Intrauterine and genetic risk factors for proliferative diabetic retinopathy

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Abbreviations and symbols

ADA	American Diabetes Association
apoA1	Apolipoprotein A1
apoE	Apolipoprotein E
BMI	Body-mass index
CIRDD	Combined Insulin Resistant and Deficient Diabetes
CKD	Chronic kidney disease
CNS	Central Nervous System
CVD	Cardiovascular disease
DOHaD	Developmental origins of health and disease
DR	Diabetic retinopathy
DR	Diabetic retinopathy
ETDRS	Early Treatment of Diabetic Retinopathy Study
FDR	False Discovery Rate
FPG	fasting plasma glucose
GAD	Glutamic acid decarboxylase antibodies
GO	Gene Ontology
GoDARTS	Genetics of Diabetes Audit and Research in Tayside Scotland
GWAS	Genome-wide association study
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein
HOMA	The Homeostasis Model Assessment
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IROD	Insulin Resistant Obese Diabetes
KEGG	Kyoto Encyclopedia of Genes and Genomes
LADA	Latent Autoimmune Diabetes in Adults
LBW	Low Birth weight
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LEARn	Latent Early-life Associated Regulation
LMM	Linear Mixed Model
MAC	Minor allele counts
MAF	Minor allele frequency
MARD	Mild age-related diabetes
MD	Mild diabetes
MD	Mild diabetes
MDH	Mild diabetes with high HDL cholesterol
MDH	Mild diabetes with high HDL cholesterol
MOD	Mild obesity-related diabetes
MODY	Maturity-Onset Diabetes of the Young

NPDR	Non-proliferative diabetic retinopathy
NU	Neuronal unit
NVU	Neurovascular unit
OGTT	Oral Glucose Tolerance Test
OR	Odds ratio
PC	Principal components
PDR	Proliferative diabetic retinopathy
PGRS	Polygenic risk scores
QC	Quality control
SAID	Severe autoimmune diabetes
SHD	Severe Hyperglycemic Diabetes
SIDD	Severe insulin-deficient diabetes
SIRD	Severe insulin-resistant diabetes
SNP	Single nucleotide polymorphisms
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VEGF	Vascular endothelial growth factor
WES	Whole-exome sequencing study
WESDR	Wisconsin Epidemiologic Study of DR
WHO	World Health Organisation
WTCCC	Welcome Trust Case Control Consortium
YOB	Year of birth

Abstract

Diabetes is a complex progressive metabolic disorder characterized by hyperglycemia and caused by different etiopathogenic factors. Individuals with diabetes have heterogeneous clinical representation and increased risk of micro- and macrovascular complications. Diabetic retinopathy (DR) is the most frequent microvascular complication of diabetes and one of the leading causes of blindness. Currently, existing treatment modalities target a severe sight-threatening form of the disease, proliferative DR (PDR), and are characterized by significant side effects. The prevailing strategy for prevention or slowing down DR progression is glucose-lowering therapy, which is not efficient enough and might be harming to older groups of patients. Risk factors for PDR include duration of diabetes, hypertension, dyslipidemia, genetics and environment, and their interplay. The adverse intrauterine environment, particularly exposure to prenatal famine, was shown to play an important role in predisposition to diverse metabolic disorders in adults such as type 2 diabetes (T2D), hypertension, and cardiovascular diseases (CVD).

In this Ph.D. thesis, we aimed to study novel diabetes subgroups based on pathophysiological characteristics of patients, highlighting subgroup(s) with an elevated risk of diabetic complications, particularly PDR. Further, we aimed to investigate the association of intrauterine exposure to famine with the risk of PDR in adult individuals with T2D. Finally, we wanted to study the molecular mechanisms linked to famine-related PDR.

In paper 1, we performed a k-means cluster analysis to identify novel subgroups of individuals with new-onset and long-term diabetes, and estimated the risks of diabetic complications using logistic regression. In paper 2, we evaluated effect of intrauterine famine exposure on the risk of PDR in individuals with T2D using logistic regression adjusted for established risk factors such as age, sex, duration of diabetes and HbA1c. In paper 3, we performed candidate gene analysis using generalized estimation equation (GEE) to study the effect of interaction between SNPs and perinatal famine exposure on the risk of PDR. In paper 4, we performed genome-wide

association (GWAS) and interaction (GWIS) studies using a linear mixed model (LMM) to investigate molecular underpinnings of famine-related PDR.

In paper 1, we identified three subgroups with severe diabetes and two subgroups with mild diabetes. The highest risk of PDR was observed in the severe autoimmune diabetes (SAID) and severe insulin-deficient diabetes (SIDD) subgroups and the lowest in the insulin-resistant obesity-related diabetes 2 (IROD2) subgroup. In paper 2, we demonstrated that individuals with T2D, who were perinatally exposed to famine had an elevated risk of PDR in adult life. In paper 3, we demonstrated a significant association between famine-associated PDR and SNPs which were located in genes with neuronal function. In paper 4, we identified diverse pathways potentially linked to famine-related PDR, among them the most significant were lipid metabolism and inflammation pathways.

In conclusion, we suggested that the altered development of neurovascular unit in the retina due to exposure to intrauterine famine may increase susceptibility to PDR later in life. Changes in metabolic adaptations during developmental programming induced by adverse early life events may affect insulin secretion and lipid metabolism, which consequently may increase predisposition to PDR under diabetes environment in adulthood. We suggested that drugs targeting these mechanisms in addition to glucose-lowering treatments may be beneficial for the prevention or slowing down the progression to PDR in the early stages of the disease.

List of Publications

Paper 1

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Paper 2

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Paper 3

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Paper 4

Fedotkina O., Ozgumus T., Åkerlund M., Sulaieva O., Nilsson P.M., Lyssenko V.

Genome-wide association and interaction analyses for severe diabetic retinopathy.

Manuscript.

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1 Introduction

1.1 Diabetes

1.1.1 The global significance and epidemiology

Diabetes is one of the largest medical battles worldwide. International Diabetes Federation (IDF) estimated that globally 463 million people between 20 and 79 years of age were living with diabetes in 2019, and this number was estimated to rise to 700 million by 2045.[1] The main factors contributing to the increased diabetes prevalence are believed to be aging of the global population, urbanization, and decreased physical activity associated with obesity.[1] Some populations are at a higher risk of diabetes. In 2019 the highest prevalence has been reported in Middle East and North America (12.2%), and at the lowest in Africa (4.7%), prevalence in Europe was reported as 6.3%. The largest numbers of people with diabetes live in China (116.4 million), India (77.0 million), and the USA (31.0 million).[1] People diagnosed with diabetes have an increased risk of micro- and macrovascular complications of diabetes: retinopathy, nephropathy, neuropathy, and cardiovascular diseases. Diabetes also increases the risk of cancer and mental illnesses including depression, Alzheimer disease, and dementia.[2-5] Development of these complications causes the decrease of quality and expectancy of life, leading to 11.3% of deaths associated with diabetes in adults aged 20-79 years.[1] In total, diabetes and its complications caused 4.2 million deaths in 2019, which is 12.8% of deaths in this age category.[1] Nearly 50% of individuals with diabetes globally and approximately 60% in South-East Asia and Africa are undiagnosed, meaning that these individuals are a high-risk group for diabetic complications without knowing that.[1,6] Diabetes prevalence in Ukraine was reported to be 8.4% in 2019, however, given a poor referral to the hospitals, this figure is most likely underestimated.[7]

1.1.2 Diabetes classification

In 1978, diabetes was suggested as a group of disorders characterized by hyperglycaemia and caused by different etiopathogenic factors.[8] Currently, the disease is classified into several subtypes with different clinical representations. According to the World Health Organisation (WHO) and the American Diabetes Association (ADA), diagnosis of diabetes is based on blood glucose level: higher than 7 mmol/l for Fasting Plasma Glucose (FPG), higher than 11.1 mmol/L for Oral Glucose Tolerance Test (OGTT), or higher than 6.5% for glycated haemoglobin (HbA1c).[1,9] Additional factors should be considered in order to classify different diabetes subtypes. These factors include age at onset, presence of obesity, presence of islet specific autoantibodies, insulin secretion capacity, diabetes predisposition in family, and genetic tests.[10,11]

Type 1 diabetes (T1D) accounts for 5-10% of all diabetes cases. Clinical manifestation typically occurs in young people below 25 years of age but could affect other age categories as well.[12] It is an autoimmune disease characterized by the attack and destruction of pancreatic β-cells by the immune system. As a result, it is believed that T1D manifests when approximately 80% of pancreatic islets are lost and patients become dependent on insulin treatment for their lifetime. The majority of individuals with T1D (92%) demonstrate the presence of autoantibodies against β-cells.[13] Among several antibodies types, glutamic acid decarboxylase (GAD) antibodies are one of the most frequent and are present in 90% of all antibody-positive individuals.[14]

The largest part of all individuals with diabetes, 80-90%, are diagnosed with type 2 diabetes (T2D). This type of diabetes usually develops after 35 years of age and is typically characterized by obesity with abnormal fat distribution and insulin resistance in combination with insufficient insulin secretion.[15-19] In individuals with insulin resistance, cells fail to respond to insulin. This leads to decreased uptake of glucose by the peripheral tissues and consequently, the concentration of glucose rises in the bloodstream, leading to chronic hyperglycemia. Obesity imposes demands on pancreatic β-cells to secrete more insulin to compensate for the degree of insulin

resistance. Those individuals who fail to increase their insulin secretion will develop T2D.

Although classical T1D and T2D have very different clinical representations, it is challenging to distinguish some individuals with mixed characteristics of both types.[20,21] For instance, T1D might develop in adults aged 20-50 years, and in this case individuals will likely be obese, and often have remaining functioning β-cell at diagnosis.[20] Moreover, some individuals with a subtype of diabetes with characteristics similar to T2D, Latent Autoimmune Diabetes in Adults (LADA), are positive for islet antibodies.[20] LADA has a similar frequency as T1D, accounting for approximately 10% of individuals with adult diabetes.[22] Therefore, to diagnose classical T2D it is essential to exclude other forms of diabetes: adult T1D or LADA, gestational diabetes, or monogenic forms of diabetes such as Maturity-Onset Diabetes of the Young (MODY).[16] The fact that currently T2D diagnosis is based on exclusions remains the main issue and makes this type of diabetes the most challenging in treatment, despite established and well-studied risk factors.

1.1.3 Risk factors for T2D

The major risk factors for T2D are obesity, age older than 45 years, hypertension, sedentary lifestyle, genetic predisposition, and ethnicity.[23] Obesity (body-mass index [BMI] ≥30 kg/m^2), being the strongest risk factor for T2D, plays an important role in insulin resistance and contributes to younger age at onset of disease and increased susceptibility of developing T2D in youth.[24] Since insulin sensitivity decreases and insulin resistance increases with age, T2D and aging are closely correlated.[25] Other risk factors for T2D include hypertension and its treatment [26-30], low high-density lipoprotein (HDL) cholesterol[31-33], high triglyceride level being suggested as independent predictor of disease development.[34-39]

1.1.4 Heterogeneity of T2D

A variety of organs and tissues are involved in T2D pathogenesis: pancreas, liver, skeletal muscle, kidneys, small intestine, adipose tissue, and brain.[40-45] Diverse pathophysiological factors including abnormalities of gut microbiota, inflammation, and dysfunction of the immune system play an important role in T2D development.[46] As a result of the complexity and heterogeneity of T2D, individuals with the disease have different metabolic responses, diverse clinical presentations, and risks of diabetic complications.[47]

For instance, some individuals have abnormal glucose metabolism characterized by impaired fasting glucose (IFG), and others by impaired glucose tolerance (IGT).[47] The fact that IFG is a product of insulin resistance in the liver and IGT is caused by insulin resistance in muscular tissue highlights the diverse manifestation of hyperglycemia.[45,48,49] In the study on the Whitehall II cohort it was shown that individuals with hyperglycemia expressed by either IFG or IGT, or both measurements together, have different cardiometabolic risks[50] and generally have different phenotypic characteristics including BMI, blood pressure, and β-cell function in different time points before diagnosis.[50-53] Classically, age at onset of T2D was considered to be 35-55 years of age, however currently the disease can be observed in younger ages due to the increasing rates of obesity in young adults.[24] Defining BMI>30kg/m^2 as a risk factor for T2D is more applicable to the European ancestry, while for East Asian individuals this value is lower (BMI>23kg/m^2) due to a higher susceptibility to T2D in this ethnicity.[47] Even though hyperglycemia is the strongest risk factor for microvascular complications, many studies suggested that for long-term diabetes there are other factors that influence the disease and, as a result, individuals with the same level of hyperglycemia have different risks of complications.[54-58] These factors include genetics, type of diabetes treatment, obesity, hypertension, also environmental factors such as smoking and alcohol consumption, and general level of life and health care.[49]

Currently, the main strategy for T2D treatment at the first step is targeting weight loss and suggesting modification of lifestyle with physical activity and diet.[59,60] At the second step, for more severe cases, glucose-lowering therapy is prescribed.[16,61] However, despite the effectiveness of glucose-lowering therapy, in some cases it could cause harmful side effects[62] and due to diabetes heterogeneity, it has no consistent effect on the progression of diabetes complications, especially in the high-risk groups.[58,63,64] Therefore, there is a great need for a patient-centered approach in diabetes therapy, which would rely on specific characteristics of the individuals. There have been several attempts to identify (classify) subgroups of patients with similar characteristics with the aim to improve clinical decision support system.

1.1.5 Modeling heterogeneity of T2D into subgroups

One of the first approaches, which was trying to address the clinical complexity of T2D and help clinicians to improve treatment effectiveness, was offered in 2010. That was the "ABCDEFG" algorithm, which stands for Age, Bodyweight, presence of Complications, Duration of disease, Empowerment Economics, degree of Frailty and Geography.[65-67] At the initial step, patients were categorized in groups based on age (young, middle, old), then in groups based on disease duration (with cutoff 10 years) and HbA1c levels (different cutoffs depending on age/duration groups), and then recommendations for treatment were prescribed depending on the level of body weight, risk of hypoglycemia and complications.[65] Empowerment, in this case, was standing for educating of patients on disease management, accounting for cognitive damage, mental state (Frailty), and differences among ethnicities and geographical location (Geography).[67]

Another suggestion, called the "palette" model, was offered in 2017 by McCarthy and had grounds in different molecular and pathophysiological factors predisposing to diabetes development.[68] This approach suggested examination of patients based on main pathophysiological processes involved in diabetes progression:

islet development, senescence, and replacement; islet function, and autoimmunity; obesity and fat distribution; insulin resistance, and incretin activity. This is based on well-recognized evidence that patients develop diabetes either due to cumulative modest defects of several processes or due to severe defects in one particular process. This view was supported by genetic studies showing increased risk of different complications in individuals with different polygenic risk scores (PGRS) for described processes.[69-72] PGRS explains risk of developing the disease or the trait and is calculated for each individual using their genetic information and the effect sizes from corresponding GWASs.[73]

With the establishment of medical registries and the collection of large amounts of clinical, biological, and genetic data of patients with T2D, several data-driven approaches were offered with the aim to identify and characterize subgroups of T2D. In 2015, Li et al. described three subgroups of individuals with T2D using topological analysis of patient-patient networks and clinical data consisting of 73 variables including laboratory measurements, vitals, medications, and diseases, however, the two main players in the diabetes pathogenesis were missing: measurements of insulin secretion and insulin resistance.[74] According to this approach, patients were assigned to one group if they showed very similar characteristics across all variables used in the analysis. The first subgroup was characterized by the highest serum glucose concentration and BMI and was associated with an elevated risk of nephropathy and diabetic retinopathy (DR). The second subgroup was characterized by the lowest weight and by an increased risk of cancer malignancy and cardiovascular disease (CVD). The third subgroup had the highest systolic blood pressure and the strongest association with CVD, neurological disorders, allergies, and human immunodeficiency virus infections. Authors also performed genetic analysis, suggesting biologically relevant genes to be associated with specific subgroups and phenotypes typical for them.[74]

In 2018, a novel classification algorithm was proposed in the study of individuals with new-onset diabetes from several Scandinavian cohorts.[75] The authors used the k-means clustering algorithm based on six clinical variables: GAD antibodies,

HbA1c, BMI, age at onset, and insulin secretion and resistance measured with The Homeostasis Model Assessment (HOMA2-B, and HOMA2-IR).[76,77] Five subgroups of patients with different clinical characteristics and complications prevalence were identified in the analysis. The first subgroup, severe autoimmune diabetes (SAID), was characterized by the presence of GAD antibodies, lower BMI, and higher HbA1c compared to other subgroups. The second subgroup, severe insulin-deficient diabetes (SIDD), included GAD-negative individuals and had the highest risk for DR, though the clinical characteristics were very similar to the first subgroup. The third subgroup, severe insulin-resistant diabetes (SIRD), was characterized by the highest insulin resistance and obesity, and the highest risk for chronic kidney disease (CKD). The fourth subgroup, mild obesity-related diabetes (MOD), also included obese individuals, but in this subgroup insulin resistance was lower than in SIRD. And the fifth subgroup, mild age-related diabetes (MARD), included the oldest patients with the mildest characteristics of all phenotypes. Subgroups were successfully extrapolated to the populations from China, North America, and Australia including cohorts with diabetes duration within 7 years.[78,79] In the German Diabetes Study, after 5 years of diabetes duration 23% of participants switched clusters, and the progression of complications in subgroups was different compared to the newly diagnosed patients. A potential explanation for this was the usage of glucose-lowering treatment and disease progression.[80]

In the study of the Indian population from the INSPIRED cohort with young-onset T2D with less than 5 years of diabetes duration, authors also applied k-means clustering on a wider set of variables including waist circumference, serum triglycerides, and HDL-cholesterol and c-peptide.[81] Two of the subgroups were similar to the original study (MARD and SIDD), and two novel subgroups with mixed characteristics were proposed: Insulin Resistant Obese Diabetes (IROD), and Combined Insulin Resistant and Deficient Diabetes (CIRDD) with an elevated risk of DR and highest risk of CKD. The combination of insulin resistance and deficiency in CIRDD cluster was explained by authors with differences in diabetes representation in the Asian Indian population compared to Europeans. The Indian population is

characterized by younger age and lower BMI at the onset of diabetes, because of higher levels of abdominal fat, and increased insulin resistance specific for this population.[81]

In another replication attempt in the mixed multiethnic cohort from the East London Database, variables from the original study were not available at the primary care and authors used age at diagnosis, sex, HbA1c, and BMI to describe subgroups. MARD and MOD subgroups were replicated and the third subgroup, Severe Hyperglycemic Diabetes (SHD), had the highest HbA1c and risk of microvascular complications.[82] Usage of the alternative variables for identification of diabetes subgroups was also confirmed in the IMI-RHAPSODY study, where because of the absence of fasting glucose, instead of HOMA authors used random or fasting c-peptide and HDL cholesterol, HbA1c, and BMI. First, the authors performed analysis without HDL cholesterol and replicated the original four T2D subgroups. After adding HDL cholesterol to the analysis, MARD was stratified into two subgroups: mild diabetes (MD) and mild diabetes with high HDL cholesterol (MDH).[83]

Despite the fact that described data-centered clustering approaches are undoubtedly clinically important, it is not clear to what extent the variables used in the analyses are causal for diabetes development. T2D has a complex nature and is characterized by developing severity of symptoms in the course of the disease, different age at onset, and heterogeneity of etiological mechanisms due to various biological pathways being involved.[84] Common complex diseases are present in more than 1-5% of the population and have polygenic nature, meaning that several genes with moderate effects are involved in their pathogenesis.[85] Studying the genetics of this complex disease may help to understand the underlying mechanisms of its heterogeneity and subtype-specific causal biological pathways, and consequently contribute to disease prevention and treatment.

1.1.6 Genetics of complex traits

The central aim of the studies on human genetics is to explain the genetic architecture of the heritable trait or disease with the aim to improve knowledge on disease etiology, prediction, and treatment.[86] The genetic architecture comprises characteristics of genetic variants, which are associated with a particular trait. These characteristics include the type and the number of genetic variants, the magnitude of their effect, and the effect of their interactions with each other and with the environment.[86]

Various studies were performed during different decades to investigate the genetic architecture of complex traits. Twin studies were conducted on monozygotic and dizygotic twins, and were aimed at investigating the contribution of environment and genetics on the trait or disease.[87] Earlier genetic studies presumed previous knowledge about possible disease-related genes and were focused on particular regions or specific genes (genetic linkage and candidate gene studies). Genetic linkage studies aimed to find the chromosomal position of a gene, which is segregating inside families with apparent mendelian transmission. Even though these studies are best compatible with monogenic disease, this approach was also used to study complex traits.[88] Candidate genes studies examined Single Nucleotide Polymorphisms (SNPs) located in previously preselected genes based on their biological relevance for association with the trait.[89] Many promising associations between diseases and SNPs were found using this approach. However, the use of the approach for complex traits was criticized for failure to replicate findings in different studies due to small sample size, diverse populations, and inability to include all possible causative genes into analysis.[89]

The most used approach for studying complex traits genetics today is genome-wide association studies (GWASs). GWAS is a hypothesis-free approach which explores the entire genome through the effects of SNPs on a trait or disease.[90,91] First GWAS was conducted on macular degeneration in 2005.[92] After this, in 2007, the Welcome Trust Case Control Consortium (WTCCC) set the stage for many GWASs to

come, and this year is considered to be the start of the GWAS era. Since then, more than 4,500 GWASs have been carried on 5,000 traits, reporting 55,000 unique loci, and providing evidence that GWAS is a powerful tool for studying the genetics of complex traits.[91] Since in GWAS studies the entire genome is tested for the association with a trait, and therefore numerous statistical tests are performed, to decrease a rate of false-positive results i.e. type 1 error, the significance threshold is adjusted for the multiple testing correction. The standard genome-wide significant threshold is $p=5\times10^{-8}$ and is calculated using Bonferroni correction for multiple testing by dividing 0.05 by one million independent SNPs in the genome.[93] Another cutoff for p-value significance in GWAS is equal to $p=10^{-5}$ and used by the National Human Genome Research Institute for the identification of putative associations.[94] GWAS studies mainly focus on studying common complex traits and the effects of common SNPs with minor allele frequency (MAF) in a population not less than 0.05.[95,96] Therefore, the majority of SNPs identified by GWASs are located in non-coding regions since most SNPs in coding regions are rare (MAF<0.01) and as such require a large sample size to have sufficient statistical power for identification the genome-wide significant signals (with $p<5\times10^{-8}$).[91] This limitation is the most important challenge in detection of robust associations. Lack of robust association is making the translation of GWAS results to biological mechanisms underlying the disease even harder.[91]

Before performing genetic studies, evidence that a trait has a strong genetic component should be provided. This can be measured using heritability, a concept defined as a proportion of variance in a trait that is explained by genetics.[97] Missing heritability, on the other side, is the topic that gained a lot of interest in genetic studies in the last couple of decades. It is defined as a discrepancy between the broad-sense heritability (a measure of the proportion of variance in the trait which is explained by all genetic factors, including gene-gene interactions and dominant effects, and other genetic variants),[98] and narrow-based heritability, also known as SNP-based heritability, defined as the proportion of variability in the trait, which is explained by an additive effect of causal SNPs.[99] Possible explanations for the missing heritability are: 1) overestimated heritability for twin studies; 2) not detected by GWAS effect of

causal rare variants; 3) effects of gene-gene and gene-environment interactions on the trait.[100-102]

Gene-environment interaction studies play an important role in complex diseases. They increase accuracy in the assessment of both effects of genetic and environmental factors, and give more insights to the biological understanding of the disease and inputs to personalized medicine.[103] These studies are based on the hypothesis that depending on the environment, the same genotype can have different effects on the trait, or, from another perspective, that environment can have different effects on the trait depending on genotype.[103] The environmental exposure can vary: either physical exposure (ecological, radiation, undernutrition), biological (virus), chemical (medications, toxic elements), behavioral (smoking, diet), or life events (stress, trauma). Several models describe a possible relation between genotype and environment on the trait:

a) the genotype increases the effect of environment on the trait, however, the environment could also affect the trait without genotype;
b) the effect of genotype increases the effect of environment, and there is no effect of this genotype in unexposed individuals;
c) the environment exposure increases the effect of risk genotype, but there is no effect of exposure in individuals without risk genotype;
d) there is the an effect on the trait only in individuals with both risk genotype and under environmental exposure;
e) there is an effect of risk genotype and of environmental exposure, but the effect of their interaction is much stronger.[103-105]

Numerous traits have been reported to be associated with gene-environment interactions. For instance, significant gene-environment interaction effects were demonstrated for smoking and bladder cancer,[106] saturated fat and BMI,[107] different environmental exposure and diverse psychiatric disorders[108], as well as diet and physical activity and T2D.[109]

1.1.7 Genetic architecture of T2D

During the past decades, the genetics of T2D was extensively studied using twin studies, linkage studies, candidate gene studies, GWASs, and whole-genome sequencing studies. The fact that T2D has a strong genetic component was initially confirmed by showing that the disease aggregates in families. The risk to develop T2D was reported to be 40% higher for individuals who had one parent with T2D and 70% higher if both parents had the disease.[110] Similarly, twin studies on T2D demonstrated 70% heritability in monozygotic and 20-30% in dizygotic twins.[111] It is important to emphasize that high estimates of heritability are also partially related to the shared environment in families and possibly to an elevated risk of obesity, which could run in families. Therefore, real estimates for the heritability of T2D might be lower.[112]

Several linkage studies in large families with mendelian segregation reported only regions spanning two genes for association with T2D: *CAPN10*[113-118] and *TCF7L2*.[119,120] Candidate gene studies demonstrated associations with the main processes involved in T2D pathogenesis: glucose and lipid metabolism, β-cell function, insulin secretion, and signaling. Numerous important genes were discovered using this approach: drug-target *PPARG*,[121-123] *IRS-1* and *IRS-2* involved in insulin signaling,[124-126] *FTO* associated with obesity,[126,127] *KCNJ11* involved in insulin secretion,[128,129] *WFS-1* involved in β-cell function,[130,131] and MODY-related genes such as *HNF1A, HNF1B*, and *HNF4A*.[132,133]

First GWAS studies for T2D replicated results from linkage and candidate gene studies and confirmed associations for *TCF7L2*, the most significant and the most frequently replicated gene until now, and also for other genes.[134-139] Even though many common variants with modest effects were detected in numerus T2D GWAS studies, only handful of them were successfully replicated. The question of the contribution of rare variants with bigger effects to the pathogenesis and heritability of T2D has been extensively debated.[140] In the paper by Fuchsberger et al. in 2016 year, the authors addressed this question by performing whole-genome sequencing study on the European population (N= 111,548) and showing that common variants explain a much

bigger fraction of T2D variability compared to rare variants.[141] This notion was also supported in the recent GWAS (N=898,130) on Europeans which was performed in 2018 and included analyses of common and rare variants.[142] Authors identified 403 SNPs nominally associated with T2D ($p<10^{-5}$), of them, 245 were genome-wide significant, including 135 novel associations. Genome-wide significant variants (MAF<0.05) explained only 1.3% of variability having odds ratio (OR) in a range 1.08-8.05 (N=80), while this number for SNPs with MAF>0.05 explained 16.3% of the variability with OR in 1.03-1.37. Authors replicated previously known T2D genes including several signals at *TCF7L2* locus with OR from 1.05-1.36. Signals in the genes *FTO, MC4R, TMEM18, SEC16B,* and *GNPDA2* had significantly different effects in BMI-adjusted and unadjusted models, highlighting that the main effect of these SNPs on T2D was driven by obesity and confirming results from the previous studies.[143-146] SNPs in genes *TCF7L2, ARAP1, JAZF1* were suggested to be associated with insulin secretion, and in *GRB14, PPARG, HMGA1, ZNF664* with a reduced capacity of fat storage and adipose tissue.[142]

The transethnic meta-analysis, which can increase power for identification causal variants that are shared among populations,[147] included cohorts from the Japanese and European populations, and replicated 60 previously known independent loci and found 28 novel loci.[148] The heterogeneous effect of T2D pathways was confirmed and the importance of β-cell dysfunction was highlighted in pathway analysis in both populations.[148] The most recent and the largest GWAS on T2D included multi-ethnic cohorts with multiple populations (N=1,4 million). In addition to the discovery of novel T2D variants, authors aimed to investigate the impact of already known T2D variants in different populations and to identify the genetic variants associated with T2D complications. In total, the authors reported 568 independent genome-wide significant ($p<5\times10^{-8}$) SNPs, 286 of them were novel. Among reported associations, several signals were strongly associated with DR including *GJA8, TCF7L2, SLC18A2,* and *SVILP1*. Authors also showed that PGRS for T2D has a strong association with diabetic complications, being the strongest for DR. Authors suggested that PGRS will help to identify individuals with T2D with increased DR risk.[149]

Notably, a recent study by Nag et al. performed on UK biobank demonstrated no evidence for gene-gene interactions influencing the risk of T2D.[150]

There were several attempts to classify and characterize the genetics of T2D based on association with related traits and risk factors.[69,70,151] In the study by Udler et al. in 2018 the authors proposed a soft clustering analysis, allowing each genetic variant to be associated with one or more clusters. The authors identified five tissue-specific clusters of T2D SNPs. Two clusters were associated with reduced insulin secretion, three other clusters were associated with insulin resistance and represented different traits: a) obesity; b) low BMI; c) low HDL-cholesterol and high triglycerides; d) low triglycerides. Results were confirmed by demonstrating that individuals with high PGRS for a particular cluster exhibit cluster-specific phenotype.[71] The study by Mansour et al. in 2020 aimed to reach a better understanding of etiology for each diabetes subgroup offered by Ahlqvist et al.[152] Authors performed GWAS analysis in the ANDIS cohort and identified that subgroups have different genetic backgrounds and heritability. SIDD and MARD subgroups showed the strongest heritability, while SIRD had the weakest estimates for heritability. PGRS for T2D SNPs had a strong association with SIDD and MOD. This association was weaker for SAID and this subgroup was significantly associated with PGRS for T1D. The SIRD was only one subgroup where no associations with *TCF7L2* or PGRS for insulin secretion were identified.[75,152]

In summary, extensive studies on the genetics of T2D are making formidable discoveries and providing novel insights into disease pathogenesis, however, there is still a large part of missing heritability being unexplained (missing heritability).[149] Additionally, more attention should be given to studying diabetes complications, and especially prediction and treatment of individuals in high-risk groups for complications. Genetic studies of specific diabetes subgroups will help to understand the underlying biological mechanisms of each subgroup.

1.2 Diabetic retinopathy

1.2.1 The global significance and prevalence of diabetic retinopathy

Diabetic retinopathy (DR) is a sight-threatening disease of the retina and the most frequent microvascular complication of diabetes.[153,154] Overall, among all individuals with diabetes, approximately 30% develop DR, and 5-10% develop sight-threatening DR, but these estimates are largely dependent on the duration of diabetes.[155,156] Individuals with T1D develop DR almost twice as frequently compared to those with T2D: 36.3% vs 19.4%.[157,158] Together with cataract, age-related macular degeneration, glaucoma, uncorrected refractive, DR takes a leading place among the causes of blindness in the working-age population in developed countries of Eastern and Central Europe.[159-162] In 2002, the WHO estimated that DR caused approximately 15%-17% of the total blindness rate in Europe and the USA.[163] The contribution of DR to the rate of blindness is estimated to increase as the number of people with T2D and their life expectancy increase.[160] Number of people with blindness caused by DR increased from 0,2 million to 0,4 million in 2015, and rates of severe vision impairment increased from 1,4 million to 2,6 million in 2015.[160]

During the last decade, several reports suggested that the prevalence of DR was decreasing in developed countries, possibly due to the regular screening and improved systematic control of DR.[156,164,165] The latest and largest meta-analysis, which included 59 population-based studies, estimated that in 2020 the total number of individuals with DR was 103.12 million globally, and with sight-threatening DR this number was 28.54 million.[166] Since the prevalence of individuals with diabetes increases, these numbers are estimated to reach 160.50 million for DR and 44.82 million for vision-threatening DR in 2045.[166] The highest prevalence was seen in South East Asia (35.9% for DR and 14.4% for vision-threatening DR) and North America and the Caribbean (33.3% and 7.8%), and the lowest in South and Central America (13.3% and 5.8%).[166] Among Europeans, the prevalence was reported to be 18.7% for DR and 5.5% for vision-threatening DR. [166]

1.2.2 Screening, diagnosis, classification, and treatment

The microvascular circulation in retinal tissue is affected in diabetes resulting in structural changes.[167] These changes induce microvascular degeneration and restricted blood supply to vessels on the retina surface, which might lead to the formation of new fragile vessels that are prone to bursting and bleeding, and as a consequence, to vision loss.[168] DR is often associated with other vascular complications of diabetes, including neuropathy,[169] nephropathy[170], and cardiovascular diseases[171]. Pathophysiology of DR involves retina blood vessels leakage, inflammation, and neuronal dysfunction in the eye.[172] DR is a progressive disease and over time it may progress from mild to severe stages depending on diabetes management, genetics, ethnicity, and environment.[168] It is essential to categorize and describe stages of the disease severity in order to provide the optimal therapy.[173]

DR is clinically classified into two broad stages based on the level of microvascular degeneration and related ischemic damage: non-proliferative diabetic retinopathy (NPDR) and advanced, proliferative diabetic retinopathy (PDR).[168,174] To distinguish and standardize DR stages more precisely, several classification systems were proposed. One of them was the Modified Airlie House Classification, which compared stereo photographs in 7 standard photographic fields with the patient's cases in the fields.[175] Later this classification was adjusted for use in the Early Treatment of Diabetic Retinopathy Study (ETDRS), where DR was classified into 13 complex stages ranging from level 10 (no retinopathy) to level 85 (severe vitreous hemorrhages or detachment of retina or macula).[176] ETDRS was perfectly suited for research, but due to its complexity, it was rarely used in the clinics.[174] To simplify the classification scale, based on scientific evidence provided by the Wisconsin Epidemiologic Study of DR (WESDR) and ETDRS, the International Clinical Disease Scale for DR was created.[177] According to this scale, there are five stages of DR:

- The first stage is called "no apparent retinopathy", which represents a healthy eye without any sights of DR.

- The second stage is called “mild NPDR” and is characterized by a few microaneurysms.
- The third stage is called “moderate NPDR” and is characterized by more microaneurysms, bleeding vessels, and hemorrhages inside the retina.
- The fourth stage is “severe NPDR” characterized by more affected areas compared to moderate NPDR, half of the cases at this stage usually require laser treatment.[178]
- The fifth stage is proliferative DR (PDR), characterized by neovascularization affecting the disc, the retina, the iris, the angle, the vitreous, and hemorrhage or the tractional retinal detachment.[177]

It is essential to perform regular screenings of individuals with diabetes to diagnose retinopathy in earlier stages and to avoid or delay the progression to PDR.[179-183] Diagnosis of DR is based on visual examination and detection of microvascular damages on the patient’s retinal fundus photography.[179] The best practice is when the examination is performed by an ophthalmologist. In recent years Artificial Intelligence (AI) using deep learning was proposed to perform DR diagnosis using an image recognition algorithm.[184-187] Since in the early stages DR is not usually characterized by any symptoms, a patient may not recognize the disease and it may be identified only by examining the fundus photography. Currently, the recommendation for screening is once per year from the time of diabetes diagnosis[179,188], and once per two years for patients with well-controlled diabetes and no signs of retinopathy[189]. The benefits of regular screening were demonstrated in many countries especially considering the evidence that treatment of the disease becomes more efficient the earlier it is diagnosed.[180,182,183,190-192]

Currently, the main treatment alternatives are targeting the severe vision-threatening stages of the disease using laser surgery and inhibitors of vascular endothelial growth factor (VEGF).[193,194] Although these approaches are efficient and have improved over the last decade,[195-197] they still have limitations and strong side effects. Laser treatment might cause visual field loss or impairment of color vision.[198]

VEGF inhibition might cause local complications such as retinal detachment and harm the remaining healthy retina.[199] Also, it might increase the risk of hypertension, proteinuria, ischemic cardiovascular disease.[200,201] These approaches do not address the problem of DR prevention or slowing down the progression from early to more severe stages, when treatment is the most effective. Currently, the most common strategy for this is good management of diabetes and controlling for risk factors such as hyperglycemia and hypertension.[202]

1.2.3 Risk factors

The strongest risk factors for DR are diabetes duration, hyperglycemia, and hypertension.[202] After six years of diabetes duration, 45% of individuals with T1D and 38% of individuals with T2D will develop DR.[203,204] These numbers increase after 20 years of duration and reaching 80% for T1D, about 80% for insulin-treated T2D, and 50% for T2D without insulin treatment.[205,206] Glycemic control, for now, remains the primary strategy for slowing down retinopathy progression. A good glycemic control (HbA1c<7%) in patients with T1D decreases the incidence of DR by 76% and progression to PDR by 54%[202], however, this target of glycemia is very difficult to achieve. Intensive glucose-lowering therapy in individuals with T2D decreases the risk of DR by 25% and the need for laser therapy by 29%.[207] Control of blood pressure (<150/85 mmHg) in T2D patients reduces the risk of progression to PDR by 34% [208]. Even though the association between plasma lipid and lipoproteins levels and DR is less strong as with hypertension[194,209-211], lipoproteins may play an important role in the prevention or progression of DR. Patients who were treated with lipid-lowering drugs have shown a slower progression of DR[212] and needed less laser treatment[211]. Obesity[213,214], inflammation[215], endothelial dysfunction,[216,217] nephropathy[218], hormones[219], oxidative stress[220] and deficiency of vitamin D[221] are additional established risk factors for retinopathy.

Despite the solid clinical evidence of the importance of the above-mentioned risk factors in the pathogenesis of DR, controlling these risk factors might not always be efficient for preventing or slowing down DR progression. The association between risk and progression of DR and glucose control has not shown consistent results and currently remains the topic of debate.[222-225] Additionally, glycemic control must be adjusted individually depending on the risk of hypoglycemia. This is particularly crucial for the older groups of patients since it could increase the risk of non-fatal and fatal cardiovascular events.[226] In the Veterans Affairs Diabetes Trial study, the effect of intensive glycemic control was beneficial for younger patients, but harmful for the older group.[227] The ADVANCE study did not show any positive effect of glucose-lowering treatment on DR.[57] Moreover, in the Diabetes Control and Complications Trial study, it was shown that glycemic exposure explains only 11% of the risk reduction, meaning that 89% of the variation are explained by other factors.[228] Furthermore, some patients with long-term diabetes do not develop DR or don't progress to PDR.[229] Thus, in Joslin Medalist study, 43% of individuals with more than 50 years of T1D duration have not developed any symptoms of PDR.[229] Cumulatively, these results suggest that other factors, such as genetics and environment, and their interaction, play a significant role in the development and progression of DR.

1.2.4 Genetic architecture of DR

The fact that DR has a strong genetic component was supported by several pieces of evidence. First, DR co-segregates in families and individuals with DR in the families are 2-3 times more likely to develop DR than those without family members with DR.[230] Second, DR prevalence differs among ethnicities as was reviewed in chapter 1.2.1. Third, no less important, remarkably some patients never develop severe stages of DR despite the same severity and duration of diabetes as their counterparts.[229] Twin studies reported a strong co-segregation of DR among twins with T1D and T2D.[231,232] Heritability of any DR was estimated as 27%, and for PDR as 52%, which means that PDR likely has a more robust genetic component.[233,234] Until now, there

were not many estimates of SNP heritability for retinopathy. In GWAS from the Genetics of Diabetes Audit and Research in Tayside Scotland (GoDARTS) cohort, narrow-sense heritability for PDR was estimated as 7%.[235]

Linkage analyses studies for DR were performed on multiple populations and identified few linkage regions, however, no specific genes were suggested.[236-238] Multiple candidate gene studies for DR suggested several genes to be associated with the disease: *VEGFA, AKR1B1, AGER, ICAM1, MTHFR*.[239-243] Nevertheless, larger sample size is needed to reach reproducible results. It was suggested that due to different pathophysiological mechanisms in DR and PDR, different genes could be associated with less and more severe forms of the disease.[244] The main consistent and the most frequently replicated result of candidate gene studies on PDR was the discovery of *VEGF* gene family, which plays an essential role in neovascularization.[244-246]

Until now, there were 11 GWASs performed for severe DR.[235,247-256] In comparison to studies in T2D, GWASs for severe DR did not provide consistent results due to the insufficient sample size. Moreover, different studies report inconsistent definitions of DR for both cases and controls. As mentioned in chapter 1.2.2, the golden standard for the diagnosis of DR is relying on fundus photography examined by ophthalmologist and then classified using International Clinical Disease Scale (ICDS) for DR.[177] However, only few studies were using this scale,[247-252,254] and other studies were relying on different classification scales.[235,253,255,256] Some studies used a wide definition of severe retinopathy cases including individuals with PDR and diabetic macular edema,[248,251] or with severe NPDR,[235,249] while other studies defined severe DR as PDR[247,250,252]. Most of the studies used individuals with T2D with NPDR or no/minor DR as controls, however, some of the studies also included non-diabetic individuals as controls[253] or used an “extreme phenotype” approach choosing only diabetic individuals with long duration and without any sign of DR[254,256].

Despite all these limitations, few promising genome-wide significant SNPs were identified. One of them was found in the biggest GWAS for PDR in the European population (N=4,666).[235] That was variant rs3913535 with (OR=1.55, 95% CI [1,48, 1,62], $p=4.05\times10^{-9}$) in the *NOX4,* gene which is involved in the mediation of VEGF receptor and was shown to increase neovascularization in the eye of a rat model for retinopathy of prematurity.[257] Another study reported genome-wide significant variant rs9896052 with (1.67, [1.30, 2.15], $p=4.15\times10^{-8}$) at the *GRB2* gene in the meta-analysis combining European and Asian populations with type 1 and type 2 diabetes (N discovery = 844 European, N replication=583 European+699 South Asian).[249] This gene was shown to be expressed in mice and human retina, and increased expression of this gene was shown to be associated with retinal stress linked to neovascular retinopathy in the mice model.[258] This gene is also involved in VEGF signaling, which additionally confirms its biological relevance.[259] In the study on the African population, which is characterized by the elevated prevalence of PDR compared to other ethnicities, Liu et al. were able to identify several genome-wide significant SNPs associated with PDR, despite having a limited sample size N=360 but using an extreme phenotype approach.[256] There were four genome-wide significant loci, and one of them was replicated in African-Americans. This was variant rs11070992 with (1.28, [1.17, 1.40], $p=4.23\times10^{-8}$) in protein-coding gene *WDR72*, which is associated with hyperglycemia, and mRNA of this gene is expressed in the retina.[260] This gene was suggested to be involved in the proliferation of new blood vessels in the retina.[256] Other genes with genome-wide significant loci were *HLA-B* (rs1065386, 1.21, [1.14, 1.28], $p=3.0\times10^{-9}$) and *GAP43* (rs10560003, 1.86, [1.50, 2.31], $p=2.1\times10^{-8}$). Both these genes are expressed in the retina and pancreas, and *HLA-B* is also associated with DR in African and Caucasian T1D individuals.[261,262]

At present, there are two whole-exome sequencing (WES) studies for DR reported. Both of them applied the "extreme phenotype approach" aiming to find large effects of rare variants, but the sample size was still limited. By making cases and controls more homogeneous, this approach decreases noise in the data and therefore increases the statistical power.[263] One of these studies in the Saudi Arabian (n=107)

cohort identified protective genome-wide significant genes *NME3* ($p=1.55\times10^{-10}$), *LOC728688* ($p=6.23\times10^{-10}$), and *FASTK* ($p=3.21\times10^{-8}$). However, not much is known about these genes, and further research is needed to clarify their role in DR pathogenesis.[264] Another WES study in African Americans (n=70) identified 44 genes (19 of them were novel), which reached a genome-wide significance level. The researchers documented that a number of variants was located on *VEGF-B* and *ApoB* genes previously associated with DR.[265]

Today, the genetics of retinopathy remains elusive. The main obstacles that hinder progress in this research field are the low statistical power due to the small sample size, the heterogeneity of phenotype definition, and the complexity of the trait. More studies with bigger sample sizes and standardized definitions of DR cases and controls are needed to discover small genetic effects and to unravel gene-environment and gene-gene interactions effects. Identification of new clinically relevant pathogenetic pathways using genetic studies will help to understand the causal mechanisms of the disease development and progression. This will contribute to personalized treatment and prediction of DR at the early stages, which will consider the heterogeneity of disease pathophysiology.[266]

1.3 Role of intrauterine environment in adult disease

1.3.1 Developmental origins of health and disease

Developmental origins of health and disease (DOHaD) is a field of research that emerged actively in 1980th and is aimed at investigating the effect of early life events on predisposition to chronic diseases in adult life.[267] The original study by Barker et al., that initiated this research theme, showed the correlation between birth weight in infants and later death in adulthood from ischemic heart disease.[268] This study led to the theory that due to diverse intrauterine factors such as undernutrition, exposure to infections, maternal stress, and hormonal deviations, fetus' organ structure and function, growth and metabolism can be impaired, and these changes might lead to disease predisposition later in life.[269-272] The importance of maternal experience on future child life was discussed for a long time starting from the 1800th.[273,274] Due to the consequences of the severe economic crisis in 1930s, the impact of early life environmental factors on long-term events in life was recognized as a significant factor in humans and animals, in addition to the earlier suggested concept of inheritance.[275-278] The Second World War, a "natural experiment" that resulted in extreme stress exposure and starvation of thousands of people, initiated many human studies on the influence of adverse environmental conditions in early life on disease development in adults contributing significantly to the DOHaD research.[279-281]

1.3.2 Intrauterine famine and adult diseases

The majority of the famine studies were performed in the Dutch Hunger Winter (1944–1945) cohort capitalized on extensively and comprehensively collected data.[280] However, various populations were exposed to famine periods in history: the Chinese famine in 1959–1961,[282] famines in Sweden (1867-1869)[283] and Finland (1866-1868)[284], seasonal famines in Bangladesh and Gambia (between 1949 and 1994)[285], Siege of Leningrad of 1941–1944 (severe and long-lasting starvation period)[286], and the Great Ukrainian Famine (1932-1933)[287]. Based on these historical famine periods,

numerous studies have reported associations of early life events with long-term diseases. Association of LBW and intrauterine undernutrition was extensively investigated in studies on Dutch famine.[280,288,289] Diverse metabolic conditions including obesity,[290-293] hypertension,[282,294] systolic blood pressure,[282,294,295] insulin-glucose metabolism,[296] hyperglycemia,[297] HDL and LDL cholesterol,[298] total cholesterol and triglycerides,[299] and CVD[300,301] have demonstrated an association with famine exposure in early life. Additionally, there is a strong consistent association of famine exposure with approximately 2-fold increased risk of Schizophrenia in Dutch and Chinese famine studies.[302-305] Risk of T2D was extensively studied, showing consistent association with prenatal famine exposure in several cohorts including Chinese, Dutch and Ukrainian populations.[287,306-309] The strong association of T2D and early life events paved the way to several theories trying to explain the mechanisms underlying the DOHaD phenomenon: how early life events could cause diseases in later life.

1.3.3 Hypotheses underlying DOHaD

The “thrifty genotype” hypothesis was proposed by James Neel in 1962. He suggested that diabetes was favored through the process of natural selection due to “thrifty genes”, which were responsible for the effective storage of extra energy and fat from food. Individuals who carried these genes were selected evolutionary as those who have more chances to survive in poor nutrition conditions.[310] This evolutionary theory was criticized mainly because populations predisposed to obesity, such as Pacific Islanders (one of the most obese and prone to diabetes population), did not have a history of significant famine periods.[311,312] Additionally, if that were true, modern hunter-gatherers would have become obese in the periods between famines, however, historical data suggest an opposite.[313] The “thrifty phenotype” followed the “thrifty genotype” hypothesis and was introduced by David Barker in 1992. Barker proposed that limited nutrition during intrauterine development affected the growth of fetus by diminishing insulin secretion and developing insulin resistance in order to survive birth.[314,315] And these adaptational changes subsequently caused T2D and metabolic

syndrome in adult life. The main criticism against this theory was particularly against the focus on the LBW as a causal explanation[316,317] and that growth-restricted infants develop insulin resistance after birth, but not during intrauterine development.[318]

In 2005 the "thrifty phenotype" hypothesis was modified by Peter Gluckman into the "Predictive Adaptive Response" hypothesis.[319-321] This theory relies on the form of "developmental plasticity" - the capacity of individuals with similar genotypes to develop different phenotypes depending on early life environment.[321] In the case of intrauterine famine, the fetus adjusts its developmental processes according to severe undernutrition. As a result, later in life an individual is biologically prepared (programmed) for conditions with limited nutrition and might react well if these conditions are the same as predicted by programming. However, if future life is rich with nutrients, the programmed body response will have a "mismatch" with real conditions and this might lead to higher risks for metabolic disorders.[319,322-324] Explanation of this theory relies on epigenetic regulations, heritable but reversible phenomena, which induce changes in gene expression without changing the nucleotide sequence and which could be induced by the environment.[325,326] The results of the Leningrad famine, where the level of nutrition was still poor after the famine, were in support of this theory. In contrast to studies on Dutch famine, where nutrition after the war was better, studies on the Leningrad famine did not show elevated risks for metabolic diseases.[279]

In summary, DOHaD is a wide and actively developing field. A variety of phenotypes were studied in different cohorts and populations discovering new links and associations. Particularly a strong association was seen between diabetes and intrauterine famine across different populations and ethnicities. It was suggested that famine could contribute to diabetes epidemics in China, and even might have an effect through generations.[315,327] However, there were no studies on the association of famine and diabetic vascular complications, particularly on DR. Interestingly, metabolic risk factors for DR such as hypertension and hyperglycemia were shown to be associated with perinatal famine. In this Ph.D. project, we hypothesized that intrauterine famine

could influence the risk of severe DR in the Ukrainian population and studied molecular mechanisms which may potentially underlie this association.

2 Aims

The aims of the current Ph.D. project were:

1. To study novel diabetes subgroups in the Ukrainian population, particularly subgroups with the high risk of proliferative diabetic retinopathy *(Paper 1)*
2. To study an effect of the extreme perinatal exposure to famine and the risk of proliferative diabetic retinopathy in adulthood *(Paper 2)*
3. To investigate genetic risk factors for famine-linked risk of PDR *(Papers 3, 4)*

3 Methodological considerations

Detailed materials and methods for each project are described in the respective paper or manuscript. In this chapter, a brief description of the cohorts and discussion of methods and their limitations will be provided.

3.1 Cohorts

The main aims of this Ph.D project were to study the influence of the intrauterine famine on PDR, to investigate genetic risk factors of famine-related PDR, and to investigate novel diabetes subgroups in the Ukrainian population. A large part of the population in Ukraine experienced a man-made famine due to economic and political problems in the country in 1932-1933.[287] Industrial eastern regions were exposed to famine, in contrast to western regions of modern Ukraine, which were part of Poland until 1939 and did not experience famine. In the current project, we used two cohorts from the Ukrainian population, which included individuals with diabetes who were born before, during, and after years of famine in exposed as well as in unexposed regions.

3.1.1 The UNDR study

The Ukrainian National Diabetes Registry (UNDR) study is an entire Ukraine-based registry of patients with diabetes who were visiting hospitals during the period from 1999 to 2013.[287] To study the association of intrauterine famine with PDR (paper 2), we selected individuals from four geographically and climatically similar regions. There were two famine-exposed eastern regions and two unexposed western regions, Chernihiv and Kyiv and Rivne and Volyn, respectively. In brief, the cohort included individuals born between 1904 and 1977. In total, there were 101,095 individuals with clinical data and information on diabetic complications after performing quality control (QC) steps, of which 53,321 individuals were from famine-exposed and 47,774 were

from unexposed regions. Diagnosis and stages of DR were based on visual examination of fundus photography performed by ophthalmologists. PDR was defined as the presence of either proliferative DR or blindness. A detailed cohort description and QC steps were defined elsewhere.[287,328] Famine exposure in the UNDR was defined based on region and year of birth. The study was approved by the ethics committee of Komisarenko Institute of Endocrinology and Metabolism in Ukraine (Dnr3/2006-11-10) and by the Norwegian ethics committee (Norway: 2019/28968).

3.1.2 The DOLCE study

The Diagnostic Optimization and Treatment of Diabetes and its Complications in the Chernihiv Region (DOLCE) is a hospital- and primary healthcare-based study, which includes patients with diabetes from Chernihiv town, the famine-exposed region of Ukraine. In addition to anthropometric measurements, the DOLCE cohort also included fasting blood glucose, plasma and serum samples, from which C-peptide, insulin, lipids, and GAD antibodies were obtained. Insulin secretion (HOMA2-B) and resistance (HOMA2-IR) were estimated using HOMA indexes based on fasting glucose and c-peptide and using HOMA2 calculator.[76,77] Individuals from this cohort were genotyped for candidate SNPs and GWAS using Infinium CoreExome24 v1 (Illumina) chip.[329] PDR was defined as either presence of PDR, blindness, or laser treatment. This cohort was used in the analysis in Papers 1, 3, and 4.[330] The study got approval from the local ethics committees (approval number for Ukraine Dnr17/2011–09–14; for Norway 2019/28968).

3.2 Methods

3.2.1 Cluster analysis

To examine novel diabetes subgroups in the DOLCE cohort in Paper 1, we applied clustering approach proposed by Ahlqvist et al. in patients with new-onset and long-term diabetes.[75] First, we selected GAD-positive individuals and assigned them to the subgroup with autoimmune diabetes (SAID). On the remaining individuals with clinically diagnosed T2D, we performed k-means clustering on the same set of variables as in the original paper: HbA1c, HOMA2-B, HOMA2-IR, BMI, and age at onset.[75] Before performing cluster analysis, we performed essential quality control (QC) steps on the data (details in Paper 1). To avoid bias due to the higher impact of units of measurements, variables were standardized (subtracted with the mean and divided by standard deviation), male and female participants were analysed separately.

K-means clustering is an unsupervised machine learning algorithm that aims to find a structure in the data based on the distance between the data points.[331] First, the algorithm randomly selects k points as centers of each cluster. In the next step, the algorithm calculates the Euclidian distance of each data point to cluster centres based on variables used for clustering and assigns each data point to the closest cluster. The most important parameters which influence the cluster algorithm performance are the number of clusters (k) and initial values of the cluster centres. Cluster algorithm performs well and identifies structure in the data correctly when data points are more similar inside clusters and differ significantly between clusters, and when the clusters are stable.[331]

An important limitation of the current method used for the long-term diabetes group was that variables used in the analysis do not remain stable through the diabetes duration. For instance, GAD antibodies measured in years after diabetes duration could be negative, even though they were positive at the onset of diabetes.[332,333] Despite the GAD antibodies being the most frequent among all diabetes antibodies, if we would

have information for other types of antibodies, it could identify more individuals with autoimmune diabetes. Although the bias due to these issues would not be very substantial, individuals with autoimmune or monogenic diabetes (because we did not perform sequencing of MODY genes in our cohort) may be misclassified and analyzed together with T2D. Moreover, all parameters used in the cluster analysis are interdependent, and in the course of the disease interdependency between parameters may change. For instance, with the progression of diabetes insulin secretion decreases, and insulin resistance and HbA1c usually increase. Additionally, these parameters are dependent on nutrition, physical activity, diabetes treatment, and BMI. The HOMA index calculation, which relies on c-peptide and fasting glucose, might also be biased with disease duration due to changing c-peptide (Ahlqvist et al., Supplementary Appendix, Figure S13).[75]

Limited sample size may also impact the performance of the clustering algorithm. Even though there are no assumptions for sample size or data distribution for the k-means clustering algorithm, a larger sample size can have an impact on the number of clusters and a clearer separation between clusters. Therefore, an increased sample size could lead to better algorithm performance and stronger cluster stability in the DOLCE cohort.

3.2.2 Epidemiological analysis

We estimated the association of PDR and intrauterine famine exposure in the UNDR cohort in Paper 2. Before performing statistical analyses, QC was performed (details in Supplementary Materials for Paper 2). In the first step, we calculated the OR for PDR in famine-exposed and unexposed regions for each year of birth using logistic regression (**Figure 1, A**). In the second step, ORs for interaction term between famine region and year of birth (YOB) were calculated for each year of birth in the entire cohort using logistic regression (**Figure 1, B)**. To avoid bias due to age by comparing the risks of PDR in individuals with younger age as a reference, we used the following age groups: individuals with YOB in 1929–1936 were compared to individuals with

YOB before 1928, individuals with YOB in 1937–1946 were compared to individuals with YOB 1936, and individuals with YOB after 1947 were compared to individuals with YOB 1946 (**Figure 1, B**). We adjusted logistic regression for sex, duration of diabetes, hypertension, and a year of diagnosis. HbA1c measurements were available only for 18,507 (~13%) individuals, therefore we used this adjustment only in the combined analysis.

In the combined analysis, we compared the prevalence and OR of PDR in individuals born in famine years (YOB in 1932-1934, 1941-1945, 1947) vs non-famine years before 1950 (YOB in 1904-1931, 1935-1940, 1946, 1948-1950) and after 1950, separately in famine exposed and unexposed regions (**Figure 1, C**). We did not find a significant difference in PDR risk between exposed and unexposed years in decades before 1950, therefore we combined YOB into decades before 1950 (famine decades) and after 1950 (non-famine decades). We defined YOB 1950 as a cutoff for decades of famine exposure (**Figure 1, D)**. Then we compared risks of PDR in individuals born in famine (YOB≤1950) vs non-famine decades (YOB>1950) separately in exposed and unexposed regions to confirm that elevated risk of PDR is attributable to famine exposure, but not to a decade of birth. We used logistic regression to calculate OR for PDR adjusting it for sex, diabetes duration, age, HbA1c, and year of diagnosis. All statistical analyses were performed using R software.[334]

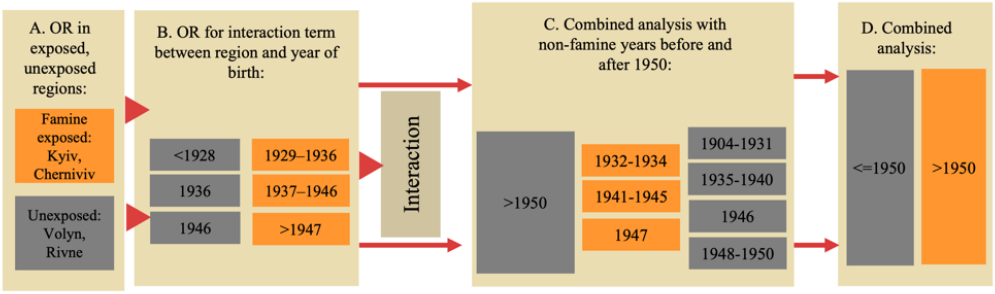

Figure 1. *Schematic analysis plan for Paper 2.*

This study design has some limitations. First, since we included in the analyses individuals who were over 60 years old and who went through adverse events in early life, we cannot exclude survival bias. Due to exposure to intrauterine famine, weaker individuals could die and stronger individuals, who possibly carry genes responsible for surviving in adverse conditions, could survive. Moreover, selective mortality could also play a role, meaning that those who were suffering from the more severe famine died earlier from the more severe representation of diabetes and other diseases, and therefore we analyzed cases with a less severe famine exposure.

Unregistered population migration could also lead to a bias in the data, if individuals who were born in unexposed regions and then migrated to famine regions were analyzed as those exposed to famine. Additionally, in our study we did not have information about registered calories intake, therefore, we could not define the severity of the famine exposure. However, this limitation is related to all famine birth cohorts, except the Dutch cohort where calories intake was registered.[335] Additionally, due to the limited sample size, we could not perform stratified analysis by famine exposure in different trimesters of pregnancy. This limitation might have impacted association results of different phenotypes with intrauterine famine exposure depending on the period of pregnancy and fetus development.[335]

3.2.3 Candidate gene analysis

We used the candidate gene approach in Papers 1 and 3 in the DOLCE study.[330] In Paper 1, we performed candidate gene analysis for 62 SNPs associated with T2D (details in Paper 1, chapter Genetics) [336] in 1,115 individuals with long-term diabetes with the goal to investigate the association of genetic variants with novel diabetes subgroups. For this, we used logistic regression adjusted for sex and age, using the insulin-resistant obesity diabetes (IROD2) subgroup as a reference.

In Paper 3, our aim was to investigate the effect of genetic risk factors in the context of intrauterine famine exposure on the risk of developing PDR in adulthood for 3,583 individuals from the DOLCE cohort. We tested 76 candidate SNPs that were associated with diabetes and related traits (details in Paper 3, chapter Genetics). Since all individuals from the DOLCE cohort were from famine exposed region, we defined famine exposure based on YOB ('famine exposed' for YOB before 1950, 'not exposed' for YOB after 1950, coded as 1 and 0 respectively). Before performing the genetic association test, we performed QC of the data (details in Supplementary Materials of Paper 3). We used the gene-environment interaction approach to test the hypothesis that the effect of genetics on the risk of PDR might be mediated by intrauterine famine exposure.[103] Since we had family relatedness in the data, we used a generalized estimation equation (GEE) adjusting for risk factors (sex, duration of diabetes, age, and HbA1c) and correcting for family relationships.[337] It is important to account for relatedness in genetic association studies to decrease the type 1 error rate which could appear due to the correlation of phenotypes between individuals with shared genetic background.[338] The traditional regression model does not address this issue since it assumes independence of all samples and therefore is not suitable for the analysis of such data.

To test that SNPs have significantly different effects in famine-exposed and unexposed groups, we analyzed the heterogeneity of effect sizes in these groups. First, GEE was performed separately in famine exposed and unexposed groups, and then using Cochran's Q-test for heterogeneity[339] we estimated the probability that variability of effect sizes between groups was larger than variability inside the groups. To obtain estimates for heterogeneity, we used I^2 index, which represents the proportion of variability across studies due to actual difference and not due to chance. I^2 was calculated based on results from Cochran's Q-test using the formula:

$$I^2 = \frac{Q - df}{Q \times 100\%}$$

Where Q is Q-value, and *df is* degrees of freedom.[340] All statistical analyses were performed using R software and plink v. 1.07.[334,341]

An important weakness of candidate gene analysis in our study was the limited sample size (N=3,583 in Paper 3 and N=1,115 in Paper 1), which may increase rate of type 2 error, i.e. false-negative results. To increase the sample size and power of the study in paper 1, we included individuals with NPDR in the control group, in addition to individuals without any DR. However, as the mean duration of diabetes in the DOLCE cohort was only six years, these persons potentially could develop PDR later with diabetes progression and therefore they could fail to be true controls. Therefore, the genetic analysis could be biased. However, a similar definition of controls was used in the recent GWAS on severe DR, where authors reported that approximately 50% of the individuals without DR at baseline will develop DR in the first five years of diabetes and 25% of individuals with NPDR will recover fully in the same period.[235]

Moreover, we compare individuals from significantly different age groups which could bring bias due to more severe diabetes and comorbidities in older people and therefore higher risk of PDR. However, this limitation is also relevant for the majority of studies on famine exposures which were usually based on historical cohorts, where data was collected at the time of exposure. Consequently, exposed and unexposed groups were segregated by the timing of exposure in the gestation stage and unexposed individuals were usually older or younger than exposed.[342] Additionally, since people who were included in analyses were over 60 years old, we could not rule out the potential effect of life events (stress, quality of food, toxins, climate, radiation, smoking, alcohol, etc.), which could contribute additionally to the increased risk of the disease.

3.2.4 Genome-wide association and interaction analysis

We performed genome-wide association (GWAS) and interaction (GWIS) studies in the DOLCE cohort with the aim to investigate the association of SNPs with

PDR (GWAS), and to test the effect of interaction between intrauterine famine and genetics on PDR (GWIS) in Paper 4.

On 551,839 directly genotyped SNPs, we applied standard QC steps for GWAS studies (details in Supplementary information in Paper 4).[343] We excluded population outliers and examined the population structure of our cohort, by performing a Principal Component Analysis (PCA). PCA is a machine learning algorithm that aims to reduce the dimensionality of the data by computing a new set of variables called "principal components" (PC) that keep maximum variability of the data.[344] This algorithm is widely used in GWAS studies with the aim to estimate homogeneity of the sample and sample ancestry. By plotting the first two PC, we demonstrated that individuals in the DOLCE cohort closely overlap with the European ancestry meaning that the European population can be used for imputation despite the potential diversity of the Ukrainian genome.[345] Imputation is a standard procedure for GWASs that predicts genotypes at SNPs which were not directly genotyped in the sample, based on correlation and linkage disequilibrium (LD) with directly genotyped variants.[346] This procedure increases the statistical power of the association study by increasing genomic coverage and is useful for performing meta-analysis combining cohorts genotyped on different platforms.[347] We imputed SNPs on Michigan Imputation Server using the reference panel with European population HRC r1.1 2016. After imputation, we filtered out SNPs with low imputation score ($INFO_{score}<0.4$) and rare and low frequency SNPs ($MAF<0.05$).

We ended up with 2,925 individuals included in the analyses (1,364 were exposed to famine, and 1,561 unexposed), 91 (3%) of them had PDR. Since data for HbA1c was not available for all individuals in the cohort, analysis with HbA1c adjustment was performed additionally ($N=2,925$, $N_{HbA1c}=2,305$). For GWAS, we used linear mixed model (LMM) adjusting for sex, duration of diabetes, and age as fixed effects and for Genetic Relationship Matrix (GRM) as a random effect to account for population structure and cryptic relatedness in order to control for type 1 error rate.[348] The GRM reflects the genetic correlation between each pair of individuals in the dataset

and is calculated based on SNP data.[349] LMM is commonly applied in human genetic studies when there are clusters or correlations in the data, since it accounts for the difference of effects within the groups (fixed effects) and between groups (random effects).[350] For GWIS we used LMM with interaction term adjusting for the main SNP and famine exposure effects. We performed complementary analysis using logistic regression and excluding related individuals (N=52), since LMM is known to produce higher rate of type 1 error especially in situations with imbalanced case/control ratio.[351] We adjusted the model for sex, age, diabetes duration, HbA1c and the first two PC to account for population structure. GWAS and PCA were performed using GCTA software,[352] GWIS was performed in GEMMA software[353], QC steps and logistic regression were performed using plink software.[341]

The main weakness of this study was the limited sample size. Due to a big number of association tests and moderate effect size of common SNPs, GWAS studies require a large sample size to be able to reach sufficient power to identify genome-wide significant SNPs (with $p<5\times10^{-8}$).[354] This issue is even more important for genome-wide interaction studies due to the higher number of statistical tests being performed.[355] Moreover, the duration of diabetes in the DOLCE cohort was short (mean 6 years), therefore the prevalence of PDR in the cohort was low (3%). This can contribute to the higher OR observed in our study as it is expected for common SNPs in GWASs where the prevalence of outcome reported to be higher than 10%. Especially it concerns GWIS since OR for the interaction term is calculated based on allele counts in cases and controls in famine exposed and unexposed subgroups. Therefore, due to stratification into eight cells, allele counts might be even more imbalanced compared to OR for GWAS, where only 4 cells are compared. Additionally, an imbalanced case-control design together with a limited sample size decreased the power of the genetic analysis and led to stricter QC in our cohort. A low proportion of cases in the cohort produced an insufficient number of minor allele counts (MAC) for SNPs with lower MAF, and therefore the chi-square test could not be performed for these SNPs. Due to this, SNPs with MAC<5 and MAF<0.1 were excluded from the analyses in each

subgroup. With this filter, we possibly also excluded SNPs which could have a significant association in cohorts with larger sample size and longer diabetes duration.

3.2.5 Gene and gene-set analyses

To observe the cumulative effect of SNPs on PDR with the goal to increase power by decreasing the number of statistical tests, we performed gene and gene-set enrichment tests based on p-values from GWAS and GWIS in Paper 4. In gene analysis, SNPs were assigned to genes based on genomic position, and then the association of phenotype and gene was tested based on SNPs summary statistics. Then, gene-set analysis (pathway analysis) combined genes in pathways and tested the association of pathways and phenotype. In our project, we used Gene Ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.[356,357] Analysis was performed using MAGMA software.[358]

3.2.6 DNA methylation and gene expression lookups

To look at potential epigenetic effects, in Paper 3, we performed DNA methylation analysis on our data and look-ups in publicly available databases. We executed methylation analysis separately in famine-exposed and unexposed participants from the DOLCE study (in total n=51) to investigate possible epigenetic changes which might be induced by famine exposure. We also did a lookup in the publicly available mother-child database ALSPAC to investigate potential methylome QTL effects and to deposit tissue biobank GTEX database to investigate gene expression for our top SNPs in different cohorts.[359,360]

3.2.7 Correction for multiple testing

When performing numerous statistical tests, it is essential to avoid false-positive results i.e. type 1 error by accounting for multiple testing corrections.[361] There are

several methods to adjust p-values for multiple testing, the most popular of them are Bonferroni correction, Holm-Bonferroni method (or False Discovery Rate, FDR), Hochberg's procedure, and Dunnet's correction.[362] For genetic analyses in papers 1, 3, and 4, we used Bonferroni correction, which is the strictest and the most conservative method commonly used in genetic studies.[363] In this method, the adjusted p-value is calculated by the division by the number of independent tests. For pathway analyses in paper 4, we used different thresholds for gene analysis and for gene-set pathways analyses dividing 0.05 by a number of genes, numbers of pathways in GO, and KEGG respectively. In paper 1, for the analysis of risks of diabetes complications we adjusted p-values using the Benjamini & Hochberg (or FDR) method. This method at first step ranks all p-values in the data and relies on the following formula:

$$FDR_i = \frac{0.05 \times N}{i}$$

where N is the total number of tests, and i is a number in ranking for this p-value.[364]

4 Summary of the papers

4.1 Paper 1: Novel Reclassification of Adult Diabetes Is Useful to Distinguish Stages of β-Cell Function Linked to the Risk of Vascular Complications: The DOLCE Study From Northern Ukraine

The aim of the first study was to describe the novel diabetes subgroups in the Ukrainian cohort and to assess performance of the clustering algorithm in the cohort with long-term diabetes. We used the clustering approach published by Alquist et al., which performs clustering of diabetes patients in five novel diabetes subgroups based on six affordable clinical variables and which was successfully replicated in several populations.[75] We used the DOLCE cohort, which included individuals with new-onset (N=887, mean±SD, mean duration=1.1±1.1 years) and long-term (N=1,253, duration=11.0±6.9 years) diabetes. We performed k-means cluster analysis and estimated the risk and prevalence of complications for new-onset and long-term diabetes groups. Additionally, we performed genetic analysis for 76 candidate SNPs reported in DIAGRAM GWAS meta-analyses to find associations with the severity of diabetes and complications.[336]

Subgroups in the new-onset group of patients from the DOLCE cohort had similar characteristics as in the original study. However, in the long-term group novel diabetes subgroups differed from the original characteristics. The prevalence of severe autoimmune (SAID) and severe insulin-deficient (SIDD) diabetes subgroups in the long-term diabetes group was approximately twice as high compared to the new-onset group. These two severe subgroups were similar to the equal new-onset diabetes subgroups, characterized with the lowest insulin secretion HOMA2-B, highest HbA1c, and low age at onset. In long-term diabetes, we did not differentiate the original insulin resistance subgroup (SIRD) and obesity diabetes (MOD) subgroups, in contrast we had

two subgroups with admixed characteristics. Severe insulin resistance obesity diabetes 1 (IROD1) in long-term diabetes had, similarly to the original SIRD, the highest insulin resistance HOMA2-IR, however, insulin secretion was lower. The severe insulin resistance obesity diabetes 2 (IROD2) subgroup had likewise elevated insulin resistance as IROD1, however insulin secretion was higher, and HbA1c was lower. In contrast to other studies, the IROD1 subgroup was not characterized by the highest prevalence of CKD while the SIDD subgroup was characterized by the highest prevalence of all complications including CKD. There was an important observation that despite high HOMA2-IR, the IROD2 subgroup had a low prevalence and risks of all microvascular complications (Figure 2.1, 2.2). Therefore, we used this group as a reference in genetic analysis and showed an association of rs7903146 in *TCF7L2* with more severe diabetes phenotypes and risk of complications.

Altogether Ukrainian new-onset subgroup had a more severe representation of diabetes and less frequently prescribed insulin treatment compared to the ANDIS cohort. Lower insulin secretion in DOLCE may be partially explained by exposure to famine in 1932-33 in Ukraine. Individuals who were born in these years and were exposed to famine in utero could have a malformation of the pancreas which could lead to a reduced number of β-cells and consequently affect insulin secretion later in life. The elevated prevalence of PDR in the SAID and SIDD clusters emphasises importance of insulin secretion additionally to hyperglycemia in retinopathy pathogenesis. Therefore, we suggest that it might be beneficial for individuals from SIDD subgroup to prescribe insulin treatment on earlier stages of diabetes to preserve β-cell function and to delay progression to PDR. Additionally, the IROD2 subgroup in long-term diabetes with preserved β-cell function and low risk of complications may represent an interesting group for investigation of possible protective mechanisms.

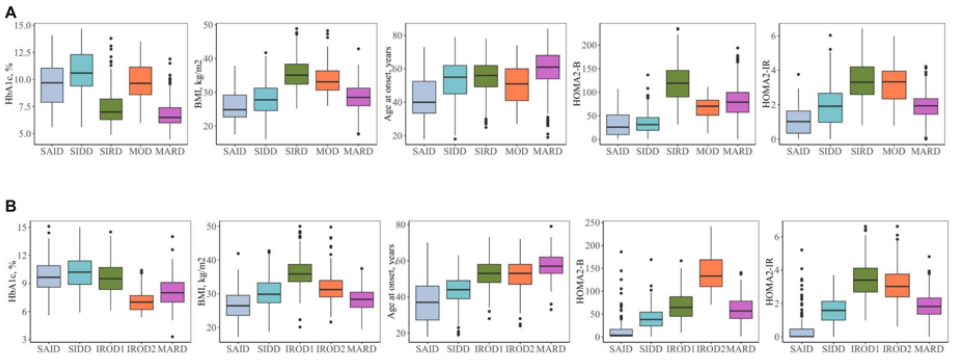

Figure 2.1. *Clinical characteristics of the clusters. (A) Individuals with new-onset adult diabetes with less than 3 years of disease duration (n = 887). (B) Individuals with long-term adult diabetes with more than 3 years of disease duration (n = 1,253).*

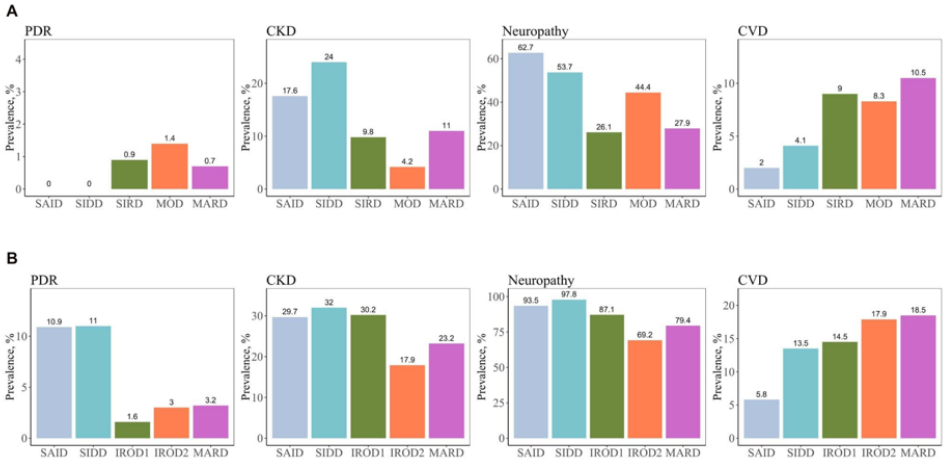

Figure 2.2. *Prevalence of macro- and microvascular complications in different clusters of adult patients with the new-onset and long-term diabetes. (A) Individuals with new-onset adult diabetes with less than 3 years of disease duration (n = 887). (B) Individuals with long-term adult diabetes with more than 3 years of disease duration (n = 1,253).*

4.2 Paper 2: Perinatal famine is associated with excess risk of proliferative retinopathy in patients with type 2 diabetes

In Paper 2, the aim was to investigate effects of the intrauterine exposure to famine on the risk of PDR in patients with T2D from Ukraine. We used the UNDR study (N=101,095) which included individuals born in famine-exposed and unexposed regions.

We demonstrated that individuals who were exposed to intrauterine famine during the Holodomor had 1.76-fold (p=0.019) increased risks of PDR, and those who were born during WWI had 3.02-fold (p=0.001) increased risk of PDR compared to individuals from unexposed regions (Figure 3). These results were independent of sex, diabetes duration, and year of diagnosis. In conclusion, we demonstrated that exposure to famine during intrauterine development is associated with the increased risk of PDR in adulthood and this was independent from HbA1c. Further studies will help to understand the biological underpinnings of this association.

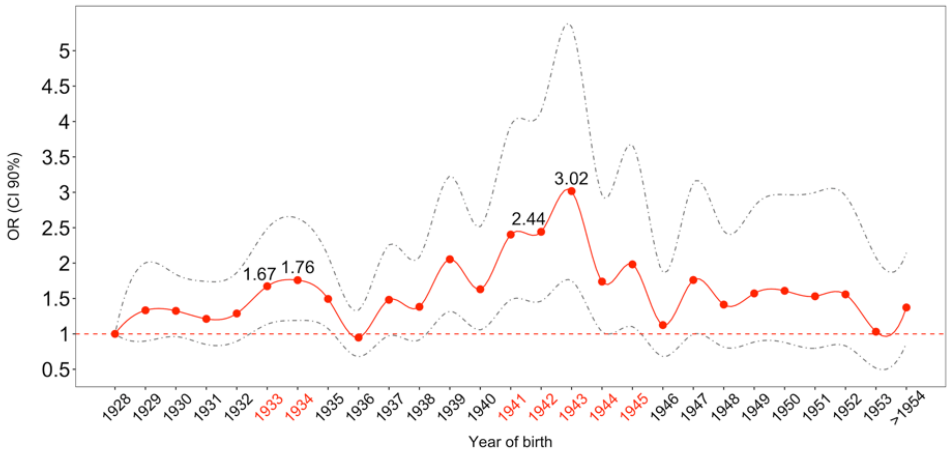

Figure 3. *Cross-over odds ratios for PDR in adulthood stratified by the years of birth (the UNDR study). Odds ratios depicted as curves above the reference line (which equals one) denote elevated risk of PDR in famine- exposed regions. Years of birth marked in red on the*

X-axis indicate periods of exposure to the Holodomor at conception (1932–1933) and WWII (1941–1945) famines.

4.3 Paper 3: Neuronal dysfunction is linked to the famine-associated risk of proliferative retinopathy in patients with type 2 diabetes

The aim of paper 3 was to investigate molecular mechanisms behind the association of exposure to intrauterine famine and increased risk of PDR in adult individuals with T2D. We used the DOLCE cohort (n=3,583) and tested 76 candidate SNPs for association with PDR under famine exposure using gene-environment interaction.

We found a significantly elevated risk for PDR in variants located in *ADRA2A PCSK9*, and *CYP2C19*2*, and a significantly decreased risk for the variant in *PROX1* gene (Table 1). Association of *ADRA2A* was significant after Bonferroni correction. For studying the functional effects, we modelled glucose starvation in embryonic retinal cells and demonstrated increased expression of *Adra2a* and *Pcsk9,* but decreased *Prox1* genes. In conclusion, famine exposure during intrauterine development may trigger a risk of PDR in adulthood in individuals with T2D and this risk may be related to the function of neurons. The results of our study highlight the importance of neuronal damage as a potential early effect of PDR, which could trigger disease development later in adult life under a diabetes environment. We suggest that neuroprotective drugs may be effective in the early stages of the disease and may contribute to prevention or protection from development of more severe forms of DR.

Gene	SNP	Exposed to famine	Unexposed	Interaction	Effect size heterogeneity	
		OR (90% CI)	OR (90% CI)	p-value	I^2	Qep
ADRA2A	rs10885122	3.67 (1.77 - 7.63)	0.45 (0.28 - 0.71)	0.003	94	0.00006*
PCSK9	rs2479409	2.27 (1.26 - 4.06)	0.59 (0.37 - 0.94)	0.021	89	0.00313
PROX1	rs340874	0.54 (0.32 - 0.89)	1.57 (1.05 - 2.35)	0.045	87	0.00642
*CYP2C19*2*	rs4244285	2.87 (1.23 - 6.68)	0.48 (0.23 - 0.99)	0.040	86	0.00826

Table 1. *Genetic variants and the risk of PDR for offspring to parents exposed and unexposed to famine in the DOLCE cohort. *significant after Bonferroni correction.*

4.4 Paper 4: Genome-wide association and interaction analyses for severe diabetic retinopathy

The aim of paper 4 was to investigate the molecular underpinnings of famine-related PDR by performing GWAS and GWIS studies. We used the DOLCE cohort with individuals from the famine-exposed region of Northern Ukraine who were born in decades with and without famine exposure. First, we performed association tests using an MLM adjusted for the risk factors and GRM. Then, based on p-values from the association tests, we performed gene and gene-set (pathway) enrichment analyses.

In GWAS, the strongest SNP rs3795299 ($p=1.05\times10^{-6}$) located in the protein-coding gene *IL22RA1,* which was also the top hit in the gene analysis ($p=3.19\times10^{-5}$). The strongest association in the gene-set analysis was a response to ketones ($p=1.27\times10^{-4}$) in GO terms and base excision repair ($p=2.0\times10^{-2}$) in KEGG pathways. In GWIS the strongest SNP was intronic variant rs1506783 ($p=1.29\times10^{-7}$) located in the *PAPPA2* gene. The most biologically interesting was rs2230805 ($p=4.44\times10^{-6}$) located on the protein-coding *ABCA1* gene, which also reached borderline genome-wide significance in the gene analysis with $p=4.31\times10^{-6}$. For GWIS the strongest pathway was DNA binding ($p=1.76\times10^{-6}$) in GO terms and tryptophan metabolism (p=

1.27×10^{-4}) and glycerolipid metabolism ($p=5.45\times10^{-4}$) pathway in KEGG pathways. In conclusion, we identified pathways that particularly underlined the importance of lipid metabolism and inflammatory processes in the PDR development for individuals exposed to intrauterine famine.

5 Discussion

By studying novel diabetes subgroups in the Ukrainian population, we confirmed the highest prevalence and risk of PDR in the severe insulin-deficient subgroup (SIDD). We also demonstrated that the lowest prevalence of PDR was in the insulin-resistance obesity diabetes 2 (IROD2) subgroup, which was characterised by the highest insulin secretion in long-term diabetes. We suggested that increased PDR risk may be associated with decrease insulin secretion additionally to hyperglycaemia. We identified an association between PDR and intrauterine exposure to famine in individuals with T2D suggesting that early life events could act as a trigger and increase the risk of PDR under diabetes environment in adult life. When investigating possible molecular links between the association of exposure to famine and PDR, we obtained results that supported the hypothesis that intrauterine factors could have an impact on neurodegenerative processes later in life and this may facilitate the development of PDR. Additionally, inflammation and lipid metabolism pathways were highlighted as the most significant contributors in the development of PDR based on GWAS results in a population with T2D that was perinatally exposed to famine.

5.1 Importance of novel diabetes subgroups for identification of individuals with a high risk of PDR

In paper 1, we performed a cluster analysis in the Ukrainian cohort of individuals with diabetes aiming to identify the subgroups at a high risk of progression to vascular diabetic complications and to assess the performance of suggested approach in individuals with long-term diabetes.[330] We used the clustering approach proposed by Ahlqvist et al., which relies on the main known clinical parameters involved in disease pathogenesis: autoimmunity, insulin secretion or β-cell function, insulin resistance, obesity, and aging.[75] As a result, we classified adult patients with diabetes into subgroups based on the underlying pathophysiology. Advantageous to the previously

offered data-driven approach,[74] this method used measurement of β-cell function, which reflects the central defect in diabetes.[46] All variables used in the analysis are affordable, not expensive to measure at clinical practice, and have strong prior evidence of being associated with the risk of diabetes development.[365,366]

The key finding of this cluster analysis was that the method has suggested two severe subgroups of T2D: insulin deficient and insulin resistant, which were linked to an increased risk of retinopathy and nephropathy. In addition, two mild subgroups of obese and elderly patients have been highlighted. Results of our analysis and other studies have demonstrated that these clusters of correlated phenotypes are well-replicated. However, these subgroups might change over time while the disease progresses, as well as the risk of associated complications.[80] To address this question, we performed analyses of cluster characteristics in patients with new-onset and long-term diabetes separately.

In the DOLCE cohort, the results of the cluster analysis in the long-term T2D group with the mean of 11 years diabetes duration showed to be somewhat different from the new-onset diabetes group. The SIDD cluster, similarly to other studies in the German, North American, Chinese, and Indian populations, was characterized by the highest prevalence of PDR, but also by the highest prevalence of neuropathy and CKD.[78-81] In patients with long-term diabetes, we could not differentiate original SIRD and MOD subgroups. In contrast, we found two insulin-resistant subgroups with admixed characteristics of SIRD and MOD. The insulin-resistant obese diabetes (IROD1) was comparable to the similar cluster in the Indian cohort and was characterized by lower insulin secretion compared to ANDIS, higher abdominal adiposity, and a high risk of CKD.[81] The most interesting finding lies in the fact that we identified a subgroup of insulin-resistant obese diabetes (IROD2), which despite the high insulin resistance had preserved β-cell function, lower HbA1c, and the lowest risks of microvascular complications. Our findings suggested that in years following the diagnosis the subgroups' characteristics may be modified by biochemical processes

in the body due to disease progression and treatment, and thus it would be reasonable to perform clustering across the different periods of disease duration.

The DOLCE cohort has certain population-specific differences. Since medical insurance is not a regular practice in Ukraine, persons who have signs of diabetes could postpone visits to the hospital and be diagnosed with diabetes at later stages, when the complications have developed into more severe forms.[7,367] The late referral of patients to the hospitals may result in an even longer actual duration of diabetes than reported. Different approaches to treatment and prescribed medications in Ukrainian hospitals may also have an effect on patients' characteristics, especially for individuals with long-term diabetes duration.[7] The genetic makeup of the Ukrainian population may differ from European cohorts.[345,368] Moreover, part of individuals from the DOLCE cohort was exposed to famine during early life,[330] which could contribute to a higher susceptibility and more severe diabetes presentation characterized by increased risks of complications, particularly CVD and possibly PDR.[287,328,369,370] It would be interesting to perform a cluster analysis on the Dutch population, which has a more similar healthcare system and level of life to Scandinavian countries, but was also exposed to famine.[371]

The clustering algorithm, therefore, needs to be optimized to account for differences in relationships between the clinical parameters due to population-specific and other ascertainment-associated factors. The k-means clustering algorithm presumes that all variables have the same weight and the same impact on the outcome (formation of clusters). However, dependency and influence of parameters might change when the disease progresses, as was mentioned in chapter 3.2.1. Additionally, in distinct populations impact of variables could be different. Therefore, it would be interesting to perform clustering assigning various degrees of importance to variables and consequently to regulate the contribution of the variables to the cluster formation. This could be done by using different scales of measurements for variables in k-means clustering algorithm.[372] Thus, for example, in our cohort from the Chernihiv region of Northern Ukraine insulin secretion may be the most important parameter. In the Indian population, which is generally characterized by higher insulin resistance and visceral

obesity, these two variables could get more weight compared to the Caucasian population. Nevertheless, the difficulty and potential inaccuracies in defining the weight of the variables would be an issue that could also bring bias to the formation of subgroups. A deeper understanding of disease pathogenesis in different populations is essential for the correct application of this method.

In summary, identification of severe clusters such as insulin deficient subgroup enables the detection of individuals at a high risk of developing microvascular complications, particularly retinopathy. In addition to our results, the SIDD subgroup was characterized by an increased risk of DR in ANDIS and in other studies, suggesting that this group is characterized by early onset of DR.[75,78-81] We further support this finding by demonstrating a low risk and prevalence of PDR in the subgroup with the highest insulin secretion, IROD2. Genetically, the significant association of the IROD2 subgroup with the lower frequency of the risk allele of rs7903146 in *TCF7L2,* a key gene linked to β-cell dysfunction, underlines the predisposition of this variant to severe T2D phenotypes and diabetes complications. This is in line with the results of the recent GWAS for T2D in the Japanese population, which demonstrated that PGRs for T2D is strongly associated with diabetes complications and particularly the significant association was detected for rs7903146 with DR.[148]

The association of the severe insulin-deficient subgroup (SIDD) with an elevated risk of PDR, and, an opposite, lowest risk of PDR in the subgroup with the highest insulin secretion (IROD2) supports the fact that β-cell secretory failure is a strong risk factor for DR additionally to hyperglacemia.[373,374] And it was even suggested that β-cell replacement may be efficient for the prevention of DR or from its progression to more severe sight-threatening forms (PDR).[374,375] Therefore, it would be beneficial to identify the SIDD subgroup with severe insulin deficiency in early stages of diabetes. The early initiation of insulin treatment in patients from this subgroup may prevent accelerated β-cell dysfunction and may prevent or delay progression to PDR. Interestingly, recent omics analyses from the Rhapsody consortia demonstrated the link of the severe insulin-deficient group to defects in leptin and growth pathways.[376] This

demonstrates the potential involvement of developmental pathologies in the pathophysiology of diabetic retinopathy and perhaps other vascular complications.

5.2 What mechanisms are triggering the progression of severe DR? Role of perinatal exposure to famine.

In paper 2, we demonstrated an association of the extreme intrauterine exposure to starvation (famine) and the increased risk of PDR in adulthood in individuals with T2D. The period of fetus development from conception to birth, when organs are developing and growing, is a critical time for the determination of future health.[267] It is well documented that early life events such as intrauterine starvation, maternal stress, exposure to extremely cold weather or to toxic food, etc have an impact on metabolic conditions in adult life. As was mentioned in chapter 1.3.2., many complex disorders in adult life were shown to be associated with diverse intrauterine exposures, particularly exposure to severe undernutrition. Several theories explaining this association were offered, starting from the evolutionary "thrifty genotype" hypothesis [310], following the "thrifty phenotype" hypothesis[315], and the Predictive Adaptive Response hypothesis.[321] Hence, the important question to address is: which mechanisms are driving PDR predisposition in patients with T2D from Ukraine who were exposed to famine during intrauterine development?

There could be several different potential mechanisms or a combination of them, which could drive this association (**Figure 4**). According to the "thrifty phenotype" hypothesis, due to the lack of nutrients in conditions of intrauterine exposure to famine, a fetus' pancreatic β-cell mass and function are reduced and the fetus adaptationally develops insulin resistance.[318] Since insulin is a major fetal growth hormone, these events may result in intrauterine growth retardation and, consequently, organs and tissues malformation and LBW of the infant.[314] Moreover, maternal body weight and size are proportionally associated with fetus' weight,[271,377] which means that the more

severe conditions of famine the mother experiences, the more severely the fetus weight can be affected. LBW may be one of the potential mechanisms explaining the increased risk of PDR (**Figure 4)**. Indeed, many adult disorders, including CVD, impaired glucose tolerance [378], T2D [379-381], hypertension [382], and end-stage renal failure [383,384] were reported to be associated with LBW. An increased predisposition to these diseases and the more severe forms of them, being risk factors for PDR, may contribute to the progression of PDR. For instance, more severe T2D characterized by an earlier age at onset and higher HbA1c, is a risk factor for progression of DR to PDR. Hypertension, being a risk factor for DR,[207] could also increase the odds of faster deterioration of DR [385,386], or risk of developing proliferative hypertensive retinopathy (**Figure 4)**[387]. Interestingly, our results have shown that the association of PDR and intrauterine famine is independent of HbA1c and hypertension (Paper 2, Fig.1C). [328]

LBW may also have a direct effect on DR without influencing disease progression through mediating risk factors (**Figure 4)**. LBW was shown to be associated with retinopathy of prematurity.[388,389] Moreover, reduced retina microvascular density in newborn kids was suggested to be associated with LBW.[390,391] Growth-related alterations in utero may contribute to the formation of fragile vessels, which could lead to developing more severe forms of DR under T2D exposure later in life. Nonetheless, the association between retinopathy and LBW in individuals with T2D has been inconclusive.[392,393] In addition, in the Dutch cohort LBW was observed only in individuals who were exposed to intrauterine famine in the first two trimesters of pregnancy.[288] Interestingly, adult disease such as intima-media thickness was shown to be associated with lower maternal calories intake independently of birth weight.[394] This evidence suggests that LBW is not the only potential explanation for this association and other factors and mechanisms may play an important role.

As described in chapter 1.3.3, according to the PAR hypothesis, metabolic processes are programmed during intrauterine development in an adaptative manner to environmental factors. If in adult life a “mismatch” to changes of the environment happens, an individual increases a chance of developing metabolic disorders.[272] Hence,

which mechanisms may drive the "intrauterine programming" predisposition to severe PDR? In other words, how developmental plasticity could influence the risk of PDR development in adulthood many years after intrauterine exposure to severe undernutrition with or without alteration in birth weight?

One explanation that deserves attention is tissue and organ-specific adaptations in metabolism during intrauterine development which may lead to lifelong irreversible changes in organ structure (**Figure 4)**.[272] During gestation and early postnatal life every organ has a critical period of active development characterized by the differentiation and maturation of its tissues.[271,395-397] While famine exposure affects the maternal environment during the period important for fetal organ formation, it could also alter organ development.[318] The programming could also be a result of altered fetus' and placenta' hormonal concentrations which modify the sensitivity of various tissues to these hormones (**Figure 4)**.[272,398,399] As a consequence, due to these alterations, the function of the organ in the future adult life may be compromised. For instance, such intrauterine environments as undernutrition and hypoxia were shown to be associated with a decreased nephrons number and therefore, an increased risk of hypertension and renal disease in adulthood.[400] Additionally, intrauterine undernutrition was shown to have an impact on a number of pancreatic β-cell and islet vascularization, and consequently increased risk for impaired glucose tolerance in adulthood.[401]

Therefore, if famine exposure affects the mother on 7-10 weeks of gestation when the fetus' retina is developing, it may influence retina structure.[402] In this case, vessels of the retina may be underdeveloped and have less density, and therefore may be more prone to further damages caused by external factors (such as high glucose levels). It may also affect the neuronal unit. Underdeveloped neurons and vessels in the retina may form a weak interconnectivity and in a diabetes environment later in life could exert high susceptibility to disruption of the entire NVU. These events consequently may increase predisposition to microvascular abnormalities and neovascularization causing the more severe forms of DR (**Figure 4)**.

Another potential mechanism involved in the disruption of NVU in adulthood could be epigenetics. There is solid evidence that epigenetics may change the development of different phenotypes depending on early life exposure and that effect may last not only in utero but also in adult life. Studies on Dutch famine revealed that methylation of several genes was decreased even 60 years after famine exposure.[403-405] Epigenetics was suggested as a potential mechanism, which underlies the increased risk of metabolic disorders after famine exposure in early life.[406] It was shown that epigenetic dysregulation may have an impact on the onset, latency period, and progression of neurodegenerative disorders.[407,408] The 'Latent Early-life Associated Regulation' (LEARn) model suggests that altered expression of genes caused by the specific environment during intrauterine development could have an impact on neurobiological disorders in adulthood, particularly in the situations when additional environmental factors, such as stress or head trauma, or poor diet in adult life, trigger the disease.[409] Moreover, the intrauterine insult on neuronal unit may have an impact on neurodegeneration later in life.[410] Altered gene expression may impact neurodegeneration in the retina (**Figure 4)**, the important mechanism in pathogenesis of PDR, which will be described in detail in the next chapter 5.3.

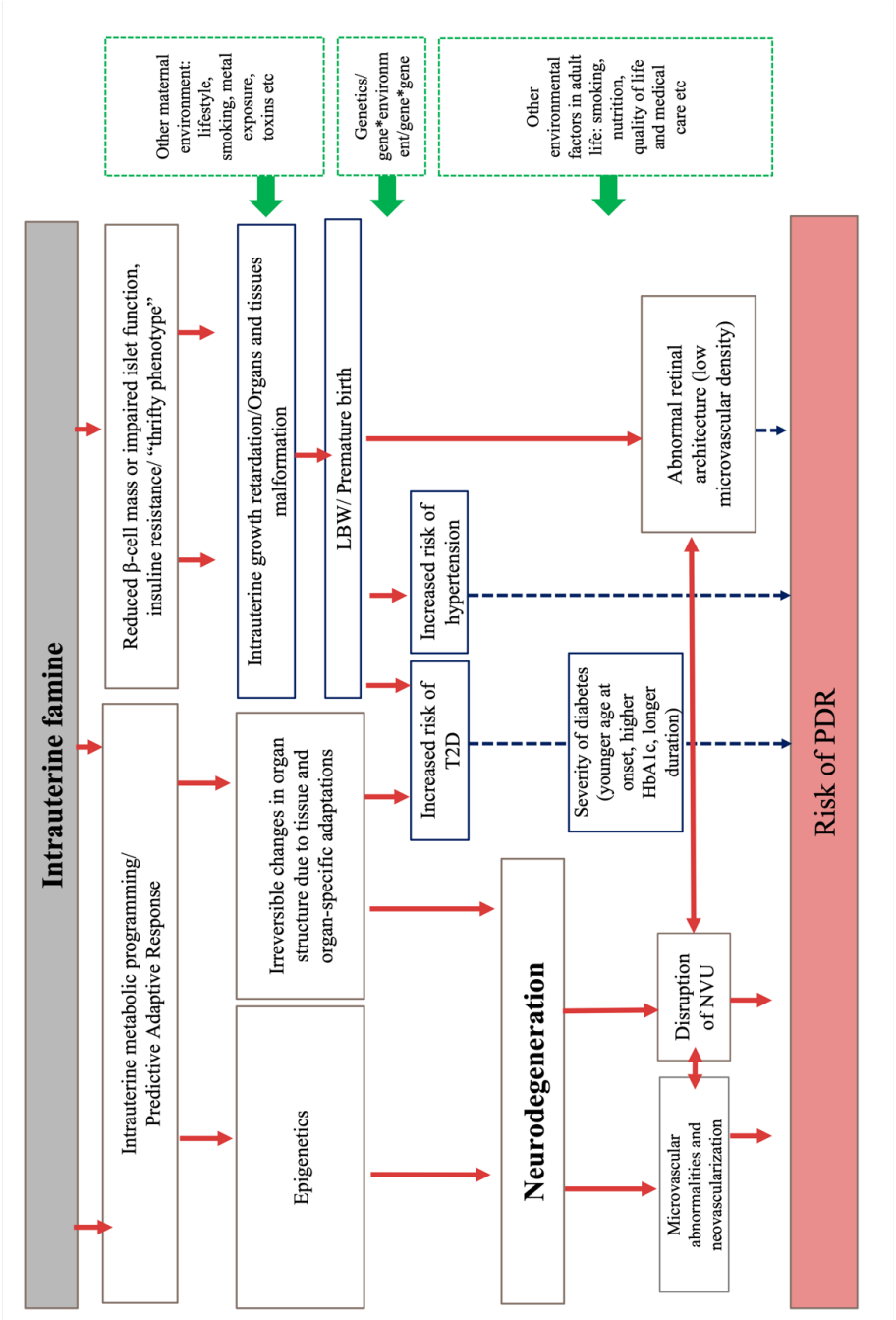

Figure 4. *Potential mechanisms which may trigger the progression of PDR under perinatal famine exposure. Red lines represent direct effects and blue dash lines represent indirect effects.*

5.3 Role of the neuronal unit in PDR pathogenesis after intrauterine famine exposure

In paper 3, our results of candidate gene analysis suggested that neuronal damage due to intrauterine starvation may be an early event that increases risks of PDR for individuals who develop diabetes later in life.[328] DR was traditionally classified as a microvascular disease since the major damages and abnormalities are related to the defects of blood vessels. In our study, we strengthen the link of neurodegeneration as a potentially early event in pathogenesis of PDR.[168] Thus, what is the importance of neuronal unit and neurodegenerative processes in PDR pathogenesis? And through which molecular mechanisms the exposure to famine in utero might induce neurodegenerative processes in many years in adult life?

Recent studies illustrated that the neurovascular unit (NVU) plays a significant role in DR pathogenesis.[411-416] The retinal NVU is characterized by a physical and biochemical relationship between neuronal and vascular units (**Figure** 5).[417,418] The neuronal unit includes mainly retinal ganglion cells, glial cells, and other neuronal cells and is responsible for energy balance and communication between cells.[266,411] The vascular unit includes endothelial cells and pericytes (**Figure 5**) and maintains vascular integrity.[194] The process when factors such as hyperglycemia impairs the structure and function of retinal neuronal cells is called neurodegeneration, and it was already described and studied in 1990.[419] Müller cell is the core of the neurovascular unit in the eye and the most frequent type of retinal glial cells that plays a central role in retina metabolism, nutrition, and signals transmission.[420] When the connection between the Müller cells and the vascular unit is affected, neovascularization in the retina is induced.[421] Müller cells start to produce more VEGF, which have a neuroprotective function and help neurons to survive.[422] However, an uncontrolled amount of VEGF might induce vessel leakage, which is one of the most crucial events in the early stages of diabetic retinopathy.[422,423] Neurodegeneration was observed in the retina of individuals with diabetes even without any microvascular abnormalities.[424,425] Thus,

neurodegeneration was suggested to be an early sign of DR which consequently may lead to the damage of the vascular unit of the retina, and was suggested to be useful in modelling for DR prediction.[194,412,416,426]

The potential evidence that the intrauterine environment can impact neurodegeneration in the retina in adult life may be explained by the connection between the brain and retina. The retina and the brain frequently show similar pathologies and have similarities in their neuronal structure.[427,428] Retina belongs to the Central Nervous System (CNS) and shares with it similar biological properties, anatomic features, and embryologic origins.[427,428] Since the retina is an extension of the CNS, the examination of the eye was offered as a non-invasive method to the diagnosis of CNS diseases.[427] Biological pathways involved in neurodegenerative processes in the brain have also been identified in neurodegeneration in DR pathogenesis.[429] In a very recent paper, it was suggested that DR has common biological pathways with neurodegenerative Alzheimer's disease, referring to the fact that both diseases have neurodegenerative nature.[430]

Interestingly, it was shown that the first processes, which lead to such neurodegenerative brain disorders as Huntington[431,432] and Parkinson[431] diseases start as far back as during early embryonic development. Additionally, studies on the Dutch and Chinese famines provided solid evidence that neurodegenerative disorders such as Alzheimer disease, dementia, and schizophrenia, despite the fact that they develop in adulthood, are strongly associated with adverse events in early life including intrauterine exposure to famine.[371,433-435] It is not surprising, since compared to the adult brain, the developing brain is more vulnerable to exposure.[436,437] In animal models, it was shown that maternal exposure to adverse events such as exposure to powerful medicines or bacteria could induce neuronal loss and apoptosis in the brain of fetus rats.[438,439] Thus, extensive studies on intrauterine exposure and brain disorders might shed light on common mechanisms involved in the development of neuronal pathology in retinal disease.

Our findings support the hypothesis that famine exposure could affect neuronal function *in utero* and then later in life in the presence of other risk factors, it could trigger neurodegeneration and consequently increase the risk of neuronal disorders. Studying the influence of genetics on the risk of PDR in individuals exposed to extreme intrauterine undernutrition helped us to identify mechanisms and genes (such as *ADRA2A*, *PCSK9*, *PROX1*), which could be involved in the pathogenesis of compromised formation and function of neuronal unit in PDR. Our findings suggest that regular screening and detailed assessments of the neuronal unit could be useful for prediction of PDR at early stages, and consequently have a preventive value in progression to more severe forms of DR. A better understanding of the neuronal unit of the retina will help to improve the treatment and prediction of DR at earlier stages.

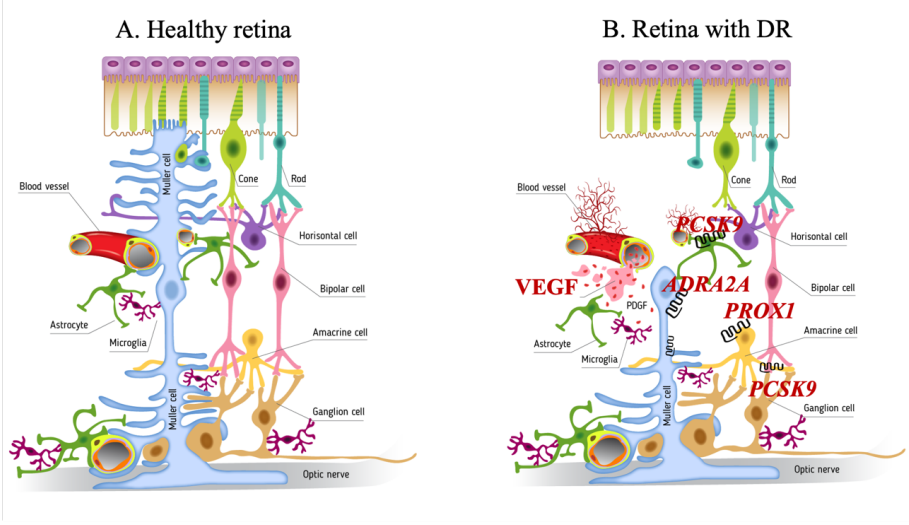

Figure 5. *The neurovascular unit of the retina. A) Neurovascular unit of the healthy retina. B) Disturbance of the vascular unit in the retina due to neurodegenerative processes during diabetic retinopathy. Neurodegeneration induces elevated production of VEGF, which leads to neovascularization. The genes ADRA2A, PCSK9, PROX1, which are expressed in neuronal tissue, were top signals in paper 2.*

5.4 Biological pathways potentially involved in the famine-related PDR

In paper 4, by performing GWAS and GWIS studies we found diverse pathways potentially associated with the risk of PDR and famine-related PDR. The most significant and biologically interesting were the pathways related to lipid metabolism and inflammation derived from the interaction model. Additionally, one of the strongest hits in the interaction model was located in the protein-coding gene *ABCA1,* which is involved in lipid metabolism. This gene was borderline genome-wide significant in the gene analysis for GWIS. These findings indicate the importance of lipid metabolism in PDR pathogenesis, but also the heterogeneous nature of PDR and highlight the complexity of the disease and the variety of biological pathways involved.

In fact, DR is a highly heterogeneous disease and is described by a complex interplay of biological processes triggered by hyperglycemia.[168] Genetic and epigenetic factors, elevated production of free radicals, VEGF, inflammation, end products of severe glycosylation may influence the disease development.[168] Roughly 30 cell types, which are almost all cell types in the retina, are affected due to diabetes.[194,440] The ADA defined DR as an extremely tissue-specific neurovascular complication of diabetes which is described by disturbance of interconnection between several types of retinal cells.[441] Even though neurodegeneration was suggested to be an important event for DR, it is not always an early sign of disease and some individuals do not express signs of neurodegeneration at the onset of DR.[442] This fact additionally highlights the heterogeneous nature of DR.

In our GWIS results, we found inflammatory pathways among the top pathways for famine-related risk of PDR. Indeed, DR pathogenesis involves not only vascular and neuronal mechanisms but also inflammatory pathways.[443] Inflammation is responsible for structural and molecular modifications in the retina, however underlying molecular mechanisms for this connection are not yet completely studied.[443] Notably, Adamis et al. even offered to consider DR as a low-grade inflammatory

disease.[444] In the Diabetic Retinopathy Genomics (DRGen) Study it was shown that genetic variants involved in inflammatory pathways may be involved in DR pathogenesis.[445] Additionally, the role of inflammation in DR was supported in the study by Meleth et al., where authors have shown that elevated level of serum inflammatory markers is associated with the severity of DR.[446] The role of the locally produced inflammation markers in the development of microvascular diabetes complications was also supported in the review by Kaul et al.[447] Moreover, in a recent Chinese famine study, inflammatory markers were shown to be elevated in the adult population exposed to intrauterine famine and were offered to be a mediator of the increased risk of liver disease.[448]

In our results from the pathway analysis based on GWIS, we supported the notion that lipid metabolism is playing a role in DR pathogenesis and progression.[449] A few decades ago, cross-sectional studies have shown an association between the severity of DR and cholesterol level.[450] Lipid-lowering dietary[451] and drugs were offered as protection against DR development and progression, showing different degrees of effectiveness in several studies.[452-456] Markedly, lipid-lowering strategies as a treatment recommendation against DR may be especially efficient for individuals who were prenatally exposed to famine and as a result in adulthood may be more prone to elevated cholesterol levels.[299]

Notably, the strongest SNP from our GWIS results is located in *ABCA1*, a gene which reached borderline significance in the gene analysis. In support of our findings, in the Dutch famine study *ABCA1* was among the top signals showing an increased level of methylation in individuals exposed to intrauterine famine compared to unexposed in approximately 60 years after exposure.[404] This gene may play an important role in DR pathogenesis due to several reasons. *ABCA1* is a member of an ATP-binding cassette transporters, known to be involved in transporting a number of molecules across intra- and extracellular membranes.[457] *ABCA1* is described to mediate the efflux of cholesterol and phospholipids to apolipoprotein A1 (apoA1) and apolipoprotein E (apoE).[458-460] Since apoE metabolism is an important process in

Alzheimer disease, *ABCA1* was offered to play an essential role in disease pathogenesis and in neurodegeneration via its control of apoE metabolism.[461-463] It was also suggested that *ABCA1* may have an effect on the increased risk of T2D through elevated cholesterol levels in β-cells.[461] Mice studies have shown that *ABCA1* may be involved in insulin secretion and pancreatic β-cells dysfunction[32,464,465], which is suggested to be associated with increased risk of DR as was mentioned in chapter 5.1. Notably, *ABCA1* was suggested to play an important role in retina neovascularization and expression levels of this gene were found to be elevated in individuals with PDR.[466] Since abnormal cholesterol metabolism is closely associated with the development of DR, *ABCA1* was found to be an attractive therapeutic target for DR.[466,467] In conclusion, main results of the paper 4 highlight the importance of lipid metabolism in the pathogenesis of PDR and specifically further support the *ABCA1* gene as a possible therapeutic target.

6 Conclusions

The main aims of this thesis were to investigate novel diabetes subgroups and to study the effects of intrauterine exposure to famine and genetic risk factors for PDR in the Ukrainian population.

In paper 1, we confirmed that subgroups in individuals with new-onset diabetes were similar to the original Scandinavian study. In long-term diabetes, our results demonstrated that SAID and SIDD subgroups were similar to the original publication and were characterized by low insulin secretion and high HbA1c. However, SIRD and MOD clusters were difficult to differentiate. We identified two severe insulin resistance obese diabetes subgroups (IROD1 and IROD2), which were characterised by increased insulin resistance and differed from each other by insulin secretion and HbA1c levels. The IROD2 subgroup in contrast to IROD1 was characterized by high insulin secretion, low HbA1c, and low risks of all complications. An important observation was that prevalence and risk of PDR were highest in subgroups that were characterized with the lowest insulin secretion (SAID and SIDD), and the lowest risk of PDR was in the subgroup with the highest insulin secretion IROD2. These observations prompt us to suggest that early insulin treatment in the SIDD subgroup may prevent progression to PDR.

In paper 2, we demonstrated the association of intrauterine exposure to famine and the risk of PDR in adult individuals with T2D. Notably, this association seemed to be independent of HbA1c. In paper 3, we further investigated possible molecular mechanisms behind this association. We found a significant association of SNPs, located in genes with neuronal function and famine-related risk of PDR. Our main conclusion was that exposure to famine in early life may act as a trigger and increase the risk of neurodegeneration in many years after exposure in adult individuals with T2D. We suggested that neuroprotective drugs may be beneficial for the treatment of DR in the early stages and may slow down the progression of the disease to PDR.

Especially this treatment may be important for individuals born with low birth weight or premature.

In paper 4 we found several important pathways that may be potentially involved in PDR pathogenesis. Inflammatory and lipid metabolism pathways were among the top results. We concluded that lipid metabolism may be an essential process for PDR development especially for individuals exposed to famine prenatally. An additional significant finding was the borderline genome-wide significant association of *ABCA1* with PDR. This gene is involved in lipid metabolism and was reported to be associated with neurodegenerative diseases and the severity of DR. We suggest that this gene may be an efficient therapeutic target for DR treatment.

In summary, by investigating the effects of the adverse intrauterine environment on the risk of PDR in adulthood, our results shed the light on supporting evidence that normal priming of neurons and vessels during early life might be a critical mechanism. The effects of disturbed developmental programming might involve abnormalities in lipid metabolism and insulin secretion, but also an immune response, predisposing to the occurrence of specific diabetes clusters linked to the elevated risk of PDR. We suggest that drugs targeting these pathways may be beneficial for DR treatment in the early stages and prevention from development to PDR.

7 Future perspectives

The clustering algorithm, which was used in this Ph.D. project to identify novel diabetes subgroups, utilized classical clinical factors and metabolic pathways known to predict diabetes development. However, it would be interesting to improve the algorithm using a broader selection of variables including additional biomarkers, genetic information (for instance, PGRS for T2D), and environmental factors associated with diabetes risk. A combination of several environmental factors such as diet, physical activity, treatment, stress, smoking, educational attainment, etc, may be used as one variable through constriction of the environment relationship matrix. Further studies on insights into the heterogeneity of diabetes may help to understand the possible optimal model and combination of variables that should be used in the clustering analysis. This algorithm may provide better precision for different diabetes subgroups, improve diabetes treatment and management of complications.

PDR is a complex disease characterized by microvascular damage and neurodegeneration. Even though neurodegeneration was proposed to be an early event in PDR pathogenesis, this process is not always assessed in individuals at disease onset nor when DR progresses, which makes it difficult to assess its contribution. Hence, to distinguish subgroups of PDR that are more prone to neuronal or vascular damage, sub-phenotyping of PDR may be beneficial for disease management and treatment. This can be achieved by extensive phenotyping, which includes Optical Coherence Tomography (OCT) with information on structural changes in the retina, vascular and neuronal layers thickness, length, density, and diameter. Identification of PDR subgroups with different pathophysiology would help to characterize and understand the genetic background of vascular and neuronal forms of the disease.

Today, the genetics of PDR remains elusive, and it is essential to discover the genetic architecture of the disease. Moreover, it would be interesting to understand the interplay between clinical risk factors, genetic predisposition, environment, and combination of these factors contributing to the disease progression. As we stand now,

more genetic studies including examining the role of rare variants, gene-gene, and gene-environment interactions should be done. It is crucial to collect more genetic data in cohorts with information about PDR and with longer diabetes duration to have sufficient statistical power to identify the causal variants. Meta-analyses in different populations with harmonized definitions of cases and controls should be done and will help to increase the power of GWAS. To verify the role of the predictive adaptive response hypothesis in the susceptibility to PDR, it would be interesting to investigate the risk of PDR depending on nutrition level in adult life in individuals exposed to intrauterine famine. Further, a genome-wide interaction study with a matrix that combines several environments could be done. Taken together, genetic studies with a large sample size, detailed phenotyping of cohorts in different populations, and harmonization of phenotypes definition, will bring new insights into the understanding of the pathophysiology of the development and progress of retinopathy.

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Papers I-IV

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Novel Reclassification of Adult Diabetes Is Useful to Distinguish Stages of β-Cell Function Linked to the Risk of Vascular Complications: The DOLCE Study From Northern Ukraine

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Background: Presently, persons with diabetes are classified as having type 1 (T1D) or type 2 diabetes (T2D) based on clinical diagnosis. However, adult patients exhibit diverse clinical representations and this makes treatment approaches challenging to personalize. A recent Scandinavian study proposed a novel classification of adult diabetes into five clusters based on disease pathophysiology and risk of vascular complications. The current study aimed to characterize new subgroups of adult diabetes using this strategy in a defined population from northern Ukraine.

Methods: We analyzed 2,140 patients with established diabetes from the DOLCE study (n = 887 with new-onset diabetes and n = 1,253 with long duration). We used the k-means approach to perform clustering analyses using BMI, age at onset of diabetes, HbA_{1c}, insulin secretion (HOMA2-B), and insulin resistance (HOMA2-IR) indices and glutamic acid decarboxylase antibodies (GADA) levels. Risks of macro- (myocardial infarction or stroke) and microvascular [retinopathy, chronic kidney disease (CKD) and neuropathy] complications and associations of genetic variants with specific clusters were studied using logistic regression adjusted for age, sex, and diabetes duration.

Results: Severe autoimmune diabetes (SAID, 11 and 6%) and severe insulin-deficient diabetes (SIDD, 25 and 14%) clusters were twice as prevalent in patients with long-term as compared to those with new-onset diabetes. Patients with long duration in both SAID and SIDD clusters had highest risks of proliferative retinopathy, and elevated risks of CKD. Long-term insulin-resistant obese diabetes 1 (IROD1) subgroup had elevated risks of CKD, while insulin-resistant obese diabetes 2 (IROD2) cluster exhibited the highest HOMA2-B, lowest HbA_{1c}, and lower prevalence of all microvascular complications as compared to all other clusters. Genetic analyses of IROD2 subgroup identified reduced

frequency of the risk alleles in the *TCF7L2* gene as compared to all other clusters, cumulatively and individually ($p = 0.0001$).

Conclusion: The novel reclassification algorithm of patients with adult diabetes was reproducible in this population from northern Ukraine. It may be beneficial for the patients in the SIDD subgroup to initiate earlier insulin treatment or other anti-diabetic modalities to preserve β-cell function. Long-term diabetes cases with preserved β-cell function and lower risk for microvascular complications represent an interesting subgroup of patients for further investigations of protective mechanisms.

Keywords: clustering, β-cell function, diabetes complications, genetics, adult diabetes

INTRODUCTION

Diabetes represents a global health problem, affecting today more than 400 million people worldwide, which is estimated to increase up to 600 million by 2030 (IDF, 2019). Diabetes in adults, of which type 2 diabetes (T2D) being the most common type, comprises around 90% of all diabetes cases. The incidence and prevalence of adult diabetes are more rapidly increasing in the low- and middle-income countries (IDF, 2019). The official prevalence of diabetes in Ukraine was reported to rise by about 25% from 2007 to 2019, reaching 8.4% in 2019 (Khalangot and Tronko, 2007; IDF, 2019). This situation is particularly alarming because nearly an equal number of people have undiagnosed diabetes in Ukraine meaning that the prevalence rate is doubled in real life (IDF, 2019; Mankovsky, 2020). Diabetes is one of the leading causes of blindness, end-stage renal disease, limb amputation, heart disease, stroke, liver cirrhosis, and premature death in working age adults (IDF, 2019). Numerous clinical and genetic studies demonstrated that adult diabetes is a highly heterogeneous metabolic disease with diverse underlying mechanisms (Tuomi et al., 2014; McCarthy, 2017). Lack of pathophysiology-based classification hampers personalized therapy targeting specific complications of diabetes (Fitipaldi et al., 2018). This shortcoming was recently addressed in a Scandinavian cohort of patients with newly diagnosed adult diabetes, by applying an unbiased unsupervised clustering approach to propose a reclassification of the disease (Ahlqvist et al., 2018). Based on six clinically affordable parameters including glycated hemoglobin (HbA_{1c}), body-mass index (BMI), age at diabetes onset, insulin resistance, insulin secretion calculated using homeostasis model assessment (HOMA2), and glutamic acid decarboxylase antibody (GADA) levels, a new five-cluster classification scheme for adult diabetes was proposed. These clusters reflected essential pathophysiological mechanisms in the disease and were named accordingly as severe autoimmune diabetes (SAID), severe insulin-deficient diabetes (SIDD), severe insulin-resistant diabetes (SIRD), mild obesity-related diabetes (MOD), and mild age-related diabetes (MARD). This novel classification has now been validated in a number of studies across Europe and Asia, emphasizing the robustness of the approach (Dennis et al., 2019; Zaharia et al., 2019; Zou et al., 2019) even though there was some ethnic heterogeneity reported in an Asian-Indian study (Anjana et al., 2020). The suggested clustering approach is attractive for clinical practice because of the opportunity to predict the risks of diabetic complications at diagnosis and tailor patients' therapy accordingly (Ahlqvist et al., 2018; Zaharia et al., 2019).

The aim of the present study was to assess the prognostic value of the new reclassification approach by Ahlqvist et al. (Ahlqvist et al., 2018) for the first time in an East-European population from Ukraine including patients with newly diagnosed and long-term adult diabetes.

PARTICIPANTS AND METHODS

Study Cohort

The Diagnostic Optimization and Treatment of Diabetes and its Complications in the Chernihiv Region, Ukraine (DOLCE) is a hospital- and primary health care-based study of individuals with diabetes and their healthy relatives, including total of 6,095 participants. The cohort consists of 785 persons with T1D, 4,297 with T2D, 62 with unspecified diabetes, and 951 healthy first- or second-degree relatives. All participants completed a questionnaire supervised by an endocrinologist and a trained diabetes nurse, which covered the person's medical history and included information of family history of diabetes, anthropometric measurements (weight, height, and blood pressure), alcohol intake, smoking, diabetes medication, antihypertensive and lipid-lowering treatments. Information of prevalent cardiovascular events, neuropathy, chronic kidney disease, and stages of retinopathy was reported by primary care physicians using patients' hospital discharge records as primary source and was used as data entry into the DOLCE database at the screening visit. Fasting blood samples were drawn for plasma glucose and HbA_{1c} measurements. Additional plasma and serum samples were stored at –80°C for C-peptide, insulin, lipids, and glutamic acid decarboxylase antibodies (GADA) measurements, which were performed at the Department of Clinical Chemistry, Scania University Hospital, Malmö, Sweden. A written informed consent form was obtained from every participant. The DOLCE study was approved by the local ethics committees (approval number for Ukraine Dnr17/2011–09–14; for Norway 2019/28968).

Measurements and Calculations

C-peptide concentrations were measured with an electro-chemiluminescence immunoassay on Cobas e411 (Roche Diagnostics, Mannheim, Germany) or a radioimmunoassay (Human C-peptide RIA; Linco, St Charles, MO, United States; or Peninsula Laboratories, Belmont, CA, United States). GADA were measured with an ELISA from the samples collected at the screening visit. Test results greater than or equal to 5 U/ml were considered as positive. The radio binding assays had 62–88% sensitivity and 91–99% specificity, and the ELISA assay had 72% sensitivity and 99% specificity (Combinatorial Autoantibody or Diabetes/Islet Autoantibody Standardization Programs 1998–2013). β-cell function (HOMA2-B) and insulin resistance (HOMA2-IR) were assessed with Homoeostasis Model Assessment 2 (HOMA2) and were calculated with the HOMA2 calculator using C-peptide and fasting glucose measurements (Levy et al., 1998; HOMACalculator, 2020). Data values for BMI, HOMA2-B, and HOMA2-IR with more than three standard deviations were excluded. Only individuals with age at diabetes diagnosis older than 18 years and complete information on BMI, age at onset, HbA_{1c}, HOMA2-B, HOMA2-IR, sex, duration of diabetes, and complication status were included in the final analysis (n = 2,140).

Definition of Diabetic Complications

Proliferative diabetic retinopathy (PDR) was defined as having one of the following conditions: (a) proliferative retinopathy, (b) laser treatment, or (c) blindness of either one or both of the eyes. Stages of PDR were based on fundus photographs and were evaluated by ophthalmologists. Chronic kidney disease (CKD) was defined as having at least one of the following conditions: (a) estimated glomerular filtration rate less than 60 mL/min/1.73 m^2 (eGFR $<$ 60), (b) clinically documented diagnosis of nephropathy, dialysis, or end-stage renal disease (ESRD). eGFR was calculated using Modification of Diet in Renal Disease (MDRD) formula as: $186 \times (\text{Creatinine}/88.4)^{-1.154} \times (\text{age})^{-0.203} \times 0.742$ for female participants; $186 \times (\text{Creatinine}/88.4)^{-1.154} \times (\text{age})^{-0.203}$ for male participants (Mula-Abed et al., 2012). Neuropathy was defined as clinically diagnosed peripheral neuropathy. Cardiovascular diseases were defined as either presence of myocardial infarction or stroke using International Classification of Diseases (ICD)-10 codes I21, I24, and I61–I64, respectively.

Statistical Analysis

In SAID cluster we included individuals with positive GADA. SIDD, SIRD, MOD, and MARD cluster analysis was performed on individuals who were negative for GADA antibodies (where GADA measurements were available) using previously defined parameters by Ahlqvist et al. (Ahlqvist et al., 2018): HbA_{1c}, BMI, age at onset of diabetes, HOMA2-B, and HOMA2-IR. The analyses were done in two groups of patients: (i) long-term diabetes with more than 3 years of disease duration and (ii) new-onset diabetes with less than 3 years of disease duration to harmonize with the Swedish ANDIS cohort employed as the reference (Ahlqvist et al., 2018). The analyses were performed using k-means clustering on the centered and scaled values. We used "kmeansruns" function from R package fpc v2.2–8 with 4 as the assigned number of clusters and default parameters: krange = 4, criterion = "asw," iter.max = 100, runs = 100, alpha = 0.001, critout = FALSE (Hennig, 2020). Male and female patients were analyzed separately to avoid sex bias, and results were merged afterward. Risks for diabetic complications in each cluster were calculated using logistic regression adjusted for age, sex, and diabetes duration with the MARD cluster used as reference. To adjust for multiple phenotype testing, we calculated a false discovery rate (FDR). FDR was calculated using p.adjust function implemented in the "stats" package in R (R Core Team, 2021). FDR $<$ 0.05 was considered statistically significant.

Genetics

Genotyping of DNA samples was available only in patients with clinically defined T2D using Infinium Core Exome Chip InfiniumCoreExome-24v1-1 (https://www.illumina.com). Imputation was done using Michigan Imputation Server and Reference Panel HRC r1.1 2016. Standard quality control steps for Genome-Wide Association Studies (GWAS) were applied (Marees et al., 2018). We analyzed SNPs associated with T2D, which were previously reported in the DIAGRAM GWAS meta-analysis (Morris et al., 2012). Logistic regression adjusted for sex and age was used to study associations between the insulin resistant obese diabetes 2 cluster and the genetic variants as compared with all other clusters in patients with long-term diabetes. SAID cluster was not included in the genetic analysis due to small sample size of individuals with genotypes in this group (n = 26). Bonferroni correction was used to adjust for multiple testing in genetic association tests. $P < 0.05$ was considered statistically significant.

RESULTS

The clinical characteristics and prevalence of macro- and microvascular complications in adult patients with (a) new-onset and with (b) long-term diabetes are shown in **Table 1**.

New-Onset Adult Diabetes

There were 887 (36% men) with new-onset diabetes (years, 1.1 ± 1.1). Oral glucose-lowering treatment was reported for 42.6% of the patients, while insulin treatment was initiated in 8.8% of the patients. The prevalence of PDR was 0.7%, and CKD was 12.4%. Peripheral neuropathy was reported in 34.3%, while non-fatal CVD occurred in 8.6% of the individuals (**Table 1**).

Long-Term Adult Diabetes

There were 1,253 persons (34% men) with long diabetes duration (mean ± SD, years, 11.0 ± 6.9). BMI in this group was similar to the new-onset diabetes group (kg/m^2, 30.9 ± 5.3 and 30.7 ± 5.6). Oral glucose-lowering treatment was reported for 51.6% of the patients, while insulin treatment was initiated in 27.7% of the patients. Glycemic control was observed to be worse in patients with long-term diabetes (HbA_{1c}%, 9.0 ± 2.0 and 7.9 ± 2.1) than in those with new-onset disease. As expected, insulin secretion

TABLE 1 | Clinical characteristics of patients with adult diabetes in the DOLCE study.

Phenotype	New-onset	Long-term
N (men,%)	887 (36%)	1,253 (34%)
Age at visit, years	56.9 (12.5)	61.2 (10.0)
Age at onset of diabetes, years	55.8 (12.4)	50.1 (10.8)
Diabetes duration, years	1.1 (1.1)	11.0 (6.9)
HbA_{1c},%	7.9 (2.1)	9.0 (2.0)
HbA_{1c}, mmol/mol	62.9 (23.4)	74.4 (21.3)
BMI, kg/m^2	30.7 (5.6)	30.9 (5.3)
Waist, cm	97.4 (14.3)	99.1 (12.7)
HOMA2-B	81.1 (44.6)	63.9 (45.9)
HOMA2-IR	2.4 (1.2)	2.2 (1.3)
C-peptide, nmol/l	0.9 (0.5)	0.8 (0.5)
Without treatment,%	46.9%	12.1%
Tablets,%	42.6%	51.6%
Insulin,%	8.8%	27.7%
Tablets and insulin,%	1.7%	8.5%
Sulfonylurea,%	27%	45%
PDR,%	0.7%	5.5%
CKD,%	12.4%	26.7%
Neuropathy,%	34.3%	85.6%
CVD,%	8.6%	14.9%

Data are represented as mean ± (SD).
HOMA2-B, homoeostatic model assessment 2 estimates of β-cell function; HOMA2-IR, homoeostatic model assessment 2 estimates of insulin resistance; PDR, proliferative diabetic retinopathy; CKD, chronic kidney disease; CVD, cardiovascular disease.

estimated with HOMA2-B was lower in the persons with long-term diabetes (%, 63.9 ± 45.9 and 81.1 ± 44.6) as compared to those with new-onset (**Table 1**). The prevalence of PDR was 5.5%, CKD was 26.7%, peripheral neuropathy was 85.6%, and non-fatal CVD was 14.9% (**Table 1**).

Characteristics of Different Clusters in Patients With Adult Diabetes

Descriptive characteristics and frequency of the clusters in the patients with long-term and new-onset adult diabetes are presented in **Figures 1**, **2** and **Supplementary Table 1**.

Severe autoimmune diabetes (SAID) was twice prevalent in patients with long-term (11 and 6%) as compared to those with new-onset diabetes (**Figure 1**). Of these, in total 74 (37 and 43%) had clinical diagnosis as T2D. Individuals in this cluster had low BMI, younger age at diagnosis, and the lowest HOMA2-B and HOMA2-IR as compared to other clusters **(Figure 2** and **Supplementary Table 1**). Individuals with SAID most frequently had insulin treatment, particularly among those with the long-term diabetes (84.1 and 58.8%), while oral antidiabetic treatment was more frequent in the new-onset group (9.4 and 27.5%) (**Supplementary Table 1**).

Severe insulin-deficient diabetes (SIDD) was almost twice as prevalent in the patients with long diabetes duration than in those with new-onset diabetes (25 and 14%) (**Figure 1**). This cluster was characterized by low insulin secretion as shown by HOMA2-B, relatively low BMI and the high HbA_{1c} than the other clusters (**Figure 2** and **Supplementary Table 1**). The frequency of insulin therapy alone (42.6 and 25.6%) or in combination with oral anti glycemic medications (17.9 and 4.1%) was higher than in all other clusters apart from SAID for the patients with long-term and new-onset disease (**Supplementary Table 1**).

Severe insulin-resistant diabetes (SIRD) cluster occurred with 27% frequency in patients with new-onset diabetes (**Figure 1**). This cluster in the new-onset group was characterized by the highest insulin-resistance index HOMA2-IR (%, 3.4 ± 1.1) and HOMA2-B (%, 121.7 ± 42.2), elevated BMI and lower HbA_{1c} (**Figure 2** and **Supplementary Table 1**). The insulin resistant obese patients with long-term diabetes differed from the original SIRD individuals. They exhibit similarly elevated HOMA2-IR (%, 3.5 ± 1.1) while insulin secretion index HOMA2-B was lower (%, 65.7 ± 27.8) and therefore we named this cluster insulin resistant obese diabetes 1 (IROD1) (**Figure 2** and **Supplementary Table 1**). IROD1 cluster also showed the highest waist circumference among all clusters, reflecting the presence of an abdominal adiposity (**Supplementary Table 1**). Oral anti-glycemic treatment was common in long-term and new-onset groups (72.2 and 48.3%), however, long-term IROD1 reported more frequent use of sulfonylurea (61%). In addition, half of the individuals with new-onset diabetes (49.1%) and only 8.6% with long-term did not receive any anti-diabetic medications.

Mild obesity-related diabetes (MOD) cluster in new-onset group occurred with the frequency of 7% and was the smallest after SAID cluster (**Figure 1**). This group in the new-onset group was characterized by elevated BMI (kg/m^2, 34.0 ± 4.9), HOMA2-IR (%, 3.3 ± 1.2), and moderately elevated HOMA2-B index (%, 66.9 ± 23.8). Patients with long-term diabetes in this cluster showed elevated BMI (kg/m^2, 32.0 ± 4.5) and HOMA2-IR (%, 3.1 ± 1.1), but did not match their original MOD counterparts in the new-onset group by markedly elevated HOMA2-B index (%, 139.4 ± 38.8), and therefore we named them insulin resistant obese diabetes 2 (IROD2) (**Figure 2** and **Supplementary Table 1**). Patients in both groups received often oral anti-hyperglycemic treatment (64.7 and 70.8%), or were controlled with diet and/or lifestyle intervention (27.9 and 23.6%) of long-term and new-onset diabetes, respectively (**Supplementary Table 1**).

Finally, mild age-related diabetes (MARD) was the largest cluster in both long-term and new-onset adult diabetes (27 and 46%) (**Figure 1**). This cluster was by definition characterized by the highest age at disease diagnosis (years, 59.7 ± 11.7 and 59.9 ± 11.7) and had similar characteristics across the two patients' groups (**Figure 2** and **Supplementary Table 1**).

Risk of Diabetes Complications in Different Clusters

We assessed the risk of macro- and microvascular complications of diabetes in each cluster using MARD as reference group, and adjusted for age, sex, and diabetes duration. In this cross-sectional study, patients with new-onset adult diabetes had few PDR events, and therefore analysis was not performed to calculate PDR risk in these patients. Only few cases (4.2%) had CKD

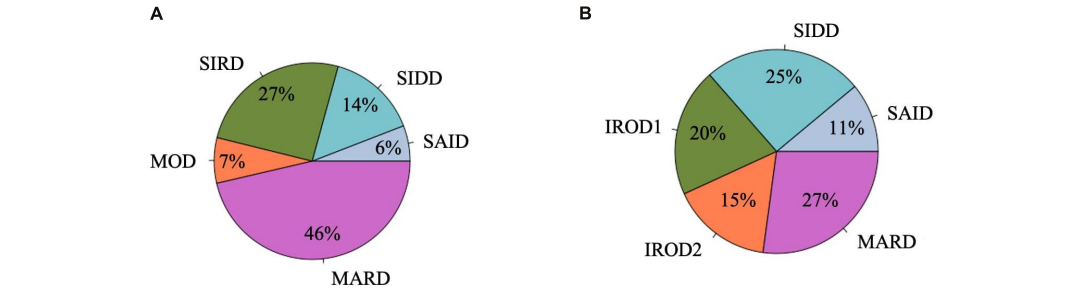

FIGURE 1 | Frequency distribution of clusters in the DOLCE study. **(A)** Individuals with new-onset adult diabetes with less than 3 years of disease duration (*n* = 887). **(B)** Individuals with long-term adult diabetes with more than 3 years of disease duration (*n* = 1,253).

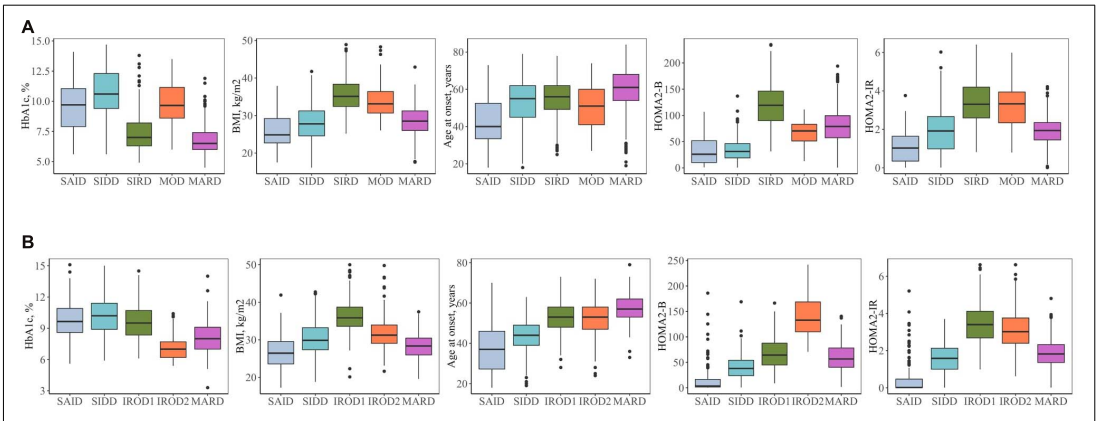

FIGURE 2 | Clinical characteristics of the clusters. **(A)** Individuals with new-onset adult diabetes with less than 3 years of disease duration (*n* = 887). **(B)** Individuals with long-term adult diabetes with more than 3 years of disease duration (*n* = 1,253). SAID, severe autoimmune diabetes**;** SIDD, severe insulin-deficient diabetes; SIRD, severe insulin-resistant diabetes; MOD, mild obesity-related diabetes; MARD, mild age-related diabetes; IROD1 and 2, insulin-resistant obese diabetes 1 and 2; HOMA2-B, homoeostatic model assessment two estimates of β-cell function; HOMA2-IR, homoeostatic model assessment two estimates of insulin resistance.

in MOD cluster with the new onset diabetes (**Figure 3** and **Supplementary Table 1**). Similar to the reference ANDIS study (Ahlqvist et al., 2018), in long-term group the SAID cluster was characterized with high prevalence of PDR (10.9%) with OR of 9.32-fold (95% CI, 2.15–40.46, p = 0.003) relative to MARD cluster. Prevalence of CVD in this cluster was detected to be lower than in the other clusters (5.8%). The SIDD cluster had similarly to SAID high prevalence and increased risk of PDR (11%, OR 2.42, 95% CI, 0.96– 6.07, p = 0.06) relative to MARD cluster (**Figure 3**, **Table 2**, and **Supplementary Table 1**). Prevalence of CKD was found to be elevated in all severe clusters, i.e., SAID (29.7%), SIDD (32%), and insulin-resistant obese diabetes 1 (IROD1) (30.2%) conferring an increased risk of 2.59-fold (1.34–5.00, p = 0.005), 2.03-fold (1.21– 3.40, $p = 7.1 \times 10^{-3}$), and 1.63-fold (1.08 –2.46, p = 0.02) relative to MARD in patients with long diabetes duration. In contrast to ANDIS, neuropathy prevalence was high across all clusters, but the relative risk was highest in SIDD cluster (OR 13.60, 95% CI 5.20–35.50, $p = 1.10 \times 10^{-7}$). In general, the IROD2 cluster exhibited the lowest prevalence of all microvascular complications, particularly CKD and neuropathy.

Genetic Analyses

We analyzed variants reported in the DIAGRAM GWAS meta-analyses (Morris et al., 2012) to provide insights on the key T2D SNPs associated with protective phenotype of insulin resistant obese (IROD2) cluster in patients with long-term diabetes relative to all other clusters (excluding SAID due to the lack of genetic information in this cluster). The top SNP was rs7903146 in *TCF7L2* (OR, 95%CI; 0.54, 0.39–0.74, p = 0.0001), which showed significantly lower frequency of the risk T-allele in IROD2 than in IROD1 (p = 0.008), SIDD (p = 0.001) and MARD (p = 0.0003) (**Table 3**). The same directionality for lower risk allele frequencies in IROD2 was also found for *KCNQ1* locus (rs163184, 0.67, 0.52–0.88, p = 0.003), *NOTCH2* locus (rs1493694, 0.49, 0.30– 0.80, p = 0.004), locus in the gene related to cell-matrix interplay *ADAMTS9* (rs6795735: 0.72, 0.56– 0.93,

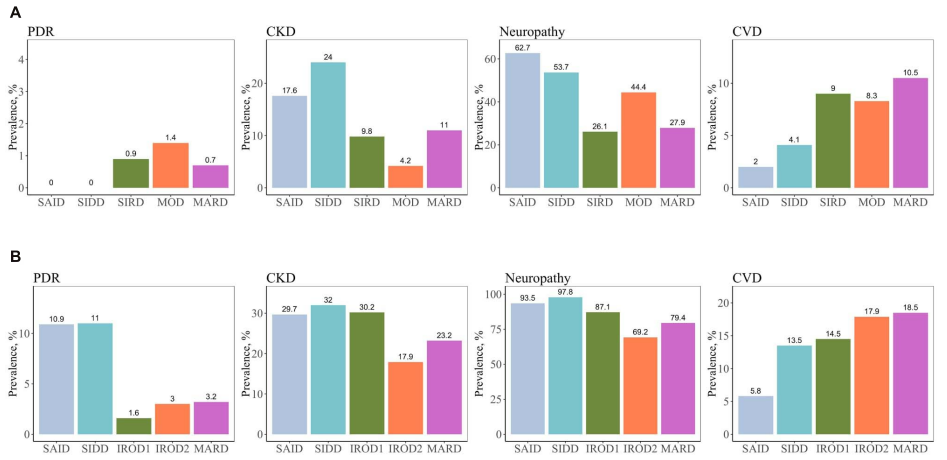

FIGURE 3 | Prevalence of macro- and microvascular complications in different clusters of adult patients with the new-onset and long-term diabetes. **(A)** Individuals with new-onset adult diabetes with less than 3 years of disease duration (n = 887). **(B)** Individuals with long-term adult diabetes with more than 3 years of disease duration (n = 1,253). PDR, proliferative diabetic retinopathy; CKD, chronic kidney disease; CVD, cardiovascular disease.

TABLE 2 | Risk of macro- and microvascular complications in different clusters in long-term adult diabetes relative to MARD.

Complications	SAID		SIDD		IROD1		IROD2	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
PDR	9.32 (2.15, 40.46)	0.003*	2.42 (0.96, 6.07)	0.06	0.53 (0.16, 1.79)	0.31	0.73 (0.23, 2.32)	0.59
CKD	2.59 (1.34, 5.00)	0.005*	2.03 (1.21, 3.40)	7.1×10^{-3}*	1.63 (1.08, 2.46)	0.02*	0.85 (0.53, 1.39)	0.52
Neuropathy	3.30 (1.29, 8.43)	0.01*	13.60 (5.20, 35.50)	1.0×10^{-7}*	2.08 (1.28, 3.37)	3.0×10^{-3}*	0.70 (0.45, 1.09)	0.12
CVD	0.61 (0.25, 1.51)	0.3	1.13 (0.60, 2.11)	0.71	1.11 (0.68, 1.8)	0.68	1.44 (0.87, 2.36)	0.15

PDR, proliferative diabetic retinopathy; CKD, chronic kidney disease; CVD, cardiovascular disease; OR, Odds Ratios; 95% CI, 95% confidence intervals; and p-values were calculated using logistic regression adjusted for sex, age, and diabetes duration.
**Significant after adjustment for the multiple testing using FDR (<0.05).*

p = 0.012), loci in genes *ZFAND6* (rs11634397: 0.73, 0.56–0.94, p = 0.016), and *ZFAND3* (rs4299828: 0.73, 0.54– 0.99, p = 0.042). On the contrary, higher frequencies were observed for the risk alleles of SNPs in the gene related to cell signaling *PTPRD* (rs16927668: 1.40, 1.03–1.91, p = 0.033). However, after adjustment for multiple testing using Bonferroni correction, only variant rs7903146 in *TCF7L2* remained statistically significant (p-value $_{IROD2vs.allclusters}$ = 0.008, p-value $_{IROD2vs.SIDD}$ = 0.048, p-value $_{IROD2vs.MARD}$ = 0.002) (**Table 3**).

DISCUSSION

The findings from this observational study demonstrate that the novel approach using SAID, SIDD, SIRD, MOD, and MARD clustering of diabetes subgroups (Ahlqvist et al., 2018) is replicated in the patients with adult diabetes from northern Ukraine. In accordance with the published studies, the SIDD cluster had the highest prevalence of retinopathy and neuropathy, and the insulin resistant subgroups were linked to high risk of CKD. In contrast to Scandinavian and German populations (Ahlqvist et al., 2018; Zaharia et al., 2019) and similar to the insulin-deficient insulin-resistant subgroup in a recently published Asian-Indian cohort (Anjana et al., 2020), SIDD cluster in this cohort also showed high risk of CKD. With longer duration of diabetes, the clusters might change, and insulin resistant obese cases could be challenging to match to the original SIRD and MOD cluster of corresponding new-onset diabetes. In general, patients with long-term diabetes and preserved β-cell function demonstrated better glycemic control measured by HbA_{1c} and lower risk of all microvascular complications than expected.

An important observation was lower insulin secretion in patients from northern Ukraine with new-onset adult diabetes compared to the Swedish ANDIS cohort. The history of Ukraine during the first half of the 20^{th} century could contribute to this difference. It has been previously reported that children born to parents exposed to the Ukrainian Holodomor famine (1932–1933) showed increased risk of developing T2D later in life (Lumey et al., 2015). Early life exposure to starvation might

TABLE 3 | Top T2D SNPs nominally associated with IROD2 cluster in individuals with long-term diabetes.

SNP	Gene	Chr	BP	Risk allele	RAF	IROD2 vs. all clusters		IROD2 vs. IROD1		IROD2 vs. SIDD		IROD2 vs. MARD	
						OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
rs7903146	*TCF7L2*	10	114758349	T	0.28	0.54 (0.39, 0.74)	0.0001*	0.61 (0.42, 0.88)	0.008	0.54 (0.37, 0.77)	0.001*	0.45 (0.31, 0.65)	0.0003*
rs163184	*KCNQ1*	11	2847069	G	0.50	0.67 (0.52, 0.88)	0.003	0.60 (0.44, 0.82)	0.001	0.74 (0.54, 1.01)	0.059	0.71 (0.52, 0.98)	0.036
rs10923931	*NOTCH2*	1	120517959	T	0.13	0.49 (0.30, 0.80)	0.004	0.52 (0.29, 0.90)	0.020	0.50 (0.29, 0.87)	0.014	0.45 (0.26, 0.78)	0.004
rs6795735	*ADAMTS9*	3	64705365	C	0.59	0.72 (0.56, 0.93)	0.012	0.68 (0.50, 0.93)	0.015	0.78 (0.58, 1.06)	0.113	0.72 (0.53, 0.97)	0.033
rs11634397	*ZFAND6*	15	80432222	G	0.66	0.73 (0.56, 0.94)	0.016	0.71 (0.51, 0.99)	0.041	0.70 (0.51, 0.96)	0.025	0.76 (0.56, 1.03)	0.081
rs16927668	*PTPRD*	9	8369533	T	0.18	1.40 (1.03, 1.91)	0.033	1.45 (1.00, 2.10)	0.047	1.53 (1.04, 2.24)	0.032	1.25 (0.85, 1.82)	0.255
rs4299828	*ZFAND3*	6	38177667	A	0.81	0.73 (0.54, 0.99)	0.042	0.65 (0.46, 0.93)	0.018	0.80 (0.55, 1.15)	0.227	0.79 (0.55, 1.13)	0.195
rs459193	*ANKRD55*	5	55806751	G	0.72	1.36 (1.01, 1.84)	0.044	1.30 (0.90, 1.86)	0.159	1.32 (0.92, 1.88)	0.128	1.44 (1.01, 2.07)	0.045

Association of T2D SNPs with IROD2 cluster as case group. OR and 95% CI were calculated using logistic regression adjusted for sex and age.
Chr, chromosome; BP, base pair; RAF, risk allele frequency.
Number of individuals in the analyses IROD2 vs. all clusters: n = 837, IROD2 vs. IROD1: n = 357, IROD2 vs. SIDD: n = 355, IROD2 vs. MARD: n = 415.
**Significant after adjustment for the multiple testing using Bonferroni correction ($p < 0.05$).*

exhibit detrimental effects, which might result in malformation of the pancreas, thereby reducing the number of β-cells (β-cell mass) and/or affecting the secretory activity of β-cells (β-cell function) (Chen et al., 2017). The decrease in insulin output as a consequence of the reduced β-cell mass could be caused by a progressive β-cell death through established starvation-induced processes on apoptosis and disrupted autophagy (Marrif and Al-Sunousi, 2016). Thus, intrauterine programming may be related to the restricted insulin secretory capacity of pancreatic islets as a response to the demands imposed by increased insulin resistance linked to obesity in adult life. This could contribute to more severe manifestation of diabetes in the Ukrainian population. Lower frequency of insulin therapy in Ukrainian patients with new-onset diabetes in adults despite more profound insulin secretory defects compared to the ANDIS cohort could indicate that many more patients might benefit from early intensified treatments targeting preservation of insulin secretion. Naturally, discovering new ways to maintain or refine β-cell mass via enhancing β-cell survival and/or reducing apoptosis is of specific interest to investigate.

SIDD was the most severe cluster in this study, which supported consistently documented high prevalence of retinopathy across all published studies, and also showed elevated prevalence of CKD. These results are opposite to ANDIS and other cohorts (Ahlqvist et al., 2018; Zaharia et al., 2019; Zou et al., 2019) showing increased risk of CKD conferred to SIRD cluster, but somewhat similar to the recently reported findings in Asian-Indian population (Anjana et al., 2020) demonstrating increased risk of CKD in the combined insulin-deficient insulin-resistant cluster. One potential explanation for this observation could reflect and support the above-mentioned effects and malformations of organs as a consequence of abnormal intrauterine programming, which may lead to increased susceptibility to vascular complications in patients with adult diabetes later in life. In support of this, studies from the Dutch famine birth cohort have reported increased risk of microalbuminuria in adults after prenatal exposure to famine (Painter et al., 2005). Researchers suggested that fetal undernutrition may lead to lower nephron endowment, reduced number of glomeruli and consequently hyperfiltration, which may cause glomerular damage and lead to a reduction in renal function.

In line with study by Zaharia et.al of German patients with adult diabetes (Zaharia et al., 2019), the highest waist circumference was observed in the IROD1 cluster of long-term diabetes. From this perspective, the data suggest that IROD1 is associated with abdominal (visceral) type of adiposity as compared to IROD2 in which subcutaneous adipose tissue is prevailing fat deposition. Abdominal adiposity is considered to be much unhealthier than subcutaneous fat accumulation (Kahn et al., 2001) and associated with increased insulin resistance, inflammation, atherosclerosis, and vascular complications (Reijrink et al., 2019) as well as increased mortality (Christakoudi et al., 2020). Similarly, in the German Diabetes Study, the patients with newly diagnosed adult diabetes in the SIRD cluster demonstrated the highest hepatocellular lipid content and the highest prevalence of hepatic fibrosis at 5-year follow-up (Zaharia et al., 2019). This supports the idea of visceral obesity contributing to elevated risks of metabolic disorders and vascular complications in SIRD.

An interesting finding of the clustering approach in long-term adult diabetes was related to the IROD2 cluster with preserved β-function. It is tempting to speculate that people in the IROD2 cluster could have escaped starvation during the

historical famines and thereby have had their β-cell function preserved relative to the people who experienced undernutrition during times of exposure. However, we do not have information on caloric intake during famine periods; hence, this hypothesis could only be tested in a population where such information exists (e.g., Dutch famine birth cohort).

To shed light on possible underlying genetic factors of associated with beta-cell function in IROD2 cluster, we compared the frequency of established T2D variants (Morris et al., 2012). The genetic results identified consistently lower frequency of risk alleles at the *TCF7L2* locus in IROD2 cluster compared to each other cluster in this group of patients. Since 2007, polymorphisms of *TCF7L2*, encoding for transcription factor-7–like 2, are considered to be guilty of β-cells dysfunction and increased risk of diabetes in different ethnic populations (Scott et al., 2007; Dimas et al., 2014). *TCF7L2* is a member of the T-cell–specific high-mobility group box-containing family of transcription factors, that acts through Wnt-β-catenin dependent and independent pathways (Karve et al., 2020) and coordinates expression of various genes regulating cell cycle and fate determination. In pancreas *TCF7L2* was shown to play a crucial role in regulation of β-cell survival and proliferation rate. Moreover, *TCF7L2* controls the expression of genes involved in insulin granule fusion at the plasma membrane through syntaxin repression, affecting insulin secretion levels (da Silva Xavier et al., 2009). According to our results, *TCF7L2* rs7903146, which is a lead among T2D susceptibility loci (Hattersley, 2007), associated with more severe T2D phenotypes, and the lower frequencies of the risk allele appear to be associated with the protection against progression toward several vascular complications. As rs7903146 has been shown to reside in islet-selective open chromatin (Gaulton et al., 2010), this clearly motivates further metabolic studies of this group to identify epigenetic factors that play multifaced roles.

Second top signal in IROD2 group of long-term diabetes suggested reduced frequency of the risk allele in the imprinting gene *KCNQ1* (rs 163,184) that has also been shown to be expressed in the pancreatic β-cells and to act through impaired islet function on the risk of future T2D (Jonsson et al., 2009). *KCNQ1* locus was first discovered as a top signal in the two GWAS for T2D from Japan (Unoki et al., 2008; Yasuda et al., 2008) and identified as a GWAS locus for parent-of-origin effects in a large family-based study from Iceland (Kong et al., 2009). Functional and analyses of imprinting status of this genomic region suggested that metabolic effects conferred by the risk alleles at the *KCNQ1* locus target the cyclin-dependent kinase inhibitor *CDKN1C* playing a key role in regulating pancreatic β-cells proliferation and development (Kassem et al., 2001). Expression of both *KCNQ1* and *CDKN1C* demonstrated to exhibit temporal effects in fetal and adult human pancreas and islets emphasizing that the diabetes risk may be mediated in early development (Travers et al., 2013). In line with this notion, unbalanced placental expression of *CDKN1C* has been associated with intrauterine growth retardation (McMinn et al., 2006). This further supports the idea of possible contributing role of intrauterine programming of reduced pancreatic β-cell function in this population.

Several other genetic loci might deserve attention such as consistently lower frequency of risk variants in *NOTCH2* rs1493694 in IROD2 cluster. *NOTCH2*, which encodes for neurogenic locus notch homolog protein 2, is known to be responsible for regulating interactions between adjacent cells. It was demonstrated that *NOTCH2* is involved in insulin secretion and sensitivity as well as growth and development of the pancreas (Jonsson et al., 2013). The protein's extracellular domain consists of multiple EGF-like repeats while intracellular domain is involved in cell signaling affecting a variety of developmental processes controlling cell fate determination. Altered *NOTCH2* expression was found to be related to diabetic complications (Rasheed et al., 2017).

It is worth discussing that comparison of cluster characteristics in patients with longer duration of diabetes demonstrated that we for sure could not tell which of the two insulin resistant obese clusters in long-term would match the original SIRD and MOD. The original SIRD cluster in the new-onset group was characterized by high HOMA2-IR and HOMA2-B. In long-term diabetes, we could not observe the original phenomenon of simultaneously elevated both indices. To avoid confusion with the original clusters, we named the groups IROD1 in which for the given high HOMA2-IR a reduced HOMA2-B was observed, and IROD2 in which for the relatively lower HOMA2-IR higher HOMA2-B was observed. One possible explanation for the long-term HOMA-B changes in IROD1 group could be related to more frequent use of sulfonylurea drugs (61 vs. 48%, $p = 0.007$), which increase insulin secretion in short term but are considered to lead to lower insulin secretion in long term (Maedler et al., 2005; Shin et al., 2012). Additionally, these clusters differed in respect to the risk of complications with IROD2 having lowest prevalence of CKD. The IROD2 cluster was characterized by better insulin secretion and reduced frequency of the risk allele in the *TCF7L2* gene in line with genetic data in the SIRD cluster from ANDIS (Aly et al., 2020). These findings emphasize that in long-term cases changes in HOMA2-IR and HOMA2-B might occur and rather a general fit to the cluster might be considered as opposed to the given preference to one of these measures, and genetic information could be beneficial to assign people to the original SIRD cluster.

Limitations

The analyses in the present study were conducted on the patients with adult diabetes from a defined population of northern Ukraine (Chernihiv and Kyiv regions) with a history of Holodomor famine. Therefore, the findings might not be generalized to the other regions of Ukraine. The cohort comprised of adult patients with established diabetes, and the blood sampling was conducted at the study visit instead of the time of diagnosis, which limits the prognostic assessment complications risk in this cross-sectional study. Follow-up data would be required to determine if the difference between the SIRD and MOD groups in new onset diabetes and the IROD1 and IROD2 groups are due to phenotype changes over time, and in that case which of the two indices of insulin resistance and

β-cell function (HOMA2-IR or HOMA2-B) in patients with long-term would have a better fit to the original insulin resistant SIRD and MOD clusters coined for ANDIS. An alternative explanation is poor performance of the clustering algorithm due to the relatively small cohort size. It has been seen also in ANDIS that the MOD and SIRD clusters are the least stable but that a larger sample size can improve stability and reproducibility of the clusters. There were more women than men in this cohort, which could potentially give gender-specific effects. However, all analyses were separately performed in men and women, and gave similar results. The K_{mean} clustering algorithm used in the current approach presumes that all the clustering variables have the same weight. Nevertheless, giving variables different weights or prioritizing importance or using another clustering algorithm might improve the approach. All patients were recruited at the primary health care centers or outpatient clinic, which minimized the bias related to recruitment of severe diabetes patients admitted to the hospital or being on ward. Although the sample size is limited for genetic analyses, the power calculations showed that significant effects ($p < 0.00014$) would be reached for variants with the Genotype Relative Risk (disease probability for individuals with 1 risk allele divided by disease probability for individuals with 0 risk alleles) above 1,8 (Skol et al., 2006; Goncalo Abecasis et al., 2017).

CONCLUSION

In conclusion, pathophysiology-based clustering is undoubtedly beneficial for diagnosing different subtypes of adult diabetes related to risk of micro- and macrovascular complications. Assessment of GADA is prerequisite to correctly re-classify SAID patients with adult diabetes, which in the clinical practice can be misclassified as T2D. It can be a clear advantage for the patients belonging to the SIDD cluster to start treatment with insulin or other therapeutic modalities at an earlier stage in order to preserve and maintain β-cell function. The persons with long-term diabetes assigned to IROD2 cluster exhibited preserved insulin secretion and lower risk for microvascular complications. Thus, this cluster represents an interesting subgroup of patients for further investigations of protective mechanisms. The current diabetes cluster approach could be further refined and optimized by including other new biomarkers derived from ongoing omics studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because of GDPR and ethical restrictions. Requests to access the datasets should be directed to valeriya.lyssenko@uib.no.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee at "Regional Hospital," Chernihiv, Ukraine (approval number Dnr17/2011-09-14) and Regional Committee for Medical and Health Research (Ethics, South-east, Panel A, approval number 2019/28968). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OF did the statistical analyses, data interpretation, and wrote the manuscript. TO, EA, and OA assisted in statistical analyses. LC, NK, TS, and TB performed recruitment of patients, data acquisition, and clinical interpretation. OS, TO, EA, LG, and PN contributed to editing of the manuscript and data interpretation. VL conceived and designed the study, planned the analyses, supervised all parts of the study, interpreted the data, and wrote the manuscript. All authors contributed to the interpretation of the data, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.637945/full#supplementary-material

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II

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Perinatal famine is associated with excess risk of proliferative retinopathy in patients with type 2 diabetes

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ABSTRACT.

Purpose: **Intrauterine undernutrition is associated with increased risk of type 2 diabetes. Children born premature or small for gestational age were reported to have abnormal retinal vascularization. However, whether intrauterine famine act as a trigger for diabetes complications, including retinopathy, is unknown. The aim of the current study was to evaluate long-term effects of perinatal famine on the risk of proliferative diabetic retinopathy (PDR).**

Methods: **We studied the risk for PDR among type 2 diabetes patients exposed to perinatal famine in two independent cohorts: the Ukrainian National Diabetes Registry (UNDR) and the Hong Kong Diabetes Registry (HKDR). We analysed individuals born during the Great Famine (the Holodomor, 1932–1933) and the WWII (1941–1945) famine in 101 095 (3601 had PDR) UNDR participants. Among 3021 (251 had PDR) HKDR participants, we studied type 2 diabetes patients exposed to perinatal famine during the WWII Japanese invasion in 1942–1945.**

Results: **During the Holodomor and WWII, perinatal famine was associated with a 1.76-fold ($p = 0.019$) and 3.02-fold ($p = 0.001$) increased risk of severe PDR in the UNDR. The risk for PDR was 1.66-fold elevated among individuals born in 1942 in the HKDR ($p < 0.05$). The associations between perinatal famine and PDR remained statistically significant after corrections for HbA1c in available 18 507 UNDR ($p_{\text{additive interaction}} < 0.001$) and in 3021 HKDR type 2 diabetes patients ($p < 0.05$).**

Conclusion: **In conclusion, type 2 diabetes patients, exposed to perinatal famine, have increased risk of PDR compared to those without perinatal famine exposure. Further studies are needed to understand the underlying mechanisms and to extend this finding to other diabetes complications.**

Key words: diabetic retinopathy – famine – intrauterine exposure – microvasculature – type 2 diabetes – undernutrition

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Introduction

Patients with type 2 diabetes are at high risk of vision-threatening retinopathy. However, the underlying causal mechanisms are not well understood, and current preventive and/or treatment modalities are far from optimal. An adverse foetal environment, associated with low birth weight, has consistently been associated with increased risk of type 2 diabetes, overt cardiovascular diseases and hypertension (Barker et al. 1989; Bonamy et al. 2005; Lawlor et al. 2005; Bonamy et al. 2007; Vaag et al. 2014; Vaiserman 2017). The underlying pathogenic mechanisms behind correlation between intrauterine undernourishment and development of type 2 diabetes have been ascribed to the shunting of energy resources to the cerebral metabolism in order to secure normal brain development at the expenses of reduced glucose uptake and utilization on the periphery (muscle, adipose tissue and liver) (Kuzawa 1998). Thus, earlier studies by Barker and colleagues reported reduced insulin secretion in persons with low weight at birth suggesting that undernutrition during fetal life could impair the development of pancreatic β-cells secreting insulin (Hales et al. 1991; Robinson et al. 1992). Recent studies also reported decreased insulin-stimulated glucose uptake as early as 25 years of age in people born with low birth weight (Jaquet et al. 2000). These defects may arise as the result of epigenetic changes occurring in fetal life, including not the least immature stem cells, permanently changing key cell functions in all the affected organs in type 2 diabetes throughout life (Vaag et al. 2012; Vaag et al. 2014). Indeed, perinatal exposure to famine was suggested to contribute to the rapid increase of type 2 diabetes prevalence in China – the current epicentre of the global diabetes epidemic (Zimmet et al. 2017). To this end, the Dutch hunger winter (1944–1945), the Chinese famine (1959–1961) and the Great Ukrainian (1933–1934) famine studies consistently confirmed the link between famine exposure at or prior to birth and the long-term adverse consequences in adults such as hyperglycaemia, obesity, dyslipidaemia, cardiovascular disease and kidney dysfunction (Roseboom et al. 2001; Lumey et al. 2015; Zimmet et al. 2017). Famine-related undernutrition, particularly during late pregnancy, was associated with intrauterine growth retardation (Stein et al. 1995). Longitudinal studies of preterm or born small for gestational age infants documented abnormal and reduced retinal vascularization (Kistner et al. 2002; Mitchell et al. 2008; Gopinath et al. 2010). Interestingly, there are even data to suggest that an adverse intrauterine environment is associated with microvascular dysfunctions per se, including diabetic retinopathy (DR) later in life (Hellstrom et al. 1998; Clough & Norman 2011). Thus, type 2 diabetes patients exposed to famine or undernutrition early in life may exhibit a disproportionately increased risk of DR compared with type 2 diabetes patients who have not been exposed to undernutrition early in life. However, we are unaware of any previous studies that have addressed this question.

Methods

Study populations

Ukrainian National Diabetes Registry (UNDR)

Details of the data ascertainment in the UNDR were reported elsewhere (Lumey et al. 2015). All patients attending healthcare centres and diabetic clinics were registered in the UNDR database. Present analyses of the UNDR involved 101 095 eligible patients with type 2 diabetes born during 1904–1977. Of these, 53 321 (34% men) were from two regions in northern Ukraine (Chernihiv and Kyiv) with a history of the Holodomor famine in 1932–1933, and 47 774 (37% men) were from two regions in western Ukraine (Rivne and Volyn) who did not experience the Holodomor famine, as these regions were a part of Poland until 1939. These northern Ukraine regions were chosen for analysis because there are geographically and climatically similar, and populations residing in these regions are ethnically homogeneous and have similar socioeconomical and nutritional profiles to western Ukraine regions. Patients from the unexposed control regions had similar clinical characteristics including age at visit, age at onset and BMI, as compared to the exposed regions. However, they had less oral and insulin treatments, and on average 1% (7 mmol/mol) lower HbA1c. Both populations experienced the WWII 1941–1945 and the postwar 1947 famines. Periods of famine exposure were defined according to the population counts from the Ukrainian national census and were confirmed using the birth year pyramids (Fig. S1), as previously reported (Lumey et al. 2015). Type 2 diabetes was defined as the age of onset over 40 years, or between 35 and 40 years, if patients did not receive insulin treatment. Stages of DR were based on fundus photography and were recorded by ophthalmologists. Primary care physicians recorded the information about different stages of DR in the UNDR cohort, with the first entry on 27 January 1999 and the last entry on 18 January 2013. Proliferative diabetic retinopathy (PDR) was defined as the presence of proliferative retinopathy, or blindness in either eye. The institutional review board of the Komisarenko Institute of Endocrinology and Metabolism (Kyiv, Ukraine) approved the use of anonymized data (approval number for Ukraine: Dnr3/2006-11-10), also study was approved by the Norwegian ethics committee (approval number for Norway: 2019/28968). A flow chart with the selection of individuals for the analyses is presented in Fig. S4A.

The Hong Kong Diabetes Register (HKDR)

The HKDR was established in 1994 at the Diabetes and Endocrine Centre, the Prince of Wales Hospital, Hong Kong Special Administrative Region (Luk et al. 2017). Patients with physician-diagnosed diabetes who attended the Centre for a comprehensive evaluation of diabetes complications were consecutively recruited. Referral sources included hospital- and community-based clinics. Detailed information, including demographics, comorbidities and medication use, was documented. Physical measurements, including vital signs and anthropometric parameters, were collected. The presence of diabetic retinopathy was assessed by fundus photography and interpreted by trained endocrinologists. Advanced diabetic retinopathy was defined by fulfilling one or more of either: reduced visual acuity, proliferative diabetic retinopathy, preproliferative diabetic

retinopathy, history of laser photocoagulation or presence of laser scar, history of vitrectomy. Fasting blood samples were obtained for plasma glucose, HbA1c, lipids and renal function tests. Written informed consent was obtained from patients at study enrolment. The HKDR was approved by the New Territories East Cluster Clinical Research Ethics Committee (reference number 2007.339). During the WWII period (1941–1945), Hong Kong experienced famine exposure as a consequence of the Japanese invasion, which lasted for three years and eight months. The data set used in the present analyses included 3021 eligible participants from HKDR as described in Fig. S4B.

Statistical analysis

The odds ratios (OR) of PDR associated with being born during the Holodomor and the consecutive WWII exposure were calculated using logistic regression adjusted for sex, duration of diabetes, and year of diagnosis and plotted for each individual year of birth for the exposed and unexposed populations. The OR of PDR for the birth years 1929–1936 were plotted with individuals born before or in 1928 as a reference group; for each birth year during 1937–1946 with individuals born in 1936 as a reference group; and for each birth year after 1947 using individuals born in 1946 as a reference group. The hypothesis that the risk of PDR was higher in the exposed regions during famine periods was tested by fitting the interaction term between the year of birth (YOB) and the region of birth using logistic regression, adjusted for sex, duration of diabetes and year of diagnosis (VanderWeele & Knol 2014). Because only 18 507 (~13%) of the UNDR participants had HbA1c measurements, subsequent episodes of the Holodomor and the WWII famines were combined into decades of births before 1950 (exposed) and after 1950 (unexposed). A significant ($p < 0.10$) interaction term (famine decades, Yes/No × exposed regions, Yes/No) between combined famine exposures and the exposed regions indicated elevated risk at specified time points attributable to the famine. In the HKDR, logistic regression was performed to test the hypothesis whether people born during the period of WWII famine (1942–1945) had higher odds of PDR as compared to the reference group of those born in 1948 (economic recovery and political independence of Hong Kong), adjusted for gender, year of assessment, diabetes duration and HbA1c (Carroll 2007). All analyses were performed using R software and Graph Prism (Adrian & Dragulescu 2018; Wickham 2018; Carey & Ripley 2019; Hadley Wickham et al. 2019; Team RC 2020; GraphPadPrism). All reported *P*-values are two-sided ($p < 0.05$) (VanderWeele & Knol 2014).

Results

To evaluate the long-term effects of perinatal famine on the risk of PDR, we analysed the data on 101 095 patients with known type 2 diabetes from the UNDR cohort. Populations exposed to the Holodomor demonstrated evident gaps for the births during the years of famine exposure, as illustrated by the demographic plots stratified by the region and YOB (Fig. S1). The overall prevalence of PDR was 4.7% in the exposed populations (Chernihiv and Kyiv) and 2,2% in the unexposed populations (Rivne and Volyn) (Table 1). Demographic characteristics of age, age-onset, BMI, diabetes duration and HbA1c for the exposed and unexposed type 2 diabetes populations are shown in Table 1. Exposed population included less men (34.2% vs 37.3%, $p < 0.0001$), had higher HbA1c (63.96 mmol/mol vs 57.19 mmol/mol, $p < 0.0001$) and exhibited higher frequency of hypertension (71.1% vs 65.08%, $p < 0.0001$). No differences were observed between groups regarding diabetes duration (Table 1). There were statistically significant differences in the clinical characteristics of retinopathy cases in famine-exposed and unexposed groups; however, these differences were clinically insignificant (Table S1).

The OR for PDR in type 2 diabetes individuals born at the time of the Holodomor and the WWII famine for each YOB and the exposed and unexposed populations are shown in Fig. 1A and Table S2a. The corresponding OR from the interaction analyses for each YOB for offspring of individuals from the exposed compared to the unexposed populations are shown in Fig. 1B. Type 2 diabetes individuals with perinatal famine during the Holodomor, the WWII and the postwar had significantly increased risk of PDR in adulthood, with the highest OR = 1.76 (90% Confidence Interval (CI), 1.19–2.63, $p = 0.019$) for the YOB 1934; OR = 3.02 (90% CI, 1.75–5.34, $p = 0.001$) for the YOB 1943; and OR = 1.76 (90% CI, 1.00–3.13, $p = 0.103$) for the YOB 1947 (Fig. 1B, Table S2a). Further adjustment for seasonality and hypertension did not change the clear association between early famine exposure and development of PDR (Fig. S3D,E). Notably, the risks for PDR were also observed to be high for persons born before Holodomor (YOB in 1904–1931), between prewar and WW2 famine periods (YOB in 1935–1941) and after WW2 famine (YOB in 1946, 1948–1950) in exposed regions (Fig. S2B), and therefore, the entire population was further stratified for the combined analyses by YOB <1950> (<1950 between and within famine periods, > 1950 non-famine, Fig. 1D). In the combined analysis, individuals from the exposed regions born during famine periods showed 1.63-fold (95% CI, 1.38–1.93, $p = 1.02 \times 10^{-8}$) increased risk of PDR compared to individuals from the unexposed regions with OR = 1.07 (95% CI, 0.84–1.37, $p = 0.60$), $p_{\text{additive interaction}} = 8.5 \times 10^{-10}$ (Fig. 1D). Adjustment for the HbA1c levels in the subset of individuals with available data ($N = 18\,507$) did not change interaction results $p_{\text{additive interaction}} = 0.009$ (Fig. S2A). Clinical characteristics of the replication HKDR cohort are shown in the Table S3. The OR for PDR associated with exposure to famine as a consequence of the Japanese invasion in Hong Kong during WWII are shown for each year of birth from 1939 to 1947 in Fig. 1C and Table S2b. In line with the results in the UNDR cohort, type 2 diabetes offsprings of individuals exposed to famine in the HKDR cohort showed significantly increased risk of PDR with the OR=1.66 (95% CI, 1.08–2.53, $p = 0.019$) among those born in 1942 adjusted for gender, year of assessment, diabetes duration and HbA1c. These results support robustness of the findings and suggest that perinatal famine was associated with excess risk of PDR in individuals with type 2 diabetes.

Table 1. Clinical characteristics of participants in the UNDR study

Phenotype	All	Exposed to Holodomor	Unexposed to Holodomor	p-value
Number of people (M, %)	101 095 (35.7)	53 321 (34.2)	47 774 (37.3)	–
Retinopathy (%)	3601 (3.5)	2552 (4.7)	1049 (2.2)	<0.0001
Hypertension (%)	62 610 (68.05)	32 275 (71.1)	30 335 (65.08)	<0.0001
Age at baseline (years)	65.83 (10.88)	66.54 (10.61)	65.04 (11.12)	<0.0001*
Age of type 2 diabetes diagnosis (years)	58.82 (10.33)	58.82 (10.09)	58.82 (10.6)	<0.0001
Duration of type 2 diabetes (years)	7.25 (7.1)	7.91 (7.18)	6.5 (6.93)	<0.0001*
BMI (kg/m^2)	28.82 (4.74)	28.73 (4.62)	28.91 (4.85)	<0.0001
Height (m)	166.56 (7.65)	166.36 (7.68)	166.75 (7.61)	0.50
HbA1c (%)	7.44 (1.77)	8 (2.21)	7.38 (1.71)	<0.0001
HbA1c (mmol/mol)	57.78 (19.37)	63.96 (24.2)	57.19 (18.74)	<0.0001
SBP (mm/Hg)	143.21 (18.75)	144.64 (19.42)	141.82 (17.97)	<0.0001
DBP (mm/Hg)	86.42 (10.12)	87.05 (10.28)	85.81 (9.92)	<0.0001
Treatment: diet	26%	15%	38%	<0.0001
Treatment: insulin	8%	7%	8%	<0.0001
Treatment: pills	58%	68%	47%	<0.0001
Treatment: pills and insulin	8%	10%	6%	<0.0001

p-value was calculated using linear regression adjusted for sex, diabetes duration, age at visit and year of diagnosis.
* Sex adjusted p-values.

Discussion

In the present study, we explored the hypothesis that the risks for PDR in adulthood were higher in type 2 diabetes offspring of parents with exposure to famine in the two independent cohorts from the Ukrainian National Diabetes Registry (UNDR) and the Hong Kong Diabetes Register (HKDR). A retrospective analysis of the UNDR cohort indicated that being born during the Great Ukrainian famine (the Holodomor, 1932–1933) was associated with increased risk of PDR in patients with type 2 diabetes independently of disease duration, HbA1c and year of diagnosis. Furthermore, perinatal exposure to the WWII (1941–1945) was associated with increased risk of PDR in the populations with previous exposure to the Holodomor famine as oppose to the populations that did not experience the Holodomor. The link between exposure to famine at birth in 1942 and increased risk of PDR in adulthood was replicated by studying patients with type 2 diabetes from the HKDR, who were exposed at birth to the WWII famine as a consequence of the Japanese invasion in 1942–1945.

Type 2 diabetes is a multiple organ disease involving defects in pancreatic insulin secretion, hepatic glucose production, muscle insulin action, adipose tissue metabolism, gut incretin functions, appetite regulation and CNS functions, as well as vascular functions influencing organ blood flow. Emerging research has shown that low birth weight, occurring as the result of an adverse intrauterine environment, may contribute to the majority or all known metabolic organ defects relevant to the development of type 2 diabetes, and even be present prior to the onset of overt disease (Vaag et al. 2012; Vaag et al. 2014). Thus, intrauterine growth restriction may lead to alterations of fetal programming in a number of organs and functions including vascular endothelium and contribute to mechanisms underpinning elevated blood pressure in people with low weight at birth (Gennser et al. 1988). This, in turn, may increase propensity of low birth infants to have increased risks of vascular complications as adults. By studying patients with diagnosed type 2 diabetes exposed to severe famine at the time of birth in two different nations, before and during the WWII, we here report evidence that susceptibility to adult PDR might be programmed early in life by mechanisms possibly not related to hyperglycaemia. Our observations indicate that during intrauterine famine, insult to vessel abnormalities may already occur perinatally, which lead to vessels being more susceptible to damage and abnormal functioning due to high glucose levels later in life. We thereby expand the concept of early life developmental programming to include the risk of severe DR among patients with diagnosed type 2 diabetes.

The crude prevalence of visual impairment and blindness caused by PDR has increased in recent years, mainly due to the increase of type 2 diabetes in low- and middle-income countries (Flaxman et al. 2017). Nevertheless, whether factors such as perinatal undernutrition, triggering an epidemic of diabetes in these high-risk populations, may also predispose to the more severe diabetes progression towards organ and vascular damage are not well studied. Our data clearly suggest that perinatal undernutrition may have a direct role in the programming of vascular structure and/or functions, predisposing to severe DR and potentially other vascular complications later in life. In support of our data, it is well established that prematurity and impaired foetal growth adversely influence retinal microvascularization in later life, as documented in children and adults (Kistner et al. 2002; Mitchell et al. 2008; Gopinath et al. 2010). In this regard, we have observed a higher prevalence of hypertension in exposed to the Holodomor population in Ukraine than in unexposed. The perinatal exposure to famine has earlier been linked to hypertension in the Dutch hunger winter (Stein et al. 2006), which is an established risk factor for retinopathy in patients with diabetes. It is therefore quite possible that integrated biological mechanisms underlying hyperglycaemia and elevated blood pressure at least in part could act as mediators of intrauterine exposure to famine and diabetic retinopathy in adulthood. Importantly, it has been reported that type 2 diabetes patients born with low birth weight exhibit excess mortality compared with type 2 diabetes patients with average birth weight (Leibson et al. 2005).

Our findings are likely to have direct clinical implications and calls for replications and expansions to include other diabetic vascular complications in the current as well as in other cohorts. DR

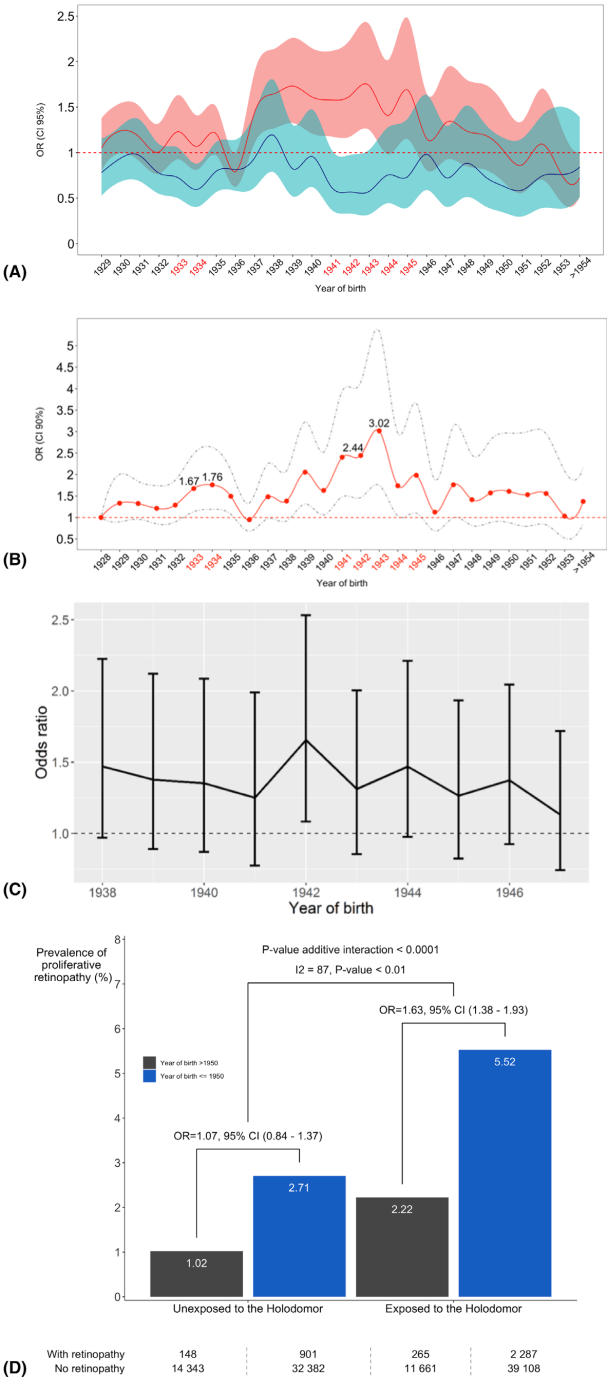

Fig. 1. (A) Odds ratios of PDR in adulthood stratified by the years of birth for exposed and unexposed to the Holodomor regions (the UNDR study). OR and 95% CI of PDR in type 2 diabetes offspring of individuals from the regions exposed to the Holodomor (n = 53 321, Chernihiv and Kyiv, red lines) and unexposed (n = 47 774, Volyn and Rivne, blue lines) from the UNDR stratified by the year of birth. Logistic regression models for PDR were adjusted for sex, diabetes duration and year of diagnosis. (B) Cross-over odds ratios for PDR in adulthood stratified by the years of birth (the UNDR study). ORs and 90% CI for PDR obtained from interaction analyses between the year and the region of birth using a logistic regression adjusted for sex, duration of diabetes and year of diagnosis. Spline function was applied to interconnect odds ratios for curve fitting (spline function, stats package, R). Odds ratios depicted as curves above the reference line (which equals one) denote elevated risk of PDR in famine-exposed regions. Years of birth marked in red on the X-axis indicate periods of exposure to the Holodomor at conception (1932–1933) and WWII (1941–1945) famines. (C) Odds ratios of PDR stratified by the year of birth in the HKDR. Risks for PDR in the HKDR of patients with type 2 diabetes. ORs and 95% CI of PDR were calculated for each year of birth, comparing with the reference group of those born in 1948, using logistic regression adjusted for gender, year of assessment, diabetes duration and HbA1c. (D) Odds ratios of PDR in adulthood in the combined analysis of famine and non-famine periods for exposed and unexposed to the Holodomor regions (the UNDR study). Association of the combined exposure at the specified time points on the prevalence of PDR in type 2 diabetes offspring of individuals with a history of the Holodomor. OR and 95% CI for the risk of PDR was obtained using logistic regression adjusted for sex and age, and duration of diabetes and year of diagnosis. The impact of the combined exposure to famine defined at specific time points on the risk of PDR between individuals from the regions exposed or unexposed to the Holodomor was assessed using interaction model and verified using I^2 heterogeneity test. HKDR = Hong Kong Diabetes Registry; PDR = proliferative diabetic retinopathy; UNDR = Ukrainian National Diabetes Registry.

is commonly observed already at the time of diagnosis in some type 2 diabetes patients, and our results raise the question of how adverse perinatal programming can contribute to this finding. It furthermore raises the question of whether glucose treatment may be as efficacious to prevent progression of DR among patients with type 2 diabetes exposed to an adverse perinatal environment as those patients without any early life nutritional or development shortcomings or deficiencies. If our findings can be reproduced and extended to other populations, perinatal famine could be contemplated as a key factor not only for the

development of DR but also for the better understanding of the subclusters in type 2 diabetes patients (Ahlqvist et al. 2018).

Our study has some limitations and strengths. Present observations suggest that there might not be a clear cut for prewar famine exposure in the regions from northern Ukraine as Stalin terror towards Ukrainian uprisings against the Soviet-Russian regime started in the early 1920s (Johnston 1986). Thus, elevated risks for PDR indicate that these regions might experience famine as a continuum moving from the less to severe or extreme exposures. However, periods of Holodomor and WWII are undoubtedly the most severe famine exposures, which evidently were confirmed by the population loss as demonstrated on the demographic pyramids. The HKDR is a much smaller data set, and a significant peak for an association of famine with severe DR during WWII year 1942 could be observed by chance. However, this peak is compatible with the highest peaks observed during Holodomor and WWII famine periods in the UNDR. The association results in the Hong Kong cohort might be more sensitive and thereby powerful due to more homogenous ascertainment as compared to the possible heterogeneity attributed to several sites included in the UNDR. However, the UNDR has a larger sample size and greater overall power to detect famine effects. In the UNDR cohort, we were also unable to disassociate the cases of laser treatment and blindness caused by other eye diseases of the elderly, such as cataracts and macular oedema. Nevertheless, sensitivity analyses restricted to only individuals with reports on concomitant PDR and blindness resulted in similar conclusions indicating a non-significant contribution of the potentially smaller part of other reasons contributing to blindness (Fig. S3A). This is comparable to the results in the Hong Kong population where cataracts and macular oedema were excluded from the analyses. Additionally, we cannot examine any minor effects of migration. However, such effects would cause an underestimation of the observed effects, why in fact the associations between starvation exposure and PDR development later in life may be even higher than found in this study. Finally, we cannot rule out the possibility of a calendar effects or effects unrelated to famine such as different overall socio-economic circumstances, persistent psychological stresses, medical treatments and other factors, which could have detrimental effects on normal course of the pregnancy in these rather specific time periods. These factors could contribute to observed differences in clinical risk profiles between patients from exposed and unexposed regions and thereby could be considered mediators between periods of famine and PDR.

In conclusion, these results indicate that patients with type 2 diabetes exposed to perinatal famine are at increased risk of severe DR compared with subjects with type 2 diabetes born during periods without societal famine exposures. Further studies are needed to understand the underlying mechanisms, as well as to confirm and extend these findings to other diabetes complications.

Data and Resource Availability

The data sets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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OF did the statistical analyses, RJ, RP, MGR, TU, CH, RS, DJ and IA involved in data interpretation and drafted the manuscript. DS assisted in statistical analyses and acquisition of the data. LC, NK, TS, TB and AMZ developed the study design and collected the data. VK, AV and MDK involved in acquisition of the data. PMN and AAV contributed to the editing of the manuscript. VL conceived and designed the study, planned the analyses, supervised all parts of the study, interpreted the data and wrote the manuscript. All authors contributed to the interpretation of the data and approved the final version of the manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Demographic plots according to the year of birth in the Ukraine National Diabetes Registry (UNDR).
Figure S2. (A) Influence of perinatal exposure to famine on the risk of proliferative diabetic retinopathy in adulthood in a subset of $n = 18\,507$ individuals with the data on longterm glycemia measured with HbA1c in the UNDR study. (B) Risks for DR in the combined analysis of YOB in the exposed and unexposed to the Holodomor populations (UNDR).
Figure S3. (A) Cross-over odds ratios for PDR in adulthood stratified by the years of birth restricted to individuals with concomitant proliferative retinopathy and blindness (UNDR). (B) Cross-over odds ratios for PDR in adulthood stratified by the years of birth, excluding blindness cases without PDR diagnosis (UNDR study). (C) Cross-over odds ratios for PDR in adulthood stratified by the years of birth and seasons for years of birth with famine exposure (UNDR study). (D) Cross-over odds ratios for PDR in adulthood stratified by the years of birth and adjusted for seasonality (UNDR study). (E) Cross-over odds ratios for PDR in adulthood stratified by the years of birth and adjusted for hypertension (UNDR study).
Figure S4. (A) Flowchart for quality control and preparation dataset for analysis in the UNDR study. (B). Flowchart for quality control and preparation dataset for analysis in the Hong Kong Diabetes Registry (HKDR).
Table S1. Clinical characteristics of participants in the UNDR study.
Table S2. (a) Crossover odds ratios for the interaction between the year and region of birth for prevalent proliferative diabetic retinopathy in the UNDR. 2b. OR for advanced DR for each year of birth in the HKDR.
Table S3. Clinical characteristics of the participants in the HKDR.
Table S4. Odds ratios of severe DR in adulthood stratified by the years of birth for exposed and unexposed to the Holodomor regions (the UNDR study).
Table S5. (a) Cross-over odds ratios for PDR in adulthood stratified by the years of birth excluding blindness cases without PDR diagnosis (UNDR study). (b) Cross-over odds ratios for PDR in adulthood stratified by the years of birth and seasons: January-June and July–December for years of birth with famine exposure (UNDR study). (c) Cross-over odds ratios for PDR in adulthood stratified by the years of birth and adjusted for seasonality (UNDR study). (d) Cross-over odds ratios for PDR in adulthood stratified by the years of birth and adjusted for hypertension (UNDR study).

Errata for
Intrauterine and genetic risk factors for proliferative diabetic retinopathy

"[Click and enter subtitle]"

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Thesis for the degree philosophiae doctor (PhD)

at the University of Bergen

24.04.2022

(date and sign. of candidate) (date and sign. of faculty)

Errata

Page 103 Incorrect “Paper I-III” – corrected to “Papers I-IV”

Page 151 in Paper 1, Figure 3 – below the picture is added legend “*Source of images: Servier Medical Art, https://smart.servier.com/*”

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