The role of incretin hormones and glucagon in patients with liver disease

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THE 4 ORIGINAL PAPERS ARE

1. **Junker AE**, Gluud L, Holst JJ, Knop FK, Vilsbøll T. Diabetic and non-diabetic patients with non-alcoholic fatty liver disease have impaired incretin effect and fasting hyperglucagonaemia. J Intern Med. 2016 May;279(5):485-93.

2. Influence of gastrointestinal factors on glucose metabolism in patients with cirrhosis. **Junker AE**, Gluud LL, Holst JJ, Knop FK, Vilsbøll T. J Gastroenterol Hepatol. 2015 Oct;30(10):1522-8.

3. Effects of glucagon-like peptide-1 on glucagon secretion in patients with non-alcoholic fatty liver disease **Junker AE**, Gluud LL, van Hall G, Holst JJ, Knop FK, Vilsbøll T. J Hepatol. 2016 Apr;64(4):908-15.

INTRODUCTION

Within the last 30 years an obesity epidemic has developed. The World Health Organisation (WHO) estimates that, worldwide, 35% of the adult population are overweight with a body mass index (BMI) of 25.0-29.9 kg/m2 and 11% are obese (BMI ≥30 kg/m2) (1). The consequence is an increase in obesity-related health problems like insulin resistance and type 2 diabetes. Obesity is also associated with non-alcoholic fatty liver disease (NAFLD) (2), which covers a spectrum from steatosis to non-alcoholic steatohepatitis (NASH) that may lead to fibrosis, cirrhosis and hepatocellular carcinoma. At present, NAFLD is the most common liver disorder in the Western world and is predicted to be the most common indication for liver transplantation by 2020 (3).
The liver plays a central role in carbohydrate metabolism. During fasting, hepatic glucose production ensures a steady supply of glucose to vital organs such as the central nervous system. In the postprandial state, hepatic glucose production is suppressed and the liver shifts it's handling of glucose to fit the anabolic state; storing of glucose as glycogen. These mechanisms contribute to the clearance of circulating glucose after a carbohydrate-rich meal (4) and disturbances may lead to impaired glucose metabolism. Thus, up to 70% of patients with type 2 diabetes have NAFLD (2) and 30% of patients with cirrhosis have type 2 diabetes (5).
Another very important mechanisms for maintaining stable plasma glucose levels is the incretin effect. The incretin effect refers to the amplification of glucose-induced insulin secretion exerted by the gut-derived incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (6).
The pathogenesis of type 2 diabetes includes reduced beta cell function and insulin resistance (7). In the last decades, a defective incretin hormone system, resulting in an impaired incretin effect, has been recognised as important in the pathogenesis of type 2 diabetes (6). However, the impact of NAFLD and cirrhosis on the incretin hormone system has only been sparsely investigated with conflicting result. One study showed impaired GLP-1 and normal GIP responses in patients with NAFLD (8). In another study, patients with NASH were characterised by prolonged GIP secretion in response to an oral fat load compared to controls (9). In patients with cirrhosis, GLP-1 and GIP secretion has been shown to be increased, but at the same time first phase insulin responses are impaired (10). The effect of the incretin hormones on insulin and glucagon secretion in patients with NAFLD and cirrhosis has not been investigated.
As alluded to above, the prevalence of glucose intolerance and type 2 diabetes is high in patients with NAFLD and cirrhosis. The objective of the present studies was, therefore, to investigate the function of the incretin hormones and glucagon in relation to glucose metabolism in patients with liver disease. Firstly, we assessed the impact of NAFLD on the incretin effect in patients with or without type 2 diabetes. Secondly, we investigated the influence of cirrhosis on incretin physiology and, finally, we examined the glucagonostatic effect of GLP-1 and its potential glucose-dependency in non-diabetic patients with NAFLD.

INCRETIN PHYSIOLOGY

In healthy glucose tolerant individuals, the incretin effect accounts for up to 70% of the insulin response after food intake (11) (Figure 1). The incretin effect describes the phenomenon that ingested glucose mediates a much greater insulin response than intravenous glucose (12). Food intake stimulates secretion of the gut-derived hormones, GLP-1 and GIP that mediate the incretin effect. GLP-1 is a 30-amino acid peptide (GLP-17-36) processed from proglucagon in enteroendocrine L cells. The endothelium in the distal ileum and parts of the colon has the highest density of L

cells. GIP consists of 42 amino acids (GIP1-42) and is processed from proGIP in enteroendocrine K cells. The mucosa in the duodenum and upper jejunum has the highest density of K cells. Both hormones are degraded by the enzyme dipeptidyl peptidase 4 (DPP-4) found in plasma and in most tissues including a soluble form in the blood. The degradation of GLP-1 in the circulation happens within a few minutes (<2 min), whereas GIP has a longer half-life (~7 min) (13-16). The result of DPP-4 degradation is the formation of inactive metabolites, which the kidneys clear (17). Because of the rapid degradation, the amount of active GLP-1 that reaches the portal circulation is only 25% of what was produced. The degradation continues in the portal vein and in the liver and, thus, only 10-15% of the active forms of GLP-1 reaches the systemic circulation (12). GIP is not degraded as extensively and fast, about 50 % of circulating GIP occurs in the intact form (18).

In the pancreas, the incretin hormones stimulate the beta cells to secrete insulin. The insulin stimulatory effects of GLP-1 and GIP are additive (19) and highly glucose dependent. Because of the latter, the hormones do not stimulate insulin secretion at plasma glucose concentrations below 4 mmol/l (20). GLP-1 and GIP most likely contribute about equally to the potentiation of glucose-induced insulin secretion during meals, however GLP-1 seems to be more efficacious at high glucose levels (21).

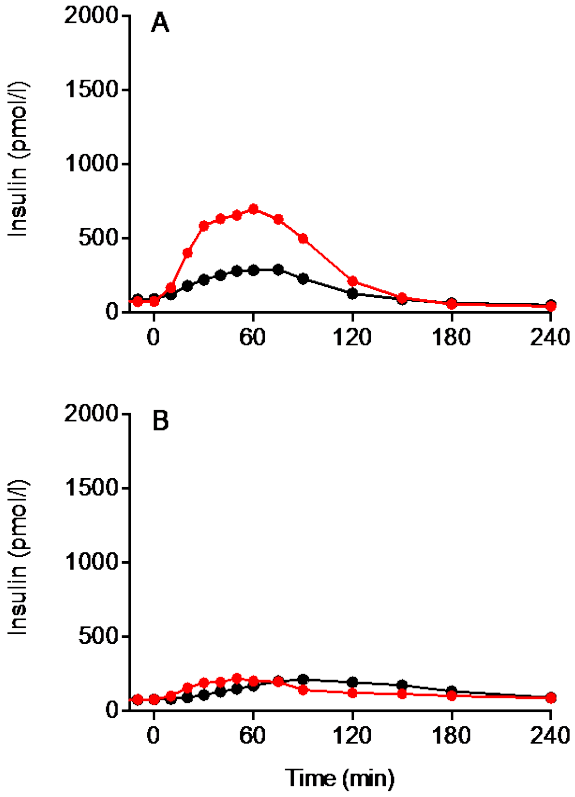
Because only small amounts of active GLP-1 reach the circulation, it is likely that other pathways mediate some of its insulinotropic effects. Evidence suggests that vagal sensory afferents in the gut and in the hepato-portal vein may be important (22). Indeed, the insulin response and activation of autonomic nerves is more pronounced after intraportal glucose infusion compared to glucose infused in a peripheral vein. Interestingly, some studies have indicated that vagal nerve endings in the hepato-portal region have GLP-1 receptors (23) and that intraportal infusion of GLP-1 receptor agonists increases glucose disposal (24). Thus, the insulinotropic effects of GLP-1 may depend on long neural reflexes between the portal vein and/or the gut and the pancreas (22).

GLP-1 also strongly inhibits glucagon secretion and gastric emptying at high plasma glucose levels, whereas GIP stimulates alpha cells to secrete glucagon when plasma glucose concentrations are low (12,25).

GLUCAGON PHYSIOLOGY

Glucagon is a peptide hormone produced by the alpha cells in the pancreas. Glucagon is insulin's major counteracting hormone and acts to secure plasma glucose homeostasis. The mechanisms underlying the regulation of glucagon secretion are complex and involve nutrients, nerves, and hormonal factors (26). Hypoglycaemia is a powerful stimulus for glucagon secretion. Glucagon stimulates hepatic glycogenolysis and gluconeogenesis and hereby releases glucose to the circulation (26). GIP, some amino acids, and the autonomic nervous system can also stimulate glucagon secretion (27-30). Following meal ingestion, a rise in plasma glucose concentrations and insulin suppresses glucagon release. Glucose may cause an increase in adenosine triphosphate (ATP) that depolarizes ATP-sensitive K+-channels in the alpha cell. The resulting depolarization inactivates Ca2+-channels and hereby supresses glucagon secretion (31). In addition, somatostatin and GLP-1 also suppress glucagon (32-35). However, the exact mechanisms by which these factors work together to regulate glucagon remain unclear. Some evidence suggests that during low plasma glucose levels, voltage-dependent sodium and calcium channels maintain glucagon secretion (36). Another theory is the intra-islet hypothesis, which states that insulin released from beta

Figure 1:



The incretin effect. Plasma insulin concentrations after a 50g oral glucose tolerance test (OGTT) (red) and an isoglycaemic intravenous glucose infusion (IIGI) (black) in healthy individuals (A) and patients with type 2 diabetes (B). ***(Study I)***

cells inhibits glucagon secretion from neighbouring alpha cells. Insulin would hereby tonically inhibit glucagon, and as insulin concentrations decrease in response to low plasma glucose levels, glucagon levels would rise (37). There is, however, evidence derived from studies of incretin hormones, which disputes the intra-islet hypothesis. As an example, studies of perfused rat pancreas show that at low glucose and insulin levels, GLP-1 can stimulate somatostatin from neighbouring delta cells located in the pancreatic islets. GLP-1 hereby inhibits glucagon release secretion via somatostatin release (33). In agreement, GLP-1 powerfully suppresses glucagon secretion in patients with type 1 diabetes without residual insulin secretion (38).

TYPE 2 DIABETES

Type 2 diabetes is characterised by an inability to adjust insulin secretion to insulin sensitivity (6). WHO estimates that 9% of the adult world population have diabetes (39). In Denmark, the prevalence is above 300.000 (40). The hallmarks of type 2 diabetes are insulin resistance and beta cell dysfunction, which progress as individuals advance from normal to impaired glucose tolerance and, finally, to overt type 2 diabetes. The pathogenesis of type 2 diabetes involves numerous genetic variants in combination with sedentary lifestyle and high caloric food (7). Ectopic fat accumulation in both muscle tissue and the liver is recognised to play a role in the development of insulin resistance. Ectopic fat distribution in muscle and especially in liver tissue is associated

with insulin resistance, but the mechanisms are unclear (see Non-alcoholic Fatty Liver Disease, Pathogenesis) (41).

Incretin pathophysiology in patients with type 2 diabetes

The pathogenesis of type 2 diabetes also involves an impaired or lost incretin effect (42). An impaired incretin effect hinders patients with type 2 diabetes from adjusting insulin secretion to their insulin need, which causes postprandial hyperglycaemia (43). In addition, a progressive increase of body mass index and reduction of glucose tolerance independently diminishes the incretin effect (44). It could therefore be argued that impaired incretin effect is innate in the diabetic state. However, first-degree relatives of patients with type 2 diabetes have a normal incretin effect, making a role for genetic factors less obvious (45). In addition, normalisation of glycaemic control was able,at least partly, to restore the insulin responses to GLP-1 and GIP in patients with type 2 diabetes (46). The incretin effect is also restored in women with gestational diabetes after giving birth (47). Thus, a reduced incretin effect seems to be fully reversible and a result of the diabetic state rather than the cause of diabetes (48). Similarly, and impaired incretin effect can be induced in healthy individuals by introducing insulin resistance and/or glucose intolerance (49).

As before mentioned, GLP-1 and GIP are the major hormones responsible for the incretin effect. It would therefore be reasonable to believe that impaired secretion of the incretin hormones cause impaired incretin effect. However, studies of GIP show both reduced (50,51) and even increased concentrations of GIP in patients with type 2 diabetes (52). Likewise, some studies find reduced (50) and others preserved secretion of GLP-1 (53,54). Two recent meta-analyses including 22 and 23 trials, respectively, concluded that GIP and GLP-1 secretion were normal in patients with type 2 diabetes (55,56). Thus, reduced incretin effect may not only be a result of reduced GLP-1 and/or GIP secretion. Evidence also points to a decreased sensitivity to the insulinotropic effects of GIP (57,58) and reduced potency of GLP-1 (59). In patients with type 2 diabetes it is possible to restore the insulinotropic effect using pharmacological doses of GLP-1 but not when using GIP (59,60).

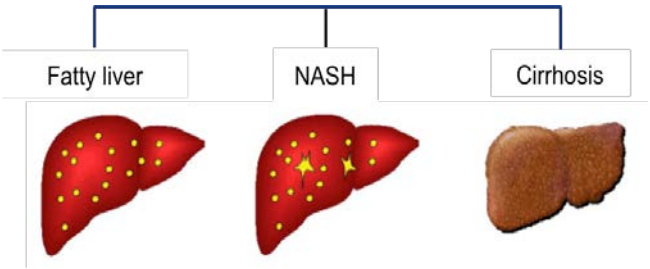
Glucagon pathophysiology in type 2 diabetes

Most patients with type 2 diabetes have fasting and postprandial hyperglucagonaemia. Hyperglucagonaemia stimulates hepatic glucose production, which contributes to hyperglycaemia both in the fasting and postprandial state. Interestingly, patients with type 2 diabetes exhibit immediate glucagon suppression to an intravenous infusion of glucose, suggesting that the alpha cell functions normally with respect to glucagon suppression. Therefore, the exaggerated glucagon responses to oral glucose may involve gastrointestinally derived factors. Alpha cell hypersensitivity to GIP has been proposed to explain the phenomenon (61). Evidence also points to a positive correlation between insulin resistance and hyperglucagonaemia in obese subjects (62,63).

NON-ALCOHOLIC FATTY LIVER DISEASE

NAFLD is defined as fat infiltration exceeding more than 5% of hepatocytes in the absence of alcohol abuse (>20g/day for women and >30g/day for men) and/or use of steatogenic drugs (e.g. amiodarone, tamoxifen and glucocorticoids) (64). The spectrum of NAFLD is wide and ranges from simple steatosis to NASH (see below) with risk of fibrosis and finally development of cirrhosis (Figure 2) (3). NAFLD is regarded as the hepatic manifestation of the metabolic syndrome (65).

Figure 2



The spectrum of NAFLD. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis

Natural history of NAFLD

Progression from steatosis to NASH occurs in around 30% of patients. NASH will lead to cirrhosis in approximately 20% of cases, and of these, 40% will experience decompensated liver failure (66). The relation between NAFLD and type 2 diabetes is complex. NAFLD is an independent predictor of type 2 diabetes; conversely, type 2 diabetes is a perfect metabolic setting for NAFLD to develop (65). Thus, type 2 diabetes, once present in NAFLD, promotes development of NASH, cirrhosis and hepatocellular carcinoma. Furthermore, NAFLD is an independent risk factor of cardiovascular disease (65).

Prevalence and diagnosis of NAFLD

The estimate of NAFLD prevalence varies according to the diagnostic method and study population (3). NAFLD is often underdiagnosed because patients are asymptomatic (67). Up to 80% of NAFLD patients have normal levels of alanine aminotransferase (68). Alanine aminotransferase also correlates poorly with NAFLD stage (69), particularly in patients with type 2 diabetes (70). In the general population, the prevalence of NAFLD, as assessed by liver function tests, is 3 to 12% (2). When NAFLD is diagnosed by ultrasound, the prevalence ranges from 10 to 24% (64). When diagnosed by magnetic resonance spectroscopy, the prevalence of NAFLD reaches 30% (68). In high-risk populations, the prevalence and severity of NAFLD increases (65). Thus, in patients with the metabolic syndrome 54% have NAFLD and the prevalence reach 94% in severely obese individuals (BMI>30 kg/m2) (2).

The prevalence of NASH in the general population is estimated to 3-16% (71,72) with a rise to 25-30% in obese patients. In obese patients with type 2 diabetes, the prevalence of NASH is more than 35% (3,65). Ultrasound is recommended as a first-line evaluation procedure if liver function tests are elevated, but has high inter-observer variability and hepatic fat infiltration fat must exceed 20 to 30% to be detected. Magnetic resonance spectroscopy is expensive and not widely available, but can identify >5.5% hepatic fat infiltration. The diagnosis of NASH requires a liver biopsy. The NAFLD fibrosis score can help identify patients with a high risk of NASH and fibrosis and thus, eligible for a liver biopsy. The score is based on body mass index, age and levels of alanine aminotransferase, aspartate aminotransferase, platelets and albumin in blood and the presence/absence of diabetes (73). A liver biopsy is the only way to distinguish simple fat infiltration from NASH.

Pathogenesis

The pathogenesis of NAFLD is complex and involves several parallel events. Insulin resistance is central to the progression from healthy to fatty liver, but is a result of several mechanisms (74,75). Delivery of free fatty acids to the liver accounts for nearly

two-thirds of hepatic lipid accumulation (76). Free fatty acids, in turn, may originate from dietary intake. A study carried out in young adults showed that a weight gain of 10% induced by high caloric food and sedentary life style increased liver fat 2.5 fold (77). Another important origin of free fatty acids is adipose tissue. In the adipose tissue, insulin resistance impairs triglyceride oxidation, which increases the flux of free fatty acids to the liver and contributes to steatosis (78). In addition to free fatty acids, increased de novo lipogenesis also contributes to lipid accumulation (76). Excess liver fat leads to deterioration of hepatic insulin signalling, which results in hepatic insulin resistance and compensatory hyperinsulinaemia (79).

Although the fatty liver increases the ability to oxidise fat, prolonged accumulation of lipids can cause lipotoxicity. Lipotoxicity promotes inflammation and apoptosis by several mechanisms. The main route of hepatic fat oxidation is the mitochondrial tricarboxylic acid (TCA)-cycle. An overactive TCA-cycle is able to stress the endoplasmic reticulum and results in formation of reactive oxidative species (ROS) that eventually will cause mitochondria dysfunction and mediate inflammation (80,81). Mitochondrial dysfunction also leads to formation of toxic and proinflammatory lipid metabolites like ceramides and diacylglycerol (82). Furthermore, insulin resistant adipose tissue may also promote inflammation by reducing release of anti-inflammatory adipokines such as adiponectin and increasing release of pro-inflammatory cytokines like interleukin 6 (IL-6) and tumour necrosis factor-alpha (TNF-α). Indeed, patients with NAFLD have decreased plasma concentrations of adiponectin, inversely related to the degree of inflammation and fibrosis (83). An imbalance of anti- and pro-inflammatory cytokines may therefore contribute to NASH and liver fibrosis. Finally, genes like, patatin-like phospholipase domain containing 3 (PNPLA3) and altered gut flora, have also been linked to development of steatosis and inflammation (65).

Incretin hormones and NAFLD

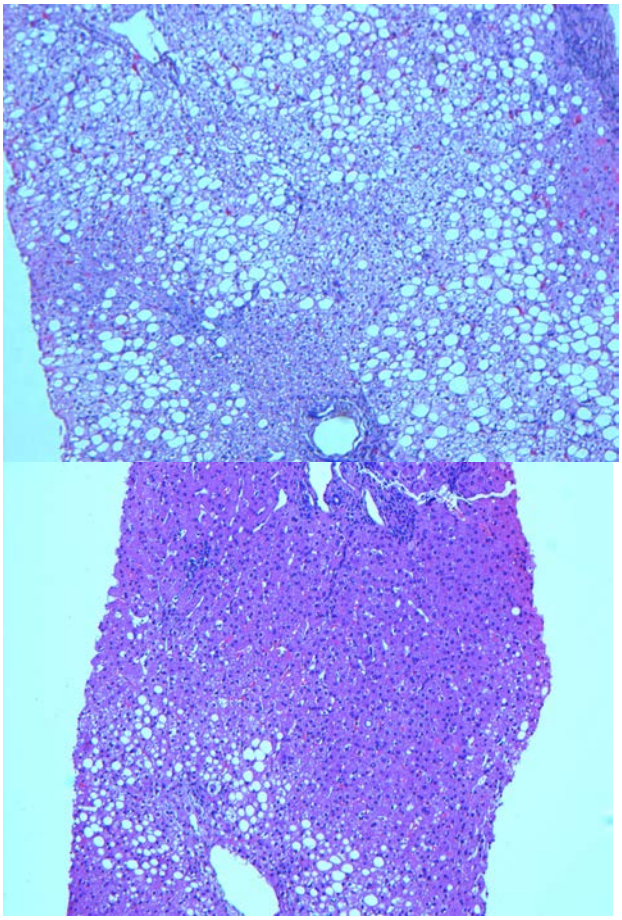
Some evidence suggests that GLP-1 may be directly or indirectly involved in the pathogenesis of NAFLD, but the underlying mechanism is unclear. The presence of GLP-1 receptors on hepatocytes is controversial. Initial reports suggested that GLP-1 receptor agonists reduce hepatic steatosis (84). Further studies identified GLP-1 receptors and GLP-1 receptor mRNA in hepatocyte cell cultures (85) and in human liver biopsies (86). Subsequent studies have confirmed that GLP-1 receptor agonists reduce steatosis by increasing hepatic lipid oxidation and reducing lipogenesis (85,87-89), but expression of GLP-1 receptor mRNA in hepatocytes has not been confirmed (89-91), suggesting that the effects of GLP-1 are indirect, possible mediated via insulin. An altered degradation of GLP-1 may be related to changes in insulin levels. Thus, there is evidence that patients with NAFLD have enhanced activity of the enzyme DPP-4 compared to healthy controls (92). Moreover, DPP-4 activity in serum and hepatic expression of DPP-4 are correlated negatively to NAFLD severity (93). DPP-4 deficient rats have decreased levels of hepatic pro-inflammatory and profibrotic cytokines and reduced hepatic steatosis compared to wild type rats (88). Taken together, NAFLD patients may have increased DPP-4 activity resulting in lower levels of biologically active GLP-1, which - probably indirectly - may contribute to steatosis.

Glucagon and NAFLD

It is recognised that lipid metabolism involves glucagon (31). Glucagon stimulates the hormone sensitive lipase in adipose tissue, which results in increased release of free fatty acids to the circulation and the liver (94) although this may not apply to humans (95). In a rodent model of hyperlipidaemia, glucagon also suppressed serum cholesterol, very low density lipoprotein (VLDL) and triglycerides levels (96). Some studies have proposed a role of glucagon in NAFLD, but the evidence is inconclusive. In one study, glucagon receptor knockout mice fed a high fat diet were resistant to steatosis (97), whereas another study demonstrated susceptibility to steatosis (98). There is evidence however, that steatosis deteriorates glucagon action in the liver. In rodents, steatosis decreases hepatic expression of glucagon receptors and hepatic glucose production is impaired in response to glucagon infusion (99,100). Thus, potentially hepatic glucagon resistance may be a consequence of steatosis (101). Furthermore, in humans high glucagon concentrations positively correlate with increased liver enzymes (102). Whether hyperglucagonaemia is directly involved in the pathogenesis of NAFLD or is a consequence of steatosis remains unanswered.

Treatment of NAFLD

Treatment options demonstrating a sustained effect in the handling of NAFLD are very limited. Weight loss is effective but difficult to obtain (and maintain) for the majority of patients. A weight loss of 3 to 5% improves steatosis and a 10% decrease in body weight has been shown to reduce hepatic inflammation (103). In line with these considerations, bariatric surgery should be considered in overweight patients with NASH (103). Metformin increases hepatic and muscular insulin sensitivity, but does not improve liver function tests or liver histology in NASH (104). Glitazones (peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists) reduce hepatic steatosis (105), but are rarely used in Europe due to adverse events including heart failure, bladder cancer and loss of bone density (106). One trial suggested that vitamin E 800 IU per day improves liver function tests, steatosis and inflammation, and therefore vitamin E was recommended for NASH in non-diabetic subjects (105). However, there is emerging evidence suggesting that vitamin E may increase mortality (107). Both GLP-1 receptor agonists and DPP-4 inhibitors improve liver function tests in patients with NAFLD and type 2 diabetes (108-110). In an uncontrolled trial of 25 patients with dysregulated type 2 diabetes, GLP-1 receptor agonist treatment reduced hepatic fat content assessed by proton magnetic resonance spectroscopy. Patients achieved hepatic fat reduction independently of weight loss, but the reduction was positively correlated to improvement in glycaemic control (111). We recently reported a case of reduction of hepatic steatosis and inflammation assessed by liver biopsy during agonist therapy. The patient had type 2 diabetes and NASH and was treated with the GLP-1 receptor agonist liraglutide for 46 weeks (112) (Figure 3). Despite emerging evidence of improved hepatic fat content, inflammation and fibrosis following GLP-1 receptor agonist treatment, no controlled trials exist



Liver histology before (upper) and after (lower) treatment with a GLP-1 receptor agonist. Light microscopy of liver tissue (haematoxylin-eosin x 100 magnification). Upper panel: Liver tissue with hepatic fat infiltration affecting >66% of hepatocytes, ballooning cells and lobular inflammation consistent with NASH (NAFLD activity score 5). Peri-cellular and peri-portal fibrosis (fibrosis score 2). Lower panel: Liver tissue with hepatic fat infiltration affecting 40-50% of hepatocytes, sparse lobular inflammation and no ballooning cells consistent with simple steatosis (NAFLD activity score 2). Peri-cellular and periportal fibrosis (fibrosis score 2)

CIRRHOSIS

Cirrhosis is the end-stage of chronic liver disease. Cirrhosis is characterised by diffuse fibrosis and nodular regeneration of hepatocytes. Alcoholic liver disease and NAFLD are the most common aetiologies of cirrhosis (113,114). Patients with cirrhosis can decompensate, which causes a series of severe complications (115), including portal hypertension, ascites, hepatic encephalopathy and varicial haemorrhage (116). Some cirrhotic patients also develop parasympathetic neuropathy and sympathetic hyperactivation (117). In addition, liver damage causes metabolic alterations of lipid, protein and glucose metabolism. Accordingly, a majority of patients with cirrhosis suffer from glucose intolerance (118). The complications to cirrhosis increase mortality and morbidity, and up to 40% die within the first year of diagnosis (119).

In hepatology, the Child Pugh score (A, B or C) is often used to assess the prognosis of chronic liver disease, mainly cirrhosis. The score is based on biochemical tests (bilirubin, albumin, prothrombin time) and the presence of ascites and hepatic encephalopathy (120).

Hepatic diabetes

The WHO estimates that up to 70% of patients with cirrhosis are glucose intolerant and 30% have manifest diabetes (121). Diabetes following cirrhosis is termed “hepatic diabetes” and represents a different entity than type 2 diabetes. Patients with hepatic diabetes are more insulin resistant, but have fewer microvascular complications (122). In addition, hepatic diabetes is associated with higher portal pressure (5,114) and with an increased risk of hepatocellular carcinoma and death (123).

The pathogenesis of glucose intolerance in cirrhosis is still poorly understood. Normally, the liver takes up one third of ingested glucose (124). However, because of reduced hepatic extraction and porto-systemic shunting in patients with cirrhosis, glucose appears more quickly in the circulation which result in various degrees of glucose intolerance. Additionally, hepatic insulin resistance is pronounced in patients with cirrhosis, which also contributes markedly to glucose intolerance (125). Several mechanisms lead to insulin resistance in cirrhosis. One mechanism is an impaired growth hormone - insulin growth factor-1 axis. The cirrhotic liver produces less insulin growth factor-1, which result in reduced negative feedback to the pituitary gland and therefore higher concentrations of growth hormone. High growth hormone concentrations impair the actions of insulin and cause insulin resistance (126). Furthermore, insulin receptors are down regulated in the cirrhotic liver, which contributes to insulin resistance. Accordingly, the beta cells compensate by increasing insulin secretion causing hyperinsulinaemia (118). This is supported by studies of pancreatic islets from patients with cirrhosis demonstrating higher proliferation and less apoptosis of beta cells together with increased sensitivity to glucose (127,128). Finally, patients with cirrhosis have decreased hepatic extraction and shunting of insulin, which also may contribute to their hyperinsulinaemic state (129,130).

Another consistent finding in cirrhosis is hyperglucagonaemia (131,132). Hypersecretion from alpha cells rather than shunting seems to cause elevated glucagon levels (133-135), but little is known about the metabolic consequences. Glucagon infusion fails to suppress hepatic glucose production in cirrhosis, which may imply decreased hepatic glucagon sensitivity (101). In summary, patients with cirrhosis are insulin resistant and have high plasma levels of insulin and glucagon - and hepatic diabetes seems to develop as beta cell failure progresses and compensatory insulin secretion diminishes.

Incretin hormones and cirrhosis

Data on GLP-1 and GIP in cirrhosis are limited. One study showed that non-diabetic patients with Child Pugh A and B (120) cirrhosis had exaggerated responses of GLP-1 and GIP after an oral glucose tolerance test (OGTT). Indeed, peak levels of both incretin hormones were 2 times higher than controls but, nevertheless, patients still had impaired first-phase insulin responses (10). Another study compared GLP-1 responses to a mixed meal in normal glucose tolerant individuals and patients with diabetes and cirrhosis. Hormones were measured directly in the portal vein, and identified no difference between diabetic and non-diabetic patients. These data suggest that impaired sensitivity of GLP-1 and GIP, and not blunted secretion, contributes to impaired glucose tolerance in cirrhosis (134).

GLP-1-based therapy may have therapeutic potential in individuals with hepatic diabetes. Pancreatic islets from rats with cirrhosis have impaired insulin responses to glucose but, interestingly, the responsiveness can be restored after incubation with GLP-1 (121). In a case study, a patient with cryptogenic cirrhosis treated with a GLP-1 receptor agonist showed clinical improvement of cirrhosis

(reduction of spleen size, increased platelet count and albumin and improvements of liver function tests) (136).

HYPOTHESES AND OBJECTIVE

The objective of this thesis was to investigate the impact of liver dysfunction on incretin and glucagon (patho)physiology in relation to glucose metabolism. The liver is pivotal to glucose homeostasis, and patients with liver disease often have glucose intolerance. We therefore hypothesised that normal glucose tolerant patients with NAFLD would develop reduced incretin effect and that NAFLD would aggravate the incretin effect in patients with existing type 2 diabetes. Thus, in study I we investigated the incretin effect and glucagon secretion in patients with NAFLD with and without type 2 diabetes and in controls. We further hypothesised that the incretin effect would be disturbed in non-diabetic patients with more severe liver disease. The objective of study II was therefore to investigate the incretin effect in patients with cirrhosis. Finally, the hypothesis in study III was that an impaired glucagonostatic effect of GLP-1 contributes to the hyperglucagonaemic state of patients with liver disease. We therefore explored the glucagonostatic properties of GLP-1 in non-diabetic patients with NAFLD.

SUMMARY OF STUDIES

Study I

In study I we explored the influence of histologically verified NAFLD on the incretin effect, GLP-1, GIP and glucagon in patients with normal glucose tolerance or type 2 diabetes.

Four age, sex and body mass index-matched groups of participants were studied: 1) normal glucose tolerance and NAFLD (n=10); 2) type 2 diabetes and NAFLD (n=10); 3) type 2 diabetes and no liver disease (n=8) and 4) healthy controls (n=10). All participants underwent a 50g-OGTT and an isoglycaemic intravenous glucose infusion (IIGI). We determined the incretin effect by relating beta cell secretory responses during the OGTT and IIGI. Controls exhibited higher incretin effect (70±30%, median±interquartile range) compared to the remaining three groups ($p < 0.001$): 55±26% in the non-diabetic NAFLD patients, 33±41% in NAFLD patients with type 2 diabetes and 5 ± 22% in patients with type 2 diabetes and no liver disease. Fasting hyperglucagonaemia was seen in both non-diabetic and type 2 diabetic NAFLD patients (7.5±3.3 and 7.5±9.9 pmol/l, $p = 0.78$) whereas patients with type 2 diabetes and no liver disease exhibited similar fasting glucagon levels as controls (4.5±3.1 and 4.5±4.3 pmol/l, $p = 0.72$). All groups had similar GLP-1 and GIP responses.

We concluded that patients with NAFLD have reduced incretin effect and impaired handling of ingested glucose. NAFLD was also associated with fasting hyperglucagonaemia independent of their type 2 diabetes.

Study II

Study II investigated the influence of gastrointestinal factors, including the incretin effect, in non-diabetic patients with cirrhosis.

We included ten patients with compensated Child Pugh A and B cirrhosis and ten matched healthy controls. Patients had both alcoholic cirrhosis and cirrhosis caused by primary biliary cirrhosis. All underwent a 4 hour 50g-OGTT and an IIGI. We calculated the incretin effect based on insulin, C-peptide, and insulin secretion rates and the gastrointestinal-mediated glucose disposal. Despite higher levels of GLP-1 and GIP, patients with cirrhosis had reduced incretin effect based on both insulin (24±42 vs. 70±30%, $p=0.002$), C-peptide (35±44 vs. 55±30%, $p=0.008$). Patients with cirrhosis also had markedly lower gastrointestinal-mediated glucose disposal than controls (30±23 vs. 52±20%, $p=0.003$). Despite findings of fasting hyperglucagonaemia, both oral and intravenous glucose suppressed plasma glucagon in cirrhosis. We therefore conclude that patients with cirrhosis have impaired handling of oral glucose and reduced incretin effect, which may contribute to their glucose intolerance.

Study III

In study III, we evaluated the glucagon-suppressive effect of GLP-1 and its potential effects on endogenous glucose production and whole body lipolysis in non-diabetic patients with NAFLD.

Ten non-diabetic patients with liver biopsy-verified NAFLD (NAFLD activity score 2.5±1.0) and 10 matched controls underwent a 2-hour intravenous GLP-1 (0.8 pmol × kg-1 × min-1) and placebo infusion on two separate days. Since GLP-1-mediated glucagon suppression has been shown to be glucose-dependent, plasma glucose was clamped at fasting level during the first hour, then raised and clamped at 'postprandial level' (fasting plasma glucose level plus 3 mmol/l) for the remaining hour. We evaluated relative plasma levels of glucagon, endogenous glucose production and whole body lipolysis rates with stable isotopes and also calculated the respiratory quotient using indirect calorimetry.

Compared to controls, patients with NAFLD were insulin resistant (homeostatic model assessment (HOMAIR): 3.8±2.2 vs. 1.6±1.5, $p=0.003$) and had higher fasting glucagon concentrations (7.5±5.3 vs. 5.8±1.5 mmol/l, $p=0.045$).

During the placebo infusions, neither group showed suppression of plasma glucagon concentrations at fasting glucose levels (-11±32 vs. 18±63 mmol/l x min-1, NAFLD vs. controls, respectively, $p=0.59$), whereas a similar suppression was observed during 'postprandial' level (-165±32 vs. -135±78 mmol/l x min-1,NAFLD vs. controls, respectively, $p=0.56$). We identified similar glucagon suppression in both groups during the GLP-1 infusion at fasting (-97±75 vs. -93±41 pmol/l × min-1 NAFLD vs. controls, respectively, $p=0.566$) and 'postprandial' plasma glucose levels (-108±101 vs. -97±53 pmol/l × min-1,NAFLD vs. controls, respectively, $p=0.196$). We also showed that patients had impaired GLP-1-induced suppression of endogenous glucose production at fasting and 'postprandial' glucose levels and impaired elevation of respiratory quotient during 'postprandial' glucose levels. The latter reflecting metabolic inflexibility due to insulin resistance.

We concluded that NAFLD patients have high fasting concentrations of glucagon, but preserved glucagonostatic effect of GLP-1, which may be important to sustain normoglycaemia. Furthermore, impaired suppresion of endogenous glucose production and metabolic inflexibility seems to be a characteristic pathological trait of NAFLD before type 2 diabetes has developed.

DISCUSSION

The studies included in this thesis provide important new knowledge about the pathogenesis of glucose intolerance in patients with liver disease by elucidating the role of the incretin hormones and glucagon.

In study I, we found that patients with NAFLD, both with and without type 2 diabetes, had reduced incretin effect. A similar study from another group showed reduced incretin effect in lean NASH patients with normal glucose tolerance (9). However, these authors did not measure plasma concentrations of GLP-1 and GIP. This raises the question whether reduced secretion or decreased action of the incretin hormones causes an impaired incretin effect. Normal GLP-1 and GIP responses in study I suggest that reduced beta cell sensitivity to the insulinotropic effect of the

incretin hormones impairs the incretin effect in patients with NAFLD. This is in line with what is found in patients with type 2 diabetes (6). The normal plasma responses of GLP-1 and GIP in patients with NAFLD from study I are in contrast to a previous study from another group (8). Interestingly, we found a smaller incretin effect in type 2 diabetic patients without NAFLD compared to those with NAFLD alone (study I). Several studies have shown that beta cell dysfunction aggravates the incretin effect (53,47,137). Accordingly, the patients with type 2 diabetes without NAFLD had lower insulin responses indicative of insufficient beta cell function. Thus, impaired beta cell function may therefore explain the difference in incretin effect between the groups with type 2 diabetes (6).

In study II we showed that non-diabetic patients with cirrhosis had reduced incretin effect. In agreement with previous findings (10), patients with cirrhosis had increased levels of GLP-1 and GIP. This suggests, similar to patients with type 2 diabetes (6), that patients with cirrhosis have impaired beta cell sensitivity to the insulinotropic effect of GLP-1 and GIP. In addition, parasympathetic neuropathy and sympathetic hyperactivity in cirrhosis (22,117) may interfere with the neural-mediated effects of GLP-1 (see above, Introduction) and hereby contribute to the reduced incretin effect. In line with this, we also found impaired gastrointestinal-mediated glucose disposal in cirrhotic patients. The mechanisms underlying gastrointestinal-mediated glucose disposal include all factors involved in oral glucose disposal: the incretin effect, the glucagonostatic effects of GLP-1, differences in hepatic glucose uptake and neural reflexes. Patients with type 2 diabetes also have impaired gastrointestinal-mediated glucose disposal mainly because of reduced incretin effect (138). Other factors may however, be more important in cirrhosis. Portal glucose and hereby also oral glucose stimulates hepatic glucose uptake. The phenomenon is probably neurally mediated (139). This neural stimulus may be affected by autonomous neuropathy causing diminished hepatic uptake of glucose and contributes to impaired gastrointestinal-mediated glucose disposal. Similar changes are seen in non-diabetic patients with impaired parasympathetic nerve function due to truncal vagotomy (140), which supports a role for autonomous neuropathy in glucose intolerance. Decreased parenchymal mass and porto-systemic shunting may also contribute to impaired gastrointestinal-mediated glucose disposal in cirrhosis. However, the patients in study II were mostly Child Pugh A and therefore unlikely to have clinical relevant shunts.

In both study I, II and III, patients with NAFLD and cirrhosis had fasting hyperglucagonaemia. A link between liver disease and high glucagon levels is supported by similar observations in patients with chronic viral hepatitis (125), and the notion that elevated liver enzymes are independently associated with fasting hyperglucagonaemia (102). Although most patients with type 2 diabetes also have fasting hyperglucagonaemia, it is not a consistent finding (53,57). Patients in study II had fasting hyperglucagonaemia independently of type 2 diabetes. This implies that NAFLD, and not type 2 diabetes, causes high fasting glucagon levels and that liver damage is central to hyperglucagonaemia. On the other hand, both non-diabetic NAFLD (study I) and cirrhosis patients (study II) had immediate suppression of glucagon in response to oral glucose compared with delayed suppression in patients with type 2 diabetes with and without NAFLD (study II). Thus, impaired action of the incretin hormones on glucagon secretion seems to be a unique pathological trait of type 2 diabetes that is not associated with liver damage. This was also confirmed in study III, where we found intact suppression of glucagon by physiological doses of GLP-1. The underlying mechanism behind hyperglucagonaemia is not known. Preclinical studies suggest that steatosis causes not only insulin, but also glucagon resistance, defined as reduced hepatic glucose production in response to a glucagon infusion (99,100). (141). Indeed, glucagon receptor knock-out mice (97) and mice with reduced glucagon receptor expression develop hyperglucagonaemia (142). Moreover, Longuet et al. showed that a 'circulation factor' produced after disturbance of hepatic glucagon signalling causes alpha cell proliferation and hypersecretion of glucagon (143). Taken together, glucagon resistance may cause compensatory secretion of glucagon or a rise in a 'circulation factor' causing alpha cell hyperplasia and elevated glucagon levels. Patients with cirrhosis also have impaired hepatic glucose production in response to glucagon infusion, which suggest that more severe damages to hepatocytes can cause glucagon resistance (101). It should be mentioned, however, that patients with cirrhosis have impaired glycogen synthesis (141). This can affect hepatic glucose production, which will be underestimated. A difference in elimination of glucagon in patients with liver disease and healthy subjects offers another explanation of hyperglucagonaemia. However, the liver does not seem to play a major role in glucagon elimination. Hyperglucagonaemia may be a factor in the pathogenesis of NAFLD. In rodents, glucagon stimulates the hormone sensitive lipase in the peripheral adipose tissues. Elevated glucagon levels may therefore increase lipolysis, which causes higher flux of free fatty acids to the liver and hereby contribute to steatosis (94).

STUDY LIMITATIONS

There are some limitations to the studies in this thesis, which should be taken into consideration. The study population was heterogeneous with regards to severity of histological liver changes. In study I and III, patients had a wide spectrum of NAFLD from steatosis to NASH and, additionally, some had stage 1A/B fibrosis (144). If we had included patients with either steatosis or NASH, or both in separate groups, we could have estimated the isolated impact of steatosis and NASH on our outcomes. The limited number of subjects in the studies increases the risk of type 2 errors. Nevertheless, we identified significant difference in all studies. We cannot, however, rule out that important difference between our studies groups have been missed.

In study II, patients had both alcoholic cirrhosis and cirrhosis caused by primary biliary cirrhosis. Although we excluded patients with on-going alcohol abuse, most patients in study II were previous heavy drinkers. Heavy drinking is associated with risk of chronic pancreatitis (145). It is known that patients with even slightly impaired glucose tolerance caused by chronic pancreatitis also have reduced incretin effect (48) and this could, therefore, explain our findings in patients with cirrhosis. The included patients had, however, no clinical or biochemical indications of chronic pancreatitis and exhibited prompt insulin responses. Thus, damage to the endocrine pancreas is not likely to have influenced our results.

Another important limitation is the wide spectrum of glucose tolerance, evaluated asresponses to OGTTs, in non-diabetic patients with NAFLD (study I) and cirrhosis (study II) compared to controls. Patients with NAFLD had fasting plasma glucose <6.1 mmol/l, 2-hour plasma glucose <7.8 mmol/l and were therefore defined as normal glucose tolerant. However, the 4-hour OGTT area under the curve (AUC) values was greater in non-diabetic NAFLD patients than controls (298±129 vs. 180±155 mmol/l × min, $p<0.001$) indicating some glucose intolerance. In study II, the difference in the 4-hour OGTT AUC value was even more pro-

nounced between patients with cirrhosis and healthy controls (609±458 vs. 180±155 mmol/l × min, p=0.005). It is recognised that patients with impaired glucose tolerance have impaired incretin effect (44) and it is possible that the differences in glucose tolerance may influence our result. Thus, better matching of glucose tolerance in non-diabetic patients would have increased the validity of our studies. Nevertheless, all non-diabetic patients had normal values of HbA1c (<43 mmol/mol) as an indication of normal glycaemic regulation.
Finally, all healthy controls in study I and III were obese and had steatosis excluded by ultrasonic and biochemical measurements. Ultrasound can only detect steatosis involving more than 20-30% of hepatocytes (146). In light of the diagnostic method used and the high prevalence of NAFLD in obese subjects, some degree of steatosis may have been missed in controls. We did not exclude steatosis by liver biopsy due to ethical concerns and magnetic resonance spectroscopy was unfortunately not available in our laboratory. However, the included controls were metabolically healthy, as assessed by HbA1c and OGTT, and were, therefore, unlikely to have had clinical significant steatosis (78). Moreover, the presence of steatosis in controls would only have led to underestimation of our findings.

CONCLUSIONS

This thesis provides important information regarding the pathophysiology of glucose intolerance in patients liver disease. We demonstrate that patients with NAFLD, in spite of normal glucose tolerance, have reduced incretin effect that is further aggravated by type 2 diabetes. We also find fasting hyperglucagonaemia in NAFLD patients, independently of type 2 diabetes. We show that cirrhosis is associated with impaired handling of oral glucose and reduced incretin effect. Finally, we find a preserved glucagonostatic effect of GLP-1 in patients with NAFLD, in spite of their hyperglucagonaemia. In light of our findings, the insulinotropic and glucagonostatic effects of GLP-1 receptor agonists might prove beneficial in patients with liver disease.

PERSPECTIVES AND FUTURE RESEARCH

This thesis provides a background for further research to clarify incretin and glucagon pathogenesis in liver disease.
The mechanism behind the consistent finding of fasting hyperglucagonaemia in patients with liver disease merits further investigations. Charbonneau et al. demonstrated that glucagon receptor expression was reduced in a rat model of steatosis (100). Hepatic glucose production was also decreased in response to glucagon infusion in rodent steatosis as an indication of glucagon resistance (99). In humans, patients with cirrhosis have a similarly impaired hepatic glucose production following glucagon infusion (147). In line with these studies, it would be interesting to investigate expression of glucagon receptors in liver biopsies from both NAFLD and cirrhotic patients. Another interesting experiment would be to examine hepatic glucose production in response to glucagon infusion in patients with NAFLD with and without type 2 diabetes. Such experiments could elucidate the role of hepatic glucagon resistance in glucose intolerance in type 2 diabetes.
As previously stated, neural reflexes between the portal vein and the pancreas may mediate some of the glucose lowering effects of GLP-1 (24,148). In study I, we hypothesised that autonomous neuropathy could interfere with this reflex. To investigate the role of autonomic neuropathy and the intraportal vein, it would be interesting to evaluate glucose disposal after intraportal infusions of GLP-1 in both non-diabetic and diabetic patients with cirrhosis. Access to the portal vein in humans may seem difficult, but the authors of a previous study were able to evaluate hormone concentrations during a meal test in patients undergoing transjugular intrahepatic portosystemic shunting (134).
Hepatic diabetes is a common clinical problem and increases the risk of hepatocellular carcinoma (149). Nevertheless, only few studies have evaluated anti-diabetic treatment of hepatic diabetes. Most oral anti-diabetic drugs are metabolised in the liver and cirrhotic patients are therefore susceptible to hypoglycaemic events (115), which makes treatment problematic. GLP-1-based therapy has some obvious advantages. GLP-1 is not metabolised in the liver and does not normally cause hypoglycaemia (25). On the other hand GLP-1 has little or no effect on insulin resistance, which is significant in cirrhosis. A randomised and double-blind clinical trial of GLP-1 receptor agonists in patients with hepatic diabetes would be interesting. The primary endpoints should be glycaemic control but also change in intraportal pressure, incidence of hepatocellular carcinoma and death.
GLP-1 has a possible role in hepatic lipid regulation (150) and GLP-1 receptor agonists can reduce steatosis in patients with type 2 diabetes (111). In addition, NAFLD increases the risk of type 2 diabetes (65). A clinical, controlled trial of the effects a GLP-1 receptor agonist in non-diabetic and diabetic patients with steatosis has not been undertaken. The endpoints should be histological assessment of steatosis, inflammation and fibrosis. Another interesting endpoint would be the ability of the GLP-1 receptor agonist to prevent development of diabetes in non-diabetic patients with steatosis.

SUMMARY

Non-alcoholic fatty liver disease (NAFLD) is defined as hepatic steatosis exceeding 5% of hepatocytes with no other reason for hepatic fat accumulation. The association between NAFLD and type 2 diabetes is strong. Accordingly, up to 70% of obese patients with type 2 diabetes have NAFLD. The spectrum of NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis with variable degrees of fibrosis and cirrhosis. Cirrhosis is the end-stage of chronic liver disease and is characterised by diffuse fibrosis and nodular regeneration of hepatocytes. Alcoholic liver disease and NAFLD are the most common aetiologies of cirrhosis. The WHO estimates that 70% of patients with cirrhosis have impaired glucose tolerance and 30% have manifest diabetes. The latter is termed hepatic diabetes and is associated with increased complications to cirrhosis and hepatocellular carcinoma.
The objective of this thesis was to study the impact of liver dysfunction on incretin and glucagon (patho)physiology in relation to glucose metabolism. We hypothesised that NAFLD patients with normal glucose tolerance would develop reduced incretin effect and that NAFLD would worsen the incretin effect in patients with existing type 2 diabetes. Thus, in study I, we investigated the incretin effect and glucagon secretion in patients with NAFLD with and without type 2 diabetes compared to controls. We also hypothesised that the incretin effect would be disturbed in non-diabetic patients with more severe liver disease. Hence, the objective of study II was to investigate the incretin effect in patients with cirrhosis. Finally, the hypothesis in study III was that an impaired glucagonostatic effect of GLP-1 contributes to the hyperglucagonaemia of patients with liver disease. We therefore explored the glucagonostatic properties of GLP-1 in non-diabetic patients with NAFLD.
The results of study I show that patients with NAFLD have normal secretion of GLP-1 and GIP and a reduced incretin effect. The groups with type 2 diabetes have the lowest incretion effect. We also find that NAFLD patients have high fasting glucagon concen-

trations regardless of their glucose (in)tolerance. We further demonstrated that patients with normal glucose tolerance and NAFLD have preserved glucagon suppression to both oral and intravenous glucose. In study II, we find that non-diabetic patients with cirrhosis have elevated concentrations of GLP-1 and GIP and a reduced incretin effect. Patients with cirrhosis also have fasting hyperglucagonaemia, but show intact glucagon suppression during both oral and intravenous glucose administration. Finally, study III demonstrates that normal glucose tolerant NAFLD patients had preserved glucagonostatic effect of GLP-1.
In conclusion, our studies offer important information regarding the pathophysiology of glucose intolerance in patients with liver disease. We demonstrate that patients with NAFLD, in spite of normal glucose tolerance, have reduced incretin effect that is further aggravated by type 2 diabetes. We also find fasting hyperglucagonaemia in NAFLD patients, independently of type 2 diabetes. We show that cirrhosis is associated with impaired handling of oral glucose and reduced incretin effect. Finally, we find a preserved glucagonostatic effect of GLP-1 in patients with NAFLD, in spite of their hyperglucagonaemia. In light of our findings, the insulinotropic and glucagonostatic effects of GLP-1 receptor agonists might prove beneficial in patients with liver disease.

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