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Glucose and aging: contribution of the model organism *Caenorhabditis elegans*

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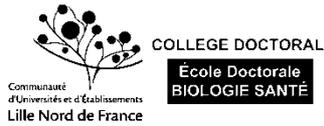
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Glucose et vieillissement : intérêt du modèle *Caenorhabditis elegans*

Présentée et soutenue

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Avant Propos

Cette thèse a été en partie financée par une bourse obtenue auprès de l'Université de Lille et l'iSite, sous la responsabilité de l'Ecole Doctorale Biologie Santé de Lille (« MD-PhD »). Ce travail est le fruit du travail effectué dans le laboratoire de métabolisme et vieillissement de Professeur Charles MOBBS à l'Icahn School of Medicine at Mount Sinai aux Etats-Unis et du laboratoire de Biologie du Vieillissement de Professeur Eric BOULANGER au sein du LIRIC (Lille International Research Inflammation Center) en France.

Ces travaux ont été initiés par Professeur Charles MOBBS et Professeur Eric BOULANGER, travaillant sur les mécanismes du vieillissement. J'ai eu la chance d'initier le travail réalisé dans le laboratoire de Professeur Charles MOBBS lors de mon année de Master 2 et financée par l'Année Recherche.

Les travaux décrits dans cette thèse ont été valorisés sous la forme de plusieurs posters commentés présentés à la réunion annuelle de la Société Française de Gériatrie et Gériatrie (SFGG) de novembre 2018, un poster présenté à la réunion annuelle de l'European Geriatric Medicine Society (EUGMS) en septembre 2017, une communication orale au congrès interrégional et transfrontalier de gérontologie (SSGC)/ Ageing Fit en février 2017 et une communication orale lors d'un colloque scientifique à New York en 2014. Les travaux permettront prochainement d'aboutir à l'écriture et la publication d'un article dans lequel je serai auteur de rang significatif.

Titre en français

Glucose et vieillissement : intérêt du modèle *Caenorhabditis elegans*

Résumé en français

Depuis les premières études démontrant les effets du glucose sur la longévité et la santé, des mécanismes sous-jacents restent à déterminer. La restriction du glucose prolonge la durée de vie, alors qu'un excès de glucose raccourcit la durée de vie de nombreux organismes. Comprendre les effets du glucose sur le vieillissement revêt un grand intérêt pour la communauté scientifique et médicale, car cela pourrait permettre de traiter de nombreuses maladies liées à l'âge, telles que le cancer, le diabète de type 2 ou les maladies neurodégénératives. Les études chez les invertébrés et en particulier chez *Caenorhabditis elegans* (*C. elegans*) ont permis de découvrir de nombreux gènes et voies impliqués dans le vieillissement, dont beaucoup sont conservés chez les mammifères. *C. elegans* peut être utilisé pour mimer *in vivo* la plupart des maladies humaines, telles que les maladies d'Alzheimer au niveau métabolique et génomique. Notre hypothèse est que le métabolisme du glucose accélère le vieillissement. La complexité du processus de vieillissement nous a amené à l'étudier sous deux angles différents: la restriction d'ingestion du glucose et l'excès d'ingestion du glucose. Des études antérieures ont montré que la restriction alimentaire (qui réduit la disponibilité du glucose) permettait le recrutement du CREB-Binding Protein (CBP) pour contrôler l'expression d'autres gènes impliqués dans la détermination de la longévité et une modification de la source principale d'énergie du glucose vers des substrats alternatifs. Lorsqu'il est inhibé par l'ARNi dans un modèle de *C. elegans* pour la maladie d'Alzheimer, le CBP réduit la longévité. Pour déterminer si cet effet était dû spécifiquement au CBP, nous avons inhibé d'autres histone acétylase transférase (HAT) avec une méthode d'ARNi dans ce modèle de *C. elegans* pour la maladie d'Alzheimer et évalué la longévité ainsi que la protéotoxicité $A\beta_{1-42}$ responsable d'une paralysie accélérée dans ce modèle due à l'agrégation de $A\beta_{1-42}$. Pour la deuxième partie de notre travail, nous avons voulu caractériser le modèle *C. elegans* pour l'étude des milieux enrichis en glucose. Ce modèle a été utilisé par le passé pour étudier les effets des régimes enrichis en glucose, mais tous les aspects du métabolisme du glucose en ce qui concerne le vieillissement ne sont pas connus et bien caractérisés. Notre objectif était de décrire les effets d'un milieu enrichi en glucose sur l'expression d'enzymes impliquées dans le métabolisme du glucose et le stress oxydatif par la qRT-PCR. Nous nous sommes également concentrés sur la production endogène de produits de glycation avancés (AGE), des études récentes de notre laboratoire les incriminant dans les mécanismes sous-jacents au vieillissement. Enfin, nous

avons développé un dispositif de criblage semi-automatisé à haut débit pour nous permettre de cribler des composés capables de retarder le vieillissement et les pathologies liées à l'âge. Nous avons utilisé notre nouveau dispositif pour analyser la bibliothèque de 2560 composés de Microsource SPECTRUM.

Nos résultats montrent que l'inhibition de CBP accélère de façon unique le vieillissement et augmente la protéotoxicité de A β ₁₋₄₂ dans un modèle de *C. elegans* de la maladie d'Alzheimer. Nous avons pu mieux caractériser le modèle *C. elegans* en tant qu'organisme avantageux pour l'étude des effets de milieux enrichis en glucose sur le vieillissement, et nous avons en particulier confirmé que la réponse au stress oxydatif et la production endogène des AGE augmente avec le temps et la concentration en glucose. Enfin, les résultats de notre criblage ont identifié l'acide phénylbutyrique, un inhibiteur de l'histone déacétylase dont l'action est corrélée à celle de CBP, comme le composé le plus efficace pour protéger contre la protéotoxicité A β ₁₋₄₂. Ceci plaide pour le rôle des modifications épigénétiques et de détection des nutriments dans la médiation des effets du métabolisme du glucose sur le vieillissement.

Titre en anglais

Glucose and aging: contribution of the model organism *Caenorhabditis elegans*

Résumé en anglais

Since the first studies demonstrating the effects of glucose on lifespan and healthspan were performed, underlying mechanisms remain to be determined. Glucose restriction is known to extend lifespan whereas excessive glucose shortens lifespan in a multitude of organisms. Understanding how glucose affects aging is of great interest to the scientific and medical communities as this could lead to targeted treatments for many age-related diseases such as cancer, diabetes type 2 or neurodegenerative diseases. Studies in invertebrates and particularly in *Caenorhabditis elegans* (*C. elegans*) have contributed to discover many genes and pathways involved in aging, many of which are conserved in mammals. *C. elegans* can be used to recapitulate most human diseases, such as Alzheimer's diseases at the metabolic and genomic level *in vivo*. Our hypothesis is that glucose metabolism drives aging. The complexity of the aging process led us to study it from two different angles, glucose restriction and excess.

Previous studies demonstrated that dietary restriction (which reduces glucose availability) induces CREB-Binding Protein (CBP) to control the expression of other genes involved in the determination of lifespan and a metabolic shift from glucose to alternative substrates. When inhibited by RNAi in a *C. elegans* model for Alzheimer's disease, CBP reduced lifespan. To

determine whether this effect was due to a specific role of CBP we inhibited other HATs with a feeding RNAi method in the same *C. elegans* model for Alzheimer's disease and assessed lifespan as well as A β ₁₋₄₂ proteotoxicity responsible for accelerated paralysis in this model. *C. elegans* has been used as a model organism to study the effects of glucose-enriched diets in the past but not all aspects of the glucose metabolism in respects to aging are known and well characterized. We aimed at describing the effects of a glucose enriched medium on expression of enzymes involved in glucose metabolism and oxidative stress by qRT-PCR. We also focused on endogenous production of advanced glycation end products as recent studies from our laboratory incriminate them in mechanisms underlying aging. Finally, we developed a high-throughput semi-automated screening device to allow us to screen compounds capable of delaying aging and age-related pathologies. We applied our newly developed device to screen the Microsource SPECTRUM library of 2560 compounds.

Our results show that the inhibition of the histone acetyl transferase CBP uniquely accelerates aging and increases A β ₁₋₄₂ proteotoxicity in a *C. elegans* model of Alzheimer's disease, expanding earlier results. We were able to better characterize and standardize *C. elegans* as a valid model organism for the study of the effects of glucose-enriched medium on aging and in particular confirmed that the oxidative stress response and endogenous production of advanced glycation end products increased with time and glucose concentration. Finally the results of our large-scale compound screen identified phenylbutyric acid, a histone deacetylase inhibitor whose action correlates with CBP's, as the most efficient compound to protect against A β ₁₋₄₂ proteotoxicity. This further argues for the role of epigenetic and nutrient-sensing modifications in mediating the effects of glucose metabolism on aging.

Résumé long Français

Introduction générale

Le glucose est la source d'énergie ultime pour la plupart des organismes vivants et il a été démontré que le glucose avait un impact sur le vieillissement. La privation en glucose, dans le cas des interventions de restriction alimentaire, augmente la durée de vie et améliore la santé de nombreuses espèces. Au contraire, les excès de glucose nuisent à la durée de vie et sont à l'origine de nombreuses maladies liées à l'âge[1]. Les mécanismes sous-jacents ne sont pas encore complètement compris. Par l'intermédiaire du modèle *Caenorhabditis elegans* (*C. elegans*), nous avons cherché à mieux déchiffrer les mécanismes expliquant le rôle du glucose dans le vieillissement.

Nos résultats ont permis de mieux appréhender les mécanismes protecteurs de la restriction calorique, caractérisé par une diminution de l'utilisation du glucose et ont également contribué à faire de *C. elegans* un organisme modèle de choix pour l'étude des régimes enrichis en glucose, responsables de l'accélération du vieillissement. Le but ultime de la recherche sur le vieillissement étant de développer des cibles potentielles ayant des effets protecteurs sur la durée de vie et la santé, nous avons également développé une méthode de criblage semi-automatique appliquée à *C. elegans*, qui nous permettra de tester des composés en nous basant sur notre compréhension des mécanismes fondamentaux du vieillissement.

Transport, métabolisme et physiologie du glucose :

Le glucose est le sucre, ou monosaccharide le plus abondant dont la formule est $C_6H_{12}O_6$ ou $H-(C=O)-(CHOH)_5-H$. Il s'agit de la source d'énergie la plus importante pour une majorité d'organismes vivants. La première étape de dégradation du glucose pour produire de l'énergie est la glycolyse, un ensemble de réactions chimiques ancestral permettant la dégradation du glucose en deux molécules de pyruvate. La glycolyse est la source de métabolites intermédiaires qui, s'ils sont produits en excès (en cas d'excès de glucose), sont détournés vers différentes voies impliquées dans la physiopathologie du diabète. Dans les cellules, la glycolyse est la séquence de dix réactions catalysées par des enzymes et se produit dans le cytosol des cellules eucaryotes[2].

La réaction globale peut être résumée comme suit :



La glycolyse comprend trois enzymes régulatrices : l'hexokinase, la phosphofructokinase et la pyruvate kinase. Ces enzymes existent aux points de régulation du processus glycolytique et

indiquent les points où les intermédiaires peuvent quitter la voie de la glycolyse pour d'autres processus.

Le transport du glucose est la première étape limitante de l'utilisation du glucose[3]. Étant donné que la bicouche lipidique des cellules eucaryotes est imperméable aux molécules hydrophiles, le glucose est absorbé par deux familles de transporteurs de glucose: les transporteurs de glucose (GLUT)[4], et le transporteur lié au sodium-glucose (SGLT)[5].

Métabolisme glucidique en situation non physiologique :

Restriction calorique : Un contrôle de la glucorégulation adapté est la marque d'un vieillissement réussi. L'une des méthodes les plus efficaces pour maintenir l'homéostasie du glucose consiste à recourir à des interventions nutritionnelles, la plus efficace étant la restriction alimentaire. Il est largement admis dans la communauté scientifique que la restriction alimentaire (restriction des apports de 20-40% sans malnutrition) augmente la durée de vie moyenne et maximale ainsi que la santé dans un large éventail d'organismes, comprenant la levure, les mouches, les vers, les poissons, les rongeurs et les singes rhésus (*Macaca molatta*)[6][7] Chez les rongeurs et les singes rhésus, la restriction alimentaire retarde la perte de fonction et réduit l'incidence des principales maladies liées à l'âge[8]. La restriction alimentaire est la méthode la plus efficace connue à ce jour pour prolonger la durée de vie de toutes les espèces testées, y compris les mammifères. L'universalité de l'extension de la durée de vie induite par les restrictions alimentaires suggère une possible conservation des voies de régulation de la longévité parmi les espèces. L'objectif de la recherche sur les restrictions alimentaires est bien sûr de comprendre son mécanisme protecteur sous-jacent et non de le promouvoir en tant que mode de vie. En effet, une restriction alimentaire sévère à long terme peut augmenter le risque d'anémie, d'arythmie cardiaque, d'altération de la fonction reproductrice et de fractures ostéoporotiques, comme cela est observé chez les patients présentant des troubles de l'alimentation tels que l'anorexie mentale et la boulimie mentale. La restriction alimentaire doit être considérée comme un outil permettant de comprendre le vieillissement humain, qui repose sur la traduction des effets chez les primates. À cet égard, les résultats des dernières études sur les primates non humains menées à l'Université du Wisconsin sont prometteurs[9].

Parmi les voies et mécanismes impliqués dans les effets protecteurs de la restriction alimentaire citons : les mécanismes de régulation énergétiques cellulaires, avec une diminution de l'activation de la voie de signalisation insuline/insulin growth factor like (IGF-1), qui inhibe le facteur de transcription FOXO, acteur reconnu dans le vieillissement des espèces[10] ; l'inhibition de la voie mTOR (mammalian Target of Rapamycin)[11], l'activation des

sirtuines[12]. Tous ces acteurs sont reconnus pour leur rôle dans les mécanismes du vieillissement. Récemment, des recherches pointent aussi le rôle des modifications épigénétiques et en particulier de l'acétylation des histones comme étant l'un des mécanismes permettant l'effet protecteur de la restriction alimentaire[13]. Plusieurs découvertes du laboratoire du professeur MOBBS impliquent le CBP (CREB Binding Protein), une histone acétyl transférase jouant un rôle majeur dans les mécanismes sous-jacents des effets protecteurs de la restriction alimentaire sur la durée de vie. Il a été montré que l'expression de la CBP était corrélée à la durée de vie de cinq souches différentes de souris[14], ce qui n'a été démontré pour aucun autre gène impliqué dans des mécanismes de restriction alimentaire tels que SIRT-1, AMPK ou mTOR. En outre, lorsque l'on inhibe l'expression de CBP dans l'organisme modèle *C. elegans* par ARNi (ARN interférence), la prolongation de la durée de vie induite par la restriction alimentaire est complètement annulée, indépendamment du protocole de restriction alimentaire utilisé[14], ce qui n'a pas été reproduit avec un autre gène à ce jour. Ces résultats ont été confirmés par trois laboratoires indépendants[15–17].

CBP pourrait être un facteur commun intégrant de multiples voies protectrices dans le vieillissement puisqu'il se lie à plusieurs protéines impliquées dans l'extension de la durée de vie, telles que FOXO, HSF-1, NRF-2. On sait que FOXO se lie à la CBP et en a besoin pour ses effets métaboliques, ce qui conforte les arguments en faveur d'une restriction alimentaire et des mécanismes de partage de la voie insuline-IGF-1 impliquant l'activité de CBP.

Dans les sections précédentes, nous avons discuté du devenir du glucose dans un contexte physiologique ainsi que dans le cadre d'une restriction alimentaire lorsque le métabolisme du glucose est réduit. Nous discuterons ici du devenir du glucose en situation d'hyperglycémie et de son impact sur le vieillissement.

La dysrégulation du glucose et l'hyperglycémie sont généralement associées à un âge avancé[18], tout comme le diabète sucré, une maladie caractérisée par une glycémie (i.e. concentration plasmatique de glucose) élevée, résultant d'anomalies de la sécrétion d'insuline et / ou de l'action de l'insuline, souvent décrite comme une maladie accélérant le vieillissement. La théorie unifiée de la toxicité du glucose a été avancée par BROWNLEE il y a plus de 30 ans[19]. En cas d'hyperglycémie, la majeure partie du glucose est métabolisée par la voie glycolytique décrite précédemment. L'excès de glucose et les points de régulation de la voie glycolytique déterminent la déviation du glucose et de ses métabolites intermédiaires vers les voies alternatives du métabolisme du glucose. L'objectif pour l'organisme est de mobiliser toutes les voies possibles pour augmenter l'élimination du glucose plasmatique. La plupart de ces voies sont utilisées de manière très limitée dans des conditions de glycémie standard. Il

s'agit de : la voie du polyol, la voie des hexosamines, la voie de la protéine kinase C, et la voie des produits de glycation avancés (AGE)[19]. Comme ces derniers présentent un intérêt particulier pour cette thèse, nous allons très brièvement passer en revue la modification post-traductionnelle protéique : la glycation.

La glycation est une réaction non enzymatique entre les sucres réducteurs et la fonction aminé d'une protéine. La réaction est irréversible et produit des AGE. Des études du laboratoire du professeur BOULANGER ont impliqué les AGE dans le vieillissement[20–22]. Le mécanisme du rôle de AGE dans le vieillissement est encore largement inconnu, mais il existe des arguments impliquant le récepteur aux AGE, RAGE, en particulier en ce qui concerne l'insuffisance rénale chronique et le développement d'autres maladies liées à l'âge, à savoir les maladies cardiovasculaires (BELMOKHTAR et al. 2019)[23].

Le vieillissement :

Le vieillissement est défini comme un déclin progressif, en fonction du temps, de fonctions physiologiques affectant tous les êtres vivants. De plus, l'âge est un facteur de risque robuste et indépendant pour toute une gamme de maladies non transmissibles telles que le cancer, les maladies cardiovasculaires et les maladies neurodégénératives. Des preuves accablantes suggèrent que les processus intrinsèques au vieillissement contribuent à la pathogenèse des maladies liées au vieillissement.

En 2013, LOPEZ-OTIN et ses collègues ont tenté d'identifier et de classer les caractéristiques cellulaires et moléculaires du vieillissement[24]. La motivation pour identifier ces caractéristiques était de conceptualiser l'essence du vieillissement et ses mécanismes sous-jacents. Neuf caractéristiques ont été identifiées et chacune remplit les critères suivants : "i) elle doit se manifester au cours du vieillissement normal ; ii) son aggravation expérimentale doit accélérer le vieillissement ; et iii) son amélioration expérimentale doit retarder le processus normal de vieillissement et, augmenter la durée de vie en bonne santé ". Les caractéristiques identifiées sont l'instabilité génomique, l'attrition des télomères, les modifications épigénétiques, une perte de protéostase, la détection dérégulée des nutriments, le dysfonctionnement mitochondrial, la sénescence cellulaire, l'épuisement des cellules souches et l'altération de la communication intercellulaire.

Pour permettre l'étude du vieillissement, les chercheurs font appel à différents organismes modèles, parmi lesquels : la levure *Saccharomyces cerevisiae*, le ver plat Plathelminthes *Macrostomum lignano*, le ver Nématode *Caenorhabditis elegans*, la drosophile, le zebrafish *Danio rerio*, le killifish turquoise ou *Nothobranchius furzeri*, et bien sûr les souris avec des modèles génétiques permettant plus précisément d'étudier le vieillissement accéléré (modèles

de maladies de vieillissement accéléré comme la progéria, la maladie de Huntington...), le vieillissement ralenti (souris Klotho) ou les maladies liées à l'âge (modèles de maladie d'Alzheimer). Le vieillissement est également étudié dans des modèles plus atypiques tels que les oiseaux ou singes *Macaca mulatta*[25].

Le modèle *Caenorhabditis elegans* :

C. elegans est un nématode, ou ver rond, acéломate, long d'un millimètre. Sa durée de vie est très courte (environ 3 semaines) et variable en fonction de différents paramètres externes telles que la température et la disponibilité de la nourriture. L'élevage en laboratoire est effectué dans des boîtes de pétri ou dans des plaques multi-puits sur un milieu géloséensemencé avec une souche de bactéries *Escherichia coli* ou dans un milieu liquide contenant également des bactéries *Escherichia coli* à une concentration donnée. Les températures optimales de culture sont comprises entre 15° et 25°. Il s'agit d'un animal hermaphrodite autofécondant, caractéristique précieuse en laboratoire car permettant d'obtenir une population clonale à partir d'un seul individu homozygote pour un caractère considéré[26]. Les avantages de ce modèle pour étudier le vieillissement comprennent: sa courte durée de vie, une connaissance parfaite de son cycle de vie et de son génome; l'autofécondation des hermaphrodites assurant une information génomique et un fond stables, sa simplicité anatomique, avec un nombre limité de cellules somatiques (959 cellules dont 302 neurones) minimisant les paramètres à prendre en compte; l'existence d'outils moléculaires pour moduler facilement et efficacement l'expression de ses gènes (notamment la technique d'interférence d'ARN, ARNi) ou pour exprimer des protéines codées par des gènes rapporteurs ou des protéines hétérologues; sa transparence, permettant des études microscopiques *in vivo*; et le fait qu'environ 40% des gènes de *C. elegans* sont des orthologues des gènes humains, plus de 80% d'entre eux étant des gènes impliqués dans les voies de signalisation. Comme tout modèle, *C. elegans* a ses limites : il n'a pas de système circulatoire ou inflammatoire par exemple ce qui rend difficile la transposition de facteurs potentiellement important dans le processus du vieillissement comme l'inflammaging[27]. Il n'empêche que des maladies humaines peuvent être modélisées chez *C. elegans* comme la maladie d'Alzheimer[28]. Pour les études présentées dans ce travail, nous avons choisi de travailler avec la souche CL2006, qui exprime un mini-gène humain β sous contrôle d'un promoteur constitutif spécifique du muscle (*unc-54*) qui conduit à l'accumulation de dépôts immunoréactifs A β et d'amyloïde intracellulaires, conduisant à un phénotype de paralysie progressive. Dans cette souche, les vers accumulent l'A β dans les muscles de la paroi du corps[29]. Le modèle *C. elegans* est depuis longtemps utilisé pour l'étude de la restriction calorique, il existe huit différents protocoles décrits[30]. Par contre en ce qui concerne les

régimes enrichis en glucose, le modèle n'est utilisé que depuis 2009[31,32]. Il a été montré que le glucose accélère le vieillissement en activant la voie de l'insuline-IGF1 ce qui a pour conséquence de réprimer le facteur de transcription DAF-16/FOXO.[33] Malgré nombre d'études ayant exploré le rôle du glucose chez *C. elegans*, le modèle reste à être homogénéisé pour permettre la comparabilité des résultats. Enfin l'un des grands avantages de *C. elegans* en tant qu'organisme modèle est son accessibilité au criblage à haut débit. Le phénotype de vieillissement de *C. elegans* peut être évalué manuellement en mesurant différents paramètres phénotypiques tels que le comptage des courbures corporelles par minute, la vitesse de déplacement, le taux de pompage pharyngial[34] ainsi que les taux de paralysie dans le modèle de maladie d'Alzheimer CL2006[29]. Bien que ces mesures manuelles aient conduit à des découvertes importantes en ce qui concerne les voies et les mécanismes moléculaires, elles sont à faible débit, demandent beaucoup de travail, prennent beaucoup de temps, limitant par conséquent le nombre d'animaux dans chaque expérience et étant sujettes aux erreurs humaines et à la subjectivité de l'analyse, ce qui rend difficile l'obtention d'une bonne significativité statistique. La communauté de chercheurs utilisant *C. elegans* doit développer une méthode de criblage reproductible, automatisée, rapide et rentable[35].

Hypothèse de travail

Notre hypothèse est que le métabolisme glucidique accélère le vieillissement. Etant donné la complexité du phénomène étudié nous l'avons abordé sous deux angles : l'étude de l'excès et de la restriction glucidique. L'objectif à long terme de la recherche sur le vieillissement est de développer des médicaments qui permettront de retarder les effets négatifs du vieillissement et des maladies liées au vieillissement / d'augmenter la santé. L'un des atouts de notre organisme modèle choisi, *C. elegans*, est la possibilité de mise en culture d'un nombre élevé de vers, ce qui permet de tester des quantités importantes de composés lors de criblages haut débit[35]. L'élément clé manquant aux méthodes de criblage existantes est la capacité d'évaluer les phénotypes fonctionnels du vieillissement et des maladies liées à l'âge, ainsi que le manque d'automatisation. Il était donc essentiel de développer une méthode de dépistage permettant une évaluation phénotypique automatisée et rapide de plusieurs médicaments potentiels. Nous avons développé une méthode de criblage semi-automatisée afin de répondre à ces questions. Notre hypothèse de travail est que le glucose et son métabolisme conduisent au vieillissement. La complexité du processus de vieillissement nous a amené à l'étudier sous deux angles différents.

La restriction alimentaire diminue l'exposition totale au glucose et conduit à un schéma particulier d'expression génique métabolique qui favorise l'utilisation de substrats alternatifs au glucose. Nous nous sommes concentrés sur le rôle de l'histone acétyl transférase CBP dans la promotion de ce modèle d'expression génique protecteur et qui tend à diminuer le métabolisme du glucose.

Les régimes riches en glucose accélèrent le vieillissement. Parmi les produits qui s'accumulent dans les organismes soumis à une alimentation riche en glucose, les recherches indiquent que les produits de glycation avancés sont potentiellement responsables des effets néfastes sur le vieillissement. En effet, ils sont associés au stress oxydatif cellulaire ainsi qu'aux complications du diabète et d'autres maladies liées à l'âge. Nous avons cherché à caractériser davantage l'organisme modèle *C. elegans* soumis à un régime alimentaire riche en glucose, en nous concentrant sur les différentes voies métaboliques activées, les produits de glycation avancée endogènes ainsi que le reflet du stress oxydant engendrés.

La première partie de notre travail, basée sur les études précédentes menées dans le laboratoire de professeur Charles MOBBS[14], consistait à confirmer le rôle protecteur unique de CBP en tant que histone acétyl transférase (HAT) sur la santé et la durée de vie d'un modèle de maladie d'Alzheimer de *C. elegans*. Il s'agissait de la souche CL2006 qui constitue un modèle bien établi de *C. elegans* pour la protéotoxicité et la maladie d'Alzheimer. Ce modèle développe une paralysie en vieillissant en raison de l'agrégation de la protéine humaine A β _{-1,42} (forme toxique du peptide β -amyloïde (A β) qui est le constituant majeur des plaques séniles, pathognomonique du cerveau des patients Alzheimer) dans la paroi musculaire du ver. Les vers transgéniques manipulés expriment A β _{-1,42} sous le promoteur constitutif des muscles de la paroi corporelle *unc-54*. Ces modèles ont permis de déterminer que la toxicité A β _{-1,42} dépend fortement de gènes induisant la longévité, tels que DAF-2 et DAF-16[28]. La deuxième partie du travail consistait à développer une méthode de criblage semi-automatique appliquée à *C. elegans* qui permettait une évaluation phénotypique et que nous avons validée avec le criblage de 2560 composés pharmaceutiques de la "banque SPECTRUM Microsource". Enfin, dans le laboratoire du professeur Eric BOULANGER, nous avons examiné le métabolisme du vieillissement sous un autre angle, en nous concentrant sur les effets d'un régime riche en glucose, dans le but de caractériser et d'homogénéiser *C. elegans* comme modèle d'étude en se concentrant sur les différentes voies métaboliques du glucose impliquées, l'expression de *fgt-1*, le seul transporteur du glucose identifié jusqu'ici[3], les signes directs et indirects de stress oxydatif et la formation de AGE endogènes.

Matériels et Méthodes

Matériels

Présentation des souches : Deux souches de *C. elegans* ont été étudiées dans nos expériences : la souche sauvage N2 et la souche CL2006, modèle de la maladie d'Alzheimer obtenues du CGC (Caenorhabditis Genetics Center) de l'Université de Minnesota.

Tous les vers sont nourris par la souche OP50 d'*Escherichia coli* obtenue au CGC également.

Synchronisation des souches : Une des caractéristiques intéressantes de l'organisme modèle *C. elegans* est la possibilité d'obtenir des populations synchronisées de vers au moyen de protocoles simples, tels que le NaOH. Avant chaque expérience une synchronisation permettant de récupérer des œufs est réalisée.

Milieus de culture : Les vers sont cultivés en milieux solide ou liquide. Le milieu naturel du ver est un milieu solide. La culture des vers dans un milieu liquide est principalement motivée par le gain de place considérable qu'elle permet, ceci était indispensable pour les expériences d'extraction d'ARN et le criblage qui nécessitent tous deux un nombre très important de vers. Grâce aux mutations génétiques, la fonction des gènes est déduite dans des organismes chez lesquels la séquence des gènes est altérée. En génétique inverse, l'étude fonctionnelle d'un gène commence par la séquence du gène plutôt que par un phénotype mutant. À l'aide de diverses techniques, la séquence d'un gène est modifiée et son effet sur le développement ou le comportement de l'organisme est analysé.

L'ARN interférence est un processus conservé au cours de l'évolution conduisant à un 'silencing' génique post-transcriptionnel déclenché par l'ARN double brin (ARNdb, duplex) ou un petit ARNdb interférant (siARN). Les siARN sont reconnus par le complexe de 'silencing' induit par l'ARN (RISC). Le complexe effecteur RISC ARNi a pour fonction de dérouler les duplex de siRNA pour permettre une liaison précise à la cible d'ARNm complémentaire, suivie du clivage du duplex de l'ARNm et de la dégradation de l'ARNm. Une deuxième caractéristique de l'ARNi qui opère chez *C. elegans* à la différence d'autres organismes, comme la drosophile ou le mammifère, est la capacité d'être appliqués à l'échelle systémique. Un ARNi dit 'systémique' fait référence à l'introduction d'ARNdb dans un tissu, et qui conduit à la désactivation d'ARN dans des cellules distantes. Ainsi fonctionne la méthode d'alimentation d'une bactérie résistante à la RNase III (souche HT115 *E. coli*) exprimant l'ARNsb de *C. elegans* pour administrer des ARNdb chez *C. elegans*. Les ARNi de ces études ont été obtenus à partir de la banque d'alimentation ARNi développée par le laboratoire Ahringer. HT115 contient de l'IPTG (β -D-1-thiogalactopyranoside d'isopropyle), un imitateur moléculaire de l'allolactose, déclenchant la transcription de lac opéron et de la séquence associée à celui-ci

inductible par l'ARN polymérase T7 (polymérase virale avec sa propre séquence promotrice) et une perturbation du gène RNase III (l'enzyme qui se lie spécifiquement à, et clive l'ARN double brin), et marqué avec la résistance à l'ampicilline. Ces bactéries produisant des ARNi ont permis de nourrir les vers à partir du premier jour de l'âge adulte. Le choix des HAT à inhiber a été déterminé comme suit : pour déterminer si CBP avait un rôle unique sur le vieillissement et la maladie d'Alzheimer dans nos organismes modèles, nous avons déterminé quelles HAT devaient être testées avec BLAST (outil de recherche d'alignement local de base). Une recherche BLAST permet à un chercheur de comparer une séquence de requête avec une bibliothèque ou une base de données de séquences et d'identifier les séquences qui ressemblent à la séquence de requête. Pour chaque HAT humaine, nous avons mené une recherche protéine-protéine BLAST afin d'identifier les protéines de *C. elegans* présentant les homologies les plus élevées à utiliser dans nos études. Les HAT testées dans nos expériences ont été : hat-1, mys-1, mys-2 et ada-2.

Développement d'un outil de criblage à haut-débit : Des méthodes de criblage à haut débit existent pour *C. elegans* mais elles ne permettent pas d'analyse phénotypique. Il s'agit d'un problème qui limite la portée des études sur le vieillissement, de nombreuses maladies associées à l'âge modélisées chez *C. elegans* étant étudiées au moyen de changements phénotypiques à mesure que la maladie modélisée s'aggrave. Nous avons pour objectif de développer un outil permettant d'analyser le phénotype de paralysie de la souche CL2006, un modèle de la maladie d'Alzheimer chez *C. elegans*. Comme mentionné précédemment, le phénotype de paralysie dans CL2006 est lié à l'accumulation de peptide A β ₁₋₄₂ humain dans la paroi musculaire du ver, conduisant à une paralysie progressive et à l'imitation du développement de la maladie d'Alzheimer. Cela signifie que l'analyse de la paralysie permettrait indirectement l'étude du développement de la protéotoxicité. La distribution des composés biopharmaceutiques dans les plaques 384 puits et l'enregistrement des expériences étaient entièrement automatisés, mais la distribution des vers et l'analyse des images ont été effectués manuellement, c'est pourquoi nous qualifions notre criblage de 'semi-automatisé'. Le microscope Lab Minion a été développé en tant qu'outil de criblage semi-automatisé pour permettre le criblage rapide à grande échelle de composés protégeant contre la protéotoxicité induite par l'A β ₁₋₄₂ humaine dans un modèle *C. elegans* de la maladie d'Alzheimer, vers CL2006.

Pour tester notre outil de criblage semi-automatisé (Lab Mimion Microscope), nous avons choisi de cribler une bibliothèque de composés SPECTRUM de Microsource à grande échelle

pour rechercher des composés protecteurs contre la protéotoxicité induite par l'A β ₁₋₄₂ chez l'homme dans un modèle *C. elegans* de la maladie d'Alzheimer, CL2006.

La collection SPECTRUM de Microsource est une bibliothèque de 2560 composés biologiquement actifs et structurellement divers, comprenant presque tous les médicaments approuvés par la FDA ainsi que des produits naturels purs [MicroSource Discovery Systems Inc., Gaylordsville, CT; <http://www.msdiscovery.com> (Blobaum 2015)]. Les composés sont dissous dans du diméthylsulfoxyde (DMSO), qui est souvent utilisé dans la recherche médicale en tant que véhicule pour des études in vivo et in vitro. La bibliothèque SPECTRUM est composée d'environ 60% de médicaments approuvés cliniquement, de 25% de produits naturels et de 15% de composés de novo. Cette banque de produits permet d'évaluer des médicaments et des outils biochimiques ayant des profils biologiques connus tout en explorant le potentiel de nouveaux produits naturels purs. Chaque composé de la bibliothèque SPECTRUM a une pureté minimale de 95%. La bibliothèque de composés est gérée par le centre de criblage haut débit de l'école de médecine Icahn au mont Sinai.

Méthodes

Études de survie de C. elegans : Des analyses de survie ont été effectuées pour évaluer l'effet des régimes enrichis en glucose, de l'inhibition du métabolisme du glucose (analyse 2DG) et de différents effets de HAT sur la durée de vie. En l'absence de tout mouvement après stimulation des vers, ceux-ci étaient considérés morts. Trois expériences indépendantes ont été effectuées avec un minimum de 30 animaux chacune.

Études de paralysie : Toutes ces études ont été effectuées avec le modèle CL2006 dans un milieu solide et liquide. Après stimulation des vers, s'ils ne parvenaient pas à mobiliser une autre partie du corps que leur tête ou qu'ils faisaient moins de deux courbes avec leur corps dans un milieu liquide, ils étaient considérés comme paralysés.

Mesures d'expression génique : qRT-PCR ; Des analyses ont été réalisées en qRT-PCR pour déterminer l'expression de différentes enzymes ayant un rôle dans le métabolisme glucidique et la réponse anti-oxidante cellulaire sur des populations de vers cultivés dans des milieux liquides plus ou moins enrichis en glucose.

Détection de carboxyméthyllysine en Western Blot et détection de protéines carbonylées : Des analyses en western blot ont été réalisées pour quantification de N(6)-Carboxyméthyllysine (CML) (un AGE) endogène en fonction de l'exposition (dose-temps) des vers au glucose. Une analyse à l'aide d'un kit a permis la mesure de protéines carbonylées produites selon l'exposition des vers au glucose.

Criblage de la banque SPECTRUM : Pour notre criblage, les différentes étapes définies par le programme de Lab Minion étaient les suivantes: 1 / filmer une lettre ou un numéro d'identification de rangée sur la plaque 384 puits, 2 / stimulation des vers par choc électrique dans un puits testé, 3 / simultanément le filmage du puits pour analyse pendant 90 secondes par puits 4 / stockage des films dans un fichier informatique 5 / le bras d'électrode passe au prochain puits du rang pour stimuler et ainsi de suite jusqu'au dernier puits du rang.

Les analyses statistiques ont été réalisées dans graphPad.

Résultats

L'inhibition de CBP accélère de façon unique le vieillissement et la protéotoxicité dans un modèle C. elegans de la maladie d'Alzheimer

Étude de paralysie : Dans cette étude, nous avons déterminé les effets de l'inhibition avec l'ARNi de différentes histones acétyl transférases (HAT) sur l'apparition de la paralysie d'un modèle de *C. elegans* pour la maladie d'Alzheimer. Il y avait six groupes de vers, chaque groupe étant constitué de 30 vers avec un total de 180 vers. Les vers ont été laissés en présence d'ARNi pendant toute la durée de l'expérience et n'ont pas manqué de nourriture pendant toute la période de l'expérience. Les vers ont été classés vivants, morts ou paralysés selon les méthodes décrites ci-dessus. L'inhibition de *cbp-1* était la seule inhibition de HAT qui a accéléré l'apparition de la paralysie dans ce modèle de *C. elegans* pour la maladie d'Alzheimer. 29,6% des vers CL2006 du groupe témoin (L4440) étaient paralysés le douzième jour de l'âge adulte. En comparaison, le groupe ARNi de *cbp-1* a considérablement accéléré la paralysie, avec une paralysie maximale de 61% au jour 12 de l'âge adulte ($p = 0,0194$). Le début moyen de la paralysie dans le groupe ARNi de *cbp-1* était le onzième jour de l'âge adulte. L'inhibition de toutes les autres HAT testées dans l'étude n'a pas accéléré de manière significative le début de la paralysie par rapport au groupe témoin L4440 ($p > 0,05$) au cours des douze premiers jours de l'âge adulte. Au jour 12 de l'âge adulte, moins de 50% des vers du groupe témoin ou des groupes *hat-1*, *mys-1*, *mys-2* et *ada-2* étaient paralysés.

Étude de survie : Dans cette étude, nous avons déterminé les effets de l'inhibition de différentes histones acétyl transférases (HAT) avec la méthode d'ARNi sur la survie d'un modèle de *C. elegans* pour la maladie d'Alzheimer. Les groupes ayant une durée de vie moyenne réduite suggèrent une paralysie accélérée et une mortalité liée à la paralysie. Les vers CL2006 ont été classés comme morts, vivants ou paralysés, comme décrit dans le chapitre précédent.

L'ARNi de *cbp-1* est la seule condition ayant entraîné une accélération significative de la mortalité par rapport au témoin L4440 ($p = 0,0006$). La durée de vie médiane après inhibition

de cbp-1 était de 16 jours, soit 15,8% de moins que dans le groupe témoin. Il n'y avait pas de différence significative entre le groupe de contrôle et les groupes mys-1, mys-2, ada-2 et hat-1. Les inhibitions de mys-2, ada-2, hat-1 par l'ARNi ont en fait augmenté la durée de vie de 13,6% par rapport au groupe témoin, bien que cela n'ait pas été significatif.

Criblage haut-débit semi-automatisé

Nous avons mis au point un outil de criblage de phénotype pour *C. elegans* semi-automatique. Le microscope motorisé Lab Minion a permis la lecture automatisée de plaques à 384 puits et la stimulation des vers à grande échelle afin d'analyser les changements de mobilité après une exposition à long terme à différents composés, l'une des parties les plus consommatrices en temps dans les expériences de criblage à haut débit. Nous avons utilisé notre outil pour cribler une banque de composés gérée par le centre de criblage à haut débit de l'école de médecine Icahn au mont Sinaï. Notre outil a permis de générer des données pour 2560 composés en moins de 2 mois. Chaque plaque de 384 puits a été divisée en trois et étudiée séparément pour faciliter l'enregistrement et la préparation expérimentale de la plaque. Nous avons appliqué notre méthode de criblage récemment mise au point pour rechercher des composés retardant la paralysie chez les adultes CL2006. Nous avons examiné une banque commerciale d'agents pharmacologiques ayant des cibles humaines connues ou présumées: la banque SPECTRUM de 2560 composés.

Chaque composé a été testé à une concentration de 6 μ M (le choix de cette concentration est un compromis entre les possibilités de concentration de produit par la machine de distribution disponible sur site et la quantité de liquide nécessaire à la survie des vers dans les puits) sur une population moyenne de 10 ± 3 vers. Nous avons testé les effets de chaque composé sur la paralysie les dixième et onzième jours de l'âge adulte. L'analyse de la paralysie a été réalisée lorsque plus de 75% des vers étaient considérés comme paralysés ou morts dans les puits témoins. Parmi les 2560 composés testés, nous avons obtenu 130 'hits' définis comme les médicaments les plus efficaces pour retarder la paralysie par rapport à notre témoin pour chaque plaque. Parmi les 20 meilleurs composés, 9 sont connus pour avoir des mécanismes d'action protecteurs dans la maladie d'Alzheimer et ont été testés comme traitement de la maladie. Le composé le plus efficace identifié dans le dépistage était l'acide phénylbutyrique, un inhibiteur d'HDAC dont l'action correspond à celle du CBP avec 88% des vers encore en bonne santé à l'âge de 12 jours.

L'excès de glucose module la réponse anti-oxidante, le métabolisme du glucose et la survie de C. elegans

Concernant les études en situation d'excès de glucose, voici l'ensemble de nos résultats : la mesure de la concentration en glucose dans les surnageants de culture et les lysats de vers et l'analyse de l'expression du transporteur de glucose confirment que l'exposition à la concentration en glucose et l'ingestion de vers sont stables pour toute la durée des différentes expériences réalisées ; la concentration de glucose dans le régime alimentaire n'affecte pas l'expression relative des enzymes des différentes voies du métabolisme du glucose hormis l'expression de sorbitol déshydrogénase; l'expression relative de la sorbitol déshydrogénase diminue avec le temps et l'exposition à la concentration excessive de glucose ; parmi les enzymes impliquées dans la réponse anti-oxidante, seule l'expression de *sod-3* et *gst-4* est augmentée de façon significative avec une exposition croissante dans le temps de glucose ; il n'y a pas de modification de l'expression relative de la glyoxalase avec le temps ou la concentration de glucose ; la carbonylation protéique dépend de la concentration et du temps d'exposition au glucose ; la glycation endogène augmente avec le temps d'exposition et la concentration de glucose ; la longévité décroît avec la concentration de glucose à laquelle sont exposés les vers.

Discussion et perspectives

Discussion

Les résultats de nos expériences menées dans l'organisme modèle *C. elegans*, ayant exploré soit une restriction du glucose, soit un excès de glucose, convergent vers les mêmes conclusions sur le rôle du glucose, et plus précisément du métabolisme du glucose dans le vieillissement.

Dans la première partie de notre travail, nous avons pu confirmer le rôle unique du CBP en tant que HAT dans le vieillissement et les maladies liées au vieillissement[14]. Ceci a des implications majeures en ce qui concerne le métabolisme du glucose car il a été démontré que CBP est responsable de la favorisation de l'utilisation de sources d'énergie alternatives au glucose, comme le montre la restriction alimentaire lorsque le glucose est rare[36]. Il est intéressant de noter que ce résultat a été corroboré dans notre criblage à grande échelle pour des composés protecteurs contre la pathologie A β ₁₋₄₂ chez *C. elegans*. Le médicament le plus protecteur trouvé était l'acide phénylbutyrique, un inhibiteur d'HDAC approuvé par la FDA (Food and Drug Administration), dont l'action amplifie celle du CBP. L'acide phénylbutyrique fait maintenant l'objet d'un essai clinique multicentrique de phase 2.

Limites

Parmi les limites de nos études, nous pouvons signaler l'absence de vérification de l'efficacité de l'inhibition génique par notre méthode d'ARNi, notre incapacité à répliquer les résultats des essais de paralysie dans un milieu liquide. Nous n'avons pas pu vérifier l'ingestion des composés pharmaco chimiques par nos vers lors du criblage haut-débit, comme cela a été fait pour les ingestions de glucose dans les milieux enrichis en glucose, cela aurait nécessité des dosages de lysats de vers pour les 2560 composés et n'était techniquement pas possible dans le temps imparti. Concernant les mesures qRT-PCR d'expressions ARN pour les enzymes impliquées dans les différentes voies métaboliques alternatives du glucose et des enzymes impliquées dans la défense anti-oxidante, nous n'avons mesuré l'expression que de quelques enzymes, ces travaux doivent être complétés pour offrir une vision plus juste et adaptée du métabolisme glucidique en situation d'excès de glucose. Nous n'avons pas testé la carbonylation de la protéine pendant les premières étapes de notre expérience, ce qui aurait été intéressant pour pouvoir émettre une hypothèse sur l'origine du stress oxydatif. Avec l'augmentation du stress oxydatif au fil du temps, nous aurions pu émettre une hypothèse de corrélation entre le stress oxydatif et l'augmentation de la glycation des protéines. Malheureusement, les contraintes de temps ne nous ont pas permis de mener à bien ces études.

Perspectives

De nombreuses perspectives s'ouvrent à nous suite aux résultats obtenus : le β -hydroxybutyrate inhibe spécifiquement les HDAC de classe I (HDAC 1, 2, 3 et 8), et les niveaux de β -hydroxybutyrate dans le sang sont fortement corrélés à l'acétylation de l'histone H3 à K9 et K14. Ces mêmes schémas d'acétylation sont induits par une restriction alimentaire, un jeûne et une perfusion d'hydroxybutyrate chez la souris[37]. Il est donc très intéressant que les principales cibles de l'acétylation de la CBP soient H3, K9 et K14. Ces études soutiennent donc fortement l'hypothèse selon laquelle les effets protecteurs de la restriction alimentaire et du régime cétogène (régime à teneur très faible en glucides compensé par une haute teneur en lipides) sont médiés par un mécanisme commun, l'inhibition des HDAC de classe I conduisant à une activité de CBP relativement accrue. Le potentiel thérapeutique d'activation de l'acétylation dans les maladies liées à l'âge semble prometteur. Comme d'autres groupes l'ont montré[31,38,39], nous confirmons qu'une exposition de *C. elegans* à un régime enrichi en glucose conduit à la production d'AGE endogène. Nous avons également caractérisé la présence plus spécifique de la CML, l'un des AGE les plus étudiés. Ce n'était pas une conclusion qu'on aurait pu attendre car la production d'AGE nécessite généralement beaucoup de temps et des températures élevées, ce qui n'est pas le cas de la culture de *C. elegans* puisque cet animal vit normalement 3 semaines et à une température de seulement 20 ° C. Dans notre laboratoire, les

résultats préliminaires montrent que l'ingestion d'AGE par notre organisme modèle, *C. elegans*, est possible et conduit à une réponse anti-oxydante. *C. elegans* semble donc être un modèle valable pour l'étude des produits de glycation d'origine endogène et exogène.

Une fois de plus, l'organisme modèle *C. elegans* a contribué à améliorer nos connaissances sur les mécanismes sous-jacents au vieillissement. En tant qu'organisme multicellulaire ayant une courte durée de vie et des voies de métabolisme du glucose similaires à l'homme, la possibilité de modéliser des maladies liées à l'âge telles que la maladie d'Alzheimer et son accessibilité à des criblages haut-débit en font un modèle utile. L'excès (comme nos mesures stables du glucose dans les lysats de vers le montrent) et le manque de glucose (*C. elegans* est l'un des modèles les mieux développés pour étudier les restrictions alimentaires[30]) sont facilement contrôlés dans cet organisme modèle par rapport à d'autres organismes utilisés pour l'étude du vieillissement à courte durée de vie, tels que la drosophile.

Nos travaux ont permis de proposer une théorie unificatrice d'un mécanisme possible du vieillissement et du développement de la maladie d'Alzheimer. Le métabolisme du glucose inhibe daf-16 / FOXO, qui recrute le CBP. Ceci conduit à une augmentation de la glycolyse, responsable de la réduction du rapport NADH / NAD⁺ ainsi que de l'augmentation de la production d'AGE et de l'activation de RAGE, ce qui entraîne une augmentation de l'inflammation et du stress oxydatif, connus pour accélérer le vieillissement et les maladies liées à l'âge.

Jusqu'à présent, aucun traitement capable de retarder (ou d'inverser) les maladies liées à l'âge n'a été développé malgré nos connaissances avancées sur les mécanismes du vieillissement. La responsabilité du métabolisme du glucose, des AGEs, du RAGE et du rôle protecteur du CBP dans le vieillissement suggère des possibilités prometteuses pour le développement de médicaments à l'avenir. Dans l'intervalle, ces résultats appellent des politiques de santé publique visant à réduire l'ingestion excessive de sucres.

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Glossary

2DG: 2-deoxyglucose
A β : β -amyloid peptide
AD: Alzheimer's disease
AGE: Advanced glycation end products
AMPK: 5' adenosine monophosphate-activated protein kinase
ATP: Adénosine tri-phosphate
CBP: CREB binding protein
CREB: cAMP response element-binding protein
DAG: Diacylglycerol
DHAP: Dihydroxyacetone phosphate
DMSO: Dimethylsulfoxide
DNA: Deoxyribonucleotide
DR: Dietary restriction
F21D5.1: *C. elegans* ortholog of the human phosphoacetyl glucosamine mutase
F344: rats Fisher 344
FOXO: Forkhead Box O
FUDR: 5-fluorodeoxyuridine
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GDP: Gross domestic product
glod-4: glyoxalase
GLUT: Glucose transporter
GNAT: General control non repressible 5-related N-acetyltransferase
GnRH: Gonadotropin-releasing hormone
GST 4, 14: Glutathione S-transferase
gsy-1: glycogen synthase,
HAT: Histone acetyl transferase
HDAC: Histone deacetylase
hvk-1: hexokinase 1
IPTG: Isopropyl β -D-1-thiogalactopyranoside
LHRH: Luteinizing hormone releasing hormone
mTOR: mammalian Target of Rapamycin
MYST: MOZ, Ybf2 (Sas3), Sas2, and Tip60 (four founding members of the MYST HAT family)
NAD: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NF κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells
NGM: Nematode growth medium
OD: Optical density
P43: growth hormone receptor knock-out mice
PI3K: Phosphatidyl inositol 3-kinase
RISC: ribonucleic acid induced silencing complex
RNAi: Ribonucleic acid interference
siRNA: short interfering ribonucleic acid = small interference ribonucleic acid = silencing ribonucleic acid
dsRNA: double stranded ribonucleic acid
SCM: S-complete medium
SOD 1, 3:Superoxidismutase

sodh-1: sorbitol dehydrogenase
SGLT: Sodium glucose transport protein
T25B9.9: 6-phosphogluconate dehydrogenase
TCA cycle: Tri carboxylic acid cycle
TGF β : Transforming growth factor beta
tps-1/2: trehalose phosphate synthase 1/2
VEGF: Vascular endothelial growth factor
WHO: World health organization

CHAPTER I INTRODUCTION AND BACKGROUND TO RESEARCH

General introduction

Glucose is the ultimate energy source for most living organisms and it has been shown that glucose impacts lifespan. Glucose excess is detrimental to lifespan and cause for many age-associated diseases such as diabetes mellitus. On the contrary, glucose deprivation, in the case of dietary restriction interventions increases lifespan and healthspan across a variety of species. The mechanisms behind this are still not fully understood. Thanks to the model *Caenorhabditis elegans* (*C. elegans*), we aimed at better understanding the mechanisms underlying how glucose modifies lifespan.

Our results further our understanding of the protective mechanisms of dietary restriction, shown to be a nutritional state of decreased glucose as well as contributed to establishing *C. elegans* as a valuable model organism for the study of high glucose diets, responsible for accelerating aging. As the ultimate goal of aging research is to develop potential targets with protective effects on lifespan and healthspan we also developed a semi-automated screening method applied to *C. elegans* which will allow us to test compounds based on our understanding of fundamental mechanisms of aging.

Background to research

This chapter is divided into three parts. In the first part, we will review glucose mechanisms, initially in a physiological setting, then in two opposite situations, one of dietary restriction (glucose deprivation) and one of glucose excess. In the second part, we will explore the state of the art for aging and in the third part, we will present our model organism: *C. elegans*.

I.1 Glucose transport metabolism and function - Introduction to carbohydrates and glucose

I.1.1 Glucose transport, metabolism and function in a physiological setting

I.1.1.1 Glucose

Glucose is a carbohydrate or sugar. The suffix -ose indicates that it is a sugar. Because glucose cannot be hydrolyzed into a smaller carbohydrate, it is a monosaccharide, also called simple sugar and is the most basic unit of carbohydrates.

Glucose is the most abundant monosaccharide, containing six carbons (making it a hexose) and an aldehyde group (making it an aldose). It is therefore an aldohexose. Many of these mono and disaccharides are referred to as sugars because of their sweet taste and accordingly, the

word glucose originates from the ancient Greek τὸ γλεῦκος glykys, sweet. Glucose was first isolated from grapes by the German chemist Andreas Marggraf in 1747 (Marggraf: *Expériences chimiques faites dans le dessein de tirer un véritable sucre de diverses plantes, qui croissent dans nos contrées*. In: *Histoire de l'académie royale des sciences et belles-lettres de Berlin* (1747), p. 90: *Les raisins secs, étant humectés d'une petite quantité d'eau, de manière qu'ils mollissent, peuvent alors être pilés, & le suc qu'on en exprime, étant dépuré & épaissi, fournira une espèce de Sucre*). The name glucose was given to the sugar isolated in raisins, starch and honey in 1838 by a committee of the French Academy of Sciences composed of French chemists and physicists Thenard, Gay-Lussac, Biot and Dumas (Comptes rendus hebdomadaires des séances de l'Académie des sciences / publiés par MM. les secrétaires perpétuels, juillet 1838, site de la bibliothèque nationale de France, <https://gallica.bnf.fr/ark:/12148/bpt6k29662/f106>).

The molecular formula of glucose is $C_6H_{12}O_6$ or $H-(C=O)-(CHOH)_5-H$. The naturally most common occurring form is D-glucose, as its rare enantiomere L-glucose is inaccessible to enzymatic breakdown.

Glucose is an aldose with hydroxyle groups -OH disposed in a determined configuration along a chain composed of six atoms of carbon. The molecule can adopt a linear or cyclical configuration. The three following figures illustrate the cyclical, linear and 3D form of glucose. The figure 1 hereafter illustrates the 2D, 3D and Fischer structure of D-Glucose.

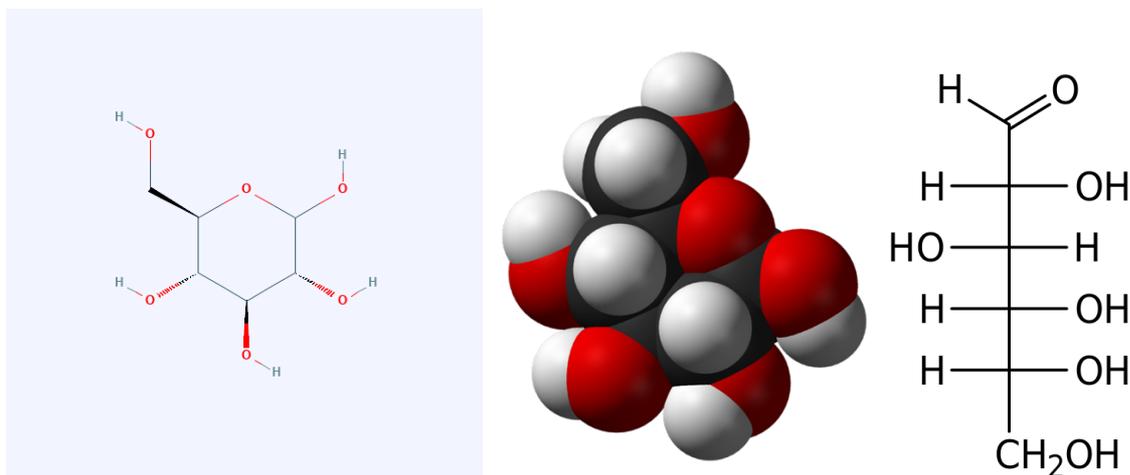


Figure 1: Schematic representations of glucose, from left to right Glucose 2D structure, 3D structure and Fischer representation, source: from pubchem.ncbi.nih.gov

Biochemical properties:

Glucose is a primary source of energy and substrate for the synthesis of macromolecules for living organisms and occurs naturally. It can be found in its free state in fruit or other plants,

where it is made from water and carbon dioxide during photosynthesis using energy from sunlight[2].

To produce energy, glucose can be metabolized through different pathways. The most common pathway in physiological conditions is glycolysis. Degradation of glucose *via* glycolysis, produces carbon dioxide and water during a cellular respiration process and constitutes a very important energy source for living cells.

In animals, there are three sources for glucose: food, the breakdown of glycogen during the glycogenolysis or synthesis in the liver and kidneys by the process of gluconeogenesis from non-carbohydrate intermediates, such as pyruvate or glycerol.

Glucose is stored in plants as starch, and in animals as glycogen, a polymer of thousands of monosaccharide units.

Among different available nutrients to living organisms, carbohydrates are the most efficient source in terms of energy production.

Glucose is the most widely used aldohexose in most living organisms, this could possibly be among other reasons, because it is the most abundant D-aldohexose allowing it to be more readily accessible to certain chemical reactions like esterification, and because glucose has a lower tendency than other aldohexoses to react nonspecifically with the amine group of proteins via a non-enzymatic reaction called glycation[40]. Glycation impairs the function of proteins when it occurs. The lower rate of glycation for glucose could be due to its more stable cyclic form compared to other aldohexoses which are more often in a reactive open-chain form.

Glucose is used as an energy source via either aerobic, anaerobic respiration or fermentation. It can be metabolized by glycolysis or the pentose phosphate pathway. In humans, through aerobic respiration it provides 3.75 kilocalories or 16 kilojoules of energy per gram (or 2871kJ per mol): this is the caloric value of glucose (Atlas de poche de physiologie Silbernagl)

1.1.1.2 Glucose degradation: Glycolysis

Physiologically, the first set of reactions allowing the conversion of glucose into usable energy is glycolysis. Glycolysis is relevant to this thesis as it is the source of intermediary metabolites which will, if in excess, be diverted to different pathways implicated in the pathophysiology of diabetes.

Glycolysis is the breakdown of glucose to convert it into pyruvate $\text{CH}_3\text{COCOO}^-$ and ATP (adenosine tri phosphate), accessible energy equivalents, without requiring oxygen, unlike the process of mitochondrial respiration. Glycolysis is a highly conserved set of reactions in the

animal kingdom and is common to virtually all cells, both prokaryotic and eukaryotic. It can also occur in the absence of enzymes, *via* metal-catalysis under oxygen-free conditions in the Archean oceans, hinting to the primeval nature of the pathway[41].

In cells, glycolysis is the sequence of ten enzyme-catalyzed reactions and occurs in the cytosol in eukaryotic cells.

The overall reaction can be summarized as follows:



The understanding of the full glycolysis pathway took more than 100 years to be complete. During his years as dean of the faculty of science in Lille, Louis Pasteur performed key experiments demonstrating that microorganisms were responsible for fermentation, thus elucidating the first steps of the glycolytic pathway and leading to our modern understanding of glycolysis[42].

The full pathway we now know as glycolysis was finally described step by step by Gustav Embden and Otto Meyerhof in the 1930s. The endeavor to determine the steps of the glycolysis pathway are due to the very short lifetime and low steady-state concentrations of the glycolytic reaction intermediates[42].

In humans, non-oxidative glycolysis occurs in erythrocytes, the kidneys, and in part in cardiomyocytes and skeletal muscles. The oxidative glucose degradation occurs mainly in the brain, partly in the skeletal muscles and in most organs.

Description of the glycolysis reaction sequence :

The glycolysis pathway has three stages well described by Berg in his Biochemistry book[2]: Stage 1 is the conversion of glucose into fructose 1,6-biphosphate and can be divided into three steps:

-glucose is phosphorylated by ATP to form glucose-6-phosphate by the enzyme hexokinase. This step adds negative charges to glucose and begins to destabilize glucose, facilitating its further metabolism. Hexokinase requires divalent ions for activity such as Mg^{2+} , catalyzes the first regulatory step of glycolysis, with a low maximum reaction speed and inhibition by high glucose-6-phosphate (a downstream metabolite) concentrations

-the next step is the isomerization of glucose-6-phosphate to fructose-6-phosphate which is the conversion of an aldose to a ketose by phosphoglucose isomerase

-finally a second phosphorylation reaction occurs where the fructose-6-phosphate is phosphorylated by ATP to fructose 1,6-biphosphate by a phosphofructokinase, an allosteric enzyme which sets the pace of glycolysis. This reaction is the first irreversible reaction. Phosphofructokinase activity increases with the $[AMP]/[ATP]$ ratio, allowing a stimulation of glycolysis as cellular energy levels decrease.

Stage 2 corresponds to the transformation of a six-carbon sugar into two three carbon fragments:

- first fructose 1,6-biphosphate is split into two three carbon fragments by an aldolase: glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP), this reaction is reversible;

-next the triose phosphate isomerase (GAPDH) catalyzes the transfer of hydrogen converting dihydroxyacetone phosphate into glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate can indeed be used by the glycolysis pathway to produce ATP whilst DHAP can be decomposed into methylglyoxal and inorganic phosphate. Thus triose phosphate isomerase suppresses an undesired side reaction by accelerating a desirable reaction so that it takes place faster than an undesirable alternative reaction.

Stage 3 allows the extraction of energy via the oxidation of three-carbon fragments:

-glyceraldehyde 3-phosphate dehydrogenase allows the conversion of glyceraldehyde 3-phosphate into 1,3-biphosphoglycerate, which has a high phosphoryl transfer potential. This step also allows the transfer of a hydride ion to a molecule of NAD^+ , producing NADH

-phosphoglycerate kinase transfers the phosphoryl group from 1,3-biphosphoglycerate to ADP, producing ATP and 3-phosphoglycerate

- phosphoglycerate mutase rearranges 3-phosphoglycerate into 2-phosphoglycerate

-2-phosphoglycerate is dehydrated and transformed into a phosphoenolpyruvate by an enolase

-finally, a pyruvate kinase irreversibly transfers a phosphoryl group from phosphoenolpyruvate to ADP, generating ATP and pyruvate. Pyruvate kinase regulation depends on the tissue but is generally inhibited by ATP levels and activated by fructose-1,6-biphosphate

There are three regulatory enzymes in glycolysis: hexokinase, phosphofructokinase and pyruvate kinase (as well as glucokinase, a hexokinase isomer present in the liver). These

enzymes exist at regulation points of the glycolytic process, and indicate points where the intermediates can leave the glycolysis pathway for other processes. This can happen, in hyperglycemic conditions and will be reviewed in the corresponding section. Also note that the first steps of glycolysis are under feedback regulation thus inhibiting excessive use of glucose[1].

After metabolizing glucose in the cytosol through glycolysis, the product of glycolysis, pyruvate is further metabolized in the mitochondria through the TCA cycle (tri-carboxylic acid cycle) also known as the Krebs cycle.

Hereafter are two schematic representations of glycolysis (figure 2 and figure 3).

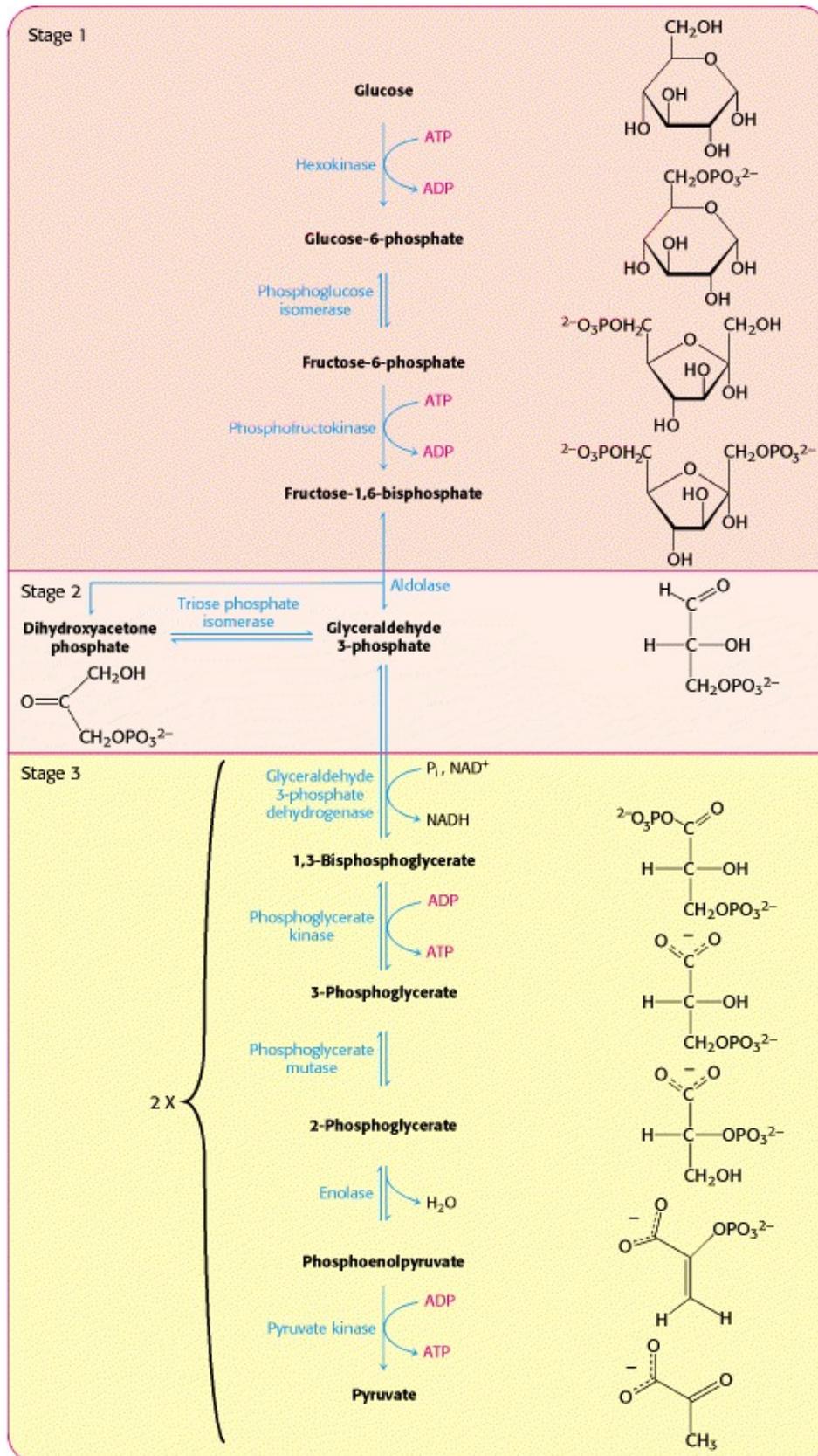


Figure 2: Glycolysis is an energy-conversion pathway in many organisms. Schematic representation of the glycolysis metabolic pathway, source from Berg JM, Tymoczko JL, Stryer L. Biochemistry. 5th edition. New York: W H Freeman; 2002. Section 16.1, available from: <https://www.ncbi.nlm.nih.gov/books/NBK22593/>

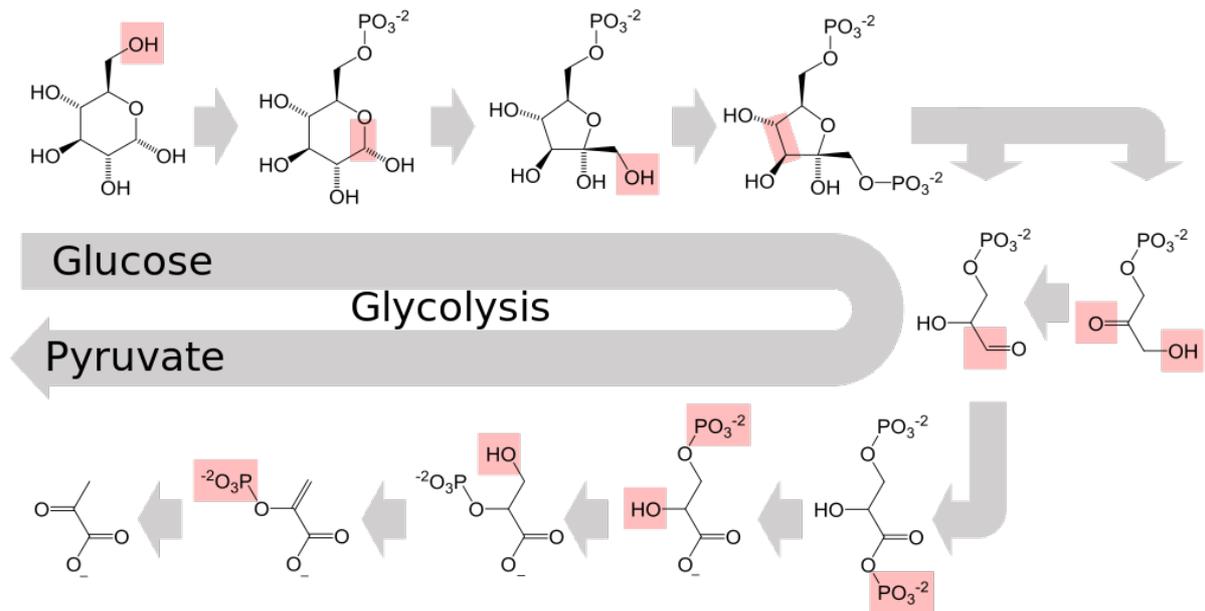


Figure 3: The metabolic pathway of glycolysis converts glucose to pyruvate *via* a series of intermediate metabolites. Each chemical modification (red box) is performed by a different enzyme, schematic representation of the glycolysis metabolic pathway, by Thomas Shafee - Own work, CC BY-SA 4.0, <https://commons.wikimedia.org/w/index.php?curid=38895499>

1.1.1.3 Glucose homeostasis in humans

Glucose homeostasis is fundamental for life, long-term health and the maintenance of glycaemia within a certain range. It is subject to a tight control thanks to two key glucoregulatory hormones: insulin and glucagon. They act amid a whole variety of hormonal, neural and substrate glucoregulatory factors released from fat tissue, or intestinal endocrine cells[43]. In physiological conditions for a healthy human being, glucose homeostasis allows a stable plasma-glucose level under various physiologic conditions such as exercise, feeding and fasting.

Glucose homeostasis is primarily dependent on a balance between glucose absorption by the small intestine, glucose production by the liver and glucose consumption by insulin dependent tissues (muscle, adipose tissue) and non-insulin dependent tissue (brain)[44].

Glucose is the main source of energy for the human brain which lacks storage capacity and therefore requires a continuous supply of glucose. Muscle's main energy source is also glucose but differs from the brain as it does not so crucially depend on external supply, being capable of glucose storage under the form of glycogen[2].

Insulin is a hormone released by the β -cells of the pancreas and causes uptake of glucose by virtually all cells in the body and reduces fat consumption as an energy source. When insulin is absent, as is the case in diabetic type 1 patients, glucose uptake is stopped and fat is used as energy[45]. Glucagon, on the other hand, is a hormone released by the α -cells of the pancreas

and acts as a counter-regulatory hormone of insulin. It is part of the counter-regulatory response system and can cause the release of glucose by the liver to rise plasma glucose in response to hypoglycemia.

1.1.1.4 Glucose transport

Glucose transport is the first limiting step to glucose utilization, and it is therefore important to understand what the key components of these transport systems are as they can participate in the health impacts of glucose.

Because the lipid bilayer in eukaryotic cells is impermeable to hydrophilic molecules, glucose is taken up by two families of glucose transporters: Glucose Transporters (GLUT) which is a facilitative class and Sodium-Glucose Linked Transporter (SGLT) which is a concentrative class. A schematic illustration of glucose transport systems in an epithelial cell is presented in figure 4.

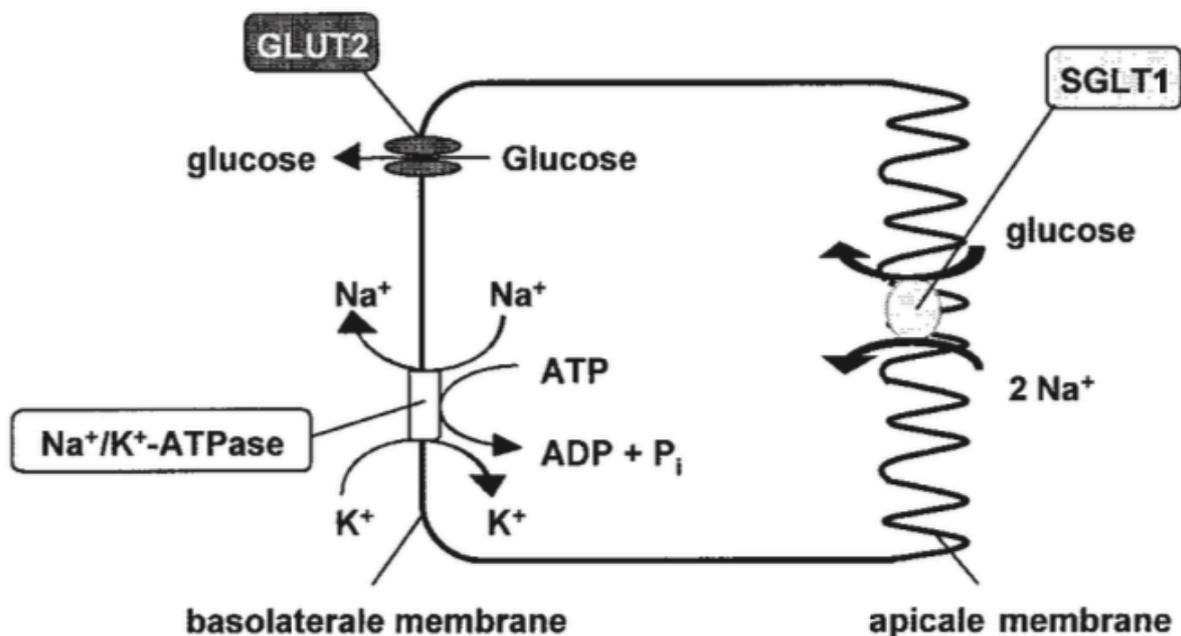


Figure 4: Transport of glucose in intestinal epithelium. Glucose enters into epithelial cells *via* the SGLT located in the apical membrane and leaves the cell *via* GLUT2 located in the basolateral membrane, source from SCHEEPERS, 2004.

The facilitative glucose transporters (GLUT or SCL2) are integral membrane proteins, they mediate passive glucose diffusion across the plasma membrane and are not coupled to energy-requiring components[46]. They can only transport a single substrate at a time down a concentration gradient by binding and releasing glucose or related hexoses according to a model of alternative conformation. The binding of glucose or related hexoses provokes a

conformational modification associated with transport and release on the other side of the cell membrane.

Fourteen GLUT have been identified in the human genome to this day[4]. They share common structure characteristics and have high sequence similarities although each transporter has different transport kinetics, pattern of tissue distribution, substrate specificity and regulatory properties. One or more GLUT proteins are expressed in every cell type of the human body. The ubiquitous expression and redundancy of this family of transporters can be explained by the critical nature of glucose as the main source of energy in humans and mammals in general, and the consequent requirement for multiple glucose transporters with different properties, expressed in a cell-type specific manner.

In mammals, insulin-signaling mediates the translocation and activation of GLUT4 and therefore controls a significant portion of glucose cellular uptake[47]. Presented below are the known mammalian glucose facilitative transporters.

Protein name	Predominant substrates	Tissue distribution and cellular/subcellular expression
GLUT1	Glucose, galactose, mannose, glucosamine	erythrocytes, brain, blood-brain barrier, blood-tissue barrier, many fetal tissues
GLUT2	Glucose, galactose, mannose, fructose, glucosamine	liver, islet of Langerhans, intestine, kidney, brain
GLUT3	Glucose, galactose, mannose, xylose	Brain, (neurons), testis
GLUT4	Glucose, glucosamine	adipose tissue (white and brown), skeletal and cardiac muscle
GLUT5	Fructose	Small intestine, kidney
GLUT6	Glucose	Brain, spleen, leucocytes
GLUT7	Glucose, fructose	Small intestine, colon, testis, prostate
GLUT8	Glucose, fructose, galactose	testis, brain, adrenal gland, liver, spleen, brown adipose tissue, lung (intracellular)
GLUT9	Urate, glucose, fructose	kidney, liver, small intestine, placenta, lung and leucocytes
GLUT10	Glucose galactose	heart, lung, brain, liver, skeletal muscle, pancreas, placenta and kidney
GLUT11	Glucose fructose	Heart, muscle
GLUT12	Glucose	heart, prostate, skeletal muscle, placenta
GLUT13	<i>Myo</i> -inositol	Brain, adipose tissue
GLUT14		Testis

Table 1 SLC2-Facilitative GLUT transporter family (adapted from[48])

The sodium dependent glucose transporters (SGLT) comprise of Na⁺-dependent glucose co-transporters (SGLT1 and 2), sensors (SGLT3) but also inositol, multivitamin transporters (SGLT4 and 6) and thyroid iodide transporter (SGLT5)[5].

For the purpose of this thesis, only the glucose devoted transporters are described in more detail. The SGLT co-transporters are integral membrane proteins that mediate the transport of glucose and with a lesser affinity galactose across the plasma membrane by an active transport mechanism[49]. The concentrative transporters are energized by either H⁺ or NA⁺ gradients, which are generated and maintained by ion pumps.

The SGLT1 is a high-affinity, low-capacity sodium-glucose symporter with a high sodium-to-glucose ration of 2:1. In comparison, the SGLT2 is a low-affinity, high-capacity sodium-glucose symporter with a sodium-to-glucose coupling ratio of 1:1.

The transporter family is mainly expressed in intestinal mucosa of the small intestine (SGLT1), and the proximal tubule of the nephron (SGLT2 and SGLT1) (SCHEEPERS 2004). In the kidneys, 98% of the glucose filtered in the glomerulus is reabsorbed along the nephron via SGLT2. In case of elevated glycaemia, glucose is excreted in urine (glycosuria) because the SGLTs are saturated with glucose and maximum transport capacities are reached.

Human SGLT3 acts as a glucose sensor by conveying information about external glucose concentration to the cell directly through membrane potential or indirectly through another protein such as a coupled G protein[5]. Presented below are the known mammalian concentrative glucose transporters.

Protein	Major expression tissue	Function
SGLT1	Kidney, intestine	Glucose re-absorption in intestine and kidney
SGLT2	Kidney	Glucose re-absorption in kidney
SGLT3	Small intestine, skeletal muscle	Glucose activated Na ⁺ channel

Table 2 Concentrative glucose transporters, adapted from ZHAO and KEATING[50]

Until now, we have focused on the physiological usage of glucose. In the next section, we will present two opposite situations, when glucose metabolism is strongly modified.

I.1.2 Glucose metabolism in a supraphysiological setting

I.1.2.1 Dietary restriction

I.1.2.1.i Brief history, health implications and examples of methods for dietary restriction in several model organisms

Improved glucoregulatory control is a hallmark of successful aging. One of the most direct methods of maintaining glucose homeostasis is through nutritional interventions, the most efficient one being dietary restriction[18].

Throughout history, numerous societies have advocated the health benefits of food limitation. Scholars, from the Ancient Greeks and Romans to Luigi Cornaro in the 15th century and Professor Maurice Gueniot, president of the Paris Medical Academy in 1931[51], have tested the hypothesis of living longer, healthier lives through restricted diet. During the Great Depression, McCay (1935)[52] rediscovered and presented this phenomenon anew to the scientific community when his team unexpectedly (they had predicted the opposite effect) found that by reducing caloric intake in rodents, their lifespan was increased in well-executed experiments.

Since then, numerous researchers have studied this effect and it has become widely accepted in the scientific community that dietary restriction (DR)—restriction in food intake (normally 20% - 40%) without malnutrition and without compromising the maintenance of all essential nutrients[53] —increases the median and maximal lifespans as well as the healthspan in a wide range of organisms[7,54] including yeast, flies, worms, fish, rodents and rhesus monkeys (*Macaca mulatta*). In both rodents and rhesus monkeys, dietary restriction delays loss of function and reduces the incidence of major age-related diseases. Dietary restriction is the most effective method known today to extend lifespan in all species where it was tested, including mammals. The ubiquitous dietary restriction-mediated lifespan extension suggests a possible conservation of longevity-regulating pathways among species[8].

Among the benefits for health induced by dietary restriction we can cite: elimination of white adipose tissue, maintained weight loss, protection against arthritis, brain atrophy delay, lower incidence and progression of cardiovascular diseases and cancer, increase in metabolic flexibility. Research has also demonstrated that dietary restriction reduces the damage mediated by Reactive Oxygen Species (ROS) by a decrease in oxidized lipids, proteins, cellular and mitochondrial DNA, and delay in the decline of cellular antioxidant defense with age[55]. Dietary restriction has been shown to increase antioxidant response pathways, as well as a down-regulate inflammatory pathways. There is also evidence for induction of autophagy, and indirect activation of lifespan mediating transcription factors such as FOXO3A[14,56]. Dietary

restriction is beneficial against age-related diseases in rodents[57]. As shown in several murine models, dietary restriction is able to inhibit spontaneous but also chemically and radiation-induced tumors[6,58,59]. Chronic nephropathies and cardiomyopathies were also shown to be prevented or delayed by dietary restriction[60,61]. This is of particular interest as cancer is the first cause and chronic kidney diseases and cardiomyopathies the second leading causes of death in rodents[57].

Although dietary restriction has been shown to extend lifespan and delay age-related diseases in most species, in nonhuman primates the impact of dietary restriction had remained controversial until recently and as such, questioned the translatability of results to humans. Indeed, two parallel studies conducted in the 1980s (National Institute of Aging - NIA - and University of Wisconsin) on rhesus monkeys reported contradictory results on lifespan and healthspan with results from the NIA study failing to show a significant difference between the control and restricted groups. When the studies were repeated at the University of Wisconsin, they showed once again that long-term dietary restriction significantly improved age-related diseases and all-cause survival[62]. The same research group compared direct longitudinal data (survival, body weight, food intake, fasting glucose levels and age-related morbidity) from both studies as well as with multicentered relational databases of primate aging and concluded that rhesus monkeys from the NIA study were effectively undergoing dietary restriction and that differences in study design explained the differences in study outcomes[9,62]. Further analysis of the data from University of Wisconsin aimed at establishing a 'primate frailty score' based on the well-known Fried criteria used in the elderly, and the results show according to their criteria that dietary restriction could reduce the incidence of frailty in nonhuman primates[63]. These results all tend towards the conclusion that the health benefits of dietary restriction are conserved in monkeys and that the mechanisms are likely translatable to human health[9].

Although to many, dietary restriction would be unfeasible in our societies, recent studies in humans are still being conducted and are uncovering the potential advantages of calorie restriction on aging and age-related diseases, translating the persistent ambivalence toward calorie restriction as a means to improve healthspan in humans. The CALERIE study, as an example, is a multicenter phase 2 study, which showed that after two years of a moderate calorie restriction, multiple cardiometabolic factors were reduced[64].

The goal of dietary restriction research is of course to understand how it works, not to promote it as a lifestyle. Indeed, long-term, severe dietary restriction can increase the risk of anemia, cardiac arrhythmia, impaired reproductive function and osteoporotic bone fractures as is observed in patients with eating disorders such as anorexia nervosa and bulimia nervosa.

Dietary restriction should be seen as a tool to understand human aging which relies on translatability of the effects in primates[62]. In that regard, results from the latest studies with nonhuman primates conducted at the University of Wisconsin are promising[9,62,63].

Here are some of the most widely used methods of dietary restriction in species commonly used to study aging:

In *Saccharomyces cerevisiae*, or baker yeast commonly used dietary restriction protocols include switching cells to a medium containing water instead of nutrients or reducing the amount of glucose in the medium from the standard 2% to 0.5 or 0.05%[6]. These methods both result in an extension of replicative and chronological lifespans (defined in the aging chapter of the manuscript).

Dietary restriction protocols performed with the fruitfly *Drosophila melanogaster* are most often implemented by diluting the concentration of the food medium and therefore of all dietary constituents (yeast, sugar mostly). It has been shown that in response to this dietary restriction, flies do not compensate by increasing food intake but rather increase the volume of their digestive tract[65]. Accurately restricting food for *Drosophila melanogaster* who only consumes up to 5 μ l of food per 24 hours is a challenge. Other methods of dietary restriction, more similar to those of rodents like intermittent feeding methods or daily provision of a limited amount of food were tested but did not show protective results on lifespan[53].

In rodents, dietary restriction increases lifespan up to 60% by delaying many chronic diseases and slowing down the physiological rate of aging[6]. If the animals serving as control have an ad libitum diet (unlimited access to food) of standard laboratory food, the animals submitted to the dietary restriction will be fed 60-70% of the daily intake of the animals in the control group. In practice, calculations are done weekly and brought back to daily intake. When the food consumption physiologically decreases with age in the control group, the quantities are kept stable in the restricted group for the remainder of the study. The severity of the caloric restriction is therefore reduced at the end of the study. Another method commonly used for dietary restriction in rodents and mammals is to determine an average daily food consumption for adult animals for a particular strain and the restricted animals are fed a predetermined percentage of that amount, the whole time of the study. In this condition, the control group is given daily food allotments known to prevent obesity and maintain a good health (usually 10% less than ad libitum food intake). It is possible to simplify the protocol by giving a double allotment every two days[66]. Finally, some researchers choose to apply intermittent fasting (every other day of ad libitum feeding) on their restricted group. Although in this case, the

overall food consumption is only moderately reduced this method of food restriction renders similar metabolic outcomes[67,68].

Dietary restriction methods used in *C. elegans* will be further discussed in a following chapter.

I.1.2.1.ii Different hypothesis for the mechanisms underlying the protective effects of dietary restriction

The relationship between caloric intake and longevity can be represented as a U-shaped curve because malnutrition and dietary excess both impact survival negatively[69].

Since 1935, many mechanisms have been suggested to explain the anti-aging effects of dietary restriction.

Some researchers initially hypothesized that dietary restriction would decrease overall metabolism. Yet, more recent publications have shown that dietary restriction induces stable or even increased mitochondrial electron transport chain function.

Hereafter, are some of the hypothesis briefly presented:

a. Harman theory of aging:

Among the first hypothesis suggested to explain the beneficial effects of dietary restriction, the Harman theory of aging (1956)[70] states that oxidative damage to macromolecules, in cells and tissues exponentially increases with aging. Metabolic use of oxygen is the greatest source of free radicals, thus would decreasing metabolism decrease free radicals? As discussed in the next paragraph, this has been shown not to be the case. The Harman theory gave rise to the oxidative stress theory of aging and finally the mitochondrial theory of aging[7], both reviewed in a later section of the manuscript. It is well established that dietary restriction delays the accumulation of oxidized molecules with age, the mechanism of which has remained controversial: increase in activity of anti-oxidant enzymes such as superoxide dismutase, catalase or glutathione peroxidase or the decrease in formation of reactive oxygen?[7]. This question remains to be answered.

b. Decreased metabolic rate:

In 1977, Sacher[71] suggested that dietary restriction extends lifespan by decreasing metabolic rate. This was based on the human studies showing a reduction in metabolic rate with decrease in food intake. Since then, several studies in different species (mice, rats, rhesus monkeys) have brought out evidence that after a brief period of reduced metabolic rate following the initiation of dietary restriction, metabolic rate was increased to a rate per gram body weight higher or equal than the control, ad libitum group and caloric restriction extends life without a long-term reduction in metabolic rate[72–74].

c. Nutrient sensing pathways and the role of glucose (or the lack of it) in mediating positive effects of dietary restriction:

These pathways are included in the illustrative figure 9 included in the chapter ‘Aging’ and in the figure 5.

It is now well established that nutrient-sensing pathways are key modulators of the aging process and modified by dietary restriction[57].

Metazoans have multiple nutrient-sensing pathways and processes that can affect lifespan: insulin/insulin-like growth factor 1 (IGF1) signaling[10], mechanistic kinase target of Rapamycin (mTOR)[11], AMP kinase[75] and sirtuins[12] among others.

Dietary restriction in mice for example is responsible for down-regulating insulin/IGF1 and mTOR pathways[57] as well as activating AMPK. Epigenetic mechanisms have been strongly linked to nutrient-sensing processes and the effects of DR, most famously those mediated by Sirtuins.

Let us review briefly some of these pathways.

Reduced Growth hormone-IGF1 axis and altered Insulin pathways:

The core of the insulin/IGF-1 signaling pathway is conserved between invertebrates and mammals but the mammalian network has increased in complexity. Three different mammalian insulin/IGF tyrosine kinase receptors have been identified: the insulin receptor (IR), IGF-1 receptor (IGF-1R), and the orphan IR related receptor (IRR). After ligand binding, the activated IGF-1 or insulin receptor phosphorylates several intracellular substrates, which in turn activate two major signaling pathways, the PI-3K-PKB/AKT pathway and the Ras-MAP Kinase pathway. This finally results in the phosphorylation of the FOXO transcription factors, thus inactivating them. Four different members of the FOXO family of transcription factors have been identified (FOXO1A, FOXO3A, FOXO4, and FOXO6)[76].

It was quickly shown that dietary restriction lowered glucose plasma levels, decreased glucose metabolism and increased insulin sensitivity[13,77]. With an increase of insulin sensitivity, less insulin is needed to maintain glucose homeostasis leading to a decrease in circulating insulin concentration. When the loss of function mutations for the insulin IGF-1 receptor and their impact on lifespan were described for several different species[78,79], interest was emphasized on the role of the insulin-GH/IGF-1 signaling pathway. If dietary restriction led to decreased circulating insulin levels and IGF-1 levels, this meant reduced insulin-GH/IGF-1 signaling and increase of nuclear FOXO activity[13].

Dietary restriction induced changes in energetics due to a reduction of glucose utilization and alternative metabolic pathways.

AMP-activated protein kinase (AMPK) senses energy availability (via [NAD⁺/NADH] and [AMP/ATP] ratios) and in consequence regulates some of the pathways or actors implicated in aging such as mTOR and SIRT1. In the case of dietary restriction and glucose scarcity, energy levels will be reduced and AMPK will be activated[75].

The mechanistic Target of Rapamycin (mTOR) is a nutrient sensing protein kinase that coordinates cell growth and metabolism from nutrient and amino acid inputs. The two mTOR complexes mTOR1 and mTOR2 affect lipid metabolism, autophagy and protein synthesis. In the case of dietary restriction, mTOR will be downregulated[11].

The sirtuin family of genes exhibit histone deacetyl transferase (HDAC) activity that remove acetyl groups to enhance expression of target proteins. Sirtuin enzymatic activity depends on the co-substrate nicotinamide adenine dinucleotide (NAD) availability which strongly connects sirtuins to metabolism. This was first shown by the Guarente laboratory in 2000, when they reported that the NAD⁺-dependent deacetylase activity of SIR2 (Silent Information Regulator) was required for the positive lifespan effect of dietary restriction in a yeast species (*Saccharomyces cerevisiae*)[12]. Several laboratories confirmed and extended these results in rodents and *C. elegans* demonstrating that the NAD⁺/sirtuin pathway modulated longevity via FOXO/daf-16 signaling[80,81]. This links sirtuin mediated lifespan extension to the insulin/IGF-1 pathway.

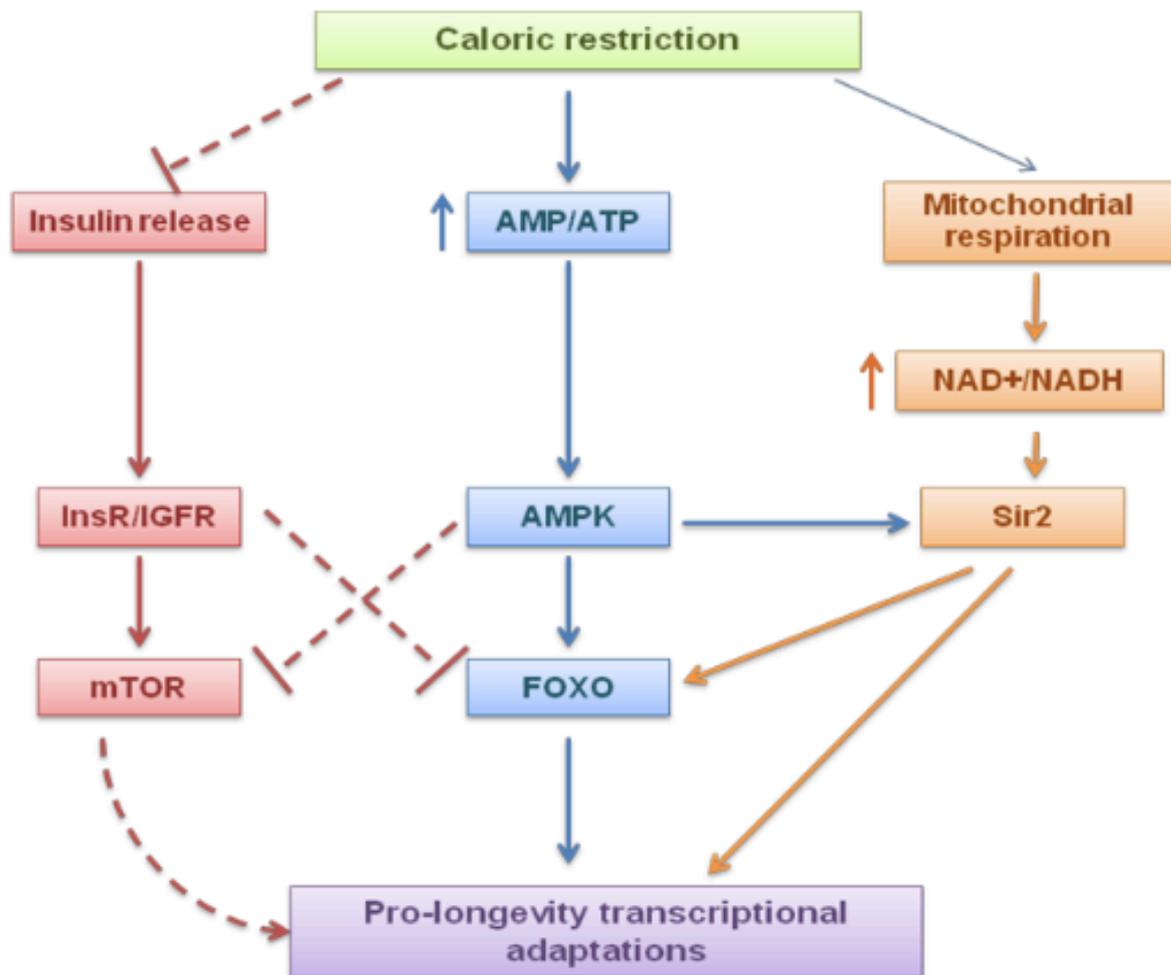


Figure 5: Main nutrient sensing pathways mediating the effects of dietary restriction, adapted from FENG PhD 2010, Study of glucose transporters in *C. elegans*, University of Bath, UK

I will present hereafter arguments for how epigenetic changes mediate protective effects of dietary restriction implicating nutrient availability and particularly glucose.

d. Epigenetic implications underlying the protective effects of dietary restriction:

The term epigenetics includes changes in organisms caused by modification of gene expression rather than alteration of the genetic code itself. These modifications are under the influence of environmental cues and in particular food and nutrient intake. For example, it has been shown that dietary restriction decreases DNA methylation and increases genomic stability during aging[82]. In addition, a recent study extended this hypothesis and showed that dietary restriction had an effect on DNA methylation which was conserved across species (rodents, rhesus monkeys and humans) and correlated with lifespan[83].

Included in the term of epigenetic modifications are histone modifications. These include acetylation, methylation, ubiquitination and ADP ribosylation.

Among these known epigenetic histone modifications, acetylation and de-acetylation are among the most studied histone modifications modifying gene expression. These modifications are mediated by specific enzymes: Histone deacetylases (HDACs) and histone acetyltransferases (HATs). These will be detailed hereafter as they are of particular interest for this thesis.

Several findings from Professor MOBBS's laboratory implicate CBP (cAMP-response element-binding protein-CREB-binding protein), a major histone acetyl transferase in the mechanisms underlying protective effects of dietary restriction on lifespan. It was shown that CBP expression correlated with lifespan in five different strains of mice[14], which has not been shown with any other gene implicated in dietary restriction mechanisms such as SIRT-1, AMPK or mTOR. Furthermore, when CBP expression was knocked-down by RNAi in the model organism *C. elegans*, lifespan extension induced by dietary restriction was completely cancelled, independently of the dietary restriction protocol used[14], this also has not been achieved with any other gene to this day. These results were replicated by three independent laboratories[15–17].

CBP could be a common factor integrating multiple lifespan extending pathways as it binds with several proteins implicated in lifespan extension such as FOXO, HSF-1, NRF-2. FOXO is known to bind to and require CBP for its metabolic effects, which supports arguments for dietary restriction and the insulin-IGF-1 pathway sharing mechanisms implicating CBP activity.

Here we will present in more detail, the histone acetyl transferase CBP:

CBP is a transcriptional co-activator and a major histone acetyltransferase (HAT). Histone acetyltransferases (HATs) are enzymes that acetylate conserved lysine amino acids on histone protein, by transferring an acetyl group to the histones, which results in important regulatory effects on chromatin structure, assembly, and gene transcription. The nucleus of eukaryotic cells contains highly organized and tightly regulated machineries involved in the control of gene transcription. Histones are the basic packaging units for DNA and they undergo post-translational modifications. Such modifications form distinct patterns that regulate the transcription of specific genes. This is in part what makes up the 'epigenetic modification'. Normal and aberrant cell function depends not only on genetic properties encoded within the DNA sequence. There is increasing evidence that specific post-translational marks on the

histones contribute to both normal and aberrant cell function. These post-translational marks are altered by histone-modifying enzymes, such as HDACs and HATs[84]. In general, histone acetylation increases gene expression. Despite the names of these enzymes, histones are not their only substrates. Non-histone substrates include other nuclear proteins like transcription and chromatin remodeling factors and diverse cytoplasmic and mitochondrial proteins. In fact, computational predictions and high throughput mass spectrometry analyses indicate that hundreds of proteins may be susceptible to acetylation[85]. The HATs can be divided into 2 classes depending on their cellular localization: cytoplasmic cytoplasm where they acetylate newly synthesized histones before they build up nucleosomes (type B) or nuclear capable of modifying histones incorporated into chromatin (type A)[86], or on the basis of their catalytic domain[87]. Type A class has been divided into five families based on similarities in their primary sequences: GNAT (General control non-repressible 5-related N-acetyltransferase) family, MYST family (closely linked to cancer), Transcription factor related, Nuclear receptor related and CBP/p300. Different HAT complexes are composed of various unique subunits. The combinations of these subunits contribute to the unique features of each HAT complex. GNAT family includes hGCN5, hPCAF, hHAT1[86].

MYST family named after its four founding members Moz, Ybf2/Sas3, Sas2, and Tip60.

These HATs are typically characterized by the presence of zinc fingers and chromodomains, and they are found to acetylate lysine residues on histones H2A, H3, and H4[87].

There are no known nuclear receptor associated histone acetyltransferase in *C. elegans*.

Presented hereafter, a table of major HATs.

Family	Members	Histone Targets
CBP/p300	CBP/p300	H2A, H2B, H3, H4
GNAT	GCN5	H3, H4
	PCAF	H3, H4
	HAT1	H4
MYST	TIP60	H2A, H3, H4
	MYST2	H4
	MYST3	H3
	MYST4	H2A, H3, H4
	MYST1	H4

Table 3 Major mammalian histone acetyl transferases, adapted from ALIBERTI, A., and BARILE, F.A.[88]

The human CBP is well conserved amongst mammals. Homologs of CBP can be found in organisms such as *Drosophila* and *C. elegans*[89].

CBP is a ubiquitously expressed nuclear protein with an approximate molecular mass of 250kDa. It was named after its initial description as an interacting partner of the transcription factor CREB (cAMP responsive element binding protein) yet, it was later found that it interacts with hundreds of proteins with different functions. It has diverse functions related to transcription activation and regulation. Thus, it is usually described as a molecular scaffold that brings different proteins together to the promoters. The large size (over 2400 amino acids) and modular organization enables interaction with several proteins at the same time.

How CBP-interacting transcriptional complexes act to increase lifespan and reduce age-related disease remains to be elucidated. Several important elements can be cited: the growing awareness of importance of CBP and other HATs in epigenetics, a more specific role of CBP in metabolic pathways, and the metabolic shift away from glucose utilization and toward beta oxidation[14].

HAT activity increases histone acetylation, probably by enhancing transcriptional flexibility, so the decreased CBP expression with age as shown by Zhang in 2009 (CBP correlation with lifespan) may contribute to age-related loss of adaptive capacity. In 2009, Zhang et al demonstrated that the CBP HAT activity was implicated in the observed protective effects on lifespan and healthspan. By inhibiting *cbp-1* with RNAi, the lifespan of wildtype *C. elegans* was significantly reduced and the effects of 3 protocols of dietary restriction on lifespan were cancelled. In the same study, an HDAC inhibitor (sodium butyrate), which unspecifically amplifies all HAT activity, was studied in *C. elegans* and it increased lifespan, in a *cbp-1* dependent manner. With this observation arose the question whether other HATs could have the same effects on lifespan as CBP.

Although numerous hypotheses have been proposed to explain the benefits of dietary restriction on longevity[90], the exact mechanisms by which DR increases lifespan and healthspan are still unclear. It would seem that caloric restriction is too complex ever to be described by a single hypothesis.

Even so, the hope to understand and discover a unique hypothesis persists and it has become increasingly plausible that the effects of dietary restriction are due to an active regulation by nutrient-sensing pathways. Dietary restriction decreases available nutrients, which in turn modulate ‘the fuel-use’ in ways which are presumably conserved across species. Dietary

restriction can be thought to influence gene expression (and lifespan/healthspan extension) in several ways: among others, through metabolism (a change in available nutrients) and epigenetics.

The first results presented hereafter contribute to present the unique role of CBP in mediating protective effects of dietary restriction.

1.1.2.2 Hyperglycemia – High glucose diet

In the previous sections we have discussed the fate of glucose in a physiological setting as well as in dietary restriction when glucose metabolism is reduced. Here we will discuss the fate of glucose in the hyperglycemic setting and its impact on aging.

Glucose dysregulation and hyperglycemia are commonly associated with advancing age[18], as is diabetes mellitus, a disease characterized by high plasmatic glucose levels, resulting from defects in insulin secretion and/or insulin action, often described as an age-accelerating disease. Indeed, as major epidemiological and intervention studies monitoring diabetic patients such as DCCT[91,92], UKPDS[93] have shown, poor glycemic control is associated with an increase of age-related diseases: cardiovascular events (stroke and myocardial infarction), chronic kidney disease[94], and dementia (vascular and neurodegenerative such as Alzheimer's disease)[95,96].

According to the WHO, in 2014 8.5% of adults suffered from diabetes worldwide, this means 422 million people, surpassing by far the earlier predictions (325 million people worldwide by 2025)[97]. High-index glucose diets, obesity, physical inactivity are among the main modifiable risk factors implying that lifestyle interventions are of major importance in the prevention and management of the disease.

The two major epidemiological concerns for diabetes are the rapid increase of diabetic populations in the middle and low income countries and the ever younger age of onset of the disease heavily related to child and teenager obesity. Diabetes type 2 is a costly disease not only for individuals and families but also for the health systems because of its chronic nature, the severity of its complications and the means required to control them[97]. The current costs linked to diabetes worldwide add up to \$1.3 trillion which translates to a share of global Gross Domestic Product (GDP) of 1.8% and are meant to double by 2030 according to a recent study by researchers from King's College London[98]. The severity of diabetes for individuals lies in its complications which are of two types: microvascular and macrovascular. The microvascular complications are retinopathy leading to blindness, nephropathy leading to kidney failure and dialysis, neuropathy leading to foot ulcers and amputations. The

macrovascular complications are coronary artery disease, stroke, high blood pressure. Diabetes is the first cause of blindness and amputation in the western world. The management of the disease challenges health care providers, the pharmaceutical industry and patients as it implies a multifactorial approach. Medication alone are insufficient and patient participation with weight loss (as little as a 5% weight loss could be enough to prevent obese patients with impaired glucose tolerance to develop diabetes) and physical activity play a major role in the disease decelerated progression. Research should also aim at helping to find solutions for preventing diabetes by preventing obesity and ways to limit the deleterious effect of high-index glucose meals.

At a metabolic level, improved glucoregulatory control is an indicator of successful aging[18]. In a recent review, KROEMER et al. describe hyperglycemia and increased glucose intake as part of general aging-inducing phenomenon and term it: “carbotoxicity”.

The review article[1] first describes the modification of human glucose consumptions across ages. KROEMER describes three major steps leading to the rise in carbohydrate consumption we know today. Indeed, in pre-agriculture communities of hunter-gatherers, diets were extremely varied and carbohydrate consisted only in 15% of the daily calorie intake. When hunter-gatherers switched to agriculture, consumption of carbohydrates showed a first increase. The second change occurs according to KROEMER et al. when mass production and consumption of refined sugars become available in the 19th century because of development of less labor-intensive methods producing sugar from beets. The eating habits in Europe were transformed.

Finally, the third surge in carbohydrate intake corresponds to the intake of ultra-processed foods after 1945.

The recommended carbohydrate intake today is 45-50%, but in many societies carbohydrate consumption can reach 65%. High carbohydrate intake is associated with a higher risk of total mortality[1].

A unified theory of glucose toxicity was first hypothesized by BROWNLEE more than 30 years ago. He reviews his findings in his Banting lecture in 2004[99].

In case of hyperglycemia, most of the glucose is metabolized in the glycolytic pathway described in the corresponding section of this manuscript. Glucose excess and regulation points of the glycolytic pathway determine the deviation of glucose and the intermediary metabolites to the alternative glucose metabolism pathways. The goal for the organism is to mobilize all

possible pathways to increase glucose clearance[100]. Most of these pathways are used in a very limited way under physiological glucose conditions.

The first alternative pathway BROWNLEE describes in his talk is the polyol pathway:

The polyol pathway is a two-step reaction which converts glucose to fructose. Glucose is first reduced to sorbitol by the aldose reductase which is in turn oxidized to fructose by the sorbitol dehydrogenase. Sorbitol is implicated in diabetic complications, particularly diabetic retinopathy.

When glucose concentration in the cell is high, the aldose reductase affinity for glucose increases. In the process of reducing glucose to sorbitol, aldose reductase consumes the cofactor NADPH[19,99], and in the second phase of the reaction, a NAD⁺ is consumed by sorbitol dehydrogenase. NADPH is the cofactor needed to regenerate the antioxidant, reduced glutathione and as mentioned earlier, NAD⁺ the necessary cofactor for sirtuins. Activation of the polyol pathway therefore depletes the cells of protective and lifespan extending cofactors.

The second pathway described in the paper is the *advanced glycation end products precursor pathway*:

When the intracellular glucose concentration is too high, during stage 2 of glycolysis, the ‘unwanted’ intermediate metabolite of the glycolytic pathway, DHAP accumulates, and the GAPDH (glyceraldehyde-3phosphate dehydrogenase) is overwhelmed, incapable of catalyzing the transfer of hydrogen from DHAP to convert it into glyceraldehyde 3-phosphate. Excess DHAP is converted to methylglyoxal, the highly reactive dicarbonyl advanced glycation end product precursor[19,99]. Methylglyoxal is a glycolytic byproduct which occurs naturally in a high glucose environment via non enzymatic reactions and leads to rapid protein modification. Among the AGE residues derived from methylglyoxal, N(6)-carboxymethyllysine is one of the most studied AGEs. The formation of methylglyoxal-derived proteins in cells is suppressed by the activity of the enzyme glyoxalase-1 that catalyzes conversion of methylglyoxal, with the cofactor glutathione, to S-D-lactoylglutathione[31]. AGEs have been implicated in many age-related diseases and it is even suspected that they have a role in aging[101]. AGE pathogenicity has different mechanisms: protein dysfunction, protein aggregation, protein reticulation secondary to the protein modification, which can lead to oxidative stress, activation of the Receptor for AGEs (RAGE) which leads to an inflammatory response and oxidative stress.

The third pathway outlined is the *PKC pathway* (protein kinase C pathway):

This is a four-step pathway also originating from DHAP excess and the overwhelming of GAPDH which produces the very toxic and reactive diacylglycerol (DAG), a physiological activator of protein kinase C. The activated protein kinase C has a variety of effects on gene expression and promotes the expression of genes which are pro-inflammatory and pro fibrotic (increase of the expression of VEGF, TGF β , NF κ B...)[19,99].

Finally the *hexosamine pathway*:

This is the most recent pathway recognized as having a role in the pathophysiological complications of diabetes and hyperglycemia. In high glycaemic situations, fructose-6-phosphate gets diverted to the hexosamine pathway and transformed in a 2-step set of reactions to N-acetyl-glucosamine.

N-acetyl-glucosamine in a process similar to phosphorylation, is capable by being added to serine and threonine transcription factor residues to induce pathologic changes in gene expression[19,99].

Presented below is a schematic representation of the described alternative pathways for glucose metabolism in hyperglycemic conditions (figure 6).

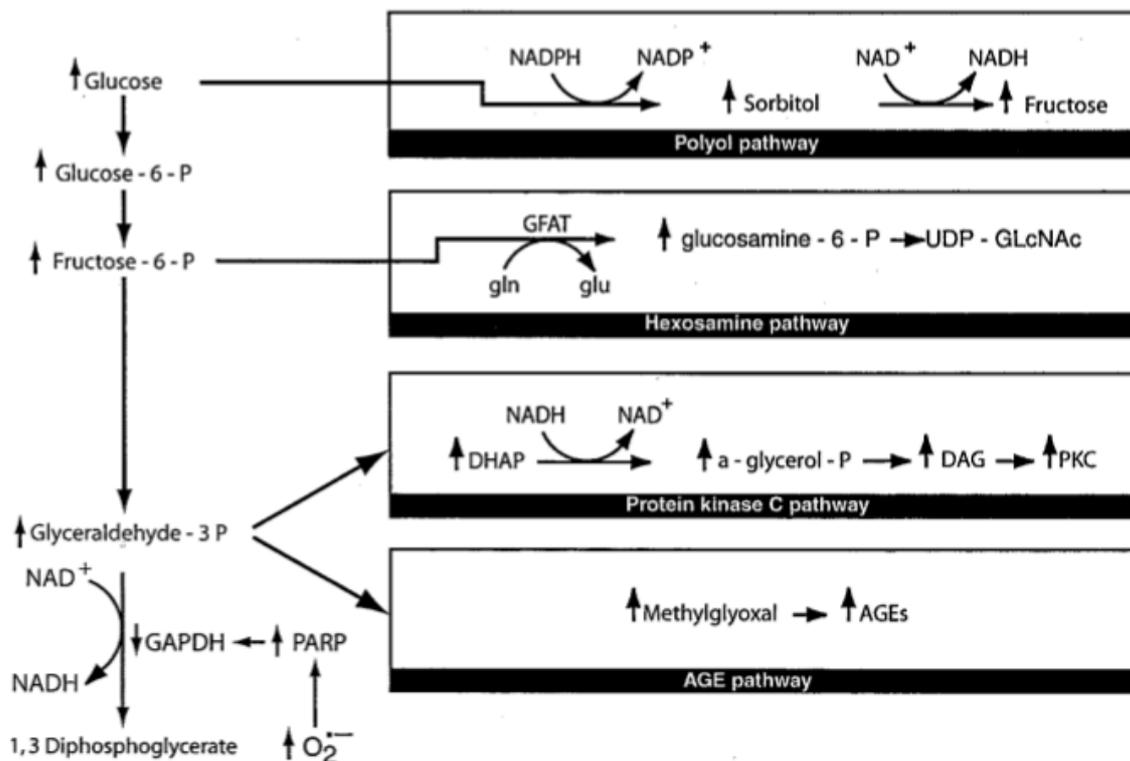


Figure 6: schematic representation of the pathobiology of diabetes Different pathways of glucose metabolism in case of hyperglycemia, from BROWNLEE[99]

In addition to the disturbed glucose metabolism, in a hyperglycemic state, proteins also go through a number of modifications. Cells routinely modify proteins to expand or specialize their function, but the modifications can also reflect cellular stress[100].

According to a review by ZHENG et al. “the modifications can be classified into two categories: irreversible and reversible. Irreversible protein modifications include carbonylation, nitration, and glycation, and reversible protein modifications include nitrosylation, acetylation, O-GlcNAcylation, ADP-ribosylation, and succination.”[100].

As it is of particular interest for this thesis we will very briefly review one of the post translational protein modifications: glycation.

Glycation is a non-enzymatic reaction between reducing sugars and amino groups of protein[102]. The reaction is irreversible and produces advanced glycation end products or AGE. This reaction was initially described by the French chemist Louis-Camille MAILLARD[101]. AGEs can be formed during the heat treatment of food [102]. They can also be produced endogenously within the organism at 37°C in conditions associated with aging, oxidation and hyperglycemia. There is currently no official classification of AGEs and the term itself can be confusing. Indeed, it is often used in literature to designate reactive metabolites, such as glyoxal and methylglyoxal, as well as the end-products of glycation. AGEs form a heterogeneous group as a result of the multiple potential combinations between various amino compounds and sugars[101]. It is important to bear this in mind as AGE characteristics differ according to their molecular precursors, their free or protein-bound form and their endogenous or dietary origin. These characteristics influence their metabolism and probably their health effects[101]. Increasing evidence is linking AGEs to various health issues, or in other words glycation level of an organ to its dysfunction. It seems that endogenous, as well as dietary AGEs could be implicated in the health effects of AGEs. Nonetheless we must stay aware that correlation does not signify a direct causal relationship. In a recent study, Dr. TESSIER showed that dietary carboxymethyllysine (CML) marked with radioactive carbon, accumulates in the tissues of mice after a diet enriched in CML. The organs most affected by CML accumulation were the kidneys, the lungs and the brain[102]. In humans, daily CML intake is estimated to range from 83µg/kg to 252µg/kg of body weight for a typical European diet[101]. Studies from Professor BOULANGER’s laboratory have implicated dietary advanced glycation end products in renal and vascular aging[20–22] and disease. Dietary CML accelerates vascular stiffness (a marker for vascular aging) in a dose dependent manner[20]. The mechanisms for AGE’s role in aging are still largely unknown. The described mechanisms to date include: accumulation in tissue, *in situ* glycation inducing protein dysfunction and tissue structure damage and finally,

interaction with RAGE (the AGE receptor) which activates major proinflammatory and pro-oxidative signaling pathways. It has been discussed that RAGE could play a role in the ‘inflammaging’ process[103]. Results from Professor BOULANGER’s laboratory conclude that RAGE could have a role in age-associated diseases, particularly in chronic kidney disease [104] In another study, it’s implication in the development of other age-associated diseases, namely cardiovascular disease as has been shown[23].

Glycation and AGEs have been proposed as a marker of aging. A recent Japanese study evaluated whether skin autofluorescence, which reflects accumulated tissue levels of AGEs, is predictive of age and lifestyle. The conclusions of the study are positive and the authors conclude that skin autofluorescence is a convenient tool to evaluate age and lifestyle[105]. Other, older studies also showed an increase in the concentration of certain AGEs in collagen with age across species [106].

In terms of markers of aging, protein carbamylation is another non-enzymatic posttranslational modification which has been more recently described and has been shown to compete with glycation for protein modification in a high glucose and kidney insufficiency setting[107]. It is an irreversible process, and as such, has been used as a biomarker for protein oxidation in disease and aging[108]. Protein carbamylation is formed by the nonenzymatic binding of isocyanate to free amino groups and preferentially occurs on the amine of lysine residues generating homocitrulline[109]. Carbamylated protein accumulation have been correlated to aging, the mechanisms remain unknown but probably occur among other things *via* the alteration of protein function. Both glycation and carbamylation induce alterations in protein and cellular properties which accumulate with age[109].

Although much has been studied in regards to glucose induced pathology in a hyperglycemic setting, questions remained unsolved concerning a direct link between hyperglycemia, aging and lifespan.

In the corresponding section of the introduction, we will review what is known on glucose excess and lifespan in the model organism *C. elegans*.

In the last section of results of this thesis, we will present our findings on activation of the different glucose metabolism pathways, glucose transport and oxidative response to hyperglycemia in the model organism *C. elegans*.

I.2 Aging

I.2.1 Definition

Aging is defined as a gradual, time-dependent loss of physiological functions, affecting all living beings[24] .

Age is a robust and independent risk factor for a range of non-communicable diseases like diabetes, cancer, cardiovascular disease, and neurodegenerative disease[110]. Overwhelming evidence suggests that processes intrinsic to aging contribute to the pathogenesis of age-related or age-associated diseases.

There has been a shift in emphasis of research in the last few years from the investigation of individual age-related diseases toward a broader context to define the basic biology of aging. A strategy to delay the aging process itself could decrease vulnerability to age-related diseases, leading to lower morbidity[69].

Diseases commonly linked to the risk of healthspan impairment and related accelerated aging include abdominal obesity, diabetes mellitus, heart and cardiovascular diseases, malignant neoplasms, Alzheimer's disease, chronic lower respiratory disease. Approximately 80% of adults above 65-year olds have at least one of the aforementioned diseases and 50% have at least two of these chronic diseases (CDC [Feb 10, 2011] Deaths: Final Data for 2005. National Vital Statistics Reports. 56, Number 10. 2008. Available at: http://www.cdc.gov/nchs/data/nvsr/nvsr56/nvsr56_10.pdf).

There is a great diversity in the aging phenotype, in animals but also among humans. Humans don't all age alike. Many factors are involved in this diversity of phenotypes, genetic as well as environmental and we are only beginning to unfold the great number of factors. To improve patient management, the Geriatric medical societies have defined three aging phenotypes: robust, frail and disability/dependence. Robust elderly people are community dwelling adults, with no geriatric syndrome. Medical management considers them as middle aged adults in terms of health objectives (blood pressure goal values, HbA1c goal value...). In some cases, chronic diseases and age-related diseases (whose frequency increases with age) lead to frailty and increasing vulnerability[111]. Frailty is a well-defined clinical syndrome in Geriatric medicine but difficult to diagnose and with no efficient screening methods to date. Indeed, although the National Institute for Aging has long defined criteria for aging biomarkers (must predict the rate of aging, must monitor the basic process underlying aging, must be able to be tested repeatedly without harming the person and must be reliable in humans and model organisms), and abundant research on the subject, no biomarker has emerged. Frailty of an

aging individual is characterized by a reduction in an organism's reserves making it more difficult for the individual to react accordingly and appropriately in case of stress (biological or psychological). As examples of frailty in an individual having a normal physiological aging: presbyopia (seeing less clearly), and presbycusis (hearing less well) impede an individual's capacity to stay active in society and to understand a conversation when many people are present. Physiological kidney and liver aging make toxics and medicine metabolism and elimination more difficult. Frailty is characterized by its reversibility, differentiating it from a dependent state. At the Lille University, Professor BOULANGER and PUISIEUX's team has defined twelve geriatric syndromes which help to better frame frailty and "geriatric state" in an elderly patient. When a patient cumulates several of these syndromes, the risk of developing a dependence increases. A geriatric syndrome is defined by 4 criteria: its frequency increases with age, it is the result of multiple factors, a consequence of the syndrome is a risk of disability, management of the syndrome requires a global multidisciplinary approach. A list of these twelve geriatric syndromes is included in the annex.

Dying of old age is not possible, one dies of sickness is what is often heard in Geriatric wards. Nonetheless, the distinction between normal and pathological aging is not always easily made. What is the threshold beyond which one is considered old? What is the threshold beyond which physiological aging becomes pathological?

In his model, Bouchon[112] was one of the first to try to answer these questions in a standardized way. Every function declines with age, but in normal physiological aging, the function always remains operative. Functional decline can be accelerated, causing pathology beyond a certain threshold in case of chronic disease or acute event. Bouchon's model is illustrated in figure 7 hereafter.

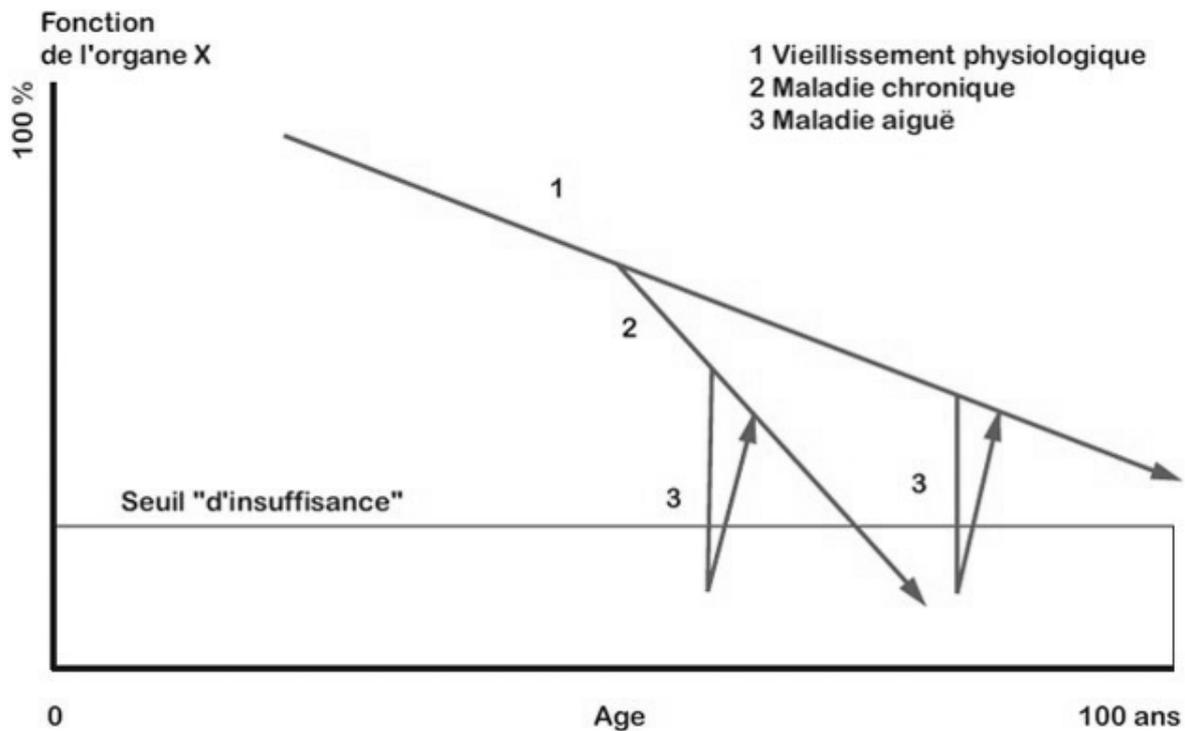


Figure 7 : « 1 + 2 + 3 Bouchon » model, diagram modeling the functional decompensation of the elderly person, source from Bouchon model 1984 Revue du praticien

I.2.2 Epidemiology

According to the United Nations population bureau, the population above 65 years old will rise from the actual 7% to 20% worldwide (<http://esa.un.org/wpp/>) before 2050.

In the European union, the proportion of the population above 65 years old rose from 16,8% to 19,2% between 2006 and 2016. Similarly in France, the population is aging and on January 1st 2018, 19,5% of the French population was above 65 years old, with the proportion of oldest old regularly and substantially increasing and soon to surpass the proportion of citizens younger than 20 years old.

Increased life expectancy as well as decline in fertility rate result in the population aging.

With increased proportions of elderly, new problems arise and must be handled: increase in chronic diseases requiring a better prevention of these chronic diseases, the need to adapt infrastructure to the different needs of an aging population,

Although life expectancy has risen thanks to major advances in medicine and hygiene, as people age, they still become more dependent due to age-associated diseases, necessitating more help for their basic activities such as eating and washing. Rates of severe disability have declined over the past thirty years, while there has been no significant change in mild to moderate disability over the same period (WHO reports, facts sheet 2018). According to WHO, the number of people who need support for activities of daily living in developing countries will

quadruple by 2050. We have succeeded to increase lifespan but not healthspan in our societies, this is the major stake for geroscience in the years to come. A longer life brings opportunities and older people can contribute positively in many ways to their families and communities, under one condition: healthy aging[113].

I.2.3 Aging-related diseases

How can we define an aging-related or age-associated disease?

First, we must differentiate age-associated diseases from aging itself. Indeed, virtually all living organisms age, but not all individuals experience age-associated diseases.

Age-associated diseases are also different from accelerated aging diseases, such as progeroid syndromes which are a group of genetic disorders. Examples include Hutchinson-Gilford progeria syndrome, Werner syndrome, xeroderma pigmentosum.

Age-associated diseases are diseases whose frequency increase with age, the main risk factor for these diseases is therefore aging. In the figure hereafter (figure 8) are presented data for cancer incidence per 100,000 people in the United States according to age. Independent of sex, the incidence of cancer, an age-associated diseases increases with age as is illustrated by the graph below.

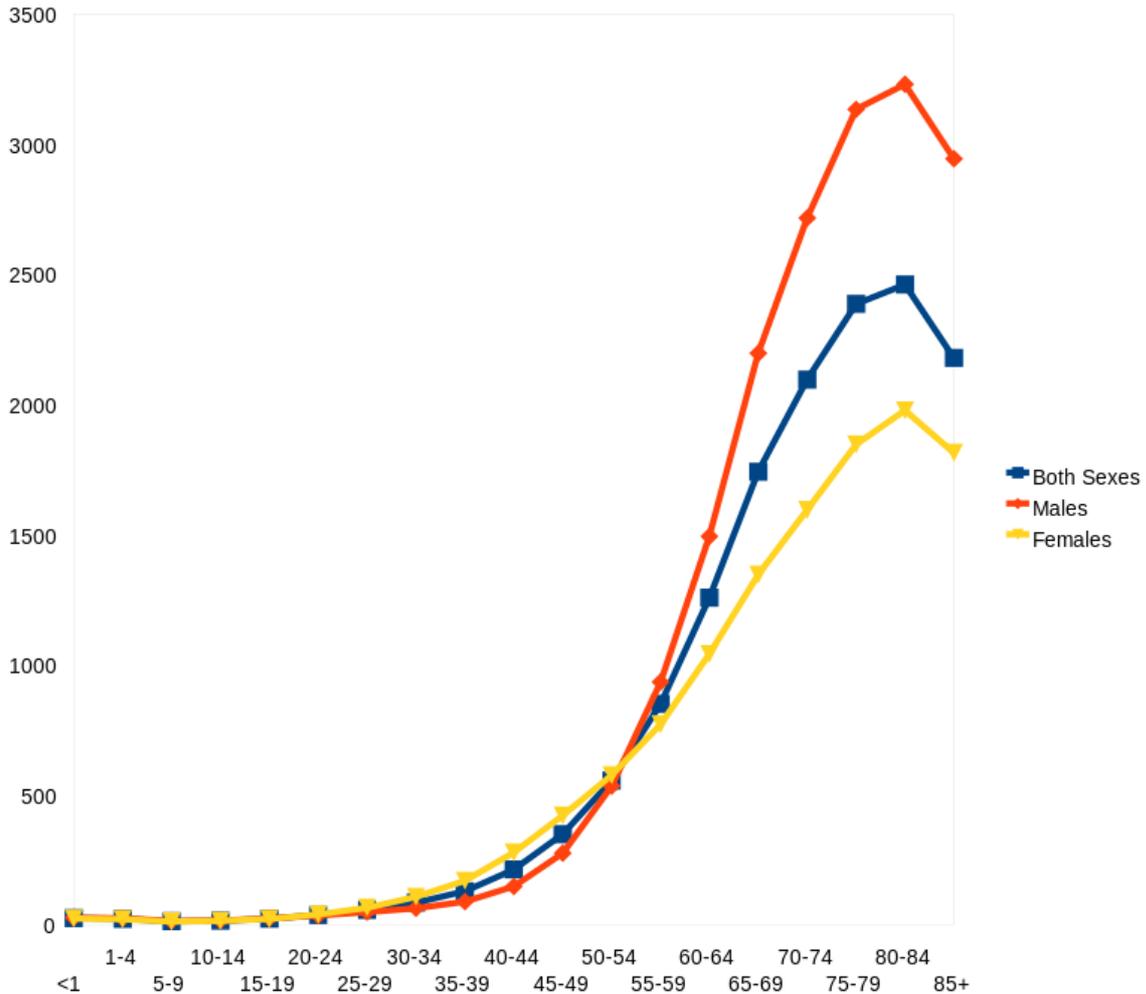


Figure 8: Cancer statistics, data adapted from CDC chronic data for the United States between 2000 and 2007, by N. Dilmen SEER. The rates are per 100,000

Among diseases associated with aging we can cite: cancer, dementia, osteoarthritis, cardiovascular diseases (including myocardial infarction, stroke and hypertension), diabetes mellitus, osteoporosis.

Commonly, these diseases associate, creating a so-called ‘multiple chronic condition’ and in an aging population, this has major implications in disease management, often leading to polypharmacy, contributing to increased disability in activities of daily living and accelerated aging.

In the United States, sixty two percent of the population above 65 have more than one chronic condition[114].

Age-associated diseases have an impact on health, quality of life but also have an important economic burden. If we consider the example of Alzheimer’s disease and related dementias,

global estimates suggest that the total economic costs caused by dementia increased from US\$279,6 billion in 2000 to \$948 billion in 2016, with an annual growth rate of 15,94%. This included costs of informal care at \$95 billion in 2000 and \$402 billion in 2016, with the annual growth rate of 21,50%[115].

Even diseases thought as less severe in middle aged populations can have dramatic impacts on independent living as people age. The most common causes for moderate and mild disabilities are sensory loss (hearing, vision), osteoarthritis, osteoporosis.

Considering glucose homeostasis, often disrupted with advancing age, recent epidemiological studies show that in the United States, more than 50% of the population above 45 years old have a glucose deregulation. The proportions rise as the population ages, and among people older than 80 years old, more than 80% in the United States show deregulated responses to high glucose levels (from CDC chronic data).

Aging is associated with disease and although freeing the elderly from disease to enjoy life, age and die *naturally* is a goal of geroscience, this is not without arising other problems, as expressed by Gems[116] through the words of the philosopher Leon Kass: “Would not the fear and loathing of death increase, in the absence of its antecedent harbingers?” And “Death would always be untimely, unprepared for, shocking”[117].

In the next section, we review a state of the art of our knowledge on the mechanisms underlying aging.

I.2.4 Nine hallmarks of aging

In 2013, in the same way that HANAHAN and WEINBERG had done it for cancer in 2000, LOPEZ-OTIN and colleagues attempted to identify and categorize cellular and molecular hallmarks of aging[24].

The motivation for picking out these hallmarks was to conceptualize the essence of aging and its underlying mechanisms.

Nine hallmarks were identified and each fulfilled the following criteria: “(i) it should manifest during normal aging; (ii) its experimental aggravation should accelerate aging; and (iii) its experimental amelioration should retard the normal aging process and, hence, increase healthy lifespan”[24].

The hallmarks are genomic instability, telomere attrition, epigenetic alterations, a loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication.

We present a summary of the 2013 landmark paper, pointing out when applicable implications for glucose metabolism.

1.2.4.1 Genomic instability

DNA damage accumulates with age because of extrinsic and intrinsic damage as the cellular repair machinery is unable to fully maintain DNA integrity. As time passes, lesions, such as somatic point mutations, deletions, integrations and chromosomal rearrangements accumulate and lead to genomic instability[118].

Complex DNA repair mechanisms exist to minimize lesions inflicted to nuclear DNA, mitochondrial DNA and to maintain length and functionality of telomeres[24].

The lesions can affect nuclear DNA, mitochondrial DNA or the nuclear architecture. Causal evidence that deficiencies in DNA repair mechanisms accelerate aging emanates from studies in mice and humans who suffer from progeroid syndromes such as Werner syndrome, Bloom syndrome, xeroderma pigmentosum, trichothiodystrophy, Cockayne syndrome, or Seckel syndrome[119]. Evidence that mitochondrial DNA damage may contribute to aging is less obvious as there are multiple mitochondrial genomes in one cell and further research is necessary to determine whether decreasing mitochondrial DNA mutations could have an impact on lifespan[24]. Nuclear lamina play a role in genomic stability by acting as a scaffold for chromatin and DNA-interacting proteins. Interest in nuclear lamina emerged when their mutations were discovered to cause accelerated aging diseases such as Hutchinson-Gilford syndrome[24].

In regards to metabolic repercussions of genomic instability, age-accelerating diseases (in particular Hutchinson-Gilford and Werner syndromes) convey evidence that chronic DNA damage can promote metabolic disorders. Patients with these syndromes frequently develop diabetes type 2[120]. The continuing DNA damage response affects anabolic reactions responsible for cell growth and proliferation driven by trophic response signals from insulin, growth hormone and IGF-1, as well as metabolic circuitries including glycolysis[121]. Progeria syndromes ataxia telangiectasia or xeroderma pigmentosum are accompanied by NAD⁺ depletion because patients display hyperactivation of the poly(ADP-ribose) polymerase 1 (involved in DNA repair) NAD⁺ dependent enzyme. As explained earlier, NAD⁺ is

fundamental to certain lifespan extension mechanisms involving sirtuins among others[122]. Genomic instability could generate metabolic adaptations that favor aging.

1.2.4.2 Telomere attrition

Telomeres protect the end of linear chromosomes and shorten with each cell division throughout life[118] as DNA polymerases are not capable of replicating the terminal ends of linear DNA strands. Telomeres are responsible for cell cycle arrest as cells reach the so-called Hayflick limit caused by critical telomere shortening[123] but also in the case of dysfunctional telomeres triggering a persistent DNA-damage response associated with accelerated aging[124]. Considering metabolism, telomere attrition promotes p53 activation which leads among other things to Ppar α (Peroxisome proliferator-activated receptor alpha) suppression[121]. Ppar α is a transcription factor which acts as a major lipid metabolism regulator in the liver. “Telomere dysfunction also enhances the requirement of glucose for the maintenance of energy homeostasis, as well as for mTor and IGF-1-dependent mitochondrial biogenesis, in mouse aging tissues”[121] as is evidenced by increased oxidative glucose metabolism in telomerase deficient mice[125].

1.2.4.3 Epigenetic alterations

The concept of epigenetics has changed several times since it was invented by Conrad Hal Waddington, a British developmental biologist in 1942[126].

Aging influences global epigenetic characteristics[13]. Epigenetics is a general term comprising nuclear architecture (e.g. lamins), telomere shortening, DNA methylation and histone modifications (histone acetylation, methylation), higher order chromatin structure modifications, non-coding RNAs[13].

Numerous chromatin modifications have been associated with aging and targeting these modifications could protect against aging and age-related diseases.

Chromatin comprises the association of DNA and histone proteins. H2A, H2B, H3 and H4 are the core histones whereas H1 and H5 are the linker histones. Histones can undergo posttranslational modifications which alter their interaction with DNA and other proteins from the nucleus. As H3 and H4 have particularly long tails, they can be modified in many different ways and locations. Known modifications include but are not limited to: methylation, acetylation, phosphorylation, ubiquitination, citrullination, beta-hydroxybutyrylation[37].

Some of these modifications are associated with diseases of accelerated aging such as H4K20 tri-methylation which is found in cells from Hutchinson-Gilford Progeria syndrome patients (a

rare premature aging laminopathy disease). Much has also been researched on DNA methylation itself as aberrant DNA methylation is found in numerous cancer cells, specific DNA methylation patterns have been described during the aging process...

In connection with metabolism, several elements link epigenetics to aging and metabolism. Intermediary metabolism generates substrates and cofactors to enzymes responsible for multiple epigenetic alterations. The first evidence already discussed earlier involves NAD⁺ and the sirtuin family of histone deacetylases. There is also evidence for transmission of epigenetic signatures of past metabolic experiences[127,128]. Of interest for this thesis, in case of dietary restriction and intermediate fasting, it has been shown that there is an increase in ketone bodies among which β -hydroxybutyrate, a powerful histone deacetylase inhibitor. This translates to global histone acetylation seen in dietary restriction. Among the genes with an induced expression, the well-known protective Foxo3a, which is part of the lifespan mediating insulin-like growth factor-1 pathway[13].

Epigenetic alterations are theoretically reversible, offering opportunities for anti-aging treatment targets[24,129].

1.2.4.4 Loss of proteostasis

Many studies have demonstrated that proteostasis is altered with aging[24]. Moreover, several age-associated diseases such as Alzheimer's Disease or Huntington's Disease are associated with aggregated proteins or chronically misfolded proteins[130].

Both the chaperones supervising/mediating correct protein folding and the proteolytic systems (autophagy-lysosomal and ubiquitin-proteasome systems) responsible for discarding dysfunctional proteins play a role in loss of proteostasis and are impaired with aging[24]. In humans, "genomic studies have revealed that polymorphisms affecting different proteasomal subunits are associated with increased susceptibility to metabolic disorders including diabetes"[121].

There is still a need to identify the metabolic factors capable of lifespan extension by preventing age-related loss of proteostasis. To date, metabolic changes associated with aging (increased glucose metabolism for example) have been shown to have inconsistent impacts on the proteostasis network[121].

1.2.4.5 Deregulated nutrient sensing

Some of the major nutrient sensing pathways have been implicated in aging mechanisms. When food is plentiful and stress levels are low, these pathways support reproduction and growth,

conversely, under stressful conditions, there is a physiological shift towards cell protection, growth arrest, reproduction delay and maintenance[10].

Among the major nutrient sensing pathways known to influence lifespan, insulin/IGF-1 signaling pathway was the first pathway shown to influence aging[78]. Inhibition of insulin/IGF-1 signaling by nutrient deprivation modifies gene expression of certain transcription factors, among which FOXO, HSF-1 or SKN-1, which in turn impact lifespan. Downstream genes of these transcription factors include stress response genes. As mentioned earlier, the effect on lifespan of the insulin/IGF-1 signaling pathway is evolutionarily conserved[10]. The insulin/IGF-1 signaling pathway and target transcription factors were discovered in the worm *C. elegans*. Variants of the mammalian transcription factor FOXO (FOXO3A) have been linked to longevity in several cohorts located throughout the world[131,132].

The insulin/IGF-1 signaling pathway participates in glucose sensing. In mammals, insulin levels rise in response to glucose. In *C. elegans*, adding glucose to the bacterial diet shortens lifespan by downregulating DAF-16, the FOXO ortholog in worms[33].

Three other nutrient sensing systems with an impact on lifespan can be cited: mTOR kinase, a major amino-acid and nutrient sensor that stimulates growth and blocks autophagy when food is plentiful[10], AMP kinase (AMPK) which senses low energy states by detecting high AMP levels and sirtuins, which detect low energy states by sensing low NAD⁺ levels[24].

In contrast to insulin/IGF-1 and mTOR signaling, AMPK and sirtuins signal nutrient scarcity and their up-regulation favors healthy aging[24]. Effective nutrient-sensing pathways are needed to suppress the progression of aging.

1.2.4.6 Mitochondrial dysfunction

Mitochondrial function has a profound impact on the aging process and is intimately linked to cellular metabolism.

Mitochondria, as the main producers of reactive oxygen species have been thought to be implicated in the aging process for many years. Multiple publications have shown that oxidative damage accumulates with age[124]. Nevertheless, the role of mitochondria has been difficult to determine and it is not clear whether oxidative damage is a cause or a consequence of aging. Although reactive oxygen species are generally implicated in cellular damage and inflammation when present at high levels, they are also important signaling molecules and can potentially increase cellular defenses through an adaptive response termed ‘mitohormesis’ when present at lower levels[124].

1.2.4.7 Cellular senescence

“Cellular senescence can be defined as a stable arrest of the cell cycle coupled to stereotyped phenotypic changes”[24].

Senescent cells accumulate during aging, this could reflect an increase in senescent cell generation or a decrease in their clearance. Recently, it was demonstrated that their pro-inflammatory secretome, referred to as the ‘senescence-associated secretory phenotype’, could induce an aging phenotype in mice and their removal was protective[133].

It was also recently demonstrated in two mouse models that senescent cells drive age-related pathologies in mice such as Alzheimer’s disease, atherosclerosis, or tumor progression[124], this stimulated the development of a new therapeutic class of drugs: senolytics. Clinical trial have only recently started in humans.

1.2.4.8 Stem cell exhaustion

Progressive stem cell exhaustion accompanies aging. Metabolic modifications with age are closely linked to this decline[121]. It has indeed been shown that intermediate metabolites from glycolytic and oxidative reactions influence the pluripotent stem cell reprogramming of differentiated cells, metabolic assessments have revealed that stem cells undergo metabolic changes as they age with significant changes in different metabolic pathways such as glycolysis[121]. Recent studies suggest that stem cell rejuvenation may reverse the aging phenotype[24].

1.2.4.9 Altered Intercellular Communication

Aging is characterized by intercellular communication deregulation, with an increase in inflammatory signaling. This aging-associated phenomenon distinguished by higher levels of inflammatory markers in cells and tissues are referred to as ‘inflammaging’[24]. Inflammaging is linked to age-associated diseases such as diabetes type 2, cardiovascular events or cancer and is now considered to be a biomarker for aging.

Inflammaging results from multiple causes such as immunosenescence (decline of the adaptive immune system function), increase of senescent cells secreting pro-inflammatory cytokines, enhanced pro-inflammatory pathways leading to increased levels of IL-1b, tumor necrosis factor and interferons production[24]. The receptor for advanced glycation end products could also be implicated in the inflammaging process[104].

Other modified aspects of intercellular communication with age include modifications in the gut microbiota, neuroendocrine signaling modifications and attenuated circadian oscillation

(responsible for decreased GnRH and LHRH secretion underscoring somatotrophic and gonadotropic axis signaling decline).

Other hallmarks have since 2013 been suggested such as protein carbamylation[109].

Hereafter a schematic representation (figure 9) of the different pathways implicated in aging mechanisms.

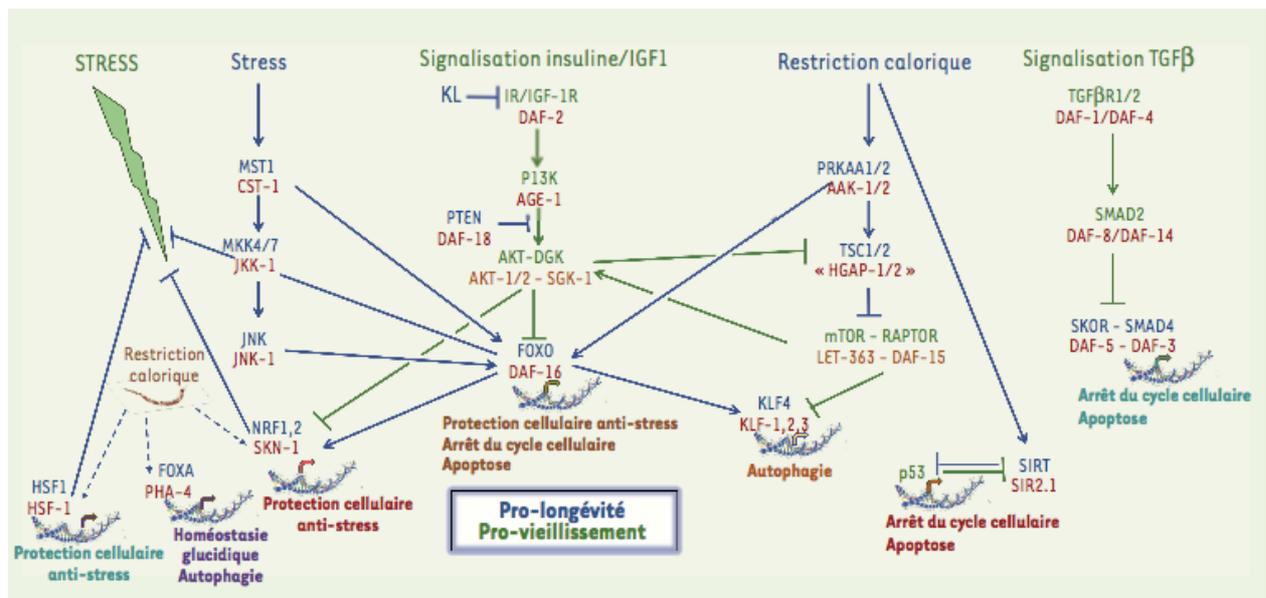


Figure 9 : Different cell signaling pathways controlling aging and longevity in mammals. The signaling pathways and the "pro-longevity" molecules are noted in blue while the signaling pathways and the "pro-aging" proteins are represented in green. The orthologous proteins of *Caenorhabditis elegans* are noted in brown. *C. elegans* does not have TSC1 and TSC2 orthologs but HGAP-1 and HGAP-2, which are orthologs of RalGAP α and RalGAP β , may have similar functions to TSC1 / 2. Dotted arrows represent the signaling pathways mainly described in the worm *C. elegans* Source: Médecine sciences Litke et al.[27]

Human relatively long lifespan does not yet allow direct mechanistic studies of aging in people. Several aging biomarkers in humans have been suggested and studied such as advanced glycation end products and protein carbamylation[108,134], nonetheless there is to date no reliable biomarker able to quantify the rate of aging. It is therefore impossible to validate different interventions as having an impact on the aging process itself in humans. Studying aging in model organisms seems as such compulsory.

I.2.5 Model organisms for studying aging and age-related diseases:

Almost all organisms age according to an aging process which follows a time progressive curve, illustrating an exponential increase in death rate with time (see figure 10 hereafter) which is remarkably consistent across species. Although one exception is known to this rule (the small freshwater cnidarian polyp *Hydra*[135] can reproduce asexually by budding indefinitely as long

as food is available), there seems to be a universal mechanism behind aging allowing us to study aging in many model organisms.

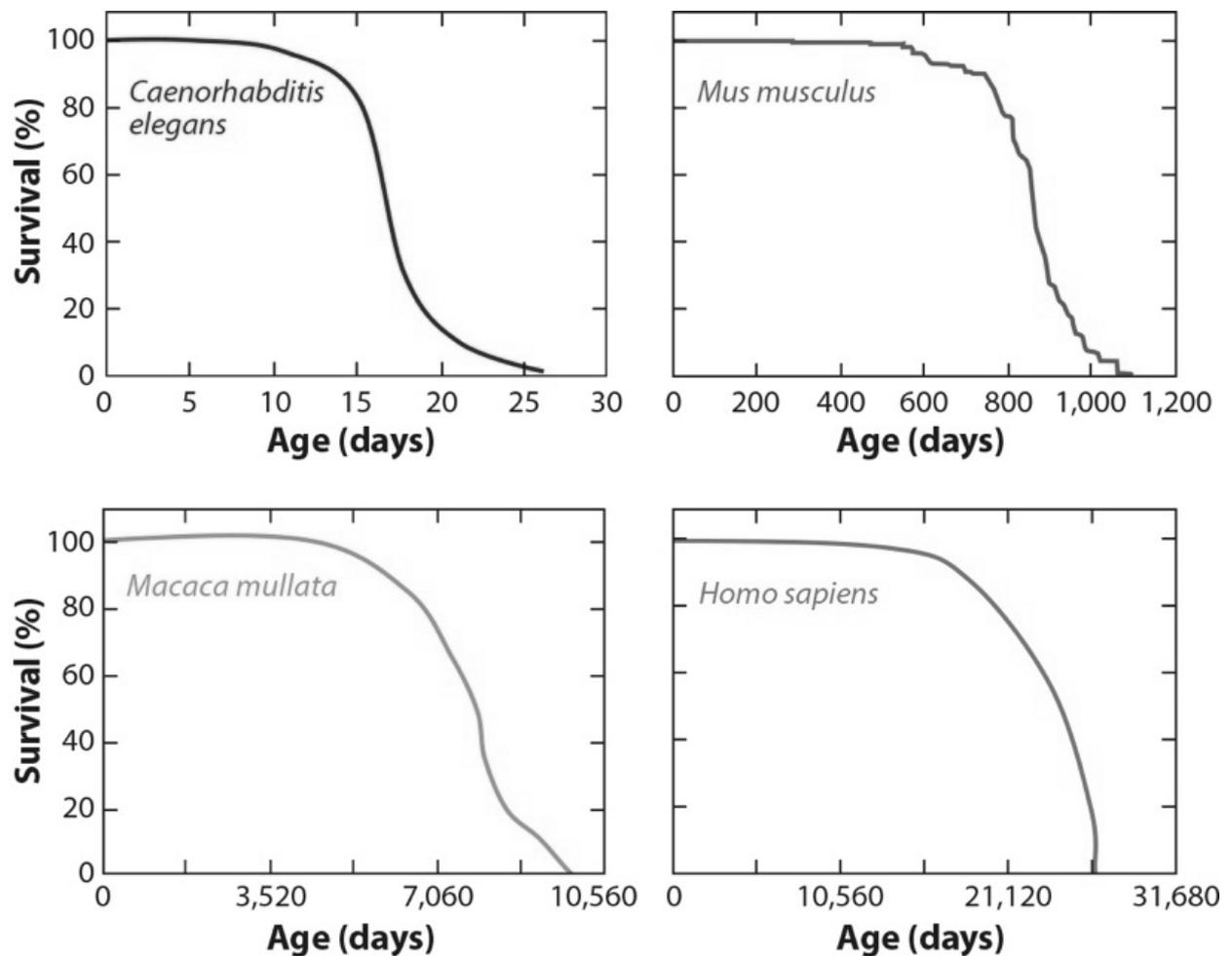


Figure 10: The universality of aging, theoretical life-span curves depicting the similarity in the aging process across model organisms relative to humans. Despite the differences in life span, the shape of the curve, often considered a measure of the health (or health span) of the organism, is similar, from Mitchell et al.[25] annual review animal biosciences

Here are some of the most commonly used model organisms used in aging research. For each one I will present, pros, cons, available tools, if dietary restriction has been studied with them and if they have been used for high glucose metabolism research.

Yeast:

Saccharomyces cerevisiae has long been used for aging research, first developed as a model organism for studying aging in 1959 with the discovery by Mortimer and Johnston of the finite replicative capacity[136].

Although it is not an animal (it is a single-celled fungus), it shares many things at the cellular and genetic levels with animals and is therefore a very useful model organism.

It presents many advantages: it is genetically tractable, has a short lifespan, there are two lifespan arms available (replicative and chronological lifespans) allowing to investigate mitotic and post-mitotic aging. There are also non-essential and essential gene deletion or conditional deletions available.

There are two ways of measuring aging in yeast and which are illustrated in figure 11. Either by measuring the replicative lifespan which equates to monitoring the number of daughter cells generating from an individual mother cell; or by measuring the chronological lifespan which equates to monitoring the survival of a population of non-dividing cells.

Although it has been and remains a very useful tool for aging research, yeast cannot model human aging because, among other things, the expression of telomerase, or the absence of p53.

Among the useful tools available in Yeast, we can cite:

-a genome database available under the link <http://www.yeastgenome.org/>

-a yeast outgrowth data analyzer facilitating analysis of chronological lifespan assays (YODA, yoda.sageweb.org), - libraries for histone mutants and multiple screens have been conducted for genes affecting lifespan[137–139].

Yeast have allowed researchers to uncover the role of sirtuins in dietary restriction[12], a hallmark finding, linking epigenetics. Interventions such as selective depletion of a single amino acid, methionine have also been tested successfully in Yeast in regards to longevity[140]. Dietary restriction is modeled in yeast by limiting glucose concentrations from 2% to 0,5% in the growth media [10].

Glucose is the major energy source in yeast.

Studies in yeast have been fruitful to help to understand the role of nutrient sensing in longevity. In *Saccharomyces cerevisiae*, nutrient signaling pathways have a pro-aging effect[141], as glucose activates these pathways, this brings a supplementary argument to the role of glucose in aging.

Below is a schematic representation of yeast replicative and chronological lifespans.

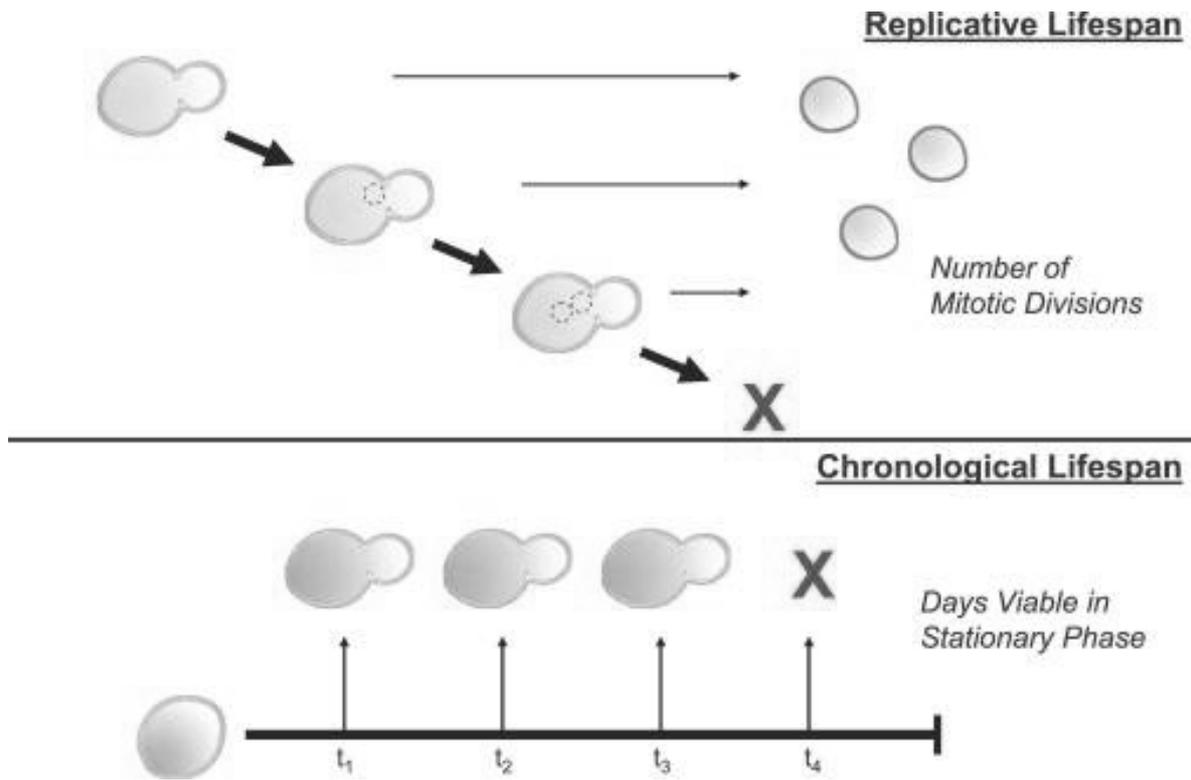


Figure 11: Schematic representation of yeast replicative and chronological lifespans, source from Kaerberlein[136]

C. elegans will be further developed in a next section as it is our chosen organism for this thesis.

The flat worm *Macrostomum lignano*

Macrostomum is a free-living, marine hermaphroditic flatworm. Among advantages of this model organism: it is transparent allowing in vivo studies, has a short generation time (about two weeks) and has a small size (1,7mm when reaches adulthood)[142]. Although a hermaphrodite, is non-self-fertilizing and has exclusive sexual reproduction.

Well-fed adult animals generate on average one egg a day, making it accessible for genetic manipulation. *In situ* hybridization and RNA interference (by soaking) protocols are established and optimized for *M. lignano*, and a number of tissue-specific monoclonal antibodies are available[142]. Among the other accessible tools, a draft genome and transcriptome are publicly available at www.macgenome.org, stable transgenic lines of *M. lignano* have recently been established[142].

Macrostomum represents a novel powerful model for molecular studies of aging attenuation[143], stem cell biology and regeneration.

Dietary restriction has been tested in *M. lignano*, conferring comparable results on lifespan as in other living organisms[144].

This model has not been used to date for the study of the impact of high glucose diets.

Drosophila melanogaster more commonly known as the fruit fly was among the first organisms used for genetic analysis and is one of the most widely used organisms for genetic research today.

Fruit flies were first used in experimental studies in 1910 by Thomas Hunt Morgan who developed the model organism. Morgan and his students elucidated many basic principles of heredity.

Among the advantages of the model: it is genetically tractable, has a short lifespan (approximately three months), and short generation time (ten days) as well as a high fecundity. Among the available tools, there is a database for *Drosophila* genetics and molecular biology (flybase.org, fruitfly.org, flymime.org), genome wide data (modencode.org), transgenic models have been developed to study age-associated diseases.

Dietary restriction has been widely studied in *D. melanogaster* although quantitative reduction of food is a challenge in this model as discussed earlier and is reviewed by Fontana et al.[6].

Increasingly, *D. melanogaster* is being used to study metabolic dysregulations, including glucose dysregulation and diabetes. Powerful interdisciplinary approaches combining *Drosophila* genetics and physiology with human biology has allowed the identification of type 2 diabetes risk genes[145]. As reviewed by GRAHAM et al.[146] *Drosophila melanogaster* is now a recognized model for the study of diabetes, diabetic complications and glucose metabolism.

Fish

Zebrafish *Danio rerio* is the most widely used fish model. It is a freshwater fish, has a lifespan of two to three years, comparable to that of rodents and a generation time of three months.

This model was first developed for scientific research by George Streisinger in the 1970s.

Among accessible tools, transgenic models have been developed and are available for distribution at the Zebrafish International Resource Center, there is a dedicated online database of genetic, genomic and developmental information the Zebrafish information network (zfin.org).

Among the advantages of the model in aging research, its remarkable ability to rejuvenate its tissues, is of interest to elucidating the mechanisms of tissue regeneration and repair[147], the embryos are transparent making them amenable to *in vivo* studies, their size make them accessible to large-scale studies.

Danio rerio has the largest share of diabetes research in fish. The advantages of the model for the study of diabetes and hyperglycemia has recently been reviewed[148].

Turquoise killifish *Nothobranchius furzeri*

This is the most short-lived vertebrate model organism to be used in the laboratory setting (13 weeks).

It is small, has a high fecundity (each female produces several hundred eggs which can resist desiccation and can be kept at room temperature for months).

Richard Furzer developed this model for research in 2002.

Among available tools, several aging biomarkers have been developed. Stable lines can be constructed by CRISPR; information on the research currently conducted on this fish is reported on a single website northobranchius.info

These fish have an increased lifespan in case of dietary restriction and after resveratrol treatment[149].

They have not to date been used to study the effect of high glucose diets.

Rodents

Rodents are commonly used to study aging and age-related diseases. They are the most commonly studied animal models to date which explains the wealth of background knowledge, genetic tools adapted to rodents.

They have been used in biomedical research since the 16th century.

Common laboratory mice, of the species *Mus musculus*

These can be divided into four separate groups: inbred mice (the most extensively used as they allow minimum genetic variability), outbred and F1 mice, wild derived mice and genetically heterogeneous mouse model. We will not elaborate further on these different groups but will take on a general point of view for rodents; a detailed review by Mitchell et al[25] is available. This vertebrate model is highly orthologous to human, on average, the protein coding regions have an 85% homology between mice and humans.

Mice have a relatively short lifespan compared to other vertebrates as it is of two to three years only.

There are multiple available high quality reliable genetic tools, knockout mouse models and inbred strains as it is the most widely used model organism in biomedical research. Stable lines can conveniently be constructed by CRISPR technology. There are also multiple premature

aging or long-lived models available (developed hereafter) Unfortunately, most strains used in aging research are inbred to a high degree which make them less genetically diverse than humans.

As mentioned above, there are multiple tools for the study of mice: mouse genome information (informatics.jax.org), published lifespan data for wildtype and inbred strains.

Common laboratory mice have been studied for both dietary restriction and high glucose diets. The results for dietary restriction are in agreement with those established in yeast, flies and worms and the most prominent pathways regulating aging are present in mice (mTOR, insulin/IGF-1, sirtuins...)[6].

The different mouse models for studying aging are well reviewed by Mitchell et al.[25]

Accelerated aging mouse models:

Models mimicking premature aging human diseases: Huntington mice, progeria mice are two examples among many.

They all have a shortened lifespan (less than 2 years).

In these models, dietary restriction has shown to have a protective effect[130], increasing lifespan and healthspan.

The artificial genetic constructs and mutations, mean the results obtained with these models must be interpreted with care, as the genetic modifications may have broader consequences than initially thought of.

Long lived mice models

The Ames dwarf, snell dwarf and Growth hormone receptor knockout mice are the classical mouse models of delayed aging. They have an exceptionally long lifespan (40-60% lifespan extension), are protected against age-related diseases including insulin resistance and present impaired insulin/IGF-1 signaling. They are therefore of great interest for the study of the insulin/IGF-1 pathway.

Most of these models show a developmental delay rather than a delayed aging. This is also true for humans with GH Receptor mutations.

The loss of function of the pathway receptor or growth hormone results in deficiencies in circulating levels of prolactin, growth hormone and thyrotropin which lead to lifespan extension. To note, there is a sex-specific difference in maximal lifespan in Ames dwarf mice (greater maximal lifespan in females). These mice show some characteristics of mice submitted

to dietary restriction: lower core body temperature, improved insulin sensitivity, enhanced antioxidant defenses, and delayed onset of neoplasia[25].

In regards to glucose metabolism, in a study performed by Wiesenborn[150] in the Ames dwarf mice, insulin action functionality is greatly enhanced and glucose homeostasis is improved. Similarly, snell mice, have reduced glucose availability[151] and GHRKO mice have a metabolic profile imitating dietary restriction and contributing to a better cognitive status during aging[152].

Klotho mice

A defect in *Klotho* gene expression in mice accelerates the degeneration of multiple age-sensitive traits identical to that observed in humans (ectopic calcification, skin atrophy, muscle atrophy, osteoporosis, arteriosclerosis, and pulmonary emphysema). Conversely, overexpression of *Klotho* in mice extends life span and has been associated with inhibited insulin and IGF-1 signaling[153]. The *klotho* gene encodes for a β -glucosidase-like protein. In the long-lived mice, an associated increased expression of glucose transporter 4 and increased insulin sensitivity have been demonstrated[154].

Fischer 344 rats

These are the most widely used general-purpose inbred rat strain. There is considerable background information on strain characteristics, including lifespan, spontaneous diseases and response to chemicals.

McCay et al presented the very first study of extended lifespan in white rats who underwent caloric restriction in 1935. Although this finding has been repeated in other rat studies as well as many other species, not all interventions are successful. Among the different outcomes in F344 rats compared to other species, Metformin extends lifespan in mice and *C. elegans* but not F344 rats[155], 2-deoxyglucose extends lifespan in *C. elegans* but not F344 rats.

Under dietary restriction conditions, glucose plasmatic concentrations are reduced by 25%[156]. These rats may develop impeded glucose metabolism and even diabetes if subjected to a high fat or high fructose diet[157].

Naked mole rats *Heterocephalus glaber*, also known as the desert mole rat.

It is the most long lived rodent known to man with a maximum lifespan of 30 years[158]. Because they are highly socialized they are commonly used to study behavioral, neurological and physiological questions. They have several age-related pathologies similar to humans

which makes them such attractive model organisms to study aging: osteoarthritis, macular degeneration, but display negligible senescence and high fecundity until death[159].

Mole-rats have low metabolic rates, but their prolonged longevity results in a lifetime energy expenditure more than 4 times that of mice[160].

Among the disadvantages there are the specific housing conditions they require: low light levels, high temperature and humidity.

Among the potential life-extending mechanisms identified in this model there is a maintenance of the antioxidant enzymes superoxide dismutase, SOD1 and 2 activity levels with age, although they do decline in other rodents[25], and the lower insulin levels as well as reduced levels of IGF-1 (this is consistent with changes reported under dietary restricted conditions)[25]. These rats seldom suffer of age associated diseases such as diabetes..

Rhesus monkeys

Macaca mulatta

The National Institute on Aging is sponsoring an extensive series of experiments to study aging and longevity using primate models including rhesus monkeys and squirrel monkeys at five primate research centers in the United States. Rhesus monkeys are particularly interesting because of their similarity to humans across a wide range of variables and their rate of aging is three times higher than that of humans.

Among the drawbacks to use this model organism, not only are they expensive model to handle because of the long lifespan (more than 30 years), but their weight, size and strict social hierarchies require special consideration and special environment enrichment[25].

The nonhuman primates have in recent years highlighted the important effects of dietary restriction among other things[62].

In case of high-fat, high-sucrose diet, *Macaca mulatta* will develop diabetes similarly to humans.

Birds

Birds have proven to be valuable model organisms for aging research because although they have relatively small body sizes, high body temperatures, high blood sugar levels and high metabolic rates, all classically associated with aging and age-related diseases they seem to age slowly and some bird species are among the longest living animals. Although their glucose levels are one-to threefold higher than most mammals, they have low insulin and high glucagon levels[161].

Recently, researchers have been able to sequence the full genome of two bird species making research with birds more feasible with the possibility to develop new genetic engineering techniques.

To note, the Japanese quail responds to dietary restriction as do mammals[162].

Senescent cell cultures

They present the advantage of offering a homogeneous system, stable lines can be constructed by CRISPR and RNAi.

The relationship between caloric intake and longevity can be represented as a U-shaped curve because extreme levels of glucose supplementation impact survival negatively whether it is too much or too little glucose[69].

This model is perhaps not the most relevant model for in vivo aging unless many senescent cells accumulate.

Among the available tools, one can cite the National Institute on Aging's Aged Cell Bank at Coriell (catalog.coriell.org)

I.3 The model organism *Caenorhabditis elegans*

I.3.1 Presentation of the model

C. elegans is a nematode, or round worm, one millimeter in length. Its lifespan is very short (about 3 weeks) and variable depending on different external variables such as temperature and food availability. Laboratory rearing is carried out in petri dishes or in multi-well plates on an agar medium inoculated with a strain of *Escherichia coli* bacteria or in a liquid medium also containing *Escherichia coli* bacteria at a given concentration. Optimum cultivation temperatures are between 15 ° and 25 °. It is a self-fertilizing hermaphrodite animal, a valuable laboratory feature because it allows to obtain a clonal population from a single individual homozygous for a given trait[26]. Hermaphrodites are determined by the presence of two X chromosomes, contrarily to males which are occasionally generated spontaneously by non-disjunction of X chromosomes during meiosis. Its ease of culture and its short lifespan are not the only assets of this organism as a model to study aging. Other benefits of this model include: perfect knowledge of its life cycle and genome; the self-fertilization of hermaphrodites assuring a stable genomic information and background, its anatomic simplicity, with a limited number of somatic cells (959 cells including 302 neurons and 95 body-wall muscles) minimizing the

parameters to be taken into account; the existence of molecular tools to easily and efficiently modulate the expression of its genes (notably the RNA interference technique, RNAi) or to express proteins encoded by reporter genes or heterologous proteins; its transparency, allowing in vivo microscopic studies; and the fact that about 40% of *C. elegans* genes are orthologs of human genes, with more than 80% of orthologs for genes involved in signaling pathways[163]. Studies conducted in *C. elegans* have led to major discoveries, including the discovery of fundamental and conserved biological processes such as programmed cell death[164] (Ellis and Horvitz 1986, Nobel Prize of 2002), and gene regulation by small RNAs[165–167] (Nobel Prize of 2006)[35].

C. elegans lifecycle: The embryo develops through a series of invariant cell divisions that occur during the first 9h of embryonic development at 22°C. After developing in the egg case, the larva hatched from the eggshell and passes through 4 larval stages (L1-L4). Between each larval stage, the animal sheds its old cuticle. Under crowded conditions and when food is limited, the L1 larvae can enter the dauer stage, in which the animal can survive for months under harsh conditions. The *C. elegans* lifecycle is illustrated figure 11 A.

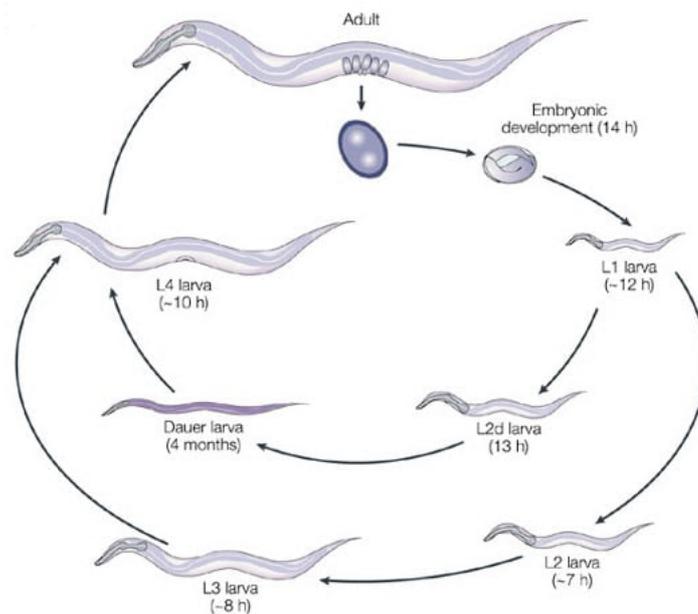


Figure 11 A: Life cycle of *Caenorhabditis elegans* from JORGENSEN and MONGO 2002[168]

As it is relatively fast to carry out lifespan assays in the worm, it is possible to analyze, within a reasonable time frame, the direct effects of genetic and environmental factors, such as nutrition. These measures are supplemented by the analysis of different aging markers in the worm. Its mobility and the activity of its pharyngeal pump diminish with age. Conversely, the presence of advanced glycation end products, and lipofuscin, a cellular pigment composed of

molecular debris and lipid granules in the intestinal tract, increase with age. A decline, and finally cessation of fertility, occur after the first third of the maximum lifespan. Since its first description as a model for the study of development more than 40 years ago by Sydney Brenner[169], the genetic manipulations that have been carried out have led to the identification of more than 200 genes having an impact on the longevity of *C. elegans*.

A first genetic screen, performed after random mutagenesis induced by EMS (ethyl methyl sulfonate), made it possible to isolate the first 5 mutants with increased longevity[170]. The genetic mapping of 4 of these mutants revealed the alteration of the same locus: age-1 (aging alteration protein 1)[171]. This discovery was a major advance in the biology of aging: for the first time, it was shown that the deletion of a single gene could have an impact on the longevity of an entire organism. Not only was the longevity of the age-1 mutant increased, but the appearance of age markers was also proportionately slowed down. The theory of the control of longevity, and therefore of aging, by certain genetic and biochemical pathways, had thus begun to be validated and questioned the passive aspect of the aging process.

In 1993, Kenyon et al. identified another gene whose deletion also increased worm longevity and slowed its aging: daf-2 (dauer abnormal formation protein 2)[78]. Mutations of daf-2 and age-1 favor the transition of larvae into the dauer stage, a form of resistance that the worm assumes when nutritional conditions are poor. In contrast to these 2 mutants, the daf-16 mutant has no dauer stage, even in an unfavorable condition[172]. DAF-16 plays a major role in the "pro-longevity" phenotype of daf-2 and age-1 mutants. Indeed, the mutation of the daf-16 gene in the daf-2 and age-1 mutants cancels this phenotype. Unlike the mutations of the age-1 and daf-2 genes, that of the daf-16 gene decreases the longevity of worms. Cloning and sequencing of the different genes made it possible to identify the functions of the corresponding proteins. AGE-1, DAF-2 and DAF-16 are in fact respectively the catalytic subunit p110 of phosphatidylinositol 3-kinase (PI3K), the insulin and growth factor receptor IGF-1 (insulin growth factor-1) (IR / IGF-1R), and a transcription factor of the FOXO family (Forkhead box protein O)[171,173]. AGE-1 and DAF-2 participate in the insulin/IGF-1 signaling pathway, whose influence on accelerated aging has been confirmed in other organisms, including yeast (via Gpr1 [G protein-coupled receptor 1]), drosophila and mice[76]. Dietary restriction, extensively studied in wildtype worms using mainly culture media containing few bacteria, main food sources of *C. elegans*, or axenic culture media, increases the longevity of this organism[30]. Conversely, diets rich in nutrients or glucose accelerate the aging of *C. elegans*.

Several signaling pathways controlling the aging of the worm, and preserved during evolution, have also been highlighted. Thus, SKN-1 (skinhead-1) / NRF1,2 and PHA-4 (defective

pharyngeal development protein 4) / FOXA (Forkhead box protein A) are important transcription factors for the "pro-longevity" phenotype of the worm. As in humans, cellular protection systems against stress are diminished with age in the worm. Conversely, mitochondrial dysfunction, which induces an increase in oxidative stress and carbonylated stress, increases[39]. The decrease with age of anti-oxidant response elements, such as the production of superoxide dismutase coded by *sod-3* (for oxidative stress) and glyoxalase 1, coded by *glod-4* (glyoxalase domain-containing protein 4) (for carbonyl stress), promotes the pro-aging effects of these stresses[174,175]. Mitochondrial dysfunction is delayed in long-lived mutants, which also develop a highly effective antioxidant system[174,176]. Loss of cellular and tissue protein homeostasis is observed in older worms, and is accelerated in worms with a pro-aging phenotype, such as the mutant of the *daf-16* gene[177]. It promotes the aggregation of proteins that limits the survival of worms. Worms with a pro-longevity phenotype, like the mutant of the *daf-2* gene, exhibit a loss of delayed protein homeostasis. With regard to epigenetics, a direct link between histone modifications and longevity has been demonstrated in *C. elegans*. Various screens using the RNAi technique have indeed made it possible to identify, in a non-exhaustive manner, genes encoding methyltransferases (*ash-2*, *set-9*, *set-15* and *set-26*) and demethylases (*mes-2*, *jmjd-2*, *lsd-1* and *utx-1*), whose expression inhibition participates in controlling the longevity of worms[178]. More concretely, the trimethylation of histone H3 on lysine 4, (K 4) (H3K4me3) increases with age, and its prevention, or the demethylation of the protein, increases longevity[127]. Conversely, trimethylation of histone on lysine 27 (H3K27me3), which directly affects the signaling pathway involving DAF-2 by inhibiting the expression of its gene, increases the longevity of worms and decreases with age in *C. elegans*.

These data, as well as those obtained with other models, made it possible to associate various histone modifications as well as different miRNAs, with the control of aging in humans.

I.3.2 Limits of the model

Like any model organism, *C. elegans* is not perfect and has certain limitations. If its simple anatomy is an asset for studying and visualizing molecular mechanisms, especially those that control aging, it is also one of its weaknesses. The worm has no vascular system, no brain (although it does have 302 neurons), heart or kidney. It is therefore impossible to specifically study the aging of these organs and some of the aging-related complications affecting them, such as cerebrovascular pathologies or renal or cardiac insufficiency. Similarly, for the skeletal system, *C. elegans* being an invertebrate, it is not suitable for research on bone remodeling and

its pathological acceleration leading to osteoporosis. Chronic low-level inflammation, which starts with age, seems to influence aging. This inflammation, better known under the term of ‘inflammaging’, is indeed a condition that could promote aging[179]. Most diseases associated with aging have an inflammatory state. However, the exact role of inflammation in the appearance of these pathologies, or their mere consequences, is not clearly determined. It therefore seems essential to know the mechanisms of appearance of this low-noise inflammatory state and to know its direct consequences on aging. However, having neither vascular system nor inflammation, the causes and consequences of inflammation cannot be studied in *C. elegans*. At the end of its larval development, the worm consists of 959 somatic cells and about 2,000 germ cells. There is no epithelial regeneration or reprogramming of the somatic cells in the worm. It is therefore not possible to study cell renewal and reprogramming, nor the impact of aging on these processes. The replicative senescence, due in part to the shortening of telomeres, cannot be explored in this organism, except for the germ line. Since it is very difficult, if not impossible, to dissect its tissues and to isolate the cells, the study of somatic cell senescence will be difficult. The factors influencing telomere shortening with age, as well as the protective role of telomerases, cannot be studied with this model.

I.3.3 *C. elegans* as a model organism for age-related diseases, example of Alzheimer’s disease

The pathological hallmark of Alzheimer’s disease is the accumulation of extracellular senile plaques and intracellular neurofibrillary tangles (NFTs) in the brains of affected individuals. The critical breakthroughs in the study of Alzheimer’s disease were the biochemical identification of the β -amyloid peptide (A β) as a primary component of senile plaques, and the microtubule-associated protein tau as the primary component of the paired helical filaments that form NFTs.

A β was subsequently demonstrated to be a proteolytic product of the Amyloid Precursor Protein (APP). Mutations in the APP gene were also found in autosomal dominant cases of familial Alzheimer’s disease (FAD) providing some evidence that A β played a role in AD (at least in familial cases) through an unknown mechanism.

C. elegans offers an efficient in vivo system to examine the toxic outcomes of over-expression of proteins and peptides that are prone to pathological misfolding[180]. The simplicity of animal models like *C. elegans* is advantageous: they do not need to recapitulate all pathological aspects of the respective diseases. The potentially confounding behavioral and cognitive responses typical of higher vertebrates are absent. Instead, rapid and clear toxic phenotypes

develop progressively, allowing unmistakably to determine the effect of a gene (here the HATs) on the A β -peptide toxicity.

Transgenic *C. elegans* models have been established for a number of (generally age-associated) neurodegenerative diseases, including Alzheimer's[29]. For the studies presented in this manuscript, we have chosen to work with the CL2006 strain. The transgenic *C. elegans* animals CL2006 express a human A β minigene driven by a constitutive unc-54 muscle-specific promoter which leads to the accumulation of intracellular A β immunoreactive deposits and intracellular amyloid, leading to a progressive paralysis phenotype[29]. In this strain, worms accumulate A β in the body wall muscles.

Many other transgenic age-associated disease models have been developed in *C. elegans*.

I.3.4 Dietary restriction in *C. elegans*

A total of eight different dietary restriction protocols have been developed in the model organism *C. elegans* and are reviewed by Greer and Brunet[30]. They are summarized in the table 4 hereafter from Greer and Brunet's article.

Method	Normal conditions	eat-2	bDR	IDR	Axenic	CDLM	DP	DD	sDR	Resveratrol
Medium	Solid	Solid	Liquid	Liquid + Solid	Liquid	Liquid	Solid	Solid	Solid	Solid
Source of food	Live <i>E. coli</i>	Live <i>E. coli</i>	Live <i>E. coli</i> (antibiotics)	Live <i>E. coli</i> (antibiotics)	Defined chemical broth	Defined chemical broth	Live <i>E. coli</i>	No <i>E. coli</i>	Live or dead <i>E. coli</i> †	Live or dead <i>E. coli</i> †
Genetic mutation		Mutation in the non- α -nicotinic acetylcholine receptor subunit	No	No	No	No	No	No	No	No
Temporal		Birth	Day 2 of adulthood	L4/young adult	Larval day 4 (L4)	Birth	Birth	Day 2 of adulthood	Day 4 of adulthood	Birth
Percentage of Lifespan extension* (%)		0–57	60–73	28	80–150	88	33	42–50	18–35	6–14
Effect on fertility		Decrease	Decrease	Decrease	Decrease	Decrease	Increase	ND	Decrease‡	No effect
Used by		[1–6]	[2,7,18]	[8]	[6,7,9]	[10]	[11]	[3,4,12]	[13]	[14–17]

*Independent laboratories calculate lifespan starting at different ages (birth vs. young adult).

†While UV-killed bacteria were used in some experiments with sDR and resveratrol, most experiments were performed with live bacteria.

‡sDR is normally initiated at a post-reproductive age (day 4 of adulthood), but when initiated at day 1 of adulthood, sDR decreases fertility (data not shown).

ND, not determined.

[1]: (Lakowski & Hekimi, 1998), [2]: (Panowski et al., 2007), [3]: (Kaeberlein et al., 2006), [4]: (Lee et al., 2006), [5]: (Hansen et al., 2007), [6]: (Houthoofd et al., 2002b), [7]: (Houthoofd et al., 2003), [8]: (Bishop & Guarente, 2007), [9]: (Houthoofd et al., 2002a), [10]: (Szewczyk et al., 2006), [11]: (Hosono et al., 1989), [12]: (Steinkraus et al., 2008), [13]: (Greer et al., 2007), [14]: (Wood et al., 2004), [15]: (Viswanathan et al., 2005), [16]: (Bass et al., 2007), [17]: (Gruber et al., 2007), [18]: (Klass, 1977).

Table 4 from GREER 2009¹⁵⁹ Eight methods of dietary restriction in *C. elegans*

Perhaps even more relevant to this thesis are the experiments in which glucose deprivation was conducted. This can be done by culturing the worms in a medium with a glucose shortage or by using 2-deoxy-D-glucose.

Complete absence of glucose is not a viable situation for *C. elegans* as experiments conducted in the VANFLETEREN laboratory demonstrated[181]. Indeed, when culturing *C. elegans* in an axenic medium, glucose is a necessary nutrient[182]. Independently of the culture medium or caloric restriction induced, a minimum amount of glucose must be present in the medium to allow viability.

2-deoxy-D-glucose is a glucose molecule which has the 2-hydroxyl group replaced by hydrogen, blocking it from undergoing further glycolysis[32,183,184]. 2-deoxy-D-glucose acts as glucose competitor in the cell by inhibiting the second step of glycolysis when the production of glucose-6-phosphate by the phosphoglucosomerase is impaired. 2-deoxy-D-glucose cannot be further metabolized after having been efficiently phosphorylated by hexokinase.

In the situation of decreased glycolysis and glucose metabolism, it has been shown that there is an increase of *C. elegans* lifespan[32].

I.3.5 *C. elegans* and enriched glucose diets

Since 2009, *C. elegans* has been used as a model organism to study high glucose toxicity.

Two landmark studies were published almost simultaneously, presenting *C. elegans* as a model organism for the study of the effects of glucose on lifespan[33] and as a model organism for the study of effects of high dietary glucose[31].

In the study by Lee, one of the detrimental mechanisms of high glucose diets on lifespan was described and implicated the insulin/IGF-1 signaling pathway. As mentioned earlier, the insulin/IGF-1 signaling pathway is a major regulator of glucose metabolism and is activated in the presence of glucose. When activated, the pathway transduces signal to downstream transcription factors which are down regulated such as DAF-16/ FOXO. In the presence of a high glucose diet, DAF-16 is downregulated and this consequently shortens *C. elegans* lifespan[33]. In the same study it was established that AQP-1 was linked to osmotic stress tolerance and lifespan regulation after exposure to high glucose concentrations LEE 2009 and this is known to affect the formation of glycerol, a metabolic intermediate of glucose in *C. elegans*.

Different metabolic pathways are enhanced in a high glucose environment and potentially negatively affect lifespan in *C. elegans*. It has been shown that the excess in glucose-6-phosphate is directed into the hexosamine pathway causing DHAP formation which is then

further degraded into methylglyoxal[31,38] an advanced glycation end product precursor. Studies by SCHLOTTERER (2009) and MORCOS give indirect arguments for this.. In case of high glucose concentration, glucose-6-phosphate excess can also be forced into the pentose phosphate pathway. As a consequence, there is an increase of NADPH levels which in turn affects the levels of reduced glutathione[185].

When interpreting results of glucose-enriched diets in *C. elegans* one must keep in mind that normal glucose intake in *C. elegans* remains unknown and that glucose-enriched diets may also affect total calorie intake and proportion of macronutrients[186].

Hormesis describes the phenomenon in which the response of an organism to a physical or chemical agent is qualitatively different dependent on the intensity of the agent.

Although there is a general consensus in the literature that glucose accelerates aging in *C. elegans*, it has also been shown that there is a hormetic profile/effect for glucose. The impact on lifespan depends indeed on the dose of glucose added to the nematode growth medium (NGM)[33]. The effect of glucose on worm lifespan, and more importantly worm healthspan does not seem to be as straightforward as first thought. It has indeed also been shown by TAUFFENBERGER et al. in 2012[187] that although a high glucose diet does have a detrimental effect on lifespan, it seems to have a protective effect on neuronal proteotoxicity. Additional work has evidenced that when worms underwent a high glucose diet during the larval stages alone, there was a positive impact on lifespan and healthspan through a probable mithormesis effect[188].

Many other examples of the hormetic effect exist in the literature, backing up the hormetic hypothesis, by which mild stress activates biological stress responses, thereby improving the health and increasing the lifespan of an organism. Activation of hormetic pathways overcome immediate stresses and may actually repair prior damage in organisms by inducing the expression of genes encoding anti-oxidant and cytoprotective enzymes.

C. elegans orthologs have been found for most of the key enzymes involved in the main eukaryotic metabolic pathways and are recorded in several databases such as Wormbase. Of course, this only provides information about the machinery. Studies have progressively established similarities in the role of the different machinery elements with studies that have established levels of gene expression and enzyme studies quantifying the different metabolites. Glucose transport in *C. elegans*: Although there are 14 known glucose facilitative transporters in mammals, only one transporter (FGT-1) has been found to mediate glucose uptake in *C. elegans* to date[3,189]. FGT-1 appears to be less substrate specific than the different GLUT

because it can also mediate the transport of other hexoses such as fructose, galactose and mannose[189].

Glucose storage in *C. elegans*: Storage of carbohydrates consists primarily of glycogen[190] although glucose and trehalose are also found in consequent amounts[191,192]. The use of the macronutrient storage molecules is regulated in part by O-GlcNac transferase and as a consequence knockout mutants for O-GlcNac transferase have a 3-fold elevation of trehalose and glycogen levels. Dauer larvae rely on their lipid reserves for long-term survival[185]. Recent studies have shown that favoring trehalose to glycogen storage, increases *C. elegans* lifespan[193].

Trehalose is found in many other organisms and is used for energy storage and stress resistance. Trehalose-6-phosphate, the precursor of trehalose is synthesized from glucose-6-phosphate by the enzymes trehalose-6-phosphate synthase TPS-1 and TPS-2[185].

I.3.6 Screening with *C. elegans*

One of the great advantages of *C. elegans* as a model organism is its accessibility to high-throughput screening.

An established method to study complex genetic pathways and discovering new biologically active compounds as well as their targets is to use model organisms or cells for high-throughput screening[35].

Cells are commonly used for high-throughput screens. The cells are usually from a single cell line. In a screening plate, one well can contain thousands of identical cells producing reliable and reproducible data. However, single cells cannot reflect the complexity of whole organisms. Screening methods have been developed for a number of model organisms, such as *C. elegans*, the zebrafish or drosophila.

The simplicity, cost effectiveness of cultivation, as well as size, possibility of culture in 384 well plates make *C. elegans* an effective *in vivo* model organism for high-throughput genetic[194,195] and drug screens[196,197] for assessment of therapeutic candidates. Using *C. elegans* for drug assays can act as a precursor to drug assays in mice and other animals. Screening drugs with *C. elegans* can help to narrow down the compounds that are actually effective, so that secondary screens done with mice or other animals can be done optimally and resourcefully. When developing a high-throughput screen several constraints need to be overcome: space, time, reproducibility. The important screen performed by PETRASCHECK et al.[197] which is the largest compound screen conducted in a whole live organism to date (88 000 compounds) did not overcome the time constraint and was labor intensive. The whole

experimental process was done manually (from plate preparation to experimentation and analysis). *C. elegans* has been used to characterize normal and disease-related molecular pathways, particularly in the fields of aging and neurodegenerative diseases[196]. The aging phenotype of *C. elegans* can be assessed manually by measuring different phenotypical parameters such as counting body bends per minute, measuring speed of movement, pharyngeal pumping rate as well as rates of paralysis in a protein aggregation disease model (as in the CL2006 Alzheimer's disease model)[27]. Although these manual measurements have led to important findings in regards to molecular pathways and mechanisms, these manual assays are low-throughput, highly labor-intensive, time consuming therefore limiting the number of animals in each experiment and prone to human errors and subjectivity of analysis, making it challenging to obtain good statistical significance. These challenges are particularly relevant for high-throughput drug screenings. There is a need for the *C. elegans* community to develop a reproducible, automated, time and cost-effective screening method for chemical compounds. Although many screening methods already exist for *C. elegans*, and chemical screens have been performed prior to the development of our protocol, these were mainly small-scale and/or candidate type approaches and none allowed automated phenotype analysis in 2014. High-scale screens are mostly based on stress resistance assays, with hits being screened for longevity effects or the direct effects of drugs or intervention on lifespan. Additionally, compared to other types of screens (mostly RNAi screens), large scale chemical screening using *C. elegans* seemed to be under-employed[198]. Not only is there a gap in methods allowing the screen of chemical compounds in *C. elegans*, there is also the need to develop methods permitting the screen of aging phenotypes more specifically. As discussed earlier, although targeting general aging mechanisms could allow delaying age-related diseases, it seems also interesting to have a means to explore mechanisms of proteotoxicity and phenotypes associated with many age-associated diseases. As discussed by BANSAL et al. it is important to examine healthspan and lifespan separately. Indeed, in a study they conducted and published in 2015, in which they examined different elements of healthspan during the normal lifespan of four different long-lived mutants they show that long-lived mutants they study extend lifespan as well as the period of frailty. This is opposed to the goal of aging research for which the goal is to extend healthspan and not lifespan alone[199].

Alzheimer's disease is the most common neurodegenerative disease and affects nearly 5 million people in the United States and 1.2 million people in France. Alzheimer's disease typically affects people 65 years of age or older. Alzheimer's disease causes deterioration of memory, mobility, and cognitive ability.

Currently, no cure exists for this disease because, among other things, of the lack of an adequate animal model allowing further experimentation and research. Although there are five drugs that have been approved by the FDA to treat Alzheimer's disease (Aricept, Razadyne, Namenda, Exelon, and Cognex), these treatments do not decrease or stop the symptoms of the disease. Instead, they increase neurotransmitters in the brain to compensate for the neurological damage caused by the progression of the disease. An alternative approach to finding a cure for Alzheimer's is to find treatments that can delay the *onset* of the disease. It is widely recognized that delaying the onset of Alzheimer's disease by 5 years would decrease the incidence of the disease by 50%[200].

With the knowledge that the paralysis phenotype in the CL2006 *C. elegans* strain reflected A β _{1,42} proteotoxicity[29], we hypothesized that drug trials for anti-Alzheimer's disease compounds have failed in humans because Alzheimer's disease entails at least two and possibly more proteotoxic processes (at least A β and tau), and successful treatments will require drugs that are more generally protective against proteotoxicity and age-related neurodegeneration. We therefore set out to screen an important number of drugs in a *C. elegans* model for Alzheimer's Disease CL2006. If a drug could delay paralysis onset, the underlying mechanism could probably entail an action against A β proteotoxicity.

CHAPTER II OBJECTIVES

II Research question

Glucose is the ultimate energy source for most living organisms. It can be found in its free state in fruit or other plants, where it is made from water and carbon dioxide during photosynthesis using energy from sunlight. In animals, there are three sources for glucose: food, the breakdown of glycogen during the glycogenolysis or synthesis in the liver and kidneys by the process of gluconeogenesis from non-carbohydrate intermediates, such as pyruvate or glycerol. In humans, high plasmatic glucose levels contribute to the development of diabetes mellitus and its complications. Although extensive research has been completed on the mechanisms behind glucose driven aging, there is still no ubiquitous explanation for the effect of glucose on aging, if one should exist. More than twenty years ago, BROWNLEE et al. presented a unified view of five optional biochemical pathways for glucose metabolism in a hyperglycemic context and their role in the development of diabetic complications. Since then, much research has been done to link the findings on the role of hyperglycemia in diabetic complications and aging mechanisms but many questions remain unanswered. Many groups around the world have been studying the role of glucose in aging but most of the focus of research has been on the role of the Insulin/Insulin-like Growth Factor-1 (IGF-1) signaling pathway, the first lifespan mediating pathway described. Little focus has been given to other glucose and alternative substrate metabolic pathways, in particular to those leading to the production of advanced glycation end products. In Professor BOULANGER's laboratory, emphasis has been put on the role of advanced glycation end products and their receptor, RAGE in aging. Moreover, excessive glucose is associated with metabolic complications and accelerated aging in several model organisms, including yeast, *Drosophila melanogaster* and *C. elegans*. To explore the mechanisms leading to aging in the context of hyperglycemia, we have chosen to work with and develop the model organism *C. elegans*.

Conversely, dietary restriction, defined as a reduction in nutrient availability in the absence of malnutrition extends lifespan and reduces age-related impairments and diseases in a variety of species, ranging from single-cell organisms to mammals. It has long been known that glucose levels and glucose metabolism is reduced during dietary restriction and could explain part of the protective effects observed. Understanding the protective molecular mechanisms of dietary restriction is an area of active research. Dietary restriction modulates the activity of multiple cellular factors, nutrient and energy sensors which regulate metabolism which include mTOR, sirtuins, 5'Adenosine monophosphate-activated protein kinase (AMPK) among others. In *C.*

elegans, separate or partially overlapping molecular pathways have been described as playing a role in a broad range of dietary restriction regimens. Although the effect of dietary restriction seems universal, no common denominator has yet been found. Studies from Professor MOBBS's laboratory have established that inhibition of CBP blocks all protective effects on lifespan and healthspan of dietary restriction in several different dietary restriction regimens. CBP's role in mediating effects of dietary restriction have been confirmed by three independent laboratories. As a transcriptional coactivator with histone acetyltransferase activity, CBP binds to different partners among which some well-known proteins implicated in lifespan extension and dietary restriction such as FOXO, HSF-1 and NRF2. This could suggest that CBP would be a common factor to multiple lifespan-mediating pathways in dietary restriction, supporting healthy aging. Recent research indicates that high glucose and lipid as well as metabolic hormone alterations may regulate the activity of CBP and that CBP is a regulator of glucose homeostasis[129,201]. To note, CBP hypothalamic expression is inhibited in diabetes[129]. Understanding how CBP regulates aging is therefore of great importance.

Aging is defined as a gradual, time-dependent loss of physiological functions, affecting all living beings. Although aging is not a disease, it is associated with an increased burden of chronological and so-called 'age-related' diseases some of which are known to be induced by high levels of glucose and delayed by interventions such as dietary restriction. Understanding the basic mechanisms of aging can help develop protective compounds and are of great interest for our aging societies.

C. elegans has been used as a model organism for biomedical research and in particular for studies of aging and metabolism. Importantly, various metabolic signaling pathways including insulin/insulin-like growth factor-1 (IGF-1) signaling are evolutionarily conserved and influence aging in *C. elegans* and complex organisms. *C. elegans* is used in many laboratories around the world to study the effects of glucose-induced toxicity as well as those of dietary restriction. Although it has been shown that high glucose diets affect fertility, growth, lifespan and aging in *C. elegans*, since the different studies were completed under a wide range of experimental conditions, it is difficult to compare results. There is therefore the need to homogenize the research conditions and characterize different elements for *C. elegans* as a valid model for the study of high glucose-induced toxicity. It has also not been yet broadly characterized in *C. elegans* if and which components of the antioxidant system are induced as well as which different glucose metabolism pathways are favored. As for the study of dietary restriction, the relative simplicity of the model organism *C. elegans* makes it a good candidate for studying the underlying mechanisms while controlling at best the potential confounders. It

has therefore long been used to study dietary restriction and as many as eight different dietary restriction regimens have been developed. *C. elegans* also offers the possibility of studying different models of age-related diseases, such as Alzheimer's disease by particularly addressing the proteotoxic processes of the disease. Indeed, among the possible pathophysiological mechanisms proposed for Alzheimer's disease is the formation and aggregation of A β peptide in brains of patients with Alzheimer's disease. Successful treatments for age-related diseases mediated by proteotoxicity will require drugs which are generally protective against proteotoxicity rather than directed against one particular misfolded or deficient protein. Although we chose to work with the CL2006 *C. elegans* strain, which accumulates the A β peptide in its muscle wall and is a well-established, worm strain with reproducible and robust aging phenotype, we could have chosen to work with strains modeling different age-related diseases which exist for *C. elegans*, such as Huntington's disease models or Parkinson's disease model. The importance here is that CL2006 represents a model for all age-related diseases where proteotoxicity plays a potential pathophysiological role.

The long term aim of aging research is to develop drugs that will be able to delay the negative effects of aging and age-related diseases/increase healthspan. One of the strengths of our chosen model organism *C. elegans* is its accessibility to culture high number of worms which allows testing important amounts of compounds in high-throughput screens. The key element missing from the existing screening methods is the ability to assess functional phenotypes of aging and age-related diseases as well as the lack of automation. Therefore, it was essential to develop a screening method for rapid automated phenotypic evaluation of multiple potential drugs.

We have developed a semi-automated screening method in order to address these questions.

Our hypothesis is indeed that glucose, and glucose metabolism drive aging. The complexity of the aging process led us to study it from two different angles.

Dietary restriction decreases total exposure to glucose and leads to a particular pattern of metabolic gene expression promoting the use of alternative substrates to glucose. We concentrated on the role of the histone acetyl transferase CBP in promoting this protective gene expression pattern and which tends to decrease glucose metabolism.

High glucose diets accelerate aging. Among the end-products which accumulate in organisms subjected to high glucose diets, research points to advanced glycation end products as potentially accountable for the detrimental effects on aging. Indeed, they are associated with cellular oxidative stress as well as mediating diabetic complications and other age-related diseases. We aimed to further characterize the model organism *C. elegans* subjected to a high-

glucose diet, concentrating on the different activated metabolic pathways, oxidative stress induced and endogenous advanced glycation end product output.

The first part of our work, based on the previous studies conducted in the MOBBS laboratory, consisted in confirming the unique protective role of CBP as a HAT on healthspan and lifespan of an Alzheimer's disease model of *C. elegans*. The second part of the work was to develop a semi-automated screening method for *C. elegans* which allowed phenotypic evaluation and that we validated with the screening of 2560 pharmaceutical compounds from the "SPECTRUM Microsource library". Finally, in Professor BOULANGER's laboratory, we examined aging metabolism with another point of view, concentrating on the effects of a high glucose diet with the goal of characterizing and homogenizing *C. elegans* as a model to study the effect of high glucose diet on aging, concentrating on the different glucose metabolic pathways involved, glucose transporter expression, direct and indirect signs of oxidative stress, and N(6)-Carboxymethyllysine (CML) formation.

CHAPTER III MATERIALS AND METHODS

III.1 Materials

III.1.1 *C. elegans* and feeding source strains

All the *C. elegans* strains were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN).

At Mount Sinai School of Medicine, New York: CL2006 worm strain and Bristol N2 wild type strain; the strain choice and purpose are described in the introduction section. Briefly, the choice to use the CL2006 strain was motivated by the fact that it is a well-established *C. elegans* model for proteotoxicity and Alzheimer's disease. This model develops paralysis as it ages because of human A β -_{1,42} aggregation in the muscle wall.

Alzheimer's disease is characterized by the presence of senile plaques and neurofibrillary tangles in the brains of affected patients[202]. The toxic form of β -amyloid peptide (A β) is the major constituent of the senile plaques. A β -_{1,42} peptide derive from the sequential cleavage of amyloid precursor protein (APP) by β and γ secretase. The precise mechanisms by which A β -_{1,42} participates in Alzheimer's disease pathogenicity is still unclear. *C. elegans* has an APP related gene, but it cannot be cleaved to produce A β peptide. Different groups set out to engineer *C. elegans* Alzheimer's disease models which express the longest most toxic A β species: A β -_{1,42} [29,203]. The engineered transgenic worms express A β -_{1,42} under the constitutive *unc-54* body wall muscle promoter. They develop progressive paralysis and intracellular A β deposits which colocalize with amyloid specific dyes (Congo Red and thioflavin S)[202]. These models helped determine that A β -_{1,42} toxicity is highly dependent on genes mediating longevity such as DAF-2 and DAF-16[204].

At the Lille University: Bristol N2 wild type strain

Feeding source for worms was the OP50 *Escherichia coli* strain which was obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). *C. elegans* is usually grown monoxenically using *E. coli* strain OP50 (Brenner, 1974). *E. coli* OP50 is a uracil auxotroph bacteria whose growth is limited on NGM plates. A limited bacterial lawn is valuable because it permits easier observation of the worms. OP50 bacteria were grown 12-16h at 37°C with continuous shaking in Lysogeny Broth (LB) medium (recipe included in the annex). The overnight culture was stored at 4°C for up to 2 weeks.

Worms were grown at 20 degrees Celsius on Nematode Growth Medium (NGM) seeded agar plates until adulthood unless described otherwise. The recipes for NGM are included in the annex section. NGM plates were seeded with 200-450 μ l of OP50 suspension and incubated

overnight at 37°C or 3 days at 20°C. Some glucose lifespan assay experiments were performed with heat-killed feeding bacteria in Lille. For this, overnight OP50 culture was concentrated 10 times and heat-killed by placing them 30 min at 65°C.

III.1.2 Worm synchronization

One of the attractive features of the model organism *C. elegans* is the possibility of obtaining synchronized populations of worms through easy protocols. This allows comparability between conditions and groups of worms which is important in lifespan and paralysis assays used for the study of aging mechanisms. To synchronize the worms, a process called axenization was used. Axenization dissolves the adult worms while preserving the eggs within them that have not yet been laid.

Before each experiment, eggs were obtained from gravid worms by axenization or hypochlorite synchronization and matured to young adult worms. The synchronization protocol followed is: gravid worms are incubated 5min at 20°C with vigorous shaking or vortexing in bleaching solution containing NaOH (500mM) and chlorine-based bleach (1% active chlorine). Worms' lysis is stopped by adding an equal volume of M9 buffer (recipe included in the annex). Eggs are recovered by centrifugation (3min at 1000g), washed twice with M9 buffer and suspended in M9 buffer (5000 eggs per ml) before incubation 16h at 20°C with continuous shaking. During incubation eggs hatch and stop development at L1 larval stage. L1 larvae are then collected and grown on NGM plates seeded with OP50 bacteria for 2 days at 20°C to develop into young adults.

III.1.3 Plate preparation solid and liquid

To grow the worms from larval stage to young adulthood we chose to plate them on solid agar medium for ease of manipulation. For the experiments, we chose to use solid and liquid medium. The natural medium/habitat for worms is a solid one although worms are able to live in a liquid medium. The experimental advantages of culturing *C. elegans* in liquid medium include: space gain, possibility of using a smaller quantity of compounds which is useful when conducting large scale drug screens.

Solid plate preparation: all assays on agar were carried out on 60mm (New York) or 100mm (Lille) NGM plates. All stock were prepared with sterile water and refrigerated at +4°C. The NGM plates were supplemented with the anti-mitotic 5- fluorodeoxyuridine (FUDR) (0.1g/ml or 150 µM) to prevent internal hatching, and egg production for all experiments. All plates were numerically labeled to ensure no scoring bias. Worms just reaching adulthood were transferred

to the experimental plates with the control (L4440 or no glucose), RNAi, 2DG (final concentration of 4mM added to the medium, purpose of 2DG described in the introduction chapter) or different concentrations of glucose (40mM, 110mM or 550mM added to the medium) depending on the given experiment. The lifespan or paralysis assay was conducted as described below[14]. Our choice for the different glucose exposure concentrations was made according to results of preliminary experiments in addition to data from the literature. SCHLOTTERER et al.[31] were the first to expose *C. elegans* to high glucose concentration to study effects on lifespan. They chose the concentration of 40mM. Many laboratories, among which professor KENYON's laboratory published results of glucose lifespan assays with glucose concentrations of 110mM being the optimal concentration to study the impact of high-glucose diets on worm lifespan[205]. Results from various laboratories are not all consistent with the first results cited previously, and the concentration of 550mM of glucose is also regularly used to study the effects of a high-glucose diet on worm lifespan.

Liquid media plate preparation:

For RNAi experiments: Bacterial concentration for the liquid assay was obtained by measuring the Optical Density (OD) at 600nm using a spectrophotometer. After obtaining the concentration of each bacterial solution containing OP50, the control empty vector L4440 or double stranded RNA, bacteria were re-suspended in S complete media (SCM) to inhibit bacterial growth (there is no metabolizable carbon source in SCM) and supplemented with 5-FUDR (0.1g/L), to make a concentration of 10^9 cells/ml (considered to be ad-libitum)[34].

Eggs were collected by standard hypochlorite treatment of gravid worms. Synchronous populations were grown on 100mm NGM plates seeded with OP50 bacteria until the L4/young adult stage. They were then washed off the agar plates with M9 buffer and transferred into the wells with 5-15 worms per well and maintained at 20 °C with gentle gyratory shaking[14].

For gene expression analysis, AGE production and protein oxidation: Day1 adult worms were grown in 6-well plates (approximately 1000 worms per well in 3ml medium) containing NGM alone or NGM plus D-glucose at a final concentration of 40mM, 110mM or 550mM. All the media were supplemented with FUdR (50 μ M) and heat-killed bacteria. Worms were incubated 3, 6, 10 and 13 days at 20°C in those different glucidic conditions. Three independent experiments were performed.

III.1.4 RNAi presentation, materials and choice

Through genetic analyses, the function of genes is investigated by studying organisms where gene function is altered. In reverse genetics, the functional study of a gene starts with the gene sequence rather than a mutant phenotype. Using various techniques, a gene's function is altered and the effect on the development or behavior of the organism is analyzed.

RNAi is an evolutionarily conserved process leading to post transcriptional gene silencing triggered by double-stranded RNA (dsRNA) or small interfering dsRNA (siRNA)[206]. siRNAs are recognized by the RNA-induced silencing complex (RISC). The RISC RNAi effector complex functions to unwind the siRNA duplexes to allow precise binding to the complementary mRNA target, followed by siRNA mRNA duplex cleavage and mRNA degradation. A second feature of RNAi that operates in *C. elegans* but apparently not in *Drosophila* or mammalian cells is systemic RNAi. Systemic RNAi refers to the introduction of dsRNA into one tissue, leading to RNA silencing in distant cells. There are four methods for dsRNA delivery in *C. elegans*: (1) micro-injection of dsRNA into any region, (2) feeding RNase III resistant bacteria (HT115 *E. coli* strain) expressing dsRNA to *C. elegans*. A genomic feeding library has been constructed and is available commercially. The library consists of genomic fragments cloned into an IPTG-inducible T7 polymerase vector producing dsRNA, (3) soaking in dsRNA, and (4) in vivo production of dsRNA from transgenic promoters. Injecting, feeding, and soaking dsRNA may result in systemic RNA silencing. The RNAi in these studies were obtained from the RNAi feeding library developed by the AHRINGER lab which has 16 757 clones made by cloning gene specific genomic fragments between two inverted T7 promoters, inserted in a L4440 plasmid. The L4440 plasmid is presented schematically hereafter in figure 12. The library uses the HT115 bacterial strain as a host for the plasmid RNAi clones. HT115 has IPTG (Isopropyl β -D-1-thiogalactopyranoside, a molecular mimic of allolactose, triggering the transcription of the lac operon and the sequence associated with it) inducible T7 RNA-polymerase (viral polymerase with its own promoter sequence) and a disruption of the RNase III gene (the enzyme which specifically binds to and cleaves double stranded RNA), and marked with Ampicillin resistance. Bacteria producing RNAi were fed to the worms from day 1 of adulthood onwards.

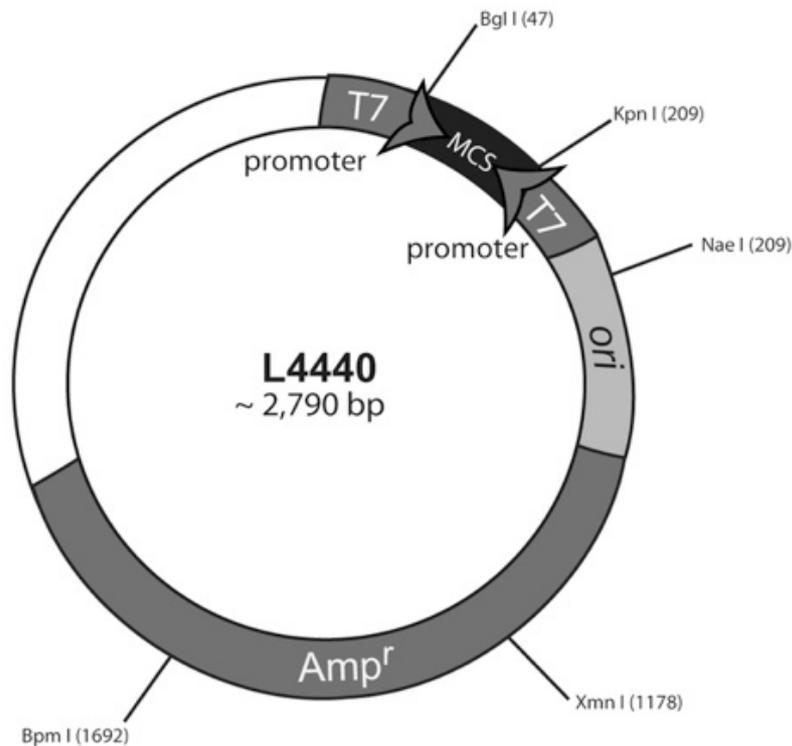


Figure 12: schematic representation of the plasmid L4440, source from addgene.org, Fire Lab

The plate preparation (solid or liquid) were identical for the RNAi experiments except for the addition of ampicillin (0.1g/L), and IPTG (0.24g/L) in the medium.

Different RNAi used included: The *cbp-1*, *hat-1*, *mys-1*, *mys-2*, *ada-2*. RNAi were obtained from the RNAi feeding library developed by the AHRINGER lab. The bacterial strain containing the L4440 plasmid was used as a control. RNAi was carried out following standard bacterial feeding methods. All RNAi experiments were conducted only in adult worms. The choice of HAT RNAi was determined as follows: to answer the question of whether CBP uniquely impacts aging and Alzheimer's disease in our model organisms, we determined which HATs needed to be tested with BLAST (Basic Local Alignment Search Tool). BLAST is a bioinformatics algorithm developed by ALTSCHUL in 1990 for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. For each human HAT, we conducted a protein-protein BLAST, to identify the proteins in *C. elegans* with the highest homologies to use in our studies.

We identified homologies with each Homo Sapiens HAT in *C. elegans* except for the Nuclear Receptor Associated (NRA) family. We were unable to do a study on TAF-1 because it was unavailable in our RNAi library.

HAT Class (A/B) and Family Type	<i>Homo sapiens</i> protein	<i>C. elegans</i> protein	Homology
A (nuclear) MYST	MYST1	MYS-2	45%
A MYST	MYST3 and MYST4	MYS-1	51%
A p300/CBP	CBP	CBP-1	51%
A GNAT	PCAF1	PCAF-1	86%
A GNAT	ADA2	PCAF-1	31%
A Transcription factor related family	TAF1	TAF-1	40%
B (cytoplasmic)	HATB	HAT-1	35%

Table 5 Presentation of different *C. elegans* HAT orthologs with corresponding homologies to human HAT (information collected via the blast.ncbi.nlm.nih.gov website) GNAT: General control non-repressible 5-related N-acetyltransferase

III.1.5 Development of the compound screening tool

High-throughput screening methods exist for *C. elegans* but in 2014, they did not allow efficient chemical screens or phenotype analysis. This is an issue and limits the scope of studies on aging as many age-associated diseases modeled in *C. elegans* are monitored *via* phenotypical changes as the modeled disease worsens. The goal that led to the creation of our protocol described hereafter was to develop a *C. elegans* screening platform that allowed the rapid screening of large chemical libraries for novel biologically active compounds, directed against aging. We aimed at developing a tool which allowed analysis of the paralysis phenotype in CL2006 strain, a model of Alzheimer's Disease in *C. elegans*. As mentioned earlier, the paralysis phenotype in CL2006 is intimately related to human A β ₁₋₄₂ peptide accumulation in the worm muscle wall, leading to progressive paralysis and mimicking Alzheimer's disease development. This meant the analysis of the paralysis would indirectly allow the study of proteotoxicity development. Compound distribution in the plates, recording of the experiments were entirely automated, but worm distribution and image analysis were done manually which is why we qualify our screen as 'semi-automated'.

III.1.5.1 Lab Minion Microscope platform design and development - 3D printing

The Lab Minion Microscope was developed as a semi-automated screening tool to allow rapid large-scale screens of compounds which protected against human A β ₁₋₄₂ induced proteotoxicity in a *C. elegans* model of Alzheimer's disease, CL2006 worms.

All the 3D-printed parts were drawn and developed by the Deezmaker 3D-printing company using *SolidWorks*. These include: parts of the three motorized arms allowing microscope movement in three spatial directions and parts of the platform holding the experimental plate. The system includes three metallic arms with rails allowing the movement of the microscope and metal stimulation device in all 3 dimensional directions, a Plexiglas stage to secure the tested plate, a small screen placed on one of the metallic arms to visualize the program steps, a computer (Mac running OSX 10.7 or higher) connected to the Minion Microscope showing the images as they are being filmed and containing software needed to control the Minion Microscope and store the images.

Associated to the Minion Microscope is software using several open source libraries to stream commands onto the machine and capture videos. The software calls the Mac versions of the open source programs Pronsole and Videosnap to do this. Pronsole is a program that streams G7code commands onto an Ardiuno7compatible processor. Videosnap captures, compresses and saves video streams. Python is the open source scripting language used by the Deezmaker team to write the code which is customizable according to the different visualization needs of the experimenter and needed to operate the recording system. The computer program defines the visioning order followed by the microscope, time of visioning and controls the electric stimulating arm.

For the purpose of our screen, the different steps defined by the program were: 1/ filming of row identification letter or number, 2/ electric shock stimulation in tested well, 3/ simultaneously the filming of well for analysis for 90seconds per well starts as the camera is placed correctly and the electrode slews away into a well on the second half of the plate for cleaning. 4/ storage of the films in computer file 5/electrode arm slews back to the next well on the row to stimulate and so on till the last well of the row.

The stimulation arm consisted in a copper electrode which was connected to a 6V battery. Samples are put in columns 1-12 only as the columns 13-24 contain a cleaning liquid for the electrode.

The microscope was a pocket pen microscope connected to a Nikon camera.

The complete device was placed on a led plastic light box.

Video is streamed over USB to a file in a hardcoded directory. Each row (wells A7P) is in one file. Video for a row is about 200 Mb for empty wells; wells with worms are more. This means that a 384-well plate (of which only 12 rows are used in the version of 2014) will generate 12 times 200 Mb or about 2.5 Gb of video. The video is compressed using the H.264 compression algorithm. The machine pauses over the beginning of each row to capture the well number on video so that the user can determine where the data came from if the files get confused.

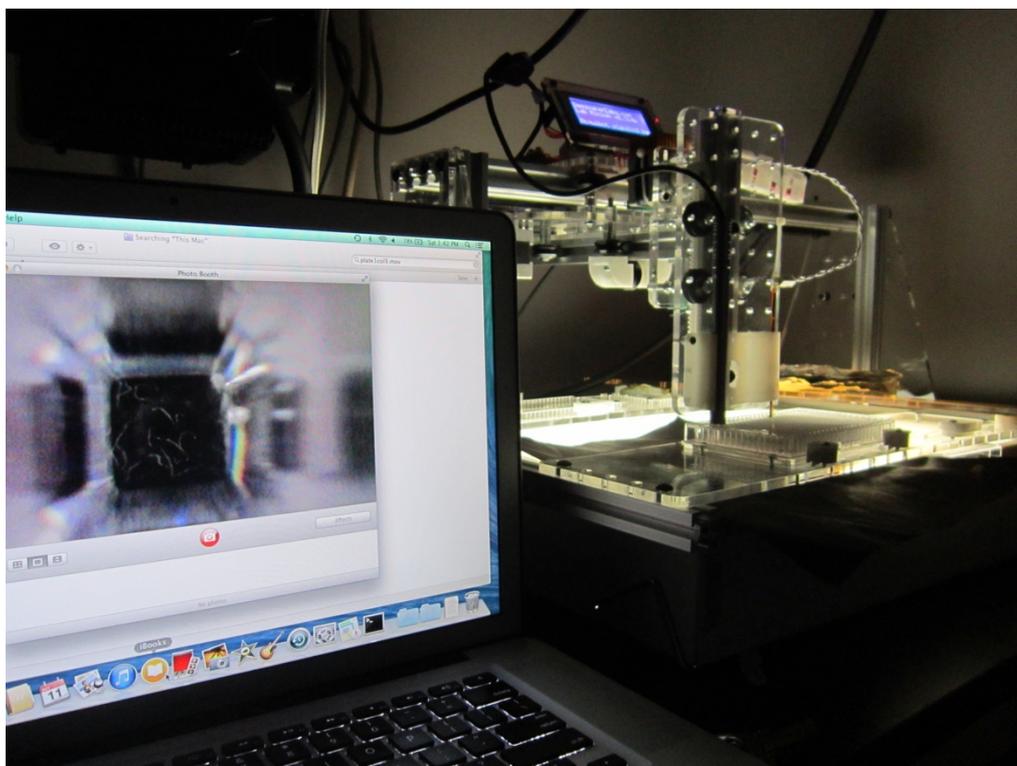


Photo of the Minion Microscope set-up

III.1.5.2 Microsource SPECTRUM compound collection

To test our semi-automated screening tool (Minion Microscope) we chose to screen a large-scale compound library for compounds which would be protective against human A β ₁₋₄₂ induced proteotoxicity in a *C. elegans* model of Alzheimer's disease, CL2006 worms.

The SPECTRUM Collection from Microsource is a library of 2560 biologically active and structurally diverse compounds including almost all FDA-approved drugs as well as pure natural products [MicroSource Discovery Systems Inc., Gaylordsville, CT; <http://www.msdiscovery.com> (Blobaum 2015)].

The compounds are dissolved in dimethyl sulfoxide (DMSO), which is often used in medical research as a drug vehicle for *in vivo* and *in vitro* studies.

The SPECTRUM library is composed of approximately 60% clinically approved drugs, 25% natural products and 15% of de-novo compounds. Such content provides the opportunity to

evaluate drugs and biochemical tools with known biological profiles and, at the same time, explore the potential of novel structural diverse pure natural products. Each compound from the SPECTRUM library has a minimum of 95% purity.

The compound library is managed by the core screening facility of the Icahn school of medicine at Mount Sinai.

III.1.5.3 Plate preparation for screening

There was one description of large scale chemical screen in *C. elegans* that was used as a basis to develop our approach: Michael PETRASCHECK's innovative and ambitious small molecule screen for life-extending molecules[197]. This study used 384-well plates and liquid cultures. Effects of the different chemicals was tested directly on their effect on lifespan. Wild type worms (strain N2) were stimulated every two days with a blue light and movement was recorded. Worms that moved were counted as alive, worms failing to move after stimulation were counted as dead. The concentration of the different compounds for this screen was 30-90 μ M. It is difficult to identify an ideal concentration to use when testing it on *C. elegans*. Indeed, it is virtually impossible to know the amount of compound which is ingested by each worm as well as the quantity of chemical which enters the organism *via* its cuticle (the worm is 'soaking' in the compound) and authors tend to recommend a concentration as high as possible. Unfortunately, the authors for the PETRASCHECK screen do not explain more in detail why they chose to test the chemicals at a concentration of 30-90 μ M[197]. In a subsequent screen by the same team, the concentration of 33 μ M was used, again with no further explanation[207]. Our initial wish was to test the compounds from the Microsource SPECTRUM library at a concentration of 30-40 μ M to match the results of this initial screen by PETRASCHECK, but this was not possible for technical reasons linked to the high-throughput core facility equipment available at the Icahn school of medicine at Mount Sinai. A compromise had to be found between our chemical concentration goal (a minimum chemical concentration was needed for an effect to be seen), the high throughput facility drug concentration capacities, and the amount of liquid needed to add the worms and OP50 in a second step of the plate preparation. As the high-throughput core facility is a shared space for all members of Mount Sinai we were not able to bring plates containing worms or OP50 because of the risk of contamination. It was therefore necessary to provide plates containing only anorganic medium to the core facility. Worms as well as feeding source (*E. coli*) were added in a final plate preparation step. The consequence of this was a supplemental dilution of the compound.

The final concentration obtained was therefore 6 μ M. The maximum chemical concentration the core facility could supply us with was 8 μ M. Because of the necessity to add the worms and bacteria only after the drugs, the final concentration of compound we obtained was 6 μ M.

SPECTRUM compounds were plated in 384-well plates at the Icahn school of medicine at Mount Sinai High-Throughput Screening Facility using an automated liquid handler. The compounds from the library were distributed in dimethyl sulfoxide (DMSO) solvent. Dr. SIVENDRAN prepared all the testing plates regarding the compounds.

The plates containing 40 μ l of SCM plus 0,1 μ l of the tested compound (concentration of 8 μ M) in each well were transported back to the Mobbs laboratory where a volume of 15 μ l containing 10 worms, OP50 *E. coli* mix and 2 μ l of FuDR were added to total 57 μ l for each well.

III.2 Methods

Hypothesis, experimental design, realization and analysis were the result of a group effort in both participating laboratories. In Professor MOBBS's laboratory at the Friedman Brain Institute, Icahn School of medicine at Mount Sinai the active participants included Emily CHEN, the Deezmaker 3D-printing company (Joan HORVATH and Rich CAMERON), Imikimabong IBIA, Dr. Fumiko ISODA, Rachel LITKE, Stephanie LUM and Emily MOBBS. In the laboratory of Professor BOULANGER, LIRIC UMR995, active participants included Dr. Chantal FRADIN, Shuting CHEN, Constance DUBOIS and Rachel LITKE.

III.2.1 Lifespan assay on agar

Lifespan assays were performed to evaluate the effect of glucose-enriched diets, the inhibition of glucose metabolism (2DG assay), and different HAT knock-down on lifespan.

Adult worms were transferred to NGM Control/RNAi/glucose/2DG plates. The worms were scored every two to three days and were categorized as dead when they failed to produce movement after repeated stimuli (gentle prodding by a platinum wire in New York or sound and light stimulation in Lille). Scoring continued until all worms were deceased. Animals were censored when they crawled off the plate, presented rupture or contained internally hatched larvae. Three independent experiments were performed with a minimum of 30 animals each. The lifespan assay experimental protocols are schematically represented in figures 13 and 14 below.

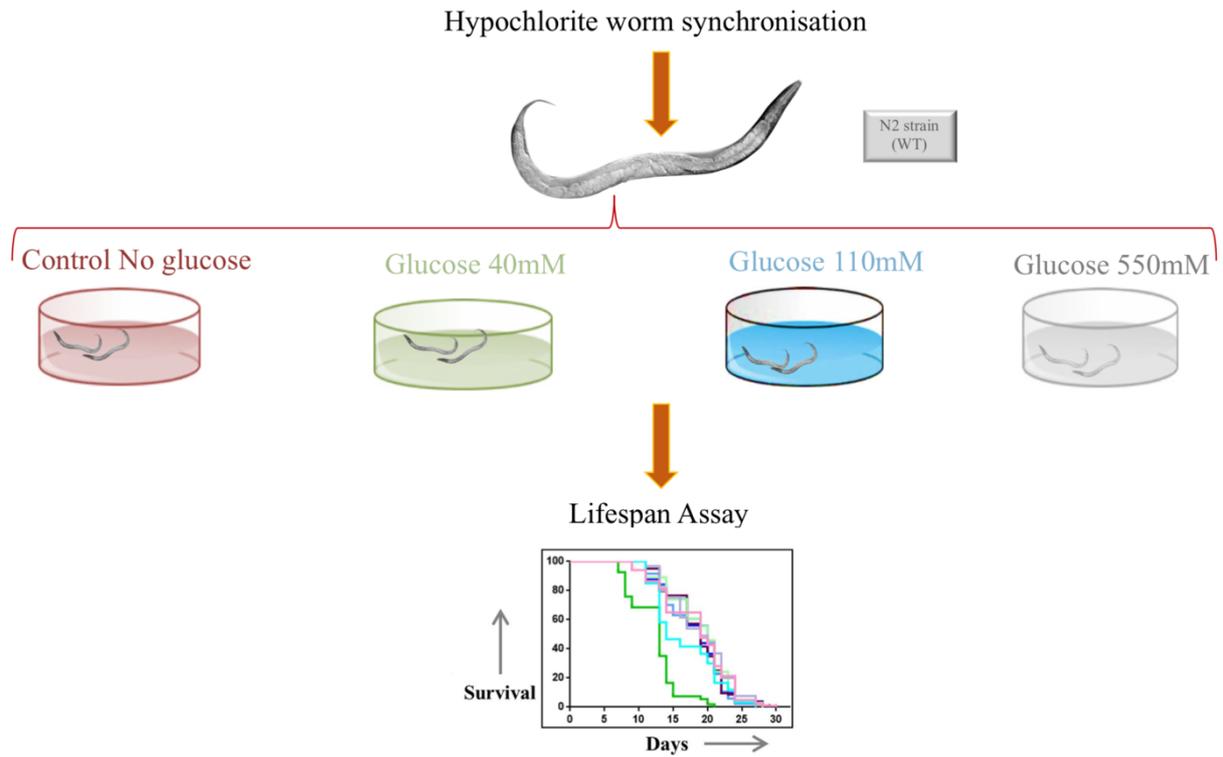


Figure 13: Schematic representation of experimental protocol for different glucose concentrations used for the lifespan assays on agar

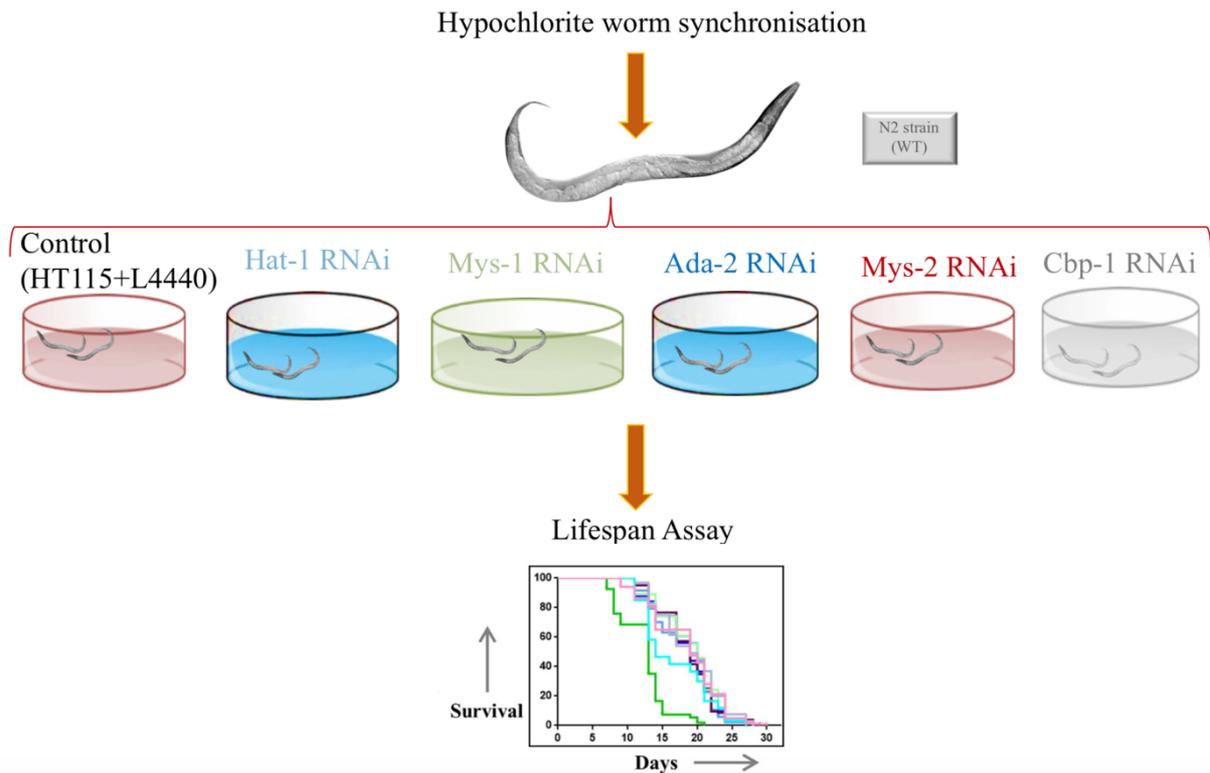


Figure 14: Schematic representation of experimental protocol for different HAT RNAi lifespan assays on agar

III.2.2 Paralysis assay

All paralysis assays were conducted with CL2006 worms. In CL2006 (at 20°C) worms develop a progressive paralysis starting at day 7-8 of adulthood which is caused by human A β -1,42 aggregation in the muscle wall. Worms were scored daily starting at day 4 until day 13. The assay was ended at an age at which wild-type worms begin to undergo age related paralysis usually defined as day 12[208]. The paralysis assay followed the method described by Cohen[204]. For all paralysis assays, we considered that the acceleration of the paralysis phenotype was an indication that the RNAi worked. In the case of a positive RNAi result (like in this study), the false positives are extremely low (<1%[209,210]). The different paralysis assay protocols are schematically represented in figure 15.

III.2.2.1 On agar

Synchronized adult worms were transferred to 60mm NGM RNAi plates with 10-15 worms on each plate. We tested the worms daily by gently prodding their head with a platinum wire. Worms that moved their heads but failed to move their bodies were categorized as paralyzed.

III.2.2.2 In liquid

For the paralysis assay in liquid, worms were filmed for 90 seconds every two days with a Nikon camera attached to the stereomicroscope and scored by eye with the video viewing in VLC.

Physiological behavior for *C. elegans* in liquid is different from that on agar. In liquid, movement is easier for the worms and it is commonly called ‘thrashing’. Even so, in liquid media, the majority of the time worms are still, making it difficult to distinguish a healthy worm from a paralyzed one. To stimulate movement, we chose to apply a small electric shock in each well with a 6V battery. A worm was considered paralyzed if it only moved its head during the 90 seconds movie or, if there were ≤ 2 thrashes (one in each direction) within the first 90 seconds after the stimulation. Worms were considered dead if no movement was visualized and healthy if they moved more than the paralyzed criteria we established.

Synchronization CL2006 worms with sodium hypochlorite

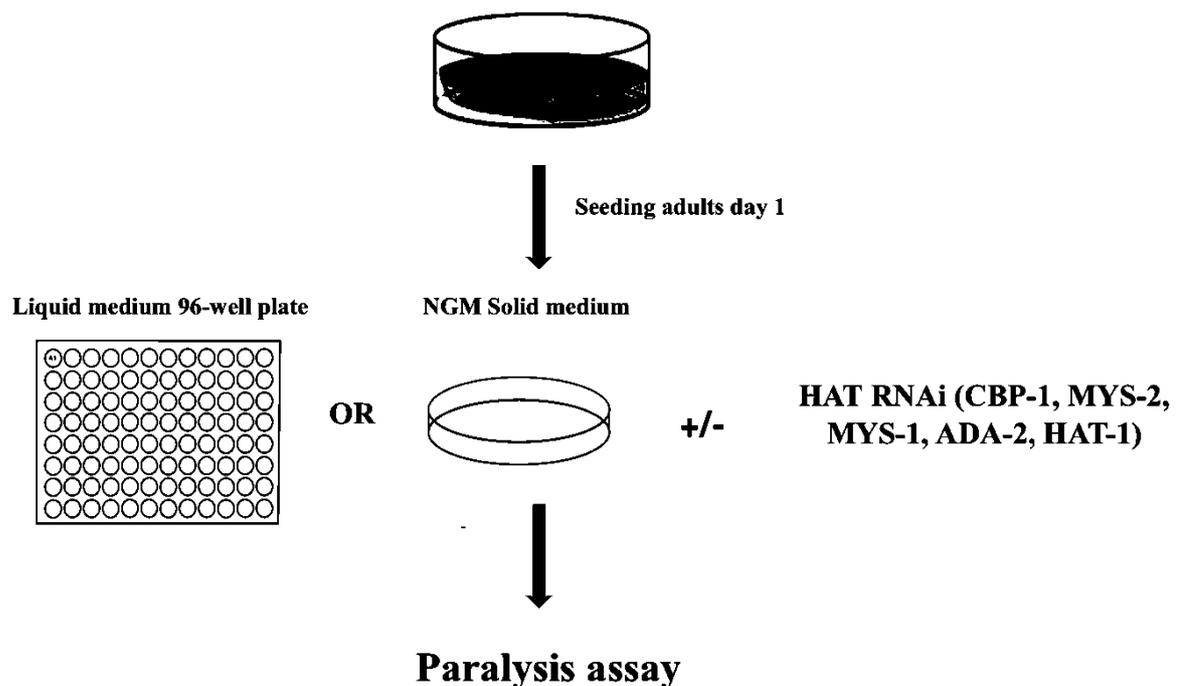


Figure 15: Schematic representation of the experimental protocol for paralysis assays on agar and in liquid medium

III.2.3 Culture conditions for analysis of gene expression, AGEs production and protein oxidation

Day1 adult worms were grown in 6-well plates (approximately 1000 worms per well in 3ml medium) containing NGM alone or NGM plus D-glucose at a final concentration of 40mM, 110mM or 550mM. All media were supplemented with FUdR (50 μ M) and heat-killed bacteria.

Worms were incubated 3, 6, 10 and 13 days at 20°C in the different glucidic conditions. Three independent experiments were performed.

For RNA extraction, worm cultures were centrifuged 2min at 500g and worms were suspended in M9 buffer before being stored at least 24h and up to 1 week at 4°C before processing.

For protein extraction, worm cultures were centrifuged 2min at 500g and worms were washed 5 times with M9 buffer before being suspended in RIPA buffer (Tris HCl 100mM pH7.4, NaCl 140mM, EDTA 5mM, sodium deoxycholate 1%, SDS 0.1% and β-mercaptoethanol 1.5%) and stored at -20°C until their lysis. Protease inhibitors (complete EDTA-free proteinase inhibitor cocktail, Sigma Aldrich) and acid-washed glass beads (425–600 μm) were added to the worms' suspension and the worms were then homogenized with Precellys®24 (Bertin Technologies) at full speed, 4 times 10 s, 3 cycles. The samples were kept at 4°C during the whole process. Soluble materials were recovered by centrifugation at 15000g for 15min at 4°C and protein concentration was determined using the Bradford assay (Sigma Aldrich). All culture supernatants were kept for determination of glucose concentration. This is schematically represented in figure 16.

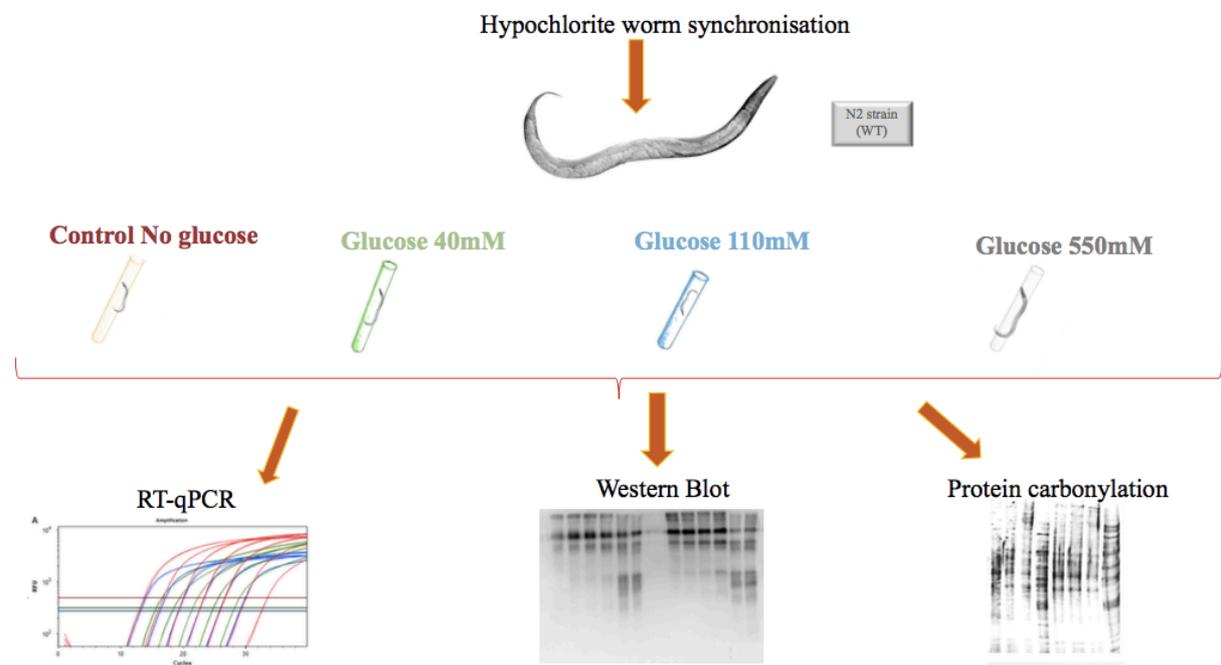


Figure 16: Schematic representation RT-qPCR, Western Blot protocol for gene expressions, AGE production and protein carbonylation depending on different glucose concentrations exposure of the worms

III.2.4 Glucose assay

Glucose concentration in culture supernatants and worm lysates was determined using OneTouch Verio IQ glucose meter and test strip (OneTouch®). The samples were compared to

a glucose standard curve within the range of 0-8mM (18-144 mg/dl). This provides a linear detection.

III.2.5 Total RNA extraction and gene expression analysis by RT-qPCR

RNAs were extracted using the acid guanidinium thiocyanate-phenol-chloroform method[211]. Briefly, the worms were homogenized in Trizol (Ambion) with acid-washed glass beads with Precellys®24 at full speed, 4 times 10s, 3 cycles. Addition of chloroform to the worms' lysates containing acidic solution and then phenol allowed separation of RNAs from DNA and proteins after centrifugation. RNAs recovered in the upper aqueous phase were precipitated by isopropanol and washed with 70% ethanol. Amount of total RNAs was measured with the NanoDrop™ (Ozyme) spectrometer.

RNAs (1µg) were treated with DNaseI (Thermo Scientific) to remove genomic DNA prior to reverse transcription to complementary DNA, cDNA (High-capacity cDNA reverse transcription kits, Applied Biosystems). Quantitative real-time PCR was performed using PowerUp™ SYBR® Green 2X Mater Mix (Applied Biosystems) with the following fast program: 2min at 50°C _to activate Uracil-DNA Glycosylase (UNG), which eliminates carry-over contamination, 2min at 95°C _to activate the DNA polymerase and; 40 cycles of 95°C _for 1s and 60°C _for 30s to amplify the target sequence. The program ended with different steps allowing the dissociation curve analysis: 95°C _for 15s (1.6°C/s), 60°C _for 60s (1.6°C/s) and 95°C _for 15s (0.15°C/s). Gene expression levels were normalized to *pmp-3* and relative expression was calculated by the 2-DDCT method. Primers are listed in Table 6 and the enzymes we chose to explore are represented figure 17 according to their localization within the glucose metabolic pathways.

<i>Gene</i>	Primer sequence (5'®3')	
<i>fgt-1</i>	Forward: TGCTCGTCACCATCGCAATT	Reverse: GGAGACTCTGGGCACAACAT
<i>gst-4</i>	Forward: TTTGATGCTCGTGCTCTTGC	Reverse: AATGGGAAGCTGGCCAAATG
<i>gst-14</i>	Forward: TTCGGGTTCGCTGGAAAAC	Reverse: CCGACTCGTTTTGCAATGAC
<i>pmp-3</i>	Forward: AGTTCAGTTGCTCTCGTTGC	Reverse: AGGCCAGCAAACATTCGAAG
<i>sod-3</i>	Forward: AAAGCATCATGCCACCTACG	Reverse: TGAATTCAGCGCTGGTTGG
<i>sod-1</i>	Forward: TCCGTCACGTAGGCGATCTA	Reverse: TCCGGCATGAACAACCATAGA
<i>hvk-1</i>	Forward: GCACCGAATGGGATGAAGTT	Reverse: TCGGGCACACTCTCCCATAT
<i>sodh-1</i>	Forward: GGTTGGAGGACACGAAGGAG	Reverse: GGCTCGTGGCCTTTCTTACA
<i>T25B9.9</i>	Forward: AGCAAATCAGCAAGGCTCTTT	Reverse: GCATCCTCCTCTCCACATCAA
<i>F21D5.1</i>	Forward: GGTGCTCCACGGTTCAGAAA	Reverse: GGCACCACATCCCTGATTCA
<i>gsy-1</i>	Forward: CCGGAGCCGTCAAGATGAAT	Reverse: CCGAGGATCACAGCGTCATT
<i>tps-1</i>	Forward: CCTGGCGTCCGACAAAGTAT	Reverse: TCTCCTCGGAGCCCATTTTG
<i>tps-2</i>	Forward: GCCTTGCCACGTCACATTTTC	Reverse: CCTCCGGAACCTTGCTTGAT
<i>glod-4</i>	Forward: GTGCCAAGACCATCGACTTT	Reverse: TCTTGCTCCATCGTCCGTTAT

Table 6 Primers list for real-time PCR Glucose transport: *fgt-1*, glucose facilitative transporter ; detoxification enzymes: *gst-4*, *gst-14* glutathione S-transferase; anti-oxidant defense : *sod-1/3*

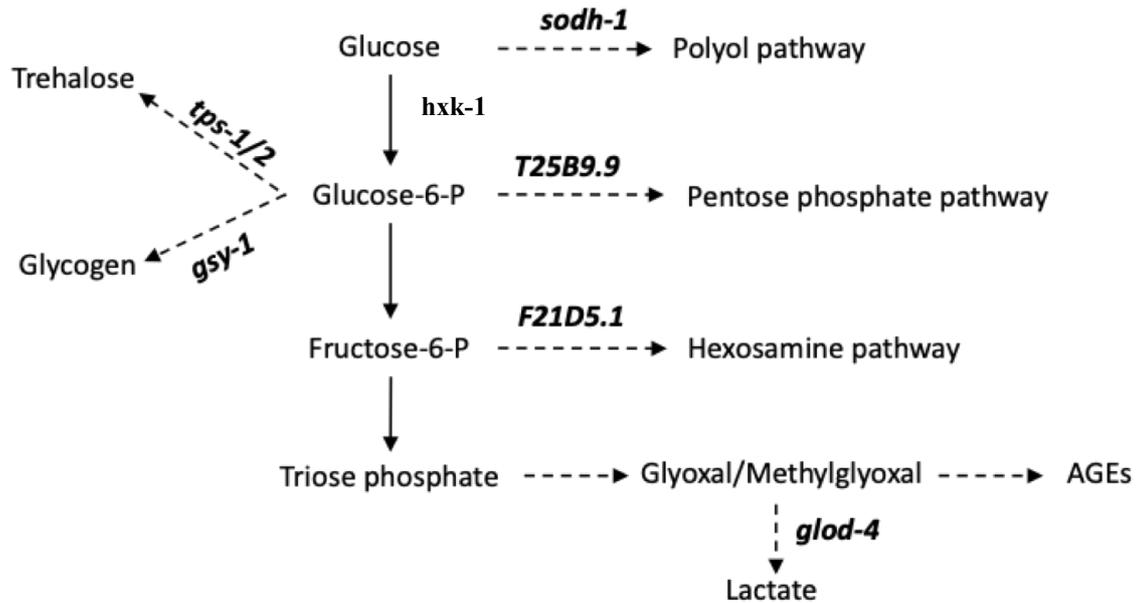


Figure 17: representation of the enzyme localization (in bold) for the RNA expression qRT-PCR measurements performed according to the glucose metabolic pathways in *C. elegans*
 hck-1: hexokinase 1, ortholog of human hexokinase which phosphorylates hexoses to hexose phosphate (first step of glycolysis)
 tps-1/2 are the trehalose phosphate synthase 1/2, catalyze the production of trehalose from glucose-6-phosphate and UDP-D-glucose in a 2-step process
 gsy-1 is the glycogen synthase, it is involved in the glycogen biosynthesis, transfers the glycosyl residue from UDP-Glucose to the non-reducing end of α -1,4-glycan
 sodh-1 is the sorbitol dehydrogenase enzyme, ortholog of human sorbitol dehydrogenase which converts sorbitol into fructose
 T25B9.9 6-phosphogluconate dehydrogenase, ortholog of the human enzyme with the same name, involved in step 3 of the pentose phosphate pathway that synthesizes D-ribulose 5-phosphate from D-glucose-6-phosphate and converts NADP⁺ to NADPH
 F21D5.1 ortholog of the human phosphoacetyl glucosamine mutase, involved in the sub-pathway that synthesizes N-acetyl-alpha-D-glucosamine 1-phosphate from alpha-D-glucosamine 6-phosphate. Catalyzes the conversion of GlcNAc-6-P into GlcNAc-1-P during the synthesis of uridine diphosphate/UDP-GlcNAc, a sugar nucleotide critical to multiple glycosylation pathways including protein N- and O-glycosylation
 glod-4 is a glyoxalase whose role is to detoxify the AGE precursor methylglyoxal into lactate.

III.2.6 CML detection by western blot analysis

Lysis supernatants (2 μ g) were denatured with lithium dodecyl sulfate (LDS) containing buffer (Life technologies) at 70°C for 10min. Samples were loaded and separated on a Bis- Tris 4-12% precast polyacrylamide gels (Life Technologies) and transferred to polyvinylidene difluoride (PVDF) membranes. Free membrane sites were blocked with nonfat dried milk diluted to a 5% concentration in TBST buffer (Tris 20mM, NaCl 150mM, Tween 20 0.1%, pH7.4): 1h at room temperature. The membranes were then incubated 2h at room temperature with either anti-CML (Abcam) or anti-actin antibodies (Sigma Aldrich) diluted 1:5000 and 1:1000 in TBST buffer containing 0.5% nonfat dried milk, respectively. After several washes with TBST buffer, membranes were incubated 2h at room temperature with alkaline phosphatase-secondary antibody conjugate diluted 1:5000 in TBST buffer containing 0.5%

nonfat dried milk. After several washes with TBST buffer and a final wash in the substrate buffer (Tris 100mM, MgCl₂ 5mM, pH9.5), membranes were incubated in the substrate solution (5-Bromo-4-chloro-3-indolyl phosphate 0.15mg/ml, nitro blue tetrazolium 0.30mg/ml) until color development. Bands were normalized to actin levels using ImageJ.

III.2.7 Detection of carbonyl groups introduced into proteins

Protein carbonylation is a major hallmark of oxidative damage[212]. The OxyBlot Protein Oxydation Detection kit (Merck) was used to analyze possible protein oxidation induced by glucose diets. The manufacturer's instructions were followed to perform the immunoblot detection of the protein carbonyl groups. Briefly, the carbonyl groups (soluble worm lysates: 0.5µg of proteins) were derivatized with 1X 2,4-Dinitrophenylhydrazine (DNPH) solution, adding DNP residues on proteins. After neutralization, proteins were loaded and separated on a Bis-Tris 4-12% precast polyacrylamide gels (Life Technologies) and transferred to PVDF membrane. Free membrane sites were blocked with nonfat dried milk diluted to a 5% concentration in TBST buffer: 1h at room temperature. The membrane was then incubated 2h at room temperature with rabbit anti-DNP antibody diluted 1:150 in TBST buffer containing 0.5% nonfat dried milk. After several washes with TBST buffer, membrane was incubated 2h at room temperature with alkaline phosphatase-secondary antibody conjugate diluted 1:5000 in TBST buffer containing 0.5% nonfat dried milk. After several washes with TBST buffer and a final wash in the substrate buffer, membrane was incubated in the substrate solution (5- Bromo-4-chloro-3-indolyl phosphate 0.15mg/ml, nitro blue tetrazolium 0.30mg/ml) until color development.

III.2.8 SPECTRUM Microsource Library screen

We applied our semi-automated screening tool (Minion Microscope) to the screening of a large-scale compound library for compounds which would be protective against human A β ₁₋₄₂ induced proteotoxicity in a *C. elegans* model of Alzheimer's disease, CL2006 worms.

All experiments were performed on CL2006 strain provided by the CGC (Caenorhabditis Genetics Center).

For practicability purposes, only a third of the wells from the 384-well plates were used per plate. This facilitated recording procedures and plate preparations.

After hypochlorite synchronization, animals were grown on Nematode Growth Medium (NGM) agar at 20°C until the stage of young adult. They were then transferred in liquid medium in the 384 well plates which already contained the tested drugs from the high-throughput core

facility. We transferred 10-12 day 1 adult worms per well in a volume of 15 μ l (a total of approximately 800 worms per plate). The total volume obtained in each well was 55 μ l. From then on the worms were exposed continuously to a single compound at the fixed concentration of 6 μ M or to the vehicle control (0,1% DMSO). Each compound was tested at a concentration of 6 μ M on an average population of 33 +/- 3 animals. In addition to the drug or vehicle, the liquid medium prepared contained FUDR (0,1g/L) to prevent self-fertilization, and S-Complete Medium (with identical composition as previously described) and OP50. The desired concentration of OP50 in each well was 1 x 10⁹ cells/ml for optimal ad libitum feeding and was determined by spectrophotometer.

We estimated the ability of the screen to detect any given percent delay of worm paralysis by testing on every plate 3 populations of worms with control DMSO 0,1% and 3 populations of worms with a compound known to extend lifespan and delay paralysis in the CL2006 strain, either mianserin (31% lifespan extension for wild type N2 strain) (PETRASCHECK 2007 and unpublished data) or sodium butyrate and α -ketoglutaric acid (unpublished data). All positive controls (mianserin, sodium butyrate and α -ketoglutaric acid) were identified as hits with delayed paralysis onset.

Once the worms were plated and counted, they were left to incubate for 10 days in a 20°C incubator, or until the second recording.

III.2.9 Paralysis assay for the screen

Worm stimulation was performed with a small 6V electric shock after multiple tries of other stimulation including shaking or shining of a blue light (as described by PETRASCHECK et al[197]). These alternative stimulation methods did not allow paralysis analysis in liquid medium in our laboratory conditions. After a brief recovery period, the worms resume normal locomotion. The criteria for scoring paralysis were the same as described earlier.

Occasionally, there were instances where worms seemed to be dead one day and alive on the next recording. In these instances, we decided to follow the rule developed in PETRASCHECK's publication[213] and assume the worms were alive.

III.2.10 Video Recording for the screen

Recordings for the SPECTRUM compound high-throughput screen were made with a Nikon camera connected with the pocket pen microscope.

For each plate, recordings were made three times, once on the day of the plating to count the number of worms per well and confirm the well-being and viability of all worms, and once on day 10 and 12 of adulthood to perform the paralysis assay. The recording of all 80 wells per session takes approximately 2 hours. At the beginning of each row the row number is filmed, so the experimenter is always aware of the row being filmed.

Recordings were done twice on two separate days to confirm paralysis in the worms.

III.2.11 Statistical Analysis for the CBP and glucose-enriched medium experiments

There were more than 100 worms used for each condition for paralysis and lifespan assays. The criterion for significance was $p < 0.05$. All statistical analysis was done using GraphPad Prism 5 Software. Survival curve p-values (statistical significance) were calculated by the Mantel–Cox log rank test using Prism 5 statistical software (GraphPad). The calculations take into account the censored observations. The median lifespan was calculated by the log rank Mantel-cox test. Prism reports the uncertainty of the fractional survival as a standard error or 95% confidence intervals. Standard errors are calculated by the method of Greenwood. The log-rank (Mantel-Cox) test is a powerful test if the assumption of proportional hazards is true. Proportional hazards means that the ratio of hazard functions (deaths per living worm per unit time interval as a function of chronological time) is the same at all time points. This test will give the same weight to events, independent of the time point.

III.2.12 Video data analysis for the large scale screen

All videos from the SPECTRUM compound screen were uploaded to our YouTube channel for analysis and visualization. Each video was visualized and analyzed by at least two viewers. The analysis were blinded. A master data sheet was completed by each viewer, so that results could be compared. Top hits were defined as any compound which delayed worm paralysis compared with the controls. This meant that on the days of the recording (day 10 and 11), top hits were defined as wells where more than 25% of worms were still healthily moving (no paralysis).

During the development of the screening assay, multiple values for each combinations were tested for their effects on *C. elegans* lifespan and movements. These tests included different OP50 concentrations, different numbers of worms per well (3 to 45), different culture volumes (40-60 μ L per well), different stimulation methods: UV lights, blue lights, shaking the plate on a cell culture platform but none were strong enough to allow paralysis analysis in our

experimental conditions. To establish the scoring rules for paralysis, we also tried out different sets of rules (time of visualization for each well, number of bends within a specific time frame...)

Different setups with the microscope for best visualization were tested: transparent 384-well plates were found to provide the best quality images with a cardboard sheet to the side of the plate but dark plates were also tested.

The values for concentrations and scoring rules indicated in the methods section were carefully selected based on statistical comparison of the different conditions tested.

The screen was blinded and code was broken for only analysis after screening was performed and images visualized and scored.

CHAPTER IV RESULTS

IV.1 CBP knock down uniquely accelerates aging and A β pathology in a *C. elegans* model of Alzheimer's disease

IV.1.1 Paralysis onset analysis in CL2006 strain on agar

In this study, we determined the effects of the inhibition with RNAi of different histone acetyltransferase (HAT) on the paralysis onset of a *C. elegans* model for Alzheimer's disease. There were six groups of worms, each group comprised of 30 worms with a total of 180 worms. The worms were left on the RNAi lawns for the whole course of the experiment and did not run out of food during the entire period of the experiment. The worms were scored as live, dead or paralyzed according to the methods described above. Figure 18 and table 7 outline the result of the paralysis assay on agar. All paralysis experiments were scored beginning day 4 of adulthood. Inhibition of *cbp-1* was the only HAT inhibition that accelerated paralysis onset in this *C. elegans* model for Alzheimer's disease. 29.6% of the CL2006 worms in the control group (L4440) were paralyzed on Day 12 of adulthood. In comparison, the *cbp-1* RNAi group accelerated paralysis significantly with a maximum paralysis of 61% on day 12 of adulthood ($p=0.0194$). The median paralysis onset in the *cbp-1* RNAi group was day 11 of adulthood. Inhibition of all the other HATs tested in the study did not significantly accelerate paralysis onset when compared with the L4440 control group ($p > 0.05$) within the first twelve days of adulthood. By day 12 of adulthood, less than 50% of the worms in the control group or *hat-1*, *mys-1*, *mys-2*, *ada-2* RNAi groups were paralyzed.

HAT RNAi Paralysis onset proportions

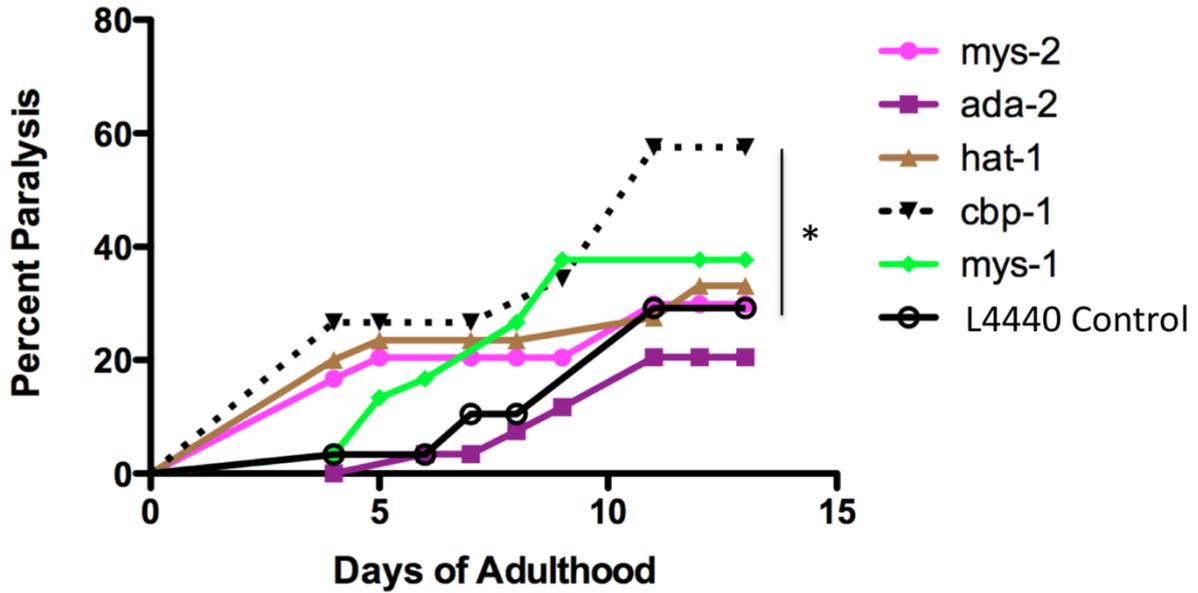


Figure 18: Paralysis onset: *cbp-1* knock-down uniquely accelerates paralysis caused by the age-dependent aggregation of transgenic human A β 1-42 (n=30/group), $*=p<0.05$ (for *cbp-1* RNAi compared to the control L4440)

HAT	<i>p</i> -value vs control
mys-2	0,677
ada-2	0,5023
hat-1	0,5772
cbp-1	0,0194 *
mys-1	0,322

Table 7: *p*-value for the difference in median age for the different HAT RNAi groups compared to control group, (significance was reached for $p < 0.05$)

IV.1.2 Lifespan analysis of CL2006 on agar

In this study, we determined the effects of the inhibition of different histone acetyltransferase (HAT) with RNAi on the survival of a *C. elegans* model for Alzheimer's disease. The groups with a decreased average lifespan suggest an accelerated paralysis and paralysis related mortality. The CL2006 worms were scored as dead, alive or paralyzed as described in the previous chapter. Figure 19 and table 8 show that *cbp-1* RNAi is the only condition that significantly accelerated mortality if compared to the control L4440 ($p=0.0006$). The median lifespan when *cbp-1* is inhibited was 16 days which was 15.8% less than in the control group.

There was no significant difference between the control group and *mys-1*, *mys-2*, *ada-2*, *hat-1* groups. *mys-2*, *ada-2*, *hat-1* inhibitions by RNAi actually increased lifespan by 13.6% relative to the control group although this was not significant.

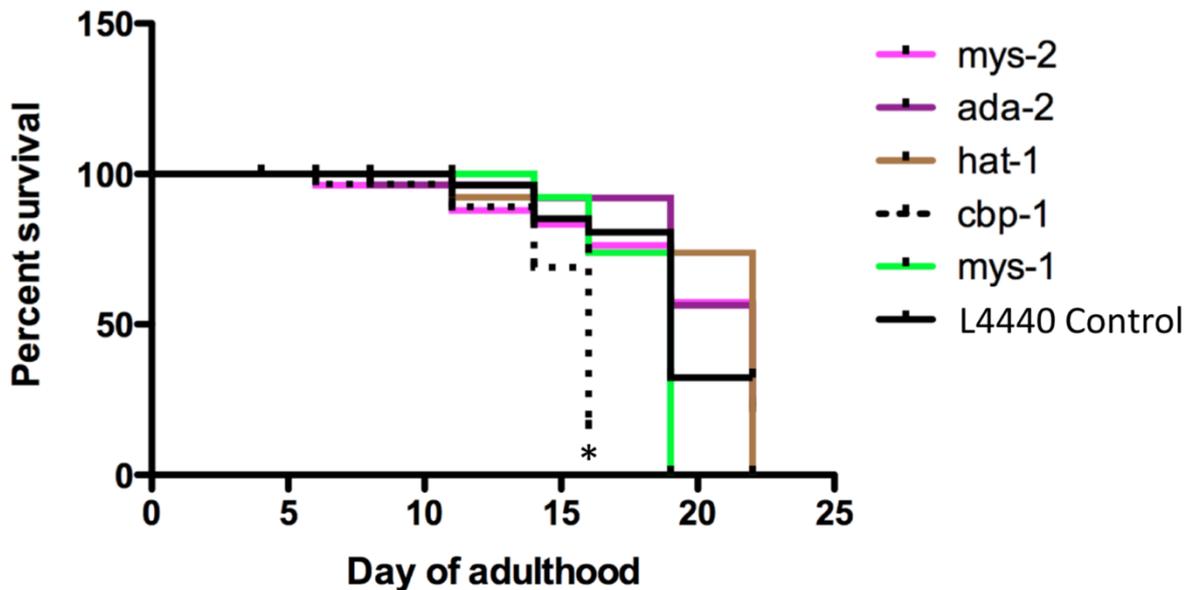


Figure 19: Survival curves: *cbp-1* inhibition uniquely and significantly reduces lifespan in the *C. elegans* model for Alzheimer’s disease (n=30/group), *= $p < 0.05$ (for *cbp-1* RNAi compared to the control L4440)

HAT	Median lifespan (days) (%relative to control)	<i>p</i> -value vs control
Control	19	-
<i>mys-2</i>	22 (+13,6%)	0,8148
<i>ada-2</i>	22 (+13,6%)	0,6849
<i>hat-1</i>	22 (+13,6%)	0,9239
<i>cbp-1</i>	16 (-15,8%)	0,0006***
<i>mys-1</i>	19 (0%)	0,5877

Table 8: *p*-value and median lifespan for the different HAT RNAi groups of *C. elegans* model for Alzheimer’s disease

IV.1.3 Censored worms on agar

Several reasons lead to censoring worms in an agar experiment: burying of the worms in the agar, crawling off of the agar onto the lid, internal hatching causing premature death and contamination. Because of a high fungi contamination of the plates, a relatively high number of worms in all groups had to be censored in the second part of the study (lifespan). Results not shown here.

IV.1.4 Paralysis onset assay in liquid analysis in CL2006

The paralysis assay in liquid allowed us to reach several goals: evaluate whether paralysis was a phenotype we could analyze in liquid medium (we were preparing for the large scale compound screen) and evaluate whether paralysis phenotype was influenced by the liquid medium when using RNAi. In liquid media, we show an acceleration of paralysis of the CL2006 strain but which is not significant compared to the control group ($p=0.0894$). There is a significant difference for *mys-2* and *ada-2*, but the knockdown of these two HATs in this situation, appear to be protective.

HAT RNAi Paralysis in liquid: Paralysis proportions

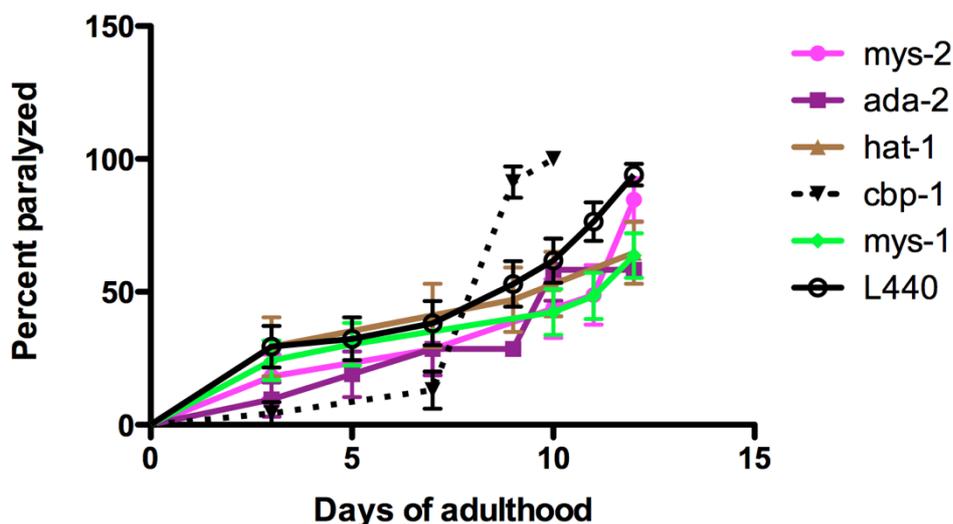


Figure 20: Paralysis assay in liquid: *cbp-1* inhibition uniquely accelerates paralysis compared to the other HATs knockdowns in liquid media ($n=30$ /group)

HAT	p -value vs control
<i>mys-2</i>	0,0617
<i>ada-2</i>	0,0202*
<i>hat-1</i>	0,066
<i>cbp-1</i>	0,0834
<i>mys-1</i>	0,0052**

Table 9: p -values for the difference in median age for the different HAT RNAi groups compared to control group in liquid medium, (significance was reached for $p < 0.05$)

The liquid paralysis assay allowed us to perfect this method in view of the development of the phenotype screening tool described in the next section.

IV.2 Development of a semi-automated high-throughput compound screening method and SPECTRUM compound library screen in a *C. elegans* model for Alzheimer's disease

IV.2.1 Development of a semi-automated phenotype screening assay for *C. elegans*

We developed a semi-automated phenotype screening assay tool for *C. elegans*. The motorized Lab Minion Microscope allowed automated 384-well plate reading and worm stimulation in a large scale screen to analyze changes in mobility after long-term exposure to different compounds which is one of the most time-consuming parts in high-throughput screening experiments. The goal of the screening method is to detect compounds capable of increasing healthspan in the model organism *C. elegans*. The opportunity we had to have certain parts made with a 3D printing technique allowed us to customize the microscope to our needs with the addition of a stimulation arm and possibility of moving the pen microscope over each part of our 384-well plates. The software developed in python by the Deezmaker team allowed automated video recordings of each well during the experiment and easy storage of the videos in addition to the stimulation of worms in each well. The videos produced were of good quality and allowed analysis of the data by different participants. All the videos were conveniently uploaded to a YouTube channel which facilitated access to the videos for all the participants of the project even if they were not physically present in the laboratory. Our stimulation method consisting in applying a 6V electric shock to the worms in each well thanks to a copper electrode allowed a reproducible stimulus of the worms and allowed effective paralysis onset analysis. This stimulus is well under that applied in studies exploring the effects of electroshock stimulation as a model for epilepsy (shock applied is usually approximately 50V[214]).

We applied our tool to screen a large-scale compound library managed by the high-throughput core facility from the Icahn school of medicine at Mount Sinai. Our tool allowed successful data generation for 2560 compounds in less than 2 months. Each 384-well plate was divided in three and studied separately to facilitate recording and experimental plate preparation.

IV.2.2 SPECTRUM library large-scale screen in a *C. elegans* model for Alzheimer's disease for 2560 compounds which delay proteotoxicity

We applied our newly developed screening method to search for compounds that delayed paralysis and therefore proteotoxicity in adult CL2006 *C. elegans*. We screened a commercial collection of pharmacological agents with known or suspected human targets: the SPECTRUM library of 2560 compounds.

Each compound was tested at a concentration of 6 μ M on an average population of 10 \pm 3 animals. We tested the effects of each compound on paralysis on Day 10 and Day 11 of adulthood. Paralysis analysis was conducted when more than 75% of worms were considered paralyzed or dead in the control wells. When more than 50% adults from the control group were still healthily moving on day 11, we extended the recordings to a session on day 12 of adulthood. We analyzed every plate separately and established hits ‘per plate’ to ensure comparability.

There were a total of 96 control wells for the screen containing 0,1% DMSO and approximately 10 worms each. On the day of the recording (day 10 and 11 of adulthood), all but 5 wells contained 75-100% paralyzed or dead worms. For five wells, there were more than 30% of worms moving (one well in plate 1 1/3 had 57%, 1 well in plate 5 1/3 had 50% moving, one well in Plate 5 3/3 had 33% moving worms, one well plate 7 3/3 had 45% moving worms and one well from Plate 8 1/3 had 67% healthily moving worms). We were not able to establish the reason for the high proportion of healthy worms in these five wells on day 10 and day 11.

Among the 2560 compounds screened, we obtained 130 primary hits defined as the most efficient drugs for delaying paralysis compared with our control for each plate. The standard quality control measures were described in the previous chapter of this thesis (chapter Methods II-8) (positive controls and vehicle controls). Top hits were defined as compounds which delayed paralysis in the worms on day 10 and 11 of adulthood compared with the control, or in other terms, wells where more than 25% of worms were moving healthily on days 10 and 11.

Compounds which delay paralysis cluster in certain pharmacological classes. We analyzed the top hit compounds based on their targets in mammals. Among the 130 compounds that reduce pathology in a transgenic model for proteotoxicity in Alzheimer’s disease (CL2006) certain pharmacological classes were more represented than others: antibiotics (6.5%), NSAIDs (2.4%), GABA receptor antagonists (1.7%), antihistamines (1.7%), anti-cancer drugs (2.9%), insecticides (4%), pesticides/herbicides (1.7%), antipsychotic drugs (1.5%). The multitude of ortholog proteins and pathways in *C. elegans* and humans suggests that many of the hit compounds might interact with ortholog targets in humans and *C. elegans*.

Among the best 20 compounds, 9 are known to have a protective action mechanisms in Alzheimer’s disease and have been tested as a treatment for the disease.

The most efficient compound identified in the screen was phenylbutyric acid, an HDAC inhibitor whose action mirrors that of CBP with 88% of worms still healthy at 12 days of adulthood.

In the table hereafter, we present the top 20 compound hits of our screen.

Compound	Healthy worms (%)	Mechanism/class	Tested for AD?
Phenylbutyric acid	88%	HDAC inhibitor	Yes (phase 2 clinical trial)
Methicillin Sodium	83%	Beta-lactam antibiotic	No
Quetiapine	82%	Atypical antipsychotic, anxiolytic dopamine, serotonin, adrenergic antagonist	No
Leoidin	75%	Natural compound, Bacteriostatic by bacterial protein synthesis?	Yes patent US20100048510A1 Amyloid fibrils dissolution
Mexicanolide	75%	Natural compound, Limonoid: phytochemicals of the triterpenoid class	Yes[215,216] restriction of aggregation of tau to fibrils
Bromopride	73%	Dopamine antagonist antiemetic Acetylcholine esterase inhibitor	No (patent to be used in association with AchE inhibitor to improve tolerance)
Bentazon	67%	Herbicide	No
Deoxyth+Khinorin	67%	Not known	No
Agelasine	67%	Natural compound, diterpenoid isolated from marine sponges, antimicrobial [217]	No
Isokobusone	67%	Natural compound from root crop	No
Nobiletin	65%	Natural compound, citrus flavonoid, antioxidant	Yes reduce amyloid aggregation, inhibit hyperphosphorylation of tau[218]

Celastrol	64%	Natural compound, Pentacyclic triterpenoid	Yes reduction of β -amyloid pathology in mice[219]
Gangaleoidin	57%	Natural compound inhibition of bacterial protein synthesis	No
Ramipril	56%	Anti-hypertensive drug, Angiotensin Converting Enzyme inhibitor	Yes[220,221]
Cisplatin	56%	Anticancer chemotherapy drug	Yes
Prednisone	56%	Anti-inflammatory	proposed
Tylosin Tartrate	54%	Antibiotic, macrolid	No
Ursolic acid	53%	Natural compound, pentacyclic triterpenoid	Yes[222,223]
Stigmasta-4,22 Dien-3-One	50%	Natural compound, antiviral activity	No
Malathion	50%	Pesticide, Acetylcholinesterase inhibitor	No

Table 10 Top 20 hits from the large scale screen Microsource SPECTRUM compound library with our semi-automated screening tool. These data are for Day 10 of adulthood. Percentages represent the percentage of worms moving healthily after electro-stimulation. For all control wells, less than 30% of the worms were moving healthily after stimulation on the same day of experiment.

The first part of the work done in Professor Mobbs's laboratory allowed to extend previous work and confirm that CBP had a unique life extending role among all HATs. As it is known that CBP plays a role in the metabolic shift away from glucose in a condition of nutrient deprivation (as is the case in dietary restriction), we can infer that the protective effects of CBP are linked to the decrease of glucose utilization by cells. Now let us expose our results from our glucose excess experiments.

IV.3 Glucose-enriched medium affects gene expression and lifespan of *C. elegans*

IV.3.1 Glucose concentration measurement in the culture supernatants and worm lysates and glucose transporter expression analysis confirm that glucose concentration exposure and ingestion of the worms is stable

To ensure that the worms were exposed to constant concentrations of glucose during the whole duration of our study we measured glucose concentration in the culture supernatants and worm lysates (approximately 6000 worms for each condition) at different time points of the study (day 3, 6, 10, 13 of adulthood) thanks to a glucometer. What we found is that the glucose concentration stays stable in time, ensuring that the worms have a stable exposition to the different tested glucose concentrations for the whole duration of the study. This is illustrated in figure 21 A. Our choice of the glucose concentrations exposure (40mM, 110mM, 550mM) was made according to preliminary experiments and data from the literature. Examples of commonly used concentrations to study the effects of a high glucose diet on *C. elegans* lifespan in the literature we based our decision on are 40mM (SCHLOTTERER et al. 2009[31]), 110mM (LEE et al. 2009[33]), 550mM (TAUFFENBERGER et al. 2012[187]).

The first limiting factor to glucose exposure for the worms is glucose transport. To make sure that worms were getting a constant glucose input we measured RNA expression of the only glucose transporter known to date in *C. elegans*: FGT-1. For each chosen time point (Day 3, 6, 10, 13 of adulthood), and each chosen glucose concentration (0, 40, 110, 550mM) 6000 worms were collected to measure FGT-1 RNA expression with qRT-PCR. The expression did not depend on glucose concentration in the medium and did not significantly vary in time (figure 21 B).

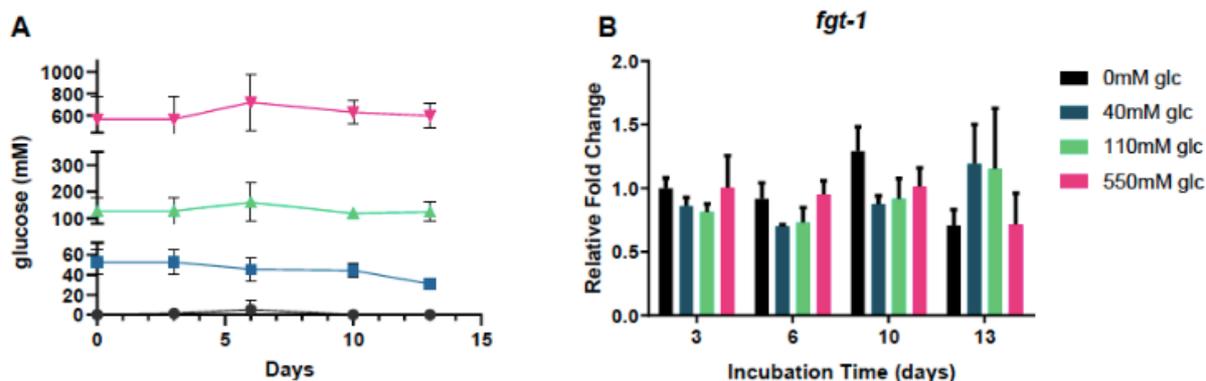


Figure 21: Glucose concentration in culture medium and expression of *fgt-1*. 6000 worms were grown in NGM containing 0mM, 40mM, 110mM or 550mM of glucose (glc). After 3, 6, 10 and 13 days of incubation at 20°C, glucose concentration was monitored in the different culture media (A) and expression of FGT-1 encoding gene by the worms was analyzed (B); *fgt-1* relative expression of each condition was compared to the relative expression in NGM with no glucose after 3 days of incubation. Data are presented as mean of relative fold change \pm SEM.

IV.3.2 Dietary glucose concentration does not affect relative expression of enzymes from glucose metabolism pathways

The relative expression of enzymes from different glucose metabolic pathways were determined with qRT-PCR for approximately 6000 worms for each condition (different time points and different glucose concentrations) in 3 independent experiments. The relative expression of *hvk-1*, *gsy-1*, *tps-1*, *tps-2*, T25B9.9 and F21D5.1 does not change with increased glucose exposure or in time as shown in figure 22.

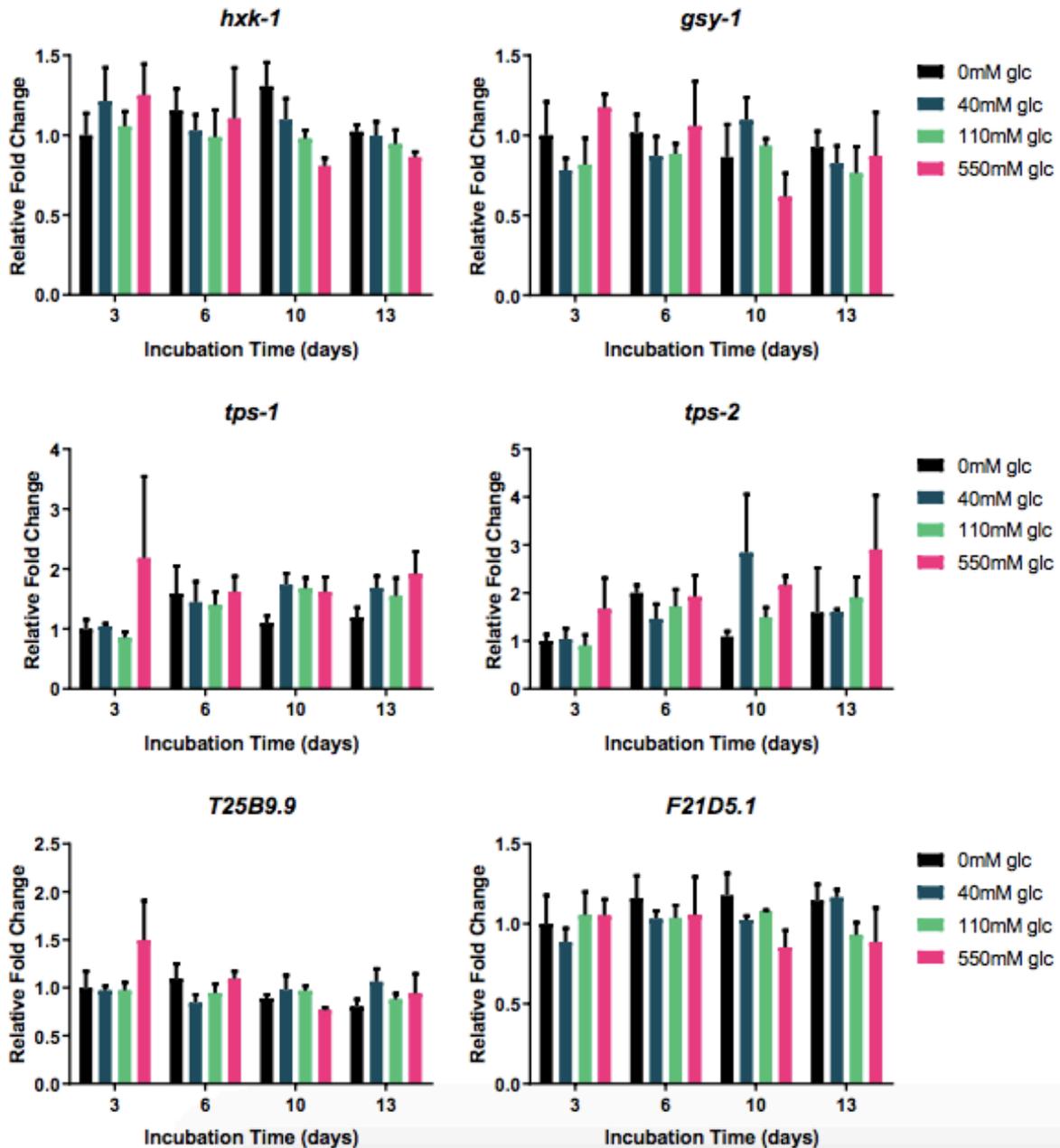


Figure 22: Relative expression of *hxx-1*, *gsy-1*, *tps-1*, *tps-2*, *T25B9.9* and *F21D5.1* after exposure of *C. elegans* to dietary glucose. 6000 worms were grown in NGM containing 0mM, 40mM, 110mM or 550mM of glucose (glc). Three independent experiments were performed. After 3, 6, 10 and 13 days of incubation at 20°C, expression of HXX-1, GSY-1, TPS1/2, T25B9.9 and F21D5.1 encoding genes was analyzed. Relative expression of each condition was compared to the relative expression in NGM with no glucose after 3 days of incubation. Data are presented as mean of relative fold change \pm SEM.

IV.3.3 Sorbitol dehydrogenase relative expression decreases with time and glucose concentration exposure

Sorbitol dehydrogenase is a cytosolic enzyme converting sorbitol (the sugar alcohol form of glucose) into fructose in the polyol pathway. Sorbitol dehydrogenase relative expression was measured with qRT-PCR in approximately 6000 worms in 3 independent experiments and

shown to decrease during the course of worm lifespan in our experiments, independently of glucose exposure, as the qRT-PCR measurements show (figure 23).

There is a significant decrease in sorbitol dehydrogenase relative expression ($p < 0.05$ for mean of relative fold change) measured with qRT-PCR as glucose concentration in the medium increases for the time points of 3, 6 and 13 days of glucose exposure but not on day 10. More precisely: on day 3 of adulthood and glucose exposure, there is a significant difference in relative expression of sorbitol dehydrogenase between the worms not exposed to glucose in the medium and the worms exposed to the highest glucose concentration in our study (550mM). On day 6 of adulthood and glucose exposure in the medium, there is a significant difference in relative expression of sorbitol dehydrogenase between the condition no glucose exposure and all the glucose concentrations in our study. For each condition described above, glucose exposure leads to the decrease of sorbitol dehydrogenase expression.

On day 10 of adulthood and glucose exposure there is no difference in relative expression between the different conditions in our study.

Finally, on the last day of measurement of the study, there is a significant decrease ($p < 0.05$) of sorbitol dehydrogenase relative expression between the no glucose condition, 40mM glucose exposure and 110mM glucose exposure.

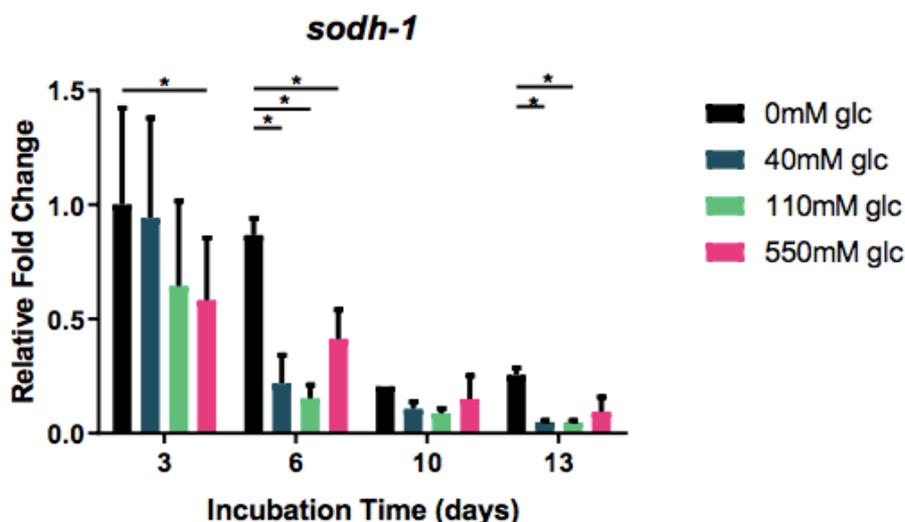


Figure 23: Relative expression of *sodh-1* after exposure of *C. elegans* to dietary glucose. 6000 worms were grown in NGM containing 0mM, 40mM, 110mM or 550mM of glucose (glc). After 3, 6, 10 and 13 days of incubation at 20°C, expression of SODH-1 encoding gene was analyzed in 3 independent experiments. Relative expression of each condition was compared to the relative expression in NGM with no glucose after 3 days of incubation. Data are presented as mean of relative fold change \pm SEM. * $p < 0.05$

IV.3.4 Relative expression of anti-oxidant defense mechanisms according to glucose exposure

The relative expression of enzymes involved in the cellular anti-oxidant defense mechanisms were measured by qRT-PCR from lysate extracts of approximately 6000 worms in three independent experiments, they are presented figure 24.

There is a significant difference ($p < 0.05$) in relative expression of extracellular SOD-3 on day 10 and 13: between the conditions no glucose and all glucose-enriched conditions for day 10, and between the no glucose condition and the most glucose enriched condition (550mM) on day 13.

Gst-4 relative expression is significantly increased on day 10 and 13 of adulthood when the worms are exposed to the highest concentration of enriched-glucose medium (550mM) in our study.

Gst-14 relative expression is significantly decreased on day 3 of adulthood between the conditions no glucose and 550mM glucose exposure.

There is no difference in *sod-1* expression between any of the conditions tested in our experiment. The expression is independent of time or glucose concentration in the medium.

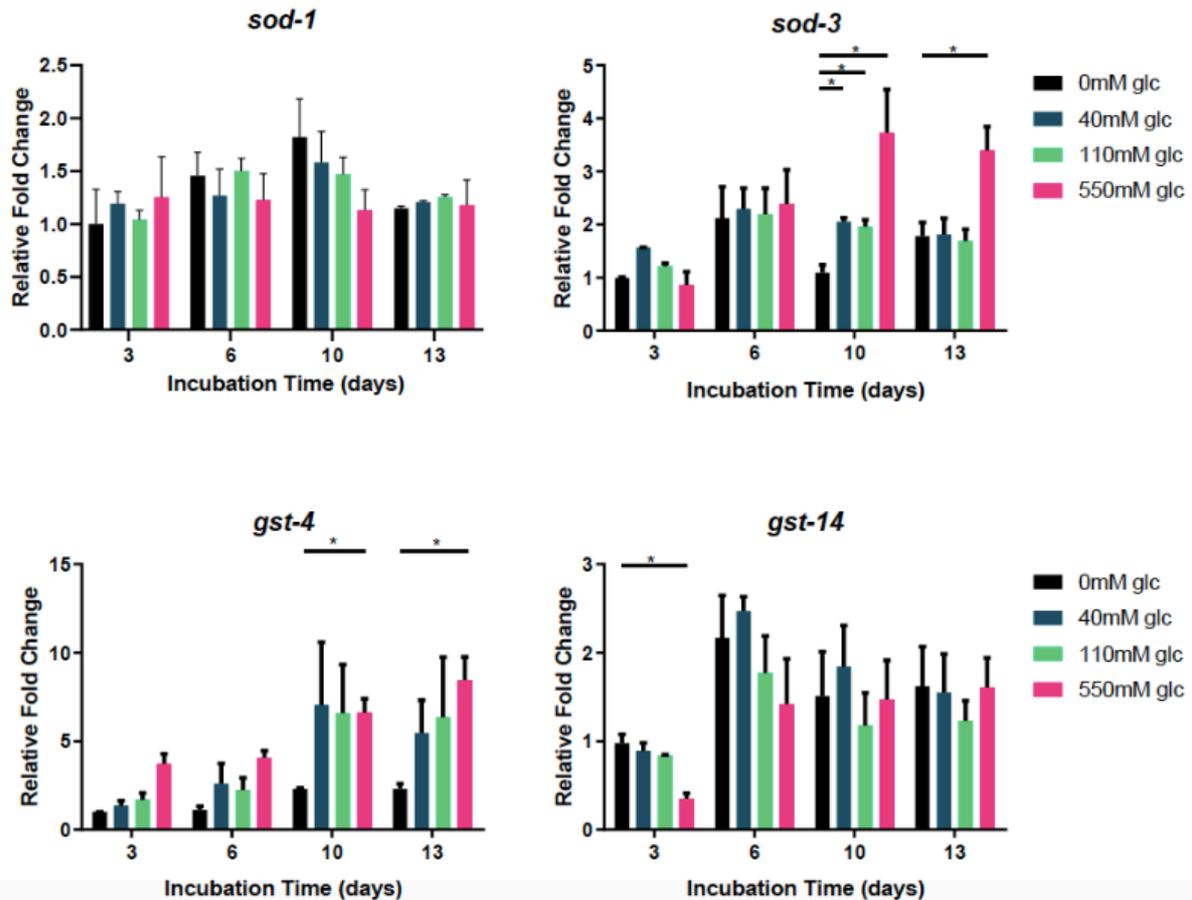


Figure 24: Relative expression of *sod-1*, *sod-3*, *gst-4* and *gst-14* after exposure of *C. elegans* to dietary glucose. 6000 worms were grown in NGM containing 0mM, 40mM, 110mM or 550mM of glucose (glc). After 3, 6, 10 and 13 days of incubation at 20°C, expression of SOD-1/3 and GST-4/14 encoding genes was analyzed in 3 independent experiments. Relative expression of each condition was compared to the relative expression in NGM with no glucose after 3 days of incubation. Data are presented as mean of relative fold change \pm SEM. * $p < 0.05$

IV.3.5 There is no significant change in glyoxalase relative expression dependent on time or glucose exposure.

Glyoxalase 1 (GLO1) is a human enzyme that participates in the detoxification of methylglyoxal and other reactive aldehydes. The ortholog in *C. elegans* is GLOD-4. We quantified relative expression of glyoxalase (*glod-4*) in lysates from approximately 6000 worms for each experimental condition (no glucose, 40mM, 110mM, 550mM glucose concentration in the medium) by qRT-PCR on days 3, 6, 10 and 13 of adulthood in 3 independent experiments. There was no significant change in glyoxalase relative expression during the course of the whole experiment. The results are shown figure 25.

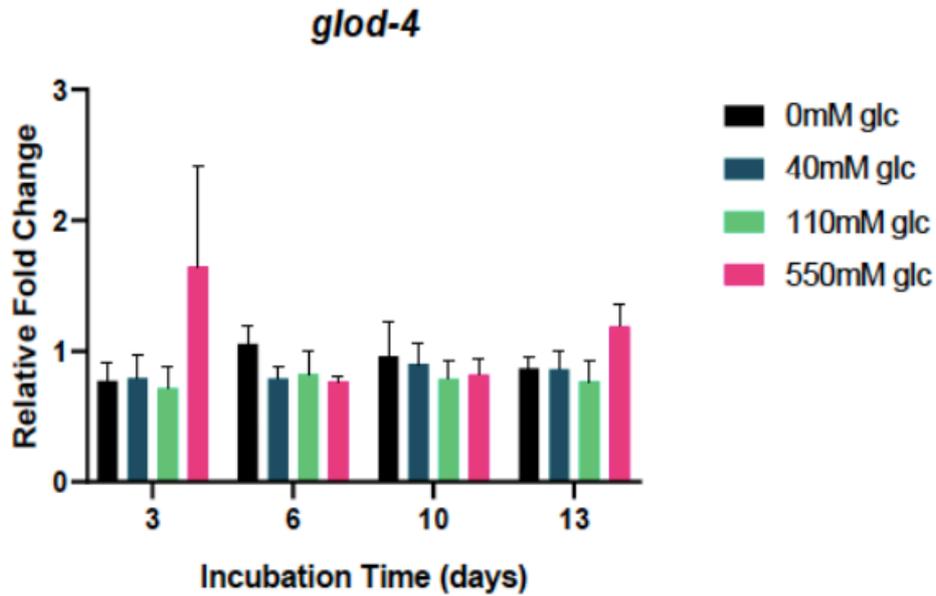


Figure 25: Relative expression of *glod-4* after exposure of *C. elegans* to dietary glucose. 6000 worms were grown in 3 independent experiments in NGM containing 0mM, 40mM, 110mM or 550mM of glucose (glc). After 3, 6, 10 and 13 days of incubation at 20°C, expression of GLOD-4 encoding gene was analyzed. Relative expression of each condition was compared to the relative expression in NGM with no glucose after 3 days of incubation. Data are presented as mean of relative fold change \pm SEM

IV.3.6 Protein carbonylation is dependent on a time and concentration glucose exposure

To analyze the impact of glucose-enriched diets on oxidative stress, protein carbonylation rate was evaluated in protein extracted from approximately 6000 worm lysates by OxyBlot Protein Oxidation Detection Kit (Merck) in 3 independent experiments.. Protein carbonylation is a major hallmark of oxidative damage[212].

Our results show an increase in protein carbonylation (and therefore indirectly of oxidative stress) with time and glucose concentration exposure.

Indeed, for days 10 and 13 the maximum number of bands (indicating the presence of carbonyl groups) are seen for the highest glucose exposure. These results were not quantified. A photograph of the best quality western blot membrane is shown figure 26.

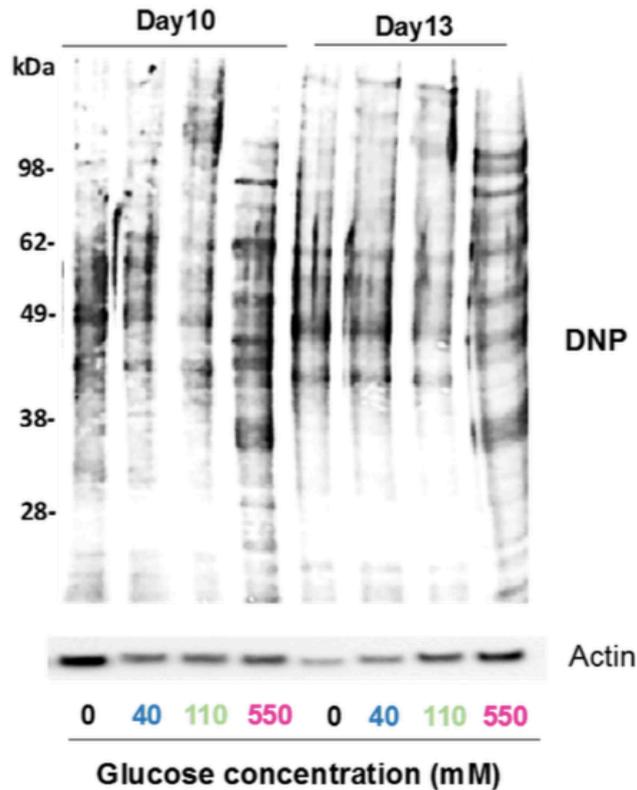


Figure 26: Carbonylation of proteins after 10 and 13 days exposure of *C. elegans* to dietary glucose. 6000 worms were grown in NGM containing 0mM, 40mM, 110mM or 550mM of glucose. After 10 and 13 days of incubation at 20°C, carbonylation of proteins were analyzed by western blot in 3 independent experiments. DNP (2,4-Dinitrophenyl) hydrazine was used to derivatize carbonyl groups on proteins. (Best image presented here)

IV.3.7 Endogenous glycation increases with time and dose glucose exposure

CML was detected in the worm lysates of approximately 6000 worms by western blot analysis. CML presence increased significantly on day 10 and 13 for the highest glucose concentration exposure compared to the no glucose condition. This translates endogenous glycation for the highest glucose concentrations within a 10 day time frame. To note, glycation increases of a factor of 3 in the no glucose condition. A picture of the best quality western blot membrane for CML is presented left and a graph of the western blot quantification is presented right of figure 27.

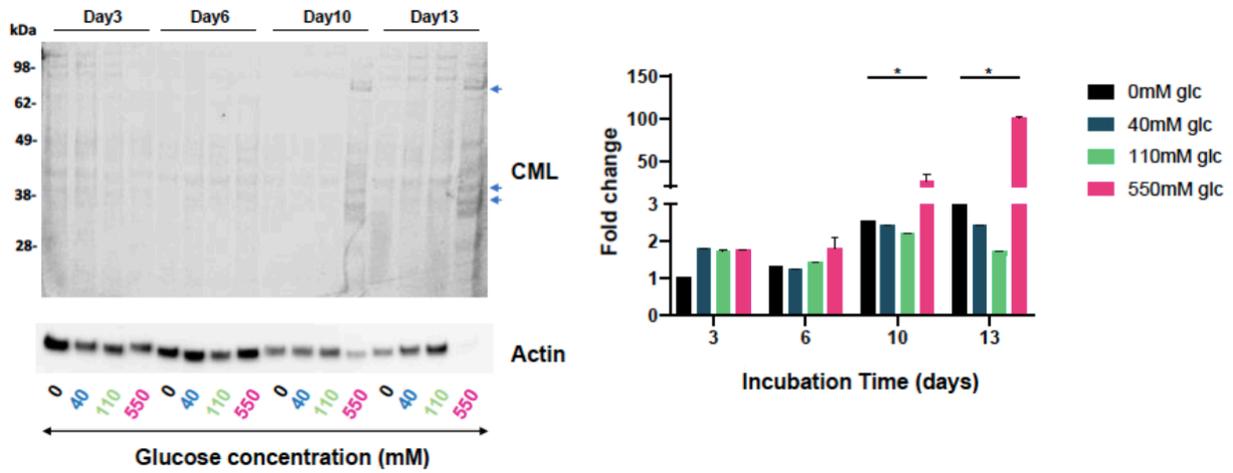


Figure 27: Endogenous glycation of proteins after exposure to dietary glucose. 6000 worms were grown in NGM containing 0mM, 40mM, 110mM or 550mM of glucose (glc). After 3, 6, 10 and 13 days of incubation at 20°C, CML modified proteins were analyzed by western blot (left panel) in 3 independent experiments (best image presented here). CML epitopes from different proteins (arrows) were quantified and results are expressed as fold change compared to the day3 condition without glucose (right panel). Data are presented as mean of relative fold change \pm SEM. * $p < 0.05$

IV.3.8 Glucose exposure affects lifespan in a dose dependent manner

We measured N2 worm lifespan (60 worms) in the presence of a glucose-enriched solid medium in 3 independent experiments. In our experimental conditions, exposure to a glucose enriched diet, significantly affects median worm lifespan negatively for a concentration of 550mM independently of the presence of live or dead bacteria. There seems to be a tendency for an increased effect with dead bacteria. Results of lifespan assays are presented figure 28.

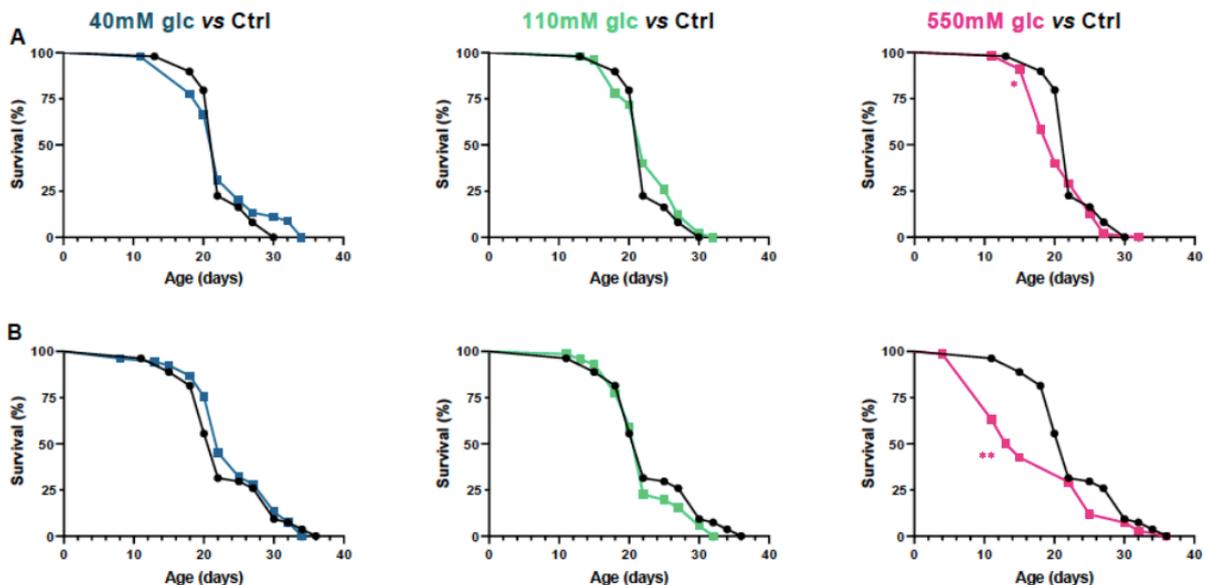


Figure 28: Survival of worms in presence of glucose. N2 wild-type strain of *C. elegans* (approximately 60 worms per condition) was grown in NGM containing no glucose (Ctrl) or 40mM, 110mM or 550mM of glucose (glc). 3 independent experiments were performed. Worms were incubated at 20°C in presence of lived (A) or heat-killed (B) bacteria. ** $p < 0.01$ and * $p < 0.05$ compared to control survival.

IV.3.9 2DG exposure extends lifespan in CL2006 worms

The addition of 4mM 2DG in the medium significantly extended lifespan in CL2006 *C. elegans* ($p=0.0046$). This is a novel concept because 2DG had only previously been proven to be effective in the wild type N2 *C. elegans* strain. This implies that glucose metabolism rather than glucose affects aging. The results of the lifespan assay are presented figure 29.

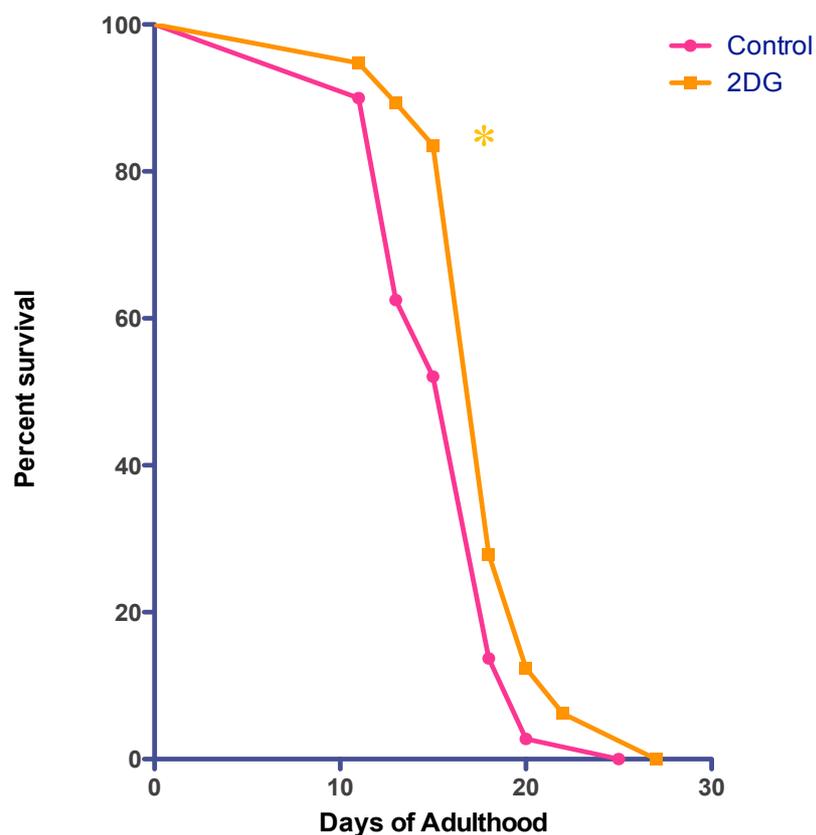


Figure 29: Lifespan assay for CL2006 worms exposed to 4mM 2DG. 60 worms on 3 experimental plates were exposed to medium containing 4mM of 2DG or to the control (NGM without 2DG). Worms were incubated at 20°C. Experiments were repeated once. * $p<0.05$ compared to control survival

CHAPTER V DISCUSSION AND PERSPECTIVES

V.1 Discussion

The results from our experiments conducted in the model organism *C. elegans* studying either glucose excess or glucose scarcity converge towards the same observations on a role of glucose, glucose metabolism and the transcription profile induced by the exposure to (or lack of) glucose in aging.

In the first part of our work we were able to confirm the unique role of CBP as a HAT in aging and age-related diseases. This has major implications in regards to glucose metabolism as it has been shown that CBP is accountable for the metabolic shift from glucose to alternative energy sources as seen in dietary restriction when glucose is scarce[14]. Interestingly, this result was corroborated in our large-scale screen for protective compounds against A β ₁₋₄₂ pathology in *C. elegans*. The most protective drug found was phenylbutyric acid, a Food and Drug Administration (FDA) approved, HDAC inhibitor whose action effectively amplifies the action of CBP and which was first used in therapies for urea cycle enzyme deficiencies, due to its ability to act as a chaperone for other enzymes[224,225]. Phenylbutyric acid is now in a phase 2 multicenter clinical trial for Alzheimer's disease with a completion date estimated in 2020 for which patients at the Mount Sinai Alzheimer's disease Research Center are participating (ClinicalTrials.gov identifier: NCT03533257). Among our top 20 hits, 12 are known to have protective action mechanisms in Alzheimer's disease and have been tested as a treatment for the disease. This argues for the validity of our screen in being able to identify compounds effective against Alzheimer pathology in *C. elegans*.

In the part of our work completed in Lille, our aim was to characterize *C. elegans* as a valid model organism to study glucose-enriched diets. We were able to explore the relative expression of glucose facilitative transporter FGT-1 (the only glucose transporter known in *C. elegans* to date), of the orthologs of enzymes from alternative metabolic pathways described in mammals as being activated in a situation of glucose excess, of enzymes involved in cellular anti-oxidant mechanisms. We also showed evidence for oxidative stress in *C. elegans* as there was an increase in carbonylated proteins for the highest concentrations of glucose after 10 and 13 days exposure and finally we showed that in a high glucose environment, after 10 days of exposure, there is protein glycation and production of CML.

CBPs protective role is unique among HATs

The protective effects of CBP on lifespan have been shown in rodents[226] as well as in *C. elegans*. Studies have reported that dietary restriction induces expression of the transcription

factor CBP, and reducing CBP by only 50% with RNAi completely blocks all protective effects of dietary restriction to increase lifespan in *C. elegans* as well as reduce proteotoxicity[14,227,228]. In our work we confirm the earlier results that *cbp-1* knock-down accelerates paralysis (proteotoxicity) in the *C. elegans* model of Alzheimer's disease in an ad lib condition[14]. The paralysis onset delays and survival rates in our control group were consistent with previous reports[14,229]. This can be an issue, as the visual analysis of paralysis can be subjective. MYS-1, MYS-2, HAT-1, ADA-2 inhibition do not have an effect on lifespan or β - amyloid toxicity in our *C. elegans* model of Alzheimer's disease on agar. This suggests the specificity of our results on lifespan and healthspan in regards to CBP.

The development of new anti-Alzheimer's diseases therapeutics is very costly and has provided medicine with no efficient drug to date. Conventional drug-development involves the identification of a suitable candidate gene or protein to target, followed by *in vitro* screening for chemical inhibitors or activators (depending on the wanted effect), and finally chemical modifications to increase *in vivo* bioavailability and efficacy. *In vivo* inefficacy or toxicity are often only revealed in the final stages of drug development, leading to a late renouncement of most candidate drugs. As we have shown in *C. elegans*, an alternative approach to drug development is to carry out unbiased chemical screening *in vivo* using appropriate Alzheimer's disease models. This approach has the advantage of being able to select compounds early on in the development process with good drug-like potential, presenting high specificity for proteotoxicity and without adverse host toxicity. This is currently unfeasible using rodent models because of time and financial constraints, but the high conservation of signaling and aging controlling pathways between mammals and *C. elegans* as well as the relative low cost and speed of screens make the worm a powerful tool to exploit for drug development. The validation of this large-scale *in vivo C. elegans* chemical screening method highlights the significant but largely underused potential of this model for drug development in the field of neurodegenerative diseases. Our platform could be adapted to screen for compounds that target other phenotypical behaviors in *C. elegans*, reflecting other human pathologies or pathophysiological mechanisms (particularly those linked with aging). The powerful molecular and genetic tools available in *C. elegans* can complete the chemical screening approach to help elucidate the underlying mechanisms of drug effectiveness. Since 2014, many digital tracking platforms have been designed and made available in order to replace manual counting of parameters such as speed of movement, bending frequency, paralysis rate, as well as more complex forms of behaviors such as omega turns. The automation is now extended to analysis

of the phenotypes in addition to the recording step. These platforms have improved the reproducibility and sensitivity of *C. elegans* studies and provided high quality data[230].

Among the drugs identified in our screen, there is an important number of antibiotics, pesticides, insecticides and herbicides. One could hypothesize that these drugs were protective against A β toxicity because of a low-dose stress on the organism which was enough to trigger a protective response. Comparably to the Petrascheck[197] screen of 88,000 compounds, our screen identified anti-psychotic drugs in the top hits of protective compounds capable of delaying impairments in our *C. elegans* model of Alzheimer's disease, such as quetiapine. This will need to be further investigated so as to understand the underlying mechanism.

Limits of the work conducted at the Icahn School of medicine at Mount Sinai

One should however keep in mind the limitations of our different experimental designs. Circumstances in which experiments are done can influence the outcome. There are different reasons why we chose to repeat the paralysis assay in liquid: for worms, growing in liquid or solid environment has an influence.

Replicating an experiment in two different environments would help confirm our findings. PETRASCHECK *et al.*[197] published results on a high throughput screening of 88 000 drugs they had performed in liquid medium. Unfortunately, these results were not replicable on agar by ZARSE *et al.* in 2008[231]. One of the explanations for the difference in results is the different worm 'living' environment. Liquid cultures are commonly used for *C. elegans* especially for pharmacology large scale studies. Since we conducted the screen and CBP experiment in 2014, it has been shown that there are significant differences in transcriptomic and phenotypical responses to rapid environmental changes such as transferring the worms to a liquid medium when they reach adulthood[232]. This could have had an impact on our phenotypical analysis of paralysis. This was avoided in the high glucose experiments conducted in 2019 by culturing worms in a liquid medium from the 'egg' stage.

In *C. elegans*, long dsRNA fragments (more than 100 bp) trigger RNAi. Their efficacy and efficiency vary spatially and temporally with the feeding method. RNAi does not accurately phenocopy the null phenotype of all genes, and may result in either partial or no loss of function. It should also be noted that if several genes have regions of identical or near-identical nucleotide sequence RNAi could target them simultaneously so that the observed phenotype may be the result of the inhibition of more than one gene[233]. It is also not possible to target the tissues/cells affected by the knock-down with the RNAi feeding method in the Nematode.

We were not able to evaluate the efficacy of our knock-down feeding RNAi approach in our studies due to a lack of time. To evaluate the knock-down proportion and efficacy in our worm population we will have to collect dsRNA-treated worms at different time-points, to extract RNA and measure mRNA levels of expression for the target genes with RT-PCR for the CBP studies. To address these limitations, other methods could have been used in our research. Another method available in a reverse genetics approach is to use the available knock-out strains for the genes of interest. Unfortunately, the CBP knock-out strain is not viable, and is therefore not available for our purpose.

Induced mutagenesis (or forward genetics) is yet another possibility for the study of a phenotype. The gene mutated is identified through standard molecular techniques after the mutant is identified. This would not have been of interest here as the goal of the study was to determine whether other HATs had an effect on amyloid induced proteotoxicity in *C. elegans*. There are additional limits to our large-scale screen. One of the disadvantages in using *C. elegans* in a compound screen, compared with a rodent model organism is the uncertainty about the amount of compound (if any) ingested by the worms in the liquid medium. Worms are indeed ‘soaked’ in the compound. With a rodent, the amount ingested can be precisely monitored by tube feeding techniques. In a large scale compound screen it is not possible to dose the compound in the worm lysates as we did for measuring glucose in the high-glucose experiments. There remains an uncertainty on amounts of compound ingestion in our screen. To overcome this uncertainty, top hit compounds are retested on solid and liquid medium more extensively.

We found a difference with the findings in literature for the proportion of paralyzed CL2006 worms on day 10. In the paper by ZHANG *et al.* in 2009[14], there were 40% paralyzed worms on day 10 of adulthood, almost two folds less than in our screen. The difference with our screen could be due to the liquid medium or the electric shock applied to the worms.

Due to technical difficulties we were unfortunately only able to expose the worms to compound concentrations of 6 μ M as opposed to PETRASCHECK’s drug screen published in 2007[197] which exposed worms to compound concentrations of 30-90 μ M. Nonetheless, we were able to evidence differences in healthspans for the different compounds tested.

Mianserin was the most efficient drug found to increase *C. elegans* lifespan in the unsurpassed screen of 88,000 compounds by PETRASCHECK *et al.* and the paper further focused on the efficacy of antidepressants. This was not confirmed in our compound screen by our criteria although we did culture worms in a liquid medium as described for the PETRASCHECK study. This could be explained by the differences in compound concentration or *C. elegans* strain used

(CL2006 vs N2 wild-type strain). On the other hand, in the PETRASCHECK screen, anti-psychotic drugs were over-represented among the protective drugs. This is also true in our screen.

Finally, in retrospect and seeing how much we learned from CBP RNAi accelerating the pathology in the CL2006 Alzheimer's disease model, we could have focused on compounds that accelerated pathology as well as drugs that delayed pathology. This would have added extra recording sessions for each plate at earlier time points.

C. elegans is a relevant and well characterized model for studying high glucose diets and its consequences

In humans, some cells are more vulnerable to hyperglycemia than others. Indeed, only a particular subset of cell types and tissues will develop damage as a consequence of the high glycemic concentration which occurs in diabetic patients. All body cells are exposed to the same hyperglycemic environment within a diabetic patient, but not all cell types are able to adapt glucose transport to glucose concentration. Cells damaged by hyperglycemia are not able to efficiently reduce glucose transport inside the cell when exposed to hyperglycemia so that the glucose concentration inside the cell stays constant. Capillary endothelial cells of the retina or mesangial cells in the renal glomerulus cannot rapidly and persistently reduce glucose transport in the cell when exposed to hyperglycemia, leading to high glucose concentrations within the cell[99,234,235].

In the case of *C. elegans*, our results also show that there is no difference in FGT-1 expression with glucose exposure or time, in particular no difference of FGT-1 expression between the no added glucose state and added glucose conditions. This implies that *C. elegans* cells are probably not capable of reducing glucose transport inside the cell when exposed to high glucose, leading to glucose induced damage and aging. Of course, these suppositions are limited by the fact that we are not yet able to separate different cell types when measuring RNA expression by RT qPCR in *C. elegans*. The results are only a reflection of total organism RNA expression. Also, we have only measured FGT-1 expression which is to our knowledge the best described and most efficient glucose transporter in *C. elegans*. It would be interesting to evaluate if there could be a reduction of 'glucose impaired lifespan' when worms are cultured with FGT-1 RNAi. It would also be interesting to measure FGT-1 expression at an earlier interval after high glucose exposure as our measurements start after three days of glucose exposure, there was maybe an adaptation of FGT-1 expression we are not aware of.

Our glucose measurements in worm lysates and supernatants are compatible with findings from others who show that glucose worm content increases with glucose exposure[236]. Although in the cited article, only one measurement was made in time for glucose worm content. Interestingly, the concentration of glucose found in worm lysates and supernatant stays stable throughout our experiment. No increase of glucose concentration is shown in time for each condition. Therefore, *C. elegans* either metabolizes and/or rejects a constant amount of glucose. The expression of enzymes from different glucose metabolic pathways in the case of glucose excess has been extensively explored in mammals, but this has not been studied to our knowledge in nematodes. A recent study has explored the effects of knock-down for the enzymes involved in the possible metabolic alternatives for the excess of glucose on lifespan[193]. Among the differences in glucose metabolism between *C. elegans* and humans, there is the fact that *C. elegans* can store excess glucose as trehalose in addition to glycogen. Trehalose is the only disaccharide characterized in nematodes[185]. It is found in lower organisms and is used as energy storage as well as a stress protectant and does not participate in spontaneous nonenzymatic reactions with proteins or lipids[193]. Trehalose has been associated with increased worm longevity, either by diet supplementation[237] or by shifting metabolism from glycogen to trehalose[193,193]. Increased levels of trehalose are described in the literature in situations of desiccation tolerance[238] and reduction of *gsy-1* function[193,239].

It is known that in case of high glucose exposure, the intermediate metabolites of the glycolysis pathway are redirected towards alternative glucose metabolic pathways (described in the introduction of this manuscript). Our results show that with glucose excess there is no significant change in relative expression in *C. elegans* of the different enzymes implicated in these alternative glucose metabolic pathways. It is known that most of the excess glucose is stored as glycogen[193]. No glucose alternative pathway seems to be favored in regards with enzyme relative expression. In regards with *sodh-1* relative expression, the second step enzyme of the polyol pathway, our results are compatible with the literature which show a decrease of *sodh-1* expression with age in *C. elegans*. In our results we show a decrease in *sodh-1* relative expression for each condition between day 3 and day 13 of adulthood. The accelerated decrease in *sodh-1* expression in the case of excess glucose exposure could be explained by a regulatory, protective downregulation by the cells. Indeed, *sodh-1* transforms sorbitol to fructose which is a much more reactive sugar than glucose. Increasing fructose levels via the polyol pathway could therefore be detrimental to the cell. Further research is needed to explore the cause of *sodh-1* accelerated reduction with an excessive glucose diet. Another hypothesis could be that

most glucose excess is metabolized through the glycolytic pathway which limits the glucose entering the polyol pathway. To note that in plants, sorbitol dehydrogenase expression and activity is enhanced by sorbitol and down regulated by sucrose[240]. Finally, *sodh-1* is a known *daf-16* target gene[241]. It has been shown that excess glucose decreases *daf-16* expression via the insulin/IGF-1 pathway mediating in part the negative effect of glucose on lifespan[33]. In the case of excess glucose, *daf-16* target genes are therefore down-regulated and this could translate the effect observed in our study.

The increase of anti-oxidant enzymes GST-4 and SOD-3 in our work can be explained by several points. Hyperglycemia has been linked to oxidative stress since the 1960s[242]. In aerobic glucose metabolism, reactive oxygen species are produced by the electron transport chain complex I, which is promoted by glucose metabolism[36,243]. In the case of excess glucose, complex I use is increased inducing increased reactive oxygen species. In response to reactive oxygen species increase, cells activate anti-oxidant enzymes to maintain a redox-balance, essential to the cell survival. In our study, *sod-3* but not *sod-1* expression is increased with glucose exposure in time. This can be due to their localization. Indeed, SOD-1 is localized in the mitochondria and SOD-3 in the extracellular space. GST-4 and SOD-3 are two enzymes which are targets of the transcription factor SKN-1. Our results show an increase in relative expression for *gst-4* and *sod-3* for the highest glucose concentrations after 10 days of exposure. This correlates with the delay of CML production as shown in our results. Incidentally, we have shown that CML enhances SKN-1 translocation in *C. elegans* (unpublished results) and this could therefore explain increase in *gst-4* and *sod-3* expression, the source of oxidative stress being AGEs.

Another explanation to the increase of *gst-4* expression could be that in the case of glucose excess, pentose phosphate shunt pathway is enhanced, which is responsible for generation of NADPH, and in turn, affects levels of reduced glutathione. This would enhance *gst-4* expression to overcome glutathione depletion[185].

We confirm that AGE production is increased in *C. elegans* in a high-glucose environment[31,193] and were able to pinpoint that CML is one of the AGEs produced in a western blot analysis.

Results from our lifespan assays for N2 wild type worms exposed to high glucose confirm the results from many other studies[31,33,193]. Our results with 2DG corroborate with and extend those of Schulz et al[32], supports the theory that the effects of high-glucose on *C. elegans* aging is due to metabolism, rather than byproducts of the glycolytic pathway/glucose itself.

Several pharmacological agents mostly originating from anti-diabetic treatments reduce carbotoxicity and have proven to have an impact on longevity of model organisms[1]. Acarbose is an anti- α glucosidase which reduces intestinal glucose absorption by blocking the breakdown of complex carbohydrates. Indeed, glucose transporters are only capable of transporting simple sugars. It has been shown in mice that acarbose increases lifespan by Harrison et al.[244]. Anti- α glucosidase occur naturally in leguminous plants which are part of the carbohydrate food group[18] and have been recommended twice a week in the diet by the last PNNS (Plan National Nutrition Santé) in France for its health benefits as a replacement for more refined carbohydrate such as white rice or pasta. The widely used anti-diabetic drug metformin, originally discovered by the Incas more than 2000 years ago, competitively inhibits the mitochondrial isoform of GPDH, thereby blocking hepatic gluconeogenesis. It has also been shown that metformin activates AMP kinase, and decreases insulin/IGF1 and mTOR pathways signaling[69] which explains part of metformin's beneficial effects on lifespan and healthspan[245].

Limits of the work conducted in Lille

As shown in a previous study from the MOBBS laboratory, CBP-1 inhibition blocks the induction of the *daf-16* target, *sod-3* in *C. elegans*. As we know that CBP-1 shifts metabolism from glucose to alternate substrates for energy, one could hypothesize that glucose excess and advancing age decreases *sod-3* expression because of a decrease in *cbp-1* and its target: *daf-16*. This does not unfortunately explain our results with an increase of *sod-3* expression in the highest glucose concentration medium after 10 days of worm exposition.

We did not test the protein carbonylation for the earlier time points of our experiment which would have been interesting in order to hypothesize on the origin of the oxidative stress. With an increasing oxidative stress over time we could have hypothesized a correlation between oxidative stress and increase in protein glycation. Unfortunately, time constraint did not allow us to complete these studies.

V.2 Perspectives

The demonstration of the specificity of CBP among the HATs in relation to lifespan and β -amyloid toxicity allows us to further hypothesize the underlying mechanisms by which dietary restriction confers health benefits. HDAC inhibitors can enhance histone acetylation. To assess CBPs HAT activity, Zhang et al assessed the efficacy of sodium butyrate (a short chain fatty

acid, which is a carboxylic acids containing up to 6 carbons in its aliphatic chain), to mimic the protective effects of dietary restriction. Butyric acid is one of the most studied short chain fatty acids due to its presence in dairy products such as cheese and butter. It is also produced endogenously in the human gut by butyric bacteria[224]. Sodium butyrate not only extended lifespan but also delayed the development of proteotoxicity in a transgenic model of Alzheimer's disease, protective effects that were completely prevented by genetic inhibition of CBP[14]. Sodium butyrate longevity therefore requires CBP. The search for HDAC inhibitors with health benefits led to the study of β -hydroxybutyrate, a ketone body (which differs from sodium butyrate by only an OH). It is an endogenous HDAC inhibitor present in organisms at millimolar concentrations during prolonged fasting and dietary restriction. Indeed, when mammals and other living organisms endure a fasting period longer than 24 hours[246] but also in dietary restriction[129], or exposed to low-carbohydrate diets, alternative metabolic pathways are activated which rely less on glucose and more on ketone body-like carbon sources. Moreover, there is evidence that substantial ketogenesis is protective and enhances resistance of a variety of cells to oxidative damage[56,247]. We could hypothesize that ketone bodies produced during dietary restriction and fasting partly mediate the positive effects of dietary restriction. The study by SHIMAZU et al. produces elements for the underlying mechanism. They demonstrated that β -hydroxybutyrate specifically inhibits class I HDACs (HDACs 1,2,3, and 8), and that β -hydroxybutyrate levels in blood are highly correlated with acetylation of histone H3 at K9 and K14. These same acetylation patterns are induced by dietary restriction, fasting, and infusion of β -hydroxybutyrate in mice (MORENO et al., unpublished data). It is therefore of great interest that the main targets of CBP acetylation are H3 K9 and K14. These studies therefore strongly support the hypothesis that protective effects of dietary restriction and the ketogenic diet are mediated by a common mechanism, the inhibition of class I HDACs leading to relatively enhanced CBP activity. As discussed in the introduction chapter, FOXO requires CBP to mediate lifespan extension in dietary restriction, linking CBP to a major lifespan mediating pathway the insulin/IGF-1 pathway[13,129,181]. In the case of dietary restriction, the decrease in glucose availability results in a decreased activation of the insulin/IGF1 pathway which itself leads to an increase in CBP recruitment by FOXO. CBP could therefore not only mediate the protective effects of the insulin/IGF1 pathway on lifespan but also reveal a central role for glucose in mediating lifespan. We have also discussed that glucose excess decreases lifespan by mediating the insulin-IGF1 signaling pathway and down-regulating DAF-16 in *C. elegans*[33]. Although enhanced expression of *cbp-1* in a high-glucose

medium has not been studied in *C. elegans*, EDWARDS et al[16]. demonstrated that β -hydroxybutyrate supplementation increased lifespan, dependent on DAF-16, SKN-1, SIR-2.1, AAK-2 and CBP-1 and more importantly for this thesis, partially prevented glucose toxicity in high glucose diet. The discovery of the therapeutic potential of activating acetylation in age-related diseases is relatively recent. Basic questions regarding the function of HDACs and HATs still need to be clarified (Do HATs other than CBP have a role in dietary restriction induced life-extension? Do they have a role in other neurodegenerative diseases such as Huntington's disease? What are the specific roles of the different HDACs and HATs in more advanced animal models and in humans?). Many challenges lie ahead, but the prospects are positive and new drugs will hopefully emerge from the current efforts.

As other groups have shown[31,39], we confirm that glucose-enriched medium exposure to *C. elegans* leads to endogenous AGE production. We have also characterized the more specific presence of CML, one of the most studied AGEs. This is not a finding one could have awaited as AGE production usually requires long periods and high temperatures and this could not be the case with *C. elegans culture* as this animal normally lives 3 weeks and at a temperature of only 20°C. The worm does not have either an inflammatory system which is another AGE production pathway (kidney insufficiency and chronic inflammation are known to increase AGE production)[101,104]. Professor BOULANGER's laboratory has studied the role of dietary AGEs in aging and shown in several models (mice and cellular) that AGEs accelerate aging (vascular and renal). New studies conducted in the laboratory charge RAGE with the role of accelerating aging in mice. Indeed, we have shown that RAGE knock out mice are protected against kidney aging and do not develop glomerulosclerosis[104]. Other studies conducted in Lille have shown that exposure to dietary AGE accelerated vascular aging in mice[20].

Among the possible therapeutic options, results from a recent study[248] suggest that circulating sRAGE could help improve healthspan and reduce 'inflammaging' by scavenging the damage and pathogen-associated molecular patterns (DAMPs and PAMPs) responsible of initiating the inflammatory responses[248].

A *C. elegans* expressing human RAGE could help explore the underlying mechanism of RAGE in aging and whether the AGE-RAGE interaction plays a role. A group at the Einstein Medical Center in New York has successfully engineered a RAGE expressing mutant[249] and is currently using it to study mechanisms of neurodegenerative diseases in aging.

In our laboratory, preliminary results show that AGE ingestion by our model organism *C. elegans* is possible and leads to an anti-oxidant response.

Considering glucose excess metabolism in *C. elegans*, we still could explore expression of the PKC (protein kinase C) pathway (enzyme pointed out is the LPIN1) and the GAPDH expression. According to BROWNLEE's unifying hypothesis, it is the increase in superoxide production by the mitochondrial complex I from the respiratory chain which inhibits GAPDH glyceraldehyde 3 phosphate dehydrogenase, leading to an increase of all upstream glycolytic intermediates. The increased levels of upstream glycolytic metabolites then activate the AGE pathway and the protein kinase C pathway, via glyceraldehyde 3 phosphate which transforms in methylglyoxal and diacylglycerol. This could help better understand how endogenous AGEs are formed. Three fields of research seem to emanate regarding the study of the role of AGEs in health using the model organism *C. elegans*: 1/ Study of endogenous glycation and AGEs, 2/ study of exogenous AGEs, 3/ study of the role of the AGE-RAGE axis in aging.

1/ By using a simplified model organism where a great majority of the features are controlled in laboratory setting, we will aim at characterizing the different AGEs formed in a full organism, their proportion, biochemical properties. We can study these features by varying the food source, sugar present in the medium (fructose?). We will also be able to study the different defense mechanisms, meant to protect cells against the presence of *C. elegans* and the different existing degradation systems. Receptors for AGEs exist in *C. elegans* such as orthologs for AGE-R1/2 and 3, or CD36. As *C. elegans* does not have the RAGE (main AGE receptor known and studied), this will allow us to study the role of these different receptors without the interference of RAGE. We will also try to determine the role of endogenous AGEs in the reduction of lifespan observed in worms grown in a medium with a high glucose concentration as shown in our work. This can be done more particularly by examining the different pathways mediating aging.

2/ Dietary AGEs are thought to affect healthspan in humans[250]. Studying the underlying mechanisms of the effects of dietary AGEs on aging are complex in mammals because of the presence of many confounding factors. As mentioned earlier, preliminary results from Professor BOULANGER's laboratory suggest that worms can ingest AGEs when present in their growth medium and that they affect lifespan negatively. Working with a 'simpler' model organism will allow to decipher the exact mechanisms underlying the deleterious effects of dietary AGEs more easily. We will aim at elucidating the how dietary AGEs are ingested, digested, metabolized by whole organisms (there is to date no good *in vivo* model for this). We will also look at the effects and interactions of dietary AGEs with the AGE receptors present in *C.*

elegans naturally. We will examine and compare the effects of different types of AGEs on aging in *C. elegans*. Indeed, the specific effects of the different AGEs is not well understood. In the same way as with the high glucose diet experiments from the thesis, we will explore anti-oxidant response, and search for evidence of increased oxidative stress when worms are exposed to dietary AGEs.

3/ Finally, as RAGE is so central to the action of AGEs in mammals, we will transfect the human RAGE in *C. elegans* by microinjection to study its physiological and pathogenic roles. This will allow the study of the AGE-RAGE axis and pathways that are activated. As we hypothesize that RAGE has a pro-aging role, we will also explore the different pathways involved in aging and whether they are activated by RAGE. If a particular phenotype is observed for worms expressing the human RAGE, we will be able to use this model to screen *in vivo* different anti-RAGE chemicals.

Conclusion

Once again, the model organism *C. elegans* has contributed to improve our knowledge on the mechanisms underlying aging. As a multicellular organism with a short lifespan and glucose metabolism pathways in many ways similar to humans, the possibility of modeling age-related diseases such as Alzheimer's disease and its accessibility to large-scale screens made it a useful model for our purposes. Both excess and limitation of glucose (one of the best developed models for studying dietary restriction) intake are readily controlled in this model organism making *C. elegans* a relevant model for the study of the effects of glucose on aging.

A unifying theory for a possible mechanism of glucose on aging can be proposed as a result of our work. Excessive glucose metabolism inhibits daf-16/FOXO, which recruits CBP. This leads to increased glycolysis, responsible for reducing the NADH/NAD⁺ ratio, increasing AGE production and RAGE activation (in mammals), which in turn will cause increase in inflammation and oxidative stress which are known to accelerate aging and age-related diseases.

Until now, no therapy has been found despite our advancing knowledge on aging mechanisms, we still have not been able to develop drugs capable of delaying (or reversing) age-related diseases. The results from our studies on glucose metabolism and protective role of CBP as well as potential role of AGEs and RAGE in aging suggest promising possibilities for drug development in the future. In the meantime, these findings call for public health policies aiming to reduce the excessive ingestion of sugars.

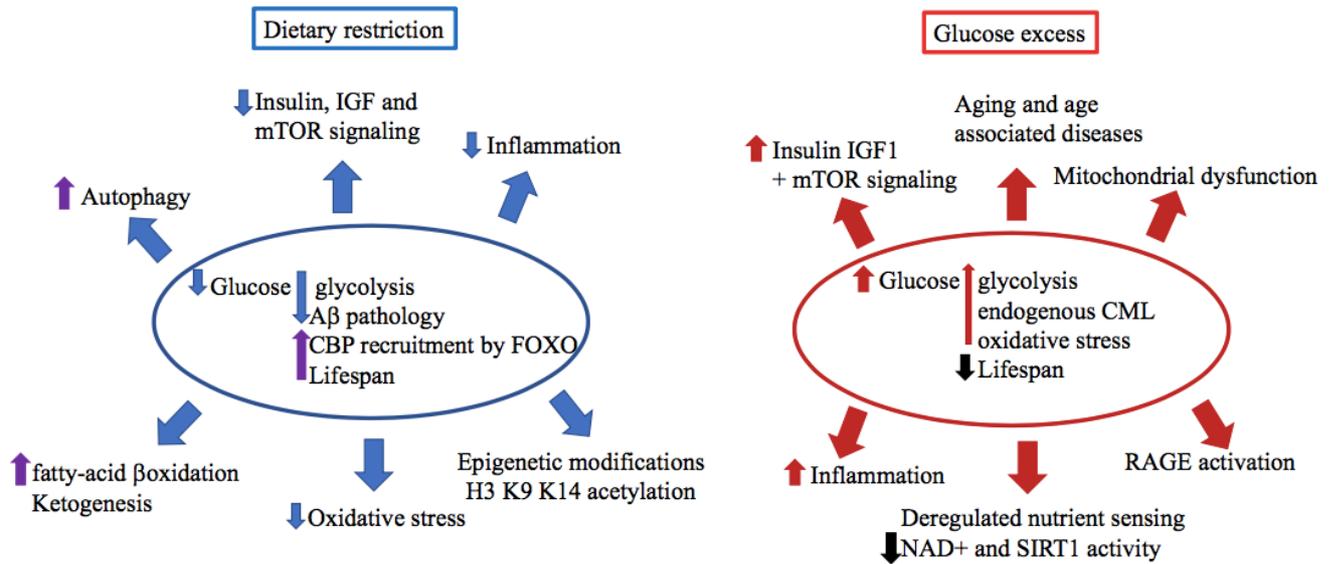


Figure 30: Summary figure This figure summarizes our arguments for the role of glucose in aging. At the center of each oval, elements from this thesis or previous work from Professor MOBBS's or Professor BOULANGER's laboratories in favor of a role for glucose in aging. The ovals are surrounded by arrows revealing possible mechanisms induced by the different levels of glucose and mediating aging.

CHAPTER VI BIBLIOGRAPHY

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ANNEX

Annex 1



Centre Hospitalier Régional
Universitaire de Lille

Les Syndromes Gériatriques (SG)

SG = 4 critères

1. Sa fréquence augmente fortement avec l'âge
2. Résulte de facteurs multiples favorisant et précipitant
3. A pour conséquences un risque de perte d'indépendance et/ou d'entrée en institution
4. Sa prise en charge est multifactorielle et requiert une approche globale

SG = 12 à rechercher à l'entrée et à évaluer à la sortie

1. Polypathologie
2. Robuste ou Vigoureux – Fragiles – Dépendants
3. Iatrogénie
4. Dénutrition et sarcopénie
5. Dépression, troubles du comportement
6. Confusion
7. Troubles cognitifs et démence
8. Troubles de l'équilibre et de la marche, ostéoporose et chute
9. Complication du décubitus: escarres, TVP, constipation, encombrement bronchique
10. Troubles sphinctériens: incontinence, rétention aigue d'urines, fécalome
11. Troubles neurosensoriels: vue, audition
12. Douleurs et soins palliatifs

Cascade gériatrique

=

Emballlement des SG et décompensation d'organes

Les données recueillies dans le cadre de l'évaluation gériatrique sont des éléments à considérer dans le cadre d'une réflexion éthique sur l'intensité des soins à prodiguer à un patient"

Annex 2

***Caenorhabditis elegans* Protocols**

Nematode Growth Medium (NGM):

NaCl (3g/l), Peptone (2.5g/l), KH₂PO₄ pH6.0 (20mM), CaCl₂ (1mM), MgSO₄ (1mM) and cholesterol (5µg/ml)

Agar (20g/l) was added to prepare maintenance and lifespan plates

Carbanecillin and Fungizone were added to the maintenance and lifespan plates prepared in New York

For the RNAi plates, Ampicillin (1ml/l), Isopropyl β-d-1-thiogalactopyranoside (IPTG) (0.24g/l), 5-fluorodeoxyuridine (5-FUDR) (100mg/l)

NB: IPTG is a molecular mimic of allolactose which triggers transcription of the lac operon. It can therefore induce protein expression where the gene is under control of the lac operator

LB medium:

Pepton (10g/l), yeast extract (5g/l) and NaCl (10g/l)

Bleaching (axenizing) solution:

NaOH (500mM) and chlorine- based bleach (1% active chlorine).

M9 buffer

NaCl (5g/l), Na₂HPO₄ (6g/l), KH₂PO₄ (6g/l) and MgSO₄ (1mM).

S-Complete Medium (for liquid culture):

S Basal [5.85 g NaCl, 1 g K₂ HPO₄, 6 g KH₂PO₄, 1 ml cholesterol (5 mg/ml in ethanol), H₂O to 1 litre. Sterilize by autoclaving.]

1 M Potassium citrate pH 6.0 [20 g citric acid monohydrate, 293.5 g tri-potassium citrate monohydrate, H₂O to 1 litre. Sterilize by autoclaving.]

Trace metals solution [1.86 g disodium EDTA, 0.69 g FeSO₄ •7 H₂O, 0.2 g MnCl₂•4 H₂O, 0.29 g ZnSO₄ •7 H₂O, 0.025 g CuSO₄ •5 H₂O, H₂O to 1 litre. Sterilize by autoclaving. Store in the dark.]

1 M CaCl₂ [55.5 g CaCl₂ in 1 litre H₂O. Sterilize by autoclaving.]

S Medium [1 litre S Basal, 10 ml 1 M potassium citrate pH 6, 10 ml trace metals solution, 3 ml 1 M CaCl₂, 3 ml 1 M MgSO₄. Add components using sterile technique; do not autoclave.]

VALORIZATION

Oral communication:

16e Congrès Interrégional et Transfrontalier de Gériatrie et Gériologie 2 and 3 February 2017 in Lille, France : Activation de SKN-1 par la CML alimentaire chez *Caenorhabditis elegans* LITKE, R., BEN SAID, I., LEYCURAS, T., BOULANGER, E., FRADIN, C.

Presented posters:

Société Française de Gériatrie et Gériologie 26-28 November 2018, in Paris, France : LITKE, R., DUNY, M., TEMPESTA, S., BOSSI, C., LEYCURAS, T., BOULANGER, E., FRADIN C. Étude de l'axe carboxyméthyllysine-RAGE dans le vieillissement chez *Caenorhabditis elegans*

R. LITKE, C. PAUL-CONSTANT, M. DUNY, A. GUILBAUD, M. HOWSAM, F. TESSIER, BOULANGER, E., FRADIN C. Analyse de l'ingestion d'une protéine glyquée par *Caenorhabditis elegans* et étude de son impact sur le vieillissement de cet organisme

Poster :

European Geriatric Medicine Society 20-22 September 2017, in Nice, France : R. LITKE, I. BEN SAID, T. LEYCURAS, C. FRADIN Modulation of SKN-1 response in *Caenorhabditis elegans* by dietary protein-bound N ϵ -Carboxymethyllysine

Publications:

FRIMAT, M., DAROUX, M., LITKE R., NEVIERE, R., TESSIER F.J., and BOULANGER E., Kidney, Heart and Brain: Three Organs Targeted by Ageing and Glycation. *Clinical Science (London, England: 1979)* 131, no. 11 (June 1, 2017): 1069–92.

LITKE, R., BOULANGER E., and FRADIN C. [*Caenorhabditis elegans* as a model organism for aging: relevance, limitations and future]. *Medecine Sciences: M/S* 34, no. 6–7 (July 2018): 571–79.

TEISSIER T., LITKE, R., TARONT-DEZITTER, S., LANCEL, S., FRADIN, C., BOULANGER, E. Involvement of the RAGE-Ligand Axis in Aging chapter 3. In *The Receptor RAGE in Vascular and Cerebral Dysfunctions*, edited by Jean-Luc WAUTIER. Cambridge Scholars Publishing, 2019.