Genetic and cellular studies of carboxyl-ester lipase (CEL), a protein involved in exocrine and endocrine pancreatic disease

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Thesis for the Degree of Philosophiae Doctor (PhD)
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Sincerely,

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Abstract

Carboxyl-ester lipase (CEL) is a digestive enzyme mainly expressed in pancreatic acinar cells, from which it is secreted into the duodenum as a component of pancreatic juice. Mutations in the human *CEL* gene have been linked to pancreatic disease. All pathogenic CEL variants identified so far affect the C-terminal region of the protein, which is very polymorphic due to repeated segments of 11 amino acids. A single-base deletion in the *CEL* repeat region causes a dominantly inherited syndrome of exocrine and endocrine pancreatic dysfunction denoted MODY8, whereas a copy number variant of the *CEL* locus, designated *CEL-HYB*, predisposes for chronic pancreatitis.

To explore the role of *CEL* variants in pancreatic disease, we examined if *CEL* CNVs and VNTR length polymorphisms could affect the risk for developing pancreatic cancer. No studies have so far linked this disease to *CEL*, despite the fact that chronic pancreatitis is a known risk factor for pancreatic cancer. Our results from CNV screening and DNA fragment analyses in two pancreatic cancer cohorts of Caucasian origin did, however, not show an association between pancreatic cancer and the investigated *CEL* variants. Still, the *CEL* gene is highly polymorphic and a role of *CEL* variants in influencing pancreatic cancer risk cannot be excluded.

To gain knowledge of how the pathogenic CEL protein variants, CEL-MODY and CEL-HYB, may cause or predispose for pancreatic disease, we started studies in cellular model systems. We found that both normal CEL and CEL-MODY most likely followed a classical secretory pathway. Their subcellular distributions did, however, differ as only CEL-MODY was observed as an aggregate at the cell surface and inside large cytoplasmic vacuoles, identified as components of the endosomal system. We further aimed to investigate the uptake of CEL protein variants in pancreatic acinar, beta and ductal cell lines and to study their effect on cell viability, as endocytosis may play a central role in disease pathogeneses. We found all CEL variants to be internalized, and compared to normal CEL, endocytosed CEL-MODY protein significantly reduced viability of all pancreatic cell line models, manifesting

as a decrease in cellular metabolism and increased caspase3/7 activity. We found that also endocytosed CEL-HYB significantly reduced the viability of pancreatic acinar and ductal cell lines, as compared to normal CEL. Moreover, we developed a co-expression model as patients are heterozygous carriers of either *CEL-MODY* or *CEL-HYB* along with one copy of the normal *CEL* gene. Interestingly, we found both CEL-MODY and CEL-HYB to affect the intracellular fate of normal CEL, whilst the cellular toxicity of the two pathogenic variants after co-endocytosis became reduced in the presence of normal CEL.

In conclusion, these studies highlight the exceedingly polymorphic nature of the human *CEL* gene and may be important for our understanding of how the pathogenic CEL variants predispose for pancreatic disease.

List of publications

Paper I

Dalva M, El Jellas K, Steine S, Johansson BB, Ringdal M, Torsvik J, Immervoll H, Hoem D, Laemmerhirt F, Simon P, Lerch MM, Johansson S, Njølstad PR, Weiss FU, Fjeld K, Molven A. Copy number variants and VNTR length polymorphisms of the carboxyl-ester lipase (*CEL*) gene as risk factors in pancreatic cancer. *Pancreatology*. 2017; 17:83-88.

Paper II

Torsvik J, Johansson BB**, Dalva M**, Marie M, Fjeld K, Johansson S, Bjørkøy G, Saraste J, Njølstad PR, Molven A. Endocytosis of secreted carboxyl ester lipase in a syndrome of diabetes and pancreatic exocrine dysfunction. *J Biol Chem*. 2014; 289:29097-29111.

Paper III

Dalva M, Lavik IMK, El Jellas K, Gravdal A, Njølstad PR, Fjeld K, Johansson BB, Molven A. Pathogenic carboxyl-ester lipase (CEL) variants are endocytosed in pancreatic cell lines and may influence properties of the normal CEL protein. *Manuscript.*

Selected abbreviations

bp	Base pair(s)
CEL	Carboxyl-ester lipase
CEL-HYB	*CEL* deletion allele encoding a CEL-CELP hybrid protein
CEL-MODY	MODY8-causing allele encoding the mutant CEL protein
CELP	Carboxyl-ester lipase pseudogene
CNV	Copy number variation
CP	Chronic pancreatitis
Del / DEL	Deletion
DUP	Duplication
ER	Endoplasmic reticulum
kb	Kilobase pair(s)
kDa	Kilodalton
MODY	Maturity-onset diabetes of the young
PEST	Proline (P), glutamic acid (E), serine (S) and threonine (T)
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VNTR	Variable number of tandem repeats
WT	Wild-type

Table of contents

1. Introduction

1.1 The pancreas

1.1.1 **Anatomical overview**

The human pancreas is a lobulated gland located in the upper left part of the abdominal cavity, partly behind the stomach. The organ is 15-20 cm of length, normally weighs 80-100 g and can be divided into a head, body and tail region (1). The duodenum surrounds the head of the pancreas in a C-shaped curve, while the narrowing pancreatic tail is located adjacent to the spleen and left kidney. Within the glandular tissue of the pancreas resides the main pancreatic duct, which increases in diameter as it extends across the organ from tail to head. The common bile duct passes through the pancreatic head region, and the two ducts unite before entering the duodenum (Fig. 1A) (2). The pancreas can be divided into two functional parts, the endocrine pancreas and the exocrine pancreas, where the latter is by far the largest with respect to tissue volume (1).

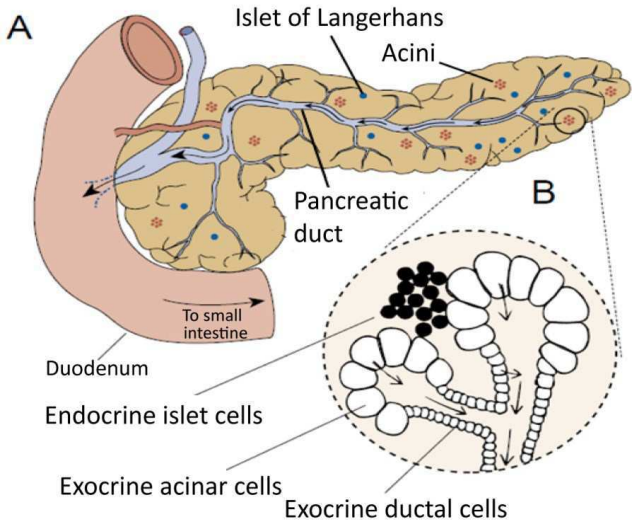

Figure 1 Anatomy of the human pancreas
A) The human pancreas consists of endocrine cell-clusters (islets of Langerhans) and exocrine acini with connecting pancreatic ducts. The ductal system drains the pancreatic juice into the duodenum. B) Schematic drawing of the functional units of the endocrine and exocrine pancreas. Endocrine islet cells produce hormones that participate in the regulation of glucose homeostasis. Exocrine acinar and ductal cells secrete digestive enzymes and bicarbonate, respectively, into the pancreatic juice. Modified from Apte et al. 1997 (3).

1.1.2 **Endocrine pancreatic function**

The endocrine part of the human pancreas represents approximately 4% of the whole organ and is scattered throughout the parenchyma. It consists of up to three million functional units called islets of Langerhans, which are cell clusters of specialized hormone-producing cells (4) (Fig. 1A and B). The islets contain mainly alpha- and beta-cells, in addition to some delta-, epsilon- and gamma-cells (5).

The beta cells, which make up about 60% of each islet (4), play a central role in regulating blood glucose levels within a narrow physiological range (6). The beta cells are responsible for the production of insulin, and upon nutrient stimulation, this hormone is secreted into the bloodstream, where it is transported to its target organs, i.e. the liver, muscle and adipose tissue. Insulin triggers glucose uptake in the cells and also promotes glucose storage in the liver in the form of glycogen, resulting in an overall reduction in blood glucose levels (1). Thus, the secretion of insulin is tightly regulated by blood glucose levels. However, other nutrients such as fatty acids, amino acids and various hormones like estrogen and leptin can also increase and regulate insulin secretion (7). The second largest cell population found in the islets of Langerhans is the glucagon-secreting alpha cells, constituting up to 30% of each islet (4). In contrast to beta cells, alpha cell secretion is stimulated as a response to low blood glucose concentrations. In addition, neurotransmitters and several neuropeptides can stimulate or inhibit glucagon secretion (8), whilst insulin have an inhibitory effect (9). Secreted glucagon stimulates liver glycogenolysis and gluconeogenesis, releasing glucose into the bloodstream. Thereby, the hormone counteracts insulin and contributes to maintain a proper glucose homeostasis (8).

The remaining hormone-secreting cells, delta-, epsilon- and gamma-cells, make up around 10% of each islet and are responsible for the production and secretion of somatostatin, ghrelin and pancreatic polypeptide, respectively (10, 11). Somatostatin can inhibit the secretion of glucagon, insulin and pancreatic polypeptide, as well as its own secretion (12). Ghrelin plays a role in metabolic regulation, energy balance (11) and appetite stimulation (1). Similarly, pancreatic polypeptide functions as regulator

of food intake and is secreted from the gamma cells in response to food ingestion (13) and upon stimulation by arginine (14).

1.1.3 **Exocrine pancreatic function**

The primary function of the exocrine pancreas is to produce and secrete digestive enzymes and bicarbonate, as components of the pancreatic juice (15). This alkaline, enzyme-rich isotonic fluid is secreted into the duodenum and is crucial for proper digestion and absorption of nutrients in the small intestine (16, 17). The human exocrine pancreas produces approximately 2-3 liter of pancreatic juice each day (17). The functional units of the exocrine pancreas are the acini and the connecting ductal system (Fig. 1A and B). The two units are tightly connected as the ductal system extends from the acini lumen to the duodenum, via intercalated, intra- and inter-lobular ducts and the main pancreatic duct (2).

Morphologically, the acini are spherical or tubular clusters of acinar cells, which are highly polarized cells responsible for synthesis, storage and secretion of digestive enzymes into the acini lumen (18). Pancreatic acinar cells may have the highest protein synthesis and secretion rate in the human body (2, 19) and contain a highly developed rough endoplasmatic reticulum (RER) in the basal part of the cells. In the apical area, newly synthesized enzymes are stored in zymogen granules (18). Upon stimulation from the gastrointestinal hormones secretin and cholecystokinin, and neurotransmitters like acetylcholine, the enzymes are secreted into the acini lumen (20). As listed in Table 1, there are four main classes of secreted digestive enzymes from the pancreas, namely lipases, proteases, nucleases and amylase. Their function, in short, is to break down dietary macromolecules, such as lipids, starch, sugars, oligopeptides and nucleic acids, into smaller molecules that can effectively be absorbed by the small intestine (2, 15, 16). Notably, the majority of the digestive enzymes are secreted as inactivated proenzymes, and activation of the enzymes occurs through proteolysis by the action of enterokinases in the duodenal lumen (2). Almost 80% of the secreted enzymes are proteases. These are tightly regulated (15) as premature activation may lead to uncontrolled proteolysis inside the pancreas,

resulting in autodigestion of pancreatic tissue and pancreatitis (21). On the other hand, digestive enzymes like lipases and amylase are often found in an active state within the zymogen granules (2).

Table 1 Major digestive enzymes secreted by pancreatic acinar cells

Enzymes	Cleavage site / hydrolytic activity	Product(s)
Proteases		
Trypsin	Endopeptidase; cleaves internal bonds after basic amino acid residues (arginine and lysine). Activates other proteases	Oligopeptides
Chymotrypsin	Endopeptidase; cleaves internal bonds after aromatic amino acid residues (phenylalanine, tryptophan and tyrosine)	Oligopeptides
Elastase	Endopeptidase; cleaves internal bonds after small uncharged amino acid residues (alanine, glycine and serine)	Oligopeptides
Carboxypeptidase (A, B)	Exopeptidase; cleaves aromatic (A) or basic (B) amino acids from C-terminal end of peptides	Amino acids and peptides
Amylases		
Pancreatic α-amylase	Hydrolyses carbohydrates like starch, simple sugars and glycogen	Carbohydrates, glucose
Lipases		
Pancreatic triglyceride lipase	Cleaves ester bond at sn-1 and sn-3 of triglycerides. Colipase-dependent	Diglyceride, carboxylate
Pancreatic lipase-related protein 2	Cleaves ester bonds of triglycerides, galacto- and phospholipids	Diglyceride, carboxylate, galactoglycerolipids
Phospholipase A2	Cleaves sn-2 acyl ester bond of phospholipids	Glycerophospholipids, carboxylate
Carboxyl-ester lipase	Cleaves ester bonds of triglycerides, galacto- and phospholipids, vitamin esters, ceramide and cholesterol esters	Diglyceride, carboxylate, cholesterol (sterols)
Nucleases		
Deoxyribonuclease and ribonuclease	Cleaves the nucleic acids DNA and RNA	Nucleotides

Based on Whitcomb et al. 2007 (15) and www.uniprot.org.

The ductal system occupies around 10% of the exocrine gland. The ductal cells are cuboidal to pyramidal epithelial cells that line the proximal pancreatic duct and are responsible for secreting bicarbonate and water, which effectively flushes out the digestive enzymes from the acini lumen (17). Moreover, bicarbonate also functions to neutralize the gastric acid, thus providing an optimal pH environment in the duodenum for the digestive enzymes to function (22).

In addition to the two functional units of the exocrine pancreas, centroacinar cells and pancreatic stellate cells are important components of the exocrine tissue. The centroacinar cells, located at the junction of the acini lumen and ducts, have ductal cell characteristics and can secrete bicarbonate and ion-rich fluid (23). Moreover, centroacinar cells are known to have an active Notch signaling promoting expression of the differentiation regulator Sox9 (reviewed in (24)), hence, they have been suggested to serve as multipotent progenitors for both endo- and exocrine cells during pancreatic regeneration and proliferation (24-26).

Pancreatic stellate cells, which have a star-shaped morphology, are mainly localized in the periacinar regions, as well as in the periductal regions (27). Normal quiescent stellate cells have the ability to synthesize extracellular matrix (ECM) proteins, and are suggested to support the structure and function of exocrine cells by maintaining the basement membrane components of the ECM (28, 29). Many physiological functions have been associated with pancreatic stellate cells (27), but their role in ECM maintenance has gained special attention. Activated stellate cells display increased levels of ECM protein synthesis and have been suggested to promote proliferation and cell migration as a result of the produced imbalance in the ECM protein turnover (30). Moreover, pancreatic stellate cells are recognized as key cells in pancreatic fibrogenesis (31, 32) and their initiating role in the pathogenesis of chronic pancreatitis and pancreatic cancer is now under scrutiny (33-37).

1.2 Diseases of the pancreas

1.2.1 **Diabetes**

Diabetes mellitus, or diabetes as it is commonly referred to, is a group of metabolic disorders resulting from deficiency in insulin secretion and/or insulin action (38). Patients suffering from diabetes fail to regulate the blood glucose homoeostasis, and the hallmark of the disease is chronically elevated levels of blood glucose (hyperglycemia). In addition, diabetes is associated with an increased risk of developing complications involving the kidneys, heart, exocrine pancreas, nerves and eyes (1). There are projections that diabetes will be the seventh leading cause of death worldwide and the fourth leading cause of death in high-income countries by the year 2030 (39).

Several forms of diabetes exists, and the most common type is Type 2 diabetes (T2D), which is estimated to account for around 90% of all cases (38, 40). T2D is characterized by slow-progression hyperglycemia and altered lipid metabolism, caused by insulin resistance and impaired insulin secretion (40). Patients can go undiagnosed for several years until symptoms arise, due to the mild manifestations at early stages, and they rarely require insulin treatment. Physical exercise, dietary interventions and oral medications are often sufficient for treatment (1). The genetics concerning T2D are complex and not yet fully understood, however, T2D is considered a multifactorial disease caused by genetic susceptibility and environmental factors. Obesity, age and lack of physical activity have all been associated with elevated risk of developing T2D (38).

The second most common form of diabetes is Type 1 diabetes (T1D), estimated to account for maximum 10% of all diabetes cases (38). T1D is defined as an autoimmune disease, characterized by pancreatic beta cell destruction, reduced insulin secretion and a daily requirement for exogenous insulin supply (38, 41, 42). The precise immunological and genetic events underlying the disease are not yet completely understood (42), and several environmental factors have been associated with elevated risk of developing T1D, though poorly defined (38). Other forms of

diabetes do also exist, including gestational diabetes mellitus (GDM), defined as diabetes diagnosed during pregnancy (43), latent autoimmune diabetes of adults (LADA), in which the patients initially are non-insulin requiring and have clinical symptoms like T2D patients (44), and monogenic diabetes (described in Section 1.2.4). These diabetes subtypes accounts for some 2-3% of all diabetes.

1.2.2 **Pancreatitis**

Pancreatitis is an inflammatory disease of the pancreas, that can be acute, recurrent acute or chronic, often with a progression from the acute to the chronic form (45). Acute pancreatitis (AP) is defined as a syndrome of sudden and transient inflammation in the pancreas, characterized by elevated levels of digestive enzymes in the blood, abdominal pain and swelling of the pancreas (46). Clinically, AP can range from a mild to severe disease, resulting in multi-organ failure, necrosis and in extreme cases, death (47). The two most common causes of AP are gallstones and alcohol abuse, whereas physical trauma, drugs, various infections, anatomic and metabolic abnormalities and genetic causes are less common (48). Some cases are also considered idiopathic (48, 49).

Over time, AP can develop into chronic pancreatitis (CP), a syndrome of persistent pancreatic inflammation, lasting more than six months (45, 50). CP is characterized by irreversible morphological changes, like fibrosis, calcification and ductal dilatation (51), which result in severe impairment of pancreatic function (35). Patients with CP often suffer from continuous attacks of abdominal pain, maldigestion and diabetes, with increased risk of developing pancreatic cancer after long-standing chronic inflammation (45, 52). The most prevalent cause of CP remains chronic alcohol abuse, in addition to genetic risk factors and a variety of environmental and metabolic factors that confer susceptibility to CP (50, 53).

CP has been found to associate with genetic mutations and polymorphisms in genes like *PRSS1* (trypsinogen *1), CFTR* (cystic fibrosis transmembrane conductance regulator), *CTRC* (chymotrypsin C), *CASR* (calcium-sensing receptor), *CPA1* (carboxyl peptidase A1), *SPINK1* (serine protease inhibitor kazal type 1) and *CEL*

(carboxyl-ester lipase) among others (Table 2) (45, 49, 53-55). The majority of the genetic risk factors are implicated in the regulation of pancreatic trypsin activation. Thus, the current model is that the risk factors are associated with premature trypsin activation, resulting in zymogen activation, autodigestion of pancreatic tissue and a triggered immune response (56).

Table 2 Overview of selected genes associated with chronic pancreatitis

Disease	Gene	Encoded protein / Type of protein	OMIM(#) /reference
Chronic pancreatitis	*PRSS1*	Cationic trypsinogen, serine protease	276000
	SPINK1	Kazal-type serine protease inhibitor-1, pancreatic secretory trypsin inhibitor	167790
	CFTR	Cystic fibrosis transmembrane conductance regulator, ABC transporter, chloride channel	602421
	CTRC	Chymotrypsin C, pancreatic secretory trypsin inhibitor	601405
	CASR	Calsium-sensing receptor, plasma membrane G protein-coupled receptor	601199
	CPA1	Carboxylpeptidase A1, pancreatic exopeptidase	114850
	CEL	Carboxyl-ester lipase , lipolytic enzyme	114840

Based on the Online Medelian Inheritance in Man (OMIM) database (https://omim.org/)

There exist several forms of CP, based upon the cause of disease (35) including a heredity variant (45). Some of the forms are briefly described below.

Hereditary chronic pancreatitis (HCP) can be defined as autosomal dominantly inherited chronic pancreatitis, or as pancreatitis associated with germline gain of function mutations in *PRSS1* (45). HCP is a rare form of CP, with an average age of onset at 10 years (57), due to defects in trypsinogen regulation (reviewed in (45)). After the first discovery of a HCP-causative mutation in *PRSS1* (58), at least 20 gain-of-function mutations have been identified within this gene (59). Some of these mutations have a high penetrance (~ 80%) and have been shown to enhance auto-activation of trypsinogen, as well as to prevent autocatalytic inactivation of trypsin

(60, 61). In addition, a triplication segment involving *PRSS1* and *PRSS2* has been associated with HCP, proposing that gene-dosage effects conferred by copy number variants of the *PRSS* locus might contribute to HCP pathogenesis (62). Another important gene associated with HCP is *SPINK1*, encoding a trypsin inhibitor (59). Missense mutations in *SPINK1* have been suggested to cause loss-of-inhibitory functions of the enzyme, resulting in autosomal recessive CP within the affected individuals (63, 64). During the last decade, environmental factors have also been considered important for disease manifestation in HCP, as well as in other subgroups of chronic pancreatitis (reviewed in (65)).

Alcohol-induced CP (ACP) is a severe complication of prolonged alcohol abuse, characterized by malnutrition, abdominal pain and diabetes (35, 66). The average onset is during the mid-thirties, and the condition is potentially fatal (67). The pathogenic mechanisms behind ACP remain mostly unknown, although several studies indicate that ACP is a multifactorial disease where, in addition to alcohol, genetic and environmental factors such as smoking (68) contribute to the pathogenesis (66, 67).

Idiopathic chronic pancreatitis (ICP) is the second-most frequent form of CP in Western countries (69), and is defined as CP in subjects with little of normal alcohol consumption, where the etiology is unknown (35, 45, 69). Several alterations within genes related to trypsinogen regulation have been found to associate with ICP (35).

Tropical calcific pancreatitis (TCP), which is mostly found in tropical areas and developing countries, is an idiopathic, non-alcoholic form of CP that is characterized by early juvenile onset, pancreatic calcification, rapid progression and severe pancreatic damage (35, 70). TCP is defined as idiopathic, however, associations with N34S mutations in *SPINK1* has been reported in several Indian TCP cases (70). In the light of recent studies, TCP is thought to be a multifactorial disease and it is questionable whether it is a disease entity separate from CP found in Western countries (71).

1.2.3 **Pancreatic cancer**

Pancreatic cancer is a common term used to describe various malignant diseases located within the pancreas. The most frequent pancreatic cancer types are the ones situated in the exocrine part of the gland, with pancreatic ductal adenocarcinoma (PDAC) accounting for approximately 95% of all pancreatic tumors (1, 72). Pancreatic cancer is an aggressive disease, with an overall mortality rate close to 100% and a 5-year survival rate of less than 5% (73). It is now the 4-5th most common cause of cancer-related deaths in Western countries (74), and several epidemiological risk factors such as smoking, diabetes, increased body mass index and alcohol abuse have been associated with the disease (reviewed in (72, 75)). Moreover, as mentioned above, chronic pancreatitis also predisposes for pancreatic cancer (73, 76).

In pancreatic cancer, somatic driver mutations such as those occurring in the *KRAS* gene are of fundamental importance (77). *KRAS* is the most frequently mutated gene in patients with PDAC: nearly 95% of the patients harbor an oncogenic form of *KRAS* (reviewed in (77)). Normal KRAS proteins, which are GDP/GTP-binding proteins, are pivotal for maintaining proper intracellular signaling that affects cellular proliferation and differentiation, thus, mutations within the oncogenic *KRAS* play a role in many cancerous diseases (78). Somatic mutations occurring in tumor suppressor genes such as *TP53* (79), *SMAD4* (80) and *CDKN2A* (81) have also been revealed as important factors driving pancreatic tumorigenesis (82, 83).

Having a family history of pancreatic cancer, has been associated with elevated disease risk as 5-10% of the patients report at least one relative with the disease (84). Still, inherited genetic mutations underlying the majority of the familial occurrences of pancreatic cancer remain unclear. However, some pancreatic cancer susceptibility genes have been identified. Rare mutations in genes like *BRCA2*, *STK11*, *CDKN2A*, *PRSS1*, *PALB2*, *ATM* (83, 85-88) are associated with a high risk, whilst polymorphisms at the *ABO* locus (89) and common mutations of the *CFTR* gene (90) are associated with a slightly increased risk (reviewed in (75, 91)).

1.2.4 **MODY - A monogenic diseases of the pancreas**

Monogenic diseases can be described as rare disorders caused by one single genetic mutation, affecting either one (dominant) or both (recessive) alleles of the gene. The pattern of inheritance can be autosomal, X- or Y-linked as well as maternal. Over ten thousand human diseases are estimated to be monogenic (92), including pancreas-associated disorders such as hereditary pancreatitis, cystic fibrosis and inherited cancer syndromes.

Among these are monogenic diabetes, estimated to account for around 2% of all diabetes cases (93-95). Monogenic diabetes is frequently caused by mutations within genes important for beta cell function, and several subgroups have been identified, such as maturity-onset diabetes of the young (MODY) (96).

MODY was first defined in 1974 by Tattersall (97), and constitutes a group of monogenic disorders characterized by autosomal dominant inheritance, early onset of non-ketotic diabetes (usually before 25 years of age) and beta cell dysfunction (93, 98). The general prevalence of MODY is difficult to assess, as clinical features often are mild or may masquerade as T1D or T2D. Taking advantage of genetic screening as a routine in diagnostics, former undiagnosed MODY patients can now be diagnosed and given better treatment and care (99, 100). Initialized by the identification of mutations in the hepatocyte nuclear factor-4alpha (*HNF4A*) gene, studies during the last two decades have shown that at least 13 genes are responsible for causing MODY (Table 3) (101-104). Most of the genes encode transcription factors or regulators of glucose metabolism (Table 3) (98). Several genes associated with MODY are accompanied by a distinct phenotype (96). Examples are MODY5, where mutations in *HNF1B* result in a syndrome of renal cysts and diabetes (105) and MODY8, a syndrome of both exocrine and endocrine pancreatic dysfunction (see Section 1.5.1 below) (106).

The remaining subgroups of monogenic diabetes; maternally inherited diabetes (107), syndromic diabetes (108) and neonatal diabetes mellitus (NDM) (109) are not within the scope of this thesis.

Table 3 MODY-causing genes

Disease (OMIM #)	Gene (OMIM #)	Encoded protein / Type of protein
1 (125850)	*HNF4A* (600281)	Hepatocyte nuclear factor-4-alpha, transcription factor, DNA-binding protein
2 (125851)	*GCK* (138079)	Glucokinase, regulator in glucose metabolism
3 (600496)	*HNF1A* (142410)	Hepatocyte nuclear factor-1-alpha, transcription factor
4 (606392)	*PDX1* (600733)	Pancreatic and duodenal homeobox 1 (insulin promoter factor-1), transcription factor
5 (137920)	*HNF1B* (189907)	Hepatocyte nuclear factor-1-beta (transcription factor-2), transcription factor
6 (606394)	*NEUROD1* (601724)	Neurogenic differentiation 1, transcription factor
7 (610508)	*KLF11* (603301)	Krueppel-like factor 11, zinc finger transcription factor
8 (609812)	*CEL* (114840)	Carboxyl-ester lipase , lipolytic enzyme
9 (612225)	*PAX4* (167413)	Paired box gene 4, transcription factor
10 (613370)	*INS* (176730)	Insulin, hormone, regulator in glucose metabolism
11 (613375)	*BLK* (191305)	Tyrosine-protein kinase BLK, tyrosine kinase
13 (616329)	*KCNJ11* (600937)	Kir6.2, integral membrane protein, subunit of ATP-sensitive K+ channels
14 (616511)	*APPL1* (604299)	Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1, adaptor protein

Based on the Online Medelian Inheritance in Man (OMIM) database (https://omim.org/)

1.2.5 **Cystic fibrosis – another monogenic disease of the pancreas**

Cystic fibrosis (CF) is caused by severe loss of function mutations in the *CFTR* gene, encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (110, 111). CFTR is a chloride channel and influence the activity of intracellular vesicle transport and other channels, critical for fluid and electrolyte secretion (Reviewed in (112)). The disease leads to dysfunctions of the lungs and other internal organs, resulting in a significantly reduced life expectancy (113). In particular, the functions of the pancreas tend to be severely affected in CF patients. In fact, the majority (85-90%) of CF patients develop pancreatic exocrine dysfunction, characterized by extensive loss of acinar cells, lipomatosis and interstitial fibrosis (114). Pancreatic dysfunction, caused by intrapancreatic duct obstruction, can also lead to cystic fibrosis-related diabetes (113, 115).

1.3 The human carboxyl-ester lipase (*CEL*) gene

1.3.1 **Structure and expression of the *CEL* locus**

The human *CEL* gene resides on band q34.3 of chromosome 9 (116). The gene spans approximately 10 kilobase pairs (kb) and consists of 11 exons (Fig. 2). The exons vary in size, from around 100 base pairs (bp) to over 800 bp (117). The *CEL* locus also includes a *CEL* pseudogene (*CELP*) located around 11 kb downstream of *CEL* (Fig. 2) (117, 118). This pseudogene, despite that it lacks exons 2-7, is highly similar to *CEL* with respect to genomic sequence (117, 118). Also, due to high sequence similarity between the promotor region of *CELP* and the mouse *Cel* gene, it has been postulated that *CELP* is the original human gene that was duplicated (118, 119). In fact, *CELP* has shown to be transcribed in various cells throughout the body, however, as the pseudogene contains a stop codon within its second exon it is not expected to result in a functional protein (120).

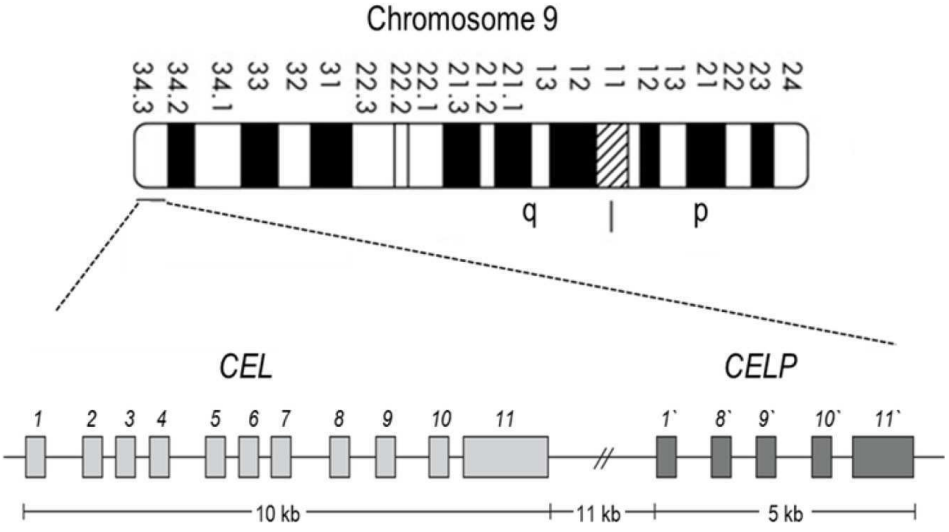

Figure 2 Location and structure of the human carboxyl-ester lipase locus
A simplified illustration of the human *CEL* locus, located on chromosome 9q34.3. The *CEL* gene consists of 11 exons, whilst the CEL-like pseudogene *CELP* contains 5 exons as the exon 2-exon 7 region are missing. Approximate gene sizes are listed in the bottom of the figure. Modified from (54, 121).

CEL has a tissue-specific expression and is highly expressed only in pancreatic acinar cells (122) and lactating mammary glands (123). Further, low levels of *CEL* expression have been documented in fetal liver (122), pituitary glands (124), vessel-wall endothelial cells (125), eosinophils (126), and in macrophages (127). Several studies have suggested that expression of *CEL* within various tissues and species are due to different regulatory systems, though, they all share one common element (128-130). This element was identified as an enhancer box motif (E-box), located at distinct upstream sites, and was shown to be essential for *CEL* expression (129). Nevertheless, the observed tissue-specific expression of *CEL* is suggested to be a result of a cooperative interaction between several elements located in the 5`-flanking region, and not conferred by the E-box alone. For instance, an additional strong pancreas-specific enhancer is needed to maintain the required high levels of *CEL* expression in the acinar cells (128).

1.3.2 **The polymorphic nature of *CEL***

Like for all other genes, single nucleotide polymorphisms (SNPs) are present and located throughout the *CEL* locus, giving rise to genetic variability. However, in the last exon of *CEL* (exon 11) there is a GC-rich variable number of tandem repeat (VNTR) region that makes the gene highly polymorphic (131, 132). Each repeat consists of nearly identical 33-bp segments, encoding 11 amino acids (133, 134). The most frequent *CEL* allele in all cohorts analyzed so far contains 16 VNTR repeats, though repeat lengths have been observed to vary from three to 23 (106, 132, 135-137). Several single-base insertion and deletion mutations have also been identified within the VNTR region (106, 138). They all lead to frameshifts that encode premature stop codons and truncated CEL proteins. Further, several types of copy number variants (CNVs) of the *CEL* locus are present (54, 132, 139). These are most probably a result of non-allelic, homologous recombination between the *CEL* and *CELP* genes (54). Taken together, *CEL* is an extremely polymorphic gene and, interestingly, some of the variants identified are associated with pancreatic disease. This is described in more detail in Section 1.5.

1.4 The human CEL protein

1.4.1 **Function and structure**

The human *CEL* gene encodes a digestive enzyme (EC 3.1.1.13) that also is referred to as bile salt-stimulated lipase (140), carboxyl-ester hydrolase (141) or bile salt-dependent lipase (142) in the literature.

As mentioned previously, CEL is manly produced in the pancreatic acinar cells and in lactating mammary glands. CEL is secreted from the exocrine pancreas into the duodenum as a component of the pancreatic juice (122, 141) and represents about 4% of the overall protein content of the juice (141). Within the duodenal lumen and after stimulation by bile salts, CEL participates in the hydrolysis of cholesteryl esters, dietary lipids and lipid-soluble vitamins (143, 144).

From the lactating mammary gland, CEL is secreted into the mother's milk where it is suggested to aid in the hydrolysis of dietary lipids in the newborn (123). Detection of breast milk CEL activity within the duodenum of newborns supports this function of CEL, although there is not sufficient evidence for assuming that CEL activity is an indispensable component of mother's milk (145, 146).

In addition to CEL's lipolytic activity, its lipoamidase activity and ability to hydrolyze ceramide have gained focus, as it may influence intestinal lipoprotein biosynthesis (147, 148). Moreover, CEL may have a role in lipoprotein metabolism and cardiovascular disease, as it has been shown to be synthesized in other tissue than acinar cells (122, 125, 127), as well as being endocytosed by enterocytes (149, 150). Moreover, it was recently suggested that CEL could utilize FAHFAs (fatty acid esters of hydroxyl fatty acids) as substrates, a group of mammalian lipids with metabolic and anti-inflammatory activities (151).

The protein structure of CEL can be divided into distinct sites and domains as illustrated in Figure 3. The amino acid sequence of CEL reveals that it belongs to the major α/β hydrolase fold family, characterized by a core structure of a strongly twisted α/β sheet of eight β-sheets and six α-helices (152, 153). CEL can also be

folded into two distinct structural domains; a globular N-terminal core domain, constituting the catalytic site, and a highly glycosylated C-terminal domain that includes the VNTR region and most likely lacks a well-defined structure (131, 154).

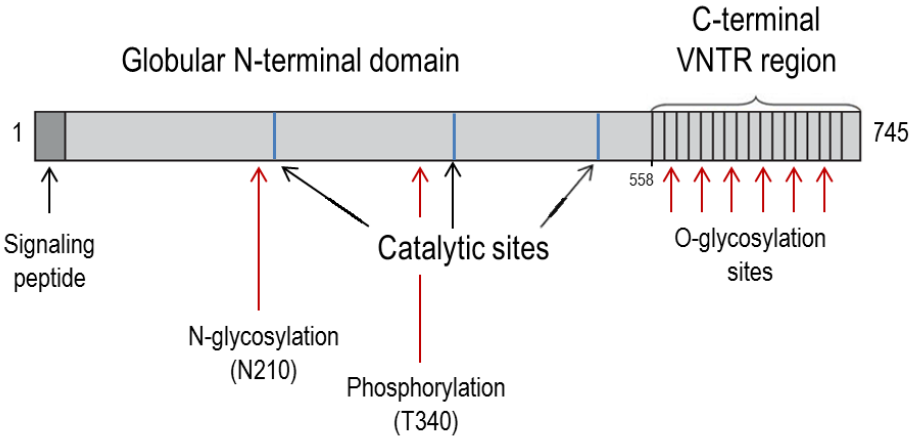

Figure 3 Schematic overview of the CEL protein structure
The most common CEL protein variant has 16 VNTR repeats as illustrated above, and consists of 722 amino acid residues, excluding the signal peptide of 23 amino acids. Some functional important sites are highlighted, including glycosylation sites, the catalytic triad sites and the VNTR region. Modified from (106).

N-terminal domain: Structure-function relations

The hydrolytic activity of CEL resides in a catalytic triad of amino acid residues, namely Ser194-His435-Asp320. These three amino acids, and the remaining N-terminal domain of CEL, have been found to be highly conserved across all vertebrate species studied (155). 3D structure analysis has revealed that the catalytic triad of amino acid residues is located centrally within CEL (153), with several mobile surface loops residing around the active site (153, 154).

Moreover, the hydrolytic activity against water-insoluble substrates, such as triacylglycerol, cholesterol ester and fatty acid chains with more than eight carbons, requires bile salt interaction of primary bile salts (140, 141, 156). It has been proposed that bile salt interactions occur at two distinct sites within the globular N-terminal domain of CEL (144): The specific binding site, located in close proximity to the catalytic triad, which is associated with CEL activation by interactions with the

7α-hydroxyl group of primary bile salts, and the less specific binding site associated with interactions toward negatively charged side chains of bile salts and aggregated micellar shaped substrates (153, 157-159).

Other features of the N-terminal domain are the signaling peptide, in addition to an N-glycosylation site and a phosphorylation site, at residues N210 and T340 respectively (Fig. 3). The functions of these features are further described in Section 1.4.2.

C-terminal domain: Structure-function relations

There is limited knowledge regarding the three-dimensional structure of the C-terminal domain of CEL as, its secondary structure suggests a flexible, open conformation (131). This domain is comprised of several 11-amino acid repeats and is enriched in the amino acid residues proline, glutamate, serine and threonine; commonly referred to as PEST-sequences (160). The number of repeats among different species can vary from none, like in fish and chicken, to 39 repeats in the gorilla (155). The most common human CEL variant has 16 repeats, but large variation among individuals has been observed (106, 132). A CEL protein with 16 repeats correlates to a mature protein of 722 amino acids, with a molecular mass of up to 140 kDa in the post-translationally modified state (54, 106, 138, 161).

There are several O-glycosylation sites residing in the mucin-like, C-terminal region, mainly on serine and threonine residues (162-164). A study on the glycosylation pattern of the human milk counterpart enzyme (BSSL), revealed that moieties such as fructose, galactose, glucosamine, galactosamine and neuraminic acids were present on the O-glycosylated carbohydrates, flanked by a O-glycosylation signaling sequence of PVPP (165). These O-linked glycans can associate with sialyl Lewis structures, known to be important for cell-cell adhesion. Moreover, a study from our group recently discovered that the O-glycome of CEL contained ABO blood group antigens, an observation that may suggest adhesive properties of CEL within the gastrointestinal tract (166).

This property could further aid circulating CEL to interact with endothelial surfaces (165, 167). Additionally, O-glycosylation might contribute to prevent the protein from intracellular proteolytic degradation by masking the PEST sequences (160) and protect against self-association caused by exposed hydrophobic regions, thereby increasing the stability and solubility of the enzyme (160, 168). However, the catalytic activity of CEL probably remains unaffected of the C-terminal region, as several studies have observed similar activity of truncated CEL protein (i.e. the version completely lacking the VNTR region) and normal CEL (54, 169, 170).

1.4.2 Synthesis, secretion and internalization of CEL

Common secretory pathway of digestive enzymes

In pancreatic acinar cells, digestive enzymes are thought to follow the classical secretory pathway (19, 171). These enzymes tend to contain an ER (endoplasmic reticulum)-signaling peptide, often found in the very N-terminal of the protein, translocating the protein-synthesizing ribosomes to the rough ER. The signaling peptide is then cleaved off, and the growing polypeptide chain enters the ER lumen. Within the ER lumen, newly synthesized peptides are subjected to co- and post-translational modifications, such as N-glycosylation, chaperone-assisted folding and other conformational changes like formation of disulfide-bonds. Correctly processed proteins are further transported through the Golgi apparatus, where additional O-glycosylation and phosphorylation takes place. Finally, the enzymes are stored in zymogen granules and upon secretory stimulation, the zymogen granules fuse with the plasma membrane and their contents are released into the acini lumen (2, 19).

Specific co- and post-translational modifications during intracellular transport of CEL

The CEL protein undergoes several steps of modification from its release into the ER lumen until it passes through the Golgi network and is secreted from the acinar cells (Fig. 4). The first step involves the removal of the 23 amino acid signaling peptide, located on the very N-terminal region of CEL. Once the CEL polypeptide is released into the ER lumen, it interacts with multiple chaperones, including GRP94, and forms

a folding complex (171). In fact, CEL is kept in close proximity to intracellular membranes during its intracellular transport through interactions with GRP94 (172, 173). This interaction is suggested to be important for proper folding of CEL, to keep CEL in an inactive state during the transport as well as to ensure proper and complete post-translational modification (172, 174).

Within the rough ER, CEL is co-translationally N-glycosylated on the highly conserved N210 residue. This modification has been suggested to be important for proper folding and secretion of CEL (175). Additionally, a recent study from our group has documented that CEL tends to aggregate intracellularly in HEK293 cells in the absence of N-glycosylation (176). However, this modification is not thought to be required for bile salt-dependent activity, despite the adjacent localization of the N-glycosylation site and the catalytic Ser194 residue (169, 170, 177). Following N-glycosylation in the rough ER, two disulfide bridges are formed (164, 165), maintaining the folded conformation of CEL prior to its transport to the Golgi network (164).

In the Golgi, the C-terminal region of CEL is excessively O-glycosylated on multiple Ser/Thr residues (162-164). The last step of modification occurs within the trans-Golgi compartment where CEL is released from intracellular membranes by phosphorylation at Thr340 (178) and stored in zymogen granules along with other digestive enzymes (141). Intracellular transport of CEL through the secretory pathway ends when the content of the zymogen granules are released into the ductal system (Fig. 4). Notable, around 30% of the secreted CEL-content in the pancreatic juice is estimated be in complex with the chaperone GRP94, whilst the remaining are free CEL molecules (179).

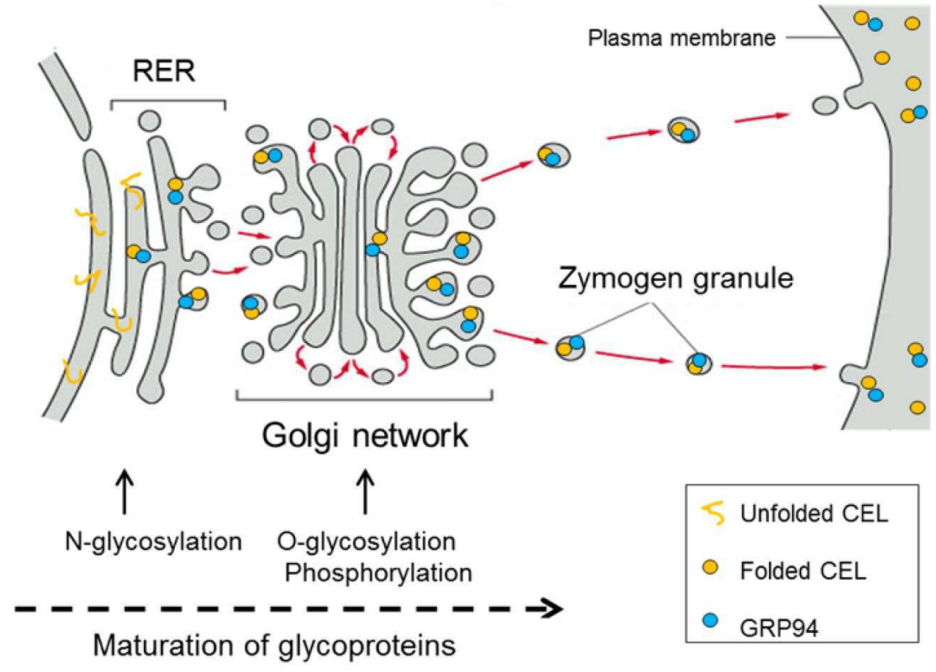

Figure 4 Secretory pathway of CEL
A simplified illustration of the secretory pathway of CEL in the acinar cells: Once the ER signaling peptide is cleaved off and the growing polypeptide enters the ER lumen, CEL is subjected to N-glycosylation and a complex with the molecular chaperone GRP94 is formed. The complex is further transported to the Golgi network, where CEL is heavily O-glycosylated. After phosphorylation, CEL is released from the membranes and co-stored along with other digestive enzymes in zymogen granules. Upon stimuli, the content of the granules are released into the duodenum lumen and a substantial part of the CEL molecules are still complexed with GRP94. Modified from (174, 180).

Internalization of CEL

Previous studies have suggested that secreted CEL, in complex with the GRP94 chaperone, can be internalized by enterocytes, i.e. by epithelial cells lining the small intestine (Fig. 5) (174, 179, 181). It was suggested that CEL recognizes specific affinity-binding sites on the microvilli membrane of enterocytes, whilst GRP94 provided CEL with the proper conformation to allow its specific interaction with the binding sites and to be endocytosed (182). A more current study has suggested that the lectin-like Ox-LDL receptor (LOX-1) is involved in the CEL/GRP94 internalization, by a mechanism of receptor-mediated endocytosis (183).

Intracellularly, the CEL/GRP94 complex has been found to dissociate within endosomal vesicles, and once the vesicles fuse with multivesicular bodies, CEL continues its way to the basolateral membrane (Fig. 5) (174, 179). Further transport within the enterocytes is through the Golgi network, before CEL reaches the basolateral membrane and is released. After its transcytosis through the enterocytes, CEL is likely to enter the blood compartment (181), as small amounts of circulating CEL has been detected in the blood stream, associated with apolipoprotein B-containing lipoproteins. Furthermore, circulating CEL has been proposed to affect cholesterol metabolism within the blood compartment, as it might modify low-density lipoprotein (LDL) structure/content (184), and play a role in atherosclerosis (181) (See also Section 1.5.4 below). CEL may be cleared from the blood circulation by renal filtration, as it is detectable in the urine of healthy subjects (185).

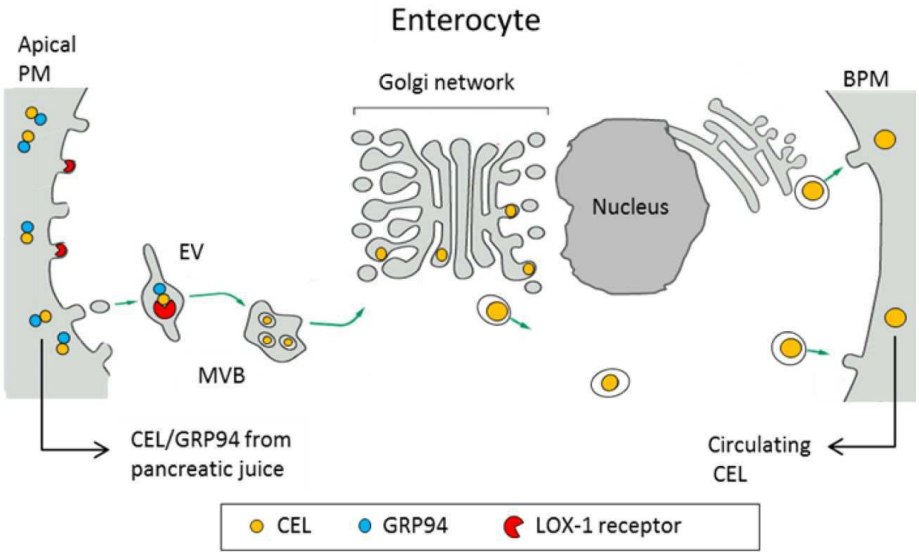

Figure 5 Endocytosis of secreted CEL by enterocytes
A simplified model for uptake of CEL by enterocytes: Secreted CEL/GRP94 complex can be taken up by enterocytes in the small intestine, via receptor-mediated endocytosis involving the lectin-like Ox-LDL (LOX-1) receptor. The CEL/GRP94 complex dissociates within the endosomal vesicle (EV), and only CEL continues the transport to the basolateral plasma membrane (BPM) after the EV has fused with the multivesicular body (MVB). After being transported through the Golgi network, CEL is released into the interstitial space and may enter the blood circulation. Modified from (174, 180).

1.5 The *CEL* gene in human disease

1.5.1 **MODY8**

MODY8 (also denoted CEL-MODY) is a monogenic disorder that follows an autosomal dominant pattern of inheritance and is characterized by pancreatic exocrine and endocrine dysfunction (106). Though MODY8 is a syndrome with a primary onset of pancreatic exocrine dysfunction, rather than beta cell dysfunction, it clinically fulfills the MODY criteria (https://omim.org/entry/606391) and hence defined as a subclass of this disorder.

This syndrome is caused by a single-base deletion mutation in the first (DEL1) or fourth (DEL4) repeat of the *CEL* VNTR region (Fig. 6A). These mutations were originally discovered in two Norwegian families (106). Since then, two other families from Europe have been found (unpublished data from our group). Mutation carriers exhibit moderate to severe fecal elastase deficiency, reduced serum levels of lipid-soluble vitamins A and E, and pancreatic lipomatosis detectable in childhood (106, 186). Moreover, the disease typically progresses over time with patients developing monogenic diabetes in their mid-thirties due to beta cell failure. From the age of 40, pancreatic cysts have been observed in all mutation carriers examined (187). Since the early clinical signs of MODY8 are limited, it has been suggested that studies of biomarkers might be important to gain better insights of the disease and improve treatment (186, 188).

When Ræder et al. first described the MODY8 syndrome in 2006 (106), it was noted that both single-base deletions (DEL1: c.1686delT; p.Val563CysfsX111 and DEL4: c.1785; p.Val605CysfsX99) lead to frameshifts and a premature stop-codon in VNTR repeat 13, affecting the next 111 (DEL1) or 99 (DEL4) amino acids (106). Thus, it is only the C-terminal domain of CEL that is changed. This result in two truncated proteins referred to in this thesis as CEL-MODY and CEL-DEL4 with a theoretical molecular mass of 73 kDa (Fig. 6B). Despite the fact that subjects carrying the DEL4 mutations display a similar phenotype as the DEL1 mutation carriers (106), it is the

functional characteristics of CEL-MODY that have been studied so far and will be further highlighted here.

Initial studies from transfected CHO cells showed that CEL-MODY exhibited similar *in vitro* catalytic activity as the normal CEL protein (CEL-WT), however, secretion of the mutant enzyme was significantly reduced, suggesting a link between disease mechanism and loss of function at the protein level (106). A study of whole-body *CEL* knock-out mice, however, reported that no alterations in pancreatic endocrine or exocrine function could be observed (189). It was therefore suggested that the MODY8 disease mechanism does not involve a simple loss of function, hence, loss of catalytic activity was not considered to explain MODY8 (189).

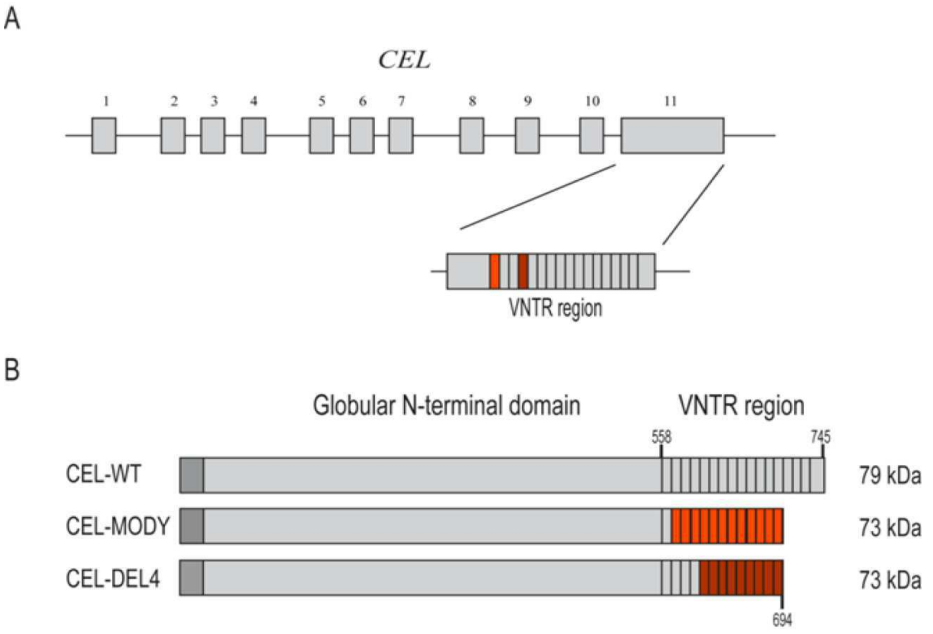

Figure 6 Schematic structures of the MODY8-associated variants
A) Localization of the pathogenic single-base deletions in repeat 1 (light red) and 4 (dark red) of the *CEL* VNTR. Grey boxes with numbers represent the *CEL* exons, whereas each VNTR repeat is illustrated by a small box within the VNTR region. B) Predicted protein structure of CEL-WT and the MODY8-causing variants, CEL-MODY (light red) and CEL-DEL4 (dark red). Normal repeats are illustrated by small grey boxes and altered pathogenic repeats are shown in light and dark red for the CEL variants. Theoretical molecular weights are listed.

In silico analysis shows that the predicted p*I* of the VNTR region increases from pH 3.3 in CEL-WT to 11.8 in CEL-MODY. It is therefore expected that the CEL-MODY protein would exhibit some altered biochemical and cellular properties compared with CEL-WT. In fact, in transfected HEK293 cells, CEL-MODY demonstrated a higher tendency to form intra- and extracellular aggregates (161). It has therefore been suggested that MODY8 is a protein misfolding disease, involving a negative gain-of-function effect conferred by the mutant protein (161). Additionally, Xiao and colleagues recently reported from studies in a rat acinar cell line that intracellular accumulation of CEL-MODY leads to cellular ER stress, followed by induction of the unfolded protein response (UPR), NF-κB activation and, finally, induction of apoptosis (190). The authors suggested that this could serve as a protective mechanism to prevent an inflammatory reaction affecting adjacent pancreatic cells (190).

1.5.2 **Chronic pancreatitis**

It has been reported by a Japanese study that the number of *CEL* VNTR repeats is associated with elevated risk of developing alcohol-induced pancreatitis, as the frequency of alleles with more than 16 repeats was increased in patient compared to healthy individuals (191). In a larger European study, however, this association could not be replicated (137). Moreover, in a recent study from our group we found a possible link between *CEL* VNTR lengths and alcoholic liver cirrhosis, but not alcoholic CP (192). The observed divergence in results between Japanese and European studies might be a consequence of methodological, ethnic or environmental factors (i.e. level of alcohol consumption) (192).

Also a CNV of the *CEL* locus has been associated with CP (54, 193). In 2015, our group identified a *CEL* deletion variant, designated *CEL-HYB*, as a novel risk factor for the disease (54). The carrier frequency of *CEL-HYB* was increased five-fold in European patients with ICP compared to healthy individuals (54). *CEL-HYB* is a hybrid gene that most likely has originated from non-allelic, homologous recombination between *CEL* and *CELP*, in which the VNTR region of *CEL-HYB* was

recruited from *CELP* (Fig. 7A) (54). *CEL-HYB* was the first lipase gene found to be associated with increased CP risk, as the majority of pancreatitis genes encode proteins of the protease-antiprotease system of pancreatic digestive enzymes (194). Moreover, the *CEL-HYB* allele was actually not detected at all in three independent Asian cohorts studied, suggesting that *CEL-HYB* is an ethnic-specific gene (195, 196). In contrast, another hybrid allele (denoted *CEL-HYB2*) has been observed in both European and Asian populations (54, 195, 196). However, *CEL-HYB2* was neither found to associate with CP nor to be translated into a functional protein, most probably due to a premature stop codon and subsequent nonsense-mediated mRNA decay (195, 196).

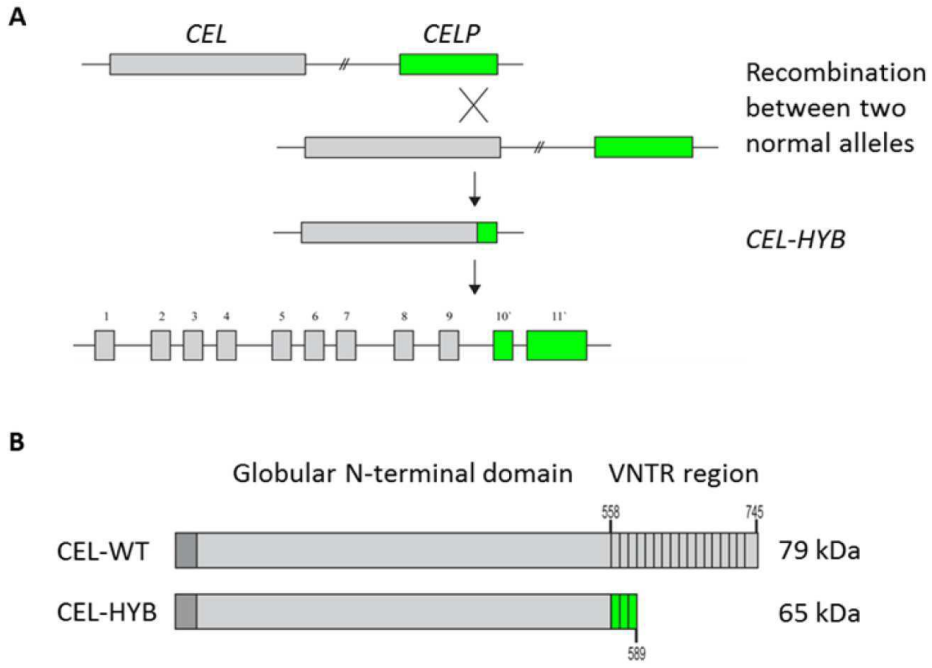

Figure 7 Schematic structure of the pathogenic CEL CNV: CEL-HYB
A) Non-allelic homologous recombination between *CEL* and its adjacent pseudogene *CELP* (green color). The crossover has likely occurred between the exon 10-exon 11 regions as illustrated by the X symbol. The large boxes illustrate the complete *CEL* and *CELP* genes, whereas the individual numbered boxes correspond to the exons of the recombined *CEL-HYB* allele. B**)** The CEL-HYB protein is similar to CEL-WT, except for the VNTR region. CEL-HYB has only three repeats, originating from the pseudogene *CELP* (green color). Consequently, CEL-HYB has a smaller theoretical molecule mass. Modified from (54).

The *CEL-HYB* gene encodes a fusion protein with only three VNTR repeats and a theoretical molecular mass of 65 kDa (Fig. 7B). CEL-HYB shows altered biochemical and cellular properties compared to CEL-WT including impaired secretion, a tendency to accumulate intracellularly and to induce autophagy. It has also been reported that CEL-HYB exhibit reduced lipolytic activity, reaching only 40% of normal CEL activity (54).

1.5.3 **Pancreatic cancer**

Fetoacinar pancreatic protein (FAPP) is a truncated glycoprotein expressed in the early development of the pancreas (197, 198). FAPP has also been reported in various pancreatic tumor-derived cell lines and in the pancreatic juice from patients diagnosed with pancreatic cancer, and it has been suggested that FAPP is an onco-fetal glycoform of CEL (199). However, additional studies have reported that CEL is neither significantly expressed nor active in neoplastic cells of the pancreas (166, 200), seriously questioning the identity of FAPP.

Moreover, a common SNP within the second repeat of the *CEL* VNTR has been suggested to be associated with pancreatic cancer (201), despite that no genome-wide association studies ever have identified *CEL* SNPs as risk factors in this disease (89, 202-204). It should be noted that the study only included a patient cohort of 36 individuals (201), which is far too small for any conclusions to be drawn.

Insertions of an extra cytosine-residue in various sites of the *CEL* VNTR region have been documented (106, 138) and one such insertion was recently proposed to associate with pre-neoplastic lesions of the pancreas (138). The reported cytosine insertion results in a truncated CEL protein and due to its possible occurrence as an early somatic mutation in PDAC, it has been suggested to be exploited as a tool within pancreatic cancer diagnostics (138).

1.5.4 **Other diseases**

Panicot and co-workers have reported a possible link between *CEL* and T1D (205). Sera from newly diagnosed T1D patients were screened for autoantibodies recognizing the C-terminal of CEL. Among patients, 73.5% showed autoreactivity in contrast to 8.4% of controls (205). Additionally, single-base insertions within the *CEL* VNTR have been associated with an increased risk of developing pancreatic exocrine dysfunction in T1D patients (106).

Furthermore, *CEL* VNTR length polymorphisms have been associated with the rate of HIV-1 disease progression (206), serum cholesterol profile (207) and cardiovascular disease (127, 130, 171, 208). Taken together, these studies indicate that there is more to CEL than being “only” a digestive enzyme and that possible alternative roles need to be further investigated.

1.6 CNVs and VNTRs in human disease

Genetic variation within the human genome exists in several different forms and may vary from single-base pair alterations (209), to kilobase pair (210, 211) and multiple kilobase pair alterations (212, 213), hence, ranging from SNPs to complex genomic rearrangements.

Copy-number variants (CNVs) are genetic variations where sections of the genome are duplicated or deleted, i.e. where there is an allele copy number different from the normal 2 copies of autosomal DNA (214). CNVs are a major source of genetic diversity in humans, and they may be formed by both recombination-based (215) and replication-based mechanisms (216-218). Studying CNVs are of high value in order to fully understand how genetic factors might contribute to phenotypic diversity (219) and human disease (220). CNVs have been reported to associate with a wide range of complex human diseases, including Alzheimer`s disease, autism, Parkinson disease and pancreatitis (see review for more details (221)), as exemplified by the discovery of the CNV *CEL-HYB*, in which predispose for CP (54). Additionally, CNVs have been directly linked to Medelian disorders like Williams-Beuren syndrome (WBS; genomic deletions in 7q11.23) (222), Charcot-Marie-tooth disease type 1A (CMT1A; duplication of the gene *PMP22*) (223) and autosomal dominant adult-onset demyelinating leukodystrophy (ADLD), which is a neurological disorder caused by gene duplications of the lamin B1 (*LMNB1*) gene (224).

A type of genetic variation related to CNVs and providing a unique source of genomic variability is variable number of tandem repeat (VNTR) regions (225). They consist of small DNA motifs which are continuously repeated several times in the genome (226). The number of repeats can differ within and between individuals, and they were originally isolated and studied for genetic mapping purposes (227). Overall, VNTR regions are estimated to represent approximately 3% of the total human genome. Although most of them are present in intragenic regions, they can also be found in functional genetic regions, such as in promotors and exons (228). The mutation rate within the VNTRs is high and the degree of polymorphism is far

larger than for other types of genetic variations such as SNPs and CNVs (225). It is therefore not surprising that VNTR polymorphisms have been linked to genetic diseases (228). For instance, functional VNTRs located within promotors or exons have been associated with diseases like attention deficient hyperactivity disorder (ADHD), T1D, T2D and polycystic ovary syndrome (228). In addition, tri-nucleotide expansions and single-base deletions in the VNTR repeats can cause monogenic diseases such as Huntington`s disease, fragile X syndrome, myoclonic epilepsy and MODY8 (see Section 1.5.1) (106, 228). Less is known about the functional relevance of VNTR polymorphisms located within introns and outside the gene coding sequence. However, they have been suggested to have an effect on alternative splicing, mRNA translocation and translation efficiency (229). Even though it is not methodologically straightforward to study these polymorphisms and their association with genetic diseases, the VNTRs of the human genome involve a substantial level of genetic variance. They should therefore be taken into account when searching for genetic susceptibility factors behind complex diseases (228).

1.7 Endocytosis in human disease

Cellular endocytosis can be described as an uptake or active transport of extracellular material into cells through the plasma membrane (230, 231). The main function of endocytosis is to take up nutrients from the environment and the process serves an essential role in normal cell physiology (232). It also contributes to the regulation of cell surface expression of receptors, transporters and other membrane proteins (232), thereby maintaining a proper balance within the cargo sorting routes running between biochemically and functionally distinct intracellular compartments (233). In addition, endocytosis plays a pivotal role in organizing, mediating, and regulating cellular signal transduction events. At the same time, the endocytic process itself is tightly controlled by several signaling pathways (233). The endocytic pathway comprises distinct intracellular compartments and systems contributing to the internalization process (233), such as the lysosomal system, a mechanism responsible for the catabolism of endo- and exogenous macromolecules (234).

Multiple pathways for mammalian cell endocytosis have been revealed (231), including clathrin-mediated endocytosis (235), caveolae uptake (236), cholesterol-sensitive clathrin- and caveolae-independent pathways (237-239) and the large capacity CLIC/GEEC pathway (240). As the endocytic process plays a vital role in normal cell homeostasis, it is not surprising that it also can be implicated in cellular pathology. Although the mechanisms are poorly understood, several reports link endocytosis to disorders such as Alzheimer's disease, cancer and atherosclerosis (232, 241). Moreover, regulation of membrane trafficking and organelle pH levels are important in the pathophysiology associated with uptake of bacteria and viruses (242). Some selected disorders in which disruption or disturbance of the endocytic process is thought to be pivotal, are shortly described below.

In protein aggregation diseases such as Alzheimer's disease, the net balance between APP (amyloid precursor protein) synthesis and degradation is disrupted. Under normal conditions, newly synthesized transmembrane APP is only to a limited degree found on the cell surface as it is continuously internalized by clathrin-mediated

endocytosis and degraded by the lysosomal system. In contrast, disturbances in the endocytic process lead to a proteolytic cleavage of APP that produces small neurotoxic Aβ fibrils. Intracellular accumulation of these aggregated fibrils, might then result in the development of Alzheimer's disease (reviewed in (232)).

In cancer, the endocytic process is also suspected to be disrupted, reducing the ability of the malignant cells to internalize, recycle or degrade membrane receptors and to properly regulate the cell surface expression of membrane proteins. The line of events that determines the fate of internalized proteins relies on intrinsic sequence motifs, posttranslational modifications and transient assemblies of phosphoinositide-binding proteins. Within cancer cells, this process is disrupted and altered, leading to an improper balance within the synthesis and degradation of key cancer drivers (reviewed in (241)).

Mutation in some genes that directly involve the lysosomal system may lead to lysosomal storage disease (LSD). This is a group of rare inherited metabolic disorders characterized by aberrant accumulation of macromolecules inside the lysosomes (243). LSDs most often occur as a result of mutations within genes encoding proteins responsible for maintaining the lysosomal system, or they are due to defects within the endocytic process. Despite being rare, over 40 LSDs have been identified, and examples are Niemann-Pick disease type C1, cystinosis, Fabry disease and Gaucher disease types 1, 2, and 3 (234, 243).

2. Aims of the present study

The *CEL* gene is highly polymorphic and has been linked to pancreatic diseases. The pathogenic *CEL* variants differ in the last exon, which contains a variable number of tandem repeat (VNTR) region. Maturity-onset diabetes of the young, type 8 (MODY8) is caused by single-base deletion mutations in the *CEL* VNTR. Furthermore, CNVs of the *CEL* locus have been identified, including a recombined deletion allele (*CEL-HYB*) that predispose for chronic pancreatitis. The CEL-MODY and CEL-HYB proteins both exhibit altered biochemical and cellular properties compared with the normal CEL protein (CEL-WT). However, the disease mechanisms involving the two pathogenic CEL variants remain unclear. As chronic pancreatitis predisposes for subsequent cancer development, it is also of interest to investigate whether CEL variants might associate with pancreatic cancer.

In the present study we therefore aimed to:

1. Evaluate the *CEL* gene as a genetic risk factor in pancreatic cancer, with a focus on CNVs and VNTR length polymorphisms (Paper I)

2. Investigate functional effects of the CEL-MODY protein in HEK293 cells, and in pancreatic acinar (266-6) and beta-like (INS 1E) cells (Paper II)

3. Extend the functional studies of Paper II to human pancreatic ductal-like cells (PANC-1) and to perform a comparison of the more recently discovered CEL-HYB protein with CEL-WT and CEL-MODY (Paper III)

4. Investigate whether co-expression of either CEL-MODY or CEL-HYB with CEL-WT could affect the functional properties of the latter (Paper III)

3. Summary of results

Paper I: Copy number variants and VNTR length polymorphisms of the carboxyl-ester lipase (*CEL*) gene as risk factors in pancreatic cancer

CNVs and the VNTR region of *CEL* have been associated with pancreatic diseases, such as chronic pancreatitis. In this study, we investigated if *CEL* CNVs and VNTR length polymorphisms could predispose for pancreatic cancer. We first analyzed a German pedigree with chronic pancreatitis and pancreatic cancer and found that at least three affected members carried the *CEL-HYB* allele. We then analyzed a series of German and Norwegian patients diagnosed with PDAC, however, we found no statistically significant association between pancreatic cancer and *CEL-HYB* or a *CEL* duplication allele. *CEL* VNTR lengths varied between 4 and 23 repeats, and as expected the 16-repeat allele was found to be by far most frequent in both cohorts and controls. Notably, the carrier frequency of the 23-repeat allele was borderline significant in Norwegian cancer cases compared to controls (1.2% vs. 0.3%; p = 0.05), although not significant within the German cohort. For all other VNTR lengths, no statistically significant difference in allele frequency was observed between cases and controls. The outcome was also negative when the *CEL* VNTR lengths were pooled into groups of short, normal or long alleles or when tested according to genotypes. These results indicate no association between pancreatic exocrine cancer and *CEL* CNVs or the VNTR region of *CEL*. Nevertheless, *CEL* is highly polymorphic and it is too early to exclude a role of *CEL* in pancreatic cancer, as novel genetic variants of *CEL* might still be revealed.

Paper II: Endocytosis of secreted carboxyl ester lipase in a syndrome of diabetes and pancreatic exocrine dysfunction

A single-base mutation within the first repeat of the *CEL* VNTR region leads to a frameshift, affecting the downstream amino acids and drastically altering the C-terminal part of CEL. The resulting mutant protein (CEL-MODY, previously denoted CEL-MUT) has been shown to exhibit altered biochemical and cellular properties compared to CEL-WT. In this study, we aimed to investigate whether the disease-causing mutation could affect intracellular distribution, internalization and

degradation of the CEL protein, using various cell line models including pancreatic acinar and beta cells. We found that both CEL-WT and CEL-MODY followed the classical secretory pathway through the ER and Golgi compartments. However, CEL-MODY was found to exhibit a higher tendency to aggregate at the outer cell surface and in large intracellular vacuoles, identified as components of the endosomal system. Additionally, secreted CEL-MODY proteins were found to be taken up by both non-pancreatic and pancreatic cell line models. Endocytosis was followed by lysosomal degradation, resulting in reduced cell viability of the exposed cells. Hence, our findings may be relevant for understanding how the pathogenic CEL-MODY protein can cause a syndrome affecting both pancreatic exocrine and endocrine cells.

Paper III: Pathogenic carboxyl-ester lipase (CEL) variants are endocytosed in pancreatic cell lines and may influence properties of the normal CEL protein

CEL-MODY is a rare protein variant of the human enzyme carboxyl-ester lipase and causes MODY8, a highly penetrant inherited disease of the pancreas. Here, both the exocrine and the endocrine compartments are affected and endocytosis has been suggested to play an important role in the pathogenesis. The pathogenic CEL variant CEL-HYB has a milder effect as it is five-fold more prevalent in cohorts of idiopathic chronic pancreatitis. Both variants exhibit altered biochemical and cellular properties when compared to CEL-WT. In this study, we aimed to analyze the uptake and effects after long-time exposure of CEL by using pancreatic acinar and ductal cell line models. Interestingly, as compared with CEL-WT, both pathogenic variants reduced the viability of acinar and ductal cells after endocytosis. Moreover, we found both CEL-MODY and CEL-HYB to affect the intracellular fate of CEL-WT when co-expressed, whilst the cellular toxicity of the two pathogenic variants after co-endocytosis became reduced in the presence of CEL-WT. Our results could provide insight necessary for understanding how the pathogenic CEL variants lead to slowly progressing pancreatic diseases.

4. General discussion

4.1 Study limitations

4.1.1 **The polymorphic nature of CEL**

In addition to SNPs located throughout the whole locus, the human *CEL* gene contains VNTR length polymorphisms and single-base insertions and deletions within the VNTR region. Due to its location adjacent to the pseudogene *CELP*, it has been postulated that *CEL* is prone to undergo recombination events that lead to CNV formation (54). In combination, all these types of variation imply that *CEL* is an unusually polymorphic gene (Fig. 8). Some of the variants have been implicated in human diseases (reviewed in (244, 245). However, the polymorphic nature of *CEL* makes it a challenging gene to study.

The highly GC-rich VNTR of *CEL* makes it very problematic to use DNA sequencing for analyzing this region. Our group has previously developed a rapid screening method for detection of single-base mutations and for determination of the total number of repeats within the VNTR (132). This method is necessary as a supplement to Sanger DNA sequencing to capture the variability of the VNTR region. When using this method in Paper I to analyze VNTR length polymorphisms in pancreatic cancer cohorts, there were still some limitations that had to be taken into account. In the presence of duplication alleles (Fig. 8D), the screening method could not distinguish between true heterozygous *CEL* samples (e.g. VNTR length = 15 and 16) and *CEL-DUP* samples of which two of the VNTRs were of equal length (e.g. 15, 16, 16). Moreover, when comparing CNV screening and VNTR length results we discovered that some samples harbored three copies of *CEL* without being identified as either *CEL-DUP1* or *CEL-DUP2* (Paper I, (246)). This indicates that other, yet to be identified, *CEL* CNVs exist. Their potential role in pancreatic disease should therefore be elucidated. Improvement of the methods for genotyping the VNTR repeats will in combination with additional CNV screening be necessary for understanding the role of *CEL* variants in human pancreatic physiology. Notably,

high-throughput methods such as next-generation sequencing and genome-wide association analysis are unlikely to cover the extensive variation of the CEL locus (54), although there is evidence that some CNVs can be detected by whole-genome sequencing (246).

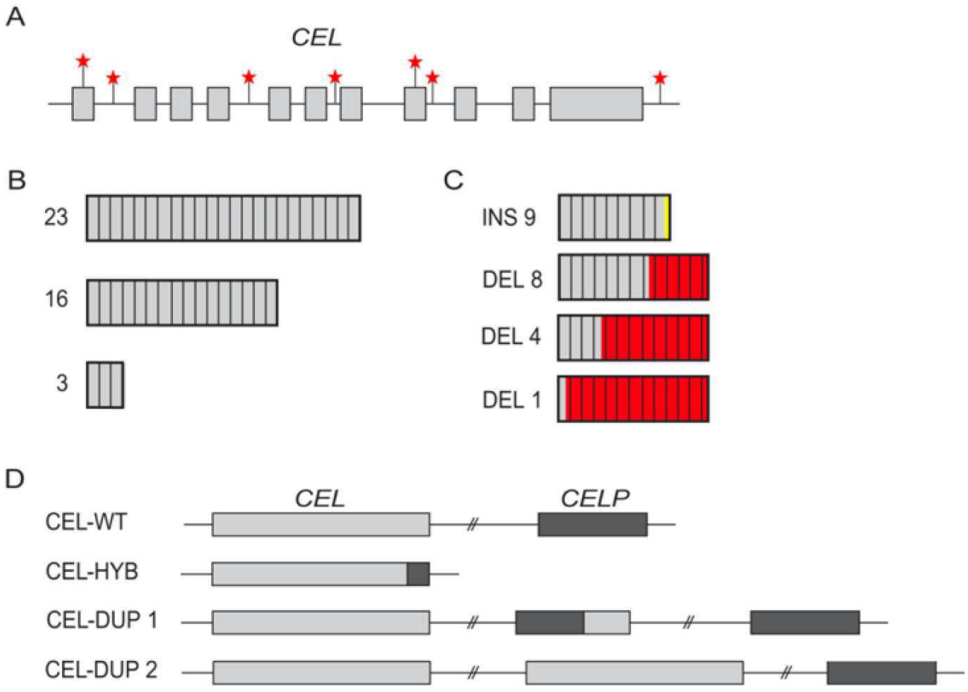

Figure 8 Genetic variability of CEL
Simplified illustration of genetic variation occurring within and around the human *CEL* gene. A) Common SNPs present in both coding and non-coding regions of *CEL*. B) VNTR length polymorphisms illustrated by alleles harboring 23, 16 and 3 repeats. Grey boxes indicate normal gene repeats. C) Single-base insertion and deletions, illustrated by an insertion in repeat 9 (INS 9) and deletions in repeat 8, 4 and 1 (DEL 8, 4 and 1, respectively). Grey boxes indicate normal gene repeats, yellow box indicate a short truncated segment due to the single-base insertion and red boxes indicate altered downstream repeats due to single-base deletions. D) CNVs of the *CEL* locus, involving the adjacent pseudogene *CELP*. Three CNVs have been identified, two duplication alleles (*CEL-DUP1* and *CEL-DUP2*) and one deletion allele (*CEL-HYB*). The large light and dark grey boxes illustrate the complete *CEL* and *CELP* genes, respectively, whereas smaller boxes illustrate segments of either gene. Modified from (54, 106).

4.1.2 Use of recombinant protein tags

The selection of various cell line models have been discussed in Paper III and will not be further elaborated on here.

The C-terminal, VNTR-containing region of CEL-MODY and CEL-HYB is where their disease-causing properties are likely to reside. Herein lies also some limitations to our expression studies as all tested CEL variants had been coupled to recombinant protein tags to simplify detection and visualization. Both pathogenic variants exhibit altered biochemical properties compared to CEL-WT (54, 161), and this may interfere with solubility and detection of the protein tags (or vice versa).

A recent study from our group have compared CEL-expression in HEK293 cells, with and without the use of the C-terminal V5/HisB tag (247). Interestingly, untagged CEL proteins displayed a stronger intracellular retention, as compared to the V5/HisB-tagged CEL proteins (247), indicating that the tag may increase the solubility of the recombinant CEL proteins. This was further supported by Xiao et al., who found that untagged CEL-MODY displayed impaired secretion when compared to CEL-WT (190). Experimental studies using V5/HisB-tagged CEL proteins may, therefore, lead to underestimation of the CEL protein variants' ability to aggregate in cellular systems.

The FLAG tag is also commonly employed for identification of recombinant proteins and several high-affinity anti-FLAG antibodies are available (248). In Paper III, we used the FLAG tag for distinguishing CEL-WT from CEL-MODY and CEL-HYB during co-expression analyses. This was successful until the CEL protein variants were to be detected in their secreted forms, either within the medium fractions or as endocytosed proteins. In fact, Schmidt et al. have shown that a tyrosine residue residing in the FLAG epitope can be subjected to post-translational tyrosine sulfation (248). This modification negatively affects binding of anti-FLAG antibodies to the epitope, making it more challenging to detect mature, secreted recombinant proteins (248). Moreover, tyrosine sulfation has been suggested to be important for protein-protein interaction and regulation of intracellular trafficking of secreted proteins

(reviewed in (249, 250)). This modification adds negative charge so interactions with positively charged amino acids are favored, potentially forming salt-bridges (251). Due to the altered VNTR region of CEL-MODY (and partially that of CEL-HYB), the net charge is shifted from negative to positive (54, 161), which may enable interactions between the CEL variants and the sulfated FLAG tag. This may interfere with detection of the tags by antibodies, as well as disturb protein-protein interactions between the CEL variants. As it may not be possible to conduct co-expression analyses without the use of recombinant protein tags, we suggest that cells in further experiments are treated with sodium chlorate prior to analyses, in order to inhibit tyrosine sulfation of the FLAG epitope (252).

4.2 CEL - a risk factor in pancreatic cancer ? (Paper I)

In addition to somatic driver mutations like those occurring in *KRAS* (77), oxidative stress and DNA damage due to life-style risk factors like smoking, diet and alcohol abuse, have been associated with pancreatic cancer development (253, 254). A history of chronic pancreatitis or diabetes has also been associated with an increased risk of developing pancreatic cancer (reviewed in (83)). In particular, subjects with hereditary pancreatitis due to *PRSS1* mutations, have a greatly increased risk of this cancer form (255).

A German pedigree with familial idiopathic CP was revealed by Fjeld et al. to carry the *CEL-HYB* allele (54). During the follow-up, the family was found to include some members with a diagnosis of pancreatic cancer (Paper I). This led to the assumption that *CEL-HYB*, which predisposes for CP, might confer risk also of pancreatic cancer development. However, we could not detect any association between *CEL-HYB* and pancreatic cancer in two cohorts from Norway and Germany (Paper I). This negative finding was later supported by the study from Shindo and colleagues (256). It has been suggested that *CEL-HYB* induces autophagy (54), which might promote pancreatic cancer development when associated with chronic pancreatitis and *KRAS* mutations (Reviewed in (245)). The carrier frequency of *CEL-HYB* is low (below 1% in tested populations) and examination of very large materials is therefore needed before a final conclusion can be drawn with regard to an association between this CNV and pancreatic cancer. Worth noting, it has been postulated that the *CEL-HYB* allele may be an ethnic-specific risk factor, as it is associated with CP risk in Caucasians but not present in Asian populations (54, 193, 195). Intriguingly, Asian individuals carry a *CEL-HYB* allele that is slightly different from that found in Europe (195), again illustrating the high genetic variability of the *CEL* locus.

In contrast to the lack of association between pancreatic cancer and *CEL* CNVs or VNTR length polymorphisms (Paper I), another study has suggested a correlation between early pancreatic carcinogenesis and the cytosine insertions within the *CEL* VNTR (138). In addition, a synonymous SNP within the second VNTR repeat of

CEL has been proposed to associate with pancreatic cancer risk, with a potential usage in pancreatic cancer diagnostics (201). However, this study was based on an extremely small cohort (n = 36, of these only 30 PDAC cases), and independent verification in larger material are needed before this SNP can be considered as a pancreatic cancer risk factor. A recent study by Tamura and colleagues has identified several rare missense mutations within *CEL* in a large cohort of patients diagnosed with pancreatic cancer (257). However, the frequency of these mutations did not reach statistical significance when compared to a control material, indicating that spurious germ line variants of *CEL* (at least not those in exons 1-10) do not associate with increased pancreatic cancer risk.

4.3 Endocytosis of CEL-MODY (Paper II+III)

From a previous study, we knew that CEL-MODY has a high tendency to form intra- and extracellular aggregates and that it can trigger a stress response in expressing cells (161). These properties would be an effect of the altered amino acid sequence of the truncated VNTR region present in the mutant protein. In this thesis, further studies on the effects of CEL-MODY in cellular models were conducted (Paper II and III).

4.3.1 **Aggregating properties of the CEL-MODY VNTR region**

The amino acid composition of the CEL-MODY VNTR has a theoretical p*I* of 11.8, compared to around 3 for the VNTR of the normal CEL protein (161). Thus, the altered VNTR is positively charged and has, because of fewer Ser and Thr residues, a reduced potential for being O-glycosylated (161). This post-translational modification has been suggested to protect the CEL enzyme from proteolytic degradation and self-association, by masking the PEST sequence and shielding hydrophobic areas located within the globular domain (160, 168). Hence, reduction in O-glycosylation may contribute to altering the physiochemical properties of CEL-MODY, thereby stimulating a tendency of the protein to accumulate and conferring cellular toxicity, as previously described (161, 190).

Additionally, the altered reading frame of the CEL-MODY VNTR introduces 10 additional cysteine residues that are not present in the VNTR of CEL-WT. These residues have been suggested to contribute to the formation of CEL-MODY aggregates by allowing multiple intra- and intermolecular disulfide bridges to form (190). Moreover, a unique 11 amino acid sequence (KEAQMPAVIRF) flanking the CEL-WT VNTR region, is absent in CEL-MODY. Although minor biological effects cannot be eliminated, we find it less likely that it is the lack of this sequence that results in the pathogenicity of CEL-MODY. In fact, cytosine insertions in the *CEL* VNTR, resulting in truncated CEL proteins lacking the unique KEAQMPAVIRF sequence, are commonly found in healthy individuals (106).

4.3.2 **How do endocytosed CEL-MODY cause cellular toxicity?**

In Paper II and III, we found CEL-MODY to be highly internalized by pancreatic and non-pancreatic cell lines, leading to decreased metabolic activity and induced caspase-dependent apoptosis in exposed cells. We know that intracellular accumulation of CEL-MODY may result in ER stress-mediated apoptosis by activating the PERK-eIF2α branch of the UPR, as increased levels of phosphorylated eIF2α (161) and elevated levels of *ATF4* and *CHOP* mRNA (190) have been documented in cells expressing CEL-MODY. However, PERK-mediated phosphorylation of eIF2α is a pathway of how intracellularly misfolded proteins induce ER stress and apoptosis (258), and it is not straightforward to explain why CEL-MODY may reduce cell viability after internalization of protein molecules from the outside (i.e. medium).

Vesicles of endocytosed CEL-MODY proteins partially co-localized with lysosomal-associated membrane proteins (LAMP1 and 2) in pancreatic and non-pancreatic cell lines (Paper II), suggesting that internalized CEL-MODY molecules are sent to the lysosomes for degradation. Since the viability of the exposed cells is negatively affected and not all CEL-MODY-positive vesicles co-localized with LAMP-positive vesicles, one might speculate that some free cytosolic CEL-MODY still could cause ER stress and trigger the PERK-eIF2α branch of the UPR, resulting in apoptosis (259) (Fig. 9). Alternatively (or in addition), the CEL-MODY molecules might negatively affect the lysosomes themselves. It has been proposed that lysosomal dysfunction is a central mechanism in pancreatitis pathology (259). Impaired autophagy, caused by defective function of the lysosomes, has been found to negatively affect the processing of cathepsins (lysosomal hydrolases) in acute pancreatitis (260). Deviating processing and activation of cathepsins can lead to increased levels of trypsin within the acinar cells, a characteristic of pancreatitis (260). Moreover, cathepsins-mediated degradation of LAMPs, which play vital roles in the function of the lysosomes, have also been linked to pancreatitis (261).

In fact, LAMP-2 deficiency has been shown to result in trypsinogen activation, acinar cell necrosis and inflammation (260, 261). Since LAMP deficiency and degradation have been associated with pancreatitis, it would be worthwhile to study LAMP co-localization further after long-time exposure to extracellular CEL-MODY, by analysing downstream cellular effects. This might elucidate why CEL-MODY triggers caspase-dependent apoptosis after endocytosis and unravel which pathways aggregated extracellular CEL-MODY can stimulate.

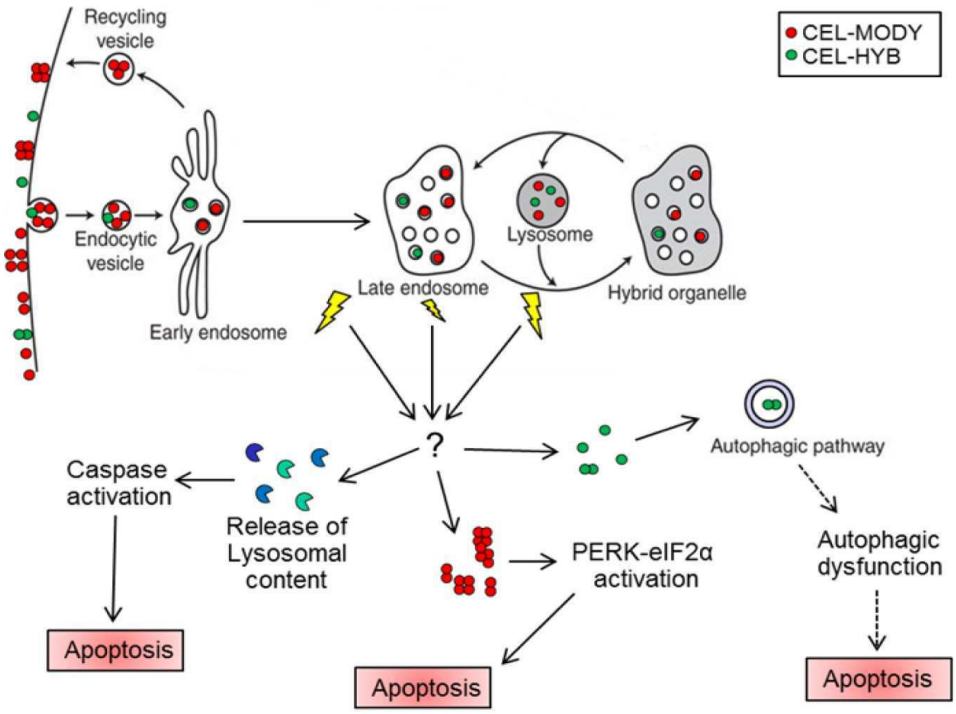

Figure 9 Internalized CEL-MODY and CEL-HYB proteins might trigger apoptosis through overlapping and distinct routes

A simplified illustration of the endocytic pathway that extracellular CEL-MODY and CEL-HYB aggregates might follow. A functional endocytic pathway will normally end with either recycling or lysosomal degradation of the endocytosed macromolecules. Disturbances of the lysosomal machinery by CEL-MODY or CEL-HYB may result in release of lysosomal contents and caspase-dependent apoptosis. Moreover, cytosolic CEL-MODY may trigger ER stress-mediated apoptosis by activation of the PERK-eIF2α branch of the UPR, whilst cytosolic CEL-HYB may cause autophagic dysfunction, by entering and exhausting the autophagic pathway. Based on (259).

4.4 Endocytosis of CEL-HYB (Paper III)

The hybrid CEL-CELP protein, designated CEL-HYB, predisposes for development of chronic pancreatitis (54). In functional testing, it displayed reduced lipolytic activity and impaired secretion, resulting in intracellular accumulation and autophagy induction. As for CEL-MODY, the pathogenic properties of CEL-HYB would be expected to result from its altered and truncated C-terminal domain.

4.4.1 **The VNTR of CEL-HYB**

The *CEL-HYB* allele most likely originated from non-allelic homologous recombination between *CEL* and its neighbouring pseudogene *CELP*, with a crossover event occurring in exon 10-exon 11 region of the *CEL* locus (54). This means that the CEL-HYB protein has a VNTR region that is derived from *CELP*. Thus, the VNTR of CEL-HYB consists of only three repeats (54) with a theoretical p*I* value of 4.8 (ExPASY p*I* calculator; https://web.expasy.org/compute_pi/) and contains two additional cysteine residues that are not present in the VNTR of CEL-WT (Fig. 3b in (54)). Moreover, it contains only three predicted O-glycosylation sites (NetOGlyc 4.0 server; http://www.cbs.dtu.dk/services/NetOGlyc/). In contrast, there are 36 predicted O-glycosylation sites present within the VNTR region of CEL-WT. Reduced O-glycosylation and the different amino acid composition may both contribute to the altered properties of the CEL-HYB VNTR region, thereby explaining why this variant is pathogenic. As for CEL-MODY, we find it less likely that it is the lack of the terminal KEAQMPAVIRF sequence that is the reason why CEL-HYB is a risk factor for pancreatic disease.

4.4.2 **How do endocytosed CEL-HYB cause cellular toxicity?**

When studying endocytosis in a pancreatic acinar cell line model, we found CEL-HYB to be taken up to a similar extent as CEL-WT although it had a significantly more negative impact on cellular viability (Paper III). Conducting similar experiments in a pancreatic ductal cell line model, we observed the same low level of uptake, however, the viability of the ductal cells were not significantly affected by

CEL-HYB when compared to CEL-WT (Paper III). It would therefore be of interest to investigate which intracellular pathways are activated in the presence of endocytosed CEL-HYB. For instance, Fjeld et al. reported that intracellular CEL-HYB retention could induce autophagy, as CEL-HYB was found to increase the level of LC3-II, an autophagy marker (54). Similar to CEL-MODY, internalized CEL-HYB could interfere with aspects of the endocytic pathway, resulting in release of lysosomal content and caspase-dependent apoptosis (259) (Fig. 9). Alternatively, free cytosolic CEL-HYB could enter the autophagy pathway and cause autophagic dysfunction (Fig. 9), which also has been implicated in the disease mechanism of pancreatitis (262).

4.5 Is CEL more than a digestive enzyme?

The VNTR region of CEL does not seem to be essential for catalytic activity. Lower vertebrates, like fish, do not contain the C-terminal sequence repeats in their CEL proteins, whereas higher vertebrates, have expanded the VNTR region along with evolution (155). Why mammals, in particular primates, evolved a mucin-like domain in CEL, is a matter of speculation. As mentioned in the Introduction, one role could relate to the solubility and stability of the enzyme. Another possibility is that CEL contributes to the gastrointestinal mucosal barrier. Intestinal mucins, which are complex glycoproteins, prevent infections by the microbiota by creating a mucosal barrier within the gastrointestinal tract (263). Through its VNTR region, CEL might participate in the interplay between the microbiota and the mucus layer (264). Intriguingly, the VNTR region can be modified by ABO blood group determinants (166), which are known to modulate gastrointestinal microbiota composition (265), lending further support to a role of CEL in the mucosal barrier.

In addition to our data on internalization by pancreatic and non-pancreatic cell lines (Paper II and III), there is evidence that secreted CEL can be endocytosed *in vivo* by enterocytes in the small intestine and released into the blood stream (174, 179, 181). These and observations related to lipoprotein metabolism (150) and atherosclerosis (127) strongly suggest that CEL may have functions outside the gastrointestinal tract. Interestingly, vascular CEL have been suggested to influence inflammatory conditions, as CEL knock-out mice were reported to be less prone to develop arthritis when compared with wild type control mice (266). Moreover, CEL have been found to inhibit transfer of HIV-1 to T lymphocytes (267) and suggested to play a role in thrombosis formation (268). Taken together, these results may implicate a role for CEL in modulating inflammatory and immunological processes.

5. Concluding remarks

In Paper I, we did not obtain evidence that linked *CEL* CNVs or VNTR length polymorphisms to increased risk for pancreatic cancer. *CEL-HYB* has been suggested to be an ethnic-specific risk factor for chronic pancreatitis, present mainly in Caucasians. Screening of cohorts of non-Caucasian origin could have yielded different results. Additionally, we expect that novel CNVs and other variants of *CEL* remain to be identified due to the highly polymorphic nature of this gene. It is therefore too early to exclude a role for *CEL* as a genetic risk factor in pancreatic cancer.

In Paper II, we concluded that both CEL-MODY and CEL-WT are likely to follow a classical secretory pathway, although CEL-MODY displayed altered intracellular distribution compared to normal CEL. Due to its different VNTR region, the CEL-MODY protein has a high propensity to form intra- and extracellular aggregates. We found that extracellular CEL-MODY was endocytosed by pancreatic acinar and beta-cell lines as well as by non-pancreatic cell lines to a higher extent than was CEL-WT. The differences most likely reflect the altered physiochemical properties of the VNTR region of CEL-MODY. Aggregation of this protein variant may arise because of less O-glycosylation, a high number of new cysteine residues, and/or charge differences that render CEL-MODY more capable of precipitating at negatively charged cell surfaces. Moreover, endocytosis of CEL-MODY was followed by degradation in the lysosomes and lead to reduced cell viability. These observations may be important for understanding how the pathogenic CEL-MODY protein can cause a syndrome affecting both pancreatic exocrine and endocrine tissue. We speculate that reuptake of this CEL variant by non-expressing cells may play an important role in MODY8 pathogenesis.

In Paper III, we expanded our functional studies both on the cellular side (by including a pancreatic ductal-like cell line) and on the protein side (by including the CEL-HYB variant). We found that CEL-MODY significantly reduced the viability of ductal cells; however, the effect was not as strong as observed in the other studied

cell lines. Moreover, the pathogenic CEL-HYB protein could be endocytosed by pancreatic acinar and ductal cell lines, although there was a less severe effect on cell viability than what was observed for the CEL-MODY variant. Both CEL-MODY and CEL-HYB appeared to affect the intracellular fate of CEL-WT, whereas their toxicity after endocytosis became reduced in the presence of CEL-WT.

The work presented in this thesis highlights the exceedingly polymorphic nature of *CEL* and illustrates how challenging it is to capture the full range of genetic variation of the human *CEL-CELP* locus. Our results indicate that it is the amino acid composition and physiochemical properties of the VNTR region, rather than its length, that confer a risk for pancreatic disease. Taken together, our findings suggest a model where both CEL-HYB and CEL-MODY have negative effects on cellular function, but where CEL-MODY is clearly the most severe of the two. This is in accordance with the observation that the *CEL-HYB* allele is a risk factor for chronic pancreatitis with intermediate effect, whereas *CEL-MODY* is a highly penetrant allele that causes the Mendelian disease MODY8. Moreover, Paper II and III may implicate proteotoxicity due to intracellular aggregation and accumulation as pivotal for initial disease development and as such a common pathogenicity mechanism for both CEL-MODY and CEL-HYB. For disease progression, endocytosis might be another common theme, although the cellular pathways involved may not necessarily be identical for CEL-MODY and CEL-HYB.

6. Future perspectives

***CEL* CNVs – Influencing susceptibility for pancreatic disease?**

More efforts are needed to explore which role *CEL* CNVs might have in pancreatic disease. In fact, our research group has recently discovered a novel *CEL* variant that contains two intact copies of *CEL* on the same allele (246). Thus, subjects heterozygous for this duplication allele exhibit an increased *CEL* gene dosage by carrying altogether three functional copies. Whether this constellation results in higher expression levels of CEL protein in the acinar cells, remains to be investigated. So far, no clear conclusion has been reached with regard to whether the new duplication allele is a risk factor for pancreatitis (246). It should be noted that some genetic variants also serve as protective factors in pancreatitis (269) and any new *CEL* CNVs should be tested with this option in mind.

On the clinical side, it will be interesting to thoroughly characterize those carriers of *CEL-HYB* that develop pancreatitis to see whether they share common phenotypic features. Such information might eventually benefit the diagnostics and follow-up of subjects with idiopathic, early-onset or familial chronic pancreatitis, which are those patients where an underlying genetic predisposition most often is found (270). Increased knowledge about the clinical phenotype associated with *CEL-HYB* and other genetic risk factors for chronic pancreatitis might subsequently pave the way for personalized medicine in this disorder.

Improved models for studying CEL-induced pancreatic disease

Patients with CEL-MODY or CEL-HYB-associated chronic pancreatitis are heterozygous carriers of the pathogenic variants (54, 106). In this thesis, we have provided some evidence that the pathogenic variants may influence the properties of CEL-WT and vice versa. Co-expression studies could provide a more realistic view on CEL disease pathogenesis and is therefore an approach that should continue.

When it comes to cellular models, we propose the usage of more representative model systems. The next step should therefore be to test how human tissue cultures like pancreatic organoids, primary acinar cells and islets of Langerhans would respond to pathogenic CEL variants. Also co-cultivation studies of macrophages or pancreatic stellate cells along with CEL-expressing acinar cells could be of interest, as both these cell-types have been associated with pancreatitis by triggering pancreatic fibrosis development (271).

Finally, mice models for the pathogenic CEL variants would represent a significant step forward. Several animal models for chronic pancreatitis are already available, but they involve disruption of the protease-antiprotease system within the pancreatic acinar cells or integrate effects of alcohol and other pathophysiological stimuli on the pancreas (272). Mice completely lacking the *Cel* gene do not display a pancreatic phenotype (189) and the first attempt to establish a mouse model for CEL-MODY was unsuccessful (273). A novel approach for making animal models for CEL-induced pancreatitis therefore needs to be taken. One option is the establishment of humanized knock-in mice models, where the endogenous mouse *Cel* VNTR has been exchanged with the VNTR sequence of CEL-MODY or CEL-HYB. Such mice are currently under construction in our group and might, if successful, serve as an intriguing tool to study the disease mechanisms of chronic pancreatitis and diabetes induced by an aggregating lipase.

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