

Monogenic diabetes and pancreatic exocrine dysfunction in mouse and man

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Abbreviations

CEL	Carboxyl-ester lipase
CELL	Carboxyl-ester lipase-like
CT	Computerized tomography
DPED	Diabetes-pancreatic exocrine dysfunction
ERCP	Endoscopic retrograde cholangio-pancreatography
EM	Electron microscopy
ER	Endoplasmic reticulum
FCPD	Fibrocalculous pancreatic disease
FED	Fecal elastase deficiency
GCK	Glucokinase
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GRP94	Glucose-regulated protein 94
GSIS	Glucose-stimulated insulin secretion
GTT	Glucose tolerance test
HDL	High-density lipoprotein
HLA	Human leucocyte antigen
HNF	Hepatocyte nuclear factor
IGT	Impaired glucose tolerance
IVGTT	Intravenous glucose tolerance test
ITT	Insulin tolerance test
KO	Knockout
LDL	Low-density lipoprotein
MODY	Maturity-onset diabetes of the young
MIDD	Maternally inherited diabetes and deafness
MRI	Magnetic resonance imaging
NASH / NAFLD	Non-alcoholic steatohepatitis / Non-alcoholic fatty liver disease
NGT	Normal glucose tolerance
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
PEST domain	Proline (P), glutamate (E), serine (S), threonine (T)
PEST	Pancreatic enzyme substitution therapy
PNDM	Permanent neonatal diabetes mellitus
TNDM	Temporary neonatal diabetes mellitus
VNTR	Variable number of tandem repeats
WT	Wild type

List of publications

- I. Mette Vesterhus, Helge Ræder, Stefan Johansson, Anders Molven, Pål R. Njølstad. Pancreatic exocrine dysfunction in maturity-onset diabetes of the young type 3. *Diabetes Care* 2008; 31 (2): 306-10
- II. Mette Vesterhus, Ingfrid S. Haldorsen, Helge Ræder, Anders Molven, Pål R. Njølstad. Reduced pancreatic volume in hepatocyte nuclear factor 1A maturity-onset diabetes of the young. *J Clin Endocrinol Metab* 2008;93 (9): 3505-9
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1. INTRODUCTION

1.1 Normal function and development of the pancreas

1.1.1 Structure and function

The pancreas is a mixed exocrine and endocrine organ that plays a central role in food digestion and glucose homeostasis. The two tissues have interacting functions; the exocrine part produces enzymes that digest polysaccharides, lipids and proteins into their basic components (monosaccharides, fatty acids and their backbones, and amino acids) thus enabling their uptake into the bloodstream, whereafter they are processed under the control of endocrine hormones. The endocrine part includes five distinct hormone-producing cell types organized into the islets of Langerhans. These islets are dispersed in the exocrine pancreatic tissue (Figure 1), consisting of acinar cells that produce and secrete digestive enzymes into ducts which transport the digestive enzymes to the intestine. There is an evolutionary variation ranging from no anatomical contact between endocrine and exocrine tissue in some groups of fish, via mixed exo-endocrine tissues in reptiles, to well-defined islets in higher vertebrates such as mammals (1).

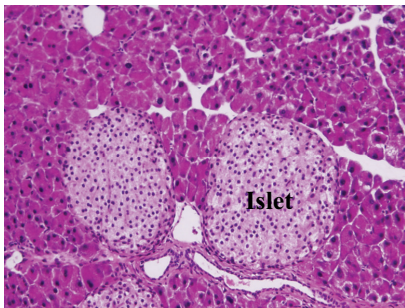


Figure 1. Hematoxylin-eosin-stained section of a normal mouse pancreas demonstrating the distinctive anatomical compartmentalization of exocrine and endocrine tissue, with the endocrine cells clustering in the islets of Langerhans. The organization in humans is similar.

The exocrine pancreas

The exocrine part of the pancreas makes up 80 % of the gland and consists of acinar cells, which are arranged in tubular or spherical cell groups with a central lumen (acini; Figure 2) (2). The lumens of the acini merge to form intralobular and then interlobular ducts that

anastomose to become the main pancreatic duct, bringing the pancreatic secretions to the duodenum. Acinar cells are highly polarized serous epithelial cells, with rough endoplasmatic reticulum occupying about 20 % of the cell volume, filling most of the basal region, whereas zymogen granules fill the apical portion of the cells. After a large meal, a decrease in both the size and the number of zymogen granules is observed in concurrence with a substantial increase in pancreatic enzyme secretion and a more extensive Golgi apparatus (3). The zymogen granules contain 12 to 15 different digestive enzymes, each granule containing the entire complement although in variable concentrations (4-6). Most of the digestive enzymes are secreted as pro-enzymes that are subsequently activated at the encounter of trypsin, following the activation of trypsin by enterokinase cleavage of trypsinogen in the duodenum (2). Centroacinar and duct cells contribute in the production of pancreatic juice by the secretion of bicarbonate and water. The bicarbonate contributes to keeping the digestive enzymes inactive, as they have a slightly acidic pH-optimum. The secreted pancreatic juice amounts to about 1500-2500 ml per day.

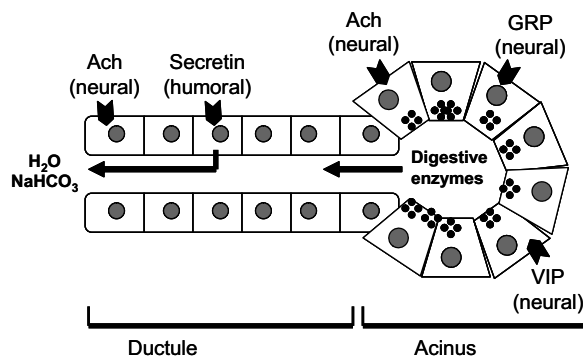


Figure 2. Schematic illustration of the functional unit of the exocrine pancreas. Acetylcholine (Ach), secretin, gastrin-releasing peptide (GRP), and vasoactive intestinal peptide (VIP) act as neural and humoral agonists to induce secretion of bicarbonate and water from the duct cells and digestive enzymes from the acinar cells. (Adapted from (2)).

In the fasting state, human pancreatic exocrine secretion is cyclical and closely correlated with upper gastrointestinal motility. Postprandially, however, enzyme delivery into the duodenum increases rapidly to reach a maximum within 30 minutes to an hour, it then decreases slightly and stabilizes, and eventually the secretory rate decreases 3-4 hours postprandially to reach the interdigestive range. However, the degree and the duration of the secretory response are dependent on the caloric content, nutrient composition and physical properties of the meal (7). The secretion of digestive enzymes and hormones from the

exocrine pancreas is mediated by fatty acids of more than eight carbons in length, monoglycerides of these fatty acids, peptides, amino acids (particularly essential amino acids; the most potent are phenylalanine, valine, methionine, and tryptophan), and, to a lesser extent, glucose through neural and humoral pathways combined (2). The intestinal gut hormones produced by entero-endocrine cells of the duodenal mucosa play an important role, particularly secretin and cholecystokinin (CCK). The latter is the major humoral mediator of meal-stimulated enzyme secretion, probably through activation of afferent neurons in the duodenal mucosa since human acinar cells do not have CCK receptors (2; 8). Secretin is the main mediator of bicarbonate secretion by the duct cells. Removal of secretin reduces pancreatic enzyme output by 50 % and the pancreatic volume and bicarbonate secretion by 80 % (9). A vasovagal enteropancreatic reflex probably contributes to enzyme secretion and augments the effect of secretin on bicarbonate secretion via the neurotransmitter acetylcholine and its G-protein coupled muscarinic (M3) receptor.

The endocrine pancreas

The Islets of Langerhans, named after Paul Langerhans who first described them in 1869, constitute the endocrine compartment of the pancreas, amounting to about 2 % of the gland by weight (2). The main function of the endocrine pancreas is the secretion of hormones regulating growth and maintaining plasma glucose levels in a tight physiological range for optimal functioning of all tissues in the body. About two million islets are found in a human pancreas (10), with the highest density in the tail region. Each islet is a rounded cluster of cells of 100-200 μm in diameter, separated from the surrounding exocrine tissue by a fine capsule of reticular fibres and a basal lamina derived from endothelial cells (11). The endocrine cells form cords in close proximity to a rich network of fenestrated blood capillaries that surround and penetrate each islet. The capillaries are arranged in a so-called *insula-acinar portal system* that conveys blood from the islets to acinar cells, thus permitting the local action of islet hormones, particularly insulin, on the exocrine pancreas. This local effect is demonstrated by the fact that peri-insular acini have larger cells, nuclei, and zymogen granule regions and different ratios of specific digestive enzymes, than acini situated further away (2). The insula-acinar portal system runs in parallel to an arterial system supplying blood directly to the acinar tissue. Both sympathetic and parasympathetic nerve fibres converge on some of the islet cells (11). The islets in the pancreatic tail, body and anterior head deriving from the dorsal bud (see below) are in general more glucagon-

rich than the PP-rich islets in the posterior head of the pancreas, deriving from the ventral bud (12).

Four major types of cells are found in the islets, and in contrast to the acinar cells they appear to be specialized into secreting a single hormone each. The beta cells (β -cells) secrete insulin, which reduces blood glucose levels by increasing glucose uptake into liver, muscle and fat, and by inhibiting glucose production in the liver. Insulin is synthesized in the form of preproinsulin, which is processed by the cleaving off of the signal peptide and the formation of disulfide bonds to yield proinsulin. Proinsulin is further cleaved into insulin (consisting of A and B chains) and the C-peptide (connecting peptide) in equimolar amounts. An increase in the serum glucose level is sensed by the beta cell and is the main stimulus for insulin secretion, resulting in a proportional increase in insulin secretion (13). The corresponding process is called the glucose-stimulated insulin secretion (GSIS; Figure 3). Insulin secretion is also stimulated by lipids (14), incretins (see below), neural input (15) and other hormones (16).

Alpha cells (α -cells) produce glucagon, which promotes the increase of blood glucose levels through activating glycogenolysis and gluconeogenesis at low blood glucose levels. The primary stimulus is hypoglycaemia. The main regulatory mechanism governing glucagon secretion is paracrine signalling, with zinc, insulin and GABA from beta cells and somatostatin from delta cells acting as inhibitors of glucagon secretion. The sympathetic nerves, the vagal nerve and several amino acids (arginine and alanine) stimulate glucagon secretion. The delta cells (δ -cells) produce somatostatin inhibiting the secretion of the other endocrine pancreatic hormones, and the PP cells produce pancreatic polypeptide (PP) that inhibits the secretion of bicarbonate and enzymes from the exocrine pancreas. It has been estimated that each islet is composed of 70-80 % beta cells, 15-20 % alpha cells, 5 % delta cells and < 2 % PP-cells, with some variation depending on in which embryologically defined part of the pancreas the islets are located (10; 17). The number of beta cells may be relatively higher in mouse than human islets (77 % versus 55 %) and the number of alpha cells may be lower in mouse than human islets (18 % versus 38 %), as indicated by a recent study (18). Traditionally, the beta cells have been regarded to form the core of the islets, surrounded by the other cell types (19), but recently this model has been modified as confocal microscopy and multiple immuno-fluorescence indicate a somewhat more scattered distribution of beta, alpha and delta cells within the islets in humans (18).

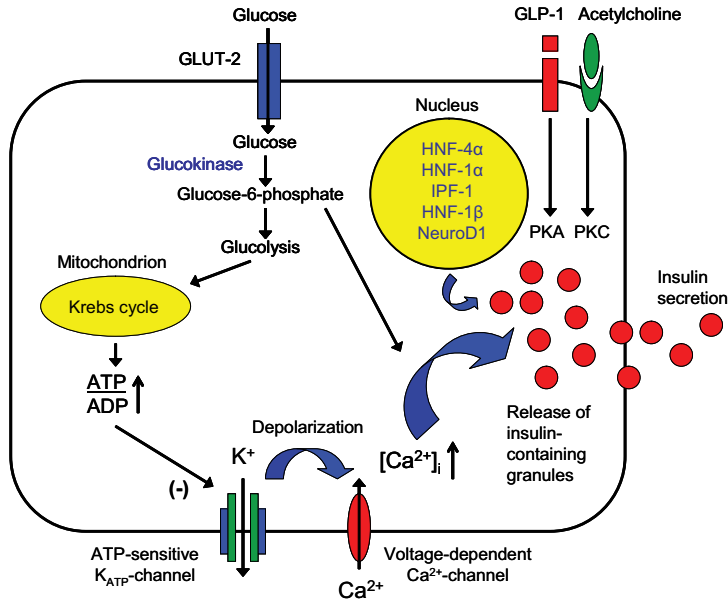


Figure 3. Schematic presentation of the beta cell and processes leading to insulin secretions, including glucose-stimulated insulin secretion (GSIS). Incretins, illustrated by GLP-1, augment insulin secretion. The MODY-associated transcription factors HNF1A, HNF1B, HNF4A, IPF1 and NeuroD1 influence insulin secretion through regulation of the transcription of the insulin gene and genes involved in the transport and metabolism of glucose. The energy level of the beta cell (ATP/ADP ratio) is the major determinant of the level of insulin secretion. An increased ratio leads to the closure of ATP-dependent SUR1 channels (K_{ATP} -channels), with subsequent depolarization of the voltage-dependent Ca^{2+} -channels, resulting in Ca^{2+} influx that signals the exocytosis of insulin.

The incretin effect

The phenomenon that a greater insulin release is achieved when nutrients are taken orally than intravenously is called the incretin effect (20). It is caused by incretins, hormones which are secreted from entero-endocrine cells and augment the glucose-stimulated secretion of insulin. In type 2 diabetes, the incretin effect has been shown to be markedly reduced (21), most likely as a consequence of the diabetic state (22). The two main incretins are glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), secreted from the proximal and distal small intestine, respectively (20). Both act on specific G-protein-coupled receptors that stimulate cAMP formation and protein kinase A (PKA) activation, promoting insulin exocytosis (Figure 3). GLP-1 also regulates proinsulin gene expression, inhibits glucagon secretion from alpha cells, promotes differentiation towards a more endocrine-like phenotype in human and rodent exocrine pancreatic cell lines, stimulate beta cell proliferation and preservation and reduces ER stress in murine islets *in vivo* (20).

ER stress is defined as conditions interfering with ER function, induced by accumulation of unfolded protein aggregates or by excessive protein traffic, resulting in decreased rate of protein translation, induction of the expression of chaperones and activation of the ER-specific protein-degrading apparatus. Whereas nutrient intake is thought to directly regulate GIP, nutrient intake is believed to regulate GLP-1 via neural or endocrine factors (20). Both incretins are degraded by dipeptidyl peptidase-4 (DPP-4), and DPP-4 inhibitors as well as incretino-mimetics have recently been approved for therapeutic use in diabetes (23). Glucagon secretion is augmented in a similar manner to the incretin effect by the entero-endocrine hormones CCK and gastrin which are increased by a protein-rich meal (24). Furthermore, receptors for GLP-1, CCK and glucagon in the brain regulate energy uptake and utilization by inhibiting food intake (24).

1.1.2 Development

Both the endocrine and the exocrine parts of the pancreas in vertebrates are derived from endoderm (reviewed in ((17; 25)). Table 1 outlines genes that are active at different phases in pancreatic development. During organ specification, two buds arise from the gut endoderm on each side of the duodenum (day 28 in humans, day 9 (E9) in mice (26)) in response to signals from the adjacent mesodermal tissues. The ventral bud becomes the uncinate process and the posterior and inferior part of the pancreatic head, whereas the dorsal bud evolves into pancreatic body and the tail and the anterior part of the head. The buds fuse as the ventral bud moves dorsally during gut rotation (by day 56 in humans, E12.5 in mice), at the same time forming the main pancreatic duct from a fusion of the dorsal and ventral pancreatic ducts made from extensive branching of the epithelial part of the pancreatic primordial. Ectopic pancreas growth is inhibited by Hedgehog signalling in the adjacent endoderm (27).

During budding, *PDX1* is expressed by ductal progenitor cells, giving rise to all adult pancreatic cells (17). *PDX1* is required for both islet and acinar differentiation (28), and its

Table 1 Some genes involved in pancreatic development

Gene name	Main developmental role
<i>Organ specification</i>	
<i>HLXB9</i>	Early pancreas development, mainly dorsal bud
<i>PTF1A</i>	Early pancreas development, mainly ventral bud
<i>SHH</i>	Early pancreatic islet development
<i>Growth</i>	
<i>IPF1/PDX1</i>	Epithelial factor required for epithelial proliferation, partly through interaction with Pbx1
<i>FGF10</i>	Mesenchymal factor stimulating the proliferation of pancreatic progenitors
<i>ISL1</i>	Growth-promoting factor in the dorsal mesenchyme
<i>CDH2</i>	Growth-promoting factor in the dorsal mesenchyme
<i>PBX1</i>	Growth-promoting factor in the epithelium and dorsal mesenchyme
<i>PTF1A</i>	Epithelial factor probably required for epithelial proliferation
<i>Specification and differentiation of endocrine and exocrine cell lines</i>	
<i>PTF1A</i>	Exocrine cell development
<i>MIST1</i>	Exocrine cell maintenance
<i>NGN3</i>	Key factor in islet cell development
<i>HES1</i>	Notch-pathway-mediated Ngn3 inhibition
<i>DLL1</i>	Notch-pathway-mediated Ngn3 inhibition
<i>HNF6</i>	Ngn3 stimulation; controls the development of pancreatic ducts
<i>NEUROD1</i>	Alpha/beta-cell development
<i>NKX2B</i>	Beta cell development
<i>NKX6A</i>	Beta cell development
<i>PAX4</i>	Beta cell development
<i>ISL1</i>	Beta cell development
<i>PAX6</i>	Beta cell development
<i>HNF1A</i>	Maintenance of differentiated beta cells
<i>HNF4A</i>	Maintenance of differentiated beta cells

(Based on (17; 25))

germline inactivation results in pancreatic aplasia (29). A recent study (30) shows that all of the Pdx1⁺ progenitors needed to make the pancreas are generated during the embryonic period spanning E8.5-12.5, and the size of this pool determines the final size of the pancreas; i.e., normal organ size cannot be restored if the progenitor pool size is reduced.

Mesenchymal-epithelial signalling is necessary for the normal growth and differentiation of the pancreatic epithelium (31). In the absence of mesenchyme or in the case of disrupted signalling, endocrine but not exocrine cells arise from the pancreatic epithelium (32; 33). Important signalling factors mediating the epithelio-mesenchymal interactions include the epidermal growth factor (EGF) and fibroblast growth factor (FGF) families (17; 33).

A recent study (34) revealed that in humans, the pancreas by 9-11 weeks consists mainly of mesenchymal tissue infiltrated by branched epithelial structures and scattered hormone-negative Neurogenin 3 (NGN3)-positive cells. *NGN3* expression is necessary and sufficient for endocrine development (35) and is repressed by the Notch pathway (36). Clusters of endocrine cells producing either glucagon or insulin are observed by 15-19 weeks, while vascularised islet-like structures appear by 20-23 weeks. By 9-10 weeks, transcripts for insulin, glucagon, somatostatin, ghrelin and pancreatic polypeptide are present, and from 11-23 weeks a progressive increase in the endocrine cell population is evident. The human equivalent of a mouse endocrine secondary transition has not been observed, neither morphologically nor on the expression level, possibly because this occurs at multiple foci without the temporal coincidence seen in mice (34). By contrast, exocrine genes show a marked transition around 11 weeks (34). *PTF1A* is necessary for exocrine cell differentiation (37), an early marker of which is carboxylester lipase (*CEL*) (38). Protoacinar structures emerge by 15-19 weeks, but amylase, a marker of the mature exocrine pancreas, is first detected at 23 weeks in typical acinar structures. *HNF6* controls the development of pancreatic ducts (25).

1.1.3 Imaging of the pancreas

Due to the high risk of complications associated with biopsy taking, precise imaging is highly important for the diagnosis of pancreatic diseases. The pancreas is traditionally evaluated by various imaging techniques including ultrasonography (US) and computed tomography (CT) (the two most commonly used techniques), endoscopic ultrasonography (EUS), endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP). Recently, technological advances have brought a host of new modalities. Table 2 describes the advantages, disadvantages and indications for the various techniques.

Imaging has long been important in the diagnosis of chronic pancreatitis, where some of the key pathological changes are irregularity and widening of the main pancreatic duct, cavities, intraductal calcifications and duct obstructions. ERCP represents the gold standard. It has been shown that MRCP correlate well with ERCP findings in patients with chronic pancreatitis including small duct disease (39; 40). Correlation between pancreatic function and imaging has been investigated in patients with clinically suspected chronic pancreatitis on US and CT as well as on EUS and ERCP (41; 42). Contrast-enhanced MRI has been reported to correlate with pancreatic exocrine function (43).

Pancreatic volume has been investigated in various conditions. A reduction of 21 % in pancreatic volume and 29 % in the area comprising beta cells has been observed in patients with chronic pancreatitis (44). Pancreatic volume and pancreatic exocrine function measured by serum immunoreactive trypsin, have been reported to correlate (45). Radiological studies have reported reduced pancreatic volume in patients with type 1 diabetes and to lesser degree in patients with type 2 diabetes (45-47). The pancreatic size reduction in type 1 and 2 diabetes is associated with diabetes duration in some (47) but not all (45; 46) studies, and reports of association of small pancreatic volume with insulin use (47) or reduced insulin secretion (46) have further been taken to support the hypothesis of reduced insulinotropic effects on the acinar cells as a mechanism for the reduction in pancreatic volume (48; 49).

Pancreatic atrophy is also a common feature of maturity-onset diabetes of the young due to mutations in *HNF1B* (*HNF1B*-*MODY*, *MODY5*) (50; 51), and subjects with diabetes and pancreatic exocrine dysfunction caused by mutations in the carboxyl-ester lipase (*CEL*) gene display pancreatic atrophy and lipomatosis (52; 53).

Total pancreatic agenesis is extremely rare and most often associated with neonatal death, but survival is possible with proper diagnosis and treatment (2). Isolated dorsal or less commonly ventral pancreatic agenesis (sometimes seen in Cumming's syndrome), on the other hand, may occur. A lack of any sign of the pancreas on a CT scan at the level of the splenic vein is usually taken into account for dorsal pancreatic agenesis. This anomaly is extremely rare and only some 20 cases have been reported (54). It may be asymptomatic and detected incidentally, but is more often associated with abdominal pain and/or diabetes and sometimes exocrine dysfunction (54-58). Familial occurrence has been reported (59).

Table 2 Methods for pancreatic imaging

Method	Advantages	Disadvantages	Indications
<i>Established methods</i>			
Abdominal US	Wide availability. Inexpensive Non-invasive. No radiation or nephrotoxic agents	Reduced visualization by body fat or bowel gas Limited accuracy for parenchymal abnormalities	First imaging modality in patients with abdominal pain, especially in children. Particularly useful in evaluating the biliary tree.
CT	Wide availability. Non-invasive	Radiation Poor sensitivity to identify ductal abnormalities or subtle parenchymal changes in chronic pancreatitis	Primary imaging modality of the pancreas in adults Useful for the diagnosis and staging of pancreatic cancer
ERCP	Allows brush cytology, stone removal and stricture dilatation	Invasive. Risk of inducing pancreatitis.	Gold standard for diagnosing chronic pancreatitis and delineating ductal anatomy
EUS	Allows fine-needle aspiration biopsies	Invasive Shortage of well trained endoscopists Operator dependency. Limited accuracy in assessing tumour vascular involvement	Most sensitive established method for detecting and local staging of pancreatic neoplasms Superior to CT for detecting malignant lymphadenopathy Detects subtle changes of early chronic pancreatitis
MRI	Non-invasive No radiation	Need for serial scans in ongoing disease. Costs.	Evaluates acute pancreatitis better than CT Contrast enhanced imaging often useful
MRCP	Non-invasive		Primarily used to diagnose bile duct stones, biliary tree abnormalities, ductal strictures or dilatation, IPMN and duct leaks. Non-invasive alternative to ERCP in selected patients with chronic pancreatitis.
<i>Novel methods</i>			
S-MRCP		Larger studies needed	Qualitative and quantitative assessment of pancreatic exocrine function; fairly good correlation with the endoscopic secretin test Evaluation of functional pancreatic duct obstruction. Improved visualization of ductal strictures, dilatations and leaks

Method	Advantages	Disadvantages	Indications
CEUS	Wide availability. Low costs. No radiation. Non-invasive. Non-toxic intravenous contrast agent that form micro-bubbles	Short circulation time of contrast imaging Operator dependency Reduced visualization by body fat or bowel gas	Provides information regarding subtle parenchymal abnormalities, correlates well with contrast CT
EUS elastography (strain imaging)		Invasive Unlikely to replace the need for tissue sampling to diagnose malignancy	Promising in the diagnosis of early chronic pancreatitis by assessing the amount of fibrous tissue present Distinguishing benign from malignant lesions
MDCT	Increased resolution Improved vascular visualization		Assessing malignancy of IPMN Improved visualization of vascular invasion in malignant disease
OCT	Extremely high resolution images of the pancreatic duct and periductal structures	Invasive (endoscope). More studies needed Unlikely to replace the need for tissue sampling to diagnose malignancy	May be useful in diagnosing early chronic pancreatitis (subtle changes in wall thickness or inflammation) Excellent accuracy for detecting neoplastic tissue
MRS	Qualitative and quantitative information on the biochemical status and physiologic processes of an organ <i>in vivo</i>	More studies and some technical improvements needed	Might be a useful imaging adjunct in the differentiation of chronic focal pancreatitis from pancreatic carcinoma

CEUS, contrast-enhanced ultrasound; CT, computed tomography; ERCP, endoscopic retrograde cholangiopancreatography; EUS, endoscopic ultrasound; IPMN, intraductal pancreatic mucinous neoplasms; MDCT, multidetector-row computed tomography; MRI, magnetic resonance imaging; MRCP, magnetic resonance cholangiopancreatography; MRS, magnetic resonance spectroscopy; OCT, optical coherence tomography; US, ultrasound.

Based on (60; 61)

1.2 Endocrine and exocrine pancreatic disease

1.2.1. Diabetes type 1 and 2

The term diabetes mellitus characterizes a heterogeneous group of metabolic disorders characterized by hyperglycemia with disturbances of carbohydrate, fat and protein metabolism, resulting from defects in insulin secretion, insulin action or both (62). Hence, diabetes results from a failure to provide sufficient insulin to supply the needs of the insulin-

sensitive organs. The onset of diabetes is defined clinically based on persistent hyperglycemia, using internationally recognized cut-off levels for fasting and stimulated blood glucose (63; 64). However, a clear preclinical phase can be detected at least for type 1 diabetes, with signs of loss of the first phase insulin response by IVGTT prior to the development of glucose intolerance (65).

More than 170 million individuals worldwide are estimated to suffer from diabetes (66), yielding a prevalence of 2.8 %. The epidemic is rapidly growing, and the prevalence is projected to double by 2025 (67). The disease is associated with considerable morbidity, being the leading cause of adult blindness and chronic renal failure as well as a major risk factor for stroke, heart disease and birth defects (68). The excess mortality attributable to diabetes was estimated to be 2.9 million deaths worldwide in 2000, equivalent to 5.2 % of all deaths, and in the United States, diabetes was the sixth most common cause of death in 2005. In Norway, a 2004 report indicated that 90 000-120 000 individuals had a diagnosis of diabetes, yielding a sex- and age-adjusted prevalence of 2.3 % (69). However, recent data suggest that more than 50 % of diabetes cases in the Norwegian population are undiagnosed at present, thus leading to an estimated true prevalence of 240 000 individuals or more (Kristian Midthjell, personal communication). The age-adjusted annual increase in the prevalence of diabetes was reported to be 0.4 % for women and 2.5 % for men in the 1990s (69), and the diabetes incidence is still increasing, particularly in men (Kristian Midthjell, personal communication).

Type 2 diabetes accounts for more than 90 % of diabetes worldwide (70). While type 1 diabetes is caused by insulin deficiency due to autoimmune destruction of pancreatic beta cells, type 2 diabetes develops as a consequence of insulin resistance; that is, impaired ability of muscle, fat and liver to respond to insulin, combined with a defect in the insulin secretory response to glucose by the beta cell (71). Longitudinal studies in high-risk individuals suggest that in type 2 diabetes, insulin resistance is an early phenomenon, occurring years before the detection of glucose intolerance, whereas the beta cell failure is a later event (72).

Type 2 diabetes is thought to result from the complex interplay of a variety of pathways under the combined control of genetic and environmental factors (Figure 4). It has been estimated that 30-70 % of the risk for type 2 diabetes is attributable to genetics (73). The

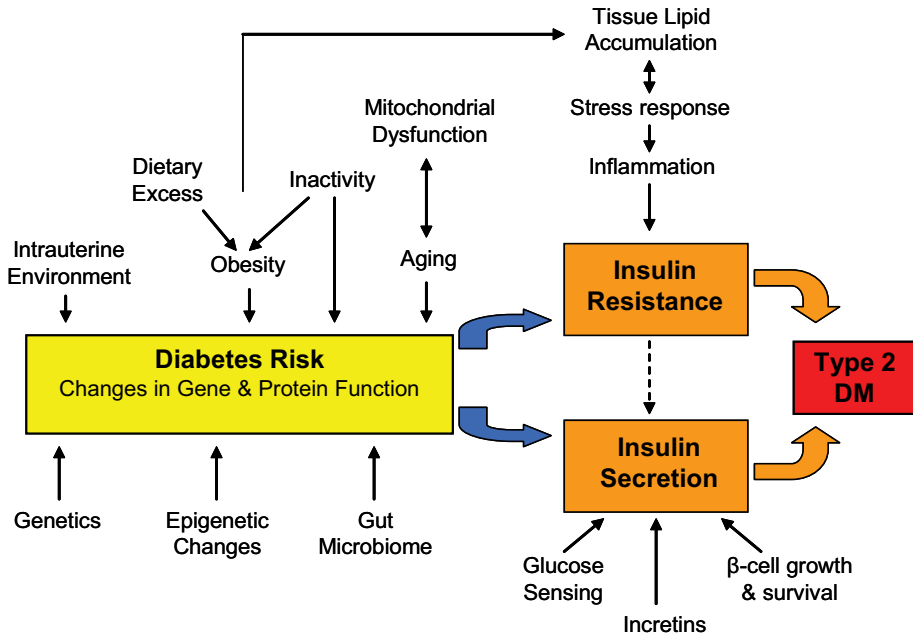


Figure 4. Schematic presentation of the current model of the complex interaction of genetic and environmental factors in the pathogenesis of type 2 diabetes. (Adapted from (73).)

importance of genetics is indicated by the higher concordance rate of type 2 diabetes in monozygotic versus dizygotic twins, the familial clustering of insulin sensitivity and insulin secretion, and the high prevalence of type 2 diabetes in certain ethnic groups such as the Pima Indians or Mexican Americans (73).

Since 2006, technological advancement allowed high-throughput genome-wide association studies (GWAS), uncovering a number of new genetic loci associated with diabetes, of which at least 20 emerged as being consistently associated with risk of type 2 diabetes across multiple studies. Many of the risk genes are expressed in beta cells (74). Surprisingly, though, most of the observed associations were for signals close to genes that would not be considered typical candidate genes and they were in noncoding regions of the gene. Generally, the signals have small effect and their predictive value for the development of diabetes in an individual is limited or non-existent (75). Furthermore, nine of the strongest loci together accounted for only 7 % of the 30-60 % increase in the risk of type 2 diabetes typically observed in siblings of type 2 diabetes probands as compared to the general population (76). These results enforced a reconsideration of the degree of genetic

heterogeneity and possibly even the role of genetics itself in the pathogenesis of type 2 diabetes (73).

Whereas type 2 diabetes is thought to be primarily heterogeneous and polygenic with low penetrance for the risk alleles discovered to date, necessitating interaction with environmental factors, subgroups exist where diabetes is transmitted with a Mendelian dominant pattern of inheritance, including maturity-onset diabetes of the young (MODY), mitochondrial diabetes, neonatal diabetes, several syndromes of severe insulin resistance and other rare genetic syndromes. It is generally accepted that, together, the monogenic forms of type 2 diabetes account for somewhere between 1 and 5 %, most likely towards the lower end of the scale (77).

1.2.2 Maturity-onset diabetes of the young (monogenic beta cell diabetes)

Maturity-onset diabetes of the young (MODY) is originally a clinical diagnosis requiring an autosomal inheritance pattern, onset before 25 years of age in at least one family member and evidence of primary beta cell dysfunction (13). Molecular studies have since the 1990's revealed the genetic background of MODY subtypes, of which at least eight are established (74). The MODY subtypes are all caused by rare mutations in the coding sequence of genes, resulting in significant amino acid substitutions or truncated proteins, leading to hyperglycemia even in the absence of other diabetogenic exposures. Subsequent to reports of a dominant mutation in sulfonylurea receptor 1 [SUR1] giving rise to diabetes fulfilling the criteria for MODY except for an atypical age of onset in adult life, the term MODY has been suggested to be substituted by "monogenic diabetes mellitus" or "autosomal dominant type 2 diabetes" (78; 79). Recently, a molecular genetic classification of the forms of monogenic beta cell diabetes has been proposed in which this group is divided into four subtypes (80): 1) diabetes diagnosed before 6 months of age (usually associated with mutations in Kir6.2, INS or SUR1, or with abnormalities in chromosome 6q24); 2) familial, mild fasting hyperglycemia (associated with glucokinase mutation); 3) familial, young-onset diabetes (associated with *HNF1A* or *HNF4A* mutations); and 4) diabetes with extrapancreatic features (associated with *HNF1B* or mitochondrial m.3243A>G mutation).

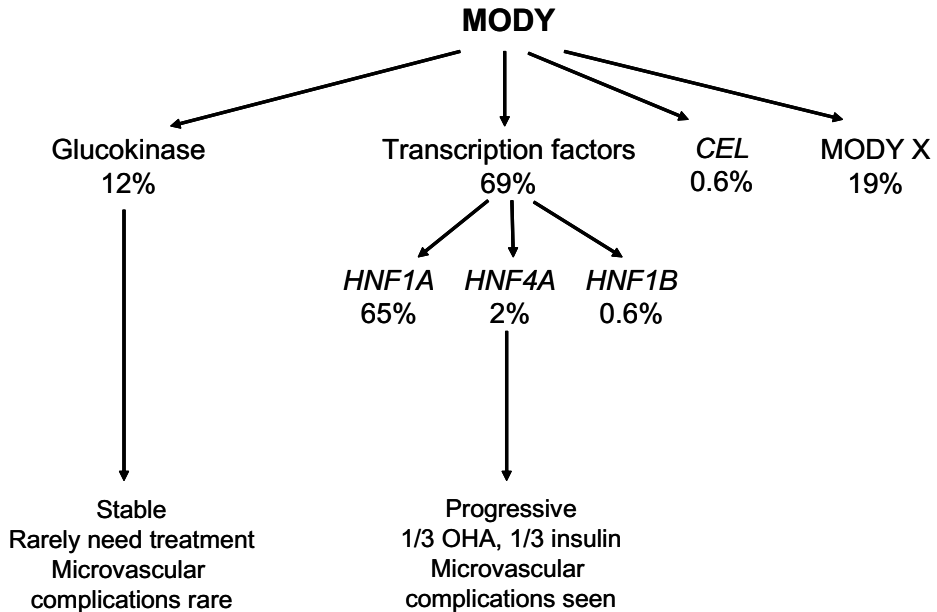


Figure 5. The relative prevalences of the MODY subgroups in Norway, based on the Norwegian MODY Registry(81).CEL, carboxyl-ester lipase; HNF, hepatocyte nuclear factor; OHA, oral hypoglycaemic agent; MODY X, maturity-onset diabetes of the young lacking molecular diagnosis.

The prevalence of MODY in Europe has been estimated to 1-2 % of non-insulin dependent diabetes (82), although population-based data are lacking. A recent study of a defined Norwegian population (the HUNT2 Study) found a prevalence of MODY of 2.2 % of the subjects with diabetes, and a minimum prevalence of *HNF1A*-MODY (MODY3) of 0.4 % of the diabetic population (77). *HNF1A*-MODY seems to be the most prevalent form in Northern Europe (77), followed by *GCK*-MODY (MODY2) (83; 84) whereas the reverse seems to be the case in Southern Europe (85; 86). Different recruitment strategies for genetic testing may contribute to the regional variation in the ratio of *GCK*-MODY to *HNF1A*-MODY, as blood glucose screening in young asymptomatic individuals would detect a higher proportion of *GCK* mutations. The relative prevalence of the MODY subgroups in Norway are shown in Figure 5.

All the MODY subtypes are due to heterozygous mutations. Table 3 outlines some of their respective characteristics. The recently discovered *CEL*-MODY is described in chapter 1.4.3. Whereas *GCK*-MODY is caused by mutations in glucokinase, the rate-limiting enzyme of glycolysis, resulting in mild fasting hyperglycemia due to defect glucose sensing

Table 3 Genes involved in monogenic beta cell dysfunction in MODY and associated pancreatic exocrine dysfunction or structural abnormalities of the pancreas

Gene name	Chrom. loc.	Cellular function	Diabetes phenotype	Exocrine pancreatic phenotype	Ref.
<i>HNF4A</i>	20q12	Transcription factor	MODY1; beta cell dysfunction, liver test abnormalities		(87)
<i>GCK</i>	7p15	Glucose phosphorylation	MODY2; mild life-long fasting hyperglycemia		(88-90)
<i>HNF1A</i>	12q24	Transcription factor	MODY3; beta cell dysfunction, diabetes phenotype similar to <i>HNF4A</i> -MODY, decreased renal threshold for glucosuria		(91)
<i>IPF1</i>	13q12	Homeodomain transcription factor	MODY4; Heterozygote: diabetes resembles type 2 diabetes, milder than <i>HNF1A</i> -MODY (rare); homozygote: pancreatic agenesis and neonatal diabetes	Pancreatic hypoplasia/agenesis	(92; 93)
<i>HNF1B</i>	17q21	Transcription factor	MODY5; Renal cysts and diabetes with genital malformations + liver test abnormalities; neonatal diabetes	Exocrine dysfunction; pancreatic atrophy	(94)
<i>NEUROD1</i>	2q32	Transcription factor	MODY6; milder diabetes than <i>HNF1A</i> -MODY, more like type 2 diabetes, insulin resistance (rare)		(95)
<i>KLF11</i>	2p25	TGF-beta inducible transcription factor	MODY7; similar to <i>NEUROD1</i> -MODY		(96)
<i>CEL</i>	9q34.3	Lipid metabolism	MODY8; beta cell dysfunction	Exocrine dysfunction; lipomatosis	(97; 98)
<i>INS</i>	11p15.5	Hormone	Rare MODY; beta cell dysfunction, permanent neonatal diabetes		(99-101)
<i>ABCC8</i> (Sur1)	11p15.1	Sulfonylurea receptor	Permanent and transient neonatal diabetes; late-onset type 2 diabetes		(102; 103)

See also Table 5. *Adapted from (73).*

(85; 88; 89), the other traditional MODY forms are associated with dysfunction of transcription factors that normally regulate the insulin gene as well as genes encoding proteins involved in glucose transport and metabolism (104) and mitochondrial metabolism (105) resulting in defects in insulin secretion leading to reduced maximal stimulated insulin secretion (106-108): *HNF4A* (MODY1) (87), *HNF1A* (91), *IPF1* (MODY4) (92; 93), *HNF1B* (MODY5) (94) and *NEUROD1* (MODY6) (95). Several of these genes interact closely in transcriptional networks controlling gene expression during embryonic development and during adulthood in tissues where they are coexpressed. Through binding sites in the predominantly beta cell specific alternative upstream (P2) promoter for *HNF4A*, transcription factors *HNF1A*, *HNF1B* and *IPF1* can activate *HNF4A*, and conversely *HNF4A* can increase expression of *HNF1A* through a binding site in the *HNF1A* promoter (109-111). Mutations in both the *HNF4A* P2 and the *HNF1A* promoter are sufficient to result in MODY, underscoring the importance of this network (110).

***HNF1A*-MODY** is characterized by a severe and progressive insulin secretion defect, a retained sensitivity to sulfonylureas, and glucosuria due to a decreased renal threshold for glucose reabsorption, which may precede the development of diabetes (112). *HNF1A* mutations have a high penetrance, with 63 % of carriers developing diabetes by 25 years of age and 96 % by 55 years (113). However, the clinical expression of *HNF1A*-MODY is highly variable even within the same family (114) and only about one-third of patients are treated with insulin after 15 years of diabetes duration, whereas others maintain glucose control by diet or oral hypoglycaemic agents (115). Patients typically present in their teens or early adult life with symptomatic diabetes. Fasting glucose levels often remain normal initially, but OGTT reveals an elevated 2h plasma glucose concentration (116) with a large increment value, typically >4.5 mmol/l. Factors contributing to an early age of onset of diabetes are mutations in the first six exons rather than the terminal exons (117), truncating rather than missense mutations (118), and intrauterine exposure to maternal diabetes (119). Sulfonylureas in low doses are recommended as the first-line treatment as the patients are very sensitive to sulfonylurea therapy (120-123), which acts on the K_{ATP} -channels to stimulate insulin release, thus bypassing the steps of glucose metabolism affected by the *HNF1A* mutation. Raised HDL-cholesterol levels are commonly observed, in contrast to the reduced levels seen in subjects with type 2 diabetes or the normal levels in subjects with type 1 diabetes (124). Still, the frequency of heart disease seems to be greater in patients

with *HNF1A*-MODY than in patients with type 1 diabetes despite a similar incidence of macrovascular complications (125).

***HNF1B*-MODY** is a multi-system disorder, but renal disease is the predominant phenotype, present in all reported probands, which is why the alternative name of Renal cysts and diabetes (RCAD) syndrome has been suggested. The renal disease phenotypes include renal cysts (most frequent), renal dysplasia, renal-tract malformations and/or familial hypoplastic glomerulocystic kidney disease (126). Renal function ranges from normal to dialysis dependent or transplanted (127); about 50 % develop end-stage renal disease (ESRD) by the age of 45 years (128). Female genital tract malformations, gout and hyperuricemia are associated with *HNF1B* mutations (127; 129), and elevated liver enzymes may be observed (50). Furthermore, pancreatic atrophy and exocrine dysfunction are usually present when *HNF1B* mutations are associated with diabetes (50; 51; 130; 131). Birth weight is reduced as a result of reduced insulin secretion *in utero* (51). Half of all *HNF1B* mutation carriers develop early-onset diabetes presenting in a similar fashion to *HNF1A* diabetes, but *HNF1B* mutation carriers are more insulin resistant (132). Because of the coexisting pancreatic atrophy and insulin resistance, *HNF1B* diabetes is not sensitive to sulfonylureas, and early insulin therapy is required (80). As 30-60 % of *HNF1B* mutations and deletions are spontaneous, a family history of renal disease or diabetes is not essential to prompt a screen for this disorder (133; 134). Even within a single pedigree there is wide variation in phenotypes, such that affected individuals with identical mutations may display different combinations and severities of organ involvement (127; 129; 134).

Other forms of monogenic diabetes include neonatal diabetes and the maternally inherited diabetes and deafness syndrome (MIDD). Neonatal diabetes is diagnosed before six months of age and characterized as transient (TNDM; linked to chromosome 6q24 abnormalities in about 70 % of cases) (135) or persistent (PNDM; in 50 % of cases caused by mutations in *KCNJ11* or *ABCC8*, encoding the Kir6.2 and SUR1 subunits, respectively, of the K_{ATP} channel; also reported in complete GCK deficiency) (90; 102; 103; 136-139). Oral sulfonylurea provides the most effective therapy (137; 140). Mutations in the insulin gene (*INS*) can cause TNDM (100; 101) and was recently reported to result in MODY (99). MIDD is caused by mitochondrial mutations, most frequently m.3243A>G, resulting in dysfunctional mitochondria and thereby disturbed ATP production, which leads to manifestations primarily in highly metabolically active organs (141).

1.2.3 Exocrine pancreatic dysfunction – diagnostic tests

Exocrine pancreatic dysfunction may lead to malabsorption, increasing the risk of fat-soluble vitamin deficiency (vitamins A, D and E) and deficiency of energy nutrients derived from fat, carbohydrates and proteins. Maldigestion and steatorrhea secondary to exocrine pancreatic insufficiency has traditionally been acknowledged to occur when postprandial secretion of pancreatic enzymes is reduced to levels below 5-10 % of normal (7; 142; 143), illustrating the large functional reserve of the exocrine pancreas. A recent review (144) argues that the believed overproduction of pancreatic enzymes is over-estimated, referring to reports showing a three orders of magnitude lower specific activity of pancreatic lipase on meal triglycerides than indicated by previous *in vitro* studies, and hypothesizing that since the pH of the small intestine is substantially reduced in pancreatic exocrine insufficiency, lipase activity is further reduced. Steatorrhea arises when excessive lipid is excreted in the faeces, defined as >7 g fat/24 hours (>5 g fat/24 hours in children) provided a balanced pre-test dietary fat intake, and this is traditionally used to define pancreatic insufficiency, i.e. uncompensated pancreatic exocrine dysfunction, which is the correlate of the end-stage exocrine pancreatic disease.

Various diagnostic tests are used to investigate pancreatic exocrine function as outlined in Table 4. Adaptations of the titrimetric method first described by Van de Kamer (145) are most commonly used to measure faecal fat excretion. To improve the validity, it is recommended to add a non-absorbable marker in the diet or to determine fat loss as a percentage of daily fat intake. Some studies report that near infrared spectrometry represent a simpler analysis and acceptable sensitivity and specificity compared to the titrimetric method to determine fecal fat in the stool collections (146). Incomplete stool collection, delayed intestinal transit or inaccurate calculation of fat intake may lead to underestimation of fat excretion, whereas overestimation can occur in infants less than six months old whose immature pancreases may excrete as much as 15 % of dietary fat (147). The method does not detect mild and moderate reductions in pancreatic exocrine function, and the secretin test remain the gold standard to measure pancreatic function. This test use intravenous delivery of the secretory stimulant secretin alone or in combination with secretagogues such as CCK to stimulate the pancreas, and assesses collected fluid volume, ions and enzymes via intubation of the duodenum. The lack of a consensus standardized procedure is a problem at

Table 4 Some pancreatic function tests

Test	Description	Advantages	Disadvantages
<i>Direct tests</i>			
Secretin (+CCK)	IV secretin +/- CCK Measure volume and HCO ₃ ⁻ secretion into duodenum +/-enzyme concentrations	Most sensitive and specific measurement of pancreatic function.	Duodenal intubation IV hormones Not widely available Not well standardized
Lundh test meal	Oral ingestion of test meal Measurement of duodenal trypsin concentration (+ amylase, lipase)	No IV hormones	Requires duodenal intubation and normal small intestinal mucosa Not widely available.
<i>Indirect tests</i>			
Fecal fat	72-hour stool collection Fat measurement in stool Preferably compared to a known dietary fat intake, e.g. a 100 g/day of fat diet	Quantitative measurement of steatorrhea	Stool collection Cumbersome analysis Compliance problems with keeping diet/reporting fat intake
Triglyceride breath test	Ingestion of 13C- or 14C-labelled triglyceride meal, measurement of labelled CO ₂ in exhaled breath	Low cost, non-invasive Measure of steatorrhea	Time-consuming (5-6 h) Limited sensitivity for mild dysfunction
Fecal elastase-1	ELISA assay measuring elastase-1 in a pea-size stool sample	Easy to perform Small, one-time stool sample	Do not detect mild or moderate dysfunction.
Fecal chymotrypsin	Enzyme assay measuring chymotrypsin in a pea-size stool sample	Room temperature No IVs or tubes	
NBT-PABA	Oral ingestion of NBT-PABA with a meal PABA in serum / urine	Simple measurements for severe pancreatic dysfunction	Do not detect mild or moderate dysfunction False positives with small bowel mucosal disease
Fluorescein dilaurate	Oral ingestion of fluorescein dilaurate with a meal Fluorescein in serum / urine		
Serum trypsin	Measure trypsin in blood sample	Simple, inexpensive Widely available Risk free	Low sensitivity False positives reported in diabetes

CCK, cholecystokinin; IV, intravenous; NBT-PABA, N-benzoyl-tyrosyl para-aminobenzoic acid.
Based on (2; 148)

least for research purposes. The Lundh test is a variant of this test, substituting a test meal for the secretagogue. Due to the invasive nature of the secretin test, and the cumbersome procedure and problems with compliance with stool collection and incorrect reports of fat intake with the Van de Kamer test, other tests have gained terrain in clinical practice.

The faecal elastase-1 assay merits a brief description as this indirect test has become increasingly used since the beginning of the 1990s (for a review, see(149)). It is an ELISA assay most often based on two monoclonal antibodies, although a test based on polyclonal antibodies also exists (150). The original reference values were established using 100 samples sent to the laboratory for microbiological culture of suspected gastrointestinal infection, and the stool water content was not reported. Since watery stools may affect elastase levels, alternative reference values have been launched based on lyophilized stool samples (151). Generally, it is agreed that a value of less than 200 μg elastase/g stool indicates mild to moderate exocrine dysfunction, whereas a value of less than 100 $\mu\text{g}/\text{g}$ indicates severe exocrine dysfunction (152). Studies have reported an intra-assay variation of 5.8-6.4, an inter-assay variation of 7.7-8.8, and an intra-patient CV of 15 % based on daily repeated measurements (152; 153). Using 200 $\mu\text{g}/\text{g}$ as cutoff, the specificity is reported to be in the range of 57-90 % compared to secretin tests or ERCP, and the sensitivity ranges 80-100 % for severe exocrine dysfunction (100 % in five out of six studies) but only 0-65 % in mild exocrine dysfunction, in both cases compared to modifications of the secretin test or ERCP/Cambridge classification (152). Thus, the test is more useful in the diagnosis of severe than mild to moderate exocrine dysfunction. Unlike the fecal chymotrypsin test, the measured fecal elastase-1 level is not affected by pancreatic enzyme supplements (148), but false positive results are reported in primary intestinal disease such as celiac disease with mucosal atrophy (149), short bowel syndrome (154), pouchitis (155), severe protein malnutrition (155), in infants less than 14 days (156) and in the case of non-lyophilized loose stools or diarrhoea (151).

1.2.4 Pancreatic exocrine dysfunction and combined exocrine and endocrine dysfunction

The combination of exocrine and endocrine pancreatic disease or dysfunction is observed in a variety of contexts. For monogenic diseases, which frequently display a combination of endocrine and exocrine pancreatic disease, a common developmental cause may be

suspected (Tables 3 and 5). Since the discovery of insulin treatment in the 1920s there have been reports of reduced exocrine pancreatic function in patients with a primary diagnosis of diabetes mellitus (157-173); and pancreatic cancer, hereditary pancreatitis, idiopathic pancreatitis and fibrocalculous pancreatitis are recognized causes of secondary diabetes as discussed below.

Celiac disease is associated with type 1 diabetes through common HLA markers (174), and the prevalence of biopsy-verified celiac disease in type 1 diabetes is estimated at around 5 % (174-177). Both primary pancreatic exocrine dysfunction, probably due to prolonged malnutrition, and dysfunction secondary to impaired release of endogenous CCK and secretin are described in celiac disease (178-180).

In pancreatic cancer, about 80 % of subjects have impaired glucose tolerance or diabetes; however, there is ambiguity as to whether pancreatic cancer causes diabetes or the conditions associated with diabetes promote the development of pancreatic cancer (reviewed in (181)).

Chronic pancreatitis is a continuing inflammatory disease which eventually leads to morphologic changes characterized by irreversible destruction and fibrosis of the exocrine parenchyma, leading to exocrine pancreatic insufficiency and progressive endocrine failure resulting in diabetes (182). Subtypes of chronic pancreatitis include hereditary pancreatitis, idiopathic pancreatitis and other causes (reviewed in (183; 184)). Diabetes is usually regarded as a secondary process to chronic pancreatitis and occurs more frequently in late-onset than early-onset pancreatitis (185; 186). The median age at onset of diabetes is 53 years in hereditary pancreatitis (187). Although alcohol is the main cause of chronic pancreatitis in most developed countries, accounting for 60-70 % of the cases in male patients, genetic alterations emerge as an important factor in the pathogenesis (182). *PRSS1* (protease, serine 1), encoding cationic trypsinogen, was the first gene in which mutations were associated with hereditary pancreatitis (188). It is proposed that pathogenic *PRSS1* mutations cause pancreatitis through enhanced auto-activation of trypsinogen to trypsin or prevent prematurely activated trypsin from being inactivated by autolysis (182). Both duplication and triplication copy number variants of *PRSS1* have been suggested to result in a gain of function in trypsin by a gene dosing effect, leading to both hereditary and idiopathic chronic pancreatitis (189; 190). In idiopathic chronic pancreatitis, heterozygous

mutations both in *CFTR* (188; 191-193) and *PRSSI* (188) have been associated with disease development. An increased risk of idiopathic pancreatitis has also been associated with variants in *SPINK1* (particularly the mutation S34N) (194), and the risk is considerably increased in combination with a heterozygous *CFTR* mutation (191). Mutations involving the calcium sensing receptor (*CASR*) have been suggested to increase the risk of chronic pancreatitis, particularly in combination with *SPINK1* mutations (195). Recently, loss-of-function mutations in *CTRC* were identified as predisposing to chronic pancreatitis (196). On the other hand, *PRSS2* mutation has been reported to be protective against chronic pancreatitis (197).

Table 5 Genes causing pancreatic exocrine dysfunction or pancreatic structural abnormalities and reported associations with diabetes

Gene name	Chrom. loc.	Cellular Function	Diabetes phenotype	Exocrine pancreatic phenotype	Ref.
<i>AIRE</i>	21q22.3		APECED	Exocrine dysfunction	(198-200)
<i>PTF1A</i>	10p12	Alpha subunit of PTF1	Permanent neonatal diabetes with cerebellar agenesis	Pancreatic agenesis	(201)
<i>CFTR</i>	7q31.2	Chloride channel; regulates other transport pathways	76 % diabetes by 30 years; TNDM (case report)	Cystic fibrosis: exocrine dysfunction	(202-205)
<i>SBDS</i>	7q11	Involved with ribosomal RNA	TNDM (case report)	Shwachman-Diamond syndrome	(206; 207)
<i>UBR1</i>	15q15-q21.1	E3 ubiquitin ligase	Early-onset DM (case report)	Johanson-Blizzard syndrome	(208; 209)
<i>PRSSI</i>	7q35	Cationic trypsinogen, protease	80 % diabetes by 80 years	Hereditary pancreatitis	(188) (210)
<i>PRSS7</i>	21q21	Initiates activation of protease proenzymes		Enteropeptidase deficiency	(211)
<i>EIF2AK3</i>	2p12	Pancreatic eIF2-alpha kinase	Wolcott-Rallison Syndrome; permanent neonatal diabetes	Pancreatic hypoplasia and fibrosis	(212)
<i>PI</i>	14q32.1	Protease inhibitor	IGT (case report)	Pancreatic fibrosis (case report)	(213; 214)

See also Table 3. APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; IGT, impaired glucose tolerance; TNDM, transient neonatal diabetes mellitus. *Adapted from (73).*

Tropical chronic pancreatitis (or tropical calcific pancreatitis) is a juvenile form of chronic calcific nonalcoholic pancreatitis seen in tropical developing countries, characterized by recurrent abdominal pain in childhood, followed by diabetes and steatorrhea one to two

decades later (215). Calculi or micro-calculi are found on radiological examination, and pathological studies demonstrate atrophy and fibrosis of the pancreatic gland and ductal metaplasia (215). The disease is believed to start in the exocrine tissue with subsequent dysfunction of the islets (215), but others suggests two separate pathogenic mechanisms leading to pancreatic exocrine dysfunction and diabetes (216). The combined condition of tropical chronic pancreatitis and diabetes is commonly termed fibrocalculous pancreatic diabetes (FCPD), although the entity is debated and classified under “diabetes due to other types” in the latest WHO classification of diabetes. FCPD is characterized by insulin requirement (217). A recent study from southern India showed a population prevalence of 0.02 % (217).

Several recent papers have reported a high prevalence of exocrine dysfunction in subjects with type 1 and type 2 diabetes based on tests for fecal elastase I to assess pancreatic exocrine dysfunction (218-228). Other studies analyzing fecal fat excretion have revealed steatorrhea in a high percentage of these patients (228; 229). Diabetes has usually been regarded as the primary process and the main mechanism is thought to be that insulin deficiency removes the trophic stimulus of insulin on acinar cells (insulo-acinar axis; insulin-pancreatic axis). Alternative hypotheses propose diabetic neuropathy or vascular damage as culprits. The clinical implications of exocrine disease secondary to diabetes have been debated (160; 230; 231).

1.2.5 Pancreatic enzyme substitution therapy (PEST)

Oral pancreatic enzyme supplements constitute the main treatment of maldigestion secondary to exocrine pancreatic insufficiency (232). Because of problems related to acid-mediated inactivation of lipase, enteric-coated microspheres are generally the preferred pharmacological formulation of pancreatic enzymes (233). Reports indicate that a minimal dose of 25 000-50 000 IU of lipase per meal is required (232), but higher doses may be needed (144). The efficacy of the treatment may be optimized by the administration of the medication along with or just after meals as opposed to just before meals (234). Others propose that further improvement can be obtained by adding H₂-blockers or proton pump inhibitors (omeprazole) to those failing to normalize their fat excretion on enzyme supplements alone (235; 236). It is recognized, however, that fat excretion is seldom normalized by PEST, although most studies evaluating this matter have been performed on

patients with cystic fibrosis. Some previous studies have observed a detrimental effect of pancreatic enzyme replacement on glycemic control in patients with chronic pancreatitis (237), but a recent prospective multicenter study examining enzyme replacement in Type 1 diabetes reported no effect on glycemic control (227).

1.3 Experimental rodent models of pancreatic disease

Most of the strains in use today derived from a limited number of founders from four subspecies of the house mouse (*Mus musculus*) that diverged approximately 1 million years ago (238). The classical studies of Coleman and Hummel (239) were the first to show that strain background affects diabetes susceptibility in mice. Strain background has been shown to influence most aspects of diabetes including insulin secretion, insulin resistance, and pancreatic characteristics such as beta cell survival and islet number; and strains also differ in their response to environmental factors like diet (238). A systematic compiling of phenotypic measurements across numerous mouse strains is found in The Mouse Phenome Database (www.jax.org/phenome).

Spontaneous mutations have given rise to several valuable animal models. The *obese (ob/ob)* mouse with a mutation in the gene for *leptin (Lep)* and the *diabetes (db/db)* mouse with a mutation in the *leptin receptor (Lepr)* gene are prominent examples (238), whereas the Akita mouse represents the only spontaneous mutation in mouse directly causing nonobese type 2 diabetes (238).

The development of gene targeted mouse models has transformed medicine by allowing the generation of disease models of human pathologies in a tractable mammalian system, thus enabling experimental dissection of disease states and identification of new therapy targets. Gene targeting has already produced more than five hundred different mouse models of human disorders. The importance of the creation of the technology for gene targeting in mice was underscored by the award of the Nobel Prize in Physiology or Medicine for 2007 to Mario R. Capecchi, Martin J. Evans and Oliver Smithies for their discoveries of “principles for introducing specific gene modifications in mice by the use of embryonic stem cells”.

Gene targeting is used to create either knockout models through inactivation of single genes, or knock-in or over-expressing models by introducing a gene and a promoter into the mouse

genome. More than ten thousand mouse genes or about half of the genes in the mammalian genome have been knocked out to date and ongoing international efforts will make knockout mice for all genes available within the near future.

The discovery by Martin Evans that chromosomally normal cell cultures could be established directly from embryonic stem (ES) cells and the demonstration by Mario Capecchi that homologous recombination could take place between introduced DNA and the chromosomes in mammalian cells thus repairing defective genes, were corner-stones in the development of genetically designed mouse models (240; 241). Tissue-specific gene knockout or over-expression enabled conditional targeting (242). *Cre-lox* technology is now widely used, and is based on the introduction of recognition sites for the enzyme Cre recombinase, so-called *lox^P* sites, into existing genes. Mice carrying such “floxed” genes are then mated with transgenic mice expressing Cre recombinase in tissues defined by the promoter placed before the Cre recombinase gene, and the target gene of the offspring is modified in the selected tissue through Cre action (243). Temporal control over expression can be exerted through the introduction of an element into the promoter which requires a ligand for introduction, for instance tetracyclin, tamoxifen or type I-interferon (244). Expression is thereby induced only by the delivery of the selected drug. Finally, *Cre-lox* technology can be used to replace an existing gene with another one (“knock-in”) (245). However, the generation of *lox^P*-flanked alleles is often time-consuming, or of limited value due to the existence of multiple copies of the gene and/or pseudogenes in the genome in which undesired targeting events may occur. The recently described RNA interference (RNAi) method may overcome such limitations, but create only knockdown models (246).

The C57BL/6 strain was created by Little in 1921 and is the most widely used of all inbred strains, accounting for about 14 % of all such uses, and it is the most commonly used reference strain in studies of diabetes. SNP analysis indicates that the strain is somewhat removed evolutionarily from the other inbred strains (238). Both protective and diabetes susceptibility alleles/phenotypes have been observed in the C57BL/6 strain. Data from the Mouse Phenome Database show that glucose and insulin levels of this strain are intermediate to those of the other strains (<http://phenome.jax.org/pub/cgi/phenome/mpdcgi?rtn=docs/home>). However, studies have shown that the C57BL/6 mouse is particularly susceptible to diet-induced obesity and hyperglycemia (238). It is less glucose-tolerant compared to C57BLKS/J, DBA/2 and 129X1 mice in a glucose tolerance

test (GTT) but more insulin-sensitive in an insulin tolerance test (ITT) than DBA/2 or 129X1 mice (247; 248), suggesting that a defect in insulin secretion is the likely cause of the reduced glucose tolerance in C57BL/6 mice. Following a high-fat, high-simple carbohydrate diet, islets from C57BL/6 mice had markedly impaired insulin secretion in response to high glucose, primarily due to a defect in second-phase insulin secretion (249; 250); and the islets were unresponsive to intermediate glucose levels (251), consistent with a beta cell defect in glucose responsiveness. Compared to other strains, lean C57BL/6 mice have a relatively low total islet mass and beta cell mass per body mass, but the highest number and the smallest size of islets, indicating a great capacity for the beta cell mass to expand under stress (238) which explains why the introduction of *Lep^{ob/ob}* on C57BL/6 background induces severe insulin resistance but only moderate and transient hyperglycemia and not diabetes (238).

The 129/Sv strain is heterogeneous and the phenotypic effects may differ between the various substrains. Substrains of 129/Sv have been employed for the majority of ES cell lines used in generating gene-targeted mice (knockout mice) (238). On both chow and high-fat-diet feeding, the 129/Sv mice maintain low insulin levels and are more glucose tolerant than other strains (252), suggesting an increased insulin sensitivity consistent with the low beta cell mass in wild-type 129/Sv mice (253). Thus, the 129 strain is relatively resistant to genetic obesity-induced diabetes. Lean 129 mice with genetically induced defects in insulin signalling due to double heterozygosity for *Irs1* and *Insr* mutations have only mild hyperinsulinemia with little insulin resistance and do not increase beta cell mass compared with wild-type 129 mice (253), in contrast to C57BL/6 mice.

A summary of some existing transgenic mouse models for pancreatic disease including diabetes is given in Table 6.

Table 6 Some mouse models of gene disruption and the associated pancreatic phenotype

Gene	Model	Phenotype	Refs..
<i>AIRE</i>	KO	Exocrine inflammation on NOD background	(254)
<i>ARNT</i>	KO	Impaired insulin secretion	(255)
<i>CFTR</i>	KO	Dilated ducts and acinar atrophy	(256)
<i>DLL1</i>	KO	Accelerated endocrine development	(257)
<i>E2F1</i>	KO	Pancreatic volume reduction and IGT	(258)
<i>E2F1/E2F2</i>	KO	Acinar atrophy, beta cell loss, fatty infiltration	(259)
<i>EIF2AK3</i>	KO	IGT, exocrine insufficiency, loss of beta cells	(260; 261)
<i>GLUT2</i>	KO	Glucose intolerance, impaired insulin secretion, diabetes	(262)
<i>HES1</i>	KO	Forms acini and islet structures from bile ducts	(263)
<i>HLXB9</i>	KO	Dorsal pancreatic agenesis, mildly disturbed endocrine-cell differentiation	(264)
<i>HNF1A</i>	KO	IGT, reduced insulin content in beta cells	(265)
<i>HNF1B</i>	KO	Embryonic lethal, pancreatic agenesis if rescued	(130)
<i>HNF4A</i>	KO	Embryonic lethal, IGT in conditional knockouts	(266)
<i>HNF6</i>	KO	IGT, aberrant islet morphogenesis	(267)
<i>INSR</i>	KO	Die within 7 days after birth from diabetic ketoacidosis	(268)
	KO	βIRKO: Loss of glucose-stimulated first-phase insulin secretion, small islets, reduced insulin content of islets	(269)
<i>INSR/IGF1R</i>	KO	IGT, loss of beta cell mass	(270)
<i>IPF1</i>	KO	Pancreatic agenesis	(271)
<i>(PDX1)</i>		Beta cell mutants: Diabetes due to reduced insulin expression and beta cell dysfunction	
	OE	Apoptosis and fatty infiltration (exocrine OE)	(272)
<i>IRS1</i>	KO	Mild insulin resistance, beta cell hyperplasia, hyperinsulinemia	(273)
<i>IRS2</i>	KO	Severe insulin resistance, reduced beta cell mass, diabetes	(274)
<i>ISL1</i>	KO	Islet and dorsal pancreatic agenesis	(275)
<i>KRT8</i>	OE	Acinar inflammation, fatty infiltration	(276)
<i>MIST1</i>	KO	Disorganized exocrine tissue	(277)
	KO	IGT and decreased islet size	(278)
<i>NEUROD1</i>	KO	Aberrant islet and entero-endocrine morphogenesis	(279)
<i>NGN3</i>	KO	Endocrine cell agenesis	(35)
	OE	Accelerated endocrine development	(257)
<i>NKX2B</i>	KO	Aberrant islet morphogenesis	(280)
<i>NKX6A</i>	KO	Reduction of beta cells after secondary transition	(281)
<i>PAX4</i>	KO	Absence of beta and delta cells	(282)
<i>PAX6</i>	KO	Absence of alpha cells	(283)
<i>PBX1</i>	KO	Hypoplasia/aberrant differentiation of pancreas	(284)
<i>PDK1</i>	KO	IGT, loss of beta cell mass	(285)
<i>PI14</i>	KO	Isolated exocrine insufficiency, acinar apoptosis	(286)
<i>PTF1A</i>	KO	Exocrine pancreatic agenesis	(287)
<i>RPH3AL</i>	KO	Defective endocrine and exocrine exocytosis	(288)
<i>SOCS1</i>	KO	Acinar inflammation, atrophy and apoptosis	(289)
<i>TGFBR2</i>	KO	Acinar inflammation, fatty infiltration	(290)
<i>TTC10</i>	KO	Disorganized exocrine tissue, ductal dilation	(291)

KO, knockout; OE, overexpressing model. *Based on the OMIM database.*

1.4 Carboxyl-ester lipase (CEL)

1.4.1 The CEL gene and protein: Structure, expression, function

The human *CEL* gene (292; 293) is located on chromosome 9q34.3 (294). The gene consists of 11 exons (295) and encodes a protein of 745 amino acids including a signal peptide. The last exon contains a VNTR encoding a highly variable number of repeats; most frequently 16 repeats of 11 amino acids (296), but repeat numbers from 7 to 21 have been observed (297; 298). The gene is well conserved in all vertebrate species examined, but the variability of the VNTR between species is high, varying from no repeats in salmon (299) through three repeats in mice (300) and four in rats (301) to as much as 39 repeats in gorilla (302). While the mouse *CEL* is a single-copy gene spanning approximately 7.2 kb on the syntenic region of the homologous mouse chromosome 2 (303), in humans a pseudogene, *CELL* (or *CELP*), is located in tandem with *CEL* 10 kb downstream of the end of *CEL*. The pseudogene lacks exon 2-7 and have differences in the coding sequence of exon 10 and 11 (295; 304), and although it is ubiquitously transcribed at the mRNA level it is not translated (292). It has been suggested that *CEL* has evolved from *CELL* by gene duplication and that the original gene has been inactivated during evolution (292).

CEL (also referred to as bile salt-dependent lipase, bile salt-stimulated lipase, bile-salt activated lipase precursor, cholesterol esterase or lysophospholipase) is an enzyme with broad specificity (292). Its main action is bile-salt dependent lipase activity to hydrolyze cholesterol esters into free cholesterol and fatty acids to facilitate their absorption in the small intestine, but a recent report demonstrates that CEL also has an important role in the absorption of dietary lipids such as triacylglycerols (TAG) and in the hydrolysis and absorption of retinyl esters, acting in concert with pancreatic triglyceride lipase (PTL) in a mutually compensatory way (292; 305). CEL also participates in chylomicron assembly and secretion in a mechanism mediated through its ceramide hydrolytic activity (293). A fraction of pancreatic CEL in the duodenum undergoes transcytosis via the lectin-like oxidized LDL receptor (LOX-1) (306) and is subsequently found in human plasma partly associated with apolipoprotein B-containing lipoproteins (293). In the circulation, CEL has been reported to be involved in several activities such as the induction of vascular smooth muscle proliferation (307), endothelial cell proliferation and chemotactic migration (308), thrombus formation through interaction with platelet CXCR4 (309) and a negative or protective effect

on atherosclerosis is debated (307; 310; 311). Furthermore, reports suggest that CEL participates directly in the selective uptake of cholesteryl esters in HDL in the liver (312). A recent report shows that CEL is removed from the circulation by renal filtration through unknown mechanisms and is eliminated in the urine of healthy persons (313).

The catalytic properties of the CEL enzyme reside in the highly conserved N-terminal part of the protein, where the active site is formed by the catalytic triad Ser194-His435-Asp320 (292). The carboxyl terminus of the protein regulates enzyme activity by forming hydrogen bonds with a surface loop partially covering the active site, thus partially shielding it (292). Bile salt binding to the bile salt binding site induces a conformational change involving the removal of the loop from the active site, enabling reaction with the substrate (314). In the absence of bile salts, crystal studies suggest a preformed catalytic site and a functional oxyanion hole explaining the activity of CEL on water-soluble esters (292). Crystal studies of truncated human CEL lacking the C-terminal PEST repeats, showed that in this protein the surface loop assumed a predominantly open conformation such that the active site was open, allowing direct access to water-soluble substances; and in the presence of a bile salt analogue the truncated protein failed to interact with bile salt, indicating that deletion of the PEST sequences may compromise the bile salt activation of CEL (315). However, other data indicate that deletions of parts of the C-terminal do not interfere with the catalytic activity of the enzyme (316; 317).

The size of the CEL enzyme is highly variable, ranging from a molecular weight of 120-140 kDa in humans to approximately 74 kDa in mouse. This size variation is due to the variable number of PEST repeats in the C-terminal end of the protein; three repeats are found in the mouse while in humans 16 repeats is most frequently found (318) although 56 % of individuals carry at least one polymorphic form of CEL (298). While associated with intracellular membranes by means of a folding complex involving the chaperone Grp94, CEL undergoes post-translational modifications first in the ER, where N-glycosylation at Asn187 is essential for correct folding and secretion, and then *O*-glycosylation of each tandem repeat of the PEST sequences in the Golgi apparatus (292) (Figure 6). The PEST sequence *O*-glycosylation is important for normal CEL secretion and for maintaining protein stability (319), and failure of *O*-glycosylation of CEL seems to lead to degradation by the proteasome-ubiquitin-dependent pathway (320). Furthermore, since the *O*-glycosylation masks the PEST sequence it is hypothesized to rescue CEL from PEST-mediated

degradation (292). In the intestine the *O*-glycosylation prevents acidic and proteolytic damage of CEL (321). The PEST repeats may also be important for heparin binding and binding to cell surface receptors and thereby involved in the suggested effects of CEL in atherogenesis (322).

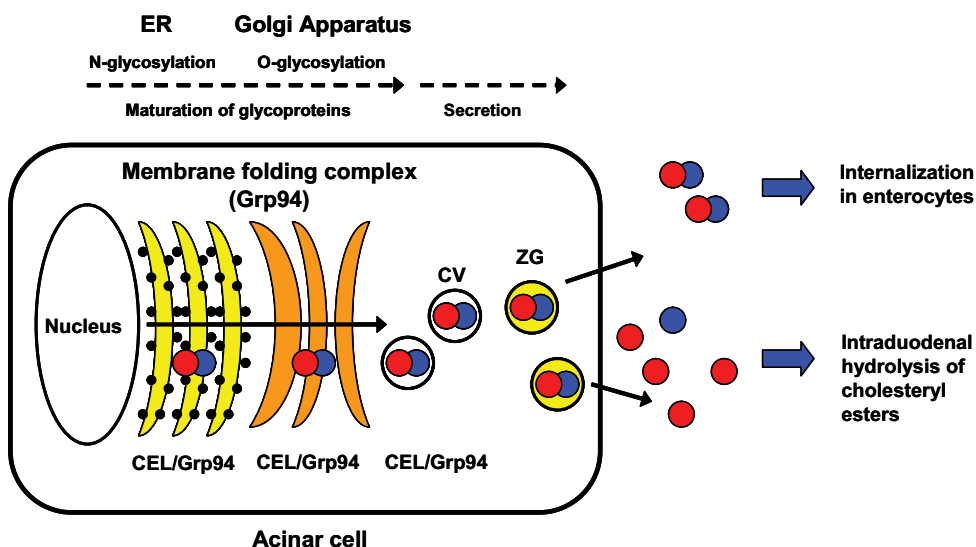


Figure 6. Schematic presentation of the secretory pathway of CEL and its association with the chaperone Grp94 in pancreatic acinar cells. Red circles, CEL; blue circles, Grp94; CV, condensing vesicles; ZG, zymogen granules. Based on (292).

CEL is mainly expressed in the acinar cells of the exocrine pancreas and in lactating mammae, but low level expression at the protein level has also been reported in human fetal liver (323) and rat liver (292); and is found in and may be synthesized by human but not mouse macrophages and endothelial cells (311; 324). In contrast, CEL is not expressed in islets or beta cells (325) and was not found by screening of laser-captured beta cells (S. Bonner-Weir, personal communication). High-level expression of human CEL in the pancreas is dependent on a pancreas-specific enhancer element found in the 5'-upstream sequence of the CEL gene in co-operation with two closely located *cis* elements, and a protein binding to a C/EBP-like motif is also necessary and probably prevents the binding of PTF1 to the partly overlapping potential PTF1 site (326). In contrast, in the mouse promoter a PTF1-binding site is mandatory for expression, and a more distal PTF1 interaction and a factor binding to the mDPE augment the effect of PTF1 binding to its site (326). Thus,

although *CEL* is expressed at comparable levels in the exocrine pancreas of both mouse and human, the most important regulatory factors in the promoters are different.

1.4.2 Pathology associated with *CEL*

The recent discovery that mutations in *CEL* cause a syndrome of diabetes and pancreatic dysfunction is discussed below. *CEL* has previously been linked to diabetes through a report of antibodies towards *CEL* in patients with type 1 diabetes (327), and Raeder *et al.* reported that single-base insertion polymorphisms in the *CEL* VNTR are associated with exocrine dysfunction in patients with type 1 diabetes (52). An association of *CEL* VNTR polymorphisms involving less than 16 repeats with lower total and LDL cholesterol levels has been observed (297; 328). Interestingly, the human *CEL* gene maps to locus q34.3 on chromosome 9 (294), close to the ABO blood group antigen locus, which is linked to lipid phenotype and cardiovascular disease in humans (329). Furthermore, a correlation of *CEL* VNTR polymorphisms with the risk of alcohol-induced pancreatitis has been reported (330). A link to the immune system has also been suggested by a report showing that variations in human milk *CEL* confers different binding capacities of dendritic cells affecting their inhibitory effect on HIV-1 transfer (331). *CEL* expression seems to be reduced in acinar cell carcinoma and is not observed in pancreatic adenocarcinoma (325).

1.4.3 The *CEL* syndrome - Diabetes and pancreatic exocrine dysfunction

Raeder *et al.* identified two different single-base deletions in the *CEL* VNTR to cause a syndrome of diabetes and exocrine dysfunction (*CEL*-MODY; denoted DPED or MODY8 in the OMIM database) through a genome-wide screen in two families and subsequent positional cloning (52). The single-base deletion in Family 1 of the article (Del1; 1686delT/C563fsX673) occurred in the first repeat on an allele of 14 repeats in which the 7th and 8th repeats were missing compared to the consensus sequence, whereas the single-base deletion in Family 2 (Del4; 1785delC/C596fsX695) occurred in the fourth repeat on an allele of 16 repeats. Both deletions were observed in poly-C tracts, which are found in every VNTR repeat of *CEL* exon 11 and which are known mutation hotspots in several genes including *HNF1A* (332). Furthermore, both alleles with single-base deletions were predicted by *in silico* studies to lead to an altered C-terminal and loss of the PEST sequence and

several glycosylation sites, but encode downstream protein segments of considerable length (100 amino acids or more) before termination by a premature stop codon. Enzyme activity assays using stably transfected CHO cells revealed reduced protein stability and secretion rate of the mutant protein, but unaffected enzyme activity.

Mutation carriers with Del1 had diabetes (N = 14), impaired glucose tolerance (IGT; N = 3), normal glucose tolerance (NGT; N=8) or uncertain glucose tolerance status (N = 8). Concerning the diabetic patients, more than half were using insulin, none had experienced ketoacidosis, and diabetes typically presented in the mid-thirties. They were further characterized by normal body mass index, moderately elevated concentrations of fasting glucose and 2-h glucose after an oral glucose tolerance test (OGTT), clearly elevated glycosylated haemoglobin (HbA1c), reduced fasting C-peptides and an impaired insulin response during an intravenous glucose tolerance test (IVGTT). IVGTT performed in mutation carriers with NGT revealed a modest but significant reduction in first phase insulin secretion on glucose stimulation, indicating an early beta cell dysfunction. The mutation carriers with IGT or NGT were generally considerably younger than the mutation carriers with diabetes. All tested mutation carriers had either severe (fecal elastase < 100 µg/g; N = 30) or moderate (fecal elastase 100-200 µg/g; N = 3) fecal elastase deficiency (FED) suggesting exocrine pancreatic dysfunction, and this was supported by findings of increased fecal fat excretion in all ten diabetic subjects tested and reduced concentrations of fat-soluble vitamins A and E in the mutation carriers compared to controls. Pancreatic lipomatosis was reported in ten adult mutation carriers with diabetes and exocrine dysfunction (52) and also in non-diabetic children with *CEL* mutations (53), indicating that lipomatosis of the pancreas may be an early event involved in the pathogenesis of the DPED syndrome.

2. AIMS OF THE PRESENT STUDY

A number of recent studies have reported a three-to-tenfold increase in the prevalence of exocrine dysfunction in subjects with type 1 or type 2 diabetes, and a considerable proportion of these patients have steatorrhea with potential for complications. This fact does not seem to have attracted the attention it deserves, and the mechanisms behind the association are not clear. Previous studies of monogenic diseases have proved useful in the understanding of molecular mechanisms involved in diabetes, and transgenic mouse models have evolved as important tools for such studies. With this in mind, and wishing to shed some light on the mechanisms of exocrine-endocrine interplay in diabetes, we aimed to:

1. investigate the existence of pancreatic exocrine dysfunction in two subtypes of diabetes, *HNF1A*-MODY and *HNF1B*-MODY (Papers I, III)
2. examine whether diabetes and/or exocrine dysfunction was associated with structural abnormalities of the pancreas in *HNF1A*-MODY and *HNF1B*-MODY (Papers II, III)
3. perform a more thorough clinical characterization of the recently discovered *CEL*-MODY syndrome of diabetes and pancreatic exocrine dysfunction (Paper IV)
4. explore the effects of pancreatic enzyme substitution on exocrine and endocrine pancreatic function in *CEL*-MODY (Paper IV)
5. investigate the clinical, pathological and molecular characteristics of two potential mouse models for *CEL*-MODY (Papers V, VI)

3. MAIN RESULTS

Paper I describes the first prevalence study of exocrine dysfunction in patients with *HNFI*A-MODY. Subjects were recruited from the Norwegian MODY Registry, and compared to patients with type 1 diabetes recruited consecutively from an out-patient diabetes clinic as well as to non-diabetic controls. The paper reports that 13 % of adult *HNFI*A-MODY patients had exocrine dysfunction as defined by a fecal elastase level less than 200 µg/g, compared to 19 % of type 1 diabetes patients and 4 % of non-diabetic controls. Thus, the prevalence of exocrine dysfunction in *HNFI*A-MODY patients was similar to that in type 1 diabetes subjects in this and previous studies, and more than three times higher than in non-diabetic controls. Fecal elastase deficiency was inversely associated with age; however, the difference between *HNFI*A-MODY or type 1 diabetes patients and nondiabetic controls was maintained after controlling for age. Fecal fat excretion was increased in all investigated *HNFI*A-MODY patients with fecal elastase deficiency, underscoring the potential clinical importance of the exocrine dysfunction.

Paper II is the first report of radiological investigations of pancreatic structure in *HNFI*A-MODY patients and studies the putative association of any structural changes with pancreatic exocrine dysfunction. We invited all of the *HNFI*A-MODY patients in which exocrine dysfunction was identified in Paper I, and matched *HNFI*A-MODY controls without exocrine dysfunction, to undergo computed tomography of the pancreas by a standardized protocol. This was compared to groups of type 1 or 2 diabetes cases with exocrine dysfunction and their age- and gender-matched controls with normal exocrine function, and to non-diabetic control subjects recruited from a CT archive. The paper reports that the *HNFI*A-MODY patients had significantly reduced pancreatic volume compared to non-diabetic controls, as did the type 1 diabetes subjects. However, the reduction in pancreatic volume was less in *HNFI*A-MODY than in type 1 diabetes. A striking atrophy such as reported in *HNFI*B-MODY, was not observed; nor were typical changes seen in chronic pancreatitis or signs of pancreatic lipomatosis as reported to be associated with *CEL*-MODY and other monogenic diseases. Pancreatic volume adjusted for body surface area was associated with fecal elastase levels, but exocrine dysfunction did not explain the reduction of pancreatic volume or the differences between pancreatic volumes in diabetes subtypes.

Paper III describes the clinical and radiological findings in five subjects from two families with *HNF1B*-MODY due to two different mutations in *HNF1B*. The paper reports agenesis of the pancreatic body and tail and a slightly atrophic pancreatic head in all of the patients as revealed by computed tomography and magnetic resonance cholangiopancreatography. Exocrine dysfunction as defined by fecal elastase deficiency was also reported in all patients, and several had vitamin deficiencies. The radiological findings strengthen the evidence for a critical role of *HNF1B* in the embryonic development of at least the dorsal pancreas.

Paper IV reports the results of a treatment study with pancreatic enzyme supplements in nine family members from Family 1 in a previous paper by Raeder *et al.* describing a new syndrome of diabetes and pancreatic exocrine dysfunction due to mutations in the *CEL* gene (*CEL*-MODY, DPED). The study showed that pancreatic enzyme substitution alleviated symptoms of malabsorption, reduced steatorrhea, and normalized blood vitamin E levels in most patients, but did not affect glycemic control despite a slight weight increase with treatment. As anticipated, exocrine function as assessed by fecal elastase levels was not affected. During the study, a more thorough clinical characterization of twelve members of both families reported in the above mentioned paper revealed neurologic pathology. Five patients had carpal tunnel syndrome, three had high-signal white matter changes on MRI, and a majority of the patients had had slowing of nerve conduction compatible with a demyelinating neuropathy which was distinct from the neuropathological pattern normally seen as a complication of diabetes.

Paper V describes the study of the pancreatic endocrine and exocrine function the carboxyl-ester lipase knockout mouse (CELKO). Based on preliminary data suggesting that a mutation causing *CEL*-MODY induced reduced stability of the CEL protein, we wanted to investigate whether a knockout of the CEL gene would recapitulate the syndrome of *CEL*-MODY in an existing CELKO mouse line. Mice of both genders were studied until twelve months of age on normal chow and on high fat diets challenging glucose homeostasis. However, only mild glucose intolerance was observed in mice with whole-body knockout of *CEL*, whereas the full phenotype of human *CEL*-MODY was not reproduced. Morphological studies of pancreatic sections did not reveal any consistent indications of preclinical disease. Our findings suggest that the pathogenic mechanisms involved in *CEL*-MODY are more complex than a simple loss of CEL function.

Paper VI describes the establishment and study of the TgCEL mouse, a transgenic mouse line expressing the human *CEL* gene carrying a delT-mutation previously demonstrated to be linked with *CEL*-MODY. The mouse line was created using Cre-Lox technology for pancreas-specific expression. Beta-gal staining and immunohistochemical investigations in offspring of cross-matings with Rosa26-beta-gal mice indicated transgenic expression restricted to the pancreatic acinar cells and 10-15 % of the beta cells. Expression of human CEL was verified by qRT-PCR. The mice were studied on normal chow diet at four, seven and nine months of age, and after a 12 weeks challenge with a 60 % high fat diet at 12 months of age. After the high fat challenge, the mice were reverted to normal chow diet and followed by physiological testing until 22 months of age. None of the mice developed diabetes, and no signs of pancreatic endocrine or exocrine abnormality were detected. Interestingly, discrete areas of abnormal acinar tissue were observed in histological sections from two male mice, of which one was a TgCEL mouse whereas there was some uncertainty concerning the genotype of the other mouse. In conclusion, the paper demonstrates the successful establishment of a mouse line expressing, at least on the mRNA level, human *CEL* carrying a disease-associated mutation; but the mice lacked a clear phenotype. This indicates that further manipulation of genetic or environmental factors should be explored in the attempt to create a transgenic mouse model for human *CEL*-MODY.

4. GENERAL DISCUSSION

Exocrine and endocrine pancreatic disease is often observed to occur together. The close co-localization of the two distinct tissues within one organ, as well as their common embryological ancestry, has given rise to hypotheses concerning the pathogenesis of pancreatic diseases. However, in many cases the molecular pathways are not fully understood, and two questions remain: Which cell type is the origin of the disease? And what is the most fundamental pathological process?

Principally, there are three possible sites of action in combined exocrine and endocrine pancreatic disease: The exocrine cells, the endocrine cells, or the common progenitor cells (Figure 7). In order to investigate the association between exocrine function and diabetes further, we have studied exocrine and endocrine function as well as pancreatic structure in three different contexts: *HNF1A*-MODY, *HNF1B*-MODY and *CEL*-MODY.

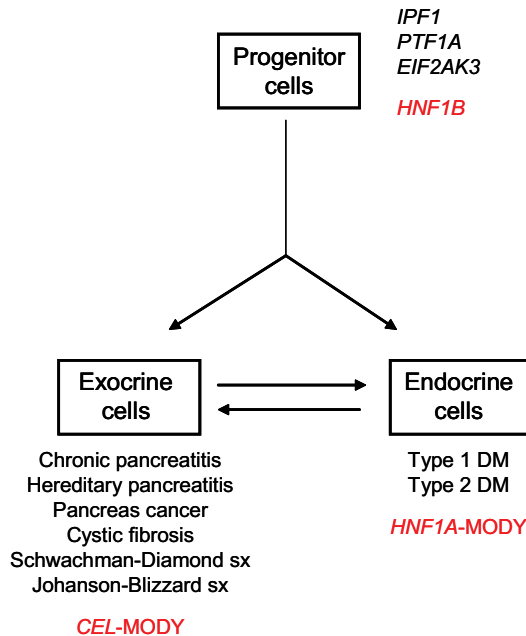


Figure 7. The schematic illustrates our current perception of the associations between diabetes and pancreatic exocrine dysfunction. The three principal sites of origin of disease are the exocrine cells, the endocrine cells or the common progenitor cells. Various causes of combined exocrine and endocrine disease are attributed to each of these tissues.

4.1 *HNF1B*-MODY: Developmental disorder and pancreas malformation (Paper III).

Considering the striking association with pancreatic atrophy reported in *HNF1B* mutation carriers based on MRI or CT imaging (50; 51; 131) and reports of exocrine dysfunction (50; 51; 333), we wanted to investigate pancreatic structure and exocrine pancreatic dysfunction in subjects with *HNF1B*-MODY. In Paper III, we describe the absence of tissue corresponding to the pancreatic body and tail as evaluated by CT and MRCP in five individuals from two families with different mutations in *HNF1B*. All of the subjects had exocrine dysfunction as assessed by fecal elastase analysis. Although our description of agenesis of the dorsal pancreas differed from the pancreatic atrophy described in previous studies, this difference might be due to alternative interpretation rather than a true variation in the structural changes. The distinction is important, however, for the understanding of the underlying pathogenetic mechanisms. Whereas agenesis and hypoplasia signify the absent or incomplete development of a tissue or organ, atrophy implies the partial or complete wasting away of an organ. Causes of atrophy include poor nourishment, poor circulation, loss of neural or hormonal stimulation of the target organ, or disease intrinsic to the tissue itself.

Mouse studies have revealed that *Hnf1b* has an essential function in the first steps of the pancreatic development as a critical regulator of a transcriptional network that controls the specification, growth, and differentiation of the embryonic pancreas (334; 335). Embryos completely deficient of *Hnf1b* exhibited a rudimentary dorsal pancreatic bud, which was only transiently present, and a ventral part of the pancreas which was not specified (130). In humans, severe hypoplasia of the pancreatic body and tail, resulting mainly from underdeveloped acini, was observed during autopsy of two fetuses carrying heterozygous mutations in exon 2 and 7 of *HNF1B*, respectively (336). Moreover, the fetuses exhibited absence of ventral pancreatic-derived tissue recognized from histological and immunohistochemical analyses, indicating ventral pancreatic agenesis in addition to dorsal pancreatic hypoplasia (336). The results of these studies seem to support a developmental defect as the cause of disease.

The findings of hypoplasia of the pancreatic head and agenesis of the body and tail in children and adult *HNF1B* mutation carriers in Paper III, are complementary with the mouse and

human fetal studies. The stronger severity of the phenotype we observed compared to that in the human fetuses, suggests that either different mutations are associated with different severity (hypoplasia vs. aplasia), paralleling observations of genotype-phenotype associations in *HNF1A*-MODY (118), but in contrast to previous reports on lacking association between genotypes and phenotypes in *HNF1B* mutation carriers (126; 131). More likely, perhaps, the pancreatic abnormalities may have a degenerative component in addition to a congenital malformation. Studies have shown that surgical pancreatectomy or autoimmune destruction of the pancreatic beta cells must remove more than 90 % of the insulin secretory capacity before resulting in diabetes (337; 338). However, others have reported an average beta cell area of 65 % in patients with overt type 2 diabetes (339). As the pancreatic volume was 24-43 % of normal in our cases, the *HNF1B* mutations could have had a direct effect on beta cell mass or insulin secretion in addition to the loss of beta cells as a result of pancreatic agenesis, in order to yield diabetes, given a relatively even distribution of beta cells within the pancreas. Interestingly, the two children in the study had not developed diabetes, although the elder child had IGT. Taken together, our observations strengthen the evidence for a critical role of *HNF1B* in the normal development and differentiation of the pancreas and support the notion that *HNF1B* mutations cause diabetes and exocrine dysfunction based on congenital malformation with a possible additional degenerative component.

Other examples of mutations in genes expressed in the progenitor cells associated with both exocrine and endocrine pancreatic disorder, include mutations in *IPF1*(93), *PTF1A* (201), and *EIF2AK3*. For all of them, pancreatic hypoplasia, atrophy or agenesis has been reported in mutation carriers in addition to either MODY-type diabetes (*IPF1*) or PNDM (*IPF1*, *PTF1A*, *EIF2AK3*) (92; 93; 201; 212). Studies have shown that targeted disruption of *Ipfl* or *Ptfla* in transgenic mice results in a failure of the pancreas to develop, i.e. pancreatic agenesis; and in addition the *Ptfla*^{-/-} null phenotype is postnatally lethal, although low insulin levels may still be detectable due to production in beta cells misallocated to splenic mesenchyme (28; 37; 271; 287). These data suggest that mutation in *IPF1* and *PTF1A* affect pancreas development, causing pancreatic agenesis leading to both diabetes and exocrine deficiency.

The loss of expression of *EIF2AK3* (eIF2a kinase 3, *PERK*) was recently identified as a new form of permanent neonatal diabetes associated with the human Wolcott-Rallison syndrome

(212). PERK KO mice have normal pancreata at birth, but develop diabetes and exocrine deficiency due to decreased beta cell proliferation and differentiation, and massive apoptosis of the exocrine tissue (260; 261; 340). *PERK* KO mice exhibit distended ER due to accumulation of proinsulin and GLUT2, and it was speculated that ER stress was the pathogenetic mechanism leading to diabetes (260). However, a later study did not detect increased ER stress markers, and a developmental defect is now considered the main cause of diabetes (340). It is still possible that ER stress leading to apoptosis of acinar tissue is the main cause of the exocrine deficiency in *EIF2AK3* mutation carriers (260).

4.2 *HNF1A*-MODY: Exocrine dysfunction and small pancreas secondary to diabetes (Papers I and II).

For *HNF1A*-MODY, information about pancreatic structure or exocrine function was, before our study started, lacking. As *HNF1A* encodes a transcription factor that is closely related to *HNF1B*, it was conceivable that a mutation in this gene could yield a similar phenotype for pancreas structure as seen in *HNF1B*-MODY. *HNF1A* is also expressed in the exocrine pancreas and might be directly associated with a defect in pancreatic exocrine function (341). In addition, exocrine dysfunction has been observed in 10-30 % of patients with type 1 or 2 diabetes (218-222), and reduced pancreatic volume as estimated by radiological imaging or autopsy studies, has been reported for type 1 diabetes patients and to some degree in type 2 diabetes in a number of papers (45-47; 342-344). Thus, if exocrine dysfunction was associated with diabetes in *HNF1A* mutation carriers, a primary process in the endocrine or exocrine tissue, or at the progenitor level, seemed possible. In Paper I we describe for the first time the prevalence of pancreatic exocrine dysfunction in a cohort of patients with *HNF1A*-MODY. Exocrine dysfunction was expressed by fecal elastase deficiency, defined by a fecal elastase level less than 200 µg/g. The data in Paper I indicated that 13 % of adult *HNF1A*-MODY patients had exocrine dysfunction. This was similar to results from the type 1 control group included in the study (19 %) and in line with previous findings in type 1 and 2 diabetic patients, but three times higher than that in nondiabetic controls (4 %). The strong association observed in *HNF1B*-MODY was not paralleled in *HNF1A*-MODY. Fecal elastase deficiency was not restricted to any particular *HNF1A* mutation, as it was observed in association with six different mutations.

In Paper II, we pursued the investigation of these patients further, and our data showed for the first time that the *HNF1A*-MODY patients had significantly reduced pancreatic volume compared to non-diabetic controls, but the reduction in pancreatic volume was less than in type 1 diabetes. We found that in both *HNF1A* mutation carriers and type 1 diabetes subjects, reduced pancreatic volume index was inversely associated with diabetes duration in agreement with some (47) but not all (45; 46) previous studies. Although reduced pancreatic volume adjusted for body surface area was associated with low fecal elastase levels, in line with a previous report observing an association of pancreatic volume and serum immunoactive trypsin (45), exocrine dysfunction did not explain the reduction of pancreatic volume or the differences between pancreatic volumes in diabetes subtypes in our study.

Pronounced atrophy of the pancreas as previously reported for *HNF1B* mutation carriers, or dorsal agenesis of the pancreas as described in Paper III, was not observed. Although *HNF1A* and *HNF1B* are closely related and interact in the same transcriptional network, *HNF1A* appears to have less pronounced effect on pancreas volume and structure than *HNF1B* (50; 126). This is agreement with findings in mouse studies delineating *Hnf1b* as an important regulator of pancreas organogenesis and differentiation, whereas *Hnf1a* primarily regulates the growth and function of beta cells (reviewed in (335)). Primary exocrine disease can also affect pancreatic volume, as demonstrated by a recent study showing a 21 % reduction of pancreatic volume in patients with chronic pancreatitis, accompanied by a 29 % deficit in beta cell area (44). Recently, it was argued that type 3c diabetes, i.e. diabetes caused by chronic pancreatitis, is much more common than generally believed, as pancreatic morphological changes are present in about 35 % of type 2 diabetes cases and in up to 50 % of type 1 diabetes subjects (345; 346). In Paper II, we found a prevalence of 19 % for fecal elastase deficiency in subjects with type 1 diabetes. The apparent discrepancy between our findings and that of some others may be due to differences in the diagnostics; most studies rely on results from one fecal elastase analysis, whereas we defined fecal elastase deficiency as fecal elastase below 200 $\mu\text{g/g}$ in two consecutive tests. Observations in subjects with diabetes include changes of the pancreatic duct system characteristic for chronic pancreatitis (347; 348). However, the structural changes characteristic of chronic pancreatitis (Table 7) were not detected in our *HNF1A* mutation carriers. Pancreatic lipomatosis has been identified as an early structural marker of pancreatic exocrine disease in *CEL* mutation carriers (52; 53), and fatty replacement has also been observed in other causes of monogenic diabetes with primary affection of the exocrine pancreas (209; 349). A negative correlation

between pancreatic fat content and beta cell function has been observed by some (350) but not by others (342). However, pancreatic x-ray attenuation was not associated with fecal elastase levels in *HNFI*A-MODY patients, and pancreatic lipomatosis was not observed.

Table 7 Exocrine function and radiological findings in some pancreatic disorders

Characteristics	T1D	T2D	<i>HNFI</i> A-MODY	<i>HNFI</i> B-MODY	<i>CEL</i> -MODY	Chronic pancreatitis
Diabetes (%)	100	100	100	50?	100?	22**
FED (%)	19	10-35	13	100	100	
Steatorrhea (%)	70	33	100	NI	78	7**
<i>Radiological findings</i>						
Pancreatic volume index reduction (%)	53	NS	25	Striking		21
Pancreatic fibrosis/lipomatosis	No	No	No	No	Lipomatosis	Fibrosis
Duct abnormalities	No *(Yes)	No *(Yes)	No	No	No	Strictures, widening, irregularities
Cavities	No	No	No	No	No	Yes
Calcifications	No	No	No	Yes/No		Yes
Pancreatic malformation	No	No	No	Dorsal agenesis	No	No

T1D, type 1 diabetes; T2D, type 2 diabetes; CEL, carboxyl-ester lipase; FED, fecal elastase deficiency, i.e. fecal elastase levels < 200 µg/g in two consecutive tests. The table is mainly based on findings in Papers I-IV, and on (44; 45; 52; 53). *Pancreatic duct changes classified as chronic pancreatitis were observed retrospectively in ERCPs in 77 % out of 156 patients with diabetes (38 subjects with IDDM) (347). **From (351).

Taken together, the findings in Papers I and II indicate that the prevalence of exocrine dysfunction as well as the structural changes of the pancreas in *HNFI*A-MODY are similar to that observed in type 1 diabetes, and different from that characterizing *HNFI*B-MODY or *CEL*-MODY (Table 7). Diabetes has traditionally been regarded as the primary process in type 1 and type 2 diabetes, leading to secondary exocrine dysfunction. The prevailing paradigm postulates that insulinopenia, resulting in deficient trophic action of insulin on acinar cells, is the main cause of impaired pancreatic exocrine function (352). In line with this, the more severe insulin deficiency seen in type 1 diabetes could explain the greater pancreatic volume reduction compared with *HNFI*A-MODY. However, alternative hypotheses for the causes of pancreatic volume reduction exist, including: 1) diabetic microangiopathy, causing pancreatic fibrosis; 2) diabetic autonomic neuropathy, leading to impaired enteropancreatic reflexes (reduced incretin effect); 3) hormone suppression caused by high levels of circulating glucagon (353) or other gut hormones such as somatostatin or

pancreatic polypeptide (354). Recently, it was suggested that pancreatic atrophy may result from the chronic inflammation associated with ongoing beta cell destruction (355). Simultaneous damage of endocrine and exocrine pancreas by a common autoimmune process has also been suggested (344; 356).

A recent review of studies reporting loss of the normally continuous interstitial matrix connection between endocrine and exocrine cells of the pancreas at the ultrastructural level in rodent models and humans with type 2 diabetes, attempts to unite several of these theories. Perivascular-periductal interstitial fibrosis is suggested to be the common denominator (357). The authors argue that A) pericapillary fibrosis in the islet capillaries and efferent islet venules might impair delivery of insulin and result in a delay of 1st phase insulin secretion; and B) exocrine pancreatic periductal fibrosis could interfere with the delivery of pancreatic enzymes to the gut, reducing digestion of nutrients, resulting in decreased stimulation of incretin secretion and a diminished incretin effect on insulin secretion; and C) islet-acinar cell-cell communication is lost, leading to defective paracrine signalling and impairing insulin's trophic effect on the acinar cells. Any of the potential mechanisms discussed above might apply to *HNF1A*-MODY as well as to type 1 and 2 diabetes. Furthermore, the possibility that primary exocrine disease is involved in the pancreatic volume reduction and exocrine dysfunction cannot be ruled out by our studies.

4.3 Clinical implications of fecal elastase deficiency in diabetes.

The fact that severe dysfunction of the exocrine pancreas is reported to be present in as many as 10-30 % of patients with type 1 and type 2 diabetes (218-222) compared to ~4 % among nondiabetic subjects, seems to have drawn little attention in clinical practice. Some even question the clinical relevance of pancreatic exocrine dysfunction as expressed by fecal elastase deficiency in subjects with diabetes, based on claims that it is moderate and nonprogressive (230; 231). This is contradicted by our finding of pathologically high fat excretion in all of the available six patients (100 %) with fecal elastase deficiency and *HNF1A*-MODY, 16 out of 23 adult type 1 diabetes subjects (70 %), four out of six children with type 1 diabetes (33 %) and one of three available type 2 diabetes subjects (33 %; Papers I and II and unpublished); as well as in seven out of nine subjects with *CEL*-MODY (78 %;

Paper IV). Our results support previous reports of a high prevalence of steatorrhea in subjects with diabetes and fecal elastase deficiency. In one large multicenter study, as many as 60 % of patients with type 1 or 2 diabetes and fecal elastase deficiency had pathologically increased fecal fat excretion (229), and in another study, 35 % of obese diabetic patients with low fecal elastase exhibited steatorrhea (222). The overall prevalence of about 42 % for impaired exocrine dysfunction as assessed by fecal elastase in subjects with diabetes, agrees with previous reports of exocrine dysfunction in diabetes patients using direct tests of pancreatic function; underscoring that fecal elastase deficiency most probably represents decreased secretion of exocrine enzymes (162; 164; 168). Moreover, an independent correlation between fecal elastase levels and, respectively, glycemic control and residual beta cell function has been observed (223).

The apparent lack of clinical symptoms of exocrine dysfunction has been used as an argument to disclaim its importance in diabetes (231). None of the subjects identified with pathologically increased fecal fat excretion in any of our studies (Papers I-IV) had spontaneously complained of abdominal symptoms to their physicians. Interestingly, on direct questioning either in a clinical interview or a questionnaire, several but not all reported symptoms such as loose stools, foul-smelling flatulence or abdominal pain. However, none of the symptoms commonly agreed to be associated with steatorrhea discriminated between subjects with and without exocrine dysfunction as they were also frequently observed in individuals with normal fecal elastase levels (Raeder and Vesterhus, unpublished). The *CEL*-MODY patients (Paper IV) in particular reported that the abdominal symptoms affected their quality of life on a daily basis. Most of them had substantially elevated fecal fat excretion with a median of 25 g/day at baseline (range 3-43). Seven out of nine patients reported immediate improvement of their abdominal symptoms with pancreatic enzyme supplement treatment (PEST).

Oral PEST constitutes the main treatment of maldigestion due to pancreatic exocrine insufficiency (for a recent review, see (358)). In Paper IV, we found a statistically significantly decreased, although still high fat excretion in the four patients who delivered stool samples at 30 months. The patients in the present study were instructed to take the capsules immediately after the beginning of the meal for maximal effect according to previous recommendations (234). It is well recognized that fat excretion is rarely normalized by PEST (358). The initial dosage of enzyme supplements of 10-20 000 IU of lipase per

meal may have been modest, as some reports indicate that a minimal dose of 25 000-50 000 IU of lipase per meal is required (232).

The effect of PEST on glycemic control in the presence of combined diabetes and pancreatic exocrine disease has been claimed to be adverse or indifferent by some and reported to improve HbA1c by others (237; 359; 360). PEST could improve glucose homeostasis through its effect on increased incretin secretion, as it has been demonstrated that GIP secretion is reduced in steatorrhea due to alcoholic pancreatitis and can be normalized by PEST (361). We found no effect on glycemic control in *CEL*-MODY patients, confirming recent results from a study examining enzyme replacement in type 1 diabetes subjects with exocrine dysfunction (227). Problems with compliance, particularly over time, complicate the evaluation of the true effect of the treatment. In addition, the motivation to collect 72-h stool samples for the assessment of the effect on steatorrhea declines, as demonstrated in our study.

The treatment of exocrine dysfunction is important because of the risk of malnutrition including deficiency of fat-soluble vitamins. A high rate of pancreatic exocrine dysfunction was reported in patients with osteoporosis and vitamin D deficiency in one study (362). Others observed normal levels of fat-soluble vitamins during a 16-week follow-up in both enzyme replacement and placebo groups of patients with diabetes and fecal elastase deficiency (227). We found pathologically reduced levels of vitamin E in all our *CEL*-MODY patients; but vitamin E increased in all patients with enzyme replacement and was restored to within normal cut-off values in five of seven patients at 12 months (Paper IV). The observed vitamin E deficiency might have contributed to the neurological manifestation of demyelinating neuropathy that was identified in a majority of the patients (363; 364). Levels of vitamins D and A were in the lower normal range in these patients, but vitamin A increased with treatment. Furthermore, osteoporosis was diagnosed in three subjects and osteopenia in two at baseline; possibly secondary to exocrine dysfunction. In *HNFB*-MODY, we also observed pathologically reduced vitamin E and vitamin D levels in two subjects but neurological examination or bone mass density measurements were not performed (Paper III).

4.4 CEL-MODY: Diabetes secondary to pancreatic exocrine disorder? The search for pathogenetic processes in two mouse models (Papers V and VI).

CEL-MODY is a syndrome of combined diabetes and exocrine dysfunction due to mutations in *CEL* (52). However, the molecular mechanisms linking genetic defects in a gene expressed in the exocrine tissue but not in islet cells, to diabetes, are unclear. Both known disease-associated alleles were predicted by *in silico* studies to lead to truncated proteins with an altered C-terminal and loss of the PEST sequence and several glycosylation sites, and to encode new C-terminal protein segments of more than 100 amino acids. Enzyme activity assays using stably transfected CHO cells revealed reduced protein stability and secretion rate of the mutant protein although enzyme activity was unaffected (52), and reduced stability of the mutant protein was confirmed in preliminary *in vitro* experiments using a rabbit reticulocyte lysate system (Vesterhus, unpublished). This could support the theory of a loss-of-function mechanism leading to disease. Alternatively, the aberrant protein could alter cellular function and trigger mechanisms that are detrimental to the tissues, such as autoimmunity or ER stress.

Mouse studies have previously proved important in the demonstration of the pathogenicity of putative disease-related alleles and the study of the associated pancreatic pathophysiology; e.g. mouse models for MODY subgroups, Johanson-Blizzard syndrome and Wolcott-Rallison syndrome (130; 209; 256; 260; 265; 271; 279; 365). Accordingly, we wanted to recapitulate the phenotype of *CEL*-MODY in an *in vivo* model with disruption of the *CEL* gene. We aimed at studying an existing global knockout mouse model (CELKO) in order to explore a loss-of-function effect; and to create and subsequently study a transgenic mouse line expressing a disease-associated human *CEL* allele (TgCEL) to investigate the possibility of an altered cellular function.

CELKO

Paper V describes the study of the pancreatic exocrine and endocrine function in CELKO mice. Phenotypes described in CELKO mice to date are listed in Table 8. The few observations that were made of differences between CELKO mice and controls in Paper V were made in females only and were compatible with mild glucose intolerance: A non-

significant tendency to reduced glucose tolerance; increased random fed blood glucose after 12 weeks on a 60 % high fat diet ($P = 0.002$); and increased islet area on 60 % high fat diet

Table 8 Phenotypes observed in CELKO mice

Phenotype	References
Independent of a low fat or high fat/high cholesterol diet:	(366)
- Increased fecal excretion cholesteryl ester	
- Decreased serum levels of cholesteryl ester	
- No difference in free cholesterol absorption or serum cholesterol levels	
- 50 % reduction in cholesteryl ester absorption	(367)
- No difference in the absorption of free cholesterol, triglycerides or retinyl ester, or in plasma lipid or lipoprotein profiles	
- Normal growth and development	
No affection of absorption of retinyl ester, chylomicron-retinyl ester, or hepatic retinoid metabolism	(368)
- Absent cholesteryl ester hydrolase activity	(369)
- Normal retinyl ester hydrolase activity	
- Normal weight gain on a high fat/high cholesterol diet	(305)
- Protection against diet-induced obesity in PTL ^{-/-} ,CEL ^{-/-} double KO mice compared to PTL ^{-/-} mice	
- Enhanced reduction in absorption of triacylglycerol and retinyl palmitate, but not cholesterol, in PTL ^{-/-} ,CEL ^{-/-} double KO mice compared to PTL ^{-/-} mice	
- Increased random fed blood glucose, tendency to reduced glucose tolerance and islet hyperplasia in female mice on a 60 % high fat diet	Paper V
- No disturbances of exocrine pancreatic function or morphology was noted	
- Increased liver steatosis	

PTL, pancreatic triglyceride lipase.

($P = 0.03$) that could be a compensatory effect of increased insulin resistance. The demonstration of a phenotype in female mice only would not be surprising even if the human syndrome seems to affect men and women alike, as restriction of the expression of diabetes to one gender is a frequent finding in rodent models (247; 370). However, an insulin secretory deficiency is the characteristic defect in *CEL* mutation carriers whereas insulin resistance is not part of the syndrome, although not formally tested; thus, the endocrine phenotype of *CEL*-MODY does not seem to be recapitulated in the CELKO model. The dietary challenge with a 60 % high fat diet, containing three times the fat in percent of calories compared to normal chow, induced changes consistent with metabolic changes promoting a diabetic phenotype, but did not provoke a full-blown *CEL*-MODY phenotype. Pancreatic exocrine dysfunction was not detected and exocrine pancreas morphology appeared normal.

Interestingly, there was a predominance of liver steatosis in female CELKO mice on a high fat diet and in male CELKO mice irrespective of diet compared to controls. Recently, liver steatosis was reported to be associated with reduced glucose tolerance and increased postprandial glucose levels, but not impaired fasting glucose, in subjects with the non-alcoholic fatty liver syndrome (371). *CEL* is expressed both at the mRNA and protein level in human liver (323). However, liver steatosis is not observed in human mutation carriers.

Instead, lipomatosis of the pancreas is an early structural marker of disease in prediabetic subjects with *CEL*-mutation (53). Recently, histopathological examinations of tumour-free pancreatic sections from a deceased *CEL*-MODY patient with mammary and pancreatic cancer, revealed insulin-staining islets, and some acini surrounded by lymphocytes, submerged in intrapancreatic fat and fibrous tissue (Immervoll, Hoem, Raeder; unpublished). These findings could implicate fat infiltration and inflammation in the pathogenesis of *CEL*-MODY. Fatty replacement of the pancreas has also been observed in cystic fibrosis, Shwachman-Diamond syndrome and Johanson-Blizzard syndrome, which are monogenic conditions with primary affection of the exocrine pancreas and frequent development of secondary diabetes (209; 349; 372). Pancreatic steatosis was recently reported to be associated with the dissemination and lethality of pancreatic cancer, supporting a role for pancreatic fat in disease development (373). No striking difference in pancreatic fatty tissue was observed between CELKO and control mice on examination of histological sections, but the differentiation of fat within or surrounding the pancreatic tissue was complicated by the less clear demarcation of the organ in mice compared to humans.

TgCEL

Paper VI describes the establishment of a mouse line exhibiting pancreas-specific expression of the human disease-associated Del1 *CEL* allele. It is worth noticing that bitransgenic *TgCEL*^{+/-}*_Cre*^{+/-} mice had a birth rate of only 11 % compared to 25 % as predicted from Mendelian laws, suggesting a certain lethality *in utero*. However, no particular pancreatic endocrine pathology was detected clinically, morphologically or at the molecular level in *TgCEL* mice fed normal chow or a 60 % by calorie high fat diet. Pancreatic exocrine dysfunction was also not detected. Principally, this could be due to either a truly normal pancreas function or a lack of sensitivity of the diagnostic methods employed. It should be noted that a stimulated insulin secretion test examining first phase insulin secretion was not

performed in mice fed the high fat diet, and histopathological examination of pancreatic sections from high fat diet mice is also pending. Therefore, a subclinical pancreatic endocrine pathology cannot yet be excluded in the high fat diet-TgCEL mice. It is also possible that an additional challenge to the pancreatic endocrine tissue such as streptozin injections would reveal a predisposition towards diabetes in TgCEL mice. We studied exocrine function in the mice by indirect methods; primarily serum amylase and fecal elastase, accompanied by investigations of fecal chymotrypsin, fat absorption and Oil Red O staining of fat in stool in some of the mice. All of the methods are previously published used in mouse studies of pancreatic exocrine function (209; 260; 374). Direct function tests are considered the gold standard in humans. It is possible that a challenge to the exocrine tissue either by *in vivo* induction of pancreatitis by cerulein injections or by cholecystokinin-stimulated amylase secretion in freshly isolated acini would unmask latent exocrine dysfunction as demonstrated for *Ubr1*^{-/-} mice (209).

Interestingly, a few discrete areas of abnormal exocrine tissue were observed by light microscopy of pancreatic sections from two male mice carrying the human *CEL* transgene. One of the mice was a TgCEL mouse, whereas the other was genotyped to lack the EL CRE transgene and thus was classified as a control mouse carrying dormant human *CEL*. However, qRT-PCR suggested some expression of human *CEL* in the pancreas of this mouse, resulting in ambiguity concerning its true genotype. The nature of the abnormality was not extensively investigated, but preliminary examination by a pathologist identified probable inclusion bodies. The implications of this are unclear. However, it is of interest that inclusion bodies were also detected during histopathological examination of pancreatic sections from a recently deceased *CEL* mutation carrier (Immervoll, Hoem, Raeder, unpublished). Further investigations are warranted, the genotype of the second mouse should be firmly established, and the finding should be replicated in a larger group of mice.

One hypothesis is that aggregation of mutant CEL protein causes pathology via ER stress-related mechanisms. Previous mouse studies have reported dilated ER cisternae as a manifestation of accumulation of aberrant proteins that could result in ER stress (260; 375). Accordingly, we examined electron micrographs of acinar and beta cells of chow-fed TgCEL and control mice of both genders, but no consistent differences in ER structure were observed (Vesterhus, unpublished).

Why did the TgCEL mouse line fail to develop a *CEL*-MODY phenotype? Given that the hypothesis is correct, there are at least four principal explanations:

1. The phenotype was present, but we failed to recognize it because
 - a. we did not do the right tests (discussed above)
 - b. the symptoms were mild and larger groups of mice would have been needed to prove a difference
2. The phenotype was latent, but did not develop because
 - a. we did not apply the right challenges (discussed above)
 - b. we did not follow the mice long enough to reveal an age-dependent phenotype
3. The design of the mouse line was not optimal, and problems could be associated with:
 - a. gene integration in the genome – yielding low or no protein expression
 - b. the elastase promoter – not ideal time- or tissue-specificity of induction of expression
 - c. the genetic background of the mouse strain – obscuring phenotype expression
4. Mice have a different metabolic system from man and might not be a suitable model system

Expression of human *CEL* was verified by qRT-PCR, and sequencing of all TgCEL mice subject to investigation confirmed the presence of the disease-associated Dell mutation. However, expression at the protein level should also be ascertained and quantified. Lacking or low expression at the protein level could explain the lacking phenotype. Only recently did we obtain an antibody that recognizes the mutant CEL protein, and we are in the process of confirming protein expression by western blot and immunohistochemical staining of pancreas sections from TgCEL mice. Pancreas-specific expression was demonstrated by beta-gal staining and immunological staining for LacZ in the offspring of EL CRE and Rosa26 mice. The elastase promoter controlling expression of the *Cre* recombinase results in transgene expression in the mature pancreas, whereas *CEL* is an early marker of exocrine cell differentiation (38). Thus, it is possible that *CEL* has as yet unknown functions during embryogenesis and that a different promoter, e.g. *PDX1*, inducing transgene expression at an earlier stage, would be better suited to promote the pathology associated with *CEL*-MODY.

A striking difference in phenotype penetrance has been demonstrated in various mouse strains based on different genetic backgrounds (247; 253; 254). Some problems associated with the interpretation of results of studies using gene targeted and transgenic mice, particularly in relationship to genetic background, are reviewed in (376). The susceptibility to impaired insulin secretion or insulin resistance varies between mouse strains as discussed in chapter 1.3. We cannot reject the possibility that the lacking phenotype might be connected to the C57BL/6-FVB genetic background of the mice, such that knock-in of the disease-associated *CEL* allele in another strain might yield other results as has been seen in mice with targeted deletions of the insulin receptor or insulin receptor substrate genes (253). Creating the TgCEL on different inbred strains (e.g. C57Bl/6, 129Sv etc) would unmask a phenotype that is influenced by one or more modifier genes close to the target locus.

The loss-of-function hypothesis versus the altered cellular function hypothesis

The evidence in favour of a loss-of-function mechanism in the pathogenesis of *CEL*-MODY relies on some previous experiments showing reduced stability of mutant *CEL* as assessed by enzyme activity assays in CHO cells or a stability assay with radioactively labelled protein using a rabbit reticulocyte lysate system for protein expression, as previously explained ((52), and Vesterhus, unpublished). However, the specific activity of mutant *CEL* could not be calculated because of the lack of an appropriate antibody, thus the results of the enzyme activity assay were uncertain. Others have reported reduced secretion rate and subsequent degradation of *CEL* synthesized in CHO IdID cells under conditions preventing proper *O*-glycosylation (319). In contrast, recent studies in our group indicated that transfected HEK293 cells secreted both wildtype and Del1 mutant protein variants into the growth medium at high levels and at similar rates (B. Johansson, unpublished). Previous papers have reported that C-terminal deletions in *CEL* removing PEST sequences and glycosylation sites do not interfere with the catalytic activity of the enzyme *in vitro* (316). Furthermore, a variant VNTR with only 3 repeats, i.e. approximately the same length of normal protein as the Del4 disease-associated allele, was observed in a Danish MODY family but is not associated with fecal elastase deficiency, although it segregates with diabetes (Torsvik *et al.*, manuscript submitted). In line with this, CELKO mice did not recapitulate the characteristics of *CEL*-MODY and the results described in Paper V do not

present new evidence in support of loss-of-function as the pathogenic mechanism in *CEL-MODY*.

Three lines of evidence suggest that the single-base deletions lead to pathology through an altered cellular function. First, the nature of inheritance: *CEL-MODY* is autosomal dominantly inherited, i.e. the syndrome appears in heterozygote mutation carriers. This is in contrast to the usually recessive nature of metabolic diseases based on enzyme defects (377). Second, the lack of a convincing phenotype in the knockout mouse model. *CELKO* mice, which should constitute the equivalent of homozygous mutation carriers, would be expected to have a stronger phenotype if the hypothesis were correct.

Third, single-base insertions leading to frameshift and premature truncation occur in *CEL* as common variants and may be associated with an increased risk of exocrine dysfunction in subjects with diabetes. Importantly, the observed *Ins4* is not associated with diabetes despite yielding the same length of normal protein as the disease-associated *Del4* mutation, although a person with *Ins4* and *Ins11* combined did have *FED* (52). The main difference between the *Ins4* and the *Del4* protein is the longer strand of altered protein downstream to the deletion versus the insertion (predicted to about 100 compared to 5 amino acids), strongly indicating that the new protein terminus is implicated in the pathogenesis. The same argument holds true for the *MODY* family without exocrine dysfunction carrying the variant *VNTR* with only 3 repeats mentioned above. The novel protein ending may include glycosylation sites that lead to aberrant glycosylation and subsequently capture of the altered protein in the quality control machinery of the cell. Previous studies have indicated that aberrant *CEL* is rapidly degraded via the ubiquitin-proteasome pathway (320). Alternatively, defective protein could accumulate or aggregate within the acinar cells, thus eliciting a cellular unfolded protein response that could trigger ER stress (320). ER stress has been previously linked to diabetes (375). In support of this hypothesis, preliminary data from our group indicate altered glycosylation in mutant compared to wildtype *CEL* resulting in a shift in the ratio of a low-molecular to a high-molecular band (B. Johansson, unpublished). Further preliminary data from immunohistochemistry studies show a changed subcellular localization from the ER and Golgi for wildtype human *CEL* to a more widespread, punctual localization to an unidentified organelle for mutant *CEL* (J. Torsvik, unpublished). Also, cells expressing mutant *CEL* display increased stress markers such as phosphorylated *eIF2A* (J. Torsvik, unpublished).

4.5 Conclusions and future directions

Papers I and II show that *HNFI1A*-MODY is associated with fecal elastase deficiency and pancreatic volume reduction in line with findings in type 1 diabetes, but with less pronounced pancreatic atrophy and a less striking association with exocrine dysfunction than in *HNFI1B*-MODY. We describe dorsal agenesis of the pancreas with associated fecal elastase deficiency to be a phenotype of *HNFI1B*-MODY, supporting a developmental role for *HNFI1B* (Paper III). Exocrine dysfunction seems to result from structural abnormality in *HNFI1B*-MODY, whereas in *HNFI1A*-MODY it is suggested to arise secondary to diabetes (Papers I-III). This underscores the heterogeneous nature of the interactions between the endocrine and exocrine pancreatic tissues that can lead to similar phenotypes of combined diabetes and exocrine dysfunction. Furthermore, our data support previous findings that fecal elastase deficiency is a relevant marker of clinically important exocrine dysfunction in diabetes as steatorrhea and deficiencies in fat soluble vitamins, particularly vitamin E, were observed in a high percentage of subjects with diabetes and fecal elastase deficiency (Papers I-IV). Based on the evidence discussed above, we suggest that whether exocrine function should be tested in all patients with diabetes, and what constitutes the criteria for enzyme substitution treatment, should be discussed as there is a distinctive risk that treatable malabsorption is currently undiagnosed in patients with diabetes.

CEL-MODY is hypothesized to be an example of monogenic diabetes arising secondary to pancreatic exocrine dysfunction. We studied the global knockout model (CELKO) and created and studied the TgCEL mouse line expressing the Del1 disease-associated human *CEL* allele in order to investigate two opposing hypotheses on the molecular mechanisms involved in the pathogenesis of the syndrome; namely loss-of-function or an altered cellular function of the mutant protein, respectively (Papers V and VI). However, no clear phenotype resembling of *CEL*-MODY was demonstrated in either CELKO or TgCEL mice. Further studies remain to be done particularly to explore the effects of mutant human *CEL* in a transgenic mouse line. Based on our current knowledge, including unpublished data, haploinsufficiency at the genetic level is a much less likely hypothesis than an altered function of the mutant protein, although it cannot be excluded.

Briefly summarized, the present work has contributed to the increasing pile of evidence concerning the nature of the association between pancreatic exocrine dysfunction and

diabetes. Future studies should investigate the pancreatic exocrine function in nondiabetic *HNF1A* mutation carriers in order to evaluate whether diabetes is in fact the primary event in exocrine dysfunction associated with *HNF1A*-MODY, or whether the exocrine pancreas has a primary role after all. Follow-up data should be collected to assess the possible development or progression of malabsorption and vitamin deficiencies and their secondary sequelae in subjects with fecal elastase deficiency and *HNF1A*-MODY, *HNF1B*-MODY, *CEL*-MODY or type 1 and type 2 diabetes. This would yield important information for the decision of whom to treat.

Future studies of *CEL*-MODY should focus on further characterization of the pancreatic exocrine dysfunction in *CEL* mutation carriers, including investigations of the incretin response and direct tests of exocrine function to differentiate between acinar and ductal damage, such as used to define the acinar pathology in Johanson-Blizzard syndrome and the ductal defects in cystic fibrosis (378; 379). Although an acinar defect is most likely, given the adult expression of *CEL* in acinar tissue and not in ductal or beta cells, embryonic expression of *CEL* might differ and affect function in adult life (323). Expanding the clinical spectrum and screening for *HNF1B* mutations in a renal registry greatly increased the prevalence of *HNF1B*-MODY (126). Likewise, screening for *CEL* mutations in materials of families with a monogenic pattern of pancreatic exocrine dysfunction as the primary clinical manifestation, as well as in MODY-X families with fecal elastase deficiency, should be done. Furthermore, sophisticated radiological modalities should be applied in an effort to further explore the morphological changes of the pancreas in *CEL*-MODY. We are currently in the process of planning such studies of both the incretin response, imaging of pancreatic morphology and function, and direct tests of exocrine function in prediabetic mutation carriers and patients with the full-blown syndrome.

Due to the polymorphic nature of the gene, sequencing of *CEL* has proved difficult. It would be of interest to develop a simple and robust method to detect insertions and deletions in the gene. The effect of gene variants as risk factors for type 1 diabetes should be further explored, and such studies are currently being pursued by our research group. Furthermore, the association of *CEL* repeat length variants with serum cholesterol levels reported by others should be confirmed (297). It would also be interesting to investigate whether *CEL* insertions are related to dyslipidemia or cardiovascular disease. Moreover, the region surrounding the gene should be studied in detail, as its highly repetitive nature provides a

reason to speculate that there might be rearrangements in the region. If so, it would be of interest to study those and to investigate whether they would be associated with disease. The post-translational processing of mutant CEL, including ubiquitination and degradation, warrants further study to improve our understanding of the pathogenesis of *CEL-MODY*.

Pursuing studies of the CELKO mice seems unlikely to yield further information, and we consider these studies finished. The TgCEL mouse line, however, should be more exhaustively studied. In particular studies in older mice and larger groups should be carried out, and a cerulein challenge to the exocrine function should be assayed. Breedings are ongoing in order to produce mice for these experiments. We are currently controlling the presence of transgene expression at the protein level by western blot and immunohistochemical analysis. As a next step, a new strategy, such as modifying the mouse model by substituting the elastase promoter with for instance a PDX1 promoter for early fetal expression of transgenic mutant *CEL*, as well as backcrossing the transgenic allele onto other mouse strains, should be considered. In parallel with the mouse studies, investigations in cell lines should be continued in the attempt to unravel the molecular mechanisms connected to the synthesis, processing and secretion of mutant CEL. Such studies are being pursued by other members of our laboratory. The establishment of an *in vivo* model that truly recapitulates the syndrome of *CEL-MODY* would be of utmost importance for the demonstration of the processes leading to disease, and to finally settle the question of which tissue is the origin of the syndrome. Ultimately, this could yield insights that would be useful for the understanding of other forms of diabetes.

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