterocytes in the intestinal epithelium. Lipids are digested with lipases, produced either in the stomach or in the pancreas. In an almost similar way, the proteins are digested with proteases secreted from the pancreas and activated at the surface of the intestine.

Besides the nutrient absorbing enterocytes, the small intestinal *villus* also consists of two other specialized cell types: mucous-secreting goblet cells and hormone-secreting EECs [57]. In the intestinal crypts in between the *villi*, stem cells reside along with Paneth cells. The stem cells of the crypts divide continuously before they move along the crypt-*villus* axis and terminally differentiate into the different specialized cell types. The turnover rate of the intestinal epithelium is high, with a complete renewal of cells every 4-5 days. Paneth cells function in immunity and antibacterial defense. A schematic representation of the small intestinal epithelium can be seen in Figure 3.

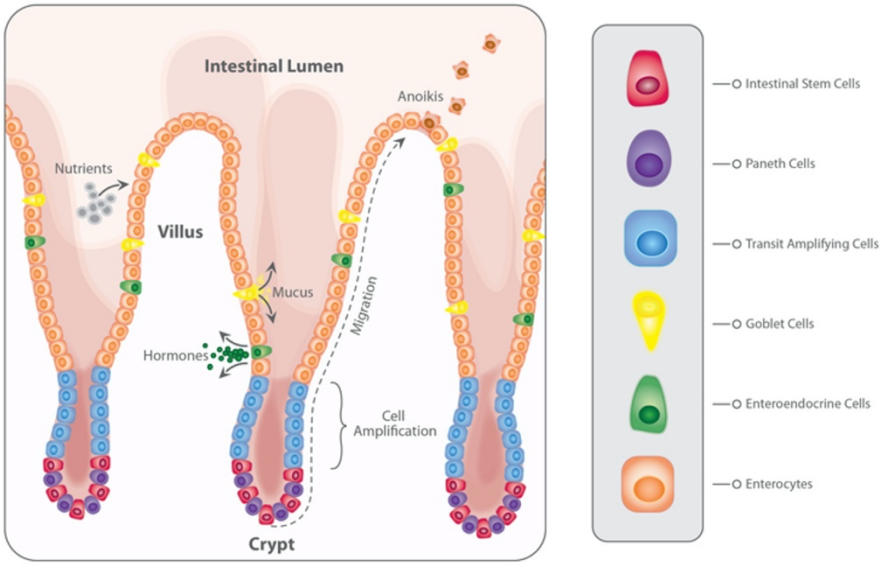

Figure 3. *Microanatomy of the jejunal epithelium showing crypts with intestinal stem cells (red) and the more specialized cells type along the villus. Nutrients are absorbed by enterocytes (orange) and hormones are secreted from enteroendocrine cells (green). Image adopted under CC-BY license from Wikimedia Commons.*

The stem cells in the intestinal crypts express the specific marker LGR5 (leucine-rich repeat-containing G-protein coupled receptor 5), a G-protein coupled receptor (GPCR) [57]. These stem cells possess the capacity to differentiate into all four types of specialized cells (enterocytes, Paneth, goblet and EECs) of the intestinal epithelium. The differentiation faith is decided by

different signaling pathways. Wnt (wingless/integrated) signaling results in a secretory lineage, while Notch signaling results in an absorptive lineage. The ligand Wnt3a has been shown to be crucial to keep the LGR5 expressing stem cells from starting to differentiate [58].

1.4.2 ENTEROENDOCRINE CELLS AND INCRETINS

Except having an essential function in the digestion and absorption of ingested nutrients, the gut also plays a crucial role in regulation of the energy metabolism [59-61]. EECs secrete hormones and peptides into the blood circulation in response to different stimuli for systemic responses. Throughout the gastrointestinal tract, from the stomach to the colon, different types of EECs are located with different functions. These functions span from regulating hunger to increasing gastric emptying.

The family of gut-hormones secreted from small intestine that have the capacity of regulating glucose homeostasis are called incretins [59]. Postprandially, incretins are secreted from EECs to regulate a vast range of processes in the endocrine pancreas. These include stimulation of glucose-dependent insulin secretion, insulin synthesis and inhibition of glucagon secretion. The incretins identified as of today are gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [60]. For each of these hormones, corresponding GPCRs exists in the target tissues.

Traditionally, the EECs secreting these two incretins have been classified as K- and L-cells, respectively [61]. GIP-producing K-cells are said to be located mainly in duodenum, while GLP-1-producing L-cells have been shown to be present along the whole gut axis but are most densely distributed in the ileum [62-64]. However, this traditional view on the distribution of K- and L-cell has recently been questioned. Recent profiling has not only shown that the distribution of K- and L-cells are highly overlapping, but also that individual EECs have the capacity of secreting both GIP and GLP-1 [65-67].

The mechanisms of action for the secretion of incretins from EECs have been extensively studied [61]. In short, the secretion of incretins from EECs upon nutrient-sensing is dependent on the entry of Ca^{2+} into the cell. When monosaccharides are transported into and metabolized by the EEC, this results in an intracellular increase of adenosine triphosphate (ATP). This in turn, results in closure of potassium channels and a depolarization of the cell membrane. When this depolarization is sufficient voltage controlled Ca^{2+}-channels open and an influx of Ca^{2+} stimulates the release of incretin

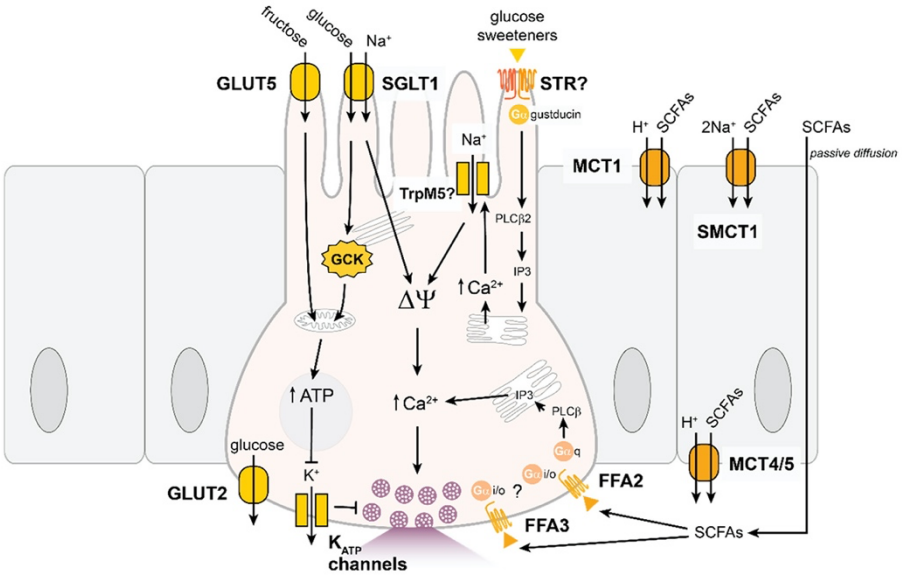

Figure 4. *Nutrient induced secretion of incretins from EECs. When monosaccharides transported into the EEC, by GLUT5 or SGLT1, is metabolized it results in increased intracellular levels of ATP and closing of basolateral potassium channels. This causes a depolarization of the membrane which opens Ca^{2+} channels and stimulate incretin secretin into the blood circulation. Intracellular levels of Ca^{2+} can also be increased by activations of FFARs. Image reprinted under CC-BY license from Lu, et al., Nutrient-induced cellular mechanisms of gut hormone secretion. Nutrients 13(3), 2021.*

hormones. The influx of Ca^{2+} can also be mediated through an activation of free fatty acid receptors (FFARs). Incretins are secreted from intracellular vesicles. Chromogranin A, a known marker of EECs, is located withing these vesicles [68]. A schematic image of nutrient-induced incretin secretion from EECs can be seen in Figure 4. FFAR1, FFAR2 and FFAR3, along with GPR109 (G-protein coupled receptor 109), have been described to be involved in the fat-induced secretion of incretin from EECs [69].

DPP-4 is the enzyme responsible for cleavage of both GIP and GLP-1 into inactive forms [59]. This enzyme is expressed in many tissues, and is active in both membrane-bound form and in circulating form.

1.4.3 BARIATRIC SURGERY AND SMALL INTESTINAL GLUCOSE CONTROL

Bariatric surgery does not only have a significant effect on weight reduction. It has also been demonstrated that the surgical procedure has an almost immediate effect on glycemic control and incretin secretion before any weight loss has occurred [70, 71]. Since the postprandial profiles of incretins are

improved already two days after surgery, before any substantial weight loss has occurred, these changes must be induced by the surgery itself. This is also supported by studies comparing the effect of equal weight loss in groups of RYGB patients to subjects with diet treatment [72, 73].

Multiples studies have reported improvements in glucose control in bariatric surgery patients with T2DM [74]. Even though it was reported over a decade ago that RYGB resolves or improves T2DM in 93% of the subjects the detailed mechanisms have yet not been fully elucidated [75]. The proposed weight-independent mechanisms include increased postprandial secretion of incretins, down-regulation of an unknown hypothesized anti-incretin factor from the proximal gastrointestinal tract, enhanced and more rapid delivery of ingested nutrients to the distal small intestine as well as changes to the nutrient-sensing mechanisms [74]. Especially the improved secretion of gut hormones following bariatric surgery has been of great interest lately and seems to be a prominent mechanism for the improved glycemic control [76, 77].

When comparing the effect of the two main bariatric surgery procedures, RYGB and SG, on glucose control both show similar long-term effects on glycaemia and insulinemia [71, 78]. RYGB however has demonstrated a better long-term preservation of the improved GLP-1 secretion levels [71].

1.4.4 DIETARY EFFECTS ON SMALL INTESTINAL GLUCOSE HOMEOSTASIS

The diet has also been shown to have effects on glucose control [79]. High ingestion of both fructose and glucose are known to induce expression of GLUT5 and SGLT1 [80, 81]. The macronutrients carbohydrate, protein and fat have all been reported to have the capacity of inducing incretin secretion from EECs [61].

1.5 INTESTINAL KETOGENESIS AND RELATED SIGNALING

Intestinal ketogenesis is a relatively new concept and few reports have previously been published on this subject in humans [82-85]. The traditional view has been that ketogenesis occurs exclusively in the liver during starvation or ketogenic diet [86]. During these conditions, when other energy sources are depleted, fatty acids from fat tissue are transported to the liver and oxidized to acetyl-CoA through β-oxidation. In mitochondria, acetyl-CoA is then through a number of enzymatically catalyzed chemical reactions converted to ketone

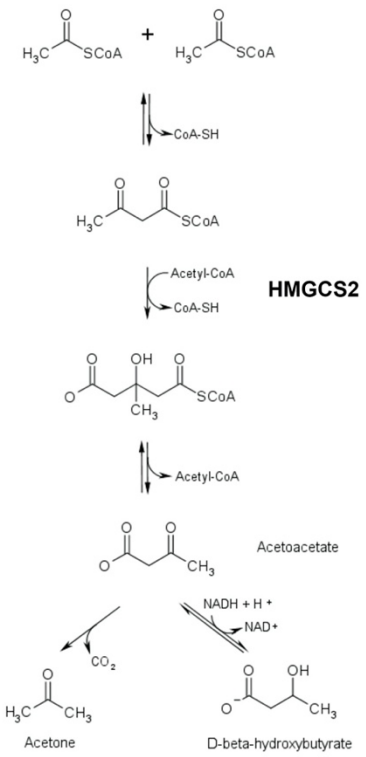

Figure 5. *Chemical pathway of ketogenesis where ketone bodies acetoacetate, acetone and βHB are synthesized from Acetyl-CoA through a number of enzymatically catalyzed steps. HMGCS2 is the rate-limiting enzyme of ketogenesis. Adopted from Wikimedia Commons under public domain.*

bodies; β-hydroxybutyrate (βHB), acetoacetate and acetone, through the process of ketogenesis. Apart from ketone bodies, ketogenesis also results in increased levels of the oxidized form of the cofactor nicotinamide adenine dinucleotide (NAD), NAD^+. The chemical reactions of ketogenesis can be seen in Figure 5. The rate-limiting enzyme of ketogenesis is 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2 or mitochondrial HMGCS, mHMGCS). After synthesis, the ketone bodies are transported via circulation to active tissues, like the brain, and metabolized to energy in the form of ATP. The rate-limiting enzyme of this extrahepatic conversion of ketone bodies into ATP is succinyl-CoA-3-oxaloacid-CoA-transferase (SCOT) [87].

The expression of HMGCS2 is known to be regulated by peroxisome proliferator-activated receptors (PPARs) in both intestinal cells and in liver tissue [84, 85, 88, 89].

Even though intestinal ketogenesis is a relatively new concept in humans, it has earlier been reported in animals. HMGCS2 has previously been shown to be expressed postnatally in the small intestine of suckling rodents and also in adult animals after a fat-dominated diet [90-93]. Intestinal ketogenesis is induced with feeding, while liver ketogenesis is induced with starvation. This interesting paradox of the intestinal ketogenesis is of key interest in this thesis.

1.5.1 SIGNALING CAPACITY OF KETOGENESIS

Ketone bodies possess a broad range of signaling capacities, from binding to receptors to inhibition of enzymes [87, 94, 95].

The receptors that the ketone body βHB can bind to are FFAR3 (also known as GPR41) and GPR109 [96, 97]. Both these receptors normally bind short-chain fatty acids, and βHB has been reported to be an antagonist of FFAR3 and an agonist of GPR109. The ketone body acetoacetate is an agonist of FFAR2 (also known as GPR43) [98]. FFAR3, GPR109 and FFAR2 are GPCRs, and more details about the signaling will be presented later in this introduction.

The ketone body βHB inhibits class I histone deacetylases (HDACs) [99]. Class I HDACs are major epigenetic regulators of gene expression through deacetylation of histones [100, 101]. Acetylation of histones result in a more relaxed histone configuration where RNA polymerase can bind and initiate transcription. Therefore, HDACs decrease gene expression. *In vitro* treatment with βHB resulted in a dose-dependent increase in acetylation of lysine 9 and lysine 14 at histone 3 [99]. Ketogenesis also has the capacity to induce deacetylation through activation of so-called sirtuins [95]. Sirtuins are NAD^+ dependent deacetylases that have the capacity to deacetylase proteins and histones [102]. Since NADH is oxidized to NAD^+ during the conversion of acetoacetate to βHB during ketogenesis, this results in elevated levels of NAD^+ during ketogenesis and increased activity of sirtuins [95].

In the intestinal epithelium, ketone bodies have been shown to induce cell differentiation and cell homeostasis through inhibition of HDAC activity [82, 83].

1.5.2 KETONE BODIES, HDACS, SIRTUINS AND GLUCOSE HOMEOSTASIS

Acetylation of histones and proteins are of great interest for regulation of metabolism and energy homeostasis [103, 104]. Tissue-specific disruption of HDAC in the mouse intestine resulted in decreased obesity and better glucose tolerance when fed a high-fat diet, compared to the control mice [105]. It has

been reported that inhibition of HDACs result in a down-regulation of GLUT1 in cancer cells [106]. Overexpression of sirtuins in intestinal cells in mice have been reported to result in improved glycemic control[107].

1.5.3 KETONE BODIES AND GPCR SIGNALING IN THE SMALL INTESTINE

As mentioned before, ketone bodies can bind to different GPCRs. GPCRs are cell surface receptors that detect extracellular signals and transduce these to intracellular signals [108]. The general principle of GPCRs is that ligand binding results in a conformational change that catalyzes the exchange from guanosine diphosphate (GDP) to guanosine triphosphate (GTP) bound to the G-proteins [109]. This triggers a dissociation between G_{α}- and $G_{\beta\gamma}$-subunits. Different α-subunits are G_s, G_q and $G_{i/o}$. The dissociated subunits activate or suppress different effectors. These effectors control two main signaling pathways: either cAMP synthesis or activation of phospholipase C. cAMP synthesis results in the activation of protein kinase A, while phospholipase C through increased Ca^{2+} influx into the cytosol activates protein kinase C. The $G_{\beta\gamma}$-subunit can also activate kinase Akt (also called protein kinase B).

Both FFAR3 and FFAR2 have been reported to be present on EECs [110]. Both these receptors have been reported to trigger GLP-1 secretion [111]. It has also been reported that activation of GPR109 results in increased GLP-1 secretion in obese subjects [112]. FFAR3 has been reported to be signal through $G_{\beta\gamma}$ activation of phospholipase C, resulting in the activation of different kinases [97, 113]. FFAR2 is coupled with either G_q or $G_{i/o}$ subunits and activation results in the inhibition of cAMP production, activation of the extracellular signal-regulated kinases (ERKs) cascade and increased levels of Ca^{2+} [114-116]. GPR109 has been described to signal via the $G_{i/o}$ subunit in adipose tissue [117].

2 AIMS

2.1 GENERAL AIM

The overall aim of this thesis was to investigate how jejunum, and the expression of different proteins in the jejunum, helps regulate glucose homeostasis in the human body.

2.2 SPECIFIC AIMS OF EACH PAPER

Paper I: Identify metabolic changes that take place in the jejunum following Roux-en-Y gastric bypass surgery of humans that contribute to the rapid increase in postprandial levels of GLP-1.

Paper II: Investigate the effects of fat- and carbohydrate dominated diets on glycemic control and insulinemic circulating markers following a meal test in a cross-over study in healthy and normal weight human subjects.

Paper III: Examine the effects of fat- and carbohydrate dominated diets on glucose absorption in the jejunal mucosa, with special focus on SGLT1 in the jejunum, in the same cohort of healthy subjects as in Paper II.

Paper IV: Explore the mechanism of the inhibitory effect of the ketone body βHB on glucose-induced secretion of GLP-1 in murine and human EECs.

3 STUDY MATERIAL AND ANALYTICAL METHODS

In this paragraph a summary of the study material and analytical methods used can be found. For further details about study materials or analytical methods, please see respective paper.

3.1 DESCRIPTION OF SUBJECTS AND STUDY MATERIALS

All study subjects gave their informed consent. All studies, as well as sample collections, were performed in accordance with the Helsinki declaration on ethical principles in research on humans and ethics applications were approved by the regional ethics committees. Details on demographics and all the ethics application numbers can be found in the respective paper.

3.1.1 PATIENTS ENROLLED FOR ROUX-EN-Y GASTRIC BYPASS

In Paper I, subjects included were patients scheduled for RYGB on clinical indication. Both subjects undergoing primary RYGB and those scheduled for conversion from vertical banded gastroplasty to RYGB were included. Three different patient cohorts were used: proteomics (n=7), confirmatory (n=9) and diet (n=12) cohort. Mucosa samples were retrieved before start of the two-week long very-low calorie diet (VLCD) (diet cohort), during RYGB (all cohorts) and 6-8 months after surgery (all cohorts). During surgery, a few centimeters of the jejunum was retrieved about 50 cm distal to the ligament of Treitz when dividing the omega-loop between the gastro-enterostomy and the jejuno-jejunostomy in order to create the Roux-en-Y construction as previously described [118]. The mucosa was separated from the musculature. Before VLCD and 6-8 months after surgery, mucosa was retrieved from jejunum/Roux limb during endoscopic examination. All mucosal tissue samples were fixed in formalin or snap-frozen in liquid nitrogen and kept at -70°C until analysis.

3.1.2 MICE FED HIGH-FAT AND LOW-FAT DIETS

In Paper I, mice of 6-8 weeks of age were fed high-fat diet (HFD) or low-fat diet (LFD).

In the first set of experiments, the mice were sacrificed after three weeks on HFD or LFD and different parts of the small intestine and liver were collected, snap-frozen in liquid nitrogen and kept at -70°C until analysis.

In the second set of experiments, the mice were fed HFD or LFD for three months. On the study day after a short fasting, an oral gavage of Intralipid was administrated containing either vehicle, HMGCS2 inhibitor hymeglusin or ketone body βHB. At 15, 30 and 120 min after administration, a venous blood sample was retrieved from the tail.

In the third set of experiments, the mice were fed HFD or LFD for three weeks. On the study day after a short fasting, an oral gavage of Intralipid was administered. 90 min after the administration, mice were anaesthetized, and blood was collected from the portal vein and from peripheral veins.

3.1.3 HEALTHY AND NORMAL WEIGHT HUMAN VOLUNTEERS FOR DIETARY STUDY

In Paper II and Paper III, healthy and normal weight participants were recruited for a single center, unblinded cross-over dietary study. The same study cohort was used for Paper II and Paper III. Inclusion criteria were voluntary participation, good self-reported general health, age between 18 and 65 years of age and BMI between 18 and 25 kg/m^2. Exclusion criteria were history of drug abuse, smoking, use of prescription medications within the last 14 days (apart from contraceptives), pregnancy or breast feeding.

16 subjects were enrolled in the study after a baseline visit, but one individual was excluded during the study due to pregnancy. Based on individual daily energy need, a high-fat diet (HFD) and a high-carbohydrate diet (HCD) were designed for each individual. For each diet, 60% of the energy content was from either fat or carbohydrates. Each dietary period lasted for two weeks and was separated by a wash-out period. For each diet, all meals, snacks, and beverages needed for the period were provided by the lab. The order of the diets was allocated randomly in blocks.

For Paper II, blood samples for biochemistry profiling were collected at the baseline visit and towards the end of each dietary period.

For Paper II, at day 12 of each dietary period the study participants visited the laboratory after an overnight fast for a mixed meal test (MMT). Before the start of the MMT start, a fasting blood sample was collected from an intravenous forearm cannula. For the MMT, the study participants were served a 600 kcal

brunch and blood was sampled through the forearm cannula at 15, 30, 45, 60, 90 and 120 min. Serum and plasma samples were prepared, aliquoted and frozen at -70°C until analysis.

For Paper III, at day 14 of each dietary period the study participants visited the hospital after an overnight fast for jejunal mucosa sample collection through enteroscopic examination. Five to ten mucosa biopsies from each subject were collected from the jejunum approximately 50 cm distal to the ligament of Treitz. Four to six biopsies were either snap-frozen in liquid nitrogen and stored at -70°C, or chemically fixed for later analysis. The remaining biopsies from each subject were prepared for functional studies in Ussing chambers.

3.1.4 CELL CULTURE

In vitro cell culture experiments were performed in Paper I, Paper III and Paper IV.

In Paper I, primary jejunal cultures were prepared from mice as previously described [119]. In short, crypts were isolated from the small intestine and seeded in Matrigel-coated 24-well plates with high glucose Dulbecco's modified eagle medium (DMEM, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 μM Y-27632). The day after seeding, wells were washed with saline secretion buffer (138 mM NaCl, 4.5 mM KCl, 4.2 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES and 0.1% BSA, pH 7.4) with 1 mM glucose for 30 min. After a first hour incubation in saline secretion buffer with 1 mM glucose, a second hour incubation was performed with saline secretion buffer with different additives: 10 mM glucose + 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), 10 mM glucose + 0.1 mM IBMX + 10 mM βHB, or 10 mM glucose + 0.1 mM IBMX + 0.1 μM somatostatin. Pertussis toxin (PTX, 500 ng/ml) was added 16 hours before the start of the experiments and included during secretion experiments when used. After each incubation, supernatants were collected and snap-frozen in liquid nitrogen.

In Paper III, Caco-2 cells at passage 48-52 were expanded on 12-well Transwell membranes (3 μm pore size) in high glucose DMEM (supplemented with 10% FBS, 1X NEAA and 1X PEST) on each side of the membrane until confluent. When confluent, cells differentiated into small intestine-like monolayers with serum-free (SF) media (high glucose DMEM, 1X NEAA, 1X PEST) on the apical side and insulin-transferrin-selenium (ITS) media (high glucose DMEM, 1X ITS, 1X NEAA, 1X PEST) on the basolateral side for 14 days. After differentiation, monolayers were cultured for 48 hours in low

glucose SF and ITS media with 10 mM butyrate and different combinations of additives on the apical side: 1 μM hymeglusin, 5 mM nicotinamide and 1 μM simvastatin. Proteins were extracted by scaping cells in ice-cold RIPA buffer, incubated on ice for 30 min, sonicated, centrifuged (10000xG, 10 min, 4°C) before finally supernatants were stored at -70°C until analysis. Cell culture media were collected for Caco-2 cells treated with 10 mM butyrate in combination with 1 μM hymeglusin and frozen at -70°C until further analysis.

In Paper IV, GLUTag cells at passage 13-15 were seeded in 24-well plates and expanded in low glucose DMEM (supplemented with 10% FBS and 1% PEST) for 3 days. For method development, GLUTag cell cultures were incubated both with and without pre-incubation, and with and without addition of 10 μM IBMX and 10 μM forskolin (fsk) during incubation. For pre-incubation, wells were treated with saline secretion buffer with low glucose concentration for 1 hour. After pre-incubation, the medium was changed to the different incubation media (saline secretion buffer with respective substance added) and incubated for 2 hours. For dose-response of glucose 0, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 mM of glucose was used. For incretin secretion inhibition experiments 0 mM glucose, 1 mM glucose, and 1 mM glucose in combination with 0.01, 0.1, 1, 10, 100 mM βHB or 0.1, 1 μM somatostatin was used. After incubation, media were saved (with addition of the 50 μM DPP-4 inhibitor) and cells were scraped of the surface in ice-cold RIPA buffer with protease inhibitor cocktail and frozen at -70°C until analysis.

In Paper IV, human jejunal enteroid cultures were established from biopsies retrieved during enteroscopic examination of the jejunum (approximately 50 cm distal to ligament of Treitz) in one healthy volunteer (23 year old, female). The technique for the establishment of enteroid cultures was developed by Sato *et al* [120]. In short, jejunal crypts were isolated, seeded embedded in Matrigel in 24-well plates and cultured in Wnt3a-supplemented expansion medium (IntestiCult Organoid Growth Medium supplemented with 10 μM Y-27632 and 0.1% gentamicin). Expanded enteroid cultures at passage 19-20 were recovered and seeded onto collagen-IV coated 24-well Transwell membranes (0.4 μm pore size) and expanded until confluent. When confluent, differentiation was achieved using the ALI (air-liquid interface) technique where the apical compartment is left empty and differentiation medium (IntestiCult Organoid Differentiation Medium supplemented with 10 μM Y-27632 and 0.1% gentamicin) is added to the basolateral compartment. Differentiated human jejunal enteroid monolayers, after the same pre-incubation as for GLUTag cells, were incubated in saline secretion buffer with 0 mM glucose, 10 mM glucose, or 10 mM glucose in combination with 10 mM βHB. Glucose was added only to the apical side of the membranes and 10 μM

IBMX/fsk was added to all groups. Apical and basolateral media were saved (with addition of 50 μM DPP-4 inhibitor) separately and frozen at -70°C until analysis.

3.2 ANALYTICAL METHODS

In this paragraph the analytical methods and the science underlying these methods are presented. A brief description of the use of each method in each study can be found, but for more details see respective paper.

3.2.1 WESTERN BLOT

Western blot (WB) is a semi-quantitative technique for relative comparison of specific proteins in protein mixtures [121]. The samples can for instance come from tissue, blood or cells from *in vitro* cell cultures. The proteins must be in solution and therefore the sample is treated with a protein extraction buffer and the sample can also be homogenized by sonication for a higher yield of protein extraction. The fundamental basis for WB is protein separation in a sample based on the size of the proteins. To achieve this, proteins are denatured in a reducing agent to remove secondary and tertiary structures and then treated with a buffer coating the proteins with negative charges, making them anionic. When loaded onto a polyacrylamide gel electrophoresis with a charge applied to it, the negatively charged proteins will migrate through the gel towards the positively charged anode. Since smaller proteins can migrate faster through the gel mesh, the proteins in a sample will be separated on the gel based on their size. After the separation based on size on the gel, the proteins are transferred to a membrane (*e.g.* polyvinylidene difluoride) by using an electrical current applied perpendicular to the charge during the gel electrophoresis to pull the negatively charged proteins from the gel towards the positively charged anode onto the membrane. After blotting, the membrane can be treated with a primary antibody specific to the protein of interest. After washing of the membrane to get rid of unbound primary antibody, a secondary antibody directed against the Fc domain of the primary antibody is applied, which recognizes the primary antibody that is attached to the antigen on the membrane. The secondary antibody is conjugated to some sort of reporter protein. This reporter is most commonly an enzyme, such as horseradish peroxidase, which allows for detection of the protein of interest using chemiluminescence. When a substrate is added to the membrane, the reporter enzyme will cleave the substrate and create chemiluminescence. This signal is proportional to the amount of secondary antibody, and therefore also proportional to the amount of protein on interest. Commercially available software can be used to quantify the signal

intensity of each sample. The membrane can be chemically stripped off of primary and secondary antibodies and re-probed with a primary antibody for a new protein of interest and procedure can be repeated. The amount of signal of the protein of interest is commonly related to the amount of signal of a housekeeper protein, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in order to normalize for differences in loading of protein in the gel electrophoresis.

WB was used in Paper I for quantification of HMGCS2 in all three human cohorts and the first set of mouse experiments, and for CPT1A, FATP4, DGAT1 and SCOT in the human diet cohort. In Paper III, WB was used for quantification of SGLT1, SGLT2, SGLT3, GLUT1, GLUT2, GLUT5, HMGCS2, H3K9ac, GPR40, GPR41, GPR43 and GPR109A in the human samples, and for SGLT1 and H3K9ac in the Caco-2 samples. In Paper IV, WB was used for quantification of phospho-ERK1/2, ERK1/2, phospho-Akt and Akt in GLUTag cells. GAPDH was used as housekeeping protein for all studies.

3.2.2 HISTOLOGY

Histology is the qualitative study of tissue and cells on a microscopic level. To achieve this, the tissue or cells of interest are chemically fixed to preserve and maintain the structure of the study material. For tissues, the samples are dehydrated and embedded in paraffin wax before cut into thin (5 μm) sections, while cells can be stained without sectioning. Since tissues and cells have little inherent contrast, they must be stained to be visualized under the microscope. The most common staining technique for visualizing tissue with light microscope is hematoxylin and eosin staining. With this technique hematoxylin stains the nuclei blueish while eosin stains the cytoplasm and other tissue elements in different shades of pink. Antibodies can also be utilized for tissue visualization in the microscope. With this method, the sample of interest is incubated with primary antibody specific to the protein of interest followed by incubation with a secondary antibody that binds to the primary antibody. The secondary antibody is conjugated to a reporter enzyme, or as used in this thesis, a fluorescent molecule that produces fluorescence upon light excitation for so-called immunofluorescence. The fluorescent molecule cannot only be conjugated to secondary antibody, but also to dyes specific to certain tissue elements (like DAPI for nuclei).

In Paper I, jejunal tissue samples from the so-called confirmation cohort were stained with hematoxylin and eosin, and with antibody against HMGCS2. In Paper III, the human jejunal samples were stained by immunofluorescence for

SGLT1, GLUT2, GLUT5 and HMGCS2. In Paper IV, differentiated human jejunal enteroid monolayers were stained with immunofluorescence against chromogranin A.

3.2.3 ELISA AND ECLIA

Enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescence immunoassay (ECLIA) are two quite similar analytical assays utilizing antibodies for antigen quantification. In ELISA, plates with capture antibodies immobilized on the well surface are used. When sample is added to the well, any specific antigen present in the sample will bind to the immobilized antibody. A detection antibody specific to the protein of interest is then added which also bind to antigen. Finally, an enzyme-linked secondary antibody is added which binds to the primary antibody and when substrate is added, emits a color signal proportional to the amount of antigen in the sample. The ECLIA technique works in a similar way as ELISA, but instead of substrate changing color proportional to antigen concentration, light is emitted proportional to antigen concentration when a current is applied to the plate.

In Paper I, ECLIA was used to quantify total GLP-1 in media secreted from *in vitro* cultures of primary jejunal cells. In Paper II, ELISA was used to quantify insulin, GLP-1 and GIP in sequentially collected blood samples during MMT. Fasting insulin was measured with ECLIA. In Paper IV, ELISA was used to quantify total GLP-1 secreted from GLUTag cells while ECLIA was used to quantify total GLP-1 secreted from differentiated human jejunal enteroid monolayers.

3.2.4 COLORIMETRIC ASSAYS AND OTHER QUANTIFICATION METHODS

Colorimetric assays function much like the ELISA and ECLIA techniques, but with one important difference; no antibodies are used. Instead, a colorimetric detector is added that changes color based on concentration of a certain substance. Alamar Blue is a variant of colorimetric assay, but instead of quantifying a specific substance, this assay quantifies the redox state of cells in cell culture. Growth and viable cells are indicated by reduced conditions and the opposite is indicated by oxidized conditions. A dye is used to quantify the redox state. Also, more simple assays can be used for faster quantification in for example blood samples. These include strips that is exposed to the sample for direct quantification using electrochemical signal and an enzyme system.

In Paper I, a colorimetric assay was used to quantify βHB in tissue homogenates collected after VLCD and 6-8 months after surgery in the so-

called diet cohort and in portal and peripheral venous blood samples following oral gavage with intralipid in the third set of mouse experiments. In the same paper, strips were used for direct quantification of βHB in sequential venous blood samples in the second set of mouse experiments. In Paper II, strips were used to quantify glucose in sequentially collected blood samples during MMT after HFD and HCD. In Paper III, a colorimetric assay was used to quantify βHB in cell culture media from Caco-2 cells. In Paper IV, a colorimetric Alamar Blue assay was used to quantify viability of the GLUTag cells.

3.2.5 PROTEOMICS

Proteomics is the study of the proteome, *i.e.* the entire collection of all proteins in a sample or several samples. This technique is usually used for broad and open exploratory research questions. To perform proteomics the complexity of the sample must first be reduced. Most commonly this is achieved by two-dimensional electrophoresis separation on gel with separation based on isoelectric focusing in one dimension and separation by protein mass in the other dimension. Differentially expressed spots in two samples, identified by protein staining on the gels, are then selected and extracted from the gels and identified using mass spectrometry to obtain the amino acid sequence. This amino acid sequence can then be compared against existing databases on known protein sequences. A list of the most probable proteins based on the amino acid sequence in the sample is the produced.

In Paper I, global proteomics was used on human jejunal samples in the so-called proteomics cohort collected at the time of surgery and 6-8 months after surgery. Details on the specific methods used have been published before [122].

3.2.6 METABOLOMICS

In parallel to proteomics, metabolomics is the study of metabolome, *i.e.* all small-molecule substrates, intermediates or end products of metabolism in a sample. In short, the sample is prepared, analyzed and then the acquired data is analyzed against reference databases for identification and statistical methods are applied for data reduction. Most commonly the analysis is performed with liquid or gas chromatography coupled with mass spectrometry or with nuclear magnetic resonance spectroscopy.

In Paper II, serum samples collected during MMT after HFD and HCD were used for metabolomics with nuclear magnetic resonance spectroscopy.

3.2.7 USSING CHAMBERS

Ussing chambers, design by Danish scientist Hans Ussing, are used to study the functional properties of epithelial membranes [123]. This method can detect and quantify transport and barrier functions of living epithelial tissues. We utilized the pulsed Ussing technique that measures the potential difference over the membrane as well as the epithelial electrical resistance after short current pulses [124]. With the potential difference and the epithelial electrical resistance known, Ohm's law can be used to calculate the ion current. Since many of the transport proteins are coupled to ion transport, the ion current can be used to quantify transport.

In Paper III, Ussing chamber experiments were used to study glucose induced SGLT1 transport in jejunal epithelial tissues collected after a two-week long HFD and HCD in healthy human subjects.

3.2.8 STATISTICS

Generally, non-parametric statistical tests were used when possible due to the relatively low number of samples. When comparing two groups, Wilcoxon signed rank test was used for paired samples and Mann-Whitney test was used for unpaired samples. When comparing more than two groups, ordinary one-way ANOVA with Dunnett's multiple comparison test was used. When comparing two or more groups with repeated measurements, two-way ANOVA with Dunnett's, Tukey's or Sidak's multiple comparison test was used. For correlation analysis, Spearman's rank correlation was used. A p-value ≤ 0.05 was considered significant. Statistical analyses were performed with GraphPad Prism for MacOS.

4 RESULTS

Below, for each paper, the results are summarized. For more detailed results, see respective printed paper.

4.1 PAPER I

Bariatric surgery, for example RYGB, has proven to be an important long-term treatment for both obesity and T2DM. Even though weight loss *per se* is probably the most important factor for improved glucose homeostasis, this does not seem to be the whole truth. RYGB surgery results in early postoperative improvements in postprandial glycemic control as well as increased release of several gut peptides, such as GLP-1, which occurs already two days after surgery, before any major weight loss has occurred [71]. Instead, it has been hypothesized that the early effect on postprandial hormone release is associated with metabolic changes in the mucosa following the anatomical reconfiguration of the gastrointestinal tract with surgery. In this study, we therefore started with an open proteomic evaluation of the jejunal and Roux limb mucosa at the time of and after surgery. The finding from this proteomic investigation was further explored in animal models and *in vitro*.

For global proteomics, mucosal biopsies were retrieved from the jejunum at time of surgery and from the Roux limb a half-year after surgery in seven patients scheduled for RYGB by the usual clinical indications. Proteomics revealed that the ketogenesis rate-limiting enzyme, HMGCS2, was the most regulated protein of all in the jejunal mucosa with a prominent (p<0.001) down-regulation following RYGB. This significant down-regulation was confirmed with WB, both in the original cohort (Figure 6A, p=0.0312) consisting of 6 patients and in a second confirmatory cohort (Figure 6B, p=0.0039) of 9 patients.

To exclude that the changes in levels of HMGCS2 were due to the VLCD that the patients undergo during the two weeks preceding bariatric surgery, a third dietary cohort was included. WB analysis of HMGCS2 in this cohort revealed that even though the VLCD had effect on other lipid metabolism proteins it had no effect on the expression of HMGCS2. In line with this, jejunal mucosa tissue levels of ketone body βHB displayed a significant decrease (p=0.0010) after RYGB surgery.

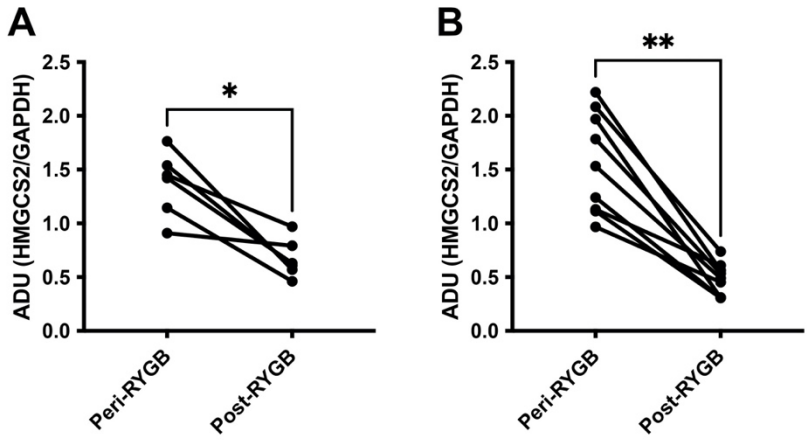

Figure 6. *WB analysis of jejunal HMGCS2 protein expression peri- and 6-8 months post-RYGB in proteomics (A) and confirmatory (B) cohorts. The densitometric signal was normalized to GAPDH protein expression. * $p \leq 0.05$, ** $p \leq 0.01$; Wilcoxon test.*

To further study the expression and function of HMGCS2, mouse models were used. In these experiments the mice were fed either HFD or LFD for an extended period of time. These dietary studies showed that intestinal HMGCS2 expression was increased with HFD and that the expression was most pronounced in the jejunal part of the small intestine (p=0.0079). The dietary studies in mice also demonstrated an increased concentration of the ketone body βHB in venous blood 120 min after an oral gavage with intralipid in mice going through HFD (p=0.0204, HFD vs LFD), and that this increase could be abolished when giving the mice HMGCS2 inhibitor hymeglusin. Prolonged HFD showed not only an increased protein expression of HMGCS2 but also an increased function. The HFD fed mice also had increased concentrations of βHB in the portal blood compared to LFD fed mice (p=0.0012). In LFD fed mice the βHB levels were significantly lower (p=0.0040) in portal blood compared to peripheral blood because the ketone bodies are exclusively produced by the liver and not present in portal, *i.e.* pre-hepatic, blood. This was not the case in the HFD fed mice, where the portal blood levels of ketone bodies were higher than the peripheral levels. This mean that after HFD, more ketone bodies were produced by the intestine than the liver. This is striking in the sense that the liver has been considered the main organ to have the capacity to increase circulation levels of ketone bodies.

To study the effect of the ketone body βHB on incretin hormone GLP-1 secretion *in vitro*, cultures of mouse primary jejunal cells were used. βHB displayed a significant (p=0.0025) inhibitory effect on GLP-1 secretion from EECs in these pilot experiments (Figure 7).

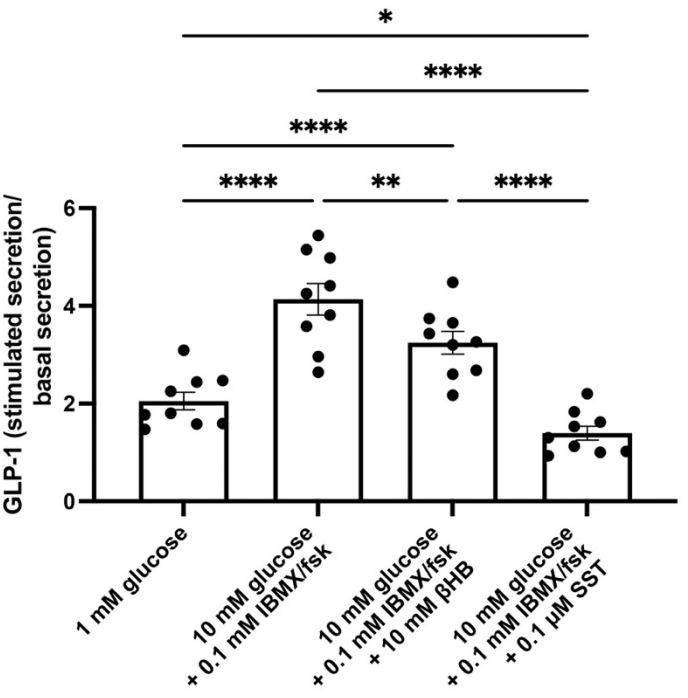

Figure 7. *GLP-1 levels secreted from mouse jejunal primary cultures after stimulation with glucose, IBMX, forskolin, βHB and somatostatin. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$; Tukey's multiple comparison test. Mean ± SEM.*

In summary, this study showed that the ketogenesis rate-limiting enzyme HMGCS2 is drastically decreased in jejunum after RYGB, but not affected by the two-week VLCD preceding the surgery. As mentioned, HMGCS2 is traditionally expressed in liver and its function has been considered to be to produce energy during starvation. This study, however, highlighted a new function of HMGCS2 and ketone body production. The enzyme was up-regulated in jejunal mucosa with prolonged HFD in mice, *i.e.* not under starving conditions but rather during high-energy consumption. This enzyme was also dramatically down-regulated after RYGB in obese humans. Further, this study demonstrated an inhibitory effect of the ketone body βHB on GLP-1 secretion. After RYGB, the substrate for ketogenesis, short-chain fatty acids, is reduced from the Roux limb due to absence of bile, and gastric as well as pancreatic lipases. Taken together this mechanism could provide an explanation to the immediately increased capability of the intestine to release GLP-1 postprandially after RYGB surgery [71]. Thus, it could be considered to be a long sought-after anti-incretin mechanism.

4.2 PAPER II

Understanding the precise effects of different dietary macronutrients, such as carbohydrates and fat, on the metabolic response and adaptation, would be of great value for patients with metabolic perturbations such as *e.g.* impaired glycemic control and diabetes. It is known that carbohydrates and fatty acids stimulate EECs to release gut hormones, such as GLP-1 and GIP, and thereby prepare the body and metabolism for the incoming nutrient load. The current understanding of how the dietary macronutrients fat and carbohydrates can influence insulin sensitivity and, in the end, result in insulin resistance and eventually T2DM, is poor. It is believed that some intermediate metabolites may influence metabolism in the long-term by causing by exerting toxic effects on pancreatic β-cells or by causing peripheral insulin resistance. The aim of this study was to investigate how a two-week HFD or HCD could affect metabolic status, particularly the metabolic intermediates that have been shown to be predictors of insulin resistance in healthy and normal weight volunteers, in a cross-over study setup.

The participants in this study started with one of the diets for two weeks according to randomization, and after a two-week wash-out period, they continued with the other diet. The two diets were iso-caloric and individually planned for each subject according to gender, age, weight, height and physical activity level. Towards the end of each dietary period the study subjects went through a MMT with a standardized meal in the form of a fatty brunch and sequentially blood sampling for 120 min. Glucose, insulin, GLP-1 and GIP were quantified in the blood samples. Metabolomics was also performed on the same samples taken during the MMT.

The quantification of glucose, insulin and GIP revealed no differences between HFD and HCD after MMT, while serum GLP-1 levels were significantly increased (p=0.0002) after HFD (Figure 8B). HFD also displayed a significantly higher (p=0.0151) level of GLP-1 at 30 min after MMT start (Figure 8A). Metabolomics analysis showed higher levels of acetoacetate (p=0.0004), βHB (p=0.0001), acetone (p=0.0002), valine (p=0.0015), creatine (p=0.0027) and leucine (p=0.0002) in the serum following HFD, while the levels of alanine (p=0.0084), methionine (p=0.0079) and glutamine (p=0.0353) were lower in serum after the HFD compared to the HCD.

In summary, this study showed that despite maintaining glycemia and insulinemia, postprandial GLP-1 levels were increased by HFD. Also, the metabolomic profiles after MMT implied a metabolomic shift towards increased risk for future impaired insulin sensitivity. These findings are of

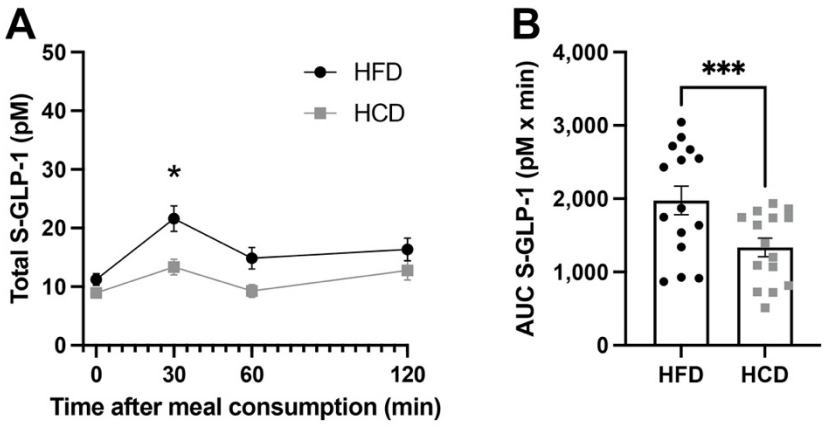

Figure 8. *Concentration profile (A) and total AUC (B) for GLP-1 during MMT in normal weight and healthy subjects after two weeks of HFD (black circles) and HCD (grey squares). * $p \leq 0.05$, *** $p \leq 0.001$; Sidak's multiple comparison and Wilcoxon signed rank test, respectively. Mean ± SEM.*

importance to lead future studies to a more complete understanding of the effects of macronutrients on metabolic regulation of insulin sensitivity.

4.3 PAPER III

To further understand the effects of dietary macronutrients on glucose homeostasis, the dietary adaption of glucose transport to HFD and HCD in the small intestinal mucosa was studied. Insights into how dietary changes influence uptake of key nutrients in the gut can be of importance for treatment of T2DM and obesity. The mechanisms for the regulation of glucose absorption by the intestinal mucosal cells upon availability of glucose in the lumen is poorly understood. Also, most human studies have been done in study groups already in the hospital for other indications. Therefore, the aim of this study was to investigate what effects diets dominated by either fat or carbohydrates have on glucose absorption in the jejunal mucosa in healthy, normal weight and non-diabetic subjects, with special focus on the main glucose transporter SGLT1.

The same cohort of subjects as in Paper II was used, but in this study the focus was put on the jejunal biopsies collected at the end of each dietary period. The jejunal biopsies retrieved were analyzed with both WB and immunofluorescence for protein expression of carbohydrate transporters. Protein expression of both SGLT1 (Figure 9A, p=0.0003) and GLUT5 (Figure 9B, p=0.0004) were significantly lower after two-weeks of HFD, compared to

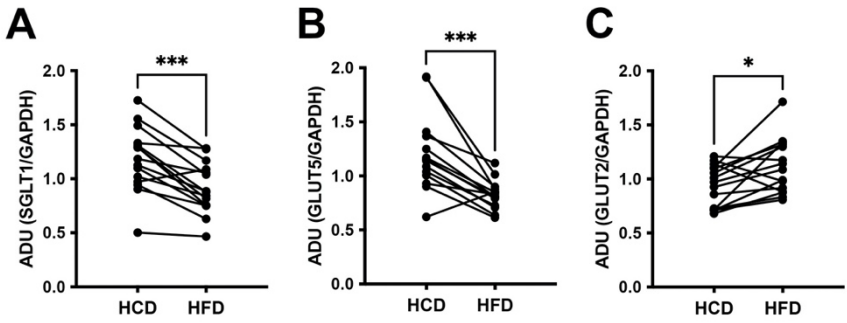

Figure 9. *WB analysis of jejunal protein expression of SGLT1 (A), GLUT5 (B) and GLUT2 (C) after two weeks of HCD and HFD. The densitometry signal of glucose transporters was normalized to GAPDH protein expression. * $p \leq 0.05$, *** $p \leq 0.001$; Wilcoxon test.*

two-weeks of HCD, while the expression of GLUT2 (Figure 9C, p=0.0151) was higher after HFD. SGLT2, SGLT3 and GLUT1 protein expressions were unchanged. Functional studies, using Ussing chambers, also displayed a significantly lower (p=0.0420) glucose-induced transport following the HFD.

After the finding of the fatty-diet induced protein expression of HMGCS2 in Paper I, this ketogenesis rate-limiting enzyme was investigated also in this dietary study in normal weight and healthy humans. Since ketone bodies are potent epigenetic modulators, the dietary effect on histone acetylation was also studied. By WB, the protein expression of HMGCS2 was found to be increased (p=0.0006) after HFD (Figure 10A) compared to HCD, while the acetylation of H3K9 was decreased (p=0.0245, Figure 10B). Expression of short-chain free fatty acid and ketone receptors FFAR3 (p=0.0002) and GPR109a

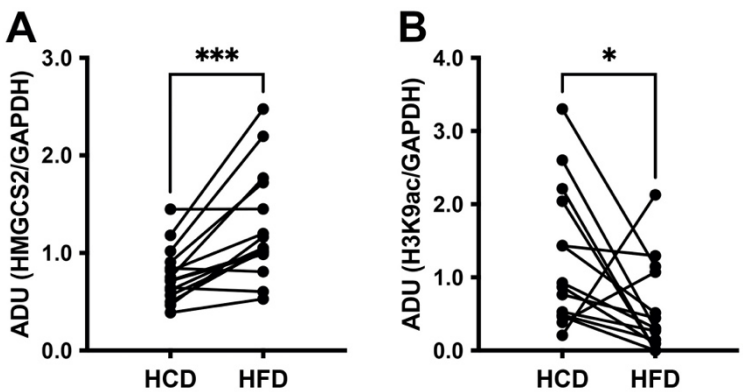

Figure 10. *WB analysis of jejunal protein expression of HMGCS2 (A) and acetylation of H3K9 (B) after two weeks of HCD and HFD. The densitometric signal was normalized to GAPDH protein expression. * $p \leq 0.05$, *** $p \leq 0.001$; Wilcoxon test.*

(p=0.0353) were increased after HFD, compared to HCD. No such effect could be observed for FFAR1 or FFAR2.

The finding that a two-week HFD resulted in lower SGLT1 expression, higher HMGCS2 expression and lower acetylation of H3K9 *in vivo* in normal weight and healthy humans was very intriguing. To further study this, *in vitro* cultures of Caco-2 cells were used. When differentiated on semi-permeable membranes, cultures of Caco-2 cells exhibit a phenotype very much resembling enterocytes of the small intestine. The differentiated monolayers of Caco-2 cells were treated for 48 hours with the short-chain fatty acid butyrate, and in combination with the HMGCS2 inhibitor hymeglusin, the sirtuin inhibitor nicotinamide or the mevalonate pathway inhibitor simvastatin.

WB quantification of SGLT1 in differentiated monolayers of Caco-2 cells revealed a significantly higher expression in cultures with hymeglusin (p=0.0403) or nicotinamide (p=0.0002), compared to cultures treated with butyrate alone (Figure 11A). For acetylation of H3K9, the pattern was the same with increased acetylation after addition of hymeglusin (p=0.0122) or nicotinamide (p=0.0008) (Figure 11B). Simvastatin had no effect on neither SGLT1 expression nor acetylation of H3K9. Quantification of the ketone body βHB in basolateral media of Caco-2 cultures displayed a clear decrease (p<0.0001) with the addition of hymeglusin, compared to butyrate alone.

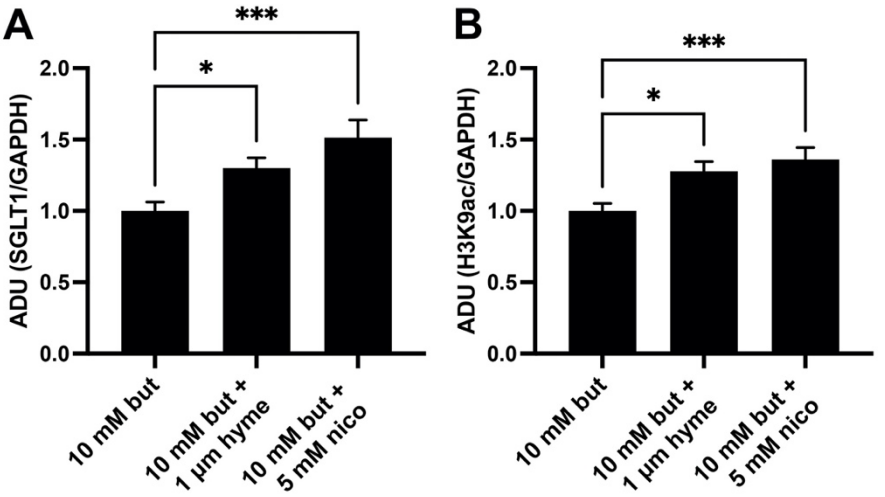

Figure 11. *WB analysis of protein expression of SGLT1 (A) and acetylation of H3K9 (B) in Caco-2 cultures after 48 hours treatment with butyrate, hymeglusin or nicotinamide. The densitometric signal was normalized to GAPDH protein expression, and each group normalized to the butyrate only-treated control group. * p ≤ 0.05, *** p ≤ 0.001; Dunnett's multiple comparison test. Mean ± SEM.*

In summary, this study showed differential effects of HFD and HCD on the expression of jejunal carbohydrate transporters. Two-week HFD decreased SGLT1 expression, while the ketogenesis rate-limiting enzyme HMGCS2 was increased. The HFD also affected the epigenetic acetylation of H3K9. *In vitro* studies of Caco-2 cells revealed a regulation of SGLT1-expression mediated through ketogenesis-induced sirtuin activity.

4.4 PAPER IV

It is well established that ingested nutrients stimulate postprandial hormone secretion from the gut. Incretins are the hormones released from the gut that stimulate a postprandial insulin release and, in the end, lower blood glucose. GLP-1 is one of the incretins, secreted from EECs in the epithelial layer of the gut. Traditionally GLP-1 is mostly secreted from L-cells residing in the distal small intestine, but more recent studies have showed that GLP-1 secreting EECs can be found throughout the small intestine. Understanding the regulation of incretin secretion might have great impact on future therapeutic options for patients with impaired glucose homeostasis and T2DM. Plasma levels of GLP-1, and also GIP, have been shown to increase rapidly after RYGB and SG surgery [71]. This elevated secretion of GLP-1 after these bariatric procedures has been linked to increased glycemic control and glucose tolerance [125]. Based on the findings in Paper I, that the ketone body βHB in primary mouse jejunal cultures inhibits GLP-1 secretion, the aim of this study was to further investigate the effects of βHB on glucose-stimulated GLP-1 secretion and explore the dose-response relationship and the mechanism for this inhibition.

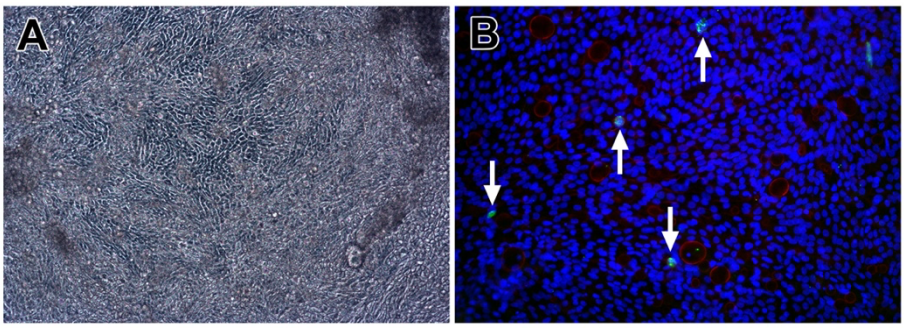

Figure 12. *Phase contrast micrograph (A) of ALI-differentiated human jejunal enteroids and immunofluorescence of the EEC marker chromogranin A (B). Positively stained (green) EECs are highlighted with arrows. Blue staining: nuclei, red staining: F-actin.*

In vitro cultures of GLUTag cells and differentiated human jejunal enteroid monolayers were used to study the inhibitory effect of βHB on glucose-induced GLP-1 secretion. Human jejunal enteroid cultures were established from crypts from a healthy and normal weight volunteer. By culturing of the isolated crypts encapsulated in Matrigel in media supplemented with Wnt3a the cell population can be enriched in jejunal stem cells. When Wnt3a is withdrawn from media, the stem cells spontaneously start to differentiate into the different cell phenotypes of jejunal mucosa. When cultured and differentiated on semi-permeable Transwell membranes, an *in vitro* cell structure similar to the native jejunum, with EECs, can be achieved (Figure 12).

For method development, GLUTag cells were cultured with and without one hour of pre-incubation in glucose-reduced media, and with and without addition of the GLP-1 secretion stimulants IBMX and forskolin during the two hour long stimulation experiment. Pre-incubation in glucose-reduced media before the start of the experiments slightly increased the glucose-induced GLP-1 levels. IBMX and forskolin also dramatically increased glucose-induced GLP-1 secretion but also the basal GLP-1 secretion. Therefore, the net effect was not changed and it was decided to use pre-incubation in glucose-reduced media, without the addition of IBMX and forskolin, for future stimulation of GLUTag cultures.

When culturing the GLUTag cells in increasing concentrations of glucose, a clear increase in GLP-1 secreted to the media was seen. Even though the

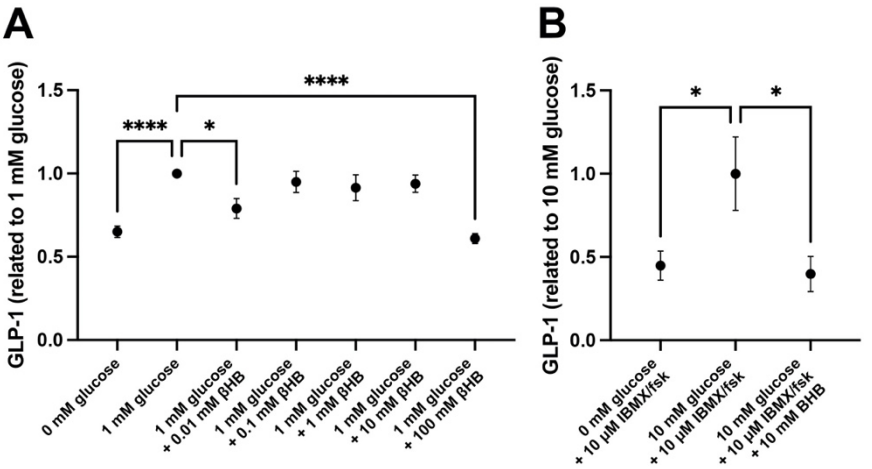

Figure 13. *GLP-1 levels secreted into media from GLUTag cells (A) and into basolateral media from differentiated human jejunal enteroid monolayers (B) after incubation with glucose in combination with ketone body βHB for two hours. Values are related to the glucose-stimulated control. * $p \leq 0.05$, **** $p \leq 0.0001$; Dunnett's multiple comparison test. Mean ± SEM.*

absolute levels of GLP-1 secretion differed between the experiments, this clearly demonstrated that GLUTag cells were a functional model to study macronutrient stimulated incretin secretion.

To ensure that the ketone body βHB was not toxic to the cells, two different doses were used in an Alamar Blue assay at various cell densities. At the highest test cell density, neither the high nor the low dose of βHB exhibited any cytotoxic effect on the GLUTag cells.

Addition of 100 mM of βHB had a significant inhibitory (p<0.0001) effect on glucose-induced GLP-1 secretion from the GLUTag cells, compared to stimulation with only 1 mM glucose (Figure 13A). Also, the lowest test dose of βHB, 0.01 mM, had an inhibitory effect (p=0.0247), while intermediate doses displayed no effect. As an inhibitory control, addition of somatostatin to glucose-induced GLUTag cells decreased GLP-1 secretion for both 0.1 µM and 1 µM (p<0.0001 for both). GLP-1 secretion from ALI-differentiated human jejunal enteroid monolayers was inhibited (p=0.0361), compared to glucose-induced GLP-1 secretion (Figure 13B).

To investigate the inhibitory regulation exhibited by βHB on glucose-induced GLP-1 secretion, some key proteins in the GPCR signaling was analyzed using WB on GLUTag cultures. Addition of 100 mM βHB to GLUTag cells significantly increased (p=0.0095) phosphorylation of kinase Akt, also known as protein kinase B, compared to the glucose-stimulated control, in the same way as the addition of 0.1 µM somatostatin (p=0.0019) (Figure 14A). ERK1/2

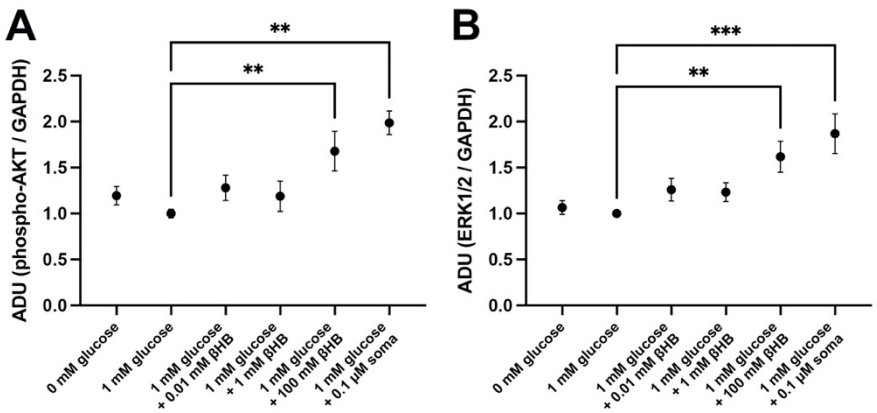

Figure 14. *WB analysis of protein expression of phosphorylated Akt (A) and expression of ERK1/2 (B) after incubation with glucose in combination with ketone body βHB for two hours. The densitometric signal was normalized to GAPDH protein expression, and each group related to the glucose-induced control group. ** p ≤ 0.01, *** p ≤ 0.001; Dunnett's multiple comparison test. Mean ± SEM*

expression also increased (p=0.0029) with addition of 100 mM βHB, as did treatment with 0.1 μM somatostatin (p=0.0007) (Figure 14B).

In summary, this study shows an inhibitory effect of βHB on glucose-stimulated secretion of GLP-1 from GLUTag cells. This study also demonstrates this inhibitory effect for the first time, to our knowledge, in an *in vitro* culture of cells of human origin. Furthermore, the results from this study suggest that the inhibitory effect of the ketone body βHB is mediated through regulation of GPCR signaling kinases Akt and ERK1/2.

4.5 OTHER RESULTS

The GLUTag and human jejunal enteroid cultures were tested for mycoplasma infection, with negative result.

5 DISCUSSION

The overall aim of this thesis was to investigate how the jejunum, and the expression of different proteins in the jejunum, aids in the regulation of glucose homeostasis in the human body, with special interest on the effect of bariatric surgery and diet induced changes.

The first, and fundamental finding, presented in this thesis was the RYGB-induced decrease in jejunal expression of the ketogenesis rate-limiting enzyme HMGCS2. Further, the results presented show, for the first time to our knowledge, the inhibitory effect of the ketone body βHB on glucose-stimulated GLP-1 secretion from EECs. The results also demonstrate a fat-induced jejunal ketogenesis effect on the glucose transporter SGLT1 in jejunal mucosa. Altogether, these findings bring new insights into the jejunal control of the glucose homeostasis and might introduce a new opportunity for the development of anti-obesity and anti-diabetic drugs, specifically targeting intestinal ketogenesis.

In both Paper I and Paper III, we show that a diet enriched in fat increases the jejunal expression of HMGCS2, both in a mouse model and in normal weight and healthy humans. Paper I displays an almost complete down-regulation of HMGCS2 expression following RYGB surgery, while in Paper III the expression of HMGCS2 in jejunum is decreased when switching from a fat-dominated to a carbohydrate-dominated diet. Interestingly, even though expression levels of HMGCS2 decreased when switching from a two-week fat-dominated diet to a two-week carbohydrate-dominated diet in the normal weight and healthy volunteers, the two-week VLCD preceding RYGB had no effect on jejunal HMGCS2 expression in the obese subjects. Possibly, this displays a chronic shift in what effect dietary changes can have. For obese patients a short-term change in diet obviously does not influence the expression of HMGCS2. Instead, it seems likely for obese patients that a more radical and direct modification with surgery is needed to reduce the expression of HMGCS2. Whether this depends on an inherent or acquired difference between the obese and the normal weight subjects remains to be investigated.

RYGB results in both an immediate and long-term reduction of jejunal ketogenesis. Immediately after surgery, the substrate for ketogenesis is depleted from what was previously the jejunum. After RYGB surgery, the Roux limb is deprived of bile, gastric and pancreatic secretions. Therefore, no short-chain fatty acids will be available as substrate for ketogenesis in the Roux

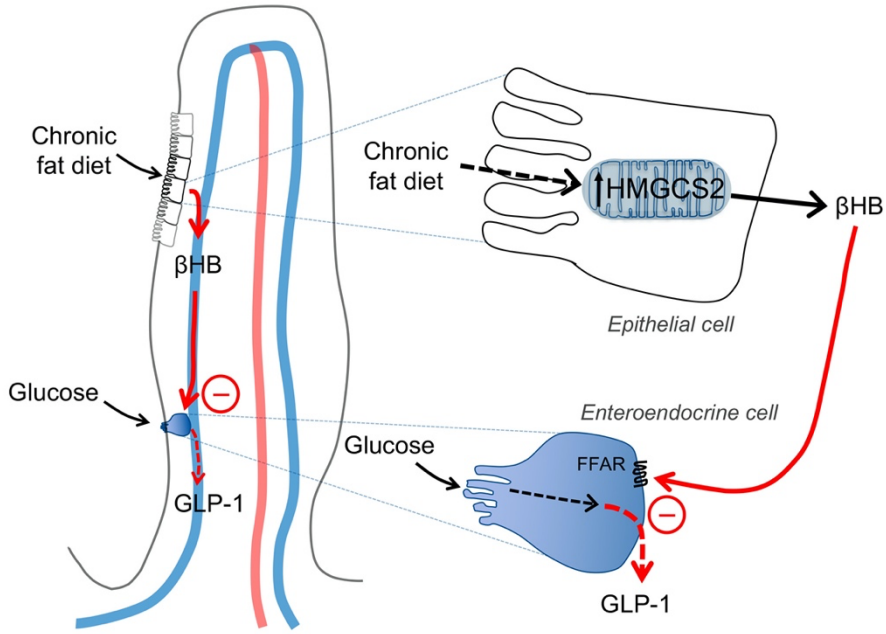

Figure 15. *A proposed mechanism for the inhibition of GLP-1 secretion from EECs targeted by RYGB. Long-term ingestion of fat result in an up-regulation of HMGCS2 and ketone body production. Ketone body βHB reaches EECs via microcirculation and exhibit effect on FFARs, ultimately inhibiting GLP-1 secretion. Figure adjusted under CC-BY-NC license from Wallenius, et al., Suppression of enteroendocrine cell glucagon-like peptide (GLP)-1 release by fat-induced small intestinal ketogenesis: a mechanism targeted by Roux-en-Y gastric bypass surgery but not by preoperative very-low-calorie diet. Gut 69(8), 2020.*

limb since no, or very little, digestion of triglycerides will occur. The bile acids and pancreatic lipases are reintroduced to the ingested food at the jejuno-jejunostomy approximately one to one and a half meters further down the small intestine. This depletion of available short-chain fatty acids in the Roux limb will result in the long-term reduction of jejunal ketogenesis. The mechanism is possibly that short-chain fatty acids are ligands to the nuclear receptor family of PPARs that are known to induce HMGCS2 gene expression in intestinal cells [84, 85].

The inhibitory effect of the ketone body βHB on glucose-induced GLP-1 secretion was shown in both Paper I and Paper IV, and a proposed mechanism can be seen in Figure 15. The exact receptor mechanism for this inhibition is not confirmed, but the results from Paper I indicate that it is inhibited through a $G_{\alpha i}$ mediated receptor signal since the inhibition was sensitive to pertussis toxin. FFAR3 is one of the $G_{\alpha i}$ coupled receptors found in small intestinal epithelial cells, and this receptor is enriched in the EECs [110, 126]. Paper IV

gives further insight in the GPCR regulation, indicating that βHB can result in differential expression and post-translational modifications of kinases ERK1/2 and Akt downstream of GPCR regulated signaling. One plausible reason why a higher dose of βHB was needed for the inhibitory effect on GLUTag cell cultures in Paper IV is the density of these cells. GLUTag cell cultures consists purely of EECs, while a normal jejunum, as well as the enteroid cultures, constitute in the range of less than 1% EECs [127, 128].

Apart from the decreased levels of short-chain fatty acids available in lumen of the Roux limb, RYGB has also been shown to result in hypertrophy [129, 130] and an increased cell proliferation in the Roux limb [118]. Both these findings indicate an increased energy expenditure in the Roux limb. Since absorption of macronutrients is abolished in the Roux limb due to the lack of digestive enzymes necessary for degradation and absorption, the energy for the mucosa itself needs to be extracted from the systemic circulation. This could be an additional explanation for the long-term effects of RYGB surgery on glucose homeostasis. The quantification of carbohydrate transporters in Paper III, displays a pattern that could be in line with this assumption. During HFD, when there is a relative shortage of carbohydrates in lumen of the small intestine, basolateral glucose transporter GLUT2 is up-regulated. This up-regulation could be due to the epithelial layer of the small intestine being in need of energy for itself. When there is a shortage of absorbable carbohydrates in the lumen, this energy is instead needs to be absorbed from the systemic circulation. Studies of changes of the glucose absorption after RYGB surgery has previously demonstrated such adaption[131, 132].

Ketogenesis might be a fat-sensory mechanism for the small intestinal epithelium. Studies in rodents have shown small intestinal HMGCS2 to be up-regulated in response to diets with high fat content [90-93]. These findings correspond well with our findings in Paper I that HFD induces HMGCS2 expression in the small intestine, and specifically in the jejunum and much less in other part of the small intestine. This fat-sensory mechanism is also demonstrated in Paper III. When the healthy individuals were given a fat-dominated diet, jejunal expression of SGLT1 decreased since the carbohydrate content of the lumen decreased and the need for transport of glucose from the lumen was decreased. Studies in Caco-2 cells showed a sirtuin dependent silencing of SGLT1 expression through fat-induced ketogenesis. A schematic presentation of this mechanism can be seen in Figure 16.

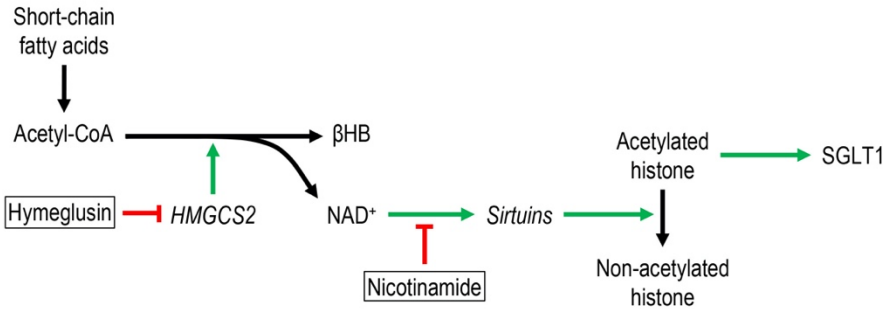

Figure 16. *Proposed mechanism for fat induction of jejunal ketogenesis activated sirtuin-controlled silencing of SGL1 expression. Black, green and red arrows indicate reactions, stimulations and inhibitions, respectively. Adopted from Elebring, et al. A fatty diet induces a jejunal ketogenesis which inhibits local SGLT1-based glucose transport – results from a randomized cross-over study between iso-caloric high-fat versus high-carbohydrate diets in healthy volunteers. In submission.*

The results of Paper III, demonstrating a ketogenesis controlled sirtuin deacetylation of histone, contrast somewhat to previous studies. It has been shown previously that addition of βHB to Caco-2 cultures has increased histone acetylation [82]. It is reported that this increased histone acetylation is achieved by βHB-dependent inhibition of HDAC enzymes. However, this effect could possibly also be because the addition of exogenous βHB shift the ketogenesis equilibrium. Addition of exogenous βHB and a possible shift in equilibrium could result in lowered NAD^+ levels and therefore also reduced sirtuin activity.

From Paper II it can be concluded that a two-week diet dominated by fat or carbohydrates does not result in any meal-induced changes in glucose nor insulin profiles. Interestingly, HFD resulted in elevated postprandial levels of GLP-1. This finding could be found contradictory to the results from Paper I and Paper IV to some extent, where it was demonstrated that ketone body βHB inhibits GLP-1 secretion. It would be reasonable to believe that the HFD, with increased jejunal ketogenesis, would result in decreased levels of GLP-1 during the meal test. The fact that this was not the case in the healthy and normal weight subjects in Paper II could be due to a compensatory effect. When exposed to a fat-dominated diet, and with increased inhibitory pressure, a short-term strategy to compensate for the inhibitory effect could be to increase the expression of EECs. We have unpublished data from immunofluorescence stainings of jejunal tissue after HFD and HCD, respectively, indicating an increased number of EECs in the jejunal mucosa after HFD. The reason why the increased levels of GLP-1 was not reflected in the insulin profiles is somewhat puzzling but could be simply because glucose

levels were kept at bay anyway, or it could be a sign of an early development of insulin resistance, also suggested by the metabolomics data in this study.

The study on normal weight and healthy volunteers for Papers II and III was designed as a clinical trial. At the time of design, other outcomes were listed as primary, and these outcomes were used for the power calculation. This power calculation might not have been optimal for the outcomes reported in Papers II and III, but still significant differences could be detected.

In conclusion, the results from the papers included in this thesis show that intestinal ketogenesis can be induced by a prolonged fat-dominated diet, and that the rate-limiting enzyme of ketogenesis, HMGCS2, is almost completely abolished following RYGB surgery. The results also display a capacity of the jejunal ketogenesis to influence glucose homeostasis by both inhibition of GLP-1 secretion from EECs and by regulation of jejunal glucose transporters.

6 CONCLUSIONS

The studies included in this thesis show that intestinal ketogenesis is induced with a fat-dominated diet, and that the rate-limiting enzyme of ketogenesis, HMGCS2, is almost completely abolished with RYGB.

A two-week HFD to healthy volunteers result in no change on the systemic glucose homeostasis after a meal test, compared to when fed a HCD but result in elevated levels of GLP-1 and metabolites reported to be related to an early onset of insulin resistance.

SGLT1 expression is decreased in healthy volunteers when fed a HFD for two-weeks, compared to a HCD. This decrease is regulated by intestinal ketogenesis through the activation of epigenetic regulation enzyme sirtuin.

The ketone body βHB inhibit GLP-1 secretion from EECs, which has been demonstrated in murine primary cells, murine cell line and in enteroids of human origin. βHB result in changed phosphorylation pattern of kinases involved in GPCR signaling and it is therefore believed that the inhibitory effect of βHB is mediated through a receptor coupled to G-protein signaling.

7 FUTURE PERSPECTIVES

The outcomes of this thesis spark interesting new research ideas for future projects. Each of the individual studies can be followed up with new studies digging deeper into the mechanisms and ideas emerging from the different studies.

A continuation of Paper I is for example to investigate the expression of HMGCS2 after the other major bariatric surgery technique used today, SG. Beneficial and immediate effects of this surgical method, as with RYGB, have been seen on incretin secretion profiles [71]. With SG, a large part of the stomach is removed along the greater curvature. This reduce the reservoir capacity of the stomach and the transition of food through the gastric tube is more rapid, reducing the time for ingested food to be digested in the stomach. Along with the removal of the stomach, most of the gastric lipase activity is also lost. Gastric lipases account from about 20% of the total lipid hydrolysis activity during digestion [133]. Thus with SG, lipase activity is decreased through less pre-incubation of the food in the stomach, as well as the removal of gastric lipases activity. Hypothetically, SG could have a similar resolution of jejunal ketogenesis inhibited incretin release as RYGB surgery, but caused by different mechanism. Both surgical techniques could immediately result in substrate depravation for ketogenesis in the jejunum and in the long-term result in decreased expression of HMGCS2. Investigation of HMGCS2 expression in small intestine after SG will bring clarity to whether this mechanism can also be attributed for SG, or if completely different mechanisms are responsible for the increased incretin secretion after SG.

For Paper II and Paper III, it would be interesting to extend the time for each diet to further study the long-term, and more chronic effects of diets on both systemic metabolic level and on local level in the gut. It would also be interesting to do meal test with serial blood collection with each of the diet types, HFD and HCD, as the test meal to see whether these would display different stimulatory effects. This might bring more insights into how chronic ingestion of diets dominated by fat or carbohydrate affect glycemia, insulinemia and incretin profiles. It is possible that the postprandial GLP-1 profiles after a test meal with each respective diet would be different. As mentioned before, it can be hypothesized that the increased levels of GLP-1 during MMT after two weeks of HFD might be due to a compensatory effect with more EECs expressed to account for the inhibitory effect of the fat-induced ketogenesis.

Since the inhibitory effect of ketone bodies is thought to be mediated through inhibition of lipid-induced receptors, FFARs, an interesting continuation of Paper IV would be to study the effect of ketone bodies on fat-induced GLP-1 secretion. It is possible that the inhibitory effect of ketones would be even greater on fat-induced GLP-1 secretion from EECs. It would also be interesting to establish jejunal enteroid monolayers from obese subjects. The response to ketone bodies of the jejunal enteroid of obese origin could be compared to the response seen in normal weight and healthy individuals in order to investigate if enteroids from obese subjects would display different responses.

A long-term extension of this thesis would be to do *in vivo* trials in animal models or in humans to further study the link between intestinal ketogenesis, incretin secretion and glucose homeostasis, and to study intestinal ketogenesis as a possible target for future anti-diabetic drugs and treatments.

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APPENDIX

Paper I - IV