Chapter 4

Impact of Oxidative Changes and Possible Effects of Genetics Polymorphisms of Glutathione S-Transferase in Diabetics Patients with Complications

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Abstract

Pancreatic β cells are more sensitive to cytotoxic stress than several other cells due to the expression of very low levels of antioxidant enzymes. Glutathione-S-transferase (GST) is a detoxification enzyme essential for a cellular protection against oxidative damage. Thus, the objective of this chapter is to verify the impact of the hypothesis of all effects of Glutathione S-transferase polymorphism in patients with diabetic complications. Diabetic nephropathy (DN) is the main secondary complication of diabetes mellitus (DM). Notably, the expression of GST genes has been described in different variations as ethnic populations. Some studies have suggested association between genetic polymorphism for GSTM1, GSTT1 and *GSTP1* and DN, but others do not. The results are still inconsistent and, therefore, more studies are needed to be performed.

Keywords: GST, diabetic nephropathy, diabetes, polymorphism, glutathione S-transferase

1. Introduction

Diabetes mellitus (DM) is defined as a heterogeneous group of metabolic disorders characterized by an unusual hyperglycemia resulting from defects in insulin action and/or secretion. An epidemic of DM is underway as result of population growth and aging, increased urbanization,

prevalence of obesity and sedentary lifestyle [1]. It is estimated that currently about 415 million individuals are diagnosed with DM worldwide and it is predicted that by 2040 these records will reach the range of 672 million [2].

Although the survival of these patients has increased in recent decades, it is known that the morbidity resulting from complications affecting the small blood vessels (microvascular) or large (macrovascular) arteries is very significant. These complications may occur as consequence of hyperglycemia that favors inadequate cellular metabolism and excessive production of reactive oxygen species (ROS). The etiopathogenesis of DM is not fully elucidated, but it is suggested that genetic and environmental factors are involved in this metabolic disorder and, in this sense, oxidative stress becomes one of the important pathways for this understanding [3].

Human cells produce many antioxidants in attempt to protect cells against damage caused by toxins from the environment. The main endogenous antioxidant defense is provided by glutathione (GSH). GSH is a linear tripeptide that arouses scientific interest because it performs multiple functions via glutathione S-transferase (GST). GSTs are one of the most important groups of phase II enzymes. It is reported that these enzymes are induced, as a protective mechanism (detoxification), under conditions of oxidative stress. GST polymorphisms were associated with increased or decreased susceptibility of various diseases, such as cancer, cardiac diseases, about everything diabetes and yours complications [4].

Some important members of the GST family stand out for having different polymorphisms between these GST mu 1 (GST M1) and GST theta 1 (GST T1) and GST Pi 1 (GSTP 1). It is reported that these GSTs subtypes are involved in the development of DM and its complications [5], so it is important to understand the impact of these oxidative changes and the possible effects of genetic polymorphisms of GSTs in diabetic patients [6].

2. Oxidative stress

Living aerobic organisms have an intracellular environment in which important biological molecules are in equilibrium, and oxidative metabolism and redox homeostasis are in sync. In these organisms, oxidative phosphorylation is a vital step of metabolism [7].

This metabolic pathway uses the energy generated by NAD^+ oxy-reduction reactions in NADH and produces adenosine triphosphate (ATP) molecules capable of storing energy for immediate consumption [8]. As consequence, free radicals are produced naturally and continuously [9]. It is important to note that the mechanism of free radical generation can also occur in cell membranes and cytoplasm with the participation of transition metals such as iron and copper [10].

In the body, free radicals can act in a beneficial way during the immune response, destroying invading pathogens and modulating the excessive inflammatory response, however, their excess may cause deleterious effects to the organism. Normally, in healthy living organisms, there is a balance between the production of free radicals and antioxidant systems [11].

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The imbalance between the production and the antioxidant defense capacity of the organism is called oxidative stress [12]. The cellular effects of this hostile environment depend on factors such as cell type, presence of surface receptors, mechanism of transduction and levels of antioxidants [7]. But it is known that prolonged exposure to oxidative stress can damage cellular components (proteins, lipids and DNA) [13], contribute to cellular aging [14], and play an important role in the pathogenesis of cancer, atherosclerosis, Parkinson, Alzheimer's and various chronic diseases such as diabetes mellitus and its complications [15–20].

3. The biological role of glutathione and glutathione S-transferases in oxidative stress

Numerous studies have shown that in order to avoid prolonged exposure to ROS produced during oxidative stress, the body has a very efficient antioxidant defense system. Glutathione S-transferases (GSTs) and glutathione (GSH) enzymes are part of this line of defense [21].

Glutathione (GSH) is a low molecular weight thiol found in all tissues, primarily in aerobic organisms. Also known as L-gamma-glutamyl-L-cysteinyl-glycine, GSH is a linear tripeptide consisting of three amino acids: glutamic acid, cysteine and glycine (**Figure 1**). Between the γ-glutamyl moiety and the free α-carboxylate group, there is a γ-peptide bond which, although unusual, prevents the hydrolysis of GSH by cellular peptidases [22].

In homeostasis conditions, GSH is the most efficient physiological reducing agent with the highest bioavailability (~ 10 mM) in the intracellular environment where it is synthesized,

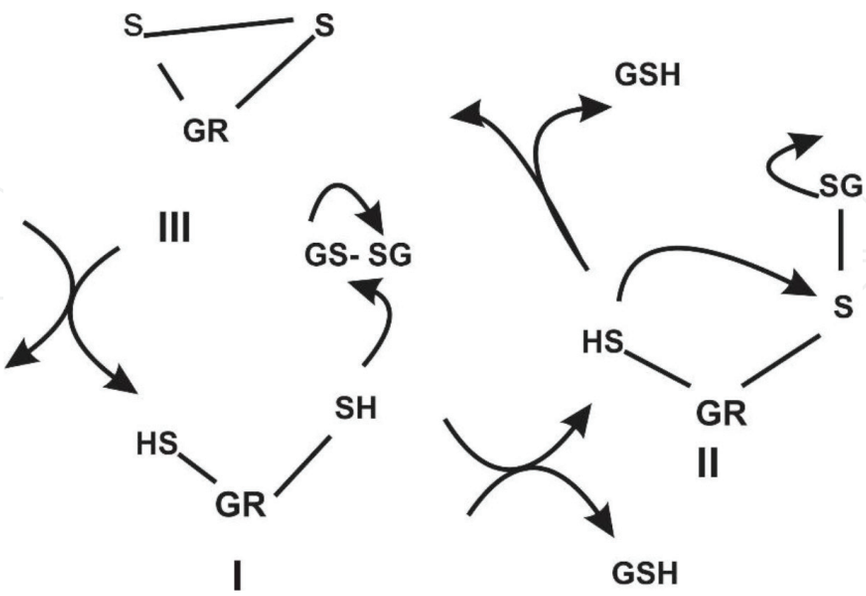

Figure 1. Schematic representation of the GSSG reduction cycle by GR.

except in epithelial cells [23]. Its synthesis occurs in two phases and counts on the action of two enzymes: γ-glutamyl-cysteine-synthetase and glutathione-synthetase [24].

In the first phase, the γ-glutamyl-cysteine-synthetase enzyme favors the formation of the peptide bond between glutamic acid and cysteine, thus forming the dipeptide γ-L-glutamyl-L-cysteine [25]. In the second phase, the enzyme glutathione synthetase binds the newly formed dipeptide to glycine, giving rise to GSH which is distributed through the bloodstream and then brought to the tissues. In both phases, consumption of ATP and Mg^{+2} occurs. The regulation of the enzyme γ-glutamyl-cysteine-synthetase is done, by negative feedback, when the GSH itself begins to be formed. This regulatory mechanism ensures that, in normal conditions, the excessive production of GSH or the intermediate γ-L-glutamyl-L-cysteine does not occur (**Figure 2**) [22, 24].

An alternative route is activated in situations where conversion of γ-glutamyl-L-cysteine into GSH is insufficient. In this case, the enzyme γ-glutamylcyclotransferase catalyzes the conversion of γ-glutamyl-L-cysteine to 5-oxoproline, favoring the occurrence of 5-oxoprolinuria, chronic metabolic acidosis and neurological disorders (**Figure 2**) [22].

During the reaction catalyzed by γ-glutamylcysteine synthetase, activation of butionin sulfoximine (BSO), an inhibitor of GSH biosynthesis, may occur. Studies suggest that this suppression of GSH by BSO may be a rather efficient strategy in cancer therapy since, during this process, there is an increase in the sensitivity of cells to ionizing radiation and to cytostatic drugs, making them more susceptible to treatment. However, the disadvantage of this technique is that the toxic effect to normal cells has potency detrimental to the individual. An alternative to limit this toxicity would be the use of localized irradiation or the topical application of cytostatic drugs, but other studies are being carried out [26].

Glutathione can be found in the intracellular medium in its reduced (GSH) or oxidized form (GSSG, dimerized form of GSH) and the GSH/GSSG ratio determines the redox state of biological systems. This is because glutathione performs a cytotoxic and genotoxic inactivation of xenobiotics and consequently promotes detoxification and cellular protection against oxidative stress and additional damage [27].

The cellular detoxification process is divided into three distinct but related phases. In phases I and II, the xenobiotic is transformed into a more soluble and less toxic product and, in phase III, are transported for cellular excretion. It is noteworthy that the efficiency of phase II depends on the action of enzymes called glutathione S-transferases (GSTs) [22].

The GSTs belong to a superfamily of multigenic enzymes that catalyze the nucleophilic attack of the reduced form of Glutathione (GSH) to compounds that present a carbon, a nitrogen or an electrophilic sulfur atom [21]. Under natural conditions, GSTs are generally found in the biological environment as homo or heterodimers. Each dimer contains two active sites with independent activities. Each site has at least two binding regions: one specific for glutathione (GSH) and the other, with less specificity, for the electrophiles (alkyl halides, epoxides, quinones, iminoquinones, aldehydes, ketones, lactones and esters, halides of aryl and aromatic nitro) [22, 28].

Mammalian GSTs are divided into families according to their location: cytosolic, mitochondrial and microsomal. The cytosolic and mitochondrial GST enzymes are soluble, unlike the microsomal GSTs that are associated with the membrane [29]. This latter family is generally

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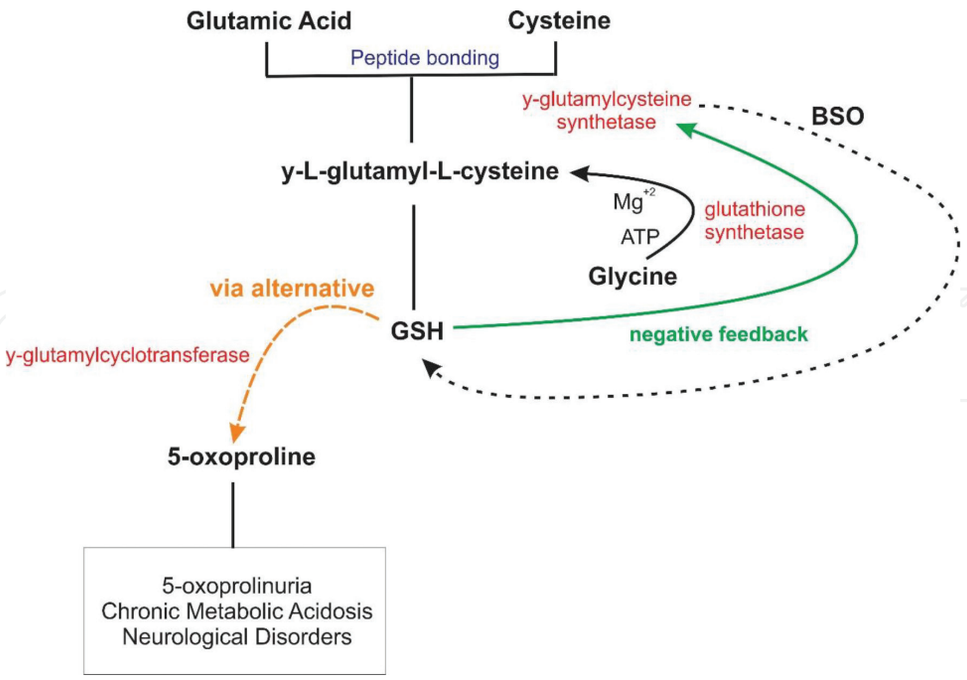

Figure 2. Scheme representing the biosynthesis and mechanism of regulation of glutathione (GSH). BSO, butionin sulfoximine; Mg^{+2}, magnesium; ATP, adenosine triphosphate.

involved in the metabolism of eicosanoids and glutathione (GSH), thus being referred to as MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) [22]. It is important to note that other families of GSTs, absent in mammals, are also described in the literature. Cytosolic and mitochondrial GSTs are expressed in different isoforms and therefore divided into classes according to the amino acid and/or nucleotide sequence, immunological properties, enzymatic kinetic parameters and/or tertiary and quaternary structure [22, 29, 30].

Based on the similarity of the amino acid sequence, GSTS found in the cytosol are called α (GSTA), μ (GSTM), θ (GSTT), π (GSTP), σ (GSTS), omega (GSTO), and zeta (GSTZ) [28, 31]. The mitochondrial GST is known as kappa (GSTK) [31]. Mammalian cytosolic GSTs are all dimeric and contain 199–244 amino acid residues in their primary structures. Mitochondrial GSTs are also dimeric proteins and their subunits typically have 226 amino acid residues. Each of these enzymes differs in their functionality [22, 33]. It is suggested that in humans, the expression of these enzymes is uniform and independent of the cell type. GSTs have long been described as originating from mitochondria; however, recent studies indicate the presence of mitochondrial GSTs in peroxisomes. These findings reinforce their participation in the detoxification processes of by-products of β-oxidation of fatty acids [22].

During the detoxification process, the GSTs catalyze the conjugation of xenobiotics with endogenous substrates, mainly GSH. This conjugate is recognized by specific transporters and is carried to the intercellular medium where it undergoes action of γ-glutamyl transpeptidase (γGT) which removes the glutamic acid residue [32]. In sequencing, the dipeptidases remove the glycine residue, leaving only the cysteine residue associated with the xenobiotic. The

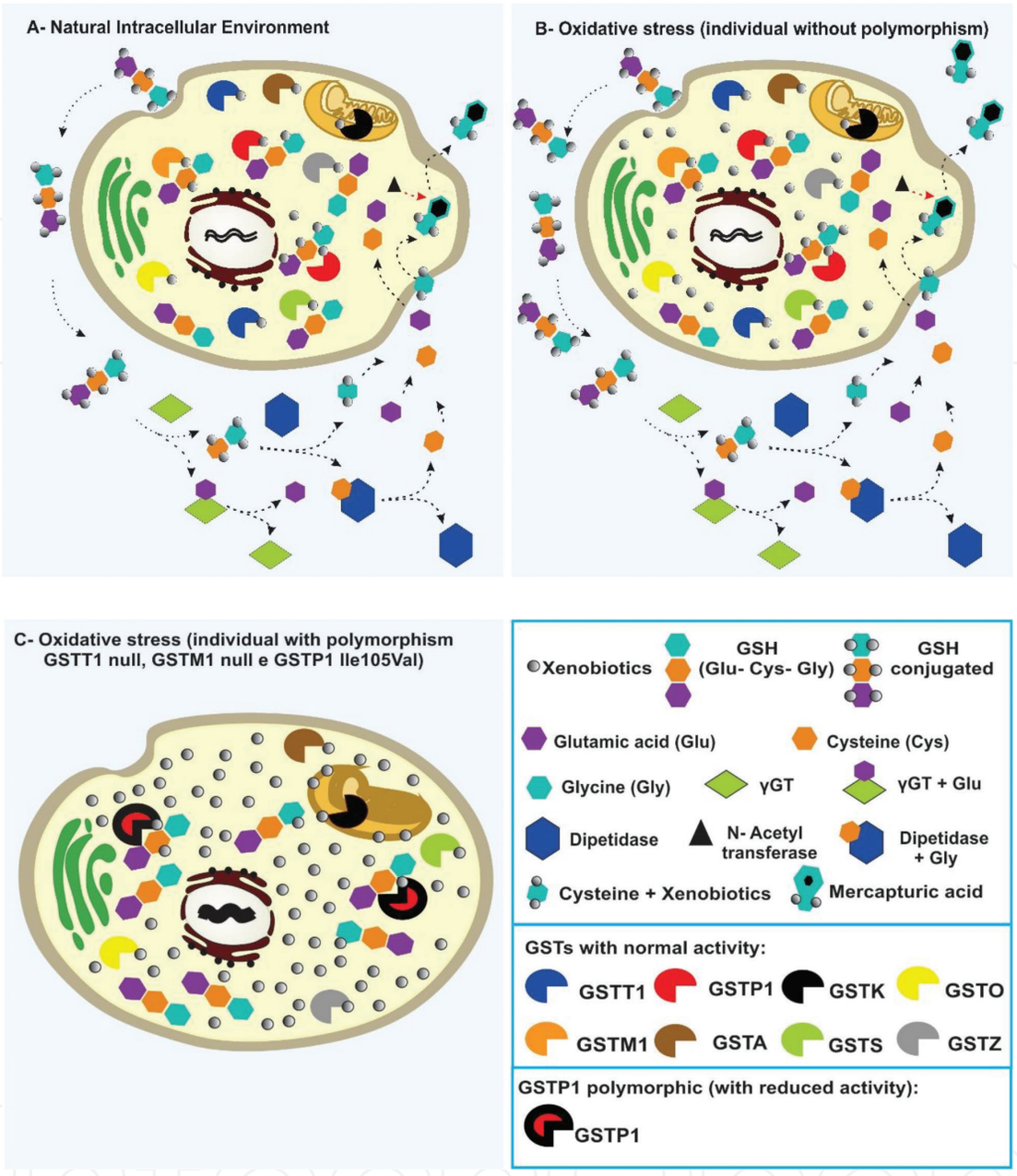

Figure 3. Schematic representation of main glutathiones S-Transferases correlated with oxidative stress in different biological conditions. A, normal intracellular environment; B, oxidative stress in an individual without polymorphism; C, oxidative stress in an individual with oxidative stress.

amino group of the cysteine residue is then acetylated by the intracellular N-acetyltransferase enzymes and thereby forms the mercapturic acid which, depending on its characteristics, is rapidly led to circulation, bile, urine or metabolized until it is eliminated (**Figure 3**) [22].

Once free, glutamate and glycine are reabsorbed by the cell and used in the regeneration of GSH through the catalytic cycle. In this stage of regeneration, three groups of enzymes are important: glutathione oxidase (GO) and glutathione peroxidase (GSH-Px), which catalyze the oxidation of GSH to GSSG, and the enzyme glutathione reductase (GR) that is responsible for the regeneration of GSH, from GSSG, in the presence of NADPH [33].

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It is important to highlight that this mechanism of detoxification via glutathione represents a fundamental biochemical evolution for the survival and guarantee of the perpetuation of many species and, although a co-transport mechanism without conjugate envelopment with glutathione has been proposed, there is no evidence experimental models that validate this model [22].

4. Oxidative alterations and the pathophysiology of diabetes and its complications

Many studies suggest that patients with diabetes present alterations in the levels of reactive oxygen species (ROS), a type of free radical whose electron is centered in the oxygen atoms [34]. This fact is justified by the toxic character of the persistent excess of glucose in the organism that ends up promoting glycation of proteins, hyperosmolarity and increase in the levels of sorbitol inside the cells [35].

Glucose is a vital source of energy for cells, and their serum levels are controlled by various organs such as intestine, liver, pancreas, skeletal muscle, adipose tissue and kidneys [36]. This regulation is facilitated by the action of hormones (glucagon and insulin), central and peripheral nervous system, as well as metabolic requirements of the body [37].

DM is defined as a heterogeneous group of metabolic disorders characterized by unusual hyperglycemia resulting from defects in insulin production and/or action [1]. In this situation, to revert the toxicity of excess glucose, this component undergoes auto-oxidation and, as consequence, ROS are generated (**Figure 4**) [37].

During auto-oxidation, excess glucose binds (protein glycation) [37] to lysine and valine residues in tissue and plasma proteins. This interaction results in the formation of Schiff's base, a labile or unstable compound that spontaneously transforms into ketoamine (glycated hemoglobin) through the *Amadori* rearrangement [35].

These oxidation and rearrangement processes, followed by further dehydration and fragmentation of *Amadori* product, promote the formation of advanced glycation end products (AGEs) (**Figure 4**) and generate other compounds with chemically active carbonyl groups. These compounds favor the oxidative stress that affects β cells of the pancreas, responsible for synthesizing and secreting insulin [8, 38].

Accumulated AGEs bind to membrane receptors on endothelial cells and promote the onset of tissue damage and the activation of the proinflammatory pathway that involves the NFκB transcription factor responsible for regulating the expression of other inflammatory cytokines (**Figure 4**) [37].

Moreover, the chronicity of this hostile environment causes the deactivation of the nitric oxide vasodilator (NO) formed by the endothelial cells [38]. This compromises the relaxation of vascular smooth muscle cells and has a degenerative effect on the vessels causing tissue death [34] and favoring the development of microvascular complications of diabetes, such as diabetic nephropathy (DN) (**Figure 4**).

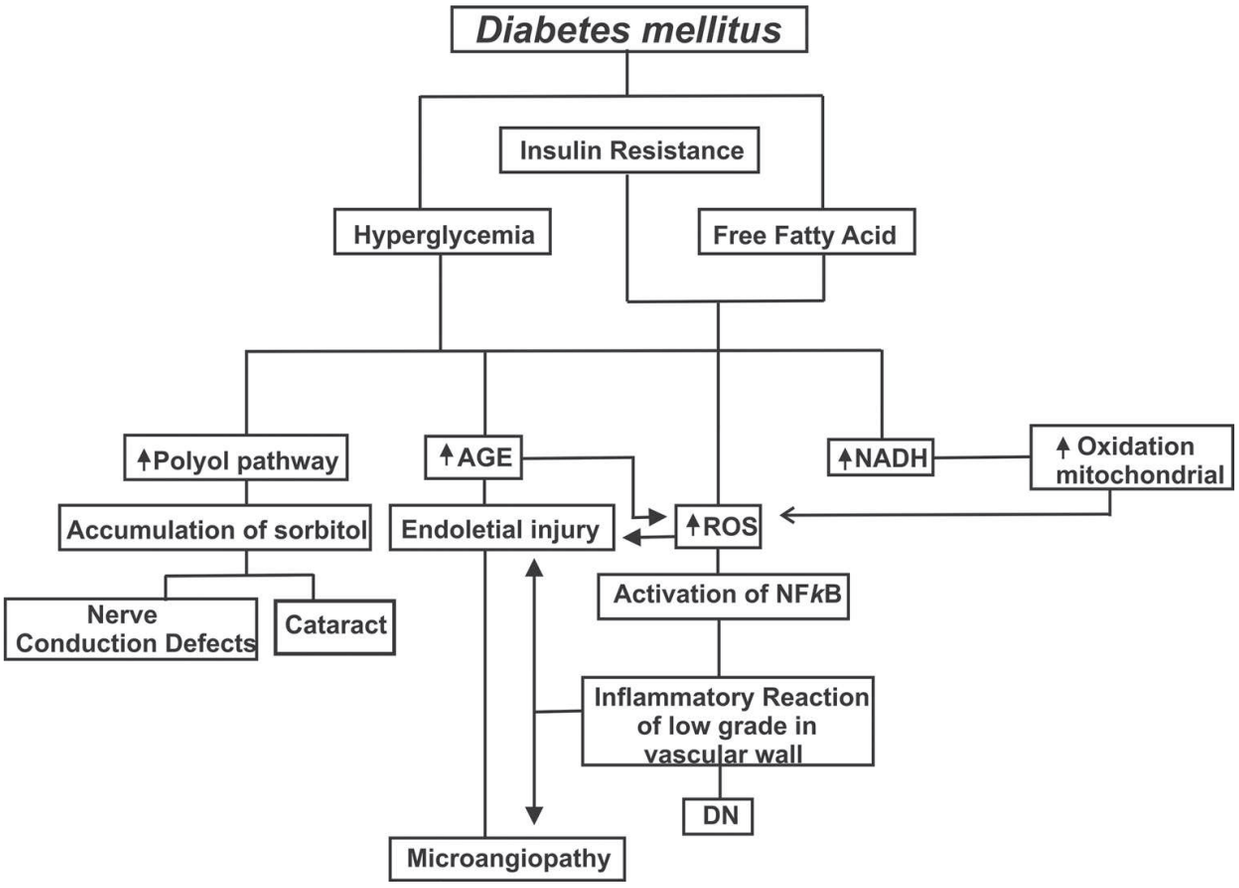

Figure 4. Main complications of *Diabetes mellitus*. NADH, nicotinamide and adenine dinucleotide; ROS, oxygen-reactive species; AGE, advanced glycation end product; DN, diabetic neuropathy.

ROS, generated by hyperglycemia, also interfere with other biochemical pathways [39]. The Krebs cycle, which, due to oxidative stress, favors the increase of the number of proton donors in the mitochondria, the main source of free radicals [37, 40]. This generates an even greater accumulation of free radicals, mainly superoxide (O_2^-) and hydroxyl compounds (OH^-) [41]. This mitochondrial production is the primary cause of long-term complications of diabetes.

The cascade signaling also suffers from oxidative stress in that it affects the activation of protein kinase C (PKC) [37], a serine/threonine kinase pathway that forms part of the mitogenic protein kinase (MAPK) [42] and plays an important role in several intracellular processes such as signal transduction, response to specific hormonal, neuronal and growth factor stimuli [28, 40].

Furthermore, hyperglycemia increases the NADH/NAD^+ ratio and decreases the NADPH/$NADP^+$ ratio (**Figure 4**). The substrates of this alteration are directed to the polyol pathway, which, at normal glucose concentrations, is not active [38]. In excess, in the polyol pathway, glucose is reduced to sorbitol, an osmotically active compound [37]. These disorders result in changes in redox homeostasis and in a variety of known effects for pathogenesis and progression of diabetes.

The accumulation of sorbitol in the ocular tissue, for example, contributes to the development of diabetic cataracts (**Figure 4**). In nerve tissue, high concentrations of this component

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decrease the uptake of myoinositol and inhibit ATPase Na^+/K^+ from the membrane, thus affecting nerve function (**Figure 4**). The accumulation of sorbitol associated with reduced hypoxia and blood flow in the nervous tissue favors the development of diabetic neuropathy [37]. This hyperglycemia may also alter gene and protein expression, endothelial cell permeability, and depletion of antioxidant molecules, including Glutathione S-transferases (GSTs), which play an important role in the cellular detoxification process [37, 41, 43–45].

5. Impact of genetic polymorphism on GSTs for patients with microvascular diabetic complications

Diabetic nephropathy (DN) is the main secondary complication of diabetes. Associated with an increased risk for cardiovascular disease and high mortality rates, DN is the leading kidney disease worldwide. Approximately 40% of diabetic patients are affected by this microvascular complication [46].

The mechanisms related to the development of DN are unclear and probably involve a number of dynamic events occurring early and with the progression of diabetes. It is known that the clinical characterization of this pathology is preceded by an established morphological renal lesion that results in imbalance of normal renal homeostasis [47]. These lesions are triggered by functional and metabolic changes. A common metabolic manifestation in the body of a diabetic individual is the picture of oxidative stress [31].

There are several factors that are involved in generating oxidative stress during diabetes. There is strong evidence that hyperglycemia results in the activation of PKC in diabetic glomeruli and, as a consequence, mesangial expansion, glomerular basement membrane thickening, endothelial cell dysfunction leading to diabetic renal disease, inflammation, apoptosis [48–50]. Diabetic renal disease, on the other hand, intensifies the formation and activation of ROS, worsening renal disease [51].

Considering that, in situations of oxidative stress, GSTs play an important role in cellular detoxification, studies of polymorphisms in the genes encoding these enzymes have been gaining prominence and arousing curiosity about a possible association with the susceptibility of this complication [52–54]. In this context, the deletions of *GSTM1* and *GSTT1* together with the *GSTP1* Ile105Val polymorphism are among the most studied isoforms in the GSTs group [55, 56].

It is described that individuals with *GSTM1* deletion polymorphisms are unable to produce the GSTM1 protein. On the other hand, the conversion of adenine to guanine at position 313 at codon 105 in the *GSTP1* gene causes the amino acid isoleucine (Ile) to be replaced with valine (val), which results in a lower activity of this isoform [56].

In the last decade, some investigations have made DM associations and their complications with the genetic polymorphism in GSTs. Notably, the expression of the GST gene has been described in different variations among ethnic populations. Studies with Egyptian children and adolescents, for example, show that the null genotype of *GSTT1* conferred a 4.2-fold

increased risk for the occurrence of DM, and in this case, associations with some biochemical variables and laboratory data were also observed (lipid profile and HbA1c). In this study, no investigation was performed when susceptibility to DN; however, the results are clear and show that gene polymorphisms encoding GSTs are associated with the development of type 1 DM and disease-related risk factors [31].

More specific studies addressing end-stage renal failure developed as a complication of DM show that this secondary complication is more common in the Asian population than in the UK population. In addition, the data are consistent and indicate that all patients of Asian origin who developed end-stage renal failure had non-insulin-dependent diabetes [57].

A meta-analysis performed by *Saadat* (2017) [58] brought together 18 studies with a total of 5483 subjects (healthy and diabetic). Overall analysis did not indicate a significant association between *GSTP1* and type 2 DM polymorphisms. Subgroup analyzes stratified by ethnicity, year of publication, and sample size also did not reveal a significant association between study polymorphism and DM2 risk.

In contrast, another meta-analysis by *Orlewski* and *Orlewska* (2015) [29] reports strong evidence of association between the genes glutathione-S-transferase (GST) and diabetic nephropathy (DN) polymorphisms. The results of this study reveal that significantly increased risks were found for the occurrence of DN in individuals with *GSTM1* genotype null. However, this same study does not observe correlation between the DN and the *GSTT1* genotype null or the presence of val alleles. Despite this, the genotype combination results indicate interaction between *GSTT1* null and *GSTM1* null, suggesting a possible summation in the deficiencies of these enzymes.

These findings differ from those found in a previous study by *Fujita* et al. (2000), where no associations between DN and genotype *GSTM1* null were observed. This study was performed with two groups of Japanese patients with or without diabetic nephropathy. Statistical analyses show that the frequency of the null genotype *GSTM1* was not significantly higher in the group of patients with nephropathy than in the group of patients without nephropathy, suggesting that the null *GSTM1* genotype does not contribute to the development of DN in this population [59].

More recent studies with the Romanian population suggest that the polymorphism of the *GSTP1* Ile105Val gene was associated with the risk of developing type 2 DM, but not with the risk of developing DN. For polymorphisms in the *GSTM1* and GSTT1 genes, the results did not indicate an increased risk of developing DM or DN [30].

Studies with the Brazilian population do not indicate an association of *GSTM1* deletion polymorphism with type 2 DM susceptibility. However, the *GSTM1* null and *GSTT1* null polymorphisms reveal an influence on some observed clinical parameters (blood glucose and blood pressure). This suggests that both polymorphisms may contribute to the clinical course of patients with type 2 DM [60].

On the other hand, other studies with the population of Central Brazil [61] suggest that individuals with null *GSTT1* polymorphism present an increased risk of approximately 2.9-fold for DN development. For the same population, no association of *GSTM1* null and DN was found. In this same study, the analysis of the influence of the deletion of *GSTT1* and *GSTM1*

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on clinical and biochemical changes did not indicate a significant association, and this suggests that the *GSTT1* null polymorphism may be associated with the risk of developing the disease, but not with the biochemical alterations analyzed.

6. Conclusion

Considering all the information described above, it is concluded that DM is among the main health concerns in the world. Hyperglycemia is the main characteristic of this pathology, and this unusual situation favors the imbalance between the reactive oxygen species and the antioxidant defense line produced by the individual. This condition is called oxidative stress and Glutathione and GSTs enzymes are fundamental for the reestablishment of redox homeostasis. The progression of diabetes and, consequently, prolonged exposure to this condition, favor the development of secondary complications of DM. DN is the main secondary complication that arises as result of DM.

Expression of polymorphic GST genes within several ethnic populations is remarkable. Some studies have suggested an association between genetic polymorphism of GSTs M1, T1 and P1 susceptibility to DM and its microvascular complications, and others do not. As the results are still scarce and inconsistent, more studies need to be done.

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