

# **GENETICS OF ENDOCRINE AND EXOCRINE DISEASES OF THE PANCREAS**

Ph.D. Thesis

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## ABBREVIATIONS

|                |   |
|----------------|---|
| cDNA           | complementary DNA   |
| CEL            | carboxyl ester lipase   |
| <i>CLPS</i>    | <i>procolipase gene</i>   |
| CTRB2          | human chymotrypsinogen B2 protein                                 |
| CTRC           | human chymotrypsinogen C protein                                  |
| Ctrb           | mouse chymotrypsinogen B protein                                  |
| Ctrc           | mouse chymotrypsinogen C protein                                  |
| <i>DEFA1</i>   | human $\alpha$ 1-defensin gene                                    |
| <i>DEFA3</i>   | human $\alpha$ 3-defensin gene                                    |
| <i>DEFB1</i>   | human $\beta$ 1-defensin gene                                     |
| DM             | diabetes mellitus   |
| DM1            | type 1 diabetes mellitus, insulin-dependent diabetes mellitus     |
| DM2            | type 2 diabetes mellitus, non-insulin dependent diabetes mellitus |
| DNA            | deoxyribonucleic acid   |
| EDTA           | ethylenediaminetetraacetic acid                                   |
| ELISA          | enzyme-linked immunosorbent assay                                 |
| <i>GAPDH</i>   | glyceraldehyde-3-phosphate dehydrogenase gene                     |
| HBD1           | human $\beta$ 1-defensin protein                                  |
| HLA            | human leukocyte antigen   |
| HNP            | human neutrophil peptide  |
| Hu1            | human cationic trypsinogen protein                                |
| IFN $\gamma$   | interferon gamma  |
| IL-1 $\beta$   | interleukin-1 $\beta$   |
| IL-6           | interleukin-6   |
| LDL            | low density lipoprotein   |
| MODY           | maturity onset diabetes of the young, monogenic diabetes          |
| <i>MPO</i>     | myeloperoxidase gene  |
| mRNA           | messenger ribonucleic acid  |
| NCBI           | National Center for Biotechnology Information, USA                |
| NF- $\kappa$ B | nuclear factor- $\kappa$ B  |
| OR             | odds ratio  |
| PAGE           | polyacrylamide gel electrophoresis                                |
| PCR            | polymerase chain reaction   |
| PMN            | polymorphonuclear immune cells                                    |
| <i>PRSS1</i>   | human cationic trypsinogen gene                                   |
| PVDF           | polyvinylidene fluoride   |
| qPCR           | quantitative (real time) polymerase chain reaction                |
| RNA            | ribonucleic acid  |
| RT PCR         | reverse transcription polymerase chain reaction                   |
| SDS            | sodium dodecyl sulfate  |
| SNP            | single nucleotide polymorphism                                    |
| TLR4           | Toll-like receptor 4  |
| TNF- $\alpha$  | tumor necrosis factor alpha                                       |

## **1. INTRODUCTION**

Disorders of the pancreas, both endocrine (e.g. diabetes mellitus) and exocrine (e.g. chronic pancreatitis or cystic fibrosis), can be triggered by different genetic factors. There exist specific mutations that can lead to the development of certain pancreatic diseases directly. A little more than half a century ago, mankind already had a significant knowledge on these disorders clinically, however at that time not much was known about their genetic background.

### **1.1. GENETIC RISK FACTORS OF DIABETES**

Based on the definitions provided by the American Diabetes Association, diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Type 1 diabetes is caused by  $\beta$ -cell destruction in the Langerhans islets of the pancreas, usually leading to absolute insulin deficiency. Characteristics of type 2 diabetes range from predominant insulin resistance with relative insulin deficiency to predominant insulin secretory defect with insulin resistance. The long term complications affect several organs including the eyes, kidneys, nerves and blood vessels, making diabetes a lifelong disease which predisposes to secondary malfunction of almost all organs in the whole body, primarily due to the metabolic changes characteristic of diabetes.

#### **1.1.1. Genetic risk factors of type 1 diabetes mellitus (DM1)**

Type 1 diabetes mellitus (DM1, OMIM: 222100) is a genetically heterogeneous autoimmune disease of glucose homeostasis that is characterized by susceptibility to ketoacidosis in the absence of insulin therapy.  $\beta$ -cells of the pancreas suffer autoimmune destruction in which several autoantibodies can have a pathogenic role against islet cells.

Based on functional, structural and genetic evidence, haplotypes DR3 and DR4 of the human leukocyte antigene (HLA) class II loci are considered strong triggering factors, while DR2 haplotype protects from DM1. Certain alleles of other genes which are involved in disease pathogenesis confer relatively low risk for development of DM1 compared to HLA class II haplotypes.

Since DM1 is known to be principally an autoimmune disease, genetic factors which influence the physiology of the immune system might have a significant role in the pathophysiology.

#### **1.1.2. Genetic risk factors of type 2 diabetes mellitus (DM2)**

Type 2 diabetes mellitus (DM2, OMIM: 125853) accounts for 90–95% of those with diabetes. This type encompasses individuals who have insulin resistance and usually have relative insulin deficiency. Although many different etiological factors can be responsible for the development of DM2, the specific causes are not known. The innate immune system is the first line of defense against microbial, fungal and viral infections. Tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6) are released when toll-like receptor 4 (TLR4) binds certain danger- or pathogen-associated molecules. These molecules can directly increase insulin resistance in fat, muscle and liver cells, which can lead to impaired glucose tolerance, the major characteristic of DM2.

## 1.2. DEFENSINS IN DIABETES

Defensins are small antimicrobial peptides of the innate immune system, which have 3 major types in vertebrates:  $\alpha$ -,  $\beta$ - and  $\theta$ -defensins. In humans only  $\alpha$ - and  $\beta$ -defensins are expressed, while the genes encoding human  $\theta$ -defensins are pseudogenes.

### 1.2.1. $\alpha$ - and $\beta$ -defensins

The human chromosome locus 8p23.1 harbors 6 different  $\alpha$ -defensin genes (*DEFA1*, *DEFA3*, *DEFA1B*, *DEFA4*, *DEFA5*, *DEFA6*) and 5  $\alpha$ -defensinpseudogenes. There are 6 different types of  $\alpha$ -defensins / human neutrophil peptides (HNP) expressed in humans. The protein product of *DEFA1*, *DEFA3*, *DEFA4* genes are HNP-1,-2,-3 and -4 derived from polymorphonuclear immune cells, while intestinal human defensins (HD-5 and HD-6) are derived from Paneth cells and are the protein products of *DEFA5* and *DEFA6* genes. The cluster of human  $\alpha$ -defensin genes include *DEFA1* (OMIM: 125220) and *DEFA3* (OMIM: 604522) lying on chromosome 8 with high variability of copy numbers.

Today, these peptides are characterized as danger signals (alarmins) that play important roles in inflammation and immunity. In addition to antimicrobial effects,  $\alpha$ -defensins have an important role in chemotaxis and induce proinflammatory cytokines. HNPs increase LDL (low density lipoprotein cholesterol) binding to the endothelial surface suggesting that  $\alpha$ -defensins may modulate the development of atherosclerosis. Neutrophil granulocytes are considered to be the primary cellular origin of HNP 1-3.

Human  $\beta$ -defensins are also antimicrobial peptides, which are thought to control the microbial flora on epithelial surfaces. According to currently accessible data from the HUGO Gene Nomenclature Committee (<http://www.genenames.org/>), 38 different genes were identified encoding potentially expressed  $\beta$ -defensins in humans, while 9 genes are referred to as  $\beta$ -defensinpseudogenes.

Several SNPs have been characterized in the *DEFB1* gene. Three frequent SNPs at positions c.-20G>A (rs11362), c.-44C>G (rs 1800972) and c.-52G>A (rs1799946) in the 5'-untranslated region (5' UTR) of *DEFB1* were described earlier. The untranslated variants influence HBD1 expression or function.

### 1.2.2. Role of defensins in diabetes mellitus

Although it has been shown that some polymorphisms in certain cytokine genes are associated either with DM1 or DM2, little is known about the genetics and the functions of  $\alpha$ - and  $\beta$ -defensins in diabetes. Infections are frequent in diabetic patients, because the antimicrobial function of their immune response is impaired. It has been reported that mRNA levels of rat  $\beta$ 1-defensin are decreased in the kidneys, which may explain the high incidence of urinary tract infections in diabetes mellitus. Recently, the effect of glucose and insulin on  $\beta$ -defensin expression has been described. However, no connection was found between genetic polymorphisms of the *DEFB1* gene and diabetes in a Brazilian study investigating diabetic children.

Increased levels of HNP1-3 in type 1 diabetic patients with nephropathy and with cardiovascular complications have recently been reported. It is tempting to speculate that copy number polymorphisms and alterations of *DEFA1/DEFA3* mRNA levels in granulocytes may influence the levels of HNP1-3 in patients with diabetes type 1 and type 2.

### **1.3. CONNECTIONS BETWEEN ENDOCRINE AND EXOCRINE PANCREATIC DISEASES AT THE GENOMIC LEVEL**

Diabetes is a multifactorial disease, with both environmental and genetic factors playing a role in its development. Mutations in only a single gene can also lead to the development of diabetes. Monogenic diabetes, also termed maturity onset diabetes of the young (MODY), develops before the age of 25, and presents as a nonketotic form of diabetes. Its inheritance follows an autosomal dominant pattern, and mutations associated with MODY cause a primary defect in the function of the beta cells of the endocrine pancreas. Exocrine pancreas function is not affected in the majority of the 11 different types of MODY. However, in MODY8 (CEL MODY), in which there is a defect in the carboxyl ester lipase (*CEL*, OMIM: 114840) gene, insufficiency of both endocrine and exocrine pancreatic function is observed.

Another example where a single malfunctioning gene may lead to both endocrine and exocrine insufficiency of the pancreas is the procolipase gene (*CLPS*, OMIM: 120105). The products of *CLPS* gene are the precursors of colipase and enterostatin. They are secreted mainly by the exocrine pancreas, but are also expressed in stomach, liver and regions of the central nervous system. The mature form of colipase is the essential cofactor of lipase, which has a significant role in the digestion of triglycerides emulsified by bile salts. Enterostatin acts as a pentapeptide hormone, which selectively down-regulates fat intake and hampers insulin secretion in animal models. Certain SNPs of the *CLPS* gene are associated with altered insulin secretion in non-diabetic Caucasians. Furthermore, *CLPS* gene mutant p.R109C was found to be associated with DM2. In a functional study, the same mutant was found to be defective in its ability to anchor triglycerides to pancreatic lipase.

The simultaneous appearance of diabetes mellitus and exocrine pancreas insufficiency is surprisingly common. In most cases they do not develop together due to a single genetic disorder as described above in the case of *CEL* MODY. Diabetes mellitus stemming from pancreatic tissue damage (pancreatitis, trauma, pancreatectomy, neoplasia, cystic fibrosis, hemochromatosis, fibrocalculouspancreatopathy or surgical pancreas resection) is classified as type 3c.

Though endocrine and exocrine malfunction of the pancreas usually have different genetic risk factors, they might have a common genetic origin due to certain mutations in *CLPS* and *CEL* genes mentioned above. Living with chronic pancreatitis leads to the development of endocrine pancreas dysfunction over time. Therefore, investigation of the mechanisms leading to chronic pancreatitis may not only result in a deeper understanding of the function of the exocrine pancreas and related diseases, but may also elucidate the mechanism of disease pathogenesis in certain types of diabetes.

### **1.4. HEREDITARY CHRONIC PANCREATITIS**

Chronic pancreatitis (CP) is defined as a relapsing or continuing inflammatory disease of the pancreas and is characterized by irreversible morphological changes, upper abdominal pain and, in some patients, permanent impairment of exocrine or endocrine function or both.

The first description of familial accumulation of chronic pancreatitis cases was provided in 1952 by Comfort and Steinberg. However, it took 44 years before the first gene associated with hereditary pancreatitis was identified by Whitcomb et al. in 1996.

Mutations in the *PRSSI* gene associated with hereditary chronic pancreatitis result in early activation of trypsinogen inside the pancreas due to altered regulation by chymotrypsin C (CTRC). High penetrance trypsinogen mutations such as p.N29I and p.R122H are associated with an autosomal dominant inheritance pattern, whereas mutations with lower penetrance (e.g. mutation p.A16V) may be found not only in hereditary but also in sporadic cases without family history.

### **1.5. MOUSE TRYPSINOGENS**

Considering the widespread use of mice in experimental studies of the pancreas, it is surprising how little is known about mouse trypsinogens. Using the currently accessible NCBI online genomic database (<http://www.pubmed.org/>) we found that eleven of the 20 genes are potentially functional (T4, T5, T7, T8, T9, T10, T11, T12, T15, T16, T20), while the other nine genes are either pseudogenes (T1, T2, T3, T14) or relic genes (T6, T13, T17, T18, T19). It remained unknown, however, which isoforms of the 11 potentially functional trypsinogen genes are expressed at the protein level in the mouse pancreas. More recently, genetic deletion of T7 indicated that this isoform may contribute to as much as 60% of pancreatic trypsinogens. It was also found that despite the presence of other trypsinogen isoforms, mice deficient in T7 did not respond to secretagogue hyperstimulation with the characteristic intra-acinar cell trypsinogen activation, which is an early event in acute pancreatitis.

## 2. AIMS

Like other members of the innate immune system, defensins can also have a role in the development of type 1 and type 2 diabetes mellitus and their macro- and microvascular complications. In order to gain deeper comprehension about the role of defensins in diabetes, the aims of this study were:

- to measure plasma levels of human neutrophil peptides 1-3 (HNP 1-3) and to examine their possible association with diabetes and / or its complications,
- to determine gene copy number polymorphism and mRNA expression levels of *DEFA1/DEFA3* genes in diabetes mellitus,
- to investigate the association of single nucleotide polymorphisms c.-20G>A, c.-44C>G and c.-52G>A in the promoter region of the  $\beta$ 1-defensin (*DEFB1*) gene with type 1 and type 2 diabetes mellitus.

Research into the pathomechanism of hereditary chronic pancreatitis has been hindered by the lack of good animal models which mimic the human disease and develop spontaneous pancreatitis. In order to create a mouse model, biochemical characterization of mouse trypsinogens was necessary. The aims of the study of mouse trypsinogens were:

- to identify the major trypsinogen isoforms expressed in the mouse pancreas,
- to characterize the autoactivation of mouse trypsinogens and study their interaction with mouse chymotrypsins,
- to investigate the biochemical characteristics of the most frequent pathogenic human cationic trypsinogen mutation p.R122H introduced into mouse trypsinogen isoforms T7 and T8.

### 3. PATIENTS AND METHODS

#### 3.1. PATIENTS AND CONTROLS IN GENETIC ASSOCIATION STUDY

All cases and controls were of Hungarian ethnic origin and were residents of Hungary. Informed consent was obtained from all subjects, and the local Ethics Committee of University of Szeged gave prior approval to the study. All subjects consented to the study and were treated according to the Patient Right Protection Act of our institutions and international guidelines.

The 221 age- and gender matched members of the control group were selected from healthy blood donors from the regional Centre of Hungarian National Blood Transfusion Service(Szeged, Hungary). Blood donors with diabetes mellitus, nephropathy, hypertension or ischemic heart disease were excluded from this study.

257 diabetic patients (122 men, 135 women) were enrolled in the study; which included 117 patients with type 1 and 140 patients with type 2 diabetes. Diagnosis of diabetes of all patients was based on the ADA criteria.

71 of the cohort had diabetic nephropathy (32 DM1 and 39 DM2), 115 patients suffered from retinopathy (47 DM1, 68 DM2). Neuropathy was diagnosed in 95 patients (35 DM1, 60 DM2).54 diabetic patients (14 DM1, 40 DM2) had previously been diagnosed with different macrovascular diseases. High number of patients (182) had treatedhypertension (50 DM1 and 132 DM2).

#### 3.2. METHODS USED IN GENETIC ASSOCIATION STUDY

To extract genomic DNA and mRNA from leukocytes and to determine  $\alpha$ -defensin levels we used anticoagulated (EDTA), centrifuged (1200 rpm/min for 15 min), peripheral blood.

We determined *DEFA1/DEFA3* gene copy number using quantitative real-time PCR according to the method used by Linzmeier *et al.* In our experiments we chose *MPO* (myeloperoxidase gene) as reference gene. Expression level of *DEFA1/DEFA3* mRNA was measured by reverse transcriptase quantitative real-time PCR. As a reference gene we used *GAPDH* in this experiment. Relative gene expression was determined using the  $\Delta\Delta C_t$  method. Genotyping of *DEFB1* SNPs (c.-20G>A , c.-44C>G and c.-52G>A) were performed using Custom TaqMan<sup>®</sup> SNP Genotyping Assays. The level of significance of the genotype frequency of different *DEFB1* SNPs was analysed by using the  $\chi^2$  test and Fischer test.

The same peripheral blood samples we used for genomic DNA and RNA extraction from patients and controls were also used for isolation of plasma, which was stored at -80°C until further analysis. Plasma HNP1-3 concentrations were determined by ELISA according to the instructions of the manufacturer. Comparisons of plasma concentrations were carried out by Mann-Whitney test and with two-tailed paired Student's test.

#### 3.3. EXPERIMENTAL PROCEDURES USED IN MOUSE TRYPSINOGEN EXPERIMENTS

Mouse trypsinogens were isolated from homogenized mouse pancreata (2-3) using ecotin affinity chromatography and cationic exchange chromatography. The eluted proteins were separated by SDS-PAGE,

transferred to PVDF membrane and individual bands were subjected to N-terminal protein sequencing by Edman degradation.

For experimental use, human cationic trypsinogen was expressed in the aminopeptidase P deficient LG-3 *E. coli* strain as fusions with a self-splicing mini-intein. Mouse trypsinogens were expressed in *E. coli* BL21(DE3), as described for human trypsinogens previously. Isolation of cytoplasmic inclusion bodies, *in vitro* refolding and purification with ecotin affinity chromatography were performed. Human CTRB2, mouse Ctrb and mouse Ctrc carrying 10His affinity tags were expressed in transiently transfected HEK 293T cells using Lipofectamine® 2000 and purified from 450 mL conditioned medium using nickel-affinity chromatography. Chymotrypsinogens were activated with trypsin and active chymotrypsin concentrations were determined by active site titration with ecotin.

Trypsinogen at 2  $\mu$ M concentration was incubated in the absence or presence of 25 nM chymotrypsin. Autoactivation was induced by 10 nM trypsin at 37 °C. At given times aliquots were withdrawn and mixed with assay buffer. Trypsin activity was measured using N-CBZ-Gly-Pro-Arg-p-nitroanilide substrate dissolved in assay buffer and following the release of the yellow p-nitroaniline at 405 nm for 1 min. Complete activation of 2  $\mu$ M trypsinogen was carried out using 140 ng/mL final concentration of human enteropeptidase.

To investigate structural changes of trypsinogens during autoactivation and degradation by chymotrypsins, aliquots were withdrawn from the reaction mixture at certain time points, protein fragments were precipitated and were separated by SDS-PAGE, transferred to PVDF membrane and individual bands were subjected to N-terminal protein sequencing by Edman degradation.

## 4. RESULTS

### 4.1. RESULTS OF HUMAN GENETIC INVESTIGATIONS

#### 4.1.1. $\alpha$ -defensins (HNP 1-3)

There was high individual variation in plasma levels of  $\alpha$ -defensin, but a significant difference between healthy subjects and both groups of diabetic patients was observed. The mean value  $\pm$  S.E.M. was  $28.78 \pm 4.2$  ng/mL in type 1 diabetes, and  $29.82 \pm 5.36$  ng/mL in type 2 diabetes, versus  $11.94 \pm 2.96$  ng/mL in controls;  $p < 0.01$  respectively. The highest concentrations of  $\alpha$ -defensin levels were found in diabetic patients with nephropathy ( $49.4 \pm 4.8$  ng/mL), and with neuropathy ( $38.7 \pm 4.8$  ng/mL) or with cardiovascular complications ( $45.6 \pm 1.45$  ng/mL) which were significantly higher in comparison with HNP1-3 plasma levels of diabetic patients without complications ( $25.4 \pm 3.5$  ng/mL).

There was no significant difference in *DEFA1/DEFA3* copy number between controls and patients or between patients in the two types of diabetes groups. No positive correlation was observed between the copy numbers and the expression levels of *DEFA1 / DEFA3* gene and also between the copy numbers and plasma levels of human neutrophil peptide 1-3.

#### 4.1.2. $\beta$ 1-defensin (*DEFB1*)

Investigating the *DEFB1* polymorphisms c.-20G>A és c.-52G>A, there was no significant difference in genotype distribution between the patients overall and the healthy controls. However, the frequency of GG genotype of c.-44C>G was significantly lower in both types of diabetes (2.5% and 2%, respectively) than in healthy controls (9%). Conversely, the prevalence of the CC genotype was 61% in the group of diabetic patients vs. 45 % of controls (Fisher test:  $p = 0.02$ , OR = 2.055, 95% CI: 1.27 – 3.745). When the patients were sorted according to their diabetic complications, lower frequency of GG genotype among the patients with nephropathy and among the patients with neuropathy was found.

### 4.2. RESULTS OF BIOCHEMICAL CHARACTERISATION OF MOUSE TRYPSINOGENS

Mice expressed 4 trypsinogen isoforms at high levels (T7, T8, T9 and T20), which were identified from homogenized whole mouse pancreata using N-terminal sequencing and mass spectrometry.

When recombinant mouse trypsinogens were incubated in 1 mM  $\text{CaCl}_2$  at pH 8.0, isoforms T7, T8 and T9 autoactivated and reached about 40-60% of potentially attainable trypsin levels. According to our experiments the T7 isoform autoactivated at the highest rate and reached the highest trypsin levels during autoactivation. In contrast, T20 did not autoactivate under these conditions. Slow cleavage of the Leu149-Ser150 peptide bond by mouse chymotrypsin C (Ctrc) in the autolysis loop was observed in T7 isoform in the absence of calcium. The Leu82-Glu83 peptide bond (corresponding to Leu81-Glu82 in human trypsinogens and other studied mouse isoforms) in the calcium binding loop was not cleaved to a detectable extent.

Mouse Ctrc almost completely inhibited autoactivation of T8 and T9 trypsinogen in 1 mM calcium and markedly reduced it in 10 mM calcium. Both isoforms were primarily cleaved at the Phe150-Gly151 peptide bond, with minimal cleavage observed at the Leu81-Glu82 peptide bond in the absence of calcium. In degradation experiments, mouse Ctrb cleaved the Phe150-Gly151 peptide bond at a more than 7-fold slower rate

than mouse Ctrc. Mouse Ctrc had essentially no effect on trypsinogen T20, except for a slight stimulation of the activation rate in 10 mM calcium.

The majority of mammalian trypsinogens do not contain Phe150 in their autolysis loop. To test whether introduction of Phe150 would reconstitute the chymotrypsin-dependent autoactivation inhibition in another mammalian trypsinogen, we mutated Ser150 in human cationic trypsinogen to Phe. Human CTRB2 at 25 nM concentration had no effect on the autoactivation of wild type cationic trypsinogen, whereas it inhibited activation of the p.S150F mutant, via cleavage at the Phe150-Gly151 peptide bond.

Mutation p.R123H protected T7 trypsinogen against degradation during autoactivation in 1 mM calcium in the presence of Ctrc; however, it had no effect on the cleavage of the Leu149-Ser150 peptide bond *per se*. Mutation p.R122H slightly stimulated autoactivation of T8 trypsinogen in 1 mM calcium, in the absence of Ctrc. In the presence of Ctrc, however, autoactivation was strongly inhibited by Ctrc, approximately to the same extent as seen with wild type T8. Consistent with the robust inhibitory effect, the Phe150-Gly151 peptide bond was cleaved by Ctrc almost as well in T8 p.R122H trypsinogen as in wild type T8, indicating that mutation p.R122H does not influence this regulatory mechanism.

## 5. DISCUSSION

### 5.1. RELEVANCE OF A-AND B-DEFENSINS IN DIABETES MELLITUS

In this work, HNP1-3 levels from the plasma of venous blood were measured. We investigated copy number variation and mRNA expression levels of *DEFA1/DEFA3* genes of the same individual. Intriguingly, our results showed significantly higher HNP 1-3 plasma levels in patients with both type 1 and type 2 diabetes mellitus compared to healthy controls. Moreover, the plasma of diabetic patients with nephropathic and neuropathic complications showed the highest concentrations of HNPs. It seems possible that the elevation of HNP1-3 in the plasma of patients with nephropathy is the consequence of the decreased renal degradation of the peptides together with advanced nephropathy. The cause of high levels of HNP1-3 in patients with neuropathy in type 1 and type 2 diabetes remains unknown.

The G allele of the c.-44C>G SNP generates a putative binding site for nuclear factor- $\kappa$ B (NF- $\kappa$ B), and induces overexpression of *DEFB1* gene. The present study has demonstrated that the distribution of *DEFB1* c.-44C>G genotypes were different between patients with diabetes and healthy controls. Our results showed that the frequency of the GG genotype was significantly higher in the control population. The presence of G allele might lead to strengthened HBD1 antimicrobial activity, which is less frequent among patients with diabetes. Conversely, in these studies subjects carrying the CC genotype were at a greater risk of acquiring infection.

Our present findings draw attention to the importance of *DEFB1* polymorphisms in diabetes, especially in case of nephropathy and neuropathy; the GG genotype could also be protective in diabetes, and the presence of CC genotype might be connected with lower expression of human  $\beta$ 1-defensin. It is noteworthy that insulin is also an important factor mediating HBD1 expression.

### 5.2. CTRC REGULATES AUTOACTIVATION OF MOUSE TRYPSINOGENS VIA CLEAVAGE OF THE AUTOLYSIS LOOP

In the past decade there were several attempts to create mouse models that develop chronic pancreatitis due to different genetic manipulations. However, none of these models attempted to manipulate genes of digestive enzymes. Human cationic trypsinogen (*PRSSI*), chymotrypsinogen C (*CTRC*) and carboxypeptidase A1 (*CPA1*) are known to have a role in the development of pancreatitis according to described plethora of biochemical and clinical studies. However, animal models that recapitulate the characteristics of human hereditary pancreatitis are still lacking.

We wanted to clarify whether introduction of trypsinogen mutations associated with human hereditary pancreatitis into mouse trypsinogens would offer a viable approach to model hereditary pancreatitis in mice. Therefore, identification of the major trypsinogen isoforms expressed by the mouse pancreas and characterization of their regulation of autoactivation by mouse *Ctrc* was necessary.

Investigating the commonly used outbred mouse strain CD-1, we found that only four trypsinogen isoforms, T7, T8, T9 and T20, are expressed to high levels in the mouse pancreas. Autoactivation of mouse trypsinogens T7, T8 and T9 was comparable, whereas T20 autoactivated more slowly, particularly in 1 mM calcium. Surprisingly, regulation of autoactivation by mouse *Ctrc* was isoform specific and mechanistically different from the actions of human *CTRC* on human cationic trypsinogen. Cleavage of the Leu81-Glu82 peptide bond in the calcium binding loop was detectable in T7 but was inefficient and resulted in minimal degradation,

when compared to the effect of human CTRC on human cationic trypsinogen. Slow cleavage of the Leu81-Glu82 peptide bond was also seen in T8 and T9, whereas isoform T20 was not cleaved by Ctrc.

In contrast, isoforms T8 and T9 were rapidly cleaved at the Phe150-Gly151 peptide bond in the autolysis loop and this cleavage resulted in marked inhibition of autoactivation without degradation. The Phe150-Gly151 peptide bond was also cleaved by Ctrb at a 7-fold slower rate. However, considering that Ctrb is the most abundant chymotrypsin in the mouse pancreas, physiological regulation of activation of T8 and T9 may be also dependent on Ctrb. Interestingly, we found that cleavage of the Phe150-Gly151 peptide bond in T8 and T9 trypsinogens also inhibits activation by enteropeptidase, the physiological trypsinogen activator in the duodenum.

Our data argue that the previously published mouse model in which a T8 transgene carrying the p.R122H mutation was introduced could not have developed the described phenotypic changes as a result of a mutation-dependent increase in trypsinogen activation. We found that mutation p.R122H did not affect inhibition of autoactivation by Ctrc in T8 trypsinogen, which stands in contrast to the robust negative effect of this mutation on CTRC-dependent degradation of human cationic trypsinogen. Thus, it seems more likely that in the published mouse model increased gene dosage or nonspecific effects of the transgene may have been the cause of the described pancreas pathology.

## 6. SUMMARY AND NEW RESULTS

### I.

1. We confirmed earlier findings of elevated HNP 1-3 plasma levels in patients with diabetes mellitus compared to healthy controls. Furthermore, we found that diabetic patients with nephropathy, neuropathy, or cardiovascular complications all had significantly higher HNP1-3 levels compared to diabetic patients without complications and also to healthy controls.
2. Our results demonstrated that the median gene copy number of human *DEFA1/DEFA3* gene is 10 per diploid genome in the Hungarian population; not only among controls but also in diabetic patients. *DEFA1/DEFA3* gene copy numbers did not correlate with mRNA expression levels in peripheral leukocytes, nor with the  $\alpha$ -defensin (HNP 1-3) levels measured in the plasma of peripheral blood. Therefore the elevated HNP 1-3 levels might not be genetically determined, or at least independent of the copy number variation of the *DEFA1/DEFA3* genes.
3. The C allele of the c.-44C>G SNP located in the promoter region of the *DEFB1* gene was found to be more frequent among diabetic patients than in healthy controls, indicating that impaired human  $\beta$ -defensin function might have a role in the development of diabetes mellitus.

### II.

1. There are 4 trypsinogen isoforms (T7, T8, T9 and T20) expressed at high levels in the mouse pancreas under physiological conditions. In 1 mM CaCl<sub>2</sub>, at pH=8.0, only T7, T8 and T9 autoactivated, while T20 did not autoactivate.
2. Ctrc and Ctrb almost completely inhibited autoactivation of T8 and T9 isoforms by enzymatic cleavage of their autolysis loop, but had no significant effect on the autoactivation of T7 isoform.
3. Introduction of the p.R123H mutation in T7, or the analogous p.R122H mutation in T8, did not significantly change the autoactivation characteristics of mouse trypsinogens. Therefore, the biochemical characteristics of these mutants did not mimic the pathogenic phenotype of the p.R122H mutation in human cationic trypsinogen.

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