UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA

EFFECTS OF GLYCATION BY METHYLGLYOXAL IN *SACCHAROMYCES CEREVISIAE*

Ana Sofia Mena Aires Pendão

Mestrado em Bioquímica
Especialização em Bioquímica Médica

Dissertação orientada por:
Professor Doutor Carlos Cordeiro
Professora Doutora Marta Sousa Silva

2020

Agradecimentos

Gostaria de começar por agradecer aos meus orientadores, Professor Carlos Cordeiro e Professora Marta Sousa Silva, por me terem dado a conhecer o tema da minha tese e terem-me acompanhado desde muito cedo no meu percurso académico. Tenho um enorme prazer em poder considerá-los mais do que professores e orientadores, obrigada pelas muitas conversas e piadas partilhadas. Sem a vossa ajuda o grupo "Fantastic 4" nunca teria tido o seu covil secreto!

Vou ter sempre uma enorme gratidão por toda a ajuda, compreensão e confiança que me deram.

Agradeço ao Professor Federico Herrera por toda a dedicação e paciência que teve na minha formação de microscopia de fluorescência, obrigada por me ter dado a conhecer esta técnica e pela sua preocupação genuína com a minha tese.

Agradeço ainda à Professora Ana Tenreiro pelo acompanhamento na citometria de fluxo, obrigada por ter estado sempre disponível e entusiasmada com os meus resultados.

Por último agradeço aos meus pais que sempre celebraram comigo as minhas conquistas e me ajudaram em todos os momentos de dúvidas e indecisões. Graças a vocês consegui alcançar mais do que as minhas expectativas esperavam e puxar para cima as minhas capacidades.

Obrigada também a todos os meus amigos e colegas que fizeram desta aventura na faculdade uma experiência incrível, cheia de pessoas amáveis e preocupadas em ajudar. Em especial, agradeço aos meus amigos mais chegados, Francisco Schäller Dias, João Luz e António Figueira - os "Fantastic 4" - e Marina Gonçalves por todos os momentos de diversão, dança e jogos. Momentos esses que foram essenciais para desanuviar de todas as preocupações de uma vida académica!

Agradeço ainda a Catarina Matias por ser uma amiga desde o berço, acompanhámo-nos uma à outra em tudo e muitos desabafos partilhámos, obrigada por acreditares em mim e por seres sempre minha vizinha, até na faculdade!

Trabalho suportado pela Rede Nacional de Espectrometria de Massa (LISBOA-01-0145-FEDER-022125), e pelo Projecto EU FT-ICR MS, fundado pela Europa e pelo programa de investigação e inovação da Union's Horizon 2020, sob acordo de concessão nr. 731077.

Work supported by the Portuguese Mass Spectrometry Network (LISBOA-01-0145-FEDER-022125), and by the Project EU_FT-ICR_MS, funded by the Europe and Union's Horizon 2020 research and innovation programme under grant agreement nr. 731077.

Abstract

Glycation is the nonenzymatic modification of biomolecules caused by reactive dicarbonyl species. *In vivo*, the major physiological glycation agent is methylglyoxal. This compound's formation occurs in all living cells, mainly nonenzymatically from dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate during glycolysis. When glycation occurs on proteins it originates advanced glycation end-products (AGEs), which will change the structure and stability of proteins, consequently impairing their functionality. AGEs tend to accumulate on cells, being associated with ageing and several human diseases, such as diabetes and amyloidotic neuropathies (Alzheimer's disease, Parkinson's disease and transthyretin amyloidosis). Hence, there is a growing interest in this post-translational modification. However, little is known about the physiologic effects of glycation, since most studies use *in vitro* approaches, which do not fully reproduce the *in vivo* process. Sac*charomyces cerevisiae* is an ideal model of *in vivo* glycation, being able to reproduce the effects that happen in higher eukaryotic cells. The *in vivo* study and use of *Saccharomyces cerevisiae* cells with different genetic backgrounds, gives us an accurate vision of a wide range of phenotypical and physiological effects caused by glycation. In the present study we show that the glyoxalase system seems to be the major detoxifying mechanism of methylglyoxal, since mutations on this pathway lead to lower growth rates. Moreover, using fluorescence microscopy and flow cytometry we found that glycation is not sufficient to alter cell morphology or its viability. However, the effects of glycation seem to be devastating on mitochondrial activity causing its dysfunction and the accumulation of reactive oxygen species (ROS). These findings provide insight to uncover knowledge of the process of glycation, necessary for better comprehension and advance in the cure and early identification of many human diseases that haunt our society.

Keywords: glycation; methylglyoxal; MAGEs; oxidative stress; Sac*charomyces cerevisiae*

Resumo

A glicação é uma modificação não enzimática de biomoléculas causada por espécies dicarbonilicas reativas. *In vivo*, a glicação ocorre predominantemente pelo metilglioxal, um composto sintetizado em todas as células vivas, principalmente de forma não enzimática a partir de fosfato de di-hidroxiacetona e 3-fosfato de D-gliceraldeído durante a glicólise. Quando a glicação ocorre em proteínas dá origem a produtos avançados de glicação (AGEs, *Advanced glycation end-products*), que irão alterar a estrutura e estabilidade destas mesmas, consequentemente prejudicando a sua função. Os AGEs tendem então a acumular-se nas células, estando associados ao envelhecimento e a várias doenças humanas, tais como a diabetes e as suas complicações, neuropatias amiloidóticas (doença de Alzheimer, doença de Parkinson e Amiloidose familiar por transtirretina) e formação de tumores. Para além disso, a acumulação de AGEs leva ao aumento da expressão de recetores destes AGEs (RAGEs), cuja ativação causa um aumento no nível de espécies reactivas de oxigénio (ROS, *reactive oxygen species*) com consequente indução de stress oxidativo, para além de também estarem associados a processos inflamatórios.

Em condições fisiológicas normais, os mecanismos de defesa de anti-glicação previnem esta acumulação de AGEs, com a proteólise lisossomal, os macrófagos e a autofagia a removerem as proteínas glicadas, a ocorrência de reparação dos nucleótidos e o com o próprio *turnover* dos lípidos a remover os productos glicados. Para além destes processos, existem ainda mecanismos de prevenção de glicação, como o sistema dos glioxalases e o sistema do aldose reductase, que evitam a acumulação de metiglioxal. No entanto, todos estes mecanismos tendem a deixar de funcionar corretamente com o envelhecimento e aparecimento de diversas patologias. Desta forma há um interesse crescente no estudo desta modificação pós-traducional e nos seus efeitos celulares.

A maioria dos estudos que envolvem processos de glicação utilizam abordagens *in vitro* que não reproduzem de forma precisa o processo *in vivo*, uma vez que a glicação se torna num processo inespecífico. É fundamental a realização de estudos *in vivo*, através da utilização de modelos celulares adequados.

No presente estudo pretendemos estudar o impacto da glicação em células eucariotas, utilizando a levedura *Saccharomyces cerevisiae* como modelo celular. Este modelo é conhecido como um modelo ideal no estudo da glicação *in vivo*, sendo capaz de reproduzir os efeitos que acontecem em células eucarióticas superiores. Para além disso, o acesso a estirpes com diferentes mutações genéticas permite consequentemente uma visão ampla e precisa com diversos efeitos fenotípicos e fisiológicos causados pela glicação.

Os estudos foram realizados em leveduras de referência (BY4741) e em estirpes deficientes em genes que codificam para enzimas envolvidos no catabolismo do metilglioxal (glioxalase I, glioxalase II e aldose redutase). Em primeiro lugar, foi estudado o impacto da glicação em determinadas características fenotípicas destas leveduras, tais como o crescimento, a morfologia celular e a viabilidade.

Para o estudo do crescimento, foram comparadas as velocidades de crescimento da estirpe de referência com as estirpes que possuíam deleções nos sistemas de destoxificação celular do metilglioxal, o sistema dos glioxalases e o sistema do aldose reductase. Submeteram-se ainda as estirpes em estudo a dois tipos de meios de crescimento, um meio *standard* YPD (condições normais) e um meio com excesso de glucose (condições de glicação). Este último irá aumentar o stress associado à glicação, uma vez que a formação de metilglioxal está associada ao fluxo glicolítico. Os resultados obtidos neste ensaio, demonstraram que a taxa de crescimento diminuía, de forma estaticamente significativa, apenas nas estirpes com deleções associadas ao sistema dos glioxalases. Desta forma, concluímos então que o

sistema dos glioxalases é o principal método para a destoxificação de metilglioxal e prevenção da glicação. Curiosamente, não houve diminuição significativa no crescimento em condições de glicação em nenhuma das estirpes, havendo, no entanto, uma subida da taxa de crescimento na estirpe deletada para o glioxalase 1 (Δglo1).

Sendo o sistema dos glioxalases o principal sistema na eliminação do metilglioxal *in vivo*, e o glioxalase I o mais relevante nesta via, para os estudos seguintes optámos por comparar apenas a estirpe selvagem com a estirpe deletada para glo1.

No estudo de morfologia celular e viabilidade recorremos a técnicas de microscopia de fluorescência e citometria de fluxo. Concluímos que, nem as condições de glicação causadas pelo meio, nem a glicação obtida pela deleção do glo1, eram suficientes para observar qualquer mudança significativa em termos destas características. No entanto, é importante referir que se consegue observar um ligeiro desvio para o estado viável mas não cultivável (VBNC, *viable but not culturable*) tanto na estirpe de referência crescida em meio com excesso de glucose tanto em qualquer dos crescimentos da estirpe Δglo1. A manutenção da viabilidade nas células, mesmo estando estas sujeitas ao stress de glicação, é possivelmente devido a haver um *shift* no balanço entre a apoptose e a autofagia, havendo já estudos que comprovam que um aumento na autofagia aumenta a protecção celular, removendo organelos e proteínas não-funcionais, bem como outras moléculas danificadas.

Após o estudo fenotípico, foi realizado um estudo fisiológico, com vista a compreender melhor a relação entre o stress causado pela glicação e o stress oxidativo. Uma vez que a maior fonte de ROS provem da cadeia respiratória mitocondrial, devido ao *leak* de protões e electrões que ocorre nesta mesma, e que tende a agravar-se com a disfunção mitocondrial, foi estudado o efeito da glicação no mitocôndrio, recorrendo novamente à citometria de fluxo, nomeadamente nos efeitos desta modificação na actividade mitocondrial e na acumulação de ROS. Demonstrámos que a glicação afecta drasticamente a actividade mitocondrial, uma vez que esta é muito superior na estirpe selvagem do que em qualquer uma das outras condições. Ainda conseguimos observar que a descida de actividade mitocondrial da estirpe selvagem crescida em condições de glicação se assemelha à descida na estirpe Δglo1, o que poderá significar que a hiperglicemia reduz a actividade do glioxalase 1. Curiosamente, apesar da disfunção mitocondrial estar normalmente associada ao aumento dos níveis de ROS, isto não se verificou na estirpe Δglo1, o que poderá significar um aumento na actividade do sistema do aldose redutase de modo a tentar contrariar o stress carboxílico, e consequentemente o stress oxidativo e acumulação de ROS. No entanto, como já mencionado, este esforço não é suficiente para manter a actividade mitocondrial. No caso da estirpe selvagem este aumento esperado de ROS foi verificado.

O conjunto destes resultados mostra os efeitos nefastos da glicação sobre as células e quão relacionada está esta modificação com o stress oxidativo. Desta forma, concluímos que a glicação parece induzir o stress oxidativo, havendo a possibilidade das células aumentarem a actividade do sistema do aldose redutase de modo a contrariar o excesso de glucose e a inibição do sistema dos glioxalases. No entanto, este esforço feito pelas células não é suficiente para combater a hiperglicemia, nem os efeitos que esta causa a nível do mitocôndrio.

O conhecimento obtido com este estudo poderá ter relevância na melhor compreensão dos efeitos da glicação no ser humano, o que será importante no avanço da cura e da identificação precoce de muitas doenças humanas que estão cada vez mais presentes na nossa sociedade.

Palavras-chave: glicação; metilglioxal; MAGEs; stress oxidativo; *Saccharomyces cerevisiae*

Index of Contents

Index of Figures

Index of Tables

Abbreviations

AGEs – Advanced Glycation End-products

BY4741 – Reference yeast strain from the Euroscarf project

CFDA – 5-carboxyfluorescein diacetate (stain), represents esterase's activity

DCFDA – 2',7'-dichlorodihydrofluorescein diacetate (stain), represents ROS accumulation

DHAP – Dihydroxyacetone phosphate, triose phosphate intermediate

FL1 – term used in flow cytometry, meaning the green channel specific fluorescence - used for green stains, such as Rhodamine, CFDA, DCFA

FL3 – term used in flow cytometry, meaning the red channel specific fluorescence - used for red stains, such as propidium iodide

FSC – term used in flow cytometry, meaning forward scatter, represents cell dimension

G3P – D-Glyceraldehyde 3-phosphate, triose phosphate intermediate

Glo1 – glyoxalase 1, lactoylglutathione lyase, EC 4.4.1.5

Glo2 – glyoxalase 2, hydroxyacylglutathione hydrolase, EC 3.1.2.6

Gly – grown on excess glucose medium, glycated/glycation conditions

Gre3 – aldose reductase, EC 1.1.1.21

GSH – glutathione

MAGEs– Methylglyoxal Advanced Glycation End-products (AGEs formed by methylglyoxal)

RAGE – receptor for AGEs/ MAGEs

ROS – reactive oxygen species

SSC – term used in flow cytometry, meaning side scatter, represents cell complexity

1.Introduction

1.1. History of Glycation

1.1.1. Maillard reaction

In 1912, a French scientist, Louis-Camille Maillard established the reaction between reducing sugars and amino groups of amino acids, leading to the formation of brown pigments. This network of reactions is known as the Maillard reaction is also termed food browning or Maillard browning, which is to this day very relevant to the food industry. For instance, the process of turning bread into toast is the result of the Maillard reaction [1, 2].

In the 1920s, an Italian scientist, Mario Amadori showed that glucose-aniline Schiff bases could be converted to isomeric products that were not glycosylamine anomers but were subsequently found to be 1-anilino-1-deoxy fructose derivatives [1, 2]. Later in 1955, an American scientist, John Hodge realized that Amadori products can arise from aliphatic amines such as amino acids, not just from aromatic amines, and that the Amadori rearrangement was a key early step (Fig.1.1, Step B) in the Maillard reaction [3]. Hodge then constructed a diagram that attempted to simplify the complete reaction network, but it was not until 1973 that the core of this diagram would be truly complete, with the work of the Japanese scientist, Namiki, whom introduced one more crucial step (Fig.1.1, Step H), the free radical degradation of Maillard intermediates [4]. This complete reaction scheme initiates with the initial stage of the Maillard reaction (Fig. 1.1, Step A), that involves the condensation of a carbonyl group from a reducing sugar, such as glucose, with a free amino group, such as the epsilon amino group of a protein lysine residue, that will do a nucleophilic attack to the electrophilic carbonyl group of an aldehyde or ketone. This reaction will result in an unstable Schiff base (aldimine), that spontaneously rearranges (Fig.1.1, step B) to form the more stable 1-amino-1-deoxy-2-ketose (ketoamine), known as the Amadori product.

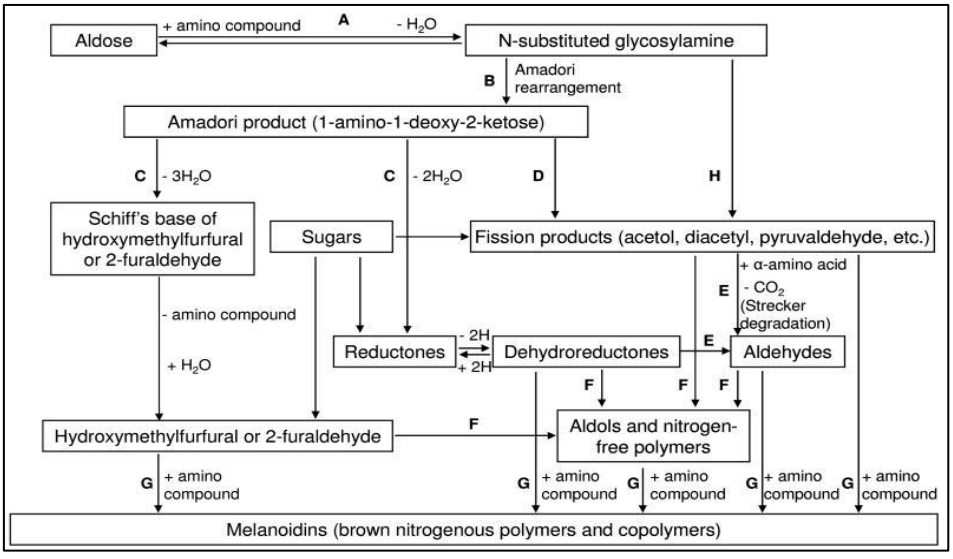

Figure 1.1 The Hodge Diagram – Maillard Reaction. **A** - initial reaction between a reducing sugar and amino group, forming an unstable Schiff base. **B -** the Schiff base rearranges to an Amadori product. **C** - degradation of the Amadori product. **D** - formation of reactive carbonyl and dicarbonyl compounds. **E** - formation of Strecker aldehydes of amino acids and aminoketones. **F** - aldol condensation of furfurals, reductones, and aldehydes produced in Steps C, D, and E without intervention of amino compounds. **G** -reaction of furfurals, reductones, and aldehydes produced in Steps C, D, and E with amino compounds to form melanoidins. **H** - free radical-mediated formation of carbonyl fission products from the reducing sugar (Namiki pathway). Extracted from: [1, 2].

It is worth to note that when the initial sugar is glucose, the Amadori product is fructoselysine [5, 6].

Amadori products are degraded through the pathways in steps C and D, which will lead to the formation of furfurals, reductones and fragmentation products (carbonyl and hydroxycarbonyl compounds). In step C, furfural formation is favored under acidic conditions, while alkaline media favors the production of reductones. In step D sugar fragmentation occurs by retro-aldolisation. The α-dicarbonyl compounds formed, such as methylglyoxal, are able to react with amino acids via the Strecker degradation (Fig1., step E), to give Strecker aldehydes of the amino acids and aminoketones.

Steps F and G are the final stages of the Maillard reaction. Step F involves aldol condensation of the furfurals, reductones and aldehydes produced in steps C, D and E without the intervention of amino compounds. While in step G the same reactions between the same intermediates occurs, but with amino compounds, leading to the formation of melanoidins [1, 7, 8].

1.1.2. The Maillard reaction in vivo & Formation of AGEs

For more than 60 years, the Maillard reaction was studied with a focus on food-like model systems. However, the human body is no less than a low heat cooking oven with an approximately 85 year cooking cycle, thus getting the attention in the late 1970s to study the Maillard reaction *in vivo* [9, 10].

For proteins, glycation is frequently used in apposition to glycosylation, with glycation being a nonenzymatic process while glycosylation is a highly regulated and specific enzymatic process forming a glycosidic bond.

In 1978, Bunn focused his studies on the elevated values of glycation in diabetes in hemoglobin A1c, which is an adduct of glucose with the b-chain of hemoglobin. It is well known that the increase in glycated proteins is a consequence of hyperglycemia and correlates with the severity of diabetic complications, glycated hemoglobin is currently being used as a biomarker for this disease [10, 11].

Protein glycation *in vivo* includes three types of reactions (Fig.1.2), [12]: early Maillard reactions (initiation) – Reactive electrophilic carbonyl groups of a reducing sugar react with nucleophilic free amino groups of proteins to form a non-stable Schiff base. Through rearrangement a more stable Amadori product is formed; intermediate Maillard reactions (propagation) – Amadori compounds are then converted into more reactive substances, such as α-dicarbonyl glyoxal compounds; late Maillard reactions (termination) – α-dicarbonyl glyoxal compounds can react with amino acid residues of peptides or proteins to form protein adducts or protein crosslinks. Alternatively, they can undergo further oxidation, dehydration, polymerization and oxidative breakdown reactions to give rise to numerous other AGEs (advanced glycation end products) [10, 13, 14].

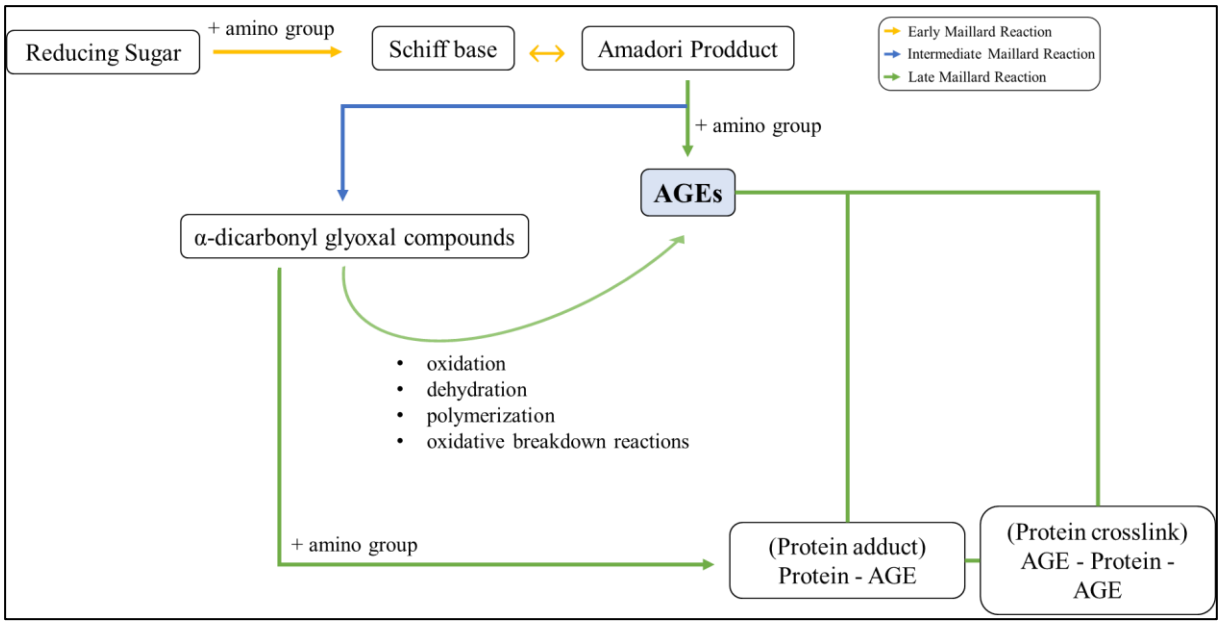

Figure 1.2 Three main types of glycation that occur in vivo. Adapted from: [10, 13, 14]

However, glycation occurs not only in proteins but also in other biomolecules with free amine groups like nucleotides and also some phospholipids. Chemically, side chains of arginine and lysine residues, the protein N-terminal amino group and the thiol groups of cysteine residues, are the main targets of glycation in proteins. Nevertheless, biologically the N-terminal is usually modified, and the glycation of cysteine thiol groups is reversible, originating unstable adducts. So, the main glycation targets in biomolecules that will originate AGEs are lysine and arginine side chains [6, 15, 16].

In the 1970s, with Bunn's investigation, the focus was on protein glycation by glucose. Nevertheless, glucose is one of the least reactive sugars and therefore less likely to perform a nucleophilic attack. Dicarbonyl compounds, such as glyoxal, 3-deoxyglucose and methylglyoxal, formed in Fig. 1.1 Step D (Fig. 1.2, intermediate Maillard reactions, propagation phase), are far more reactive [15, 17].

Among these dicarbonyl compounds, methylglyoxal is one of the most reactive glycating agents *in vivo*, which is present in all cells and has affinity to react with several amino acids including arginine and lysine residues to produce MAGEs (methylglyoxal advanced glycation end products), which exert intracellular oxidative stress and loss of function and structure [18]. Oxidative and carbonyl stress are the key factors in many pathological complications such as diabetes and neurodegenerative disorders of the amyloid type [1, 19–21].

1.2. Methylglyoxal

1.2.1. Methylglyoxal Formation

Methylglyoxal is produced as a result of both enzymatic and non-enzymatic reactions. Its production is inevitable and tightly coupled to glycolysis. However, the rate of methylglyoxal production depends on the organism, tissue, cell metabolism, and physiological conditions [22]. As such, this compound can arise mainly as a by-product of glycolysis and by different metabolic pathways, including the Wolff pathway (Figure 3, green), in which the acyclic form of glucose undergoes autooxidation to form glyoxal

or break down to 3-deoxyglucosone which in turn degrades to glyceraldehyde and methylglyoxal; the Namiki pathway (Figure 3, purple), glucose reacts with amine groups on proteins to form a Schiff base which undergoes a similar breakdown generating glyoxal and methylglyoxal; the acetoacetate metabolism (Figure 3, blue), via acetone and acetol and catalyzed by acetone monooxygenase (EC 1.14.13.226) and acetol monooxygenase (EC 1.14.13.M34); the L-threonine metabolism (Figure 3, yellow), which involves oxidation of threonine by threonine dehydrogenase (EC 1.1.1.103) to 2-aminoacetoacetate, followed by spontaneous decarboxylation to aminoacetone, that will be then catalyzed by monoamine oxidase (EC 1.4.3.4) and converted to methylglyoxal. Additionally, this compound can also be formed through lipoperoxidation (Figure 3, cyan) [23–26].

As mentioned, in eukaryotic cells, methylglyoxal is mainly produced during glycolysis, by the β-elimination of the phosphate group from the triose phosphate intermediates glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), (Figure 3, orange). This reaction was initially reported in 1930s by Meyerhof and Lohmann, but it was overlooked by referring to it as an experimental artifact. Later in 1993, Richard investigated the mechanism of formation of methylglyoxal from triose phosphates and showed that it had a physiological significance [22, 24]. In this reaction at the physiological pH, there is more tendency for the loss of α-carbonyl protons from the phosphorylated triose than the non-phosphorylated trioses, producing an enediolate phosphate intermediate possessing a low energy barrier, due to the phosphate group elimination. It is thus the deprotonation followed by the spontaneous cleavage of phosphate group of the triose phosphates that leads to the formation of methylglyoxal [23–26].

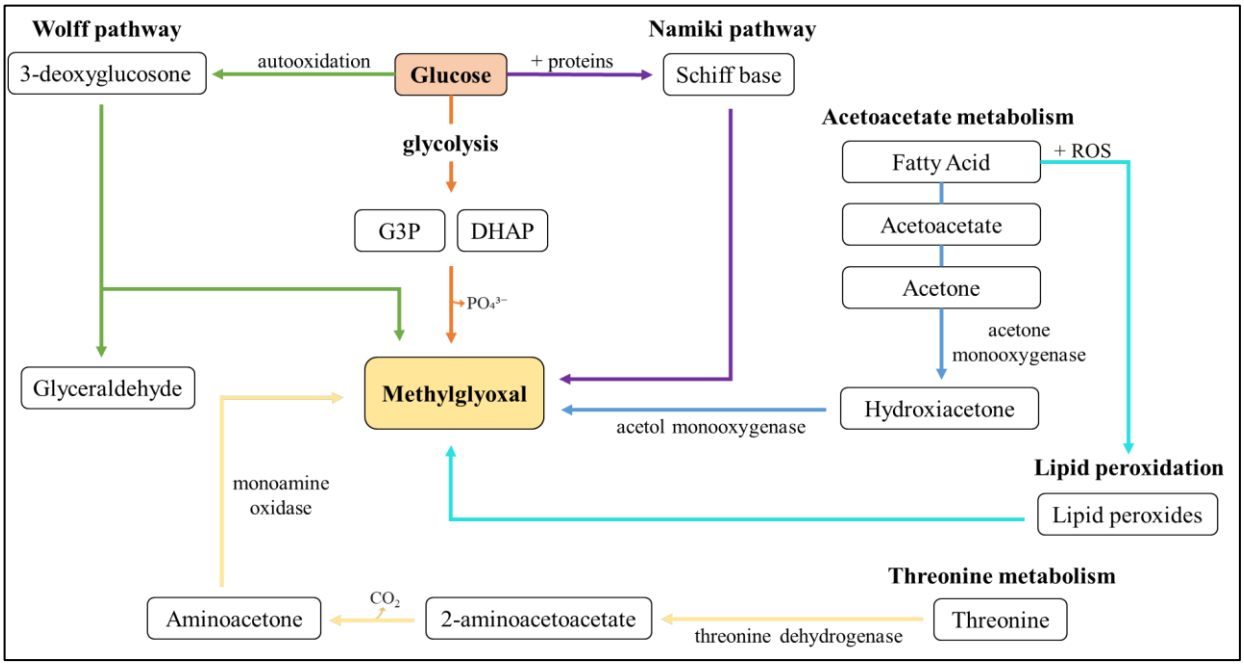

Figure 1.3 Metabolic origins of methylglyoxal. Adapted from: [20, 26]

1.2.2. Catabolism of Methylglyoxal

As previously mentioned, methylglyoxal can react with proteins, nucleic acids and lipids, being the most significant agent of glycation *in vivo*, making it's accumulation highly deleterious for the cell [27].

Methylglyoxal production is inevitable *in vivo*, so to avoid its toxic effects, cells possess different detoxifying mechanisms such as the glyoxalase, aldose reductase, aldehyde dehydrogenase and carbonyl reductase pathways [28–30]. Of these protective enzymatic mechanisms, the glutathione-dependent glyoxalase system and the NADPH-dependent aldose reductase system are the main detoxifying mechanisms for methylglyoxal, playing a major role the cellular defense against glycation [25, 26, 31].

1.2.2.1. The Glyoxalase System

The glyoxalase system is a two-step pathway comprising glyoxalase I (Glo1, lactoylglutathione lyase, EC 4.4.1.5) and glyoxalase II (Glo2, hydroxyacylglutathione hydrolase, EC 3.1.2.6) enzymes, which act sequentially to convert methylglyoxal into D-lactate, using glutathione as a co-factor [27].

First, Glo1 catalysis the isomerization of hemithioacetal, formed by the non-enzymatic reaction of reduced glutathione (GSH) with methylglyoxal, to S-D-lactoylglutathione. Then Glo2 catalysis this thioester hydrolysis to D-lactate regenerating GSH in the process [32, 33]. This was thought to be the termination of the glyoxalase system, but in 1968 the scientists George Long and Nathan Kaplan determined that in biological systems D-lactate is converted to pyruvate, with the help of the enzyme D-lactate dehydrogenase [22, 34, 35].

The compound S-D-lactoylglutathione, produced by the first step of the glyoxalase system, is a non-toxic compound, making this a crucial step for methylglyoxal detoxification. Therefore, Glo1 activity indirectly determines methylglyoxal toxicity and the rate of MAGEs formation. Consequently, decreased concentrations of this enzyme, caused by aging and oxidative stress, are associated with increased glycation and tissue damage [36, 37].

One should also consider that the recycling of GSH, necessary for the proper functioning of the glyoxalase system, occurs as S-D-lactoylglutathione is metabolized to D-lactate. As such, if there is a large amount of methylglyoxal, it may result in S-D-lactoylglutathione accumulation, keeping the limited amounts of GSH trapped in this step and reducing its availability for other cellular processes, such as defense against oxidative stress [27, 38].

Glo2 has been found to have a significant role in Huntington's and Parkinson's disease, being also associated with inhibitors of apoptosis and tumorigenesis [39, 40]. Glo1 has been found to be down-regulated in ageing, hyperglycemia, diabetes and Alzheimer's disease [25, 41–44].

1.2.2.2. Aldose Reductase System

Aldose reductase (EC 1.1.1.21, Gre3 in yeast), an aldo-keto reductase, is part of the rate-limiting step in the polyol pathway, which converts glucose to sorbitol. However, methylglyoxal is a preferred substrate of this enzyme.

In the presence of physiological concentrations of GSH, methylglyoxal is significantly converted into hemithioacetal, and catabolized by the glyoxalase system[28, 30, 45, 46]. Nonetheless, in the presence of high GSH concentrations, the efficiency of reduction of methylglyoxal, catalyzed by aldose reductase, increases instead. In addition, the site of reduction switches from an aldehyde to a ketone carbonyl. Thus, glutathione converts aldose reductase from an aldehyde reductase to a ketone reductase. This enables aldose reductase to catalyze the NADPH-dependent reduction of methylglyoxal to produce D-lactaldehyde and hydroxyacetone. A second reduction is also catalyzed by aldose reductase which further reduces these products to propane-l,2-diol [47]. If the concentration of GSH decreases to low

levels, aldose reductase catalyzes the NADPH reduction of methylglyoxal at the aldehyde carbonyl to produce acetol [28, 30, 45, 46].

1.3. Pathological significance of AGEs

AGEs are known to be mainly associated with aging, neurodegenerative disorders, diabetes and its complications, atherosclerosis, renal failure, immunological changes, retinopathy, osteoporosis, and progression of some tumors [48–51].

The presence of AGEs in proteins alter their structure and function. Nucleotides and lipids are also particularly vulnerable targets to this modification, which can favor DNA mutations or a decrease in cell membrane integrity and associated biological pathways [52]–[54]. In mitochondria, glycation can affect bioenergy production [55].

Under physiological conditions, anti-glycation defenses are enough to prevent the nefarious effects of this modification, with lysosomal proteolysis preventing accumulation of glycated extracellular proteins and autophagy eliminating glycated components inside the cell, while lipid turnover clears glycated products and nucleotide excision repair removes glycated nucleotides. Moreover, there are also detoxifying mechanisms for the glycation agents, such as the glyoxalase system and the aldose reductase system, as described above. If these clearance mechanisms are impaired, glycation damage accumulates and pathologies may develop [48, 56–59].

1.3.1. Consequences of glycation on amino groups

When proteins are glycated, their structural and physical characteristics are altered. For instance, when methylglyoxal reacts with arginine residues of proteins to form hydroimidazolone derivate (MG-H), argpyrimidine, and THP (tetrahydropyrimidine) [60]. In addition, the cross-linking between lysine residues and methylglyoxal leads to the formation of CEL [Nε-(carboxyethyl)lysine] and MOLDs (methylglyoxal–lysine dimers) [61, 62].

The proteins can also become resistant to proteases for which they would previously act as the substrate, resulting in an accumulation of that protein. Likewise, glycated intracellular proteins become less sensitive to proteolysis (due to glycation preventing ubiquitination) [48, 63–67]. As such, AGEs tend to accumulate over time in tissues, where glycation will result in protein aggregates due to the bonds created. Chemical bridges formed by AGEs also result in the reticulation of proteins and their cross-linking, a phenomenon that occurs within the extracellular matrix and significantly increases the structural rigidity [13, 48, 65].

It is also worth to note that, AGE accumulation is dependent on protein turnover rate, therefore long-lived proteins and proteins which exhibit a slow turnover, are thought to be mainly modified by glycation [13, 68].

If the protein in question acts as an enzyme, glycation can cause conformational changes near the active site, making it non-functional, which can be highly deleterious for the cell [48, 65, 69, 70].

When glycation affects amino groups in nucleotides there will be breaks to the DNA strand and mutations that lead to production of abnormal proteins or total suppression in the synthesis of a specific protein [52–54].

Modified proteins by any of this process can also cause an immune response, where antibodies against these proteins will be produced. In addition, the autoantibodies can bind to circulating AGEs and form immune complexes [13, 46, 48, 71, 72].

1.3.2. Glycation and oxidative stress

Glycation and oxidative stress are closely linked due to the fact that many glycation products, such as Amadori products and AGEs, react with oxygen producing significant quantities of free radicals. In addition, the overexpression and activation of RAGEs, receptors of AGEs, caused by the increase of glycation products, also causes production of reactive oxygen species (ROS), [48, 73–75]. Furthermore, this AGE receptor can not only be activated by AGEs, but also by other molecules, such as the family of S100/calgranulin proteins, which includes pro-inflammatory and pro-oxidizing polypeptides [51, 76]; β-amyloid peptides, which are implicated in Alzheimer's disease [77]; high-mobility group box-1 (HMGB) proteins, a nuclear protein secreted in stressful conditions. Its binding to RAGEs promotes the growth and invasive quality of glial tumors [51].

All these ligands make the activation of RAGEs play an important role not only in oxidative stress, but also in sustaining inflammation and increasing vascular endothelial growth factor production, all associated with diabetic complications and neurodegeneration [48, 73].

Moreover, when methylglyoxal concentrations exceed the detoxifying capacity of the cell's system, cells suffer from oxidative stress, which depletes the pool of glutathione and results in an accumulation of AGEs [78].

1.3.3. Elimination of AGEs

The repair of glycated proteins and the clearance of AGEs is still unknown and a matter of debate in the scientific community. There are a few hypotheses regarding potential mechanisms that repair glycated proteins. Two of the most accepted intracellular anti-glycation systems only act on Amadori products/ Schiff's bases and cannot change AGEs. The first, known as transglycation, is a non-enzymatic process that separates the carbohydrate portion from the glycated protein and transfers it to a low-molecular weight intracellular nucleophile, made up of free amino acids, such as glutathione. Removing this portion allows the protein to regain its original shape and consequently some of its function. The second repair mechanism uses fructosamine-3-kinase (FN3K), an enzyme that catalyzes the repair of the glycation intermediaries fructoselysines. Phosphorylation of fructoselysine on proteins by FN3K to fructoselysine-3-phosphate (FL3P) produces a compound that is intrinsically unstable and that decomposes spontaneously to lysine, 3-deoxyglucosone, and inorganic phosphate, thereby regenerating an unmodified amine such as a lysine residue in proteins. The fructosamine-3-kinase enzyme thus limits the formation of glycated hemoglobin in erythrocytes [48, 79].

Regarding the clearance of AGEs, glycated proteins can be taken up by macrophages via the AGE receptor, which is similar to the macrophage scavenger receptor. Moreover, a number of waste macromolecules, including AGEs are taken up from the circulation mainly via scavenger receptor-mediated endocytosis in the liver scavenger cells, such as liver sinusoidal endothelial cells and Kupffer

cells - resident macrophages located in the liver [80–82]. Moreover, extracellular glycated products can also be broken down by proteases. Their elimination through the kidneys is directly dependent on renal function [48, 82]. However, AGEs are scarcely degraded, and these clearance mechanisms tend to deteriorate and mal-function with ageing and other pathologies, consequently leading to accumulation of AGEs in different tissues [48, 80–82].

1.4. Oxidative stress

Under physiological conditions, the steady-state formation rate of reactive oxygen species (ROS) is normally balanced by a similar rate of consumption by antioxidants. Indeed, in physiological concentrations, ROS act as signaling molecules and play an important role in cell proliferation, hypoxia adaptation and cell fate determination [83].

An overproduction of free radicals in the organism that exceeds the endogenous antioxidant capacity for them to be eliminated results in oxidative stress [84].

Overproduction of ROS can damage NADH dehydrogenase, cytochrome *c* oxidase, and ATP synthase, resulting in mitochondrial damage - including mutations in mitochondrial DNA; damage to the mitochondrial respiratory chain and mitochondrial membrane permeability; and disruption to Ca^{2+} homeostasis [84–87]. Furthermore, ROS are capable of activating cellular signaling cascades that lead to the transcription of genes that facilitate the development of diabetic complications [19, 83]. In addition, oxidative stress and oxidative protein damage can accelerate the formation of toxic protein oligomers and aggregates in the nucleus and cytoplasm of nerve cells, which contributes to the pathogenesis of many neurodegenerative diseases [84, 87]. Despite the distinct causative factors and clinical symptoms of neurodegenerative diseases, these diseases have common pathogenetic features such as mitochondrial dysfunction, which is implicated in excessive ROS accumulation, impairment in proteostasis network, and neuroinflammation [88].

1.4.1. Mitochondria and production of ROS

In 1966, the production of ROS by the respiratory chain was reported [89]. This finding was followed by the work of Chance and colleagues who showed that isolated mitochondria produced hydrogen peroxide (H_2O_2) [90–92]. Later, it was assessed that this H_2O_2 originated from the dismutation of superoxide ($O_2^{-\bullet}$), which in turn was produced within mitochondria [93]. With these findings we now know that the main source of ROS is the mitochondrial electron transport chain.

The mitochondrial respiratory chain complexes consist of five complexes: complexes I (nicotinamide adenine dinucleotide (NADH)-coenzyme Q), II (succinate dehydrogenase-coenzyme Q), III (coenzyme Q-cytochrome *c* reductase), IV (cytochrome *c* oxidase) and V (ATP synthase), [94].

Complex I, II, III, and IV as well as the electron transporters ubiquinone and cytochrome *c* constitute the electron transport chain (ETC). There are two electron transport pathways in the ETC (Fig. 1.4): Complex I/III/IV, with NADH as the substrate and complex II/III/IV, with succinic acid as the substrate [84, 94].

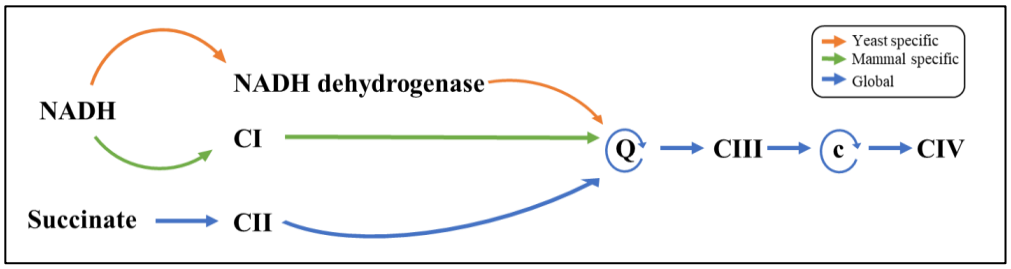

Figure 1.4 Two electron transport pathways present in the electron transport chain (yeast and mammal). CI – complex I; CII – complex II; Q – CoQ/ reduced $CoQH_2$; CIII – complex III; c – cytochrome *c*/ reduced cytochrome *c*; CIV – complex IV. Adapted from: [179, 95, 96]

Coenzyme Q (CoQ) accepts electrons released from the NADH-CoQ reductase complex (CI, complex I) and the succinate-CoQ reductase complex (CII), forming the reduced $CoQH_2$ [94].

Reduced $CoQH_2$, donates two electrons to the $CoQH_2$-cytochrome *c* reductase complex (CIII), regenerating oxidized CoQ. Within this complex the released electrons are transferred to an iron-sulfur protein and to two b-type cytochromes, then to cytochrome *c*1. Finally, the two electrons are transferred to two molecules of the oxidized form of cytochrome *c*, forming reduced cytochrome *c* [84, 94].

After being reduced by the $CoQH_2$-cytochrome *c* reductase complex (CIII), cytochrome *c* transports electrons to the cytochrome *c* oxidase complex (CIV). Within this complex, electrons are transferred firstly to a pair of copper ions, then to cytochrome *a*, then to a complex of a second copper ion and cytochrome *a*3, and lastly to O_2, the ultimate electron acceptor, yielding H_2O [84, 94].

Regarding the yeast *Saccharomyces cerevisiae*, the only difference from this global electron transport mechanism is that, in contrast to most eukaryotes, yeast does not possess a complex I, but rather three NADH dehydrogenases associated with the inner mitochondrial membrane [95, 96].

Nevertheless this exergonic process is used, both in yeast and humans, to pump protons from the mitochondrial matrix into the intermembrane space, creating an electrochemical gradient known as the protonmotive force, Δp. Δp drives protons back into the matrix through the ATP synthase (CV), driving the conversion of ADP and inorganic phosphate to ATP and thereby coupling substrate oxidation and ADP phosphorylation [97].

However, this coupling of ATP synthesis and substrate oxidation is not complete, as protons can return to the matrix independently of ATP synthase. The processes by which this occurs are named as proton leak [94, 97–100].

On the other side of the spectrum, electron leakage occurs when electrons passed down the respiratory chain exit prior to the reduction of oxygen to water at cytochrome *c* oxidase. The non-enzymatic production of $O_2^{-}\cdot$ occurs when a single electron is directly transferred to oxygen by reduced coenzymes, prosthetic groups or by xenobiotics previously reduced by certain enzymes [55].

As such, proton and electron leak are intricately linked, as superoxide production is highly sensitive to the decrease in Δp due to proton leak. At low concentrations, superoxide production may be involved in cellular signal transduction, but at high concentrations the radicals cause oxidative damage due to their high reactivity towards other cellular compounds [97].

Even though $O_2^{-\bullet}$ is not a strong oxidant, it is a precursor of most other reactive oxygen species, and it also becomes involved in the propagation of oxidative chain reactions. Dismutation of $O_2^{-\bullet}$ produces H_2O_2, which in turn may be fully reduced to water or partially reduced to hydroxyl radical ($OH^{\bullet}$), one of the strongest oxidants in nature. $OH^{\bullet}$ may also be re-reduced by $O_2^{-\bullet}$, propagating this process. In addition, $O_2^{-\bullet}$ may react with other radicals including nitric oxide ($NO^{\bullet}$), leading to the formation of a very powerful oxidant [55, 94, 101].

1.4.2. ROS Elimination

As previously mentioned, under physiological conditions, the steady-state formation of ROS is normally balanced by a similar rate of consumption by antioxidants. As such, there are a number of ways the cells can tackle ROS formation.

In mitochondria there are two main H_2O_2 degrading pathways, the glutathione (GSH) and thioredoxin (Trx) systems [102–105].

Glutathione has a crucial role in antioxidant defenses either by reacting directly with reactive oxygen, acting as a donor of electrons, or by acting as an essential cofactor for GSH S-transferases and glutathione peroxidases, having a role in converting vitamin C and E back to their active forms [104, 105]. Vitamin C provides intracellular and extracellular antioxidant capacity primarily by scavenging oxygen free radicals. It converts vitamin E free radicals back to vitamin E. Vitamin E, thus functions as a powerful antioxidant that interferes with the propagation of free radical-mediated chain reactions [105, 106]. Glutathione S-transferase catalyzes the formation of glutathione S-conjugates between reduced glutathione and ROS. Glutathione peroxidase catalyzes the reduction of H_2O_2 to water and oxygen, as well as the reduction of peroxide radicals to alcohols and oxygen, and in turn oxidizes glutathione to glutathione disulfide. The Glutathione peroxidase / glutathione system is thought to be a major defense in low-level oxidative stress [107, 108].

Thioredoxin (Trx) can reduce target proteins via cysteine thiol-disulfide exchanges. Trx-dependent peroxidase can also directly scavenge ROS (mainly H_2O_2), [102, 103].

Nevertheless, the main ROS produced is superoxide, and for this compound the most significant agent of the detoxifying pathway is the enzyme superoxide dismutase (SOD). SOD catalyzes the dismutation of superoxide anion ($O_2^{-\bullet}$) to O_2 and H_2O_2, which then undergoes elimination by the pathways mentioned [105].

Apart from these major detoxifying systems, it is known that the cell has other helpers for the elimination of ROS, namely cytochrome *c*, coenzyme Q and catalase.

Reduced cytochrome *c* can transfer electrons to the terminal oxidase. Thus, some of the electrons that escaped the respiratory chain producing $O_2^{-\bullet}$ may re-reduce cytochrome *c* and still contribute to energy production by providing the energy needed to pump H^+ through Complex IV [103, 109].

Reduced coenzyme Q (CoQ), can act as a reducing agent in the elimination of various peroxides in the presence of succinate [110]. Thus, CoQ is a source of $O_2^{-\bullet}$ when partially reduced (semiquinone form) and an antioxidant when fully reduced.

Catalase is also a major H_2O_2 detoxifying enzyme present in mitochondria [111]. Catalase-ferricatalase (iron coordinated to water) catalyzes the conversion of two H_2O_2 molecules to oxygen and water. This reaction requires H_2O_2 to bind at the active site in order to generate catalase compound I (iron coordinated with an oxygen atom), which reacts with a second molecule of H_2O_2 [55, 105, 112].

Under normal physiological conditions these detoxifying pathways put at bay ROS accumulation however, when the mitochondria is damaged proton and electron leak increase, generating an excess of superoxide [75, 102, 103].

1.5. Studying glycation in yeast

Although it may seem that yeast and humans have little in common, yeast is a eukaryotic organism, sharing many basic biological properties with humans, such as, cell cycle progression and regulation; nucleic acid transcription and translation; protein targeting and secretion; cytoskeletal structures; and organelle biogenesis, which are essentially the same in yeast as in other eukaryotes [113]. Interestingly, approximately 30% of yeast genes have counterparts in the human genome, and two-thirds of all yeast genes share at least one conserved domain with human genes [114, 115]. Many of these similar genes are known to have a role in human diseases, suggesting that such diseases result from the disruption of basic cellular processes [113, 116] .

The yeast *Saccharomyces cerevisiae* has long served as a model eukaryote by virtue of its facile genetics, scalability for biochemical analysis, short generation time and availability of large-scale screening methods [86, 113, 117–119]. In 1996, with the *Saccharomyces cerevisiae*'s genome fully sequenced, efficient homologous recombination was possible, giving researchers the opportunity to either replace any gene with a mutant allele or completely delete the gene by replacing it with a selectable marker. This enabled a collection of deletion mutant strains to be formed and used to study many cellular processes relevant to human diseases [120], DNA damage [121], oxidative stress [122], and organelle physiology [113, 123–125].

S. cerevisiae is an outstanding model to study protein glycation, for the ability to use mutant strains with different glycation phenotypes and the identification of specific protein glycation targets [60, 126, 127]. Yeast cells growing on D-glucose show a high glycolytic flux and, consequently, a high rate of methylglyoxal formation [126]. Additionally, *in vivo* glycation conditions can be simulated by increasing D-glucose concentration in growing media, allowing the study of the effects of this proteins' modification *in vivo* [126].

1.6. Motivation & Objectives

Glycation is at the core of many of the human diseases that haunt our society today, this stress state is thought to be deeply associated with diabetes and its complications, progression of tumors, a wide range of amyloidogenic neuropathies and ageing. The culprit is the accumulation of AGEs/ MAGEs, mainly formed by methylglyoxal, a compound tightly coupled with glycolysis and therefore with inevitable formation. Under physiological conditions, anti-glycation defenses are sufficient to put glycation damage at bay, with lysosomal proteolysis preventing accumulation of glycated extracellular proteins and autophagy eliminating glycated components inside the cell, while lipid turnover clears glycated products and nucleotide excision repair removes glycated nucleotides. However, when this does not occur, glycation damage accumulates, and pathologies may develop.

Despite the importance of glycation in our biology, little is known about the *in vivo* consequences of this nonenzymatic modification. Therefore, the main goal of the present work was to investigate the differences that occur when glycation occurs in eukaryotic cells. To achieve this, different strains of Sac*charomyces cerevisiae*, with different glycation phenotypes, were used. The strain's phenotypical and physiological characteristics were tested under growth in normal glucose medium and excess glucose medium. Growth rate was assessed by growth curve analysis, while morphology of the cell and viability were analyzed by fluorescence microscopy and flow cytometry. Regarding physiological characteristics, the goal was to tackle a matter that is of growing interest in the scientific community, and that is the correlation between glycation stress and oxidative stress, due to the fact that AGEs react with oxygen producing significant quantities of free radicals. To achieve this, flow cytometry was used to assess mitochondrial dysfunction and ROS accumulation in glycated yeast cells, comparing them with non-stressed yeast cells.

These findings partially uncovered the veil in glycation, getting us closer to reveal the true effects of this modification, which are necessary for better comprehension and advance in the cure and early identification of many human diseases

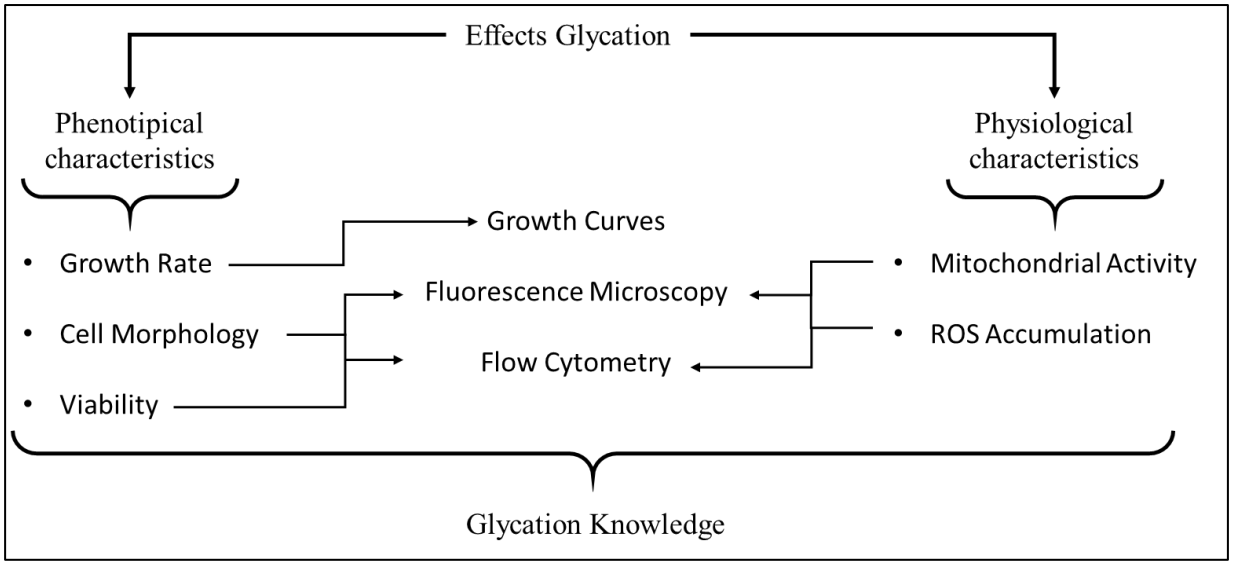

Figure 1.5 Global aims of this project

2.Materials & Methods

2.1. Yeast strains and culture conditions

Saccharomyces cerevisiae strains, Euroscarf (European *Saccharomyces cerevisiae* ARchive for Functional analysis) collection, were: BY4741 (genotype BY4741 MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0); ΔGLO1, a glyoxalase I-deficient strain (isogenic to BY4741 with YML004c::KanMX4); ΔGLO2, a glyoxalase II-deficient strain (isogenic to BY4741 with YDR272w::KanMX4) and ΔGRE3, an aldose reductase-deficient strain (isogenic to BY4741 with YHR104w::KanMX4).

Strains were kept in YPD (0.5% yeast extract - BD, 1% peptone - BD and 2% D-glucose - MERCK; 100mM glucose) agar slopes (2% agar - nzytech) at 4 ˚C and cultured in 20mL of liquid YPD medium 100mM glucose in 100mL Erlenmeyers overnight, at 30˚C with constant shaking.

Yeast strains under glycation conditions were kept in YPD (0.5% yeast extract - BD, 1% peptone - BD and 2% D-glucose - MERCK; 100mM glucose) agar slopes (2% agar - nzytech) at 4 ˚C and cultured in 20mL of liquid YPD medium 250mM glucose in 100mL Erlenmeyers for approximately 18h, 30˚C with constant shaking.

2.2. Growth curves

To 5mL of liquid YPD medium (100mM glucose for control strains; 250mM glucose for glycated strains) 250μL of each yeast strain was added to create pre-inoculums of similar Absorbance at λ640nm (0.3-0.7).

Control and glycated yeast strains from the pre-inoculum were grown in 2mL of liquid YPD medium 100mM glucose or liquid YPD medium 250mM glucose, respectively, in cuvettes of 3mL with constant shaking at 30˚C. Absorbance measures at λ640nm were taken in intervals of 3min for approximately 14h. Growth curves were smoothed by the Savitzky Golay method and then derivatized, the growth rate being the maximum of the first derivative.

2.3. Fluorescence microscopy

Yeast samples for fluorescence microscopy analysis were prepared using 2mL of each yeast strain, control and glycated. Samples were centrifuged 7000g, 4˚C for 3min. The supernatant was discarded and 1mL of PBS pH 7.4 (NaCl, KCl, Na_2HPO_4, KH_2PO_4 – MERCK) and 1μL of 1000x concentrated stain, Hoechst (Biotium) and Calcofluor-white (Biotium), was added to the cell pellet, in case of Live-or-Dye (Biotium), 2μL of 500x concentrated stain was added instead. After vortexation the samples were incubated in the dark at room temperature for 20min. Then a centrifugation 7000g, 4˚C for 3min was done and repeated 3 times to remove the excess stain, always washing the cell pellet with 1mL of PBS pH 7.4. In the final centrifugation 50μL of PBS pH7.4 and 50μL of glycerol were added, to stop the movement of yeast cells.

For fluorescence microscopy analysis, 10μL of each sample were analyzed. Samples with the same staining were treated with the same exposure time for comparison of results to be possible. Hoechst and Calcofluor-white stained samples were excited with UV cube (340-380nm) and visualized in the blue

channel. Live-or-Dye stained samples were excited with Leica Y5 cube (590-650nm) and visualized in the red channel. To have a significant pool of cells 10 images of each stain were taken, using fluorescence microscope Leica TCS SPE with an objective of 63x, and analyzed (150 to 250 cells), using Fiji: ImageJ software [128, 129]. The images were split by color channels and then adjusted with a color threshold to make Fiji recognize only the true signal in each cell. Taking into account that the size of a single yeast cell can vary between 5 microns to 10 microns and that the nucleus is as small as 1 micron, fluorescence analyses were performed in this particle range, for the nucleus morphologic analysis particles between 0,1 $pixel^2$ and 5 $pixel^2$ were chosen instead.

2.4. Flow Cytometry

Yeast samples for flow cytometry were prepared using 2mL of each yeast strain, control and glycated. Cells were centrifuged 7000g, 4˚C for 3min. The supernatant was discarded and 1mL of PBS pH 7.4 was added to the pellet. This process was repeated twice to obtain a clear sample without presence of growth medium.

Yeast samples were stained with Rhodamine (Biotium) by adding 1μL of 1000x concentrated stain was added to the clean sample. Then the samples were incubated with constant shaking at room temperature in the dark for 1h. A centrifugation 7000g, 4˚C for 3min was done and repeated 3 times to remove the excess of stain, always adding to the pellet 1mL of PBS pH 7.4. For the analysis a 1:100 dilution was made.

Yeast samples stained with DCFDA (2',7'-dichlorodihydrofluorescein diacetate) (Biotium) were prepared by adding 1μL of 1000x concentrated stain to the diluted 1:100 sample. Then the samples were incubated with constant shaking at 37˚C in the dark for 30min.

Yeast samples stained with CFDA (5-carboxyfluorescein diacetate) plus propidium iodide by adding 1μL of each 1000x concentrated stain to the diluted 1:100 sample. Then the samples were incubated at room temperature in the dark for 10min.

Flow cytometry analysis were performed CyFlow PARTEC and 30 000 cells/events were analyzed, with a sample concentration of 5.70-6,90x10^5 cells/mL. The reference sample was the yeast strain BY4741, diluted 1:100 and prepared as mentioned above, without stain. A gating was applied in the channel FSC versus SSC to ensure there were no interferences from excess stain or other residue particles. Data from FL1, green channel – DCFDA and CFDA; and FL3, red channel – propidium iodide, was collected. Results were analyzed using FlowJo software [130].

3.Results

3.1. Yeast strains control vs glycated - Similar but not Equal

To assess if the single-gene mutated strains (Δglo1, Δglo2 and Δgre3) or the glycated conditions (yeast strains grown in enhanced glucose medium) could be distinguished from one another and from the wild-type (BY4741), we performed several phenotypical and physiological assays to evaluate the characteristics of the strains, specially BY4741, wild-type, and Δglo1, single gene mutated yeast on glyoxalase I.

We choose to continue this study only with BY4741 and Δglo1 since it has been reported that the glyoxalase system is the main mechanism of detoxification of methylglyoxal. Moreover, the enzyme glyoxalase 1 is the major enzyme of this pathway, being down-regulated in many pathologies (ageing, hyperglycemia, diabetes and Alzheimer's disease) [25, 41–44].

The single gene mutated strains Δglo1, Δglo2 and Δgre3 have deletions in genes related to the catabolism of methylglyoxal (glyoxalase I, glyoxalase II, and aldose reductase, respectively).

3.1.1. Phenotypical Characteristics

3.1.1.1. Growth Curves

We first investigated yeast growth (Fig. 3.1) to verify if we could distinguish the strains by comparison of their growth rate.

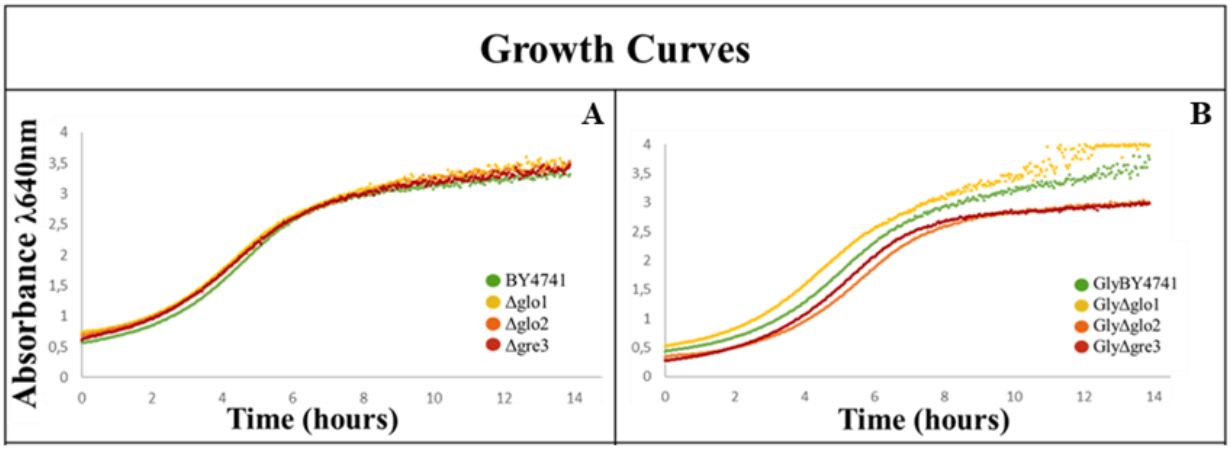

Figure 3.1 Growth Curves. A - Growth comparison between yeast strains (BY4741, Δglo1, Δglo2 and Δgre3) grown in normal glucose medium. **B-** Growth comparison between yeast strains (BY4741, Δglo1, Δglo2 and Δgre3) grown in excess glucose medium (glycated conditions, Gly).

After smoothing of the growth curves by Savitzky Golay method, the curves where derivatized. The growth rate is the highest first order derivative, located at the inflexion point (Fig. 3.2).

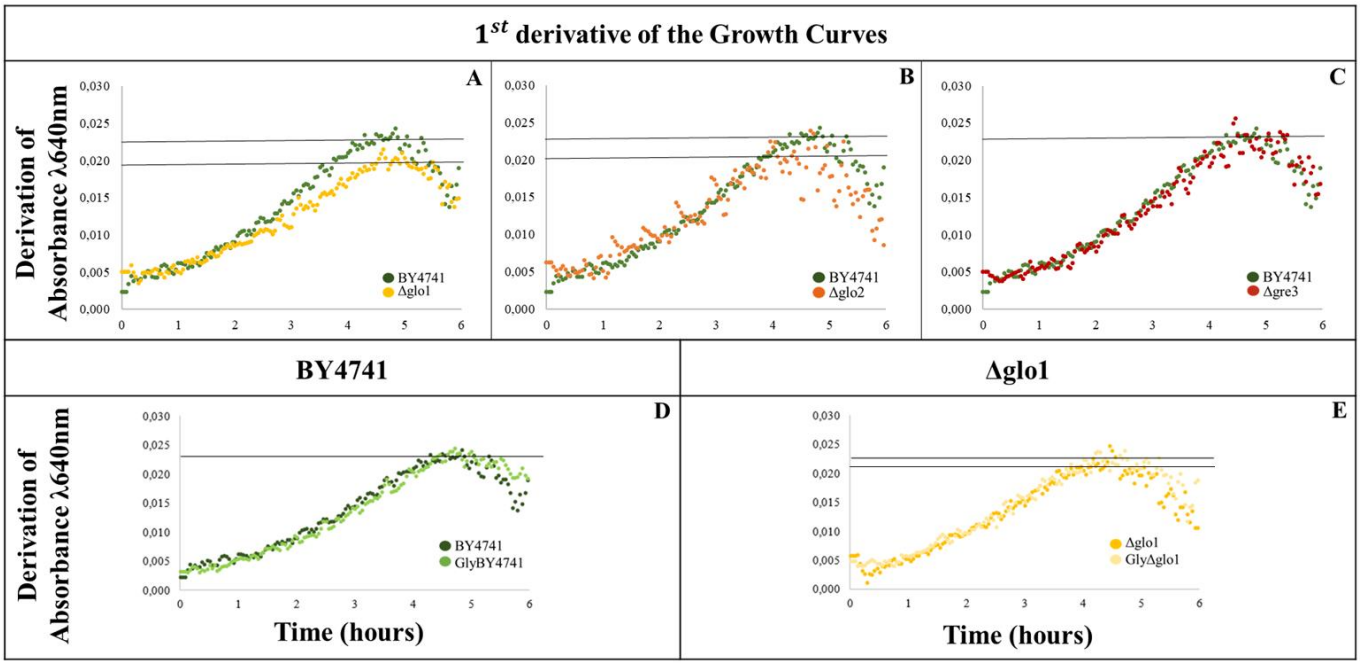

Figure 3.2 1st derivative of the Growth Curves. A, B, C – Smoothed 1st derivative growth curve comparison between yeast strains (Δglo1, Δglo2 and Δgre3, respectively) grown in normal medium and the wild type strain BY4741. **D** – 1st derivative growth curve comparison between control BY4741 and BY4741 grown in excess glucose medium, glycated conditions (GlyBY4741). **E** – 1st derivative growth curve comparison between control Δglo1 and Δglo1 grown in excess glucose medium, glycated conditions (GlyΔglo1).

The growth rates between the single-gene mutated yeast strains (Δglo1, Δglo2 and Δgre3) and wild type (BY4741); and between the control and glycated yeast were then statistically analyzed by two tailed t-test α = 0.05 (Table 1).

Table 3.1 1st derivative of the Growth Curves. Two tailed t-test α = 0.05 statistical analysis of the highest first order derivative, located at the inflexion point, between the single-gene mutated yeast strains (Δglo1, Δglo2 and Δgre3) and wild type (BY4741); and between the control and glycated conditions of these yeast strains. The values underlined represent the p-value values that are inferior to the α, meaning these results are statistically different from the control BY4741.The results are a mean ±STD from three biological independent experiments.

BY4741		**Δglo1**		**Δglo2**		**Δgre3**	
Control	**Glycated**	**Control**	**Glycated**	**Control**	**Glycated**	**Control**	**Glycated**
0.0244	0.0230	0.0218	0.0230	0.0220	0.0226	0.0233	0.0234
T-test (α=0.05) Control BY4741 *vs* Δ		**<u>0.006</u>**		**<u>0.012</u>**		0.212	
0.717		**<u>0.036</u>**		0.390		0.953	

From this analysis we observed that the growth rates of the single-gene mutated yeast strain Δgre3 cannot be statistically distinguished from BY4741, being Δglo1 and Δglo2 the mutations that seem to significantly affect the growth rate, having a lower growth rate than BY4741. However, when Δglo1 is grown in glucose enhanced medium it seems to have a higher growth rate than when grown in normal medium. The other yeast strains (BY4741, Δglo2 and Δgre3) don´t appear to have any difference in the growth-rate between the non-glycated and glycated conditions.

3.1.1.2. Morphology

For the cell morphology analysis, we used Flow Cytometry to assess if the dimension and complexity of the cells was maintained between the single-gene mutated yeast strain (Δglo1) and wild type (BY4741); and between the control and glycated conditions. Data was analyzed in the FlowJo software as a density plot, which displays two parameters (in this case, SSC – cell complexity *vs* FSC – cell dimension) as a frequency distribution. Color was used to code the different frequencies of events in the density plot, being the color red the highest density of events and blue the lowest [131], (Fig. 3.3).

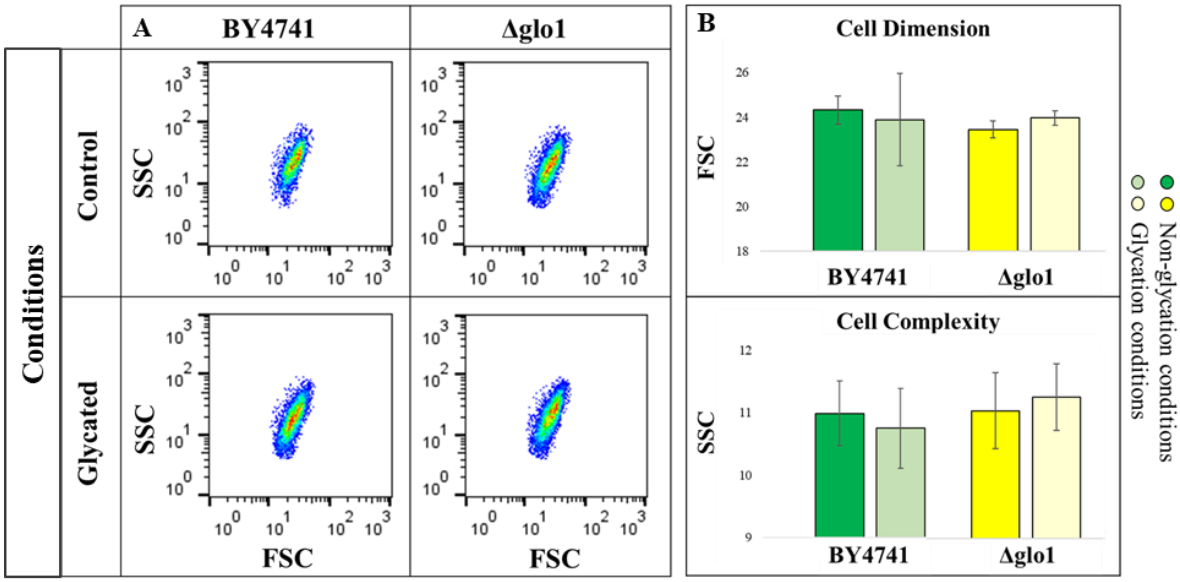

Figure 3.3 Cell's Morphology. A – Flow cytometry density plot (SSC vs FSC) of BY4741 and Δglo1, non-glycated (control) and glycated growth conditions. **B** – Geometric mean values of the frequency of the events for FCS (cell dimension) and SSC (cell complexity). Error bars represent ±STD from three biological independent experiments and three technical replicates.

The geometric mean values of the frequency of the events for FCS (cell dimension) and SSC (cell complexity) were then statistically analyzed by Two tailed t-test α = 0.05 (Table 2).

Table 3.2 Cell's Morphology. Two tailed t-test α = 0.05 statistical analysis of the frequency of the events for FCS (cell dimension) and SSC (cell complexity) between yeast strain BY4741 and Δglo1 and between the control and glycated condition of this yeast strains. The results are a mean ±STD from three biological independent experiments and three technical replicates.

FSC (cell dimension)				**SSC (cell complexity)**			
BY4741		**Δglo1**		**BY4741**		**Δglo1**	
Control	**Glycated**	**Control**	**Glycated**	**Control**	**Glycated**	**Control**	**Glycated**
24.32	23.88	23.44	23.95	10.98	10.75	11.05	11.27
T-test (α=0.05)							
0.0540				0.822			
0.436		0.060		0.519		0.496	

From this analysis we observed that the cell complexity and dimension of the single-gene mutated yeast strain Δglo1 cannot be statistically distinguished from BY4741. Nor the glycated and non-glycated growth conditions could be distinguished from one another in any of the yeast strains.

Next, to assess the morphology of the cell's nucleus we used fluorescence microscopy to detect the fluorescence of the stain Hoechst, which binds to the minor groove of the DNA with a preference for sequences rich in adenine and thymine. Using Fiji: ImageJ software we measured the shape descriptors, Circularity ($4\pi \times \frac{\text{area}}{\text{perimeter}^2}$ – a value of 1 indicates a perfect circle, as the value approaches 0, it indicates an increasingly elongated shape; captures perimeter smoothness); Aspect Ratio ($\frac{\text{major axis}}{\text{minor axis}}$ – higher values indicates an ellipse shape); Roundness ($4 \times \frac{\text{area}}{\pi \times \text{major axis}^2}$ – indicates a value of Circularity correlated with Aspect Ratio; overall shape); and Solidity ($\frac{\text{area}}{\text{convex area}}$ – lower values indicate a convex shape) (Fig.3.4) [129, 132].

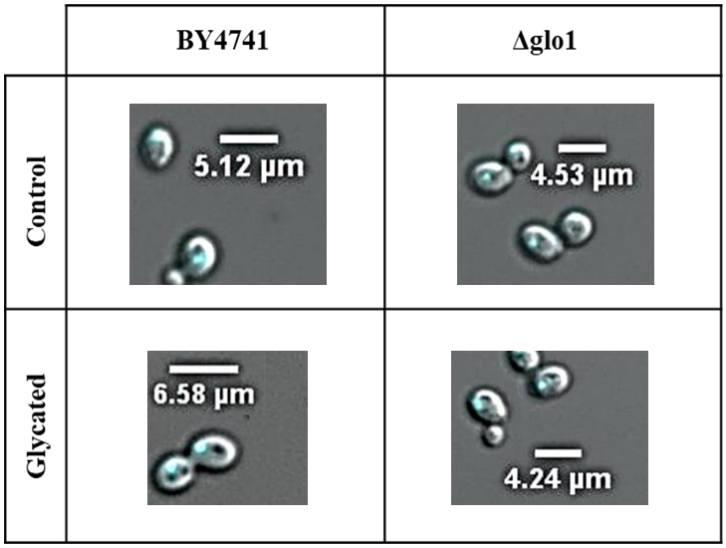

Figure 3.4 Nucleus Morphology. Fluorescence microscopy images of samples (BY4741 in non-glycated and glycated conditions; Δglo1 in non-glycated and glycated conditions) stained with Hoechst.

The values of the shape descriptors were then statistically analyzed by two tailed t-test $\alpha = 0.05$ (Table 3).

Table 3.3 Nucleus Morphology. Two tailed t-test α = 0.05 statistical analysis of the values of the shape descriptors between the yeast strain BY4741 and Δglo1 and between the control and glycated conditions of these yeast strains. The results are a mean ±STD from three biological independent experiments.

	Nucleus Morphology			
	BY4741		**Δglo1**	
	Control	**Glycated**	**Control**	**Glycated**
Circularity	0.940	0.932	0.947	0.947
Aspect Ratio	1.380	1.461	1.382	1.382
Roundness	0.749	0.728	0.748	0.747
Solidity	0.883	0.868	0.873	0.869
T-test (α=0.05)				
Circularity	0.438			
	0.660		0.992	
Aspect Ratio	0.957			
	0.321		0.998	
Roundness	0.972			
	0.406		0.961	
Solidity	0.105			
	0.116		0.577	

From this analysis we observed that the nucleus morphology of the single-gene mutated yeast strain Δglo1 cannot be statistically distinguished from BY4741. Nor the glycated and non-glycated growth conditions could be distinguished from one another in any of the yeast strains.

3.1.1.3. Viability & Vitality

For the viability and vitality analysis between the single-gene mutated yeast strain (glo1) and wild-type (BY4741); and between the control and glycated conditions, we used Flow Cytometry analysis using CFDA and propidium iodide to stain the samples. CFDA is a membrane-permeant dye that fluoresces after being processed by intracellular esterases. Once processed the stain requires cell membrane integrity to retain fluorescence within the cell, having fluorescence in the green channel (FL1). Therefore, CFDA can be used to monitor if cells are metabolically active, which translates to their vitality [133]. Propidium iodide is cell impermeable and is excluded from viable cells. Thus, propidium iodide can monitor the cells viability, having fluorescence in the red channel (FL3), [134].

To perform this assay, we analyzed the data in the FlowJo software as a density plot, FL3 *vs* FL1. In the left upper corner (Q2) we observe the frequency of dead cells; in the right upper corner (Q1) we observe the frequency of cells with a compromised membrane; in the left down corner (Q3) we observe the frequency of cells that are viable but don't have conditions to be cultured, having membrane integrity but only residual metabolic activity; in the right down corner (Q4) we observe the frequency of live cells (Fig. 3.5 A).

We also used fluorescence microscopy for the analysis of cell viability, in which samples were stained with Live-or-Dye. This stain only enters dead cells that have a compromised membrane integrity and upon entering it covalently labels free amines on intracellular proteins. Using Fiji: ImageJ software we were able to count the total number of cells and dead cells and therefore calculate the viability ($\frac{total\ number\ of\ cells - dead\ cells}{total\ number\ of\ cells} \times 100$) (Fig.3.5 B), [129, 135].

Calcofluor-white was used to easily identify the cells, being a stain that binds to cellulose and chitin in cell walls [136].

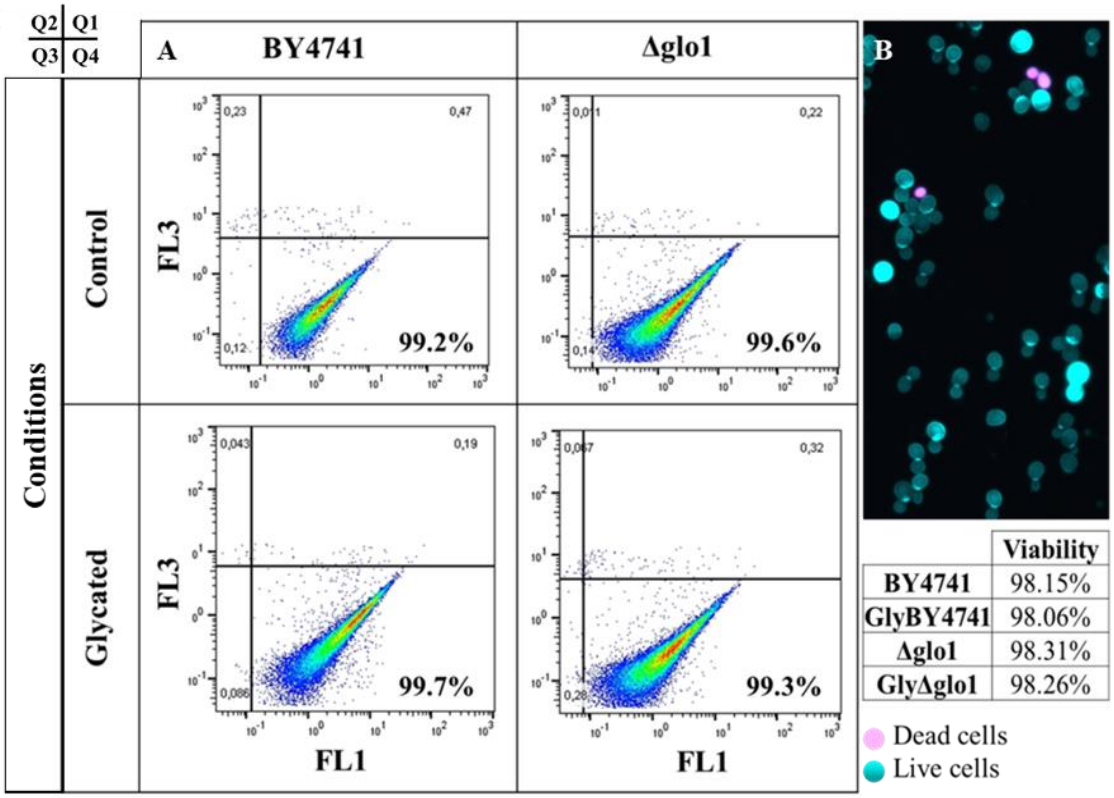

Figure 3.5 Viability and Vitality of the cells. A – Flow Cytometry analysis of viability and vitality, FL1 vs FL3, of BY474, control and glycated growth conditions, and Δglo1, control and glycated growth conditions. FL1 represents the fluorescence of the green channel (CFDA); FL3 represent the fluorescence of the red channel (propidium iodide). **B** – Representative image of the Fluorescence Microscopy results of viability of samples stained with Live-or-Dye. The results are a mean ±STD from three biological independent experiments.

From this analysis we observed that both the viability and vitality are not influenced by neither the single-gene mutations nor the glycation growth conditions, having always a high viability and vitality above 98%.

3.1.2. Physiological Characteristics

3.1.2.1. Mitochondrial Activity

To compare the mitochondrial activity between the single-gene mutated yeast strain (glo1) and wild-type (BY4741); and between the control and glycated conditions, we used Flow Cytometry analysis using Rhodamine 123 to stain the samples. Rhodamine 123 is positively charged fluorochrome at physiological pH which selectively stains mitochondria in living cells, depending on the mitochondrial membrane potential. Thus, rhodamine, is a useful probe for monitoring the abundance and activity of mitochondria, having fluorescence in the green channel (FL1) [137–139].

To perform this assay, we analyzed the data in the FlowJo software as a histogram, FL1 *vs* count. A histogram is a single parameter plot where the x-axis (FL1) represents the fluorescence intensity in a given channel, the y-axis (count) represents the number of events which have the same value of fluorescence intensity. More intense signals are displayed to the far right of the x-axis while dimmer signals appear in the left side [131].

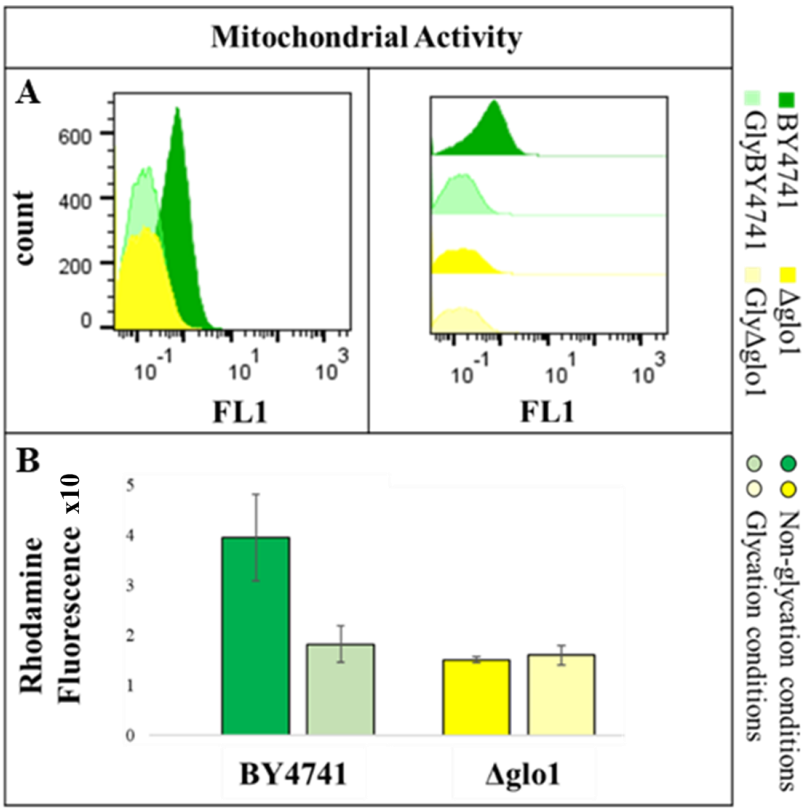

Figure 3.6 Mitochondrial Activity. A – Flow Cytometry analysis of mitochondrial activity, FL1 vs count, of BY474, control and glycated conditions, and Δglo1, control and glycated conditions. FL1 represents the fluorescence intensity of the green channel (Rhodamine); count represents the number of events of a given fluorescence value. **B** – Geometric mean values of the frequency of the events for Rhodamine fluorescence (mitochondrial activity). Error bars represent ±STD from three biological independent experiments and three technical replicates.

The geometric mean values of the frequency of the events for FL1 (Rhodamine fluorescence, mitochondrial activity) were then statistically analyzed by two tailed t-test $\alpha = 0.05$ (Table 4).

Table 3.4 Mitochondrial Activity. Two tailed t-test $\alpha = 0.05$ statistical analysis of the geometric mean values of the frequency of the events for FL1 (Rhodamine fluorescence, mitochondrial activity – original values) between the yeast strain BY4741 and Δglo1 and between the control and glycated condition of these yeast strains. The results are a mean ±STD from three biological independent experiments and three technical replicates.

Mitochondrial Activity			
BY4741		**Δglo1**	
Control	**Glycated**	**Control**	**Glycated**
0.394	0.182	0.151	0.160
T-test (α=0.05)			
Control	1.50×10^{-4}		
1.66×10^{-4}		0.27	
Glycated	0.25		

Mitochondrial activity of the yeast strains BY4741 and Δglo1 is statistically different. The single-gene mutated yeast strain Δglo1 has a lower mitochondrial activity than its wild-type counterpart. Nevertheless, between glycated conditions of the yeast strain Δglo1 we observe that the mitochondrial activity is maintained. In contrast to the mitochondrial activity of BY4741 glycated and non-glycated, where we observe that when glycated BY4741 has a lower activity. It is also worth to note that there seems to be no statistical difference between the mitochondrial activity of glycated BY4741 and glycated Δglo1.

3.1.2.2. ROS Accumulation

For the analyzes of the ROS accumulation between the single-gene mutated yeast strain (Δglo1) and wild-type (BY4741); and between the control and glycated conditions, we used Flow Cytometry analysis using DCFDA to stain the samples. DCFDA is a fluorogenic dye that measures hydroxyl, peroxyl and other ROS within the cell. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by flow cytometry in the green channel (FL1), [140].

Data was analyzed in the FlowJo software as a histogram, FL1 *vs* count.

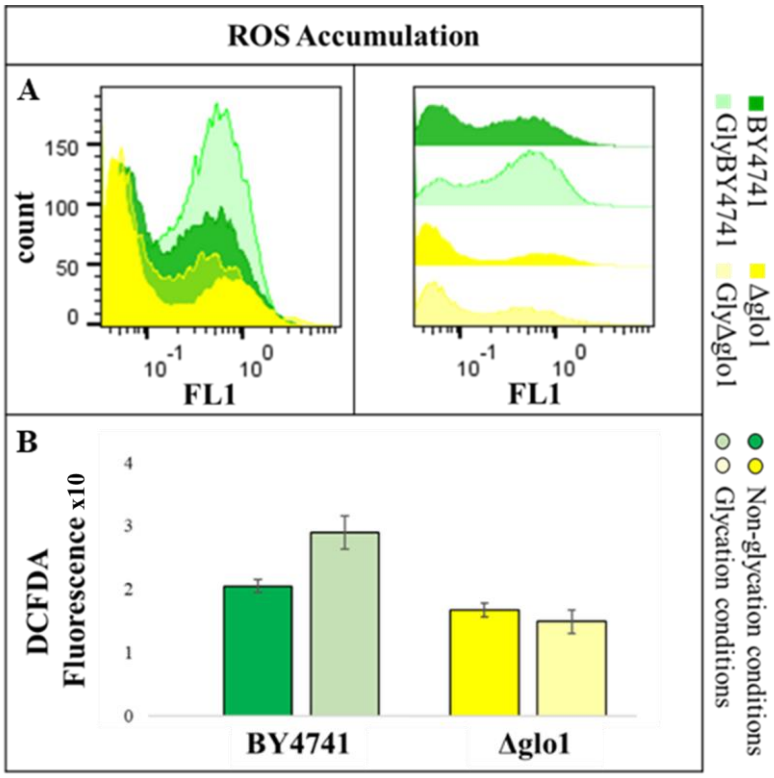

Figure 3.7 ROS Accumulation. A – Flow Cytometry analysis of ROS accumulation, FL1 vs count, of BY474, control and glycated conditions, and Δglo1, control and glycated conditions. FL1 represents the fluorescence of the green channel (DCFDA); count represents the number of events of a given fluorescence value. **B** – Geometric mean values of the frequency of the events for DCFDA fluorescence (ROS accumulation). Error bars represent ±STD from three biological independent experiments and three technical replicates.

The geometric mean values of the frequency of the events for FL1 (DCFDA fluorescence, ROS accumulation) were then statistically analyzed by two tailed t-test $\alpha = 0.05$ (Table 5).

Table 3.5 ROS Accumulation. Two tailed t-test $\alpha = 0.05$ statistical analysis of the geometric mean values of the events for FL1 (DCFDA fluorescence, ROS accumulation – original values) between the yeast strain BY4741 and Δglo1 and between the control and glycated condition of these yeast strains. The results are a mean ±STD from three biological independent experiments and three technical replicates.

ROS accumulation			
BY4741		**Δglo1**	
Control	**Glycated**	**Control**	**Glycated**
0.205	0.290	0.167	0.149
T-test (α=0.05)			
2.96×10^{-6}			
2.04×10^{-5}		0.35	

Since DCFDA fluorescence is dependent on esterase's activity we then analyzed the fluorescence of CFDA of the samples in the green channel (FL1), which gives us a dependent measure of the esterase's activity after being processed by these enzymes. This way we can better evaluate the true significance of DCFDA fluorescence results.

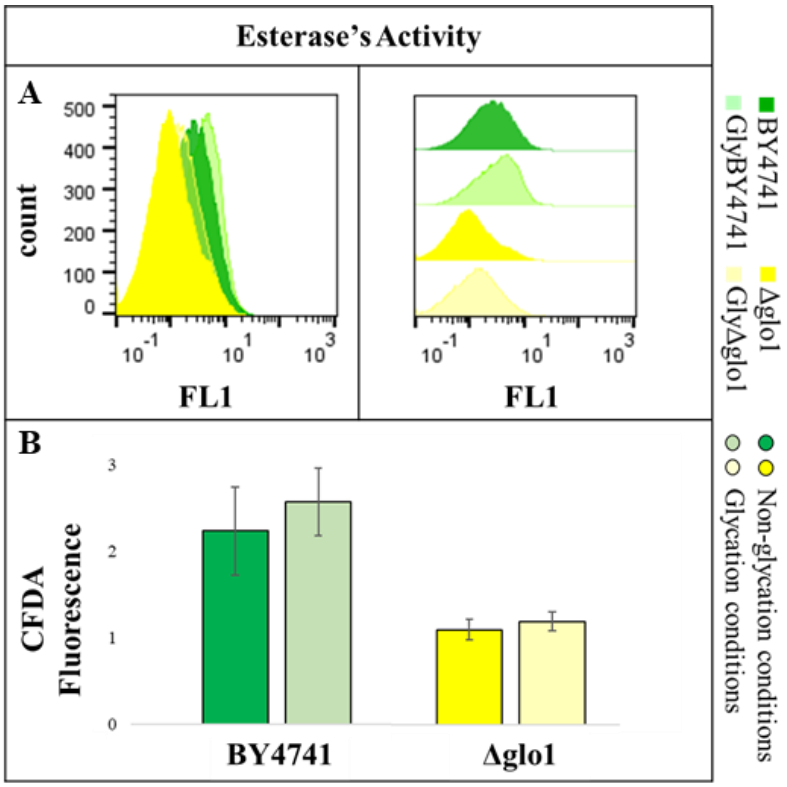

Figure 3.8 Esterase's Activity. A – Flow Cytometry analysis of esterase's activity, FL1 vs count, of BY474, control and glycated conditions, and Δglo1, control and glycated conditions. FL1 represents the fluorescence of the green channel (CFDA); count represents the number of events of a given fluorescence value. **B** – Geometric mean values of the frequency of the events for CFDA fluorescence (esterase's activity). Error bars represent ±STD from three biological independent experiments and three technical replicates.

The geometric mean values of the frequency of the events for FL1 (CFDA fluorescence, esterase's activity) were then statistically analyzed by Two tailed t-test $\alpha = 0.05$ (Table 6).

Table 3.6 Esterase's Activity. Two tailed t-test $\alpha = 0.05$ statistical analysis of the geometric mean values of the events for FL1 (CFDA fluorescence, esterase's activity) between the yeast strain BY4741 and Δglo1 and between the control and glycated condition of these yeast strains. The results are a mean ±STD from three biological independent experiments and three technical replicates.

Esterase's Activity			
BY4741		**Δglo1**	
Control	**Glycated**	**Control**	**Glycated**
2.238	2.572	1.155	1.197
T-test (α=0.05)			
0.014			
0.330		0.348	

Esterase's activity of the yeast strains BY4741 and Δglo1 are statistically different. The single-gene mutated yeast strain Δglo1 has a lower esterase's activity than its wild-type counterpart. Nevertheless,

between the glycated and non-glycated conditions of these yeast strains we observe that the esterase's activity is similar.

With these results we can now better compare ROS accumulation between the different strains and growth conditions.

The accumulation of ROS differs from yeast strain (BY4741 and Δglo1) and from glycated condition (control and glycated growth medium). However, we cannot compare the accumulation of ROS between non-glycated BY4741 and Δglo1, due to the fact that the esterase's activity of Δglo1 is lower than that of BY4741, so even though the accumulation of ROS in the Δglo1 strain seems statistically lower than in BY4741 strain this will not be representative of the correct values, since Δglo1 would have a bigger accumulation of ROS than that we verify if the esterase activity was identical to BY4741 strain. Nevertheless, between the glycated and non-glycated conditions of these yeast strains the esterase's activity is maintained, so a comparison of ROS accumulation is possible. We observe that when glycated BY4741 has a higher accumulation of ROS. In contrast, in the yeast strain Δglo1 we observe that the accumulation of ROS is not influenced by growth in excess glucose medium.

4.Discussion

4.1. Phenotypical Characteristics

4.1.1. Growth Curves

Although protein glycation has been primary associated with complex organisms and long-lived proteins exposed to high levels of glycation agents, this modification also affects organisms like yeast.

Yeast cells evolved to use D-glucose efficiently; for this, they have a very high glycolytic flux and consequently an unavoidably high methylglyoxal production rate. Therefore, throughout evolution, these cells have developed defense mechanisms against glycation, such as the elimination of methylglyoxal, which is the primary agent of glycation in vivo.

When growing the yeast strains in enriched glucose YPD medium (250 mM D-glucose) we ought to see phenotypical or/ and physiological differences due to glycation. Moreover, we are testing this condition not only in a wild type strain (BY4741), but also in null mutant yeast strains for genes involved in methylglyoxal catabolism (Δglo1, Δ glo2, and Δgre3). These null mutants, as they have fewer defenses against methylglyoxal would be expected to be more glycated than wt (BY4741).

By analyzing the growth rate of these yeast strains in non-glycated and glycated conditions, we observed that the wt (BY4741) and the null mutant yeast strain Δgre3 have a similar growth rate. Furthermore, we also observed that increased glycation doesn't affect their growth rate. The similar growth rate of the null mutant strain Δgre3 comparative to the wt could be explained by an enhanced activity of the glyoxalase system, since the aldose reductase system functions as a secondary detoxifying pathway [27, 28, 141, 142], with no consequences in the growth rate.

The appropriate control of cell growth rate is central to the long-term survival of species, particularly microorganisms, like yeast. A fast growth rate is a competitive advantage when environmental conditions are favorable, while a slower growth may allow survival under stress conditions.

Glycation and therefore, formation of AGEs, exert profound effects on proteins, DNA and lipids, causing intracellular oxidative stress and loss of function and structure [8, 19, 27, 60, 143, 144]. However, as mentioned, these effects seem not suffice to change the growth rate of the previously mentioned strains. Nonetheless, this is not true for the null mutants of the glyoxalase system, which have a lower growth rate than the wt. These mutant yeasts could be controlling the uptake of glucose to have less methylglyoxal and therefore less glycation and stress, resulting in a lower growth rate.

Our results are in agreement with previous reports concluding that the glyoxalase system, an ubiquitous enzymatic pathway, is the main detoxifying system for methylglyoxal and other reactive dicarbonyl compounds in eukaryotic cells, thereby playing a major role in the cellular defense against glycation and oxidative stress. Moreover, it has been showed that, when glyoxalase I is eliminated, the impact of glycation is higher than that of the elimination of glyoxalase II, suggesting that glyoxalase I plays a major role on the glyoxalase system [27, 28, 141, 142]. Interestingly when Δglo1 is further glycated we observed an increase in the growth rate.

4.1.2. Morphology

The yeast biological membrane is composed primarily of phospholipids, glycosphingolipids, ergosterol, and proteins [145–148].

Phospholipids are the primary structural component of the membrane and are essential to the viability of the cell. Their asymmetry of distribution in the yeast cell membrane is essential to maintain membrane surface potential and membrane protein activity [145–148].

The proteins present in the membrane are embedded integrally or peripherally in the membrane and are responsible for the well-functioning of the cell, being involved with nutrient transport, ionic and pH homeostasis, and cellular signaling and transduction [145–148].

The same principles are applied in the membranes of higher eukaryotic cells, as the glycerophospholipids found on yeast are similar to those. Nevertheless, in contrast to higher eukaryotic cells, in which cholesterol is the most abundant sterol, the yeast plasma membrane contains mainly ergosterol and minor amounts of zymosterol, both precursors for cholesterol [145, 148].

In vitro studies on the Maillard reaction have demonstrated that Amadori product autoxidation may lead to the formation of reactive oxygen species. The reactive oxygen species are likely to cause peroxidation of unsaturated fatty acid residues in membrane lipids, which propagate free radical reactions and lead to phosphatidylcholine hydroperoxide (PCOOH) formation. Thus, leading to disordered cellular integrity with angiogenic stimulation, which has been linked to atherogenesis, diabetes, and aging [149, 150].

Furthermore, any protein can be affected by glycation but long-lived proteins, such as matrix and structural proteins are more prone to form AGEs, being already reported that membrane fluidity is related to the extent of glycation of these proteins [146, 151–154].

These factors come into play when considering the membrane morphology of yeast. So, we performed an assay to test whether the morphology of the yeast was maintained during glycation.

Analyzing cell dimension and cell complexity we observed that glycation appears to not have influence in these characteristics despite of the nefarious effects of this process. Moreover, when exploring further into the cell and examining the nucleus membrane morphology, we concluded that, once again, the glycated yeast could not be distinguished from the non-glycated control yeast.

4.1.3. Viability & Vitality

AGEs can induce cell death through various pathways that involve activation of transcription factors. Such as, the NF-κB cascade where the interaction between AGEs and receptor for AGEs (RAGE) activates transcription factor NF-κB, contributing to cell proliferation by regulating genes that promote the growth and survival of cells, being involved in tumorigenic response and inflammatory responses [155–157]. The NF-AT cascade, where AGE acts through its cell surface receptor and degranulates vesicular contents, including interleukin-8, starting the cascade, which will culminate in cell death via activation of cysteine proteases, caspases that execute apoptosis [155–157]. And also, the AP-1 cascade, where AGEs increase the phosphorylation of p38 MAPK (mitogen-activated protein kinase) and JNK (c-Jun N-terminal kinase), beginning signaling pathways that regulate a variety of biological processes through multiple cellular mechanisms. This cascade also leads to increased expression of pro-apoptotic proteins and decreased expression of anti-apoptotic proteins [158–161].

Nevertheless, a novel contribution of AGEs has emerged in recent years, it has been shown that AGEs can induce autophagosome formation and consequently autophagy. This process is known to partially protect cells from death by removing old organelles, misfolded and non-functional proteins, and damaged molecules. In fact, autophagy has been often found to be impaired in diseases, such as diabetes and many neurodegenerative disorders. Therefore, cell survival is a balance between autophagy and cell apoptosis [155–157]. This balance has been hypothesized to depend on the ability of autophagy to clear ROS and to alleviate damaged mitochondria. A study found that induction of autophagy, by treating cells with AGEs for less than 12h, was accompanied by a decrease in both ROS levels and mitochondrial damage [156].

However, in our studies we have reported that despite growing yeast cells for 18h in enriched glucose YPD medium, which promotes glycation and consequently, AGEs formation, the viability and vitality of the yeast mutants or growth conditions seem to not be influenced by these factors, having all a viability and vitality above 98%. This could possibly be responsible by the protective role of autophagy, that can be more enhanced than apoptosis, even though the cultures where exposed to AGEs for more than 12h. Acquired resistance to glycation stress could be a key factor on why this happens, since the cultures were grown from the start in a rich glucose medium, or in case of the mutant Δglo1 grown in standard medium, it could have adapted to cope with the glycation stress induced by the mutation. Yet, it is worth to note that a shift to Q3 (frequency of cells that are viable but don't have conditions to be cultured, having membrane integrity but not being metabolically active) appears to happen in the wt grown in glycation inducing medium and, even more pronounced, in the null mutant Δglo1 grown in either one of the mediums (Fig. 3.5 A). This cell state, also called VBNC (viable but not culturable) has been exhaustively studied in bacteria. In contrast, it has received much less attention in other microorganisms. Nevertheless, the existence of a VBNC-like state has been suggested for yeast *Saccharomyces cerevisiae* [162].

As inhabitants of a dynamic environment, microorganisms respond to changes and environmental uncertainty by being able to adapt to certain physical and chemical stresses. Survival mechanisms are activated following the detection of these shifts and employ a complex adaptive response that leads to a state of tolerance and thus survival under sub-optimal conditions. When the environmental conditions threaten their survival or prevent them from living in optimal conditions, the cells are described as stressed [162–166].

To tackle this instability, many microorganisms maintain subpopulations with the capability to enter a temporary state of dormancy during which cells exhibit reduced growth rates and metabolic demand. So, different physiological states have been described between the unstressed state and death: viable and culturable; injured, with a compromised membrane; viable but non culturable and dead. These physiological adaptations require a variable response time depending on the intensity and abruptness of exposure to the stress-inducing factor [162–166].

The VBNC state is characterized by an incapacity of the cells to grow on culture media, even though they are still viable and maintain a measurable metabolic activity, having the cytoplasm molecularly crowded and spatially organized and an internal homeostasis guaranteed [162]. When the environment becomes permissive again, dormant cells can then resuscitate and subsequently regain growth. The VBNC population occurs during exposure to a stressful condition as a method of survival and many factors can induce entry into this state, such as, temperature; nutrient concentration; salinity; osmotic pressure; pH or in this case, stress induced by glycation [162–166]. Thus, even though the induced glycation stress was not enough to significantly affect the viability and vitality of the cells it appears that with prolonged exposure a shift towards the VBNC state would happen. These results may also point to a possible adaptation and increased resistance of the cultures to glycation stress.

4.2. Physiological Characteristics

4.2.1. Mitochondrial Activity & ROS Accumulation

Glycation mainly takes place intracellularly as α,β-dicarbonyls are produced during the breakdown of triose phosphates generated during glycolysis. As such, it can also occur within organelles like mitochondria [25].

In conditions such as, hyperglycemia, ageing, cancer, and neurodegeneration there is increased production of glycation precursors, such as methylglyoxal, leading to elevated levels of AGEs, both intracellularly and extracellularly, as well as increased levels of oxidative stress [167–169].

Oxidative stress is the redox state resulting from an imbalance between the generation and detoxification of ROS. This imbalance may be a consequence of mitochondrial dysfunction, as this condition increases ROS production [25, 144].

There are estimated to be approximately 1500 proteins in the mitochondrial proteome, seven of those proteins have, to date, been suggested as susceptible targets of advanced glycation and AGE residue formation [144]. These are Enoyl-CoA hydratase - involved in fatty acid *β*-oxidation [143]; Core protein I of complex III – involved in electron-transport activity [170]; Mitochondrial electron flavoprotein *β*-subunit – involved in electron transfer [171]; Cytochrome c_1 (part of the cytochrome bc_1 complex of complex III, a membrane-bound *c*-type cytochrome protein of mitochondria) - functions as an electron donor to cytochrome *c* [172]; Glutamate dehydrogenase [173]; Subunits of complex I and F_1-ATPase. These few, but crucial, proteins play an important role in maintaining a stable mitochondrial activity.

In our results we show how striking glycation can affect mitochondria. The wild-type yeast grown in normal glucose YPD medium has a much higher mitochondrial activity than any of the other conditions, which most likely suffer from mitochondrial dysfunction due to glycation. Furthermore, the effects of growing BY4741 in a glucose enriched medium seem to have the same level of mitochondrial activity reduction as the total inhibition of glyoxalase I. These results show that even though we cannot conclude if hyperglycemia reduces glyoxalase I activity we can infer that the end result for the mitochondria activity is analogous.

The glyoxalase system is one of the major detoxifying methods to remove methylglyoxal. Reduced activity of glyoxalase I has been reported as an effect of ageing and activation of RAGE, which further raises glycation levels, consequently leading to the formation of AGEs [28, 31, 43].

Normally mitochondrial dysfunction is accompanied by a rise in ROS levels, as proton and electron leak increase [55, 94, 97–100]. This goes into accordance with our data, since when the wild type strain is grown in a enriched glucose medium it appears to have a bigger ROS accumulation than its counterpart grown in normal conditions, with these data we can assume that mitochondrial dysfunction is taking place. Nevertheless, when comparing both conditions of the glyoxalase I mutant strain we see no difference in ROS levels. This data could suggest that the mutant yeast could have developed an enhanced activity of aldose reductase to cope with the absence of the primary detoxifying mechanism. Nonetheless, this adaptation is not sufficient to maintain normal mitochondrial activity.

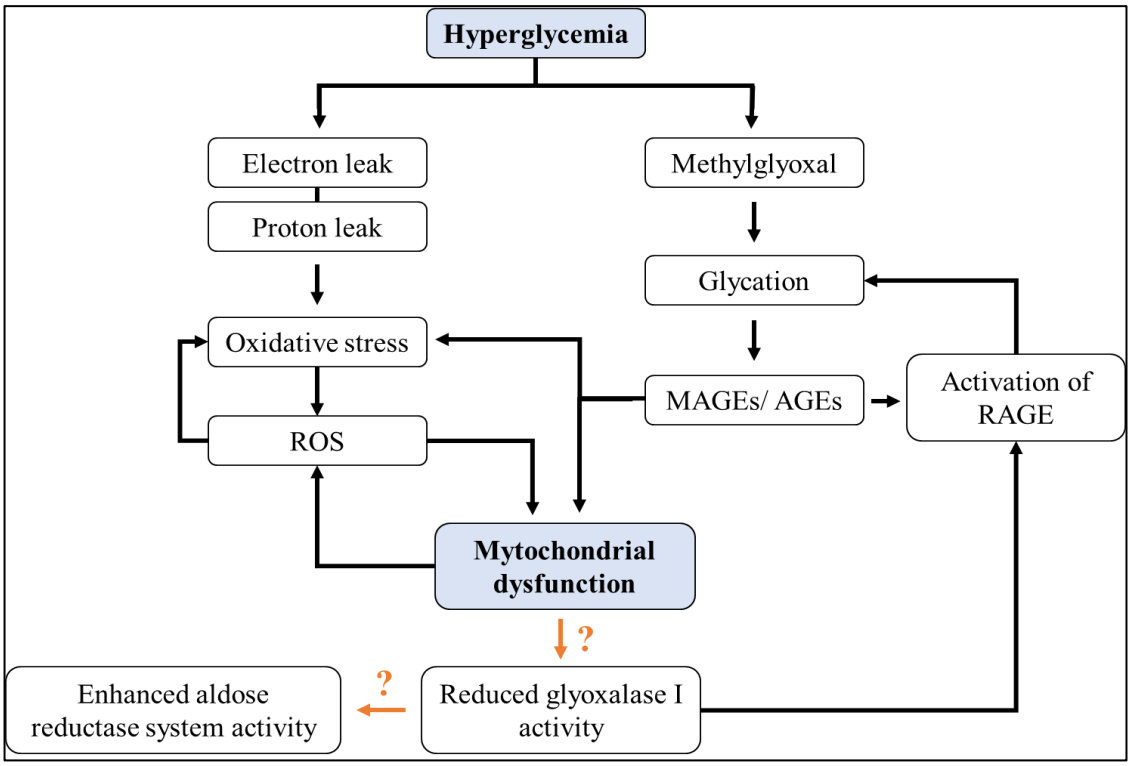

Figure 4.1 Schematic of the possible effects caused by hyperglycemia and glycation.

5.Concluding Remarks

Protein glycation has been associated to several human pathologies, such as diabetes and its complications, neurodegenerative disorder of the amyloid type and physiological processes as ageing. When proteins are glycated, they lose both structural and functional properties. These modified proteins are difficult to eliminate and tend to accumulate in a wide variety of tissues, causing the pathologies mentioned above [1, 29, 87, 169]. Therefore, it is imperative to continue to uncover more knowledge about glycation and its consequences *in vivo*. For this effect, in the present work, we investigated protein glycation by methylglyoxal in *S. cerevisiae*. This cellular model is easy to manipulate genetically, is a well-characterized eukaryotic cell with a wide collection of gene deletion mutants available that enable us to study different disease phenotypes [86, 120, 126], and has already been used to investigate protein glycation *in vivo* [60, 126, 127].

First, we assessed the impact of glycation on phenotypical characteristics, such as growth rate, morphology and viability. We observed that the growth rate decreased in the yeast mutants where the glyoxalase system was compromised. Specifically, the growth rate of glyoxalase I deficient mutant presented a bigger decrease than when glyoxalase II was deleted. This suggests that the glyoxalase system is of major importance in the elimination of methylglyoxal, with glyoxalase I being pivotal to a well-functioning mechanism [17, 27, 28, 31, 33, 174]. Interestingly, our results showed that when the yeast mutated for glyoxalase I was grown in enriched glucose medium the growth rate increased. Therefore, a subsequent study could be to assess what mechanisms are enhanced in glycation stress that could cause this reposition of the growth rate to normal values.

Regarding the cell and nucleus morphology we showed that glycation does not induce changes in the membranes, despite lipids being targets of glycation. It would be interesting to know if the lipid on these membranes were indeed affected by glycation, and if they were why did the global morphology was not affected, would it be possible for the lipid turnover to be sufficient to tackle the glycation damage or is another mechanism enhanced?

Our results also showed that the viability and vitality of the yeast cells affected by glycation stress was maintained. We suggested that these results were due to a shift in the balance of apoptosis and autophagy, with the former being enhanced. Where this to be true, future studies should sought to investigate whether the lipid and glucose metabolism are affected, as autophagy is pivotal to lipid storage and glucose is one of the regulators of autophagy, with mal-function of these metabolism being deeply connected with diabetes [155, 156, 175–177].

Then, we observed the impact of glycation on mitochondria, specifically on mitochondrial activity and ROS production. With this study we wanted to correlate glycation stress with oxidative stress and how they interacted. We showed that glycation stress is devastating for mitochondria, with mitochondrial activity being drastically reduced. It would be interesting to assess if hyperglycemia can reduce glyoxalase I activity and if this reduced glyoxalase system activity enhances aldose reductase system, as ROS accumulation showed to be equal in yeast glyoxalase I mutated cells despite being grown in standard or enriched glucose medium. However, ROS accumulation increased when the wild type yeast cell was grown in hyperglycemic conditions, showing that oxidative stress is taking part on this process [85, 88, 144, 173, 174, 178].

With these results we conclude that glycation stress induces oxidative stress, possibly leading to a reduced glyoxalase system activity and an enhanced aldose reductase system activity, to cope with the

conditions of excess glucose. Nevertheless, it is safe to assume that despite of whichever mechanisms the cells enhance to survive hyperglycemia, these are not enough to put at bay oxidative stress and ROS accumulation, being both of this stress conditions deeply connected and affecting profoundly the biology of the cells. The characterization of the yeast cells' metabolome grown under normal and glycation conditions is clearly the future work to be followed, to understand these profound cellular modifications related to oxidative stress and ROS accumulation, caused by protein glycation.

This increased knowledge will be useful to improve therapeutic strategies and enable an improved early identification of many human disorders associated with glycation.

6.References

[1] Q. Zhang, J. M. Ames, R. D. Smith, J. W. Baynes, and T. O. Metz, "A Perspective on the Maillard Reaction and the Analysis of Protein Glycation by Mass Spectrometry : Probing the Pathogenesis of Chronic Disease research articles" no. Figure 2, pp. 754–769, 2009.

[2] J. M. Ames, "Control of the Maillard reaction in food systems" Trends Food Sci. Technol., vol. 1, no. C, pp. 150–154, 1990, doi: 10.1016/0924-2244(90)90113-D.

[3] J. E. Hodge, "The Amadori Rearrangement" Adv. Carbohydr. Chem., vol. 10, no. C, pp. 169–205, Jan. 1955, doi: 10.1016/S0096-5332(08)60392-6.

[4] M. Namiki, T. Hayashi, and S. Kawakishi, "Free radicals developed in the amino-carbonyl reaction of sugars with amino acids" Agric. Biol. Chem., vol. 37, no. 12, pp. 2935–2936, 1973, doi: 10.1080/00021369.1973.10861104.

[5] F. G. Njoroge and V. M. Monnier, "The chemistry of the Maillard reaction under physiological conditions: a review." Progress in clinical and biological research. 1989.

[6] M. E. Westwood and P. J. Thornalley, "Induction of synthesis and secretion of interleukin 1β in the human monocytic THP-1 cells by human serum albumins modified with methylglyoxal and advanced glycation endproducts" Immunol. Lett., vol. 50, no. 1–2, pp. 17–21, 1996, doi: 10.1016/0165-2478(96)02496-0.

[7] M. B. Johansen, L. Kiemer, and S. Brunak, "Analysis and prediction of mammalian protein glycation" Glycobiology, vol. 16, no. 9, pp. 844–853, 2006, doi: 10.1093/glycob/cwl009.

[8] P. Ulrich and A. Cerami, "Protein glycation, diabetes, and aging" Recent Prog. Horm. Res., vol. 56, pp. 1–21, 2001, doi: 10.1210/rp.56.1.1.

[9] J. Blackledge, "Originalarbeiten The Maillard reaction *in vivo*" no. December, 2015, doi: 10.1007/BF01910730.

[10] V. M. Monnier, D. R. Sell, S. Miyata, R. H. Nagaraj, P. Odetti, and A. Lapolla, "Advanced Maillard reaction products as markers for tissue damage in diabetes and uraemia: relevance to diabetic nephropathy" Acta Diabetol., vol. 29, no. 3–4, pp. 130–135, Sep. 1992, doi: 10.1007/BF00573477.

[11] M. J. McDonald, R. Shapiro, M. Bleichman, J. Solway, and H. F. Bunn, "Glycosylated minor components of human adult hemoglobin. Purification, identification, and partial structural analysis" J. Biol. Chem., vol. 253, no. 7, pp. 2327–2332, 1978.

[12] V. M. Monnier, D. R. Sell, S. Miyata, R. H. Nagaraj, and P. Odetti, "Diabetologica 9" pp. 130–135, 1992.

[13] P. Gkogkolou and M. Böhm, "Advanced glycation end products: Keyplayers in skin aging?" Dermatoendocrinol., vol. 4, no. 3, 2012, doi: 10.4161/derm.22028.

[14] G. Abbas, A. S. Al-Harrasi, H. Hussain, J. Hussain, R. Rashid, and M. I. Choudhary, "Antiglycation therapy: Discovery of promising antiglycation agents for the management of diabetic complications" Pharm. Biol., vol. 54, no. 2, pp. 198–206, Feb. 2016, doi: 10.3109/13880209.2015.1028080.

[15] T. W. C. Lo, M. E. Westwood, A. C. Mclellan, T. Selwood, and P. J. Thornalleys, "Binding and Modification of Proteins by Methylglyoxal under Physiological Conditions" 1994.

[16] M. E. Westwood and P. J. Thornalley, "Molecular Characteristics of Methylglyoxal-Modified Bovine and Human Serum Albumins . Comparison with Glucose-Derived Advanced Glycation Endproduct- Modified Serum Albumins" vol. 14, no. 5, 1995.

[17] P. J. Thornalley, “Pharmacology of Methylglyoxal : Formation , Modification of Proteins and Nucleic Acids , and Enzymatic Detoxification-A Role in Pathogenesis and Antiproliferative Chemotherapy” vol. 27, no. 4, pp. 565–573, 1996.

[18] A. Okado et al., “Induction of Apoptotic Cell Death by Methylglyoxal and 3-Deoxyglucosone in Macrophage-Derived Cell Lines” vol. 224, pp. 219–224, 1996.

[19] H. Zong, M. Ward, and A. W. Stitt, “AGEs , RAGE , and Diabetic Retinopathy” no. May, pp. 244–252, 2011, doi: 10.1007/s11892-011-0198-7.

[20] K. Diseases, “American Journal of Kidney Diseases” vol. 35, no. 3, pp. 365–380, 2000.

[21] E. Konova, S. Baydanoff, M. Atanasova, and A. Velkova, “Age-related changes in the glycation of human aortic elastin” vol. 39, pp. 249–254, 2004, doi: 10.1016/j.exger.2003.10.003.

[22] G. K. Pandey, “Elucidation of abiotic stress signaling in plants: Functional genomics perspectives” Elucidation Abiotic Stress Signal. Plants Funct. Genomics Perspect., vol. 1, no. January, pp. 1–415, 2015, doi: 10.1007/978-1-4939-2211-6.

[23] P. B. L. Pun and M. P. Murphy, “Pathological significance of mitochondrial glycation” International Journal of Cell Biology. 2012, doi: 10.1155/2012/843505.

[24] J. P. Richard, “Mechanism for the formation of methylglyoxal from triosephosphates” Biochem. Soc. Trans., vol. 21, no. 2, pp. 549–553, 1993, doi: 10.1042/bst0210549.

[25] P. B. L. Pun and M. P. Murphy, “Pathological significance of mitochondrial glycation” Int. J. Cell Biol., vol. 2012, 2012, doi: 10.1155/2012/843505.

[26] L. Mathias, “Linking TNF-induces dysfunciton and formation of methylglyoxal derived AGEs in endothelial cells” 2008.

[27] I. Allaman, M. Bélanger, and P. J. Magistretti, “Methylglyoxal, the dark side of glycolysis” Front. Neurosci., vol. 9, no. FEB, pp. 1–12, 2015, doi: 10.3389/fnins.2015.00023.

[28] P. J. Thornalley, “The glyoxalase system in health and disease” Mol. Aspects Med., vol. 14, no. 4, pp. 287–371, 1993, doi: 10.1016/0098-2997(93)90002-U.

[29] M. P. Kalapos, “Methylglyoxal in living organisms - Chemistry, biochemistry, toxicology and biological implications” Toxicol. Lett., vol. 110, no. 3, pp. 145–175, 1999, doi: 10.1016/S0378-4274(99)00160-5.

[30] D. L. Vander Jagt and L. A. Hunsaker, “Methylglyoxal metabolism and diabetic complications: Roles of aldose reductase, glyoxalase-I, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase” Chem. Biol. Interact., vol. 143–144, pp. 341–351, 2003, doi: 10.1016/S0009-2797(02)00212-0.

[31] P. J. Thornalley, “Glyoxalase I - Structure, function and a critical role in the enzymatic defence against glycation” Biochem. Soc. Trans., vol. 31, no. 6, pp. 1343–1348, 2003, doi: 10.1042/bst0311343.

[32] E. M. CROOK and K. LAW, “Glyoxalase; the role of the components.” Biochem. J., vol. 52, no. 3, pp. 492–499, Nov. 1952, doi: 10.1042/bj0520492.

[33] E. RACKER, “The mechanism of action of glyoxalase.” J. Biol. Chem., vol. 190, no. 2, pp. 685–696, 1951.

[34] G. L. Long and N. O. Kaplan, “D-lactate specific pyridine nucleotide lactate dehydrogenase in animals” Science (80-.)., vol. 162, no. 3854, pp. 685–686, 1968, doi: 10.1126/science.162.3854.685.

[35] E. A. Pratt, L. W. M. Fung, J. A. Flowers, and C. Ho, “Membrane-Bound D-Lactate

Dehydrogenase from Escherichia coli: Purification and Properties†" Biochemistry, vol. 18, no. 2, pp. 312–316, 1979, doi: 10.1021/bi00569a013.

[36] A. Wong et al., "Advanced glycation endproducts co-localize with inducible nitric oxide synthase in Alzheimer's disease" Brain Res., vol. 920, no. 1–2, pp. 32–40, 2001, doi: 10.1016/S0006-8993(01)02872-4.

[37] B. Kuhla, H. J. Lüth, D. Haferburg, K. Boeck, T. Arendt, and G. Münch, "Methylglyoxal, glyoxal, and their detoxification in Alzheimer's disease" Ann. N. Y. Acad. Sci., vol. 1043, no. 0, pp. 211–216, 2005, doi: 10.1196/annals.1333.026.

[38] R. Dringen, J. M. Gutterer, and J. Hirrlinger, "Glutathione metabolism in brain: Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species" Eur. J. Biochem., vol. 267, no. 16, pp. 4912–4916, 2000, doi: 10.1046/j.1432-1327.2000.01597.x.

[39] S. Willingham, T. F. Outeiro, M. J. DeVit, S. L. Lindquist, and P. J. Muchowski, "Yeast Genes that Enhance the Toxicity of a Mutant Huntingtin Fragment or α-Synuclein" Science (80-.)., vol. 302, no. 5651, pp. 1769–1772, 2003, doi: 10.1126/science.1090389.

[40] A. C. McLellan, P. J. Thornalley, J. Benn, and P. H. Sonksen, "Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications" Clin. Sci., vol. 87, no. 1, pp. 21–29, 1994, doi: 10.1042/cs0870021.

[41] L. Jiang et al., "Role of the Glyoxalase System in Alzheimer's Disease" Journal of Alzheimer's Disease, vol. 66, no. 3. IOS Press, pp. 887–899, 2018, doi: 10.3233/JAD-180413.

[42] M. Morcos et al., "Glyoxalase-1 prevents mitochondrial protein modification and enhances lifespan in Caenorhabditis elegans" Aging Cell, vol. 7, no. 2, pp. 260–269, Apr. 2008, doi: 10.1111/j.1474-9726.2008.00371.x.

[43] I. Allaman, M. Bélanger, and P. J. Magistretti, "Methylglyoxal, the dark side of glycolysis" Frontiers in Neuroscience, vol. 9, no. FEB. Frontiers Research Foundation, 2015, doi: 10.3389/fnins.2015.00023.

[44] D. Schumacher et al., "Compensatory mechanisms for methylglyoxal detoxification in experimental & clinical diabetes" Mol. Metab., vol. 18, pp. 143–152, Dec. 2018, doi: 10.1016/j.molmet.2018.09.005.

[45] P. R. Steinmetz, C. Balko, and K. H. Gabbay, "The Sorbitol Pathway and the Complications of Diabetes" N. Engl. J. Med., vol. 288, no. 16, pp. 831–836, 1973, doi: 10.1056/NEJM197304192881609.

[46] D. L. Vander Jagt, R. K. Hassebrook, L. A. Hunsaker, W. M. Brown, and R. E. Royer, "Metabolism of the 2-oxoaldehyde methylglyoxal by aldose reductase and by glyoxalase-I: Roles for glutathione in both enzymes and implications for diabetic complications" Chem. Biol. Interact., vol. 130–132, pp. 549–562, 2001, doi: 10.1016/S0009-2797(00)00298-2.

[47] D. L. V. Jagt, B. Robinson, K. K. Taylor, and L. A. Hunsaker, "Reduction of trioses by NADPH-dependent aldo-keto reductases. Aldose reductase, methylglyoxal, and diabetic complications" J. Biol. Chem., vol. 267, no. 7, pp. 4364–4369, 1992.

[48] M. Fournet, F. Bonté, and A. Desmoulière, "Glycation damage: A possible hub for major pathophysiological disorders and aging" Aging Dis., vol. 9, no. 5, pp. 880–900, 2018, doi: 10.14336/AD.2017.1121.

[49] Q. Ding and J. N. Keller, "Evaluation of rage isoforms, ligands, and signaling in the brain" Biochimica et Biophysica Acta - Molecular Cell Research, vol. 1746, no. 1. Biochim Biophys Acta, pp. 18–27, 30-Oct-2005, doi: 10.1016/j.bbamcr.2005.08.006.

[50] A. Raucci et al., "A soluble form of the receptor for advanced glycation endproducts (RAGE) is

produced by proteolytic cleavage of the membrane-bound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10)" FASEB J., vol. 22, no. 10, pp. 3716–3727, Oct. 2008, doi: 10.1096/fj.08-109033.

[51] J. Xue et al., "The receptor for advanced glycation end products (RAGE) specifically recognizes methylglyoxal-derived AGEs" Biochemistry, vol. 53, no. 20, pp. 3327–3335, May 2014, doi: 10.1021/bi500046t.

[52] A. R. Mir, M. Uddin, K. Alam, and A. Ali, "Methylglyoxal mediated conformational changes in histone H2A-generation of carboxyethylated advanced glycation end products" Int. J. Biol. Macromol., vol. 69, pp. 260–266, 2014, doi: 10.1016/j.ijbiomac.2014.05.057.

[53] H. Pageon, "Reaction of glycation and human skin: The effects on the skin and its components, reconstructed skin as a model" Pathol. Biol., vol. 58, no. 3, pp. 226–231, 2010, doi: 10.1016/j.patbio.2009.09.009.

[54] M. J. Roberts, G. T. Wondrak, D. C. Laurean, M. K. Jacobson, and E. L. Jacobson, "DNA damage by carbonyl stress in human skin cells" Mutat. Res. - Fundam. Mol. Mech. Mutagen., vol. 522, no. 1–2, pp. 45–56, Jan. 2003, doi: 10.1016/S0027-5107(02)00232-4.

[55] J. F. Turrens, "Mitochondrial formation of reactive oxygen species" J. Physiol., vol. 552, no. 2, pp. 335–344, 2003, doi: 10.1113/jphysiol.2003.049478.

[56] M. Yokota and Y. Tokudome, "The Effect of Glycation on Epidermal Lipid Content, Its Metabolism and Change in Barrier Function" Skin Pharmacol. Physiol., vol. 29, no. 5, pp. 231–242, Dec. 2016, doi: 10.1159/000448121.

[57] P. Maher, "Protein Glycation and the Proteasome: Role in Neurological Diseases and Aging."

[58] C. Nigro et al., "Methylglyoxal-glyoxalase 1 balance: the root of vascular damage" International Journal of Molecular Sciences, vol. 18, no. 1. MDPI AG, 18-Jan-2017, doi: 10.3390/ijms18010188.

[59] D. E. M. Maessen, C. D. A. Stehouwer, and C. G. Schalkwijk, "The role of methylglyoxal and the glyoxalase system in diabetes and other age-related diseases" Clinical Science, vol. 128, no. 12. Portland Press Ltd, pp. 839–861, 2015, doi: 10.1042/CS20140683.

[60] N. Ahmed, O. K. Argirov, H. S. Minhas, C. A. A. Cordeiro, and P. J. Thornalley, "Assay of advanced glycation endproducts (AGEs): Surveying AGEs by chromatographic assay with derivatization by 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate and application to Nε-carboxymethyl-lysine- and nε-(1-carboxyethyl)lysine-modified albumin" Biochem. J., vol. 364, no. 1, pp. 1–14, May 2002, doi: 10.1042/bj3640001.

[61] R. A. Gomes et al., "Protein glycation *in vivo*: functional and structural effects on yeast enolase" Biochem. J., vol. 416, no. 3, pp. 317–326, Dec. 2008, doi: 10.1042/BJ20080632.

[62] R. A. Gomes et al., "Protein glycation and methylglyoxal metabolism in yeast: Finding peptide needles in protein haystacks" FEMS Yeast Res., vol. 8, no. 1, pp. 174–181, Feb. 2008, doi: 10.1111/j.1567-1364.2007.00337.x.

[63] A. Goldin, J. A. Beckman, A. M. Schmidt, and M. A. Creager, "Advanced glycation end products: Sparking the development of diabetic vascular injury" Circulation, vol. 114, no. 6. Circulation, pp. 597–605, Aug-2006, doi: 10.1161/CIRCULATIONAHA.106.621854.

[64] P. Alavi, R. Yousefi, S. Amirghofran, H. R. Karbalaei-Heidari, and A. A. Moosavi-Movahedi, "Structural analysis and aggregation propensity of reduced and nonreduced glycated insulin adducts" Appl. Biochem. Biotechnol., vol. 170, no. 3, pp. 623–638, Jun. 2013, doi: 10.1007/s12010-013-0207-1.

[65] S. Chesne, P. Rondeau, S. Armenta, and E. Bourdon, "Effects of oxidative modifications induced

by the glycation of bovine serum albumin on its structure and on cultured adipose cells" Biochimie, vol. 88, no. 10, pp. 1467–1477, Oct. 2006, doi: 10.1016/j.biochi.2006.05.011.

[66] J. Chen et al., "Advanced glycation endproducts alter functions and promote apoptosis in endothelial progenitor cells through receptor for advanced glycation endproducts mediate overpression of cell oxidant stress" Mol. Cell. Biochem., vol. 335, no. 1–2, pp. 137–146, Feb. 2010, doi: 10.1007/s11010-009-0250-y.

[67] H. F. Bunn and P. J. Higgins, "Reaction of monosaccharides with proteins: Possible evolutionary significance" Science (80-.)., vol. 213, no. 4504, pp. 222–224, 1981, doi: 10.1126/science.12192669.

[68] N. Verzijl et al., "Effect of collagen turnover on the accumulation of advanced glycation end products" J. Biol. Chem., vol. 275, no. 50, pp. 39027–39031, Dec. 2000, doi: 10.1074/jbc.M006700200.

[69] M. Mastorikou, B. Mackness, Y. Liu, and M. Mackness, "Glycation of paraoxonase-1 inhibits its activity and impairs the ability of high-density lipoprotein to metabolize membrane lipid hydroperoxides" Diabet. Med., vol. 25, no. 9, pp. 1049–1055, Sep. 2008, doi: 10.1111/j.1464-5491.2008.02546.x.

[70] R. Blakytny and J. J. Harding, "Glycation (non-enzymic glycosylation) inactivates glutathione reductase" Biochem. J., vol. 288, no. 1, pp. 303–307, 1992, doi: 10.1042/bj2880303.

[71] J. S. Flier, L. H. Underhill, M. Brownlee, A. Cerami, and H. Vlassara, "Advanced Glycosylation End Products in Tissue and the Biochemical Basis of Diabetic Complications" New England Journal of Medicine, vol. 318, no. 20. pp. 1315–1321, 19-May-1988, doi: 10.1056/NEJM198805193182007.

[72] Z. Turk, S. Ljubic, N. Turk, and B. Benko, "Detection of autoantibodies against advanced glycation endproducts and AGE-immune complexes in serum of patients with diabetes mellitus" Clin. Chim. Acta, vol. 303, no. 1–2, pp. 105–115, 2001, doi: 10.1016/S0009-8981(00)00389-2.

[73] X. Wang et al., "Advanced glycation end products induce oxidative stress and mitochondrial dysfunction in SH-SY5Y cells" Vitr. Cell. Dev. Biol. - Anim., vol. 51, no. 2, pp. 204–209, Nov. 2014, doi: 10.1007/s11626-014-9823-5.

[74] P. Younessi and A. Yoonessi, "Advanced glycation end-products and their receptor-mediated roles: Inflammation and oxidative stress" Iranian Journal of Medical Sciences, vol. 36, no. 3. Shiraz University of Medical Sciences, pp. 154–166, Sep-2011.

[75] M. T. Coughlan et al., "Rage-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes" J. Am. Soc. Nephrol., vol. 20, no. 4, pp. 742–752, 2009, doi: 10.1681/ASN.2008050514.

[76] E. Leclerc, G. Fritz, S. W. Vetter, and C. W. Heizmann, "Binding of S100 proteins to RAGE: An update" Biochimica et Biophysica Acta - Molecular Cell Research, vol. 1793, no. 6. Elsevier, pp. 993–1007, 01-Jun-2009, doi: 10.1016/j.bbamcr.2008.11.016.

[77] S. S. Yan, D. Chen, S. Yan, L. Guo, and J. X. Chen, "RAGE is a key cellular target for Aβ-induced perturbation in Alzheimer's disease" Front. Biosci. - Sch., vol. 4 S, no. 1, pp. 240–250, Jan. 2012, doi: 10.2741/s265.

[78] K. M. Desai et al., "Oxidative stress and aging: Is methylglyoxal the hidden enemy?" Canadian Journal of Physiology and Pharmacology, vol. 88, no. 3. pp. 273–284, Mar-2010, doi: 10.1139/Y10-001.

[79] B. S. Szwergold, S. K. Howell, and P. J. Beisswenger, "Transglycation - A potential new mechanism for deglycation of Schiff's bases" in Annals of the New York Academy of Sciences, 2005, vol. 1043, pp. 845–864, doi: 10.1196/annals.1333.097.

[80] N. Araki et al., “Macrophage Scavenger Receptor Mediates the Endocytic Uptake and Degradation of Advanced Glycation End Products of the Maillard Reaction” Eur. J. Biochem., vol. 230, no. 2, pp. 408–415, 1995, doi: 10.1111/j.1432-1033.1995.0408h.x.

[81] P. Yang, J. Feng, Q. Peng, X. Liu, Z. Fan, and M. Luca, “Advanced Glycation End Products: Potential Mechanism and Therapeutic Target in Cardiovascular Complications under Diabetes” Oxidative Medicine and Cellular Longevity, vol. 2019. Hindawi Limited, 2019, doi: 10.1155/2019/9570616.

[82] D. Svistounov et al., “Hepatic disposal of advanced glycation end products during maturation and aging” Exp. Gerontol., vol. 48, no. 6, pp. 549–556, Jun. 2013, doi: 10.1016/j.exger.2013.03.005.

[83] S. Sifuentes-Franco, F. P. Pacheco-Moisés, A. D. Rodríguez-Carrizalez, and A. G. Miranda-Díaz, “The Role of Oxidative Stress, Mitochondrial Function, and Autophagy in Diabetic Polyneuropathy” J. Diabetes Res., vol. 2017, no. c, 2017, doi: 10.1155/2017/1673081.

[84] C. Y. Guo, L. Sun, X. P. Chen, and D. S. Zhang, “Oxidative stress, mitochondrial damage and neurodegenerative diseases” Neural Regen. Res., vol. 8, no. 21, pp. 2003–2014, Jul. 2013, doi: 10.3969/j.issn.1673-5374.2013.21.009.

[85] A. C. Andreazza, L. Shoo, J. F. Wang, and L. Trevor Young, “Mitochondrial Complex i Activity and Oxidative Damage to Mitochondrial Proteins in the Prefrontal Cortex of Patients with Bipolar Disorder” Arch. Gen. Psychiatry, vol. 67, no. 4, pp. 360–368, Apr. 2010, doi: 10.1001/archgenpsychiatry.2010.22.

[86] G. Giaever et al., “Functional profiling of the *Saccharomyces cerevisiae* genome” Nature, vol. 418, no. 6896, pp. 387–391, Jul. 2002, doi: 10.1038/nature00935.

[87] M. P. Mattson and T. Magnus, “Ageing and neuronal vulnerability” Nature Reviews Neuroscience, vol. 7, no. 4. Nat Rev Neurosci, pp. 278–294, 16-Mar-2006, doi: 10.1038/nrn1886.

[88] N. T. Moldogazieva, I. M. Mokhosoev, T. I. Mel’nikova, Y. B. Porozov, and A. A. Terentiev, “Oxidative Stress and Advanced Lipoxidation and Glycation End Products (ALEs and AGEs) in Aging and Age-Related Diseases” Oxid. Med. Cell. Longev., vol. 2019, p. 3085756, 2019, doi: 10.1155/2019/3085756.

[89] P. K. Jensen, “Antimycin-insensitive oxidation of succinate and reduced nicotinamide-adenine dinucleotide in electron-transport particles I. pH dependency and hydrogen peroxide formation” BBA - Enzymol. Biol. Oxid., vol. 122, no. 2, pp. 157–166, Aug. 1966, doi: 10.1016/0926-6593(66)90057-9.

[90] B. Chance, H. Sies, and A. Boveris, “Hydroperoxide metabolism in mammalian organs” Physiol. Rev., vol. 59, no. 3, pp. 527–605, 1979, doi: 10.1152/physrev.1979.59.3.527.

[91] A. Boveris and B. Chance, “The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen” Biochem. J., vol. 134, no. 3, pp. 707–716, 1973, doi: 10.1042/bj1340707.

[92] G. Loschen, L. Flohé, and B. Chance, “Respiratory chain linked H2O2 production in pigeon heart mitochondria” FEBS Lett., vol. 18, no. 2, pp. 261–264, Nov. 1971, doi: 10.1016/0014-5793(71)80459-3.

[93] M. P. Murphy, “How mitochondria produce reactive oxygen species” Biochem. J., vol. 417, no. 1, pp. 1–13, 2009, doi: 10.1042/BJ20081386.

[94] H. Lodish, A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell, “Electron Transport and Oxidative Phosphorylation” 2000.

[95] P. K. Sharma, N. Mittal, S. Deswal, and N. Roy, “Calorie restriction up-regulates iron and copper transport genes in *Saccharomyces cerevisiae*” Mol. Biosyst., vol. 7, no. 2, pp. 394–402, Feb. 2011, doi: 10.1039/c0mb00084a.

[96] H. Schägger, “Respiratory Chain Supercomplexes” IUBMB Life (International Union Biochem. Mol. Biol. Life), vol. 52, no. 3–5, pp. 119–128, Sep. 2001, doi: 10.1080/15216540152845911.

[97] M. Jastroch, A. S. Divakaruni, S. Mookerjee, J. R. Treberg, and M. D. Brand, “Mitochondrial proton and electron leaks” Essays Biochem., vol. 47, pp. 53–67, 2010, doi: 10.1042/BSE0470053.

[98] “Electron transfer pathway - Bioblast.” [Online]. Available: https://www.bioblast.at/index.php/Electron_transfer_pathway. [Accessed: 10-Nov-2020].

[99] E. Gnaiger, “Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology” International Journal of Biochemistry and Cell Biology, vol. 41, no. 10. pp. 1837–1845, Oct-2009, doi: 10.1016/j.biocel.2009.03.013.

[100] A. M. Cordero-Reyes et al., “Freshly isolated mitochondria from failing human hearts exhibit preserved respiratory function” J. Mol. Cell. Cardiol., vol. 68, pp. 98–105, Mar. 2014, doi: 10.1016/j.yjmcc.2013.12.029.

[101] J. S. Beckman and W. H. Koppenol, “Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly” American Journal of Physiology - Cell Physiology, vol. 271, no. 5 40-5. Nov-1996, doi: 10.1152/ajpcell.1996.271.5.c1424.

[102] H. Li et al., “Thioredoxin 2 offers protection against mitochondrial oxidative stress in H9c2 cells and against myocardial hypertrophy induced by hyperglycemia” Int. J. Mol. Sci., vol. 18, no. 9, pp. 1–10, 2017, doi: 10.3390/ijms18091958.

[103] Q. Huang et al., “Thioredoxin-2 inhibits mitochondrial reactive oxygen species generation and apoptosis stress kinase-1 activity to maintain cardiac function” Circulation, vol. 131, no. 12, pp. 1082–1097, 2015, doi: 10.1161/CIRCULATIONAHA.114.012725.

[104] G. Morris et al., “The Glutathione System: A New Drug Target in Neuroimmune Disorders” Mol. Neurobiol., vol. 50, no. 3, pp. 1059–1084, 2014, doi: 10.1007/s12035-014-8705-x.

[105] E. Birben, U. M. Sahiner, C. Sackesen, S. Erzurum, and O. Kalayci, “Oxidative stress and antioxidant defense” World Allergy Organization Journal, vol. 5, no. 1. BioMed Central Ltd., pp. 9–19, 2012, doi: 10.1097/WOX.0b013e3182439613.

[106] A. J. L. Ham and D. C. Liebler, “Vitamin E Oxidation in Rat Liver Mitochondria” Biochemistry, vol. 34, no. 17, pp. 5754–5761, May 1995, doi: 10.1021/bi00017a007.

[107] F. Tabet and R. M. Touyz, “Reactive Oxygen Species, Oxidative Stress, and Vascular Biology in Hypertension” Compr. Hypertens., pp. 337–347, 2007, doi: 10.1016/B978-0-323-03961-1.50033-7.

[108] M. Fanucchi, The Lung - Development of Antioxidant and Xenobiotic Metabolizing Enzyme Systems., vol. Chapter 11. 2004.

[109] S. Srinivasan and N. G. Avadhani, “Cytochrome c oxidase dysfunction in oxidative stress” Free Radical Biology and Medicine, vol. 53, no. 6. NIH Public Access, pp. 1252–1263, 15-Sep-2012, doi: 10.1016/j.freeradbiomed.2012.07.021.

[110] A. Bindoli, L. Cavallini, and P. Jocelyn, “Mitochondrial lipid peroxidation by cumene hydroperoxide and its prevention by succinate.” Biochim. Biophys. Acta, vol. 681, no. 3, pp. 496–503, Sep. 1982, doi: 10.1016/0005-2728(82)90192-x.

[111] R. Radi, J. F. Turrens, L. Y. Chang, K. M. Bush, J. D. Crapo, and B. A. Freeman, “Detection of catalase in rat heart mitochondria” J. Biol. Chem., vol. 266, no. 32, pp. 22028–22034, 1991.

[112] J. P. Kehrer, J. D. Robertson, and C. V. Smith, “Free Radicals and Reactive Oxygen Species” in Comprehensive Toxicology: Second Edition, vol. 1–14, Elsevier Inc., 2010, pp. 277–307.

[113] M. G. Smith and M. Snyder, “Yeast as a Model for Human Disease” Curr. Protoc. Hum. Genet., vol. 48, no. 1, p. Unit 15.6, Jan. 2006, doi: 10.1002/0471142905.hg1506s48.

[114] D. Botstein, S. A. Chervitz, and J. M. Cherry, “Yeast as a model organism” Science, vol. 277, no. 5330. pp. 1259–1260, 29-Aug-1997, doi: 10.1126/science.277.5330.1259.

[115] M. W. Walberg, “Applicability of yeast genetics to neurologic disease” Arch. Neurol., vol. 57, no. 8, pp. 1129–1134, Aug. 2000, doi: 10.1001/archneur.57.8.1129.

[116] “Why use yeast in research? | Facts | yourgenome.org.” [Online]. Available: https://www.yourgenome.org/facts/why-use-yeast-in-research. [Accessed: 24-Mar-2020].

[117] R. J. Cho et al., “A genome-wide transcriptional analysis of the mitotic cell cycle” Mol. Cell, vol. 2, no. 1, pp. 65–73, 1998, doi: 10.1016/S1097-2765(00)80114-8.

[118] P. T. Spellman et al., “Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization” Mol. Biol. Cell, vol. 9, no. 12, pp. 3273–3297, 1998, doi: 10.1091/mbc.9.12.3273.

[119] H. Zhu et al., “Global analysis of protein activities using proteome chips” Science, vol. 293, no. 5537, pp. 2101–2105, Sep. 2001, doi: 10.1126/science.1062191.

[120] M. Aouida, N. Pagé, A. Leduc, M. Peter, and D. Ramotar, “A Genome-Wide Screen in *Saccharomyces cerevisiae* Reveals Altered Transport as a Mechanism of Resistance to the Anticancer Drug Bleomycin” Cancer Res., vol. 64, no. 3, pp. 1102–1109, Feb. 2004, doi: 10.1158/0008-5472.CAN-03-2729.

[121] G. W. Birrell, G. Giaever, A. M. Chu, R. W. Davis, and J. M. Brown, “A genome-wide screen in *Saccharomyces cerevisiae* for genes affecting UV radiation sensitivity” Proc. Natl. Acad. Sci. U. S. A., vol. 98, no. 22, pp. 12608–12613, Oct. 2001, doi: 10.1073/pnas.231366398.

[122] G. W. Thorpe, C. S. Fong, N. Alic, V. J. Higgins, and I. W. Dawes, “Cells have distinct mechanisms to maintain protection against different reactive oxygen species: Oxidative-stress-response genes” Proc. Natl. Acad. Sci. U. S. A., vol. 101, no. 17, pp. 6564–6569, Apr. 2004, doi: 10.1073/pnas.0305888101.

[123] C. J. Bonangelino, E. M. Chavez, and J. S. Bonifacino, “Genomic screen for vacuolar protein sorting genes in *Saccharomyces cerevisiae*” Mol. Biol. Cell, vol. 13, no. 7, pp. 2486–2501, Jul. 2002, doi: 10.1091/mbc.02-01-0005.

[124] K. S. Dimmer et al., “Genetic basis of mitochondrial function and morphology in *Saccharomyces cerevisiae*” Mol. Biol. Cell, vol. 13, no. 3, pp. 847–853, Mar. 2002, doi: 10.1091/mbc.01-12-0588.

[125] E. S. Seeley, M. Kato, N. Margolis, W. Wickner, and G. Eitzen, “Genomic analysis of homotypic vacuole fusion” Mol. Biol. Cell, vol. 13, no. 3, pp. 782–794, Mar. 2002, doi: 10.1091/mbc.01-10-0512.

[126] R. A. Gomes, M. S. Silva, H. Vicente Miranda, A. E. N. Ferreira, C. A. A. Cordeiro, and A. P. Freire, “Protein glycation in *Saccharomyces cerevisiae*: Argpyrimidine formation and methylglyoxal catabolism” FEBS J., vol. 272, no. 17, pp. 4521–4531, Sep. 2005, doi: 10.1111/j.1742-4658.2005.04872.x.

[127] R. A. Gomes et al., “Yeast protein glycation *in vivo* by methylglyoxal: Molecular modification of glycolytic enzymes and heat shock proteins” FEBS J., vol. 273, no. 23, pp. 5273–5287, Dec. 2006, doi: 10.1111/j.1742-4658.2006.05520.x.

[128] “ImageJ.” [Online]. Available: https://imagej.net/Welcome. [Accessed: 10-Nov-2020].

[129] “ImageJ Guide.” [Online]. Available: https://imagej.nih.gov/ij/docs/menus/analyze.html. [Accessed: 10-Apr-2020].

[130] “Home | FlowJo, LLC.” [Online]. Available: https://www.flowjo.com/. [Accessed: 10-Nov-2020].

[131] A. Adan, G. Alizada, Y. Kiraz, Y. Baran, and A. Nalbant, “Flow cytometry: basic principles and applications” Critical Reviews in Biotechnology, vol. 37, no. 2. Taylor and Francis Ltd, pp. 163–176, 17-Feb-2017, doi: 10.3109/07388551.2015.1128876.

[132] “Hoechst 33342 Staining Dye Solution (ab228551) | Abcam.” [Online]. Available: https://www.abcam.com/hoechst-33342-staining-dye-solution-ab228551.html. [Accessed: 10-Apr-2020].

[133] “5-CFDA (5-Carboxyfluorescein diacetate, single isomer) | Biotium.” [Online]. Available: https://biotium.com/product/5-cfda-5-carboxyfluorescein-diacetate-single-isomer/. [Accessed: 10-Apr-2020].

[134] “Propidium Iodide (PI) | Biotium.” [Online]. Available: https://biotium.com/product/propidium-iodide-pi/. [Accessed: 10-Apr-2020].

[135] “Live-or-Dye™ Fixable Viability Staining Kits | Biotium.” [Online]. Available: https://biotium.com/product/live-or-dye-fixable-viability-staining-kit/. [Accessed: 10-Apr-2020].

[136] “Calcofluor White Stain for microbiology | Sigma-Aldrich.” [Online]. Available: https://www.sigmaaldrich.com/catalog/product/sial/18909?lang=pt&region=PT. [Accessed: 18-Apr-2020].

[137] P. X. Petit, “Flow cytometric analysis of rhodamine 123 fluorescence during modulation of the membrane potential in plant mitochondria” Plant Physiol., vol. 98, no. 1, pp. 279–286, 1992, doi: 10.1104/pp.98.1.279.

[138] X. Ronot, L. Benel, M. Adolphe, and J. C. Mounolou, “Mitochondrial analysis in living cells: the use of rhodamine 123 and flow cytometry” Biol. Cell, vol. 57, no. 1, pp. 1–7, Jan. 1986, doi: 10.1111/j.1768-322X.1986.tb00458.x.

[139] J. E. O’Connor, J. L. Vargas, B. F. Kimler, J. Hernandez-Yago, and S. Grisolia, “Use of Rhodamine 123 to investigate alterations in mitochondrial activity in isolated mouse liver mitochondria” Biochem. Biophys. Res. Commun., vol. 151, no. 1, pp. 568–573, Feb. 1988, doi: 10.1016/0006-291X(88)90632-8.

[140] “DCFDA / H2DCFDA - Cellular Reactive Oxygen Species Detection Assay Kit.” [Online]. Available: https://www.abcam.com/dcfda--h2dcfda-cellular-ros-assay-kit-ab113851.html. [Accessed: 17-Apr-2020].

[141] P. Pir et al., “The genetic control of growth rate: A systems biology study in yeast” BMC Syst. Biol., vol. 6, p. 4, Jan. 2012, doi: 10.1186/1752-0509-6-4.

[142] M. P. Kalapos, “The tandem of free radicals and methylglyoxal” Chemico-Biological Interactions, vol. 171, no. 3. Chem Biol Interact, pp. 251–271, 15-Feb-2008, doi: 10.1016/j.cbi.2007.11.009.

[143] M. G. Rosca et al., “Glycation of mitochondrial proteins from diabetic rat kidney is associated with excess superoxide formation” Am. J. Physiol. - Ren. Physiol., vol. 289, no. 2 58-2, Aug. 2005, doi: 10.1152/ajprenal.00415.2004.

[144] N. Rabbani and P. J. Thornalley, “Dicarbonyls linked to damage in the powerhouse: Glycation of mitochondrial proteins and oxidative stress” in Biochemical Society Transactions, 2008, vol. 36, no. 5, pp. 1045–1050, doi: 10.1042/BST0361045.

[145] G. Daum, N. D. Lees, M. Bard, and R. Dickson, “Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*” Yeast, vol. 14, no. 16. Yeast, pp. 1471–1510, Dec-1998, doi: 10.1002/(SICI)1097-0061(199812)14:16<1471::AID-YEA353>3.0.CO;2-Y.

[146] C. M. Henderson and D. E. Block, “Examining the role of membrane lipid composition in determining the ethanol tolerance of *Saccharomyces cerevisiae*” Appl. Environ. Microbiol., vol. 80, no. 10, pp. 2966–2972, 2014, doi: 10.1128/AEM.04151-13.

[147] J. Cerbon and V. Calderon, “Generation modulation and maintenance of the plasma membrane asymmetric phospholipid composition in yeast cells during growth: their relation to surface potential and membrane protein activity” BBA - Biomembr., vol. 1235, no. 1, pp. 100–106, Apr. 1995, doi: 10.1016/0005-2736(94)00311-C.

[148] E. Zinser, C. D. M. Sperka-Gottlieb, E. V. Fasch, S. D. Kohlwein, F. Paltauf, and G. Daum, “Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*” J. Bacteriol., vol. 173, no. 6, pp. 2026–2034, 1991, doi: 10.1128/jb.173.6.2026-2034.1991.

[149] J. H. Oak, K. Nakagawa, and T. Miyazawa, “Synthetically prepared Amadori-glycated phosphatidylethanolamine can trigger lipid peroxidation via free radical reactions” in FEBS Letters, 2000, vol. 481, no. 1, pp. 26–30, doi: 10.1016/S0014-5793(00)01966-9.

[150] J. H. Oak, K. Nakagawa, S. Oikawa, and T. Miyazawa, “Amadori-glycated phosphatidylethanolamine induces angiogenic differentiations in cultured human umbilical vein endothelial cells” FEBS Lett., vol. 555, no. 2, pp. 419–423, Dec. 2003, doi: 10.1016/S0014-5793(03)01237-7.

[151] T. Miyazawa, K. Nakagawa, S. Shimasaki, and R. Nagai, “Lipid glycation and protein glycation in diabetes and atherosclerosis” Amino Acids, vol. 42, no. 4. Springer, pp. 1163–1170, 19-Apr-2012, doi: 10.1007/s00726-010-0772-3.

[152] P. D. Winocour et al., “Reduced membrane fluidity in platelets from diabetic patients” Diabetes, vol. 39, no. 2, pp. 241–244, 1990, doi: 10.2337/diab.39.2.241.

[153] G. Wolf, K. Sharma, and F. N. Ziyadeh, “Pathophysiology and Pathogenesis of Diabetic Nephropathy” in Seldin and Giebisch’s The Kidney, Elsevier Inc., 2008, pp. 2215–2233.

[154] E. Zinser, C. D. M. Sperka-Gottlieb, E. V. Fasch, S. D. Kohlwein, F. Paltauf, and G. Daum, “Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae”* J. Bacteriol., vol. 173, no. 6, pp. 2026–2034, 1991, doi: 10.1128/jb.173.6.2026-2034.1991.

[155] S. Mahali, N. Raviprakash, P. B. Raghavendra, and S. K. Manna, “Advanced glycation end products (AGEs) induce apoptosis via a novel pathway: Involvement of CA 2+ mediated by interleukin-8 protein” J. Biol. Chem., vol. 286, no. 40, pp. 34903–34913, Oct. 2011, doi: 10.1074/jbc.M111.279190.

[156] L. Yang, H. Meng, and M. Yang, “Autophagy protects osteoblasts from advanced glycation end products-induced apoptosis through intracellular reactive oxygen species” J. Mol. Endocrinol., vol. 56, no. 4, pp. 291–300, May 2016, doi: 10.1530/JME-15-0267.

[157] K. C. Lan et al., “Advanced glycation end-products induce apoptosis in pancreatic islet endothelial cells via NF-κB-activated cyclooxygenase-2/prostaglandin e2 up-regulation” PLoS One, vol. 10, no. 4, p. 124418, Apr. 2015, doi: 10.1371/journal.pone.0124418.

[158] J. Yue and J. M. López, “Understanding MAPK signaling pathways in apoptosis” International Journal of Molecular Sciences, vol. 21, no. 7. MDPI AG, 01-Apr-2020, doi: 10.3390/ijms21072346.

[159] A. L. Silvers, M. A. Bachelor, and G. T. Bowden, “The role of JNK and p38 MAPK activities in

UVA-induced signaling pathways leading to AP-1 activation and c-Fos expression" Neoplasia, vol. 5, no. 4, pp. 319–329, 2003, doi: 10.1016/s1476-5586(03)80025-8.

[160] K. Nonaka et al., "Advanced glycation end-products increase IL-6 and ICAM-1 expression via RAGE, MAPK and NF-κB pathways in human gingival fibroblasts" J. Periodontal Res., vol. 53, no. 3, pp. 334–344, Jun. 2018, doi: 10.1111/jre.12518.

[161] J. Zhou et al., "Increased JNK phosphorylation and oxidative stress in response to increased glucose flux through increased GLUT1 expression in rat retinal endothelial cells" Investig. Ophthalmol. Vis. Sci., vol. 46, no. 9, pp. 3403–3410, Sep. 2005, doi: 10.1167/iovs.04-1064.

[162] M. Salma, S. Rousseaux, A. Sequeira-Le Grand, B. Divol, and H. Alexandre, "Characterization of the Viable but Nonculturable (VBNC) State in *Saccharomyces cerevisiae*." PLoS One, vol. 8, no. 10, 2013, doi: 10.1371/journal.pone.0077600.

[163] M. Ayrapetyan, T. Williams, and J. D. Oliver, "Relationship between the viable but nonculturable state and antibiotic persister cells" Journal of Bacteriology, vol. 200, no. 20. American Society for Microbiology, 01-Oct-2018, doi: 10.1128/JB.00249-18.

[164] M. Ayrapetyan, T. C. Williams, R. Baxter, and J. D. Oliver, "Viable but nonculturable and persister cells coexist stochastically and are induced by human serum" Infect. Immun., vol. 83, no. 11, pp. 4194–4203, Nov. 2015, doi: 10.1128/IAI.00404-15.

[165] R. R. Colwell, "Viable but nonculturable bacteria: A survival strategy" Journal of Infection and Chemotherapy, vol. 6, no. 2. Springer Japan, pp. 121–125, 2000, doi: 10.1007/PL00012151.

[166] F. Codony, G. Agustí, and A. Allué-Guardia, "Cell membrane integrity and distinguishing between metabolically active and inactive cells as a means of improving viability PCR" Mol. Cell. Probes, vol. 29, no. 3, pp. 190–192, Jun. 2015, doi: 10.1016/j.mcp.2015.03.003.

[167] D. Dobler, N. Ahmed, L. Song, K. E. Eboigbodin, and P. J. Thornalley, "Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification" Diabetes, vol. 55, no. 7, pp. 1961–1969, Jul. 2006, doi: 10.2337/db05-1634.

[168] H. Li et al., "N2-carboxyethyl-2′-deoxyguanosine, a DNA glycation marker, in kidneys and aortas of diabetic and uremic patients" Kidney Int., vol. 69, no. 2, pp. 388–392, Jan. 2006, doi: 10.1038/sj.ki.5000064.

[169] A. J. Oudes, C. M. Herr, Y. Olsen, and J. E. Fleming, "Age-dependent accumulation of advanced glycation end-products in adult Drosophila melanogaster" Mech. Ageing Dev., vol. 100, no. 3, pp. 221–229, Feb. 1998, doi: 10.1016/S0047-6374(97)00146-2.

[170] P. GELLERFORS, M. LUNDÉN, and B. D. NELSON, "Evidence for a Function of Core Protein in Complex III from Beef-Heart Mitochondria" Eur. J. Biochem., vol. 67, no. 2, pp. 463–468, 1976, doi: 10.1111/j.1432-1033.1976.tb10711.x.

[171] A. R. Parker, "A single arginine residue is required for the interaction of the electron transferring flavoprotein (ETF) with three of its dehydrogenase partners" Mol. Cell. Biochem., vol. 254, no. 1–2, pp. 91–100, Dec. 2003, doi: 10.1023/A:1027349303797.

[172] D. Xia et al., "Crystal structure of the cytochrome bc1 complex from bovine heart mitochondria" Science (80-.)., vol. 277, no. 5322, pp. 60–66, Jul. 1997, doi: 10.1126/science.277.5322.60.

[173] M. Hamelin, J. Mary, M. Vostry, B. Friguet, and H. Bakala, "Glycation damage targets glutamate dehydrogenase in the rat liver mitochondrial matrix during aging" FEBS J., vol. 274, no. 22, pp. 5949–5961, Nov. 2007, doi: 10.1111/j.1742-4658.2007.06118.x.

[174] M. Fournet, F. Bonté, and A. Desmoulière, "Glycation damage: A possible hub for major pathophysiological disorders and aging" Aging and Disease, vol. 9, no. 5. International Society

on Aging and Disease, pp. 880–900, 2018, doi: 10.14336/AD.2017.1121.

[175] F. Moruno, E. Pérez-Jiménez, and E. Knecht, “Regulation of Autophagy by Glucose in Mammalian Cells” Cells, vol. 1, no. 3, pp. 372–395, Jul. 2012, doi: 10.3390/cells1030372.

[176] D. Bhattacharya, M. Mukhopadhyay, M. Bhattacharyya, and P. Karmakar, “Is autophagy associated with diabetes mellitus and its complications? A review” EXCLI Journal, vol. 17. Leibniz Research Centre for Working Environment and Human Factors, pp. 709–720, 24-Jul-2018, doi: 10.17179/excli2018-1353.

[177] L. Galluzzi and G. Kroemer "Cell-wide metabolic alterations associated with malignancy." vol.543, pp. 358, 2014.

[178] X. Leverve, “Oxidative, stress and antioxidants?” Cah. Nutr. Diet., vol. 44, no. 5, pp. 219–224, 2009, doi: 10.1016/j.cnd.2009.09.001.

[179] Y. Hatefi, A. G. Haavik, L. R. Fowler, and D. E. Griffiths, “Studies on the electron transfer system. XLII. Reconstitution of the” J. Biol. Chem., vol. 237, pp. 2661–2669, 1962.