

## GENETIC PREDISPOSITION FOR TYPE 1 DIABETES MELLITUS – THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN HUMAN DISEASE ETIOPATHOGENESIS

GENETSKA PREDISPOZICIJA ZA TIP 1 DIABETES MELLITUSA – ULOGA STRESA  
ENDOPLAZMATSKOG RETIKULUMA U ETIOPATOGENEZI OBOLJENJA ČOVEKA

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**Summary:** The increasing incidence of diabetes mellitus worldwide has prompted a rapid growth in the pace of scientific discovery of the mechanisms involved in the etiopathogenesis of this multifactorial disease. Accumulating evidence suggests that endoplasmic reticulum stress plays a role in the pathogenesis of diabetes, contributing to pancreatic beta cell loss and insulin resistance. Wolfram syndrome is an autosomal recessive neurodegenerative disorder accompanied by insulin-dependent diabetes mellitus and progressive optic atrophy. The pathogenesis of this rare neurodegenerative genetic disease is unknown. A Wolfram gene (WFS1 locus) has recently been mapped to chromosome 4p16.1, but there is evidence for locus heterogeneity, including the mitochondrial genome deletion. Recent positional cloning led to identification of the second WFS locus, a mutation in the CISD2 gene, which encodes an endoplasmic reticulum intermembrane small protein. Our results were obtained by the analysis of a families belonging to specific population, affected by Wolfram syndrome. We have identified the newly diagnosed genetic alteration of WFS1 locus, a double non-synonymous and frameshift mutation, providing further evidence for the genetic heterogeneity of this syndrome. Newly identified mutations may contribute to the further elucidation of the pathogenesis of Wolfram syndrome, as well as of the complex mechanisms involved in diabetes mellitus development.

**Keywords:** diabetes mellitus, endoplasmic reticulum stress, genetic susceptibility

**Kratak sadržaj:** Stalni porast incidence diabetes mellitusa u svetu i u našoj zemlji, predstavlja značajan stimulans za ubrzanje naučnih otkrića koja doprinose uvidu u kompleksne mehanizme uključene u etiopatogenezu ovog multifaktorijalnog oboljenja. Brojna istraživanja ukazuju da je stres endoplazmatskog retikuluma značajan faktor u patogenezi dijabetesa, koji doprinosi apoptozi beta ćelija pankreasa, kao i rezistenciji na insulin. Wolfram sindrom predstavlja autozomno recesivno neurodegenerativno oboljenje, koje karakteriše razvoj insulin-zavisnog diabetes mellitusa i progresivne atrofije optičkog nerva. Wolfram sindrom je retko neurodegenerativno genetsko oboljenje, nepoznate patogeneze. Gen za wolframin (WFS1 lokus) mapiran je na hromozomu 4p16.1, međutim postoje značajni dokazi za genetsku heterogenost, uz prisustvo delecija u mitohondrijalnom genomu kod malog procenta pacijenata. Analize sprovedene primenom strategije pozicionalnog kloniranja dovele su do identifikacije drugog lokusa (WFS2) i uzročne mutacije CISD2 gena za WFS2, na hromozomu 4q24, koji kodira mali intermembranski protein lokalizovan u endoplazmatskom retikulumu. Naši rezultati su dobijeni analizom porodica koje pripadaju specifičnoj populaciji, sa članovima obolelim od Wolframovog sindroma (WFS1). Identifikovali smo novu genetsku alteraciju WFS1 gena, dvostruku »non-synonymous & frameshift« mutaciju, kao dodatnu potvrdu genetske heterogenosti ovog sindroma. Novoidentifikovane mutacije doprinose razumevanju patogeneze Wolfram sindroma, kao i utvrđivanju kompleksnih mehanizama uključenih u nastanak diabetes mellitusa.

**Ključne reči:** diabetes mellitus, stres endoplazmatskog retikuluma, genetska predispozicija

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### Epidemiology of type 1 diabetes mellitus

The increasing incidence of diabetes mellitus worldwide has prompted a rapid growth in the pace of scientific discovery of the mechanisms involved in

the etiopathogenesis of this multifactorial disease. Type 1 diabetes mellitus (T1D) is the most common complex autoimmune organ-specific endocrine disorder, characterized by T cell infiltration and production of autoantibodies directed at the pancreatic islets, resulting in their dysfunction and destruction (1–3). Autoimmune destruction of the pancreatic beta cells leads to insufficient insulin production and is the result of multiple genetic and environmental influences (4, 5). A common explanation has been that the interaction of genetic susceptibility with the changes in the environment must be contributing to the increase in disease. In particular, environmental exposures to dietary antigens and viruses have been implicated (2). However, no single pathogenic environmental agent has been identified that explains all cases. One way to address this complexity is to focus on the central processes that control gene expression in response to environmental conditions. Epigenetic regulation is one such process by which mammals respond to environmental exposures (4). Beta cell development, maintenance, metabolism, and regeneration can all be influenced by epigenetic mechanisms. Also, immune responses, including the activation of T cells and induction of T regulatory cells, rely on appropriate epigenetic regulation. Furthermore, insulin and glucose metabolism influence the epigenome of tissues such as the liver, and potentially the pancreas, which could contribute to T1D associated pathologies. Clearly, information on the epigenetic mechanisms involved in the development of T1D is extremely limited and new studies are required. The hope is that this information will provide new understanding of the pathogenesis, potential treatments, or even prevention of T1D (4).

Genetic susceptibility to T1D is dependent on the degree of genetic identity with the proband, and the risk of diabetes in families has a non-linear correlation with the number of alleles shared with the proband. It has long been known that the likelihood of a person developing T1D is higher the more closely he/she is related to a T1D patient, such that first-degree relatives of cases are at 15 times greater risk of T1D, than a randomly selected member of the general population (6). However, monozygotic twins are concordant for T1D at a frequency of approximately 50%, and the incidence of T1D has been increasing in Western countries, with a doubling of incidence in the USA over the past 30 years.

Unlike type 2 diabetes mellitus, a late onset disease, resulting from the interaction of two mechanisms: 1) abnormal insulin secretion due to pancreatic beta cell defects and 2) insulin resistance in skeletal, muscle, liver, and adipose tissue, T1D typically presents in childhood and has a much stronger genetic component. It primarily arises as a consequence of autoimmune destruction of pancreatic beta cells, resulting in insufficient production of insulin; in addition, syndromes of insulin-requiring beta cell

failure in the absence of clinically evident autoimmunity also fall under the definition of T1D. This disorder accounts for approximately 10% of all cases of diabetes and is most prevalent in populations of European ancestry, with about 2 million people affected in total across Europe and North America. It is well recognized that there is an approximately 3% increase in the incidence of T1D globally per year, at least partly due to a decreasing average age of onset, and it is expected that the incidence will be 40% higher in 2010 than in 1998 (7).

Significant variations in the incidence of T1D in Europe have been detected. The incidence rates are high in the Northern and North West Europe, and low in the Central, Southern, and Eastern Europe (8). The highest incidence was registered in Finland – 42.9/100,000/yr (9), and the lowest in the Former Yugoslav Republic of Macedonia – 3.6/100,000/yr (10). Sardinia, an island in the Mediterranean, is a notable exception to this pattern with the incidence of T1D – 38.8/100,000/yr (11). The standardized incidence of type 1 diabetes (age group 0–14 yr) in Serbia in 2006 was 12.5/100,000 (12), in 1997–2006 in Montenegro 13.4/100,000 (11), in Croatia 8.9/100,000, in Slovenia 11,1/100,000, Bosnia and Herzegovina 6.9/100,000, Former Yugoslav Republic of Macedonia 3.6/100,000 children per year (10). The incidence of childhood diabetes in Montenegro and Serbia in the last 5 years are amongst the highest in comparison with other former Yugoslav republics. Therefore, it would be considerably important to carry out the genetic, immunologic, nutritional and ecologic studies, in order to explain the rapid increase of T1D incidence in children under the age of 15 in Serbia.

### **Overview of the genetic factors involved in T1D etiopathogenesis**

T1D is mostly caused by an autoimmune process, characterized by T cell-mediated destruction of pancreatic beta cells. The destruction is caused by infiltration of the islets of Langerhans by dendritic cells (DCs), macrophages and T lymphocytes (both CD4+ and CD8+) and is specific for the insulin-producing beta cells, not affecting glucagon (alpha) or somatostatin (delta) cells. The disease typically affects young individuals, with onset as early as one year of age; most cases are diagnosed before the age of 18. The autoimmune process starts even earlier, as evidenced by the presence of autoantibodies in the serum against T1D-specific antigens. The three major autoantigens involved are insulin itself, GAD65 (glutamic acid decarboxylase, 65 kDa isoform) and IA2 (insulin autoantigen 2, an intracellular phosphatase). These autoantibodies are merely markers of the destruction, which is T-lymphocyte-mediated. Both the CD4+ (helper) and the CD8+ (cytotoxic) T-lymphocyte subsets are important in the T1D process.

The former recognize extracellular antigens and promote inflammation through cytokine release, whereas the latter respond to endogenously synthesized (e.g. viral) antigens and directly kill target cells. By the time symptoms that lead to clinical diagnosis appear, most of the beta cell insulin-secretory capacity has been lost. Therefore, prediction and prevention of T1D are of crucial importance, and it is hoped that better knowledge of the genetic underpinnings of T1D will greatly facilitate both (5).

Common allelic variants at the human leukocyte antigen (HLA) class II loci account for the major T1D genetic risk in children and young adults (13). Susceptibility to type 1 diabetes (T1D) is determined by complex interactions between several genetic loci and environmental factors. Alleles at the human leukocyte antigen (HLA) locus explain up to 50% of the familial clustering of T1D, and the remainder is contributed to multiple loci, of which only four were known until recently (5). The HLA region on chromosome 6p21 accounts for about half of the familial clustering of T1D through a large variety of protective and predisposing haplotypes. Other important loci associated with T1D with much smaller effects than HLA involve the insulin gene (INS) on 11p15, PTPN22 on 1p13, CTLA4 on 2q31, the interleukin-2 receptor  $\alpha$  (CD25, encoded by IL2RA) locus on 10p15, IFIH1 (also known as MDA5) on 2q24 and, most recently, CLEC16A (KIAA0350) on 16p13, PTPN2 on 18p11 and CYP27B1 on 12q13. In addition, recent genome-wide association (GWA) studies have uncovered two additional solidly replicated loci that, however, have not yet been mapped to individual genes (14).

### Endoplasmic reticulum and stress signalling

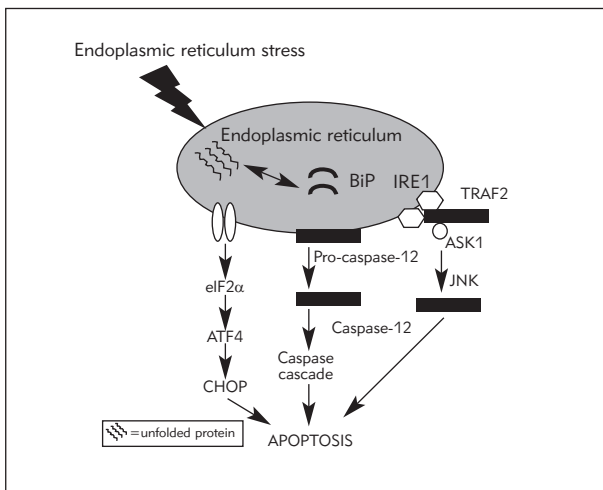
The endoplasmic reticulum (ER) is an organelle that plays several vital functions in multiple cellular processes that are required for cell survival and normal cellular functions (15, 16). The ER is the site of protein synthesis in the rough ER, and correct post-translational modifications of proteins, including glycosylation, disulfide bond formation and proper chaperone-mediated folding and assembly of many proteins, destined for secretion or display on the cell surface. The ER provides a high fidelity quality control system to ensure that only correctly folded proteins can be transported out of ER, while unfolded or misfolded proteins are retained in the ER and eventually degraded (17). In addition, because of its role in protein folding and transport in the secretory pathway, the ER is also rich in calcium-dependent molecular chaperones, such as glucose – regulated protein, 78kDa (GRP78), GRP94 and calcireticulin, which help stabilize protein-folding intermediates. The lumen of ER constitutes a unique cellular environment, with the highest concentrations of calcium

within the cell, and it acts as an indispensable source for fast physiological signalling, being a dynamic calcium ions ( $\text{Ca}^{2+}$ ) reservoir, which can be activated by both electrical and chemical cell stimulation. Endoplasmic reticulum also has vital roles in lipid-membrane biosynthesis and in controlling the production of cholesterol and other membrane lipid components (15).

Several patho-physiological stimuli, such as those that cause ER calcium depletion, altered glycosylation, nutrient deprivation, oxidative stress, DNA damage, high-fat diet, viral infections or energy perturbation can cause accumulation of unfolded proteins in the ER, triggering an evolutionarily conserved response termed the unfolded protein response (UPR) (15). Disturbances in cellular redox regulation caused by hypoxia, oxidants or reducing agents, interfere with disulphide bonding in the lumen of the ER, the chaperones become overloaded, and the ER fails to fold and export newly synthesized proteins, leading to protein unfolding and misfolding, and ER stress. The accumulation of unfolded proteins in the ER occurs most probably by exhausting proteasome capacity and causing an accumulation of unfolded proteins scheduled for degradation, via retrograde translocation from the ER into the cytosol for ubiquitylation (15).

Once ER stress is provoked in the cells, various pathways are activated (18). The consequences of triggering the UPR because of ER stress in mammalian cells can be grouped into three types of effector functions: adaptation, alarm and apoptosis (15). The initial intent of the UPR is to re-establish homeostasis and normal ER function, and adaptive mechanisms predominantly involve activation of transcriptional programs that induce expression of genes that are capable of enhancing the protein folding capacity of the ER and genes for ER-assisted degradation (ERAD). This helps clear the ER of unfolded proteins and export them to the cytosol for degradation. Translation of mRNAs is also initially inhibited for a few hours, thereby reducing the influx of new proteins into the ER until mRNA encoding UPR proteins are produced. These adaptive aspects of the UPR probably have essential roles in the normal physiology of some types of cells, including professional secretory cells, such as pancreatic beta cells, plasma cells and hepatocytes, which exert high demands on their ER (15, 18).

The *Figure 1* depicts the three main pathways of ER stress-induced apoptosis identified in human cells: (1) the proapoptotic pathway of CHOP/GADD153 (GADD153 = ATF4) transcription factor which is mainly induced via PERK/eIF2. CHOP down-regulates the anti-apoptotic factor B cell lymphoma-2 (Bcl-2), but also upregulates Ero-1, a thiol oxidase that promotes protein folding in the ER but also generates reactive oxygen species (ROS), and thereby promotes



**Figure 1** ER stress induced apoptosis (modified from (19)).

apoptosis; (2) IRE1-mediated activation of apoptosis signal-regulating kinase 1 (ASK1)/c-Jun NH<sub>2</sub>-terminal kinase (JNK). IRE1 interacts with TRAF2 (TNF receptor associated factor-2) and ASK1 (19). This leads to activation of ASK1 and JNK, followed by apoptosis; and (3) activation of the ER localized cysteine protease, caspase 12. Caspase 12 is activated by m-Calpain in the cytoplasm. Activation of m-Calpain is a consequence of Ca<sup>2+</sup> efflux out of the ER upon ER stress. These three pathways all end in caspase cascade activation, the execution phase of ER stress-induced apoptosis (19).

In eukaryotic cells, monitoring of the ER lumen and signaling through the canonical branches of the UPR are mediated by three ER membrane-associated proteins, PERK (PKR-like eukaryotic initiation factor 2 $\alpha$  kinase), IRE1 (inositol requiring enzyme 1), and ATF6 (activating transcription factor-6). In a well-functioning and »stress-free« ER, these three transmembrane proteins are bound by a chaperone, BiP/GRP78, in their intraluminal domains (amino-terminal of IRE1 and PERK and carboxy-terminal of ATF6) and rendered inactive. There may also be additional mechanisms controlling the activity of each UPR sensor and simple disruption of the interaction with BiP may not always result in constitutive activation. Accumulation of improperly folded proteins and increased protein cargo in the ER results in the recruitment of BiP away from these UPR sensors. This and potentially other yet to be discovered luminal events result in oligomerization and activation of the two kinases, PERK and IRE1, and engage a complex downstream signaling pathway (19, 20). Activation of the third branch of the UPR requires translocation of ATF6 to the Golgi apparatus where it is processed by the serine protease site-1 protease (S1P) and the metalloprotease site-2 protease (S2P) to produce an active transcription factor. ATF6 is reduced in response to ER stress, and only the reduced monomeric ATF6 can reach the Golgi apparatus, indicating that redox

status is also a potential determinant of ATF6 activation. Together these three arms mitigate ER stress by reducing protein synthesis, facilitating protein ubiquitylation and proteasome-mediated degradation, and increasing production of chaperones that help proteins in the ER lumen to fold. The result is that the ER stress resolves, and if it does not then the cell is functionally compromised and may undergo caspase-dependent apoptosis (19, 20).

It is becoming increasingly apparent that ER stress induces autophagy, or autophagocytosis, a catabolic cellular process involving the lysosome-dependent degradation of macromolecules, organelles and other cell components. Autophagy plays house-keeping roles in protein degradation, complementing the proteasome-based protein degradation system. Autophagy can also promote cell survival in many contexts, during times of nutrient deprivation and hypoxia, removing misfolded or aggregated proteins, clearing damaged organelles, as a survival mechanism (21). Its deregulation has been linked to non-apoptotic cell death and is induced in some cases by endoplasmic reticulum stress, being associated with disease pathophysiology. Autophagy promotes cellular senescence and cell surface antigen presentation, protects against genome instability and prevents necrosis, giving it a key role in preventing diseases such as cancer, neurodegeneration, cardiomyopathy, diabetes, liver disease, autoimmune diseases and infections (22).

### Endoplasmic reticulum stress and the molecular basis of human disease

The endoplasmic reticulum (ER) is the principal cellular organelle in which correct folding and maturation of transmembrane, secretory, and ER-resident proteins occur. Research over the past decade has demonstrated that mutations in proteins or agents/conditions that disrupt protein folding adversely affect ER homeostasis, leading to ER stress (23, 24). This in turn initiates the unfolded protein response (UPR), an integrated intracellular signalling pathway that responds to ER stress by increasing the expression of ER-resident molecular chaperones, attenuating global protein translation and degrading unfolded proteins. Failure to relieve prolonged or acute ER stress causes the cell to undergo apoptotic cell death. Recent groundbreaking studies have provided compelling evidence that ER stress and UPR activation contribute to the development and progression of human disease, including neurodegenerative disorders, diabetes, obesity, cancer, and cardiovascular disease. Furthermore, the ability of the UPR to modulate oxidative stress, inflammation, and apoptosis provides important cellular clues as to how this evolutionarily conserved cellular-stress pathway maintains and responds to both normal physiologic and pathologic processes. In this article, many

aspects of the UPR are reviewed in the context of how ER stress and UPR activation influence human disease. This current information provides a solid foundation for future investigations aimed at targeting the UPR in an attempt to reduce the risk of human disease (25).

Endoplasmic reticulum (ER) stress is a phenomenon that occurs when excessive protein misfolding takes place during biosynthesis. ER stress triggers a series of signalling and transcriptional events known as the unfolded protein response (UPR). The UPR attempts to restore homeostasis in the ER but if unsuccessful can trigger apoptosis in the stressed cells (26). Primary amino acid sequence contains all the information for a protein to attain its final folded conformation. However, many folding intermediates exist along the folding pathway, and some of these intermediates can become irreversibly trapped in low-energy states and activate the UPR. Clearance of such misfolded species requires a functional ER-associated degradation (ERAD) pathway, which is regulated by the UPR. Proteasomal degradation of ER-associated misfolded proteins is required to protect from UPR activation. Proteasomal inhibition is sufficient to activate the UPR, and, in turn, genes encoding several components of ERAD are transcriptionally induced by the UPR. Therefore, it is to be expected that UPR activation and impaired ERAD function might contribute to a variety of diseases and that polymorphisms affecting the UPR and ERAD responses could modify disease progression. The following examples provide the best available evidence linking the UPR pathway to the natural history of human diseases (27).

There are numerous genetic misfolding diseases that are likely influenced by UPR signalling. Because BiP release from IRE1, PERK, or ATF6 can activate the UPR, the expression of any wild-type or mutant protein that binds BiP can have a similar effect. In contrast, misfolded proteins that do not bind BiP are unlikely to activate the UPR. For example, cystic fibrosis is due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Approximately 70% of patients with this disease carry a common mutation, deletion of Phe508, that results in a molecule that is retained in the ER and eventually degraded by the proteasome (28).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, characterized pathologically by the deposition of amyloid-L protein (AL), formation of neurofibrillary tangles, and neuronal death in brain lesions. It is recognized that increased production, oligomerization and aggregation of amyloid-peptides are the crucial factors in the onset of AD (29, 30). Neurons are vulnerable to different genetic and environmental insults which affect the homeostasis of ER function via the accumulation of unfolded proteins and disturbances in redox and  $Ca^{2+}$

balances. Therefore, it is not surprising that a number of studies have demonstrated that ER stress is present in several neurodegenerative diseases. Evidence of activated UPR signalling has been detected in Alzheimer's, Parkinson's and Huntington's diseases, and in ALS (amyotrophic lateral sclerosis). Furthermore, cerebral ischemia can trigger the UPR, although a concomitant drastic decline in protein synthesis clearly decreases the level of UPR. A large body of evidence indicates that the accumulation of intracellular amyloid- and phosphorylated tau proteins, along with the perturbation of  $Ca^{2+}$  homeostasis, plays a prominent role in the pathogenesis of AD (29, 31).

Parkinson's disease is the most common movement disorder, affecting about 1% of individuals aged 65 years or older. Autosomal recessive juvenile parkinsonism (AR-JP) results from defects in the Parkin gene, which encodes a ubiquitin protein ligase (E3) that functions with ubiquitin-conjugating enzyme UbcH7 or UbcH8 to tag proteins for degradation. Overexpression of Parkin suppresses cell death associated with ER stress. Inherited Parkinson's disease is associated with the accumulation in the ER of dopaminergic neurons of PAEL-R, a putative transmembrane receptor protein that is detected in an insoluble form in the brains of AR-JP patients. The accumulation of PAEL-R results from defective Parkin that does not maintain the proteasome-degrading activity necessary to maintain ER function (32, 33).

Hyperhomocysteinemia (HHcy) is considered an independent risk factor for cardiovascular disease, including ischemic heart disease, stroke, and peripheral vascular disease. Mutations in the enzymes and/or nutritional deficiencies in B vitamins required for homocysteine metabolism can induce HHcy. Studies using genetic- or diet-induced animal models of HHcy have demonstrated a causal relationship between HHcy and accelerated atherosclerosis. Oxidative stress and activation of proinflammatory factors have been proposed to explain the atherogenic effects of HHcy. Recently, HHcy-induced endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) have been found to play a role in HHcy-induced atherogenesis (34).

### **Endoplasmic reticulum response in cancer**

During tumorigenesis, the high proliferation rate of cancer cells requires increased activities of ER protein folding, assembly, and transport, a condition that can induce physiological ER stress (35, 36). Moreover, as the tumour grows, cancer cells experience increasing nutrient starvation and hypoxia, which are strong inducers for the accumulation of unfolded or misfolded proteins in the ER and the activation of the UPR pathways (37). Indeed,

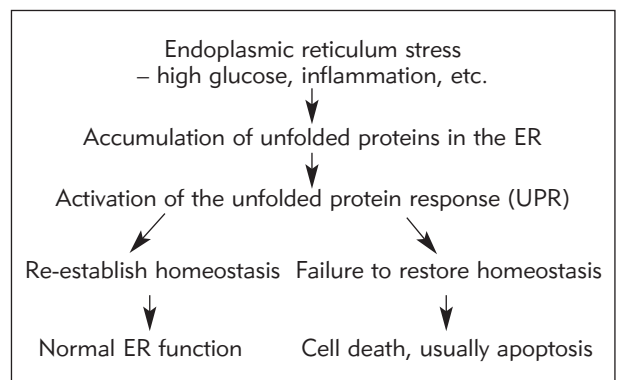
accumulating evidence has demonstrated that the UPR is an important mechanism required for cancer cells to maintain malignancy and therapy resistance. Additionally, the possibility of targeting the UPR signalling as a novel therapeutic strategy has greatly inspired the cancer research community and pharmaceutical industry (35).

Cancer cells possess rapid glucose metabolism and fast growth rate, which leads to poor vascularisation of tumour mass, low oxygen supply, nutrient deprivation, and pH changes (38). On the other hand, cancer cells can express mutant proteins that cannot be correctly folded, and experience insufficient ER energy supply, alteration of the redox environment, and viral infection (35). All of these can cause ER stress and activation of the UPR. Increasing evidence suggests that the UPR provides survival signalling pathways required for tumour growth. Indeed, increased expression of the UPR components, including the UPR transactivators XBP1 and ATF6, ER stress-associated proapoptotic factor CHOP, as well as ER chaperones GRP78/BiP, GRP94, and GRP170, have been detected in breast cancer, hepatocellular carcinomas, gastric tumours, and esophageal adenocarcinomas (39). Cancer cells may adapt to ER stress and evade stress-induced apoptotic pathways by differentially activating the UPR branches.

### Endoplasmic reticulum stress in diabetes mellitus pathogenesis

The metabolism of glucose is tightly controlled at the levels of synthesis and utilization through hormonal regulation. Glucose not only promotes the secretion of insulin, but also stimulates insulin transcription and translation. Pancreatic islet beta-cell death by apoptosis has been implicated in the pathogenesis of type 1 diabetes mellitus (T1D) and T2D by causing absolute or relative insulin deficiency, respectively. Histology of islets from both types of diabetic patients shows different degrees of inflammation with the presence of immune cell infiltration, proapoptotic cytokines, and apoptotic cells. Accumulating evidence suggests that endoplasmic reticulum (ER) stress, a cellular response triggered by disturbance of the ER homeostasis and accumulation of unfolded proteins, contributes to beta-cell death in both T1D and T2D (Figure 2) (19, 40).

Beta cells have a highly developed ER due to insulin production. Normal function of the ER requires a high concentration of free  $\text{Ca}^{2+}$  in the ER lumen. The resting free ER  $\text{Ca}^{2+}$  concentration is three to four orders of magnitude higher than in the cytosolic compartment. This allows activation of  $\text{Ca}^{2+}$ -dependent chaperones enabling posttranslational modifications like glycosylations, folding, formation of disulfide bonds, and subunit assembly of



**Figure 2** The relation between ER stress and ER stress induced apoptosis in the development of diabetes (modified from (19)).

newly formed proteins. The  $\text{Ca}^{2+}$  gradient is generated by sarcoendoplasmic reticulum  $\text{Ca}^{2+}$ ATPase (SERCA) proteins that pump  $\text{Ca}^{2+}$  into the ER and the inositol-(1,4,5)-trisphosphate and ryanodine receptors that release  $\text{Ca}^{2+}$  from the ER. Compared with other cell types, beta cells show marked sensitivity to apoptosis induced by SERCA blockers, and ER stress transducer proteins, such as inositol-requiring enzyme- $\alpha$  (IRE1 $\alpha$ ) and protein kinase-like ER kinase (PERK), are highly expressed in pancreatic beta cells, suggesting a tight control of protein synthesis and vulnerability toward ER stress. Dysregulation of ER  $\text{Ca}^{2+}$  homeostasis elicits ER stress and subsequently activates ER stress signalling pathways, collectively known as the unfolded protein response (UPR) (41).

Transcriptional induction of ER chaperones, like calnexin/calreticulin (lectin chaperones) and BiP/GRP94 (part of the heat-shock protein family), is an immediate response to ER stress, which is initiated as a first line of defense to restore protein folding capacity. The mechanism is mediated by two types of basic leucine zipper (bZIP)-containing transcription factors, ATF4/6 and X-box binding protein 1 (XBP-1) that activates expression of ER stress response genes. ER stress can also lead to activation of apoptotic pathways in cases of prolonged or pronounced disruption of ER homeostasis. Among the best described ER stress-induced proapoptotic pathways are not only activation of the transcription factor CCAAT/enhancer-binding protein homologous protein (CHOP) and cleavage of caspase-12 (22), but also activation of Bax, nuclear factor  $\kappa\text{B}$  (NF $\kappa\text{B}$ ), and c-Jun N-terminal kinase (JNK) have been reported to have a pro- or antiapoptotic effect depending on cell type and context. Furthermore, pro- and antiapoptotic Bcl-2 proteins are localized to the ER-enriched microsomal fractions and ER stress-induced cell death has been shown to signal via the proapoptotic Bcl-2 member Bax in non-beta-cell systems (42). Thus, Bax may provide a link from ER stress to proapoptotic signalling in the mitochondria. It is believed that Bax

is activated by ER stress-induced IRE1 $\alpha$  and CHOP signalling. The importance of ER stress-induced regulation of these pathways in beta-cell apoptosis remains to be elucidated (43).

ER stress and the UPR also play an important role in the pancreas by acting on islet survival and function. Indeed, this mechanism may be a potential cause of rare forms of juvenile diabetes (44). For example, PERK deficient pancreatic beta cells are more susceptible to ER stress induced apoptosis. PERK-deficient mice develop severe hyperglycemia soon after birth due to defects in islet proliferation and increased apoptosis (45). Interestingly, different models of conditional PERK deletion in islets suggest that this role of PERK may be more important during beta cell development than in the adult (46). Preventing eIF2 $\alpha$  phosphorylation in pancreatic beta cells also results in development of diabetes, potentially due to oxidative damage (47). The absence of p58 (IPK), an ER chaperone, also promotes beta cell failure (48). The PERK-eIF2 $\alpha$  pathway is also critical for islet survival and function in humans. A loss-of-function mutation in PERK causes a heritable form of juvenile diabetes called Wolcott-Rallison syndrome, characterized by severe defects in pancreatic beta cells (49). Additionally, mutations in the WFS1 gene in humans, which encodes the ER transmembrane protein wolframin, have been linked to an increased incidence of diabetes in patients with Wolfram syndrome (50).

In the case of type 1 diabetes, in which insulin-producing beta cells are lost, pancreatic beta cells have an extremely well-developed ER, which reflects their function in secreting large amounts of insulin and other glycoproteins. This secretory function of beta cells may explain why mice lacking PERK are susceptible to diabetes, showing apoptosis of their beta cells and progressive hyperglycaemia with ageing (51). Moreover, PERK gene mutations in association with infant-onset diabetes have been described in humans with the autosomal recessive disorder Wolcott-Rallison syndrome (49), in which patients at autopsy exhibit massive beta-cell loss, resembling the pathology of Perk<sup>-/-</sup> mice. Similarly, EIF2 (Ser51Ala) knock-in mice suffer from beta-cell depletion beginning *in utero*, suggesting a more rapid course than Perk<sup>-/-</sup> mice (15). The failure of Perk<sup>-/-</sup> to phenocopy EIF2 (Ser51Ala) raises the possibility that other kinases besides PERK participate in the inhibition of EIF2 during ER stress. Another hereditary disorder in which type 1 diabetes develops is Wolfram syndrome, in which defects in the ER-stress-inducible WFS1 protein occur (50). WFS1 protein expression is normally induced by stimuli that trigger insulin secretion, and silencing of the WFS1-encoding gene induces ER stress and apoptosis of beta cells (52).

### Therapeutic targeting of ER dysfunction

An important question when considering therapeutic opportunities is the relevance of observations made in cells or animals to human disease. One critical proof of principle implicating ER function in human metabolic disease comes from genetic studies of human diseases such as Wolcott-Rallison syndrome (49) or Wolfram syndrome (50). Recent studies provide strong evidence in support of a role for ER stress in human metabolic disease. Currently, much of the data on the regulation of ER stress in human metabolic disease has focused on the involvement of the IRE1 and PERK branches of the UPR, whereas the ATF6 branch has not been as well studied due to lack of effective reagents for studying this pathway in human cells. In light of this, it is important to note that genetic variation at the ATF6 and ORP150 loci has been linked to insulin resistance, further supporting a role for ER stress in human metabolic disease (53). The ER is an attractive potential therapeutic target, in part because ER adaptive responses have not had time to evolve to deal with the chronic stresses encountered due to recent changes in lifestyle, such as excess nutrient availability and obesity. Thus, maintenance or enhancement of proper ER function may be able to prevent chronic metabolic disease. Such »organelle therapy« may also be needed to disengage stress pathways from insulin signalling or other metabolic responses.

Hotamisligil et al. (20) demonstrated that two chemical chaperones, phenyl butyric acid and tauroursodeoxycholic acid, that relieve ER stress could protect liver cells from chemically induced ER stress and whole animals from obesity-induced ER stress. These chaperones increased systemic insulin sensitivity, established normoglycemia, and reduced fatty liver disease in obese mice (54). These treatments suppressed ER stress and inflammatory kinase signalling and enhanced insulin receptor signalling in adipose and liver tissues. At least in experimental models of obesity and diabetes, these agents exhibit therapeutic efficacy; however, additional work is needed to definitively link their activity to the ER. Additionally, studies in lipid-induced models of ER stress have shown that apoB100 secretion is inhibited in the liver by ER stress, an important contributor to hepatic steatosis (55). Interestingly, this inhibition of apoB100 secretion in liver insulin resistance can also be prevented by chemical chaperones *in vitro* and *in vivo* (55). Whether these approaches can be translated into treatments for human disease remains unknown, but there are certainly limitations with these chemicals due to the high doses required to produce the desired effect and relatively undesirable pharmacokinetics.

More studies are needed to elucidate how enhancing the activity of endogenous chaperones and boosting protein folding in metabolically active

tissues affects the adaptive capacity of the UPR under metabolic stress, and to develop specific strategies to do so. However, the nature and magnitude of the response to elevated chaperone levels under metabolic stresses such as obesity remain to be determined. It will be exciting to investigate individual molecular chaperones and the transcription factors that control them, such as ATF6 and XBP1, in the liver, adipose tissue, muscle, and pancreas. Concerted up-regulation of protein-folding chaperones or the programmes leading to their coordinated regulation may prove beneficial to a metabolically overloaded cell and may be a powerful approach for treating chronic metabolic diseases (23, 56, 57).

### **Unfolded protein response and endoplasmic reticulum stress in Wolfram syndrome**

Wolfram syndrome (WS) is an autosomal recessive neurodegenerative disease characterized by various clinical manifestations, including diabetes mellitus, optic atrophy, diabetes insipidus, deafness, neurological symptoms, renal tract abnormalities, psychiatric disorders and gonadal disorders. The most frequent of these disorders are early onset diabetes mellitus, with a low prevalence of ketoacidosis, and optic atrophy, which is considered a key diagnostic criterion in this syndrome. Diabetes insipidus usually develops later. This syndrome manifests in childhood, hampering diagnosis and treatment. The syndrome has variable presentation and complications are widespread. Full characterization of all clinical and biological features of WS is difficult because, with the exception of a few series, the number of patients in most reports is small. Morbidity and mortality are high and the quality of life is impaired due to neurological and urological complications. The disease is rare with an estimated prevalence of one in 770,000, and a carrier frequency of one in 354; it is believed to occur in one out of 150 patients with juvenile-onset insulin-dependent diabetes mellitus. The prevalence varies worldwide with the highest prevalence of 1 in 68,000 reported in Lebanon. This has been proposed to be due to high rates of consanguinity, prevalent in that region. The prevalence of Wolfram syndrome in patients diagnosed as having type 1 diabetes mellitus (DM) in the aforementioned study was estimated to be 4.8% as against 0.57% in the UK. It is classified as a progressive neurodegenerative disease and usually results in death before age 50 years (58, 59).

Polymeropoulos et al. first reported a nuclear gene as responsible for the disorder and localized it to 4p16.1 using linkage analysis in 11 families (60). Although the illness is genetically heterogeneous, mutations in WFS1 gene have been identified in 90% of patients (61). The wolframin protein has been localized to the endoplasmic reticulum (ER) and plays a role in calcium homeostasis (62, 63). Wolframin-deficient cells have been shown to have an altered

calcium homeostasis (63). This affects the ability of the ER to process and fold new proteins normally (63). Therefore, it is hypothesized that ER stress, which normally triggers the unfolded protein response (UPR), is exaggerated in cells that are wolframin deficient. The exaggerated UPR impairs the cell cycle progression and increases apoptosis, followed by the results that have suggested that it is inhibited proliferation that leads to beta-cell deficiency in patients with Wolfram syndrome, rather than active destruction (50).

WFS1 encodes wolframin, a transmembrane glycoprotein localized to the ER, with high expression in pancreatic beta cells, neurons and some other tissues, where it regulates  $Ca^{2+}$  homeostasis (64). To date, WFS1 mutations have been implicated in WFS, low-frequency non-syndromic hearing loss and psychiatric diseases, and common variants have recently been shown to be associated with T2D (65). Medlej et al. (66) reported 31 Lebanese patients with WS belonging to 17 families. Central diabetes insipidus was found in 87% of the patients, and sensorineural deafness confirmed by audiograms was present in 64.5%. Other less frequent features included neurological and psychiatric abnormalities, urodynamic abnormalities, limited joint motility, cardiovascular and gastrointestinal autonomic neuropathy, hypergonadotropic hypogonadism in males, and diabetic microvascular disease. New features, including heart malformations and anterior pituitary dysfunction, were recognized in some of the patients and participated in the morbidity and mortality of the disease (66). El-Shanti et al. (67) found that three families linked to 4q (WFS2) contained several patients with profound upper gastrointestinal ulceration and bleeding.

Our study of 408 nuclear families from Lebanon, which had been ascertained through a patient with insulin-dependent juvenile-onset diabetes mellitus (JOD), with a total of 455 JOD patients, including nonsyndromic and syndromic cases, shows evidence that some WFS1 mutations may result in nonsyndromic JOD, and that WFS1 mutations are responsible for a large proportion of JOD in some population subgroups, reaching 12.1% of probands in Lebanese consanguineous families (68). By sequencing WFS1 exons in WFS and non-syndromic DM probands and their parents from multiplex, consanguineous or extended families, and in probands from simplex non-consanguineous families, we identified 173 WFS1 DNA variants, 46 of which were predicted to affect the primary sequence of the protein, 38 of which were novel. A double mutation, WFS1<sup>L1B</sup>, associating the 707VI non-synonymous change and the F884fs951X frameshift on the same haplotype was homozygous in 7/17 (41.2%) of the WFS probands, and 10/27 of all WFS patients. F884fs951X frameshift affects the most C-terminal endoplasmic reticulum (ER) luminal domain, replacing the last seven amino acids by 67 other amino acids, while 707VI is



predicted to have no major functional consequence on protein function (68).

This situation results from the combination of population specific founder effects responsible for the high prevalence of WFS1<sup>LIB</sup> mutation in Lebanese patients, the non-syndromic DM phenotype frequently associated with this mutation, and the high rate of consanguinity. Our results are providing strong evidence for the combined impact of consanguinity and founder effect, resulting from the religious and socio-cultural ethnic endogamy in the Lebanese population. Based on our sequence screening survey, 5.5% (22/399) of all Lebanese JOD probands (including all WFS and non-WFS syndromic cases) were homozygous or compound heterozygous for WFS1 mutations (68).

Our findings extend the role of WFS1 mutations as a frequent monogenic determinant of JOD in some population subgroups and suggest the existence of significant genetic heterogeneity between patients with JOD, strongly dependent on populations and family structure. To our knowledge, our observation of non-syndromic DM caused by a specific recessive WFS1 mutation constitutes the first description of recessive monogenic inheritance in diabetes, which is not syndromic or neonatal (68). Based on these results, we anticipate that in some populations, or in particular subgroups of patients, monogenic causes

of diabetes may largely exceed the estimation of 1–2%, with important consequences for diagnosis and patient management (69).

## Conclusion

Significant progress has been made in elucidating the mechanism and role of the ER stress response in the pathogenesis of human diseases and therapeutic potential. The related findings have raised an exciting possibility of targeting the UPR components as an effective strategy for disease therapy. For future research, it is important to delineate the distinct roles of the UPR branches that may provide survival or death signal in human cells. The related information will be essential for pharmaceutical design toward controlling diseases through modulating UPR signalling. Research in this topic will significantly advance our understanding of the mechanisms involved in human diseases. A more complete understanding of ER stress will open up promising avenues for the development of clinically useful drugs.

## Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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