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Thibault Teissier

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# THESIS

For the degree of  
Doctor of Philosophy of the University of Lille

*École Doctorale Biologie Santé de Lille*

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## Implication of the receptor for advanced glycation end-products (RAGE) during inflammation and ageing

Thesis directed by Professor **Éric BOULANGER**  
and co-directed by Doctor **Christelle CAUFFIEZ**

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Publicly presented and defended by

**Thibault TEISSIER**

20<sup>th</sup> September 2019

### Jury

|                                 |           |                                  |                    |
|---------------------------------|-----------|----------------------------------|--------------------|
| Mrs. <b>Lynne COX</b>           | Professor | University of Oxford, UK         | <i>Rapporteur</i>  |
| Mr. <b>Ivan BAUTMANS</b>        | Professor | Vrije Universiteit, Brussels, BE | <i>Rapporteur</i>  |
| Mr. <b>Bertrand FRIGUET</b>     | Professor | Sorbonne University              | Examiner           |
| Mrs. <b>Christelle CAUFFIEZ</b> | Doctor    | University of Lille              | Thesis co-director |
| Mr. <b>Éric BOULANGER</b>       | Professor | University of Lille              | Thesis director    |





*« Ô RAGE ! ô désespoir ! ô vieillesse ennemie ! »*

*Le Cid de Pierre Corneille*

*Acte 1 , Scène 4*

*« Nothing in Biology makes sense except in the light of evolution »*

*Theodosius Dobzhansky, 1973*

*À papa*



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## Résumé

Le vieillissement est défini par l'accumulation d'événements menant à une perte d'efficacité des fonctions des organes et à une augmentation de la probabilité de mourir avec le temps. Ce processus touche tout le règne animal et bien que sa vitesse varie largement entre les espèces, modifiant grandement la longévité, ses mécanismes sont quant à eux bien conservés. Chez l'humain, l'espérance de vie a continuellement progressé au cours du siècle dernier, s'accompagnant d'une augmentation de personnes atteintes de maladies liées à l'âge et dépendantes, devenant ainsi un problème majeur de société.

La glycation est une réaction non-enzymatique menant à une interaction irréversible entre des composés carbonylés, tels que les sucres, avec des nucléophiles, comme la lysine ou l'arginine, produisant des produits de la glycation avancée (AGE). L'accumulation des AGE avec l'âge dans le corps suggère que ce processus est particulièrement impliqué dans le vieillissement. Cependant, le rôle de la consommation d'AGE alimentaires sur le vieillissement est beaucoup moins connu. Leur digestion implique d'importantes modifications structurelles et ceux-ci ne peuvent qu'avoir des effets indirects. Notre équipe a démontré qu'une consommation prolongée d'un régime enrichi en carboxyméthyllysine (CML), un des AGE les plus abondants, induisait un vieillissement vasculaire accéléré chez des souris d'âge moyen. Cependant cet effet était complètement dépendant de l'expression du récepteur aux AGE, RAGE.

RAGE est un récepteur multiligand et son activation est principalement définie par une réponse pro-inflammatoire auto-alimentée qui a été impliquée dans des maladies liées ou non à l'âge telles que les complications du diabète, des maladies cardiovasculaires, la maladie d'Alzheimer ou différents cancers. Étant donné le lien entre les AGE et RAGE et leur implication dans le vieillissement, nous émettons l'hypothèse que RAGE tient un rôle important dans le vieillissement physiologique et accéléré par les AGE. De plus, notre équipe a également démontré que la CML alimentaire s'accumulait principalement dans les reins chez la souris. Par conséquent, nous cherchons à déterminer si la CML alimentaire accélère également le vieillissement rénal chez les souris et si la suppression de RAGE empêche cet effet et a un impact sur le vieillissement normal.

Des souris sauvages (WT) et  $RAGE^{-/-}$  de 2 mois ont été nourries pendant 18 mois avec un régime contrôle ou enrichi en CML (200 $\mu$ g CML/g<sub>nourriture</sub>). La répartition de la CML a été déterminée par immunohistochimie et en HPLC-MS/MS. Le vieillissement du rein a été évalué en mesurant des marqueurs de sa fonction, de ses lésions, d'amylose ainsi que d'inflammation, d'oxydation et de vieillissement. Enfin, nous avons également évalué la fonction motrice chez de vieilles souris (~22 mois) en utilisant des tests de la locomotion.

Bien que la CML s'accumulait dans les reins de souris nourries avec le régime enrichi en CML, celui-ci n'avait que peu d'effets sur les paramètres étudiés alors que les souris ne possédant pas RAGE étaient fortement protégées contre les lésions rénales liées au vieillissement, l'amylose sénile rénale et l'inflammation à bas bruit alors que des voies pro-longévité étaient renforcées. Nous montrons ensuite que certaines fonctions motrices des vieilles souris  $RAGE^{-/-}$  pourraient être mieux conservées que chez les vieilles souris WT, supposant une sarcopénie moins importante chez les souris  $RAGE^{-/-}$ .

L'impact conséquent de RAGE sur le vieillissement et sur l'inflammation chronique à bas bruit, associé à ses caractéristiques intrinsèques, suggèrent fortement que RAGE est un récepteur de reconnaissance de motifs moléculaires (PRR) et est une preuve de principe que « l'inflammaging » est un moteur important du vieillissement qui reste néanmoins modulable, génétiquement ou pharmacologiquement.

**Mots-clés :** vieillissement, RAGE, produits de la glycation avancées, rein, inflammaging

## Abstract

Ageing is defined by the accumulation of events leading to a reduction in the efficacy of organ functions and an increased probability of death with time. This process affects all the animal kingdom and while the pace of ageing varies significantly among species, greatly affecting longevity, the mechanisms of ageing itself are widely conserved. In humans, as life expectancy at birth has been steadily increasing for over a century, the amount of people with age-related diseases and dependency has greatly increased and is becoming a major concern.

Glycation is a non-enzymatic process leading to the irreversible interaction of carbonyl compounds, such as sugars, with nucleophiles, including lysine or arginine, forming advanced glycation end-products (AGEs). This process is thought to be involved in ageing as AGEs accumulate in the body with age. However, the role in ageing of consuming AGEs produced during cooking processes is much less understood. Digestion vastly modifies their structure and they can only have indirect an impact. Our group has shown that the long-term consumption of a diet enriched with carboxymethyllysine (CML), one of the most abundant AGEs, induced an accelerated vascular ageing in middle-aged mice. However, this effect was entirely dependent on the expression of the receptor for AGEs, RAGE.

RAGE is a multiligand receptor and its activation is primarily characterised by a self-sustaining pro-inflammatory response which has been implicated in both age-related and age-independent disorders including complications of diabetes, cardiovascular diseases, Alzheimer's disease or cancers. Given the relationship between AGEs and RAGE and their respective role in ageing or age-related disorders, it was hypothesized that RAGE has an important role in both physiological and AGE-accelerated ageing. In addition, our group has demonstrated that dietary CML mostly accumulates in mice kidneys, which age slower than vessels. Therefore, a key aim of this thesis was to investigate whether dietary CML also induces accelerated kidney ageing in older mice and whether the deletion of RAGE prevents this effect and has an impact on normal ageing.

Two-month-old wild-type (WT) and RAGE<sup>-/-</sup> mice were fed a control or a CML-enriched diet (200µg CML/g<sub>food</sub>) for 18 months. CML distribution was assessed by immunohistochemistry (IHC) and HPLC-MS/MS. Kidney ageing was assessed by measuring markers of its function, lesions and amyloidosis, as well as of inflammation, oxidation and ageing. In addition, motor function in old (~22 month-old) mice was also assessed using locomotion tests.

Firstly, it was demonstrated that although CML accumulated in the kidneys of mice fed the CML-enriched diet, this diet had little effect upon the studied parameters while mice deprived of RAGE were largely protected against age-related renal lesions, renal senile amyloidosis and exhibited decreased inflammation and improved pro-longevity pathways. Thereafter, it was shown that some of old RAGE<sup>-/-</sup> mice motor functions might be better preserved than in old WT animals, suggesting a reduced sarcopenia in RAGE<sup>-/-</sup> mice.

The significant impact of RAGE on ageing and on low-grade and chronic inflammation, associated with its intrinsic characteristic, strongly suggest that RAGE is a pattern recognition receptor and is a proof of principle that inflammaging is an important motor of ageing which may be modulated through genetic or possibly pharmacologic interventions.

**Keywords:** ageing, inflammation, RAGE, advanced glycation end-products (AGEs), kidney, inflammaging



## **Implication du récepteur aux produits de la glycation avancée (RAGE) pendant l'inflammation et le vieillissement**

---

### **Introduction**

#### **Le vieillissement**

Le vieillissement se caractérise par une accumulation d'événements menant à une baisse de l'efficacité des fonctions des organes ainsi qu'à une augmentation de la probabilité de mourir avec le temps. Cette mortalité, dite intrinsèque, s'oppose à la mortalité extrinsèque, réunissant la survenue de la mort par de multiples sources externes : famine, prédation, maladies, accidents etc. Les dommages s'accumulent très tôt dans la vie, l'étude du vieillissement ne se limite donc pas à l'étude de la vieillesse mais plutôt à des événements qui débuteraient dès la naissance.

L'espérance de vie humaine à la naissance a considérablement augmenté au cours du siècle passé. En France, par exemple, l'espérance de vie à la naissance était de 45 ans en 1900 alors qu'elle se situe désormais aux alentours de 82 ans. Cependant, de larges différences entre les pays demeurent, notamment entre les pays en voie de développement et les pays développés. La récente diminution de la mortalité infantile dans les pays en voie de développement a beaucoup plus contribué à l'augmentation de l'espérance de vie dans ces pays que dans les pays développés. En effet, bien que l'espérance de vie continue d'augmenter dans les régions développées, la mortalité infantile a quant à elle très peu évolué, voire est désormais stable dans des pays comme la France. Ainsi, cette augmentation de la longévité doit pouvoir s'expliquer par d'autres biais.

Les périodes de famines, de maladie ou les conflits majeurs peuvent également significativement affecter cette espérance de vie. En France, les guerres Napoléoniennes, la guerre de 1870 ainsi que les deux guerres mondiales ont considérablement réduit cette espérance de vie à la naissance, et ce, beaucoup plus largement chez les hommes qui sont les



premières victimes de ce genre d'événements. Nous pouvons également citer la grippe espagnole qui se produisit de 1918 à 1919, directement à la suite de la première guerre mondiale, et fit 50 à 100 millions de morts dans le monde. Celle-ci eut des conséquences notables sur l'espérance de vie à la naissance des hommes comme des femmes. En revanche, depuis la fin de la seconde guerre mondiale, aucun événement similaire n'a eu lieu sur le territoire français. Ainsi, en France, l'augmentation continue de l'espérance de vie depuis cette date doit être expliquée par d'autres facteurs qui peuvent inclure des facteurs environnementaux (la vaccination, une meilleure hygiène, un meilleur accès à de l'eau et à une alimentation de qualité, etc.) et le développement d'outils et de techniques permettant de limiter ou de soigner un ensemble de pathologies, telles que les cancers, les maladies pulmonaires ou cardiovasculaires, permettant un meilleur niveau de vie à un âge avancé et limitant la dépendance des personnes âgées. Ainsi, il y a de grandes chances que les différences d'espérance de vie entre les pays développés et en voie de développement s'expliquent essentiellement par des facteurs environnementaux et sociétaux plutôt que par l'éventuelle existence d'un polymorphisme génétique.

L'extrême longévité, caractérisée par les centenaires (100 ans et plus), ou les super-centenaires (110 ans et plus), est essentiellement décrite dans les pays développés. Cependant, il est important de noter qu'un ensemble de preuves est nécessaire pour confirmer l'âge de possibles centenaires ou super-centenaires. L'accès à celles-ci étant plus difficile dans les pays en voie de développement, nous y retrouvons mécaniquement moins de centenaires. Cependant, il ne faut pas faire l'erreur de penser qu'une faible espérance de vie à la naissance signifie qu'il n'y aurait pas de personnes d'un âge avancé dans ces régions. Ces deux données ne sont pas forcément liées et l'espérance de vie à la naissance y est notamment très affectée par la mortalité infantile. Par conséquent, il est tout à fait possible que certaines personnes puissent vivre jusqu'à un âge très avancé dans ce type de région mais que nous manquons simplement des éléments de preuve nécessaires pour le confirmer.

Ainsi, l'immense majorité des super-centenaires se retrouve dans les pays les plus riches et sont très majoritairement des femmes puisqu'elles regroupent plus de 90 % de ceux-ci. Le record de longévité se retrouve chez les femmes et se situe à 122 et 164 jours pour la française Jeanne Calment alors qu'il est de « seulement » 116 ans et 54 jours chez les hommes, avec le japonais Jiroemon Kimura (11<sup>ème</sup> personne à avoir vécu le plus longtemps). Cette disparité se retrouve également lorsque l'on s'intéresse à l'espérance de vie à la naissance puisqu'elle existe dans tous les pays du monde, néanmoins à différents degrés, et augmente globalement en même

temps que l'augmentation de l'espérance de vie. Néanmoins, cette différence peut grandement varier significativement entre deux pays dont l'espérance de vie moyenne est similaire, suggérant que bien que cette différence repose sur une base biologique connue et retrouvée dans de nombreuses espèces, elle est aussi grandement affectée par les conditions environnantes.

Finalement, la question de l'existence d'une limite à la longévité humaine reste une question ouverte tant qu'un consensus scientifique n'aura pas été établi. Les données existantes demeurent insuffisantes pour arriver à une conclusion satisfaisante et fiable.

Dans le monde du vivant, la longévité varie d'une manière plus que considérable mais celle-ci semble être définie par des paramètres communs entre toutes les espèces. Chez les animaux, la longévité est fortement associée aux capacités reproductives de l'espèce concernée. Ainsi, chez les mammifères, le temps de gestation corrèle très bien avec la longévité des espèces, ce qui reflète les concepts décrits dans la théorie du soma jetable, énoncée par T. Kirkwood à la fin des années 50. Cette théorie suggère qu'il y a un échange entre la reproduction et la longévité qui est gouverné par l'allocation des ressources. Il est considéré que dans un contexte où la mortalité extrinsèque prédomine, il est inutile et coûteux de surinvestir dans l'entretien du corps (le soma) et que, du point de vue de l'évolution, il faut seulement y investir suffisamment de ressources pour éviter une mort prématurée, c'est-à-dire une mort intrinsèque survenant avant la reproduction. Il est surtout essentiel d'assurer la transmission de la lignée germinale et le soma ne serait que le transporteur de celle-ci. Il est ainsi expliqué pourquoi, du point de vue de l'évolution, les espèces avec des capacités reproductives élevées ont une longévité bien plus courte que celles avec des capacités reproductives moins importantes. L'entretien du soma a été renforcé chez ces dernières afin d'assurer la transmission de la lignée germinale.

Quelques contradictions ont été émises vis-à-vis de cette théorie, comme l'existence des espèces eusociales où ce sont les individus se reproduisant le plus qui ont la longévité la plus importante ou comme avec l'existence de la vie post-reproductive. Cependant ces contradictions peuvent s'expliquer en considérant les premiers comme un super-organisme (le soma étant représenté par les individus stériles et la lignée germinale par les reines) et les hypothèses de la grand-mère et de la mère permettent d'expliquer que la vie post-reproductive permet aux grand-mères d'apporter des soins à leur descendance, renforçant leur chance de survie, tout en évitant la compétition avec les mères. D'autre part, l'apparition de la ménopause permettrait d'éviter une mort à l'accouchement, dont le risque augmente avec l'âge. Ainsi ces contradictions ne le sont qu'en apparence et ne font que complexifier la théorie du soma jetable.

La question du vieillissement dans le règne végétal est bien plus complexe puisque les végétaux ont un développement modulaire changeant radicalement l'impact du vieillissement dans ces espèces. Ainsi, il n'est pas rare de rencontrer des spécimens d'arbres de plusieurs milliers d'années et il convient même de se demander s'il est pertinent de parler de vieillissement dans ces espèces puisque la mortalité semble parfois complètement indépendante de l'âge mais plutôt dépendante de la taille et de l'accès aux nutriments. Une question similaire s'est posée chez l'hydre d'eau douce, parfois déclarée comme immortelle, notamment grâce à un mode de développement similaire. Cependant les résultats sont encore débattus.

Les mécanismes propres au vieillissement ont fait et font toujours l'objet de recherches approfondies. Il ne semble pas pouvoir exister de gènes dédiés purement et simplement à la limitation de l'espérance de vie, en revanche, il existe des gènes dont la fonction est à double-tranchant : elle promeut une fonction essentielle tôt dans la vie, comme la croissance, mais a des effets délétères sur le maintien du soma plus tard dans la vie. Cette dualité est ce que l'on appelle la pléiotropie antagoniste : un gène donné peut avoir plusieurs fonctions dont les effets peuvent être bénéfiques ou délétères selon le contexte. Parmi ces gènes l'on retrouve notamment la voie de l'insuline et de l'*insulin-like growth factor 1 (IGF-1)*. Cette voie est fortement impliquée dans la croissance, mais son inhibition permet d'augmenter considérablement la longévité chez toutes les espèces étudiées, en l'échange d'une croissance et d'une fertilité réduites, en accord avec la théorie du soma jetable. D'autres voies possédant des effets similaires ont été répertoriées, et la voie associée à *target of rapamycin (TOR)* semble également avoir un rôle majeur.

Bien qu'il existe des gènes pouvant moduler le vieillissement et la longévité, cela n'explique pour autant pas *comment* les organismes vieillissent. Au cours de ces dernières années, neuf mécanismes fondamentaux ont été décrits comme étant à l'origine du vieillissement : l'instabilité génomique, le raccourcissement des télomères, les altérations épigénétiques, la perte de la protéostase, un dérèglement de la perception des nutriments, la dysfonction mitochondriale, la sénescence cellulaire, l'épuisement en cellules souches et l'altération de la communication entre les cellules. Cependant, ces événements, ne sont pas tous sur le même niveau hiérarchique et quatre d'entre eux (l'instabilité génomique, le raccourcissement des télomères, les altérations épigénétiques et la perte de la protéostase) sont considérés comme des marqueurs primaires qui vont être la cause des autres. Enfin, même les marqueurs les plus bas de la hiérarchie auront finalement une influence aggravante sur les marqueurs primaires.

Les dommages occasionnés par ces événements mènent ensuite à ce que l'on appelle le vieillissement physiologique : une baisse de fonction générale de l'ensemble des organes du corps. Cependant, tous les organes ne vieillissent pas de la même façon et alors que certains vieillissent relativement rapidement (comme le système cardiovasculaire), pour d'autres, les conséquences sont beaucoup moins flagrantes (comme pour le système hépatobiliaire). Le vieillissement du rein est relativement hétérogène dans la population, tandis qu'une partie non-négligeable développe une insuffisance rénale, l'autre partie semble maintenir une fonction rénale (représentée notamment par le débit de filtration glomérulaire) normale, suggérant l'influence de facteurs génétiques, environnementaux et comportementaux. Ce vieillissement rénal est caractérisé sur le plan histologique par différentes lésions glomérulaires (glomérulosclérose), une atrophie tubulaire, une hyalinose artériolaire et une fibrose interstitielle.

Enfin le vieillissement est souvent accompagné par ce que l'on appelle les maladies liées à l'âge qui peuvent être caractérisées par une augmentation de leur survenue avec l'âge ou dont le facteur de risque principal identifié est l'âge. On retrouvera un certain nombre d'atteintes cardiovasculaires, des maladies neurodégénératives (maladies d'Alzheimer ou de Parkinson), des atteintes de l'os, le diabète de type 2 et ses complications et enfin un certain nombre de cancers.

La recherche fondamentale dans la biologie du vieillissement a enfin permis de démontrer que le vieillissement n'est pas immuable et un certain nombre d'interventions a démontré que l'on pouvait significativement ralentir le vieillissement, augmenter la longévité, maintenir les animaux en bonne santé plus longtemps voire provoquer un rajeunissement. Parmi ces interventions nous retrouvons des modulations au niveau cellulaire (suppression de la lignée germinale, transplantation de jeune cellules souches, d'exosomes ou suppression de vieilles mitochondries), des modèles génétiques (provoquant la mort des cellules sénescents), des interventions pharmacologiques (inhibiteurs de mTOR, utilisation de sénolytiques, ou supplémentation en *nicotinamide adenine dinucleotide* (NAD<sup>+</sup>)) et enfin des modulation comportementales (restriction calorique).

## La glycation et les produits de la glycation avancée

Les produits de la glycation avancée (AGE) sont le résultat de la glycation, une réaction portant également le nom de réaction de Maillard car décrite par Louis Camille Maillard en 1912. La glycation est la réaction non-enzymatique entre des composés carbonylés et des nucléophiles. Parmi les composés carbonylés nous retrouvons communément les sucres réducteurs, tels que le glucose, mais aussi des dicarbonyles comme le glyoxal ou le méthylglyoxal. Ils sont majoritairement décrits pour leur glycation des acides aminés (principalement les lysines et arginines) au niveau de leurs amines, mais ils peuvent également glyquer les acides nucléiques et les lipides.

La glycation est une réaction comprenant plusieurs étapes et plusieurs voies possibles sont décrites. Les composés intermédiaires sont généralement réversibles tandis que les produits finaux, les AGE, sont jusqu'ici considérés comme irréversibles. La voie décrite la plus courante est la réaction du glucose avec une lysine, menant à un acide de Schiff, qui à la suite de réarrangements produit un produit d'Amadori pour enfin mener à la production d'un AGE par le biais de leur fragmentation, par exemple. La variété des voies de glycation existantes indique qu'il y a également une grande variété d'AGE possibles. Cette réaction survient *in vivo* à 37°C comme *in vitro*, notamment pendant la cuisson des aliments à diverses températures. La température, avec la présence d'eau, le pH et la demi-vie de la cible modifient grandement les cinétiques de réaction. Ainsi, certains AGEs se retrouvent exclusivement dans la nourriture car les conditions nécessaires à leur formation n'existent pas *in vivo*.

*In vivo*, la glycation peut être limitée par des enzymes, notamment par les glyoxalases (de GLO1 à GLO3) ou les *aldo-keto reductases* (AKR), qui détoxifient les dicarbonyles. Les produits d'Amadori peuvent quant à eux être pris en charge et retransformés en acides aminés par la *fructosamine-3-kinase* (FN-3K). Enfin, une fois formés, les AGE peuvent être excrétés par la voie rénale.

Parmi les AGE nous allons donc retrouver des AGE dérivés de lysines ou d'arginines avec des formes plus ou moins complexes. Les formes les plus complexes surviennent lorsque des lysines ou des arginines modifiées interagissent ensuite avec d'autres lysines ou arginines, formant ainsi des réticulations au sein des protéines ou entre différentes protéines. La carboxyméthyllisine (CML) est un des AGE les plus abondants et celui-ci a été retrouvé dans tous les tissus. Cet AGE peut soit provenir de l'alimentation ou être formé *in vivo*. Notre groupe a ainsi démontré que lorsque des souris étaient nourries avec un régime enrichi en CML, celle-ci

s'accumulait très préférentiellement dans les reins, suggérant que les reins puissent être l'organe le plus affecté par ce type de régime.

Les effets délétères des AGE sont en général très bien connus. Il existe tout d'abord des effets qui sont inhérents à cette réaction puisqu'elle modifie irrémédiablement, et de manière non contrôlée, la structure des protéines impliquées. Ainsi, les protéines glyquées vont soit être détruites, devenir dysfonctionnelles ou non-fonctionnelles, s'accumuler ou former des réticulations avec d'autres protéines. Ces modifications sont surtout importantes concernant les protéines avec une longue durée de vie telles que le collagène, ce qui rigidifie le tissu concerné. Enfin les AGE peuvent également interagir avec des récepteurs aux AGE.

Ces caractéristiques suggèrent que les AGE ont un rôle dans le vieillissement. Cette idée est soutenue par la mise en évidence d'une accumulation des AGE, comme la pentosidine ou la CML, avec l'âge dans tous les tissus, par une corrélation entre le taux de formation des AGE et la longévité et enfin par l'observation que les personnes, après 65 ans, ayant le plus haut niveau de CML dans le plasma ont une mortalité augmentée. Ainsi, les AGE semblent faire partie intégrante du processus normal du vieillissement. Cependant le rôle des AGE d'origine alimentaire dans le vieillissement doit encore être approfondi car les mécanismes en jeu ne sont que partiels et ne peuvent impliquer que leur interaction avec les récepteurs aux AGE. Notre groupe a toutefois démontré qu'un régime enrichi en CML induisait un vieillissement vasculaire accéléré chez des souris de 11 mois après 9 mois de régime. Et nous avons également montré que ce vieillissement accéléré était complètement dépendant d'un récepteur aux AGE, RAGE.

Ainsi, les AGE ont également été mis en cause dans des maladies liées au vieillissement dont principalement les complications du diabète (néphropathies, neuropathies, rétinopathies et cardiomyopathies), dans des maladies neurodégénératives (maladie d'Alzheimer et de Parkinson) ou encore dans des maladies osseuses ou dans la sarcopénie.

## Le récepteur pour les produits de la glycation avancée - RAGE

Il semble que, à l'image de l'impact du régime enrichi en CML sur le vieillissement vasculaire, les récepteurs pour les AGE soient cruciaux dans la perpétuation d'un certain nombre de leurs effets délétères. Il existe tout un ensemble de récepteurs ayant été décrits à ce jour, dont des récepteurs éboueurs, mais un récepteur en particulier semble être à l'origine de cette signalisation néfaste, RAGE.

RAGE est un récepteur transmembranaire multiligand dont l'expression est ubiquitaire. Il est composé d'un domaine variable V ainsi que de deux domaines constant C1 et C2, puis d'un seul domaine transmembranaire et d'un domaine cytoplasmique. RAGE peut être retrouvé sous différentes formes, notamment la forme complète (FL-RAGE ou RAGE), majoritaire, une forme sans domaine cytoplasmique (DN-RAGE), une forme sans le domaine V ( $\Delta$ N-RAGE) et enfin la forme soluble (sRAGE), dépourvue des domaines cytoplasmiques et transmembranaires à la suite d'un épissage alternatif (esRAGE) ou d'un clivage enzymatique (cRAGE). Bien que l'expression de RAGE soit ubiquitaire, elle reste relativement faible en conditions homéostasiques dans la majorité des tissus adultes, sauf dans le poumon où son expression y est très élevée. Son expression est notamment augmentée dans un contexte pro-inflammatoire grâce à la présence de sites de liaison à l'ADN de NF- $\kappa$ B au niveau du promoteur de *RAGE*.

RAGE possède au moins une vingtaine de ligands qui ont tous des origines, structures et rôles différents. Ceux-ci incluent bien évidemment les AGE, mais aussi d'autres produits de protéines ou peptides modifiés tels que les produits d'oxydation avancée de protéines (AOPP), la phosphatidylsérine ou les fibrilles amyloïdes. Des protéines impliquées dans des processus inflammatoires peuvent aussi se lier à RAGE (comme *high-mobility group box 1* (HMGB1) ou les protéines S100) tout comme des composants des parois bactériennes comme les lipopolysaccharides (LPS), comme des petites molécules incluant les acides quinoliniques ou lysophosphatidiques ou enfin comme les acides nucléiques. Beaucoup de ces ligands peuvent être considérés comme motifs moléculaires associés aux pathogènes (PAMP) ou aux dégâts (DAMP) et/ou induisant une réponse pro-inflammatoire. Toutes ces caractéristiques suggèrent fortement que RAGE est un récepteur de reconnaissance de motifs moléculaires (PRR).

La signalisation de RAGE est complexe et comporte de nombreuses voies. Sa stimulation entraîne notamment le recrutement de protéines adaptatrices telles que *toll-interleukin 1 receptor domain-containing adaptor protein* (TIRAP), *myeloid differentiation primary*

*response 88* (MyD88) ou *mammalian diaphanous 1* (mDia1). Celles sont vont pouvoir initier des voies de signalisation telles que la voie de l'AKT, celles des *mitogen-activated protein kinases* (MAPK), celles des *Janus kinase* (JAK)/*signal transducers and activators of transcription* (STAT) ou encore celle de *cell division control protein 42 homolog* (CDC42) et *Ras-related C3 botulinum toxin substrate 1* (RAC1). Cette signalisation est premièrement caractérisée par une réponse pro-inflammatoire et pro-oxydative mais aussi par une stimulation de la migration cellulaire, de l'angiogenèse ou même de l'apoptose.

Malgré toutes les recherches menées pendant ces 25 dernières années, le rôle de RAGE est toujours inconnu. Un rôle est suspecté dans le poumon, étant l'organe exprimant le plus RAGE, mais il n'est pas évident que les souris RAGE<sup>-/-</sup> aient une fonction pulmonaire diminuée. Une étude a démontré un rôle protecteur de RAGE contre *M. tuberculosis* mais d'autres études ont montré l'inverse pour des pathogènes différents. Enfin, alors que RAGE est surexprimé dans la grande majorité des cancers, son expression est réduite dans le cancer du poumon.

Malgré ces éléments de réponse, la suppression ou le blocage de RAGE s'est révélé particulièrement protecteur dans un nombre important de pathologies, non-liées à l'âge (maladies inflammatoires chroniques de l'intestin, sepsis, atteintes aiguës du poumon ou asthme) et liées à l'âge. Parmi les maladies liées à l'âge, nous retrouvons toutes les maladies où les AGE étaient impliqués, notamment les complications du diabète, suggérant que RAGE est le possible lien entre les AGE et ces maladies. Il est également possible que le rôle de RAGE soit exacerbé ou soit impliqué dans d'autres maladies par le biais d'autres ligands de RAGE qui s'accumulent également avec l'âge, de manière circulante ou dans les tissus.



## Objectifs

Les AGE et RAGE ont un rôle dans de nombreuses pathologies dont des maladies liées à l'âge suggérant un rôle, de l'un et de l'autre, dans le vieillissement. Les AGE endogènes semblent être une des causes directes du vieillissement alors que la CML alimentaire semble nécessiter RAGE pour provoquer le vieillissement vasculaire accéléré observé par notre groupe chez des souris d'âge moyen. Ainsi, étant donné l'accumulation rénale de la CML alimentaire, l'objectif de cette thèse a été de **déterminer l'impact d'un régime prolongé enrichi en CML sur le vieillissement rénal ainsi que le rôle de RAGE sur le vieillissement des souris, notamment rénal, en présence ou non du régime enrichi en CML.**

## Résultats

Pour répondre à nos objectifs, des souris sauvages (WT) et RAGE<sup>-/-</sup> de 2 mois ont été nourries pendant 18 mois avec un régime contrôle ou enrichi en CML (200µg CML/g<sub>nourriture</sub>). La distribution de la CML a été déterminée par immunohistochimie et en HPLC-MS/MS. Le vieillissement rénal a été évalué en mesurant des marqueurs de sa fonction, de ses lésions, d'amylose ainsi que d'inflammation, d'oxydation et de vieillissement. La fonction motrice a également été évaluée chez de vieilles souris (~22 mois) en utilisant des tests de la locomotion.

Nos résultats, publiés en 2019 dans la revue *Aging Cell*, ont permis de démontrer qu'à la suite du régime enrichi en CML, de la CML libre et protéique s'accumulait dans les reins des souris exposées, principalement au niveau des tubules. L'expression de RAGE n'affectait pas cette distribution. En revanche ce régime n'a pas produit d'effets significatifs sur le vieillissement rénal, contrairement à l'expression de RAGE. Tout d'abord, nos résultats démontrent que, indépendamment du régime, le vieillissement vasculaire physiologique était largement prévenu chez les vieilles souris RAGE<sup>-/-</sup>. Nous démontrons ensuite qu'en l'absence de RAGE, les vieilles souris RAGE<sup>-/-</sup> semblaient avoir une fonction rénale préservée, caractérisée par une diminution significative de l'atrophie tubulaire, de l'artériosclérose, de la fibrose interstitielle et plus particulièrement de la glomérulosclérose, contre laquelle les souris RAGE<sup>-/-</sup> étaient quasiment complètement protégées. Cette glomérulosclérose était notamment accompagnée d'une hypertrophie chez les souris WT, absente chez les souris RAGE<sup>-/-</sup>, et corrélait très fortement ( $r = 0.9223$ ,  $p < 0.0001$ ) avec une amylose glomérulaire causée par des dépôts d'apolipoprotéine A-II. Nous avons également pu mettre en évidence une forte augmentation de l'expression de marqueurs d'inflammation à l'état basal chez les souris WT avec l'âge, alors que ceux-ci étaient nettement moins exprimés chez les vieilles souris RAGE<sup>-/-</sup>. L'expression de l'antioxydant *Sod2* étant au contraire diminuée avec l'âge chez les vieilles souris WT, mais significativement moins chez les vieilles souris RAGE<sup>-/-</sup>. Enfin, alors qu'il y a une possible surexpression de SIRT1 chez les souris RAGE<sup>-/-</sup>, les activités de mTORC1 et mTORC2, respectivement représentées par la phosphorylation de S6RP et d'AKT, étaient fortement réduites chez les vieilles souris RAGE<sup>-/-</sup> comparé aux vieilles souris WT.

Enfin, dans des résultats complémentaires, nous retrouvons notamment une vitesse de course plus élevée chez les vieilles souris RAGE<sup>-/-</sup> comparées à de vieilles souris WT, alors que l'évaluation de mouvements plus lents (comme l'endurance) n'a pas révélé de différences.

## Discussion

Nos résultats démontrent que contrairement à ce que nous avons observé concernant le vieillissement vasculaire chez des souris d'âge moyen, la CML alimentaire semble ici avoir très peu d'effets sur les paramètres étudiés. Nous suggérons notamment qu'à partir d'un certain âge, la CML alimentaire rentre en compétition avec l'accumulation d'autres ligands de RAGE et que ceux-ci, ayant des affinités plus importantes pour RAGE, rendent complètement invisible l'effet CML-dépendant dans ces conditions. Aussi, contrairement à l'effet du régime, l'effet du génotype semble quant à lui être considérable vis-à-vis des paramètres du vieillissement rénal étudiés.

Ensuite, nous suggérons que l'amylose observée puisse être la cause de la glomérulosclérose présente chez les souris WT et qu'il y a un possible lien à établir avec le vieillissement du foie ou d'autres types d'amylose.

Nos résultats concernant le métabolisme sont très intéressants étant donné le lien établi entre TOR et le vieillissement. En revanche, il est absolument nécessaire d'approfondir l'étude de ces voies chez les souris RAGE<sup>-/-</sup> afin d'en déterminer l'implication précise.

Finalement la piste la plus intéressante et la plus sûre semble être celle de l'inflammation. Le statut inflammatoire est un des meilleurs marqueurs du vieillissement et l'inflammation chronique, à bas bruit et stérile décrit ce que l'on appelle « l'inflammaging », qui est à la fois une cause et une conséquence du vieillissement. Ainsi, en associant nos données à celles de la littérature, tout porte à penser que RAGE est un acteur central de l'inflammaging. C'est ce même inflammaging qui pourrait expliquer que les souris RAGE<sup>-/-</sup> présentent potentiellement une sarcopénie moins avancée.

Enfin, la nouveauté de ce travail ne réside pas simplement dans la mise en évidence de l'intérêt de l'utilisation de molécules antagonistes de RAGE dans le cadre de thérapies de certaines maladies inflammatoires ou liées à l'âge, mais surtout dans une perspective de prévention de voies physiologiques, responsables du vieillissement. Cette perspective est tout particulièrement cruciale dans un contexte de vieillissement de la population où le déclin lié à l'âge et la dépendance deviennent des enjeux de société majeurs.



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# Summary

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# Nomenclature

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For the writing of this manuscript, common guidelines were followed:

## Genes

Human: characters are all italicized and in upper-case (*e.g.* *GFP*).

Mice and rats: characters are all italicized with only the first letter in upper-case (*e.g.* *Gfp*), with the exception of the knock-out for *Rage* where the common format found in literature is followed (*i.e.* *RAGE<sup>-/-</sup>*).

*Caenorhabditis elegans*: characters are all italicized and in lower-case (*e.g.* *gfp*).

RNA names follow the same format as genes.

## Proteins

For all species, protein symbols are not italicized and all letters are in upper-case (*e.g.* GFP). There are some exceptions as different nomenclature exists in the literature (*i.e.* FoxO). The full-name of a protein is normally provided at its first occurrence except when it does not arise from a simple abbreviation (*e.g.* AKT).

In the text, italicized characters are used for the emphasis of words or expressions or for the writing of non-English words, except in an italicized text (*e.g.* in legends) where they are romanized.

# List of abbreviations

4-OHT: 4-hydroxytamoxifen

$\alpha$ -SMA:  $\alpha$  smooth muscle actin

$\gamma$ -IRE:  $\gamma$ -interferon response element

$\Delta$ N-RAGE: N-truncated RAGE

## A

A $\beta$ : amyloid  $\beta$

AD: Alzheimer's disease

ADAM10: A disintegrin and metalloproteinase domain-containing protein 10

AGE: Advanced glycation end-product

AGER: AGE receptor

AKR: Aldo-keto reductase

AH: Heavy chain amyloidosis

AIM2: Absent in melanoma 2

AL: Light chain amyloidosis

AMPK: Adenosine monophosphate-activated protein kinase

AnAge: Animal ageing and longevity database

AOPP: Advanced oxidation protein products

AP: Apurinic/aprimidinic

ApoA-II: Apolipoprotein A-II

ApoE: Apolipoprotein E

ASC: Apoptosis-associated speck-like protein containing CARD

ATG: Autophagy related

ATP: Adenosine triphosphate

ATTR: TTR amyloidosis

## B

BNIP3: BCL2 interacting protein 3

BSA: Bovine serum albumin

BubR1: Bub1-related kinase

## C

CAM: Cell adhesion molecule

CD: Cluster of differentiation

CDC42: Cell division control protein 42 homolog

CEL: N<sup>ε</sup>-carboxyethyllysine

CKD: Chronic kidney disease

CML: N<sup>ε</sup>-carboxymethyllysine

cRAGE: Cleaved RAGE

## D

D: Day

DAMP: Damage-associated molecular patterns

DAPI: 4',6-diamidino-2-phenylindole

DN-RAGE: Dominant-negative RAGE

DNA: Deoxyribonucleic acid

DJ-1: Protein deglycase

## **E**

EGFP: Enhanced green fluorescent protein

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic reticulum

ERK: Extracellular-signal-regulated kinases

esRAGE: Endogenous soluble RAGE

## **F**

FBS: Foetal bovine serum

FEEL: Fasciclin, EGF-like-, laminin-type EGF-like, and link domain-containing scavenger receptor

FH: Formin homology

Fig.: Figure

FL-RAGE: Full-length RAGE

FN-3K: Fructosamine-3-kinase

FoxO: Forkhead box O

## **G**

gDNA: Genomic DNA

GFR: Glomerular filtration rate

GLO: Glyoxalase

GnRH: Gonadotropin-releasing hormone

GOLD: Glyoxal lysine dimer

GRG: Gerontology Research Group

## **H**

HBSS: Hanks' balanced salt solution

HDL: High density lipoprotein

HEK: Human embryonic kidney

HMD: Human Mortality Databases

HMGB1: high-mobility group box 1

HP1 $\alpha$ : Heterochromatin protein 1 $\alpha$

HPLC-MS/MS: High-performance liquid chromatography-tandem mass spectrometry

HSP70: Heat shock protein 70

## **I**

IGF-1: Insulin-like growth factor 1

IGF-1R: IGF-1 receptor

IGFBP-1: IGF binding protein-1

IL: Interleukin

IL-R: IL receptor

INED: Institut national d'études démographiques

## **J**

JAK: Janus kinase

JNK: c-Jun N-terminal kinase

## **K-L**

KIM-1: Kidney injury marker 1

LEB: Life expectancy at birth

LPA: LPS lipid component A

LPS: Lipopolysaccharide

LOX-1: Lectin-like oxidized LDL receptor-1

## **M**

MAC: Membrane attack complex

Mac-1: Macrophage-1 antigen

MAPK: Mitogen-activated protein kinases

mDia1: Mammalian diaphanous 1

MG-H1: Methylglyoxal-derived hydroimidazolone 1

MHC: Major histocompatibility complex

miRNA: microRNA

MKK: MAPK kinase

MMP: Matrix metalloproteinase

MOLD: Methylglyoxal-lysine dimer

MRAD: maximal reported age at death

mRNA: Messenger RNA

mtDNA: Mitochondrial DNA

mTOR: Mechanistic TOR

mTORC: mTOR complex

MyD88: Myeloid differentiation primary response 88

## **N**

NAD<sup>+</sup>: Nicotinamide adenine dinucleotide

NADPH: NAD phosphate

NF-IL6: A nuclear factor for IL-6 expression

NGAL: Neutrophil gelatinase-associated lipocalin

NLRP3: NLR pyrin domain-containing 3

NMR: Nuclear magnetic resonance

NOX1: NADPH oxygenase 1

NuRD: Nucleosome remodelling deacetylase

## **O-P**

OST-48: Oligosaccharyltransferase 48

PAMP: Pathogen-associated molecular pattern

PARK7: Parkinsonism associated deglycase 7

PBS: Phosphate buffered saline

PBS-T: PBS-Tween 20

PD: Parkinson's disease

PGC1 $\alpha$ : PPAR $\gamma$  co-activator 1 $\alpha$

PI3K: Phosphoinositide 3-kinase

PKC: Protein kinase C

PrP: Prion Protein

PRR: Pattern recognition receptor

PTEN: Phosphatase and tensin homolog

## **R**

RAC1: Ras-related C3 botulinum toxin substrate 1

RAGE: Receptor for AGEs

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT-qPCR: Real-time quantitative polymerase chain reaction

## **S**

S6K: S6 kinase

S6RP: S6 ribosomal protein

SAA: Serum amyloid A

SASP: Senescence-associated secretory phenotype

SOD: Superoxide dismutase

SP1: Specificity protein 1

SPR: Surface plasmon resonance

sRAGE: Soluble RAGE

SR: Scavenger receptor

STAT: Signal transducers and activators of transcription

## **T**

TERT-ER: Telomerase reverse transcriptase-oestrogen receptor

TIRAP: Toll-interleukin 1 receptor domain-containing adaptor protein

TLR: Toll-like receptor

TNF- $\alpha$ : Tumour necrosis factor- $\alpha$

TNFR: TNF receptor

TOR: Target of rapamycin

TTR: Transthyretin

## **U**

ULK1: Unc-51 like autophagy activating kinase 1

USA: United States of America

## **W**

WHO: World Health Organization

WT: Wild-type

WT-1: Wilms' tumor 1

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# Foreword

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Ageing is becoming a major societal concern as the population is getting older and the psychological, human, medical and economic burden of age-related diseases and dependency is increasing. Ageing was once thought of as a universal and immutable process which appeared to be beyond our control. However, the constant increase in human lifespan has proved this to be wrong, with recent research in fundamental biology of ageing providing credible insights into the very mechanisms of ageing, while many interventions in the laboratory have substantially altered the course of ageing. Therefore, we are progressively able to promote not simply longevity but also healthy ageing.

The Maillard reaction, or glycation, was discovered in 1912 by the French chemist Louis-Camille Maillard. This reaction, first described during cooking, is characterised by a browning resulting from the reaction of sugars with proteins, notably at high temperature. It was rapidly proposed that this reaction could also occur *in vivo* at 37°C. This was soon confirmed, notably in the case of diabetes where chronic hyperglycaemia strongly favours this reaction and glycation products became a reliable marker of this disease. It was only towards the end of the 20<sup>th</sup> century that glycation products were also shown to accumulate in multiple tissues with age, firstly being described in skin and eyes, then discovered in most tissues. *In vivo*, this reaction is *by nature* detrimental as it fundamentally alters proteins, nucleic acids and lipids, preventing their proper functioning. In addition, the accumulation of glycated products was shown to be involved in a number of pathologies, often associated with increased inflammation, and including the complications of diabetes and various age-related diseases. The question as to whether the consumption of glycated products could lead to similar effects remains unanswered, but the most recent research has demonstrated that their over-consumption may have worrying consequences including the acceleration of some features of ageing. Ultimately, many of the deleterious effects of glycation products, either produced *in vivo* or originating from the diet, have been linked with a receptor for advanced glycation end-products, RAGE.

RAGE was first discovered in the early 1990s and was isolated from bovine lungs thanks to its ability to strongly bind glycation products, hence its name. It was subsequently found that this molecule was a membrane receptor not only expressed in the lungs, but in most cell types, and present in all mammals. Its involvement in diabetes rapidly became clear as its expression was increased in this condition and was further characterised as the putative link between glycation products and the complications of diabetes. But the action of RAGE is not limited to glycation products as many different molecules are able to bind to RAGE and often with an even greater

affinity. Thus, RAGE is increasingly described as the messenger of many various signals, often involved in cellular damage or pathogen-associated molecules. Thereafter, different roles have been attributed to RAGE: its expression and signalling mainly promoting inflammation, but also cell migration or cell-cell interactions, it has come to be considered as causative in a number of pathologies and age-related diseases.

The establishment of these relationships between ageing, pathologies, glycation products and RAGE is the foundation of the work in this thesis. In addition, our research group has demonstrated that a diet enriched in an advanced glycation end-product, carboxymethyllysine (CML), induced an accelerated vascular ageing in mice and we further showed that this dietary CML preferentially accumulated in the kidneys.

Therefore, this thesis aims to investigate whether dietary CML also increases kidney ageing, whether RAGE has a role in this process, and ultimately whether the absence of RAGE has an impact on physiological ageing.

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# Introduction

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## 1 | Ageing

### 1.1 | Definition

#### Box 1 – Ageing

- What is ageing?
- What is the demography of ageing?
- What is the evolutionary origin of ageing?
- What are the mechanisms and outcomes of ageing?
- Can we modulate ageing?

Ageing is characterised by an accumulation of events which lead to a reduction in the efficacy of organ functions and an increased probability of death with time. This intrinsic mortality is opposed to extrinsic mortality which groups deaths from multiple external sources: starvation, predation, diseases, accidents, etc. The study of ageing is not simply the study of old age, however, since damage begins to accumulate very early in life so that ageing might be said to begin as soon as birth. Thus, the study of ageing is the study of a number of inevitable events occurring between birth and death.

Increased probability of death is not necessarily inevitable, however, as in several organisms, including hydras, there is no real evidence of increased probability of death with time<sup>1</sup>. This specificity will be discussed later in this manuscript.

We propose below definitions of terms related to the study of ageing that will be used hereafter:

**Life expectancy** is the remaining number of years of life at a given age. The most common use of life expectancy is the life expectancy at birth (LEB). The estimation of life expectancy of living people is calculated using the death rate of people of each age at a given year. The life expectancy is then defined by the average age at death while assuming the risk of death of people of the same age is constant. Thus, the LEB of a given year is defined by calculating the average age at death considering all ages during this year, while the life expectancy at an age X considers death rates for ages equals or superior to X. It is therefore a projection and since the risk of death is constantly changing in humans, there is a good chance that a cohort's lifespan exceeds its LEB.

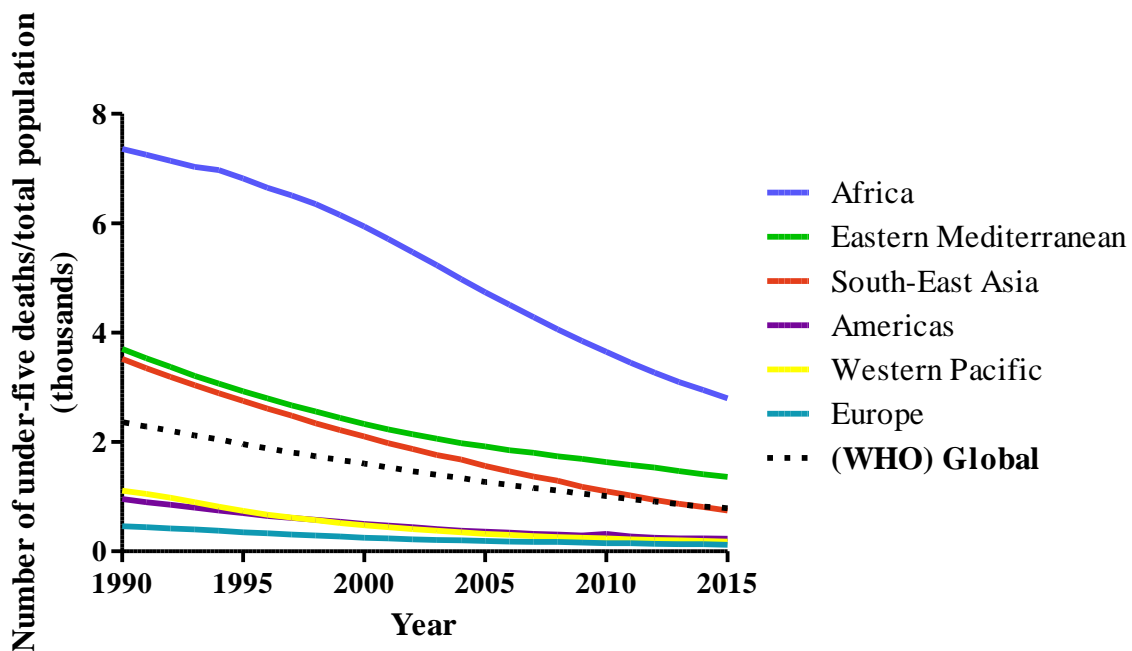
**Lifespan** is a calculated organism's length of life and can be used to determine the average or maximal length of life of a group. While life expectancy is a statistical prediction, lifespan is a calculated duration.

**Longevity** is a polysemous word referring to different and general aspects of life length, often referring to especially long or maximal lifespan.

## 1.2 | Demography of ageing

### 1.2.1 | Human longevity

Human longevity has evolved considerably during the last century and increased worldwide. As an example, LEB was 45 years old in France in 1900 and is now around 82 years old<sup>2</sup>. However, there are important differences between developing and developed countries in this regard. First, a greater percentage of this increase is explained by a diminution of infant mortality rates in developing than in developed countries<sup>3,4</sup>. In Africa (World Health Organization (WHO) region) there were around 7.4 child deaths (under 5 years old) for 1000 inhabitants in 1990, but this quickly decreased to 2.8 by 2015. By comparison in Europe (WHO region), a wealthier region, this ratio was 0.46 in 1990 and 0.12 in 2015 (**Fig. 1**). This evolution is similar when considering the number of births instead of total population<sup>5,6</sup>. As a consequence, as child mortality has been very low in rich regions for decades, its evolution (if it exists) has proportionally significantly less influence on overall LEB compared to that of developing regions.



**Figure 1. Child mortality in the world.** Number of under-five deaths reported to total population (thousands) in regions defined by the WHO (full lines) and globally (dotted line) each year from 1990 to 2015

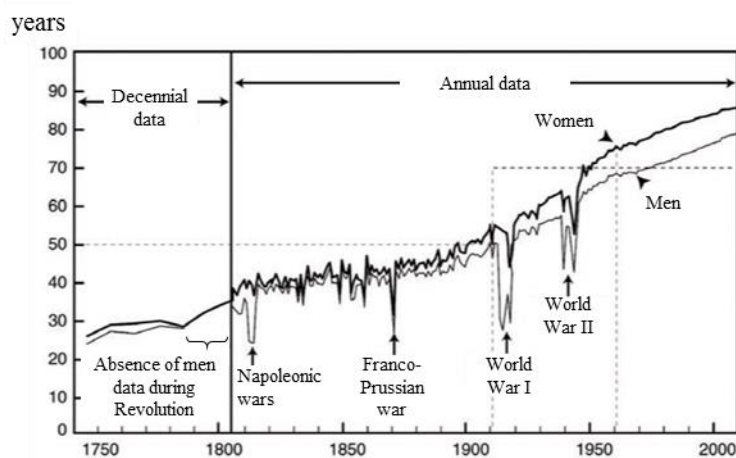
Despite the important and rapid amelioration of child mortality, LEB in developing countries remains lower than in developed countries<sup>7</sup> (**table 1**). In Africa, between 2000 and 2015, there were 3.14 less child deaths per 1000 inhabitants and LEB rose from 50.8 to 61.2 years. In the

same period in Europe, LEB increased from 72.5 to 77.2 years while there were only 0.13 less child death for 1000 inhabitants. This lack of proportionality and the significant increase in longevity in Europe despite no massive change in child mortality, as seen in Africa, suggests that there are other parameters that strongly influence LEB.

**Table 1. Life expectancy at birth in 2000 and 2015 in WHO regions and globally.**

| Year | Africa | Eastern Mediter-ranean | South-East Asia | Americas | Western Pacific | Europe | (WHO) Global |
|------|--------|------------------------|-----------------|----------|-----------------|--------|--------------|
| 2000 | 50.8   | 65.5                   | 63.5            | 73.6     | 72.8            | 72.5   | <b>66.5</b>  |
| 2015 | 61.2   | 68.8                   | 69.2            | 76.6     | 76.7            | 77.2   | <b>71.7</b>  |

Famines, diseases, accidents and conflicts also have a large impact on a country's LEB. It is widely reported that world wars had significant impact on countries' LEB, as in France<sup>2</sup>, and more especially on men's LEB as they were the first victims of such events (**Fig. 2**).



**Figure 2. Evolution of the life expectancy at birth in France since 1740.** Modified after Institut National d'Études Démographiques (INED), accessed 21 May 2019<sup>2</sup>

The Spanish flu, occurring in 1918-1919, at the end of the World War I, had devastating consequences and it is estimated that 50 to 100 million people died worldwide<sup>8</sup>. Thus, it contributed to the second drop in LEB seen during the World War I. As evoked earlier, there is little evolution in infant mortality in France, which is now considered as stable<sup>9</sup>, and there have not been any major conflicts in France's territory for decades. But as shown in **Fig. 2** there is, however, a steady increase in LEB. This increase must therefore be explained by other factors

such as different environmental factors (spreading of pathogens limited by vaccination, better hygiene, ameliorated accessibility to high quality food and water, etc.) and the development of tools and techniques to limit or cure some pathologies, including cancers, cardiovascular diseases or pulmonary diseases, that alleviates the burden of old age and dependency<sup>10-12</sup>. Thus, lower LEBs in developing countries might very well be explained by societal and environmental factors and there are no current data suggesting the existence of genetic variance that would explain such differences.

It should also be stated that while LEB remains lower in developing countries, this does not mean that there are no centenarians (100 years old and above) or super centenarians (110 years old and above) as LEB is not representative of maximal reported age at death (MRAD). According to the Gerontology Research Group (GRG), there are, however, no or very few supercentenarians in developing countries<sup>13</sup>. But it is very important to note that these are *reported* cases and it is very likely that a number of centenarians and supercentenarians have never been reported or that they cannot be validated because of a lack of official documents. Indeed, GRG states that to be verified, a supercentenarian needs to provide documents of early-, mid- and late-life to prove their age.

In 2015, 1739 supercentenarians had been reported and most of them were born in developed countries such as the United States of America (USA, 698), Japan (264), the United Kingdom (159), France (150) or Italy (145) (**Table 2**).

**Table 2. Supercentenarians in the world.**

| <b>Birthplace</b>   | <b>Number of supercentenarians (F/M)</b> | <b>Oldest supercentenarians (years and days)</b>                              |
|---------------------|------------------------------------------|-------------------------------------------------------------------------------|
| Australia           | 21 (19/2)                                | F: Cock C. (114 y. and 148 d.)<br>M: Lockett J. (111 y. and 123 d.)           |
| Austria-Hungary     | 20 (20/0)                                | F: Marinic K. (110 y. and 307 d.)<br>M: Vietoris L. (110 y. and 309 d.)       |
| Belgium             | 19 (16/0)                                | F: Ledent A. (111 y. and 192 d.)<br>M: Goossenaerts J. (111 y. and 143 d.)    |
| Brazil              | 1 (1/0)                                  | F: Gomes Valentim. M. (114 y. and 347 d.)                                     |
| British India       | 2 (2/0)                                  | F: Farrell E. (112 y. and 35 d.)                                              |
| British West Indies | 2 (1/1)                                  | F: Brown V. (114 y. and 297 d.)<br>M: Sisnett J. (113 y. and 90 d.)           |
| Canada              | 47 (45/2)                                | F: Meilleur M.-L. (117 y. and 230 d.)<br>M: Saint-Amour J. (110 y. and 18 d.) |
| Cape Verde          | 1 (1/0)                                  | F: Domingues A. (114 y. and 183 d.)                                           |
| Channel Islands     | 1 (1/0)                                  | F: Wood E. (110 y. and 215 d.)                                                |

|                |                     |                                                                                                  |
|----------------|---------------------|--------------------------------------------------------------------------------------------------|
| Colombia       | 1 (0/1)             | M: Guzman-Garcia D. (111 y. and 105 d.)                                                          |
| Denmark        | 4 (3/1)             | F: Svensson J. (111 y. and 125 d.)<br>M: Mortensen M. (115 y. and 252 d.)                        |
| Ecuador        | 1 (1/0)             | F: de Capovilla M. E. (116 y. and 347 d.)                                                        |
| France         | 150 (142/8)         | <b>F: Calment J. (122 y. and 164 d.)</b><br>M: Flocquet M. (111 y. and 320 d.)                   |
| Germany        | 60 (54/6)           | F: Holtz A. (115 y. and 79 d.)<br>M: Dörnemann H. (111 y. and 279 d.)                            |
| Greece         | 2 (1/1)             | F: Lambropoulos I. (110 y. and 25 d.)<br>M: Pandazes G. (110 y. and 341 d.)                      |
| Italy          | 145 (129/16)        | F: Manfredini D. (115 y. and 257 d.)<br>M: Frau G. (112 y. and 172 d.)                           |
| Japan          | 264 (234/30)        | F: Okawa M. (116 y. and 302 d.)<br><b>M: Kimura J. (116 y. and 54 d.)</b>                        |
| Mexico         | 2 (2/0)             | F: Mexia S. (114 y. and 17 d.)                                                                   |
| Netherlands    | 26 (23/3)           | F: van Andel-Schipper H. (115 y. 62 d.)<br>M: Bos J. P. (111 y. and 156 d.)                      |
| New Zealand    | 1 (1/0)             | F: Booth E. (110 y. and 55 d.)                                                                   |
| Newfoundland   | 1 (0/1)             | M: McCouberg J. (111 y. and 40 d.)                                                               |
| Norway         | 16 (13/3)           | F: Bolette Torp M. (112 y. and 61 d.)<br>M: Smith-Johannsen H. (111 y. and 204 d.)               |
| Peru           | 1 (1/0)             | F: Dougherty J. (110 y. and 106 d.)                                                              |
| Portugal       | 9 (7/2)             | F: de Jesus M. (115 y. and 114 d.)<br>M: Moreira de Oliveira A. (112 y. and 130 d.)              |
| Puerto Rico    | 4 (3/1)             | F: Trinidad Iglesias-Jordan R. (114 y. and 272 d.)<br>M: Mercado del Toro E. (115 y. and 156 d.) |
| Russia         | 15 ( <b>12/3</b> )  | F: Steinberg G. (114 y. and 63 d.)<br>M: Arvonon A. (111 y. and 150 d.)                          |
| Saint Kitts    | 1 (1/0)             | F: Hill R. (110 y. and 294 d.)                                                                   |
| South Africa   | 1 (1/0)             | F: Booysen J. (111 y. and 151 d.)                                                                |
| Spain          | 46 (37/9)           | F: Castro M. A. (114 y. and 220 d.)<br>M: Riudavets-Moll J. (114 y. and 81 d.)                   |
| Sweden         | 16 (15/1)           | F: Zachrisson A. (113 y. and 0 d.)<br>M: Engberg A. (111 y. and 128 d.)                          |
| Switzerland    | 1 (1/0)             | F: Duvoisin E. (111 y. and 87 d.)                                                                |
| United Kingdom | 159 (151/8)         | F: Jennings A. (115 y. and 8 d.)<br>M: Allingham H. (113 y. and 42 d.)                           |
| USA            | <b>698</b> (641/57) | F: Knauss S. (119 y. and 97 d.)<br>M: Beard M. (114 y. and 222 d.)                               |

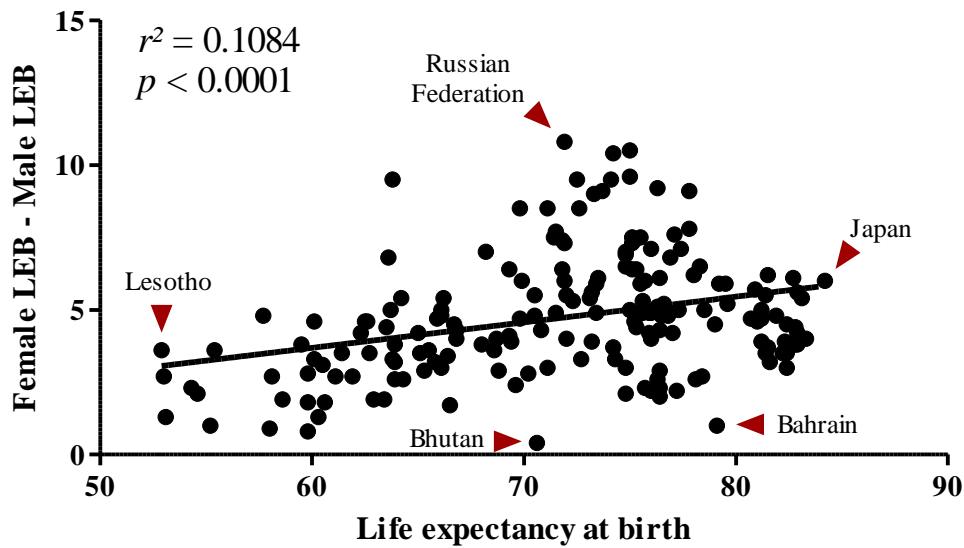
*F: female; M: Male; Bold: highest number of supercentenarians; Highest F/M ratio; Longest lived F and M. Data gathered from the 2015 GRG database on validated supercentenarians*

Thus, only five countries account for more than 80% of all known supercentenarians that are living or have so far been recorded. They are all developed countries with similar gross domestic product per capita, or purchasing power parity, according the International Monetary Fund<sup>14</sup>, suggesting similar accessibility to health systems. Differences between those countries



might first be explained by higher or lower population size and by different exposure to major conflicts. The USA, as an example, has the largest population and is the country which suffered the least number of casualties during World War II. It should also be noted that **Table 2** only takes into account the place of birth: people might have moved during their life and some countries emerged or changed during the previous century, hence the absence of many developed countries such as those emerging from the break-up of the Soviet Union. Differences with developing countries can therefore be explained by harsher living conditions and conflicts, but also merely because of a lack of enough documents to verify claims. Thus, it is likely that the number of supercentenarians in such countries will increase in the future, not only thanks to better living conditions but also to improved availability of documentation. In this regard, GRG states that less than one-third of supercentenarian claims above 110 years old are validated and only 2% of claims for 115 years old and above are validated.

There is also an important difference between women and men for both LEB and MRAD worldwide. First, there are only 160 men for 1579 women reported to be 110 years old or higher (**Table 2**). Thus, while the current longevity record is held by Jeanne Calment, a French woman who lived until the age of 122 years and 164 days, Jiroemon Kimura, a Japanese man, “only” lived until the age of 116 years and 54 days and holds the record for males but is ranked 11<sup>th</sup> among all the oldest reported persons. Worldwide, male LEB is also consistently lower than female LEB. Data provided by the WHO<sup>15</sup> indicate that the highest LEB in 2016 was 84.2 years, in Japan, but women had a LEB of 87.1 years while men had a LEB of 81.1 years. The same year, the lowest LEB was found in Lesotho with an average of 52.9 years (54.6 years for women and 51 years for men). Countries that have the highest Women LEB – Men LEB difference are the Russian Federation (10.8 years), Lithuania (10.5 years) and Belarus (10.4 years). On the other hand, Bhutan (0.4 years), Guinea (0.8 years) and Mali (0.9 years) are the countries with the lowest difference. Globally, there is a correlation between LEB and this difference ( $r^2 = 0.1084$ ,  $p < 0.0001$ ) but some countries like Bahrain have a high LEB (79.1 years) but a small difference between women and men (1 year) (**Fig. 3**).



**Figure 3. Differences of life expectancy at birth (LEB) between countries and males and females.** Data of LEB were gathered from the WHO database for each country and male LEB was subtracted to female LEB to provide a linear regression. Arrowheads indicate countries of interest that have the lowest and highest LEB and the lowest and highest difference regarding male and female LEB

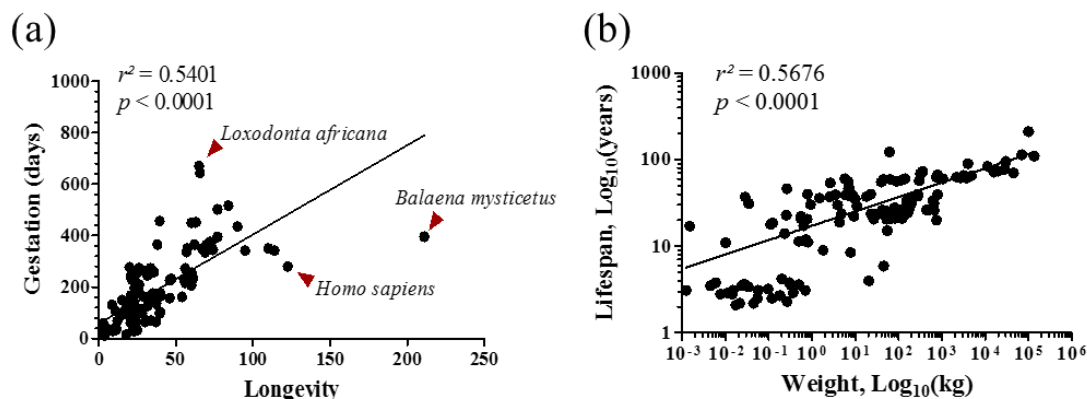
These observations are in line with data in other mammals regularly showing longer lifespan in females than in males<sup>16,17</sup>. However, even though a part of this difference might be explained by biological differences, variations between countries suggest that other parameters need to be taken into consideration to fully explain such a difference in humans. First of all, in countries with lower LEB, such biological differences might be too weak in comparison to other parameters to induce major significant differences between men and women. Events of great famine, diseases and conflicts significantly outclass biological differences between men and women and affect them similarly. This potentially explains why in countries where these events are more frequent, leading to a lower LEB, differences in LEB between men and women are lower. Similar patterns are seen when looking at a country's history of LEB. In France, the LEBs of women and men were very similar during the 19<sup>th</sup> century but the gap increased at the end of the 19<sup>th</sup> century. It has steadily increased from around 2.5 years in 1900 to 5.6 years currently. The increase of this gap in France was notably associated with a reduction of a number of diseases and conflicts<sup>18,19</sup>. On the other hand, countries having similar LEB, like Bhutan (70.6 years) and the Russian Federation (71.9 years), can also have very dissimilar LEB differences (0.4 and 10.8 years, respectively), suggesting that living conditions might have a significant influence on this gap<sup>20</sup>.

A recent paper suggested that there was evidence for a limit to human lifespan<sup>21</sup>. This study suggests that in France (and in other countries), the highest rate of change in survival since 1900 was observed in populations aged between 80 and 100 years old. After a peak at 100 years old, this rate plummets. Thereafter the authors suggest that the yearly MRAD has increased until the late 1990s and has now reached a plateau. They finally show that the annual average age at death for supercentenarians has not increased since 1968 and conclude that human lifespan is limited. However, this study has received much criticism and has been intensely debated through a number of brief communications arising<sup>22–26</sup>. The first critiques were based on the use of the Human Mortality Databases (HMD) in which data for 90-year-old people and above are smoothed, and sometimes assumed, because of the scarcity of the data. The rate of change is also greatly affected by data transformations that are needed for the use of logarithm and the peak of the rate of change can vary a lot following this transformation and the period considered<sup>22</sup>. The analysis of the yearly MRAD received most of the criticism. The authors chose to define two periods, 1972–1994 and 1995–2015 to provide two linear regressions. Such analysis can favour many biases and one outlier point (such as the MRAD of Jeanne Calment) may significantly influence a regression, especially in such a small sample size. Other authors have provided a number of models to depict how such data manipulation can have serious consequences on the interpretation of this dataset<sup>23,24,26</sup>. Finally, the data used for the analysis of the annual average age at death in supercentenarians has also received criticism, mostly because of the lack of data before 1980 and because of the asynchronous addition of countries to this dataset<sup>23</sup>. Therefore, these communications suggest that the MRAD could still be increasing. What this debate shows is that we are currently unable to say definitively whether there is an intrinsic biological limit to human lifespan; if it does exist, we are still unable to define it, mostly because of the scarcity of the available data.

### 1.2.2 | Longevity in other species

Longevity in other species has been described and varies immensely, ranging from a few hours to thousands of years. The study of lifespan in many species has shown that there are links between fertility, the size of progeny and longevity. It has also been shown that when lifespan increases, fertility decreases and *vice-versa*<sup>27–29</sup>. Strikingly, data from 133 mammal species, gathered from the animal ageing and longevity database (AnAge)<sup>30,31</sup>, show that there is a clear correlation in mammals between gestation time and their longevity ( $r^2 = 0.5401$ ,  $p < 0.0001$ ) (**Fig. 4a**). *Balaena mysticetus*, the longest-lived mammal with an estimated age of 211 years ( $\pm 35$  years) for the oldest specimen<sup>32,33</sup>, is a clear outlier in this regard and its extreme longevity

might be explained by factors that are specific to this species. Similarly, the longevity of *Homo sapiens*, reported to be the second longest-lived mammal, is also higher than anticipated by this correlation and can again be mostly explained by the specific living conditions that allow greater longevity than in other species. While we can therefore predict the longevity of a species in general terms, it is probably not possible to determine the longevity of an individual as intra- and inter-species variations in longevity or lifespan are probably governed by different mechanisms. In addition, while correlation of longevity and average adult weight of a species is very strong in the heaviest mammals, there is a large variation in smaller ones (**Fig. 4b**). Thus, longevity is not simply bound to the complexity of an organism but is also linked to its reproductive capabilities.



**Figure 4. Correlation of gestation time and adult weight with longevity in mammals.** Correlation of gestation time (days) and longevity (years) of mammals according to the AnAge database, including the longest- and shortest-living mammals. Linear regression (a). Correlation of adult weight ( $\text{log}_{10}$ , kg) and longevity ( $\text{log}_{10}$ , years) of mammals. Spearman correlation (b)

This observation is important to the Disposable Soma theory of ageing proposed by T. Kirkwood, which suggests that there is a trade-off between reproduction and longevity governed by resource allocation<sup>34,35</sup>. In a context where most deaths in nature result from predation, starvation and disease, high investment in somatic maintenance is inefficient as extrinsic mortality prevails, but it must remain sufficient to avoid premature death, *i.e.* early intrinsic mortality caused by random damage (“error catastrophe” theory) and ensure reproduction. Thus, the theory suggests that, from an evolutionary perspective, the soma is merely a carrier of the germline and is primarily designed to support germline transmission, hence the “disposable soma”. Therefore, species with high reproductive capacities are short-lived while the maintenance of the soma has been improved in species that produce fewer progeny. Thereafter, a model has been proposed to predict the optimum investment in somatic maintenance with regard to fecundity<sup>36</sup>.

There are however some discrepancies between this theory and observations made in several species. Eusocial animals, for example, seemingly directly contradict this view. Eusociality is defined by a division and a specialisation of roles between members (forming separate groups including reproductive and non-reproductive castes), overlapping generations and cooperative care of juveniles. A typical example of eusociality is ants that have sterile workers, fertile males and a queen. In a given species, lifespan is not fixed at birth and it has been shown to vary following sexual activity. In fruit flies, for example, sexual activity has been associated with reduced male lifespan<sup>37</sup>. On the other hand, in eusocial hymenopterans queens live much longer than sterile workers and queens mated with fertile males, but not with sterilized males, live much longer than virgin queens<sup>38,39</sup>. Similar observations have been made in the less-common eusocial mammals, including various kinds of mole-rats where breeding is associated with longer lifespan<sup>40-42</sup>. Instead of a trade-off between reproduction and longevity proposed by the disposable soma theory, the opposite is observed: reduced lifespan and reproductive capacity for workers which is the opposite for the queens. In fact, mole-rat workers are most exposed to extrinsic mortality and, therefore, investment in somatic maintenance would be quickly wasted. On the other hand, as there are very few queens dedicated to reproduction, early intrinsic death must be avoided at all cost, hence a higher investment in somatic maintenance in this very specific case. It has been proposed that such colonies should rather be considered as a “superorganism” where workers are the disposable soma while the queens are the germline. As such, the trade-off is found when considering the quality (*i.e.* the capacity of an organism to withstand destruction) and quantity of offspring<sup>39</sup>.

Another critique of the disposable soma theory is the existence of post-reproductive lifespan. If longevity is designed merely to answer reproductive needs, why are some species, such as some primates (including humans) and cetaceans<sup>43-45</sup>, able to live sometimes far beyond their reproductive lifespan? Different answers can be given to this question. The first and simplest answer is that lifespan is not so precisely defined by evolutionary processes and it might therefore be an artefact of lifespan increase. However, subtler hypotheses have also been proposed, namely the grandmother and mother hypotheses. The grandmother hypothesis stipulates that although older females are no longer able to reproduce, they still have an influence on the selection of their own genes. Indeed, the care they provide to their progeny and the progeny of their children increases the chance of survival and reproduction of these progenies and, as a consequence, increases the transmission of genes that may originate with the grandmother<sup>46,47</sup>. Therefore, the higher the maintenance of the soma during post-

reproductive lifespan, the higher the chance of ensuring the transmission of the grandmother's genes, without competing with the mothers<sup>43</sup>. On the other hand, the mother hypothesis suggests that menopause is an evolutionary tool that decreases mortality in children and the risk of death during childbirth<sup>47,48</sup>. Nonetheless, these hypotheses have limitations and the effects of grandmothing may not be as important as suggested<sup>49-51</sup>. Other data suggest that, for their part, men do not gain extra fitness through grandfathering<sup>52</sup>.

Despite these ostensibly contradictory observations, it should be noted that the trend evoked earlier remains valid. Therefore, rather than simply stating that these examples invalidate the disposable soma theory, we should rather consider that they add complexity to the theory. In this way we can see that while this trade-off exists in most social organisations, some very specific social organisations both allow and require lifespan to increase with sexual activity.

Although most data presented above relate to mammals, longevity has been studied in a number of other species. Thus, the AnAge database describes organisms with extreme longevity, living beyond 1 000 years: *Cinachrya antartica* (1 550 years, porifera)<sup>53</sup>, *Pinus longaeva* (5 000 years, pinophyta)<sup>54,55</sup> and *Scolymastra joubini* (15 000 years, porifera)<sup>53</sup>. OldList, a database dedicated to old trees, lists dozens of referenced tree species living between 1 000 and 5 000 years<sup>56</sup>. Elsewhere, *Buellia frigida*, a lichen, has been estimated to live up to 6 517 years old<sup>57</sup>, the largest known clone of *Larrea tridentate*, also known as the Creosote bush, has an estimated age of 11 700 years<sup>58</sup>, while there is much speculation about "Pando" the largest clonal colony of *Populus tremuloides*, and possibly the largest living being, which could be at least 10 000 years old<sup>59</sup>. Finally, although data potentially refer to a colony rather than an individual, 400 000 to 600 000 year-old Siberian actinobacteria have shown metabolic activity and DNA repair at subzero temperatures and are suspected of being able to survive over half a million years without being dormant<sup>60</sup>.

It is widely reported that plants have, on average, much longer lifespans than most animal species. The question of ageing is perhaps even more complex than in animals. Although we share a number of essential cellular mechanisms with plants, their modular development, life history and breeding are significantly different. With time, plant growth tends to decrease and there is accumulation of mutations in meristems, which resembles ageing. However, as shown by grafting studies<sup>61-63</sup>, it has been argued that reduced growth is rather the result of a restraint, owing to higher demands on nutrients and water with increasing height and girth, enhancing competition between modular structures<sup>64</sup>. Regarding somatic mutations in meristems, it has

been shown that not only deleterious mutations are efficiently removed by intraorganismal selection, but also that beneficial mutations can be selected to provide adaptation to variable environments<sup>65</sup>. These kinds of observations have even raised the question of whether whole-plant ageing exists at all<sup>66,67</sup>.

Similar to this question, negligible senescence (*i.e.* absence of evidence of senescence) has also been suspected in several organisms described in the AnAge database. Among them we find the above-mentioned *Pinus longaeva*, *Arctica islandica* (ocean quahog clam), *Strongylocentrotus franciscanus* (red sea urchin), *Sebastes aleutianus* (roughey rockfish), *Terrapene carolina* (eastern box turtle), *Emydoidea blandingii* (blanding's turtle) and *Proteus anguinus* (olm)<sup>68</sup>. Extrinsic death aside, this would make them therefore potentially immortal as the probability of dying does not increase with time. However, we should note that the absence of evidence is not necessarily evidence of absence and that there could simply be a lack of data on measurable markers of biological ageing in these species. Despite this, one study on hydra ageing has often been cited as a case of immortality in animals. This work shows that contrary to other organisms, reproductive and death rates do not change with time, suggesting a lack of senescence, possibly permitted by a complete and constant renewal of tissues, specific to this species<sup>1</sup>. However, the study by Martinez has been criticised because it compared budding rates of a control group to budded animals from the same group and concluded that even though there were fluctuations in budding rates, the similarity between each group precluded ageing as a cause. But it has been argued that asexual reproduction does not produce younger animals but merely clones of the same age. The control group should therefore be compared with a group of animals produced by sexual reproduction to determine whether there is a decline in budding rate with age<sup>69</sup>.

So far, we have seen the extent to which lifespan and longevity varies in the current living world and have examined clues as to *why*, from the evolutionary perspective, ageing exists, or rather why lifespan is flexible and modulated across species. Some of these clues indicate that ageing, or at least the mechanisms of ageing, while perhaps not universal, are at least shared across the animal kingdom. But we have still not seen *how* ageing is modulated. What follows is a description of the currently-known and suspected mechanisms of ageing.

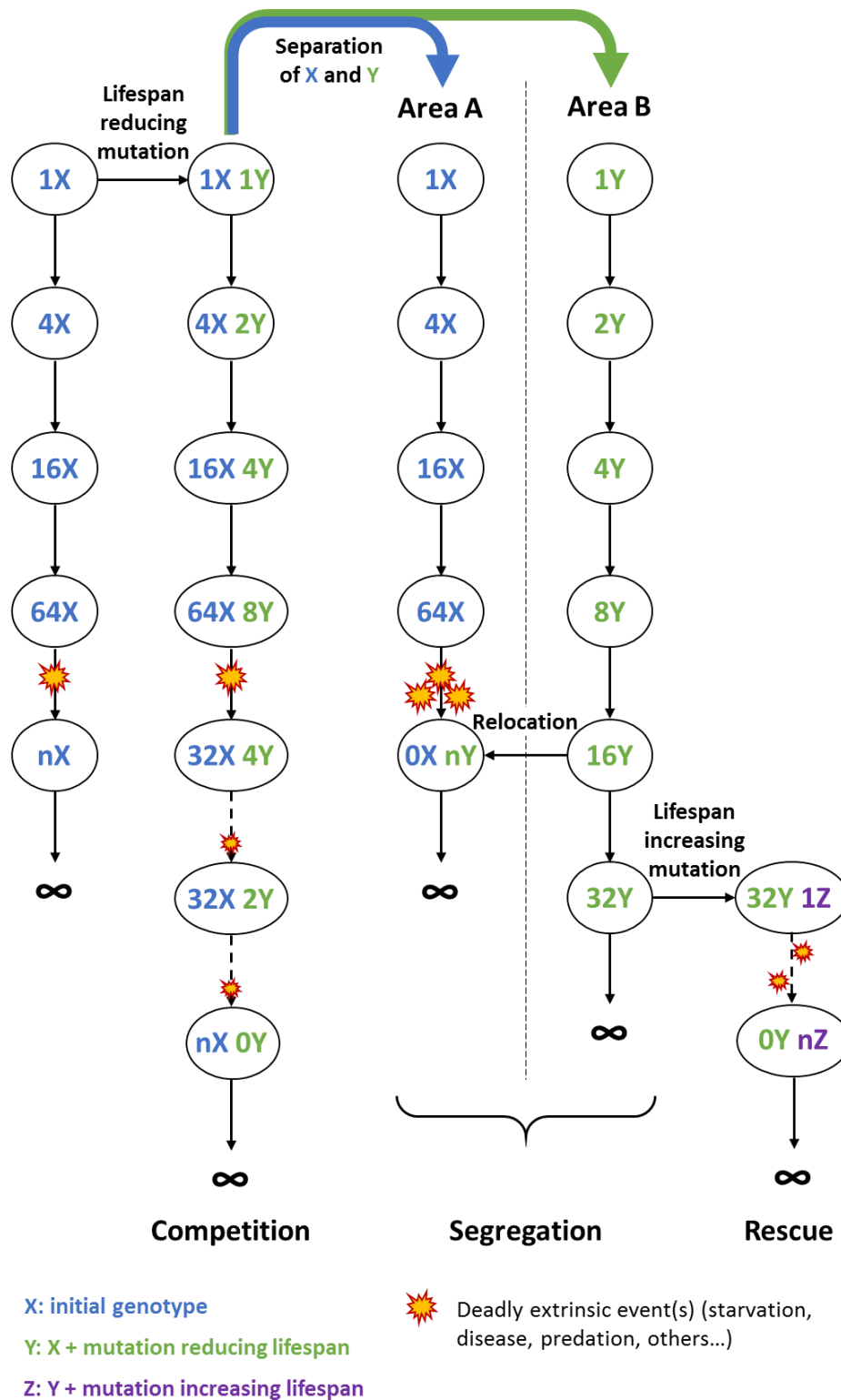
### 1.3 | Mechanisms of ageing

#### 1.3.1 | The evolution of ageing

Current research has not shown genes dedicated to ageing. As discussed below, every current deletion of genes that have an impact on lifespan always involve drawbacks. Hence, there is no known program of ageing, *i.e.* a genetic program designed to limit a species' lifespan. The tremendous difference in longevity between species suggests, however, that there are some “programs of longevity”, *i.e.* genes that have evolved to meet requirements that ensure the sustainability of a species. Complex organisms that require long gestation or egg incubation times need correspondingly longer lifespans that correspond to their reproduction capabilities. On the other hand, species that have higher reproduction capacities have a shorter lifespan – but it does not mean that they have evolved mechanisms that restrict their lifespan. Rather, in accordance with the law of parsimony, it is more sensible to think that mechanisms that extended lifespan could not have been conserved because they would have caused local overpopulation and thereby limited nutrient accessibility. Thus, it would not be viable to select modifications that increase longevity in highly proliferative organisms, and shorter lifespan in such organisms is the result of an *absence* of selection for modifications that increase it since there is no evolutionary benefit and hence no selection pressure. This hypothesis is simpler and requires less assumptions than a hypothesis suggesting selection of mechanisms that limit lifespan.

The following model gives insights into how deleterious mutations such as the reduction of lifespan can or cannot be selected through evolution (**Fig. 5**).





**Figure 5. Basic model of survival of deleterious traits in evolution.** Model representing the selection of traits, X, Y and Z, in different situations. From left to right: initial situation with X only; competition between X and Y where Y is shorter-lived than X but otherwise identical; segregation of X and Y and into two different areas; rescue, where Z is longer-lived than Y but otherwise identical. X, Y and Z have their own growing rate, owing to their lifespan, represented by the increasing number from top to bottom. n is an integer. This number can be affected by extrinsic events, represented by the star-like symbol

The selection of a deleterious trait through evolution is deemed very unlikely. As such, a mutation which merely confers a shorter lifespan has little or no chance of being selected as a primary trait during evolution. One could think that it would confer the ability to better survive in a situation of nutrient limitation, but this ignores the context of permanent competition during evolution. Figure 5 attempts to describe this situation. In the initial situation, members of a group (X), reproduce and die at a constant rate. They can thrive indefinitely but extrinsic events, such as nutrient limitation, can limit or reduce the size of this group.

In the case of the apparition of a mutation leading to a reduced lifespan, a second group (Y) will appear in the same area (heterozygous organisms are not detailed here but conclusions remain similar) (**Fig. 5, Competition**). By definition, if the lifespan of individuals of the Y group is shorter they will reproduce less during their lifetime, hence a smaller population size of this group at each generation. If there are no major extrinsic events, there will be a coexistence of X and Y but X will rapidly become massively dominant and will prevail even in the absence of selection pressure. In the case of extrinsic events affecting the size of these groups, X and Y being similar they would be equally affected and there would be a proportional reduction of each group's size. Given the speed of growth of X, with successive deadly extrinsic events, it will become increasingly dominant while Y will eventually fade out.

Only one very specific situation can support the selection of lifespan reduction as a primary trait: when X and Y are physically separated and not competing with each other (**Fig. 5, Segregation**). Even though this event is rarely seen, it will allow each group to thrive independently. However, there is still the risk that X eventually invades the area where Y is located. Therefore, the only situation that could allow Y selection is the complete absence of competition between X and Y and possibly the extinction of X because of extrinsic events to ensure the selection of Y. However, this situation is completely reversed if a mutation *increasing* lifespan occurs in Y, either correcting the initial mutation or enhancing other survival mechanisms, which will repeat the competition process (**Fig. 5, Rescue**). As a consequence, although this model remains simplistic with regards to the complexity of evolution, it is almost impossible to select a trait that *primarily* induces a reduction in lifespan and it is thus very unlikely that there are genes or mechanisms dedicated to ageing and spread throughout the animal kingdom.

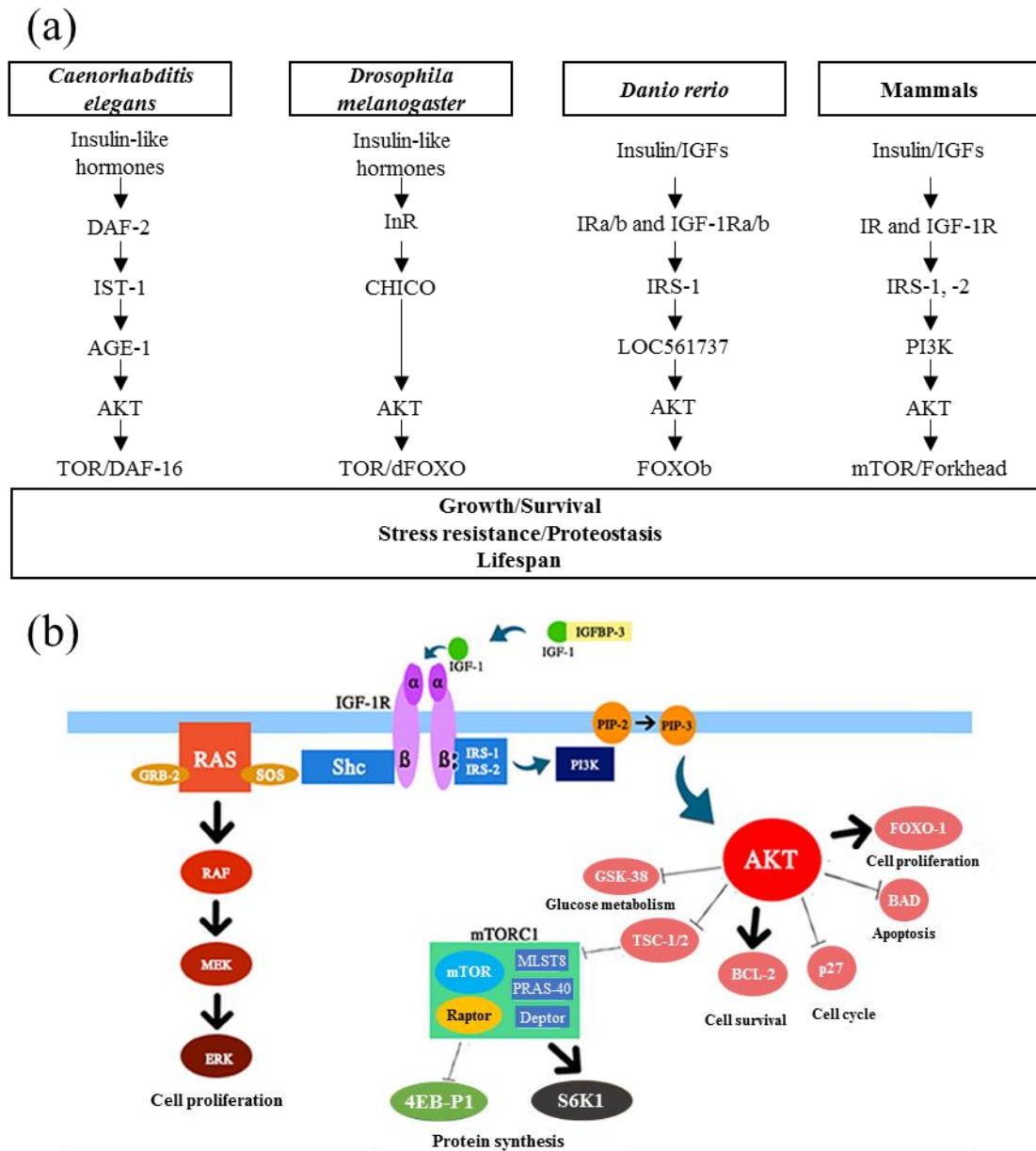
However, the emphasis on *primarily* is essential in this regard, as there exist means to select a reduced lifespan as a *secondary* trait. A shorter lifespan can arise if a trait is beneficial but

involves a diminution of lifespan. The trait is selected if it outweighs the burden of a shorter lifespan. For example, in the above-mentioned situation, if the Y mutation also confers a substantial resistance to starvation, then it would give it the potential to become dominant and be selected through evolution. This duality directly refers to the antagonistic pleiotropy hypothesis.

### 1.3.2 | The antagonistic pleiotropy hypothesis

The antagonistic pleiotropy hypothesis is one of the main hypotheses in the biology of ageing purporting to explain the existence of lifespan-limiting genes. This hypothesis notably suggests that evolution selects some genes of which expression is very beneficial early in life at the cost of a more negative impact in later life. Their selection during evolution would be allowable owing to their beneficial effects on survival and reproduction being expressed during a crucial period of natural selection<sup>70</sup>. Thus, several genes have been identified as important during development but which become deleterious in adults, often possessing pro-ageing properties in later life.

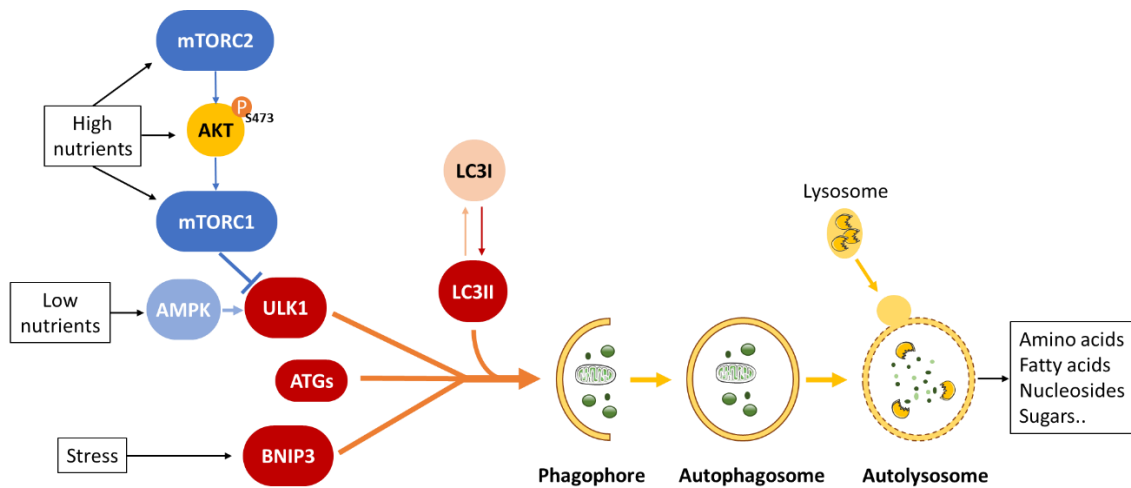
The insulin/insulin-like growth factor 1 (IGF-1) signalling (IIS) is involved in cell survival and proliferation and in protein synthesis, is highly conserved through evolution, and is indubitably the best representative of obvious antagonistic pleiotropy (**Fig. 6a**). Knock-out or knock-down of members of this pathway always leads to a consequent alteration of longevity and is always associated with developmental consequences. In *Caenorhabditis elegans*, knock-out of *daf-2*, the ortholog of the human IGF-1 receptor (IGFR-1R), doubles or triples median and maximal lifespan<sup>71,72</sup> but with a concurrent ~20% reduction in fertility<sup>73</sup>. Although reduction in fertility is quite low, *daf-2* mutants are nonetheless quickly outcompeted by control worms, suggesting an overall reduced fitness<sup>73</sup>. Although lifespan increase is smaller, identical results are found in hypomorphic mutations of *age-1*<sup>74,75</sup>. Similar results, but to different extents, have been reported in *S. cerevisiae*, *D. melanogaster* and *M. musculus*<sup>76-79</sup>. Interestingly, the growth hormone (GH), produced by the pituitary in mice, controls IGF-1 secretion. Alteration of the pituitary development, such as in the Ames and Snell dwarf mice, significantly increases lifespan while growth and fertility are greatly reduced<sup>80-82</sup>. In humans, people with homozygous mutations or deletion on the GH receptor gene exhibit the Laron syndrome. They present multiple deficiencies in development leading to dwarfism but are notably well-protected against pathologies such as cancer and diabetes<sup>83,84</sup>. However, no increase in life expectancy has so far been reported in these people.



**Figure 6. Insulin/Insulin-like growth factor-1 (IGF-1) signalling (IIS) pathway.** Evolutionary conservation of the insulin and insulin-like pathway in *C. elegans*, *D. melanogaster*, *M. musculus* (a). Schematic representation of the IGF-1 pathway (b). Modified after O'Neill et al., 2012<sup>85</sup>, Khan et al., 2019<sup>86</sup> and Vitale et al., 2019<sup>87</sup>

The target of rapamycin (TOR) is also currently one of the most-studied and promising representatives of antagonistic pleiotropy associated with ageing. TOR is a member of a protein complex, very well conserved across evolution, and two complexes have been described in mammals: mechanistic TOR complex 1 (mTORC1) and mTORC2. They are mainly involved in metabolism (such as nutrient sensing, protein synthesis or autophagy<sup>88,89</sup> (Fig. 7)) and are linked to the above mentioned IGF-1 pathway<sup>87,90</sup> (Fig. 6b). However, mTORCs can be activated by a number of signals and should therefore be considered partly independently of the

IGF-1 pathway<sup>91</sup>. The pro-ageing consequences of the mTOR pathway have been widely described, however it also has a pivotal role in development and in simple physiological tasks<sup>92,93</sup>. As such complete inactivation of TOR or the S6 kinase (S6K), one of its substrates, results in embryonic lethality at least in fruit flies and in mice<sup>94,95</sup>. As presented in a following section (1.5.3 | Pharmacologic interventions), tight regulation might, however, be beneficial as long as vital functions are maintained.



**Figure 7. Interplay between nutrients, mTOR pathway and autophagy.** The mTOR pathway is activated in the presence of nutrients and growth factors, which inhibits the initiation of autophagy through *unc-51* like autophagy activating kinase 1 (ULK1). Low nutrients activate ULK1 via AMPK while *BCL2* interacting protein 3 (BNIP3) is activated by stresses such as hypoxia. ULK1 and BNIP3 are subsequently associated with a range of autophagy related (ATG) proteins to initiate the formation of a phagophore, with the help of the active form of microtubule-associated proteins 1A/1B light chain 3B (LC3B), LC3II. The phagophore encapsulates debris or organelles in an autophagosome, which is then fused with lysosomes, forming an autolysosome, to degrade its content and provide nutrients

Together they form the most-reported pathways of antagonistic pleiotropy associated with ageing. Along the same lines, in *C. elegans*, at least 24 other genes involved in development similarly affect lifespan and fertility<sup>96</sup>. Many other gene mutations which positively affect longevity have been reported, however there is a paucity of data on potential trade-offs on fertility or other aspects of fitness<sup>73,97</sup>. In addition, laboratory and field conditions vary greatly and some trade-offs are only visible in the wild<sup>73</sup>.

Some authors suggest that the adenosine monophosphate-activated protein kinase (AMPK) is another pathway associated with longevity. However, most of its effects are linked to IIS or the mTOR pathway. They also suggest that there is enough evidence to consider limiting the  $\beta$ -

adrenergic system to extend both lifespan and healthspan, but results are not as obvious as the above-mentioned pathways<sup>86,98</sup>.

In these situations, increased longevity is always associated with reduced or delayed reproduction, indicating reduced fitness. Therefore, if we associate increased or an earlier onset of fertility to mutations which decrease longevity in our model (**Fig. 5**), Y is very likely to become dominant and be selected. This association of longevity and fertility is highly consistent and is probably no coincidence. Taking into consideration what has been described so far, it is very likely that processes which carefully and simultaneously balance sustainability and reproduction, or other parameters, have been selected through evolution.

### 1.3.3 | The hallmarks of ageing

While antagonistic pleiotropy explains how altering the expression of some genes modulates longevity, and the associated disposable soma theory offers explanations as to why longevity varies across species, we have yet to discuss *how* ageing occurs. When the disposable soma theory was first formulated, it presupposed that the maintenance of the soma was in conflict with what was then called the “error catastrophe” theory. This theory suggests that ageing is at least partly caused by cumulative inaccuracy in protein synthesis due to an accumulation of mutations<sup>35,99–101</sup>. Although this theory might be true, current knowledge of ageing has extended the number of mechanisms thought to be fundamental for ageing and they form a broadly-accepted framework of understanding known as “the hallmarks of ageing”<sup>102</sup>.

Nine hallmarks were described in a review describing this framework<sup>102</sup>: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication. What follows is a brief and updated description of these hallmarks.

#### **Genomic instability**

Genomic instability is defined by instability of nuclear DNA, mitochondrial DNA (mtDNA) and the nuclear architecture. In line with the above-mentioned error catastrophe hypothesis which proposes that accumulating mutations are the primary cause of ageing, many kinds of somatic mutations do increase with ageing in most tissues from humans and other species<sup>103</sup>. Accordingly, impairment of DNA repair mechanisms involves major ageing-related defects and limited lifespan<sup>104,105</sup>. Aneuploidy, which can have even more serious consequences, has also been reported to increase with ageing but is reduced in mice overexpressing a mitotic regulator, Bub1-related kinase (BubR1), while healthspan is also ameliorated<sup>106,107</sup>. mtDNA is considered

as more susceptible to mutations as there are no histones and DNA repair mechanisms are less efficient<sup>108</sup>. Moreover, despite the large number of mtDNA copies, there is a trend toward homoplasmy with accumulating mutations<sup>109</sup>. Deletion of the mitochondrial DNA polymerase  $\gamma$  in mice is associated with accumulating mutations and subsequently impaired mitochondrial function, premature ageing and reduced lifespan<sup>110–114</sup>. Finally, the nuclear architecture has a key role in chromosomal integrity: deleterious mutations in the gene coding for the nuclear lamina are major sources of accelerated ageing<sup>115,116</sup> and aberrant isoforms increase with age<sup>117,118</sup>.

### **Telomere attrition**

Telomere attrition is characterised by a shortening of telomeres after each DNA replication owing to the inability of DNA polymerase to replicate the lagging strand<sup>119–121</sup>. This telomere attrition could be further worsened by the formation of G overhangs at leading-end telomeres through 5' end resection<sup>122,123</sup>. This mechanism is thought to be one of the major causes of replicative senescence and forms the basis of the Hayflick's limit<sup>124</sup>. With reducing telomere length the shelterin complex that initially protects the telomeres cannot no longer bind and telomeres are eventually recognized as double-strand breaks<sup>125,126</sup>. A subsequent DNA-damage response is engaged, leading to cell cycle arrest *via* p53<sup>127–129</sup> and p21 activation<sup>130,131</sup>. This telomere shortening can be avoided or limited by telomerase activity but telomerase is not expressed in most somatic tissues<sup>132,133</sup>. Although this mechanism is universal, its kinetics and significance differ between species<sup>134,135</sup>. As an example, mice telomeres are 50 to 100 kb long while human telomeres are 5 to 15 kb long<sup>136</sup>. Dysfunctions in shelterin complex components have been associated with multiple conditions and accelerated ageing<sup>137–140</sup>, and are important for the recruitment of telomerase to telomeres<sup>141</sup>. Telomerase deficiency has been linked to an increased susceptibility to various diseases<sup>142,143</sup>. Importantly, telomerase reactivation in aged telomerase-deficient mice leads to tissue rejuvenation<sup>144</sup>.

### **Epigenetic alterations**

Epigenetic alterations are the result of several kinds of modifications, either on DNA or on histones, that alter transcription<sup>145</sup>. Different patterns of histone methylation or acetylation have been linked to ageing which can be either increased or decreased<sup>146,147</sup>. These modifications can be regulated or reversed, notably by deacetylases such as sirtuins. However, even though multiple studies have described a role for them in ageing, the extent of their impact remains a matter of debate<sup>148</sup>. Glycation and carbamylation of histones have also been reported<sup>149–153</sup> and are both associated with ageing<sup>154,155</sup>. DNA is globally hypomethylated with ageing, however

hypermethylation of 353 CpG sites has been described as one of the best markers of ageing<sup>156,157</sup>. CpG sites being located in promoters, their methylation is known to have consequences for transcription<sup>158,159</sup>, this pattern therefore very likely contributes to changes in transcription with age. There is also a global diminution with ageing of proteins involved in chromatin remodelling such as heterochromatin protein 1 $\alpha$  (HP1 $\alpha$ ), the nucleosome remodelling deacetylase (NuRD) complex and polycomb group proteins<sup>160–163</sup>. These modifications are associated with increased transcriptional noise<sup>164,165</sup>, changes in gero-miRNAs transcription<sup>166</sup> and altered rate and accuracy of translation<sup>167,168</sup>.

### **Loss of proteostasis**

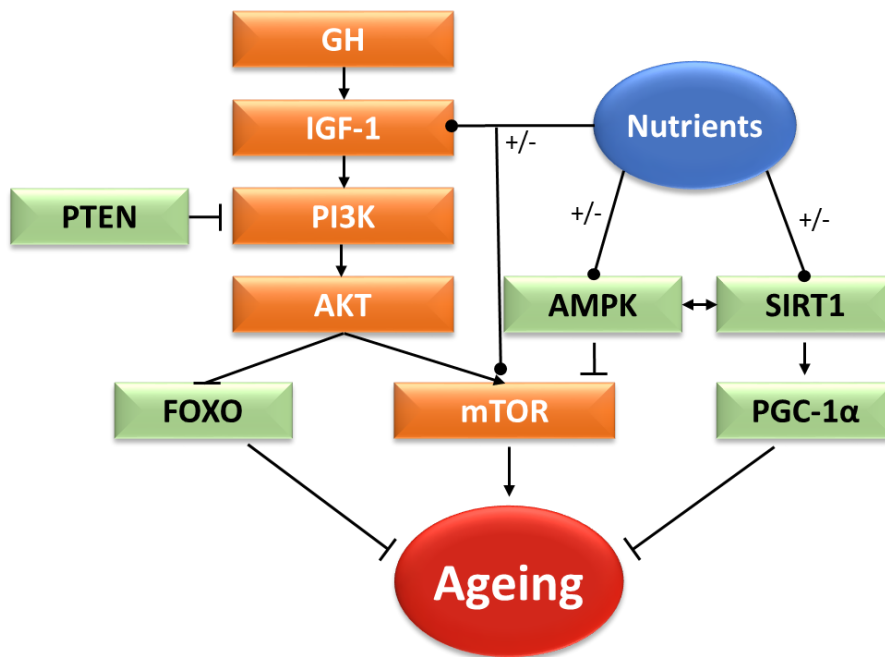
Loss of proteostasis occurs when stability and functionality of the proteome are impaired. This proteostasis is mainly regulated by chaperones which ensure a correct protein folding while degradation systems, such as the ubiquitin-proteasome system or the autophagy-lysosomal system, degrade misfolded and damaged proteins<sup>169</sup>. Importantly, these two mechanisms are impaired with age<sup>170–173</sup>. Failures in these mechanisms lead to the aggregation of unfolded or misfolded proteins. The proteins are known to engage the unfolded protein response which notably leads to endoplasmic reticulum (ER) stress unless quickly resolved<sup>174</sup>. This stress can lead to cell apoptosis or senescence in the long run and is associated with several age-related diseases<sup>175–177</sup>. Accordingly, deficiency in stability induces accelerated ageing<sup>178,179</sup> whereas overexpression of chaperones increases longevity<sup>180–184</sup>. Similar results have been obtained regarding degradation systems<sup>185–187</sup>.

### **Deregulated nutrient sensing**

Deregulated nutrient-sensing is closely related to IIS pathway proteins, including mTOR, AMPK, described earlier, and sirtuins (**Fig. 8**). Although we have indicated that activities of the IIS pathway and mTOR are pro-ageing, GH and IGF-1 levels diminish with normal and accelerated ageing<sup>188</sup>. This is considered by some authors as a protective answer to ageing when damage is accumulating<sup>189</sup>. However, an excessive reduction of these mechanisms, as seen when phosphoinositide 3-kinase (PI3K) or AKT are absent, is lethal<sup>190</sup>. Another study has reported that inactivation of mTOR resulted in an early mortality in mice embryos<sup>94</sup>, however knock-out of S6K1, one of its main substrates, was reported to be associated with an extended longevity in female mice<sup>191,192</sup>. It should be noted that a double knock-out of S6K1 and S6K2 is fatal<sup>193</sup>. mTOR signalling has been reported to be augmented with age in some tissues and decreased in others<sup>194–197</sup>, potentially reflecting IGF-1 diminution. Thus, mTORC1 overactivation during diabetes might induce pro-ageing mechanisms<sup>198</sup>. Contrary to IIS and



mTOR, AMPK and sirtuins are activated when energy levels are low and are usually considered to be pro-longevity molecules. AMPK notably prevents mTOR activation<sup>199–201</sup> which might explain its pro-longevity properties<sup>202,203</sup>. Sirtuins can deacetylate other proteins than histones, notably PPAR $\gamma$  co-activator 1 $\alpha$  (PGC1 $\alpha$ ) which has an important role in metabolic response and whose activity is reduced with age<sup>204–206</sup>. Overexpression of SIRT6 increases lifespan in male mice and is associated with decreased levels of IGF-1 and increased levels of IGF binding protein-1 (IGFBP-1)<sup>207</sup>.



**Figure 8. Nutrient sensing and ageing.** The somatotroph axis, involving GH, and the IIS pathway favour ageing (orange) while molecules regulating this pathway (Phosphatase and tensin homolog (PTEN), AMPK), regulated by this pathway (FOXO) or involved in nutrient sensing by other pathways (SIRT1, PGC-1 $\alpha$ ) limit ageing (green). Nutrients positively or negatively modulate the activity of several of these actors. Modified after López-Otín et al., *Cell*, 2013

### Mitochondrial dysfunction

Mitochondrial dysfunction is at the centre of many non-age-related and age-related disorders and might also be a cause of ageing. As mentioned earlier, mitochondria accumulate mutations with time and there is a shift towards homoplasmy. This supports the mitochondrial free radical theory. Dysfunctional mitochondria, partly resulting from this homoplasmy, produce reactive oxygen species (ROS) because of electron leakage through the respiratory chain which, in turn, increase mitochondrial and cell damage<sup>208,209</sup>. The manganese superoxide dismutase (SOD), regulated by SIRT3, can limit net ROS production but activity and expression of these two proteins are reduced in old age<sup>210–213</sup>. ROS are considered by many as a direct cause of ageing

since they are able to oxidise many cell components such as proteins, nucleic acid and lipids. However, this view is increasingly debated and contradictory results are accumulating regarding the real impact of ROS on ageing: the real role of mitochondrial dysfunction in ageing might therefore reside in other mechanisms<sup>214,215</sup>. Up to a certain concentration, ROS might serve as a stress signal which promotes a pro-survival response. It is only above this tipping point that ROS concentrations provoke deleterious consequences that surpass their beneficial effects<sup>102</sup>. The role of mitochondrial dysfunction in ageing remains, however, unquestioned. Indeed, it is not limited to increased ROS, but includes altered apoptosis, increased inflammation or disrupted crosstalk among organelles<sup>209,216,217</sup>. In addition, adenosine triphosphate (ATP) production is also reduced because of respiratory chain leakage<sup>209</sup> and because of the reduced activity of key proteins such as PGC1 proteins<sup>218</sup>.

### **Cellular senescence**

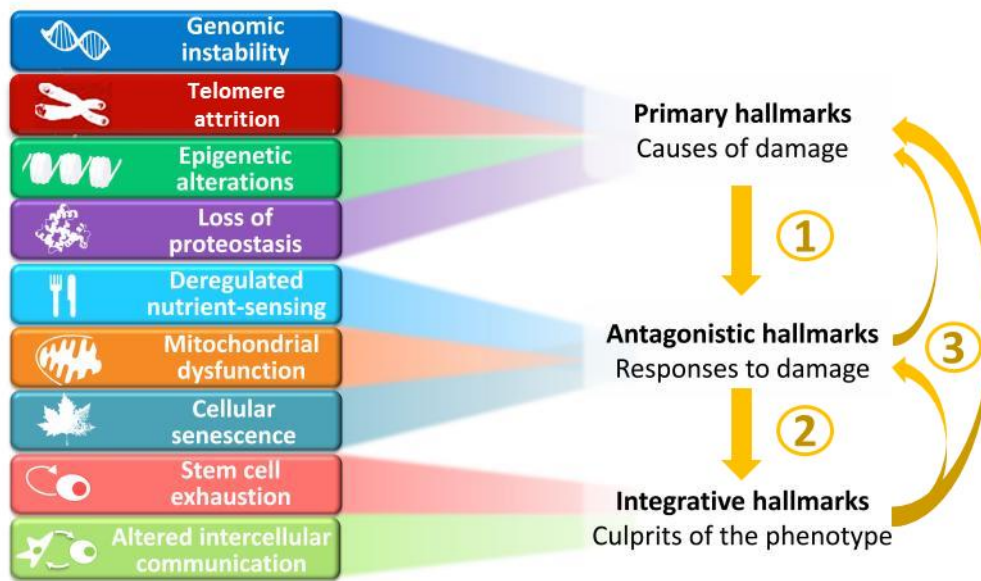
Cellular senescence is an irreversible cell cycle arrest, regularly associated with ageing, that is accompanied by a senescence-associated secretory phenotype (SASP)<sup>219</sup>. As described earlier (Telomere attrition section), cellular senescence mainly results from attainment of the Hayflick's limit which engages cell cycle arrest because DNA breaks are identified. De-repression of the INK4/ARF locus or non-telomeric DNA damages, both increasing with ageing, can also trigger senescence<sup>220</sup> and p16<sup>INK4a</sup> levels correlate strongly with chronological age<sup>221,222</sup>. However, *in vitro* studies have shown that the state of senescence could also be achieved through oncogene-induced senescence<sup>223,224</sup>, oxidative stress-induced senescence<sup>225–227</sup> or through sirtuin inhibition<sup>228</sup>. The rate of accumulation of senescent cell varies among both organisms and organs and this rate increases with age<sup>229–231</sup>. Senescent cells accumulate damage which is responsible for their aberrant secretory phenotype. The SASP is mainly characterized by the secretion of pro-inflammatory molecules such as interleukins and chemokines, but also growth factors and metalloproteinases<sup>219,232</sup>. The SASP is therefore an important contributor to so-called inflammaging, which is a sterile, chronic and low-grade inflammation, actively contributing to ageing and which might be a better predictor of successful ageing than telomere length<sup>233–235</sup>. The inflammatory status of senescent cells is further enhanced by cytosolic chromatin fragments, a common feature of senescent cells<sup>236,237</sup>. Interestingly, telomere dysfunction and subsequent DNA damage response can be induced by chronic inflammation<sup>238</sup>. Accordingly, transplantation of senescent cells into young tissue promotes age-related changes<sup>239,240</sup>.

**Stem cell exhaustion**

Stem cell exhaustion leads to a lower rate of tissue renewal which quickly becomes detrimental for somatic maintenance. Although stem cells are supposed to alternate between quiescence and self-renewal, this process is not perfect and they eventually accumulate damage that limits self-renewal and proliferation<sup>241,242</sup>. This exhaustion occurs in most, if not all, tissues containing niches<sup>243–245</sup>. Hematopoietic stem cells, mainly providing circulating cells, are similarly affected<sup>246–248</sup>, notably leading to a reduced clonality of T cells<sup>248</sup>. This exhaustion is the result of both intrinsic<sup>249–252</sup> and extrinsic age-related defects. Indeed, the state of the stem cells' compartments plays an important role in the maintenance of the niche<sup>253–255</sup>. Inflammaging likely favours stem cells' ageing while systemic factors from young mice have been shown to improve age-related changes<sup>256–258</sup>.

**Altered intercellular communication**

Altered intercellular communication is the result of the many mechanisms of ageing which alter cellular and organ function as signals become either over- or under-expressed, or else are under- or mis-interpreted. Inflammaging, as stated above, is one clear example of this altered intercellular communication. In addition, the heightened inflammatory status of the elderly further impairs the capacity of the immune system to clear out inflammatory cells<sup>259–261</sup>. This lack of clearance of pro-inflammatory tissue damage and senescent cells by inefficient immune cells allows further accumulation, thus engaging a vicious circle. Most pro-inflammatory pathways converge to nuclear factor- $\kappa$  B (NF- $\kappa$ B) which acts as a central hub in inflammatory responses<sup>262,263</sup>. Thus, conditional inhibition of NF- $\kappa$ B successfully leads to rejuvenation in old mice skin<sup>264</sup>. The already mentioned GH from the pituitary and IGF-1 are also good representatives of this altered communication as their expression and function are both affected by, and affect, ageing. We could add to this list the gonadotropin-releasing hormone (GnRH) whose expression is reduced with age with consequent age-related changes such as skin atrophy, muscle weakness or bone fragility. Type-2 diabetes is a good example of a deficient response to a stimulus as cells become less and less responsive to insulin. While type-2 diabetes can occur quite early in life, its increasing prevalence with age suggests that it could be considered as an age-related disease<sup>265–267</sup>. Finally, organ dysfunction can induce whole organism dysfunction. As an example, kidney failure not only impairs waste removal but also has dramatic consequences on blood pressure and subsequent damage including heart failure.



**Figure 9. Hierarchy of the hallmarks of ageing.** The nine hallmarks of ageing are divided into three categories: Primary hallmarks, antagonistic hallmarks and integrative hallmarks. The primary hallmarks are the initial damages of ageing, they reinforce antagonistic hallmarks (1) which can be beneficial in some conditions, similar to hormesis, but quickly become deleterious and integrative hallmarks are the phenotypical consequence of higher hallmarks (2). Lower hallmarks can subsequently worsen higher hallmarks (3). Modified after López-Otín et al., *Cell*, 2013

None of these hallmarks exist in isolation – on the contrary, they are interconnected and influence each other. Going further we can even describe a hierarchy among these hallmarks, meaning that some of these hallmarks occur prior to others and primarily induce other hallmarks. López-Otín *et al.* have reported this hierarchy, defining Primary hallmarks, Antagonistic hallmarks and Integrative Hallmarks (**Fig. 9**). The Primary hallmarks therefore initiate ageing which is then maintained by the subsequent hallmarks that can directly or indirectly accelerate the rate of ageing by increasing multiple stresses, notably genomic instability, telomere attrition, epigenetic alterations or loss of proteostasis.

These hallmarks broadly summarise the current knowledge of the biology of ageing. However, as suggested elsewhere<sup>268,269</sup>, ageing not only affects proteins and nucleic acids, as described so far, but it should be noted that alterations in lipids with ageing have also been reported but were not described in the review of López-Otín *et al.*

## 1.4 | Age-related decline and disorders

The mechanisms of ageing described above are the root cause of a global organismal decline. This decline is characterised by a reduced organ efficacy, an increased risk of developing age-related diseases and an increased probability of death with time. Below is a description of the major age-related dysfunctions.

### 1.4.1 | Physiological systems age-related decline

The physiological decline is defined here as age-related changes that are widely shared in the population, as opposed to age-related diseases which only concern a minority of elderly people and therefore considered pathological. It should be noted, however, that the course of ageing can vary significantly in humans and some changes may occur much later, or even not at all in some people, thereby highlighting the limits of their definition as “physiological”. Similarly, some age-related changes that involve a minority of elderly people, and hence could be considered pathological, can become common if people become old enough; in this situation, the delimitation between “physiological” and “pathological” becomes blurred.

Virtually every function of the organism is affected by ageing but not necessarily at the same pace. There is also a great variation between people, due to gene polymorphism, lifestyle and some diseases. We will briefly describe here the most common features of ageing of major systems such as the musculoskeletal system, the cardiovascular system, the respiratory system, the gastrointestinal system, the hepatobiliary system, the nervous system and the immune system.

#### **Musculoskeletal system**

Sarcopenia is one of the most obvious consequence of ageing and results in increasing and massive loss of muscle associated with decreased muscle strength<sup>270</sup>. This is obviously associated with reduced mobility, gait speed and grip strength<sup>271,272</sup>. It is characterized by a decline in size and number of fibres, mainly type 2 fibres, and an infiltration of adipose and fibrous tissues<sup>273</sup>. This musculoskeletal system condition is further worsened by age-related diminution of bone mass and density<sup>274</sup>. While basal mechanical properties of tendons are maintained with age, their healing is impaired<sup>275</sup>. On the other hand, mechanical properties of ligaments might be impaired with age<sup>276</sup>.

### **Cardiovascular system**

The function of the cardiovascular system is adversely affected by ageing and has been thoroughly investigated as cardiovascular diseases are the primary cause of death globally<sup>277,278</sup>. Both diastole and systole are affected, due to changes in ventricles, valves and blood vessels. Myocytes that form the ventricular walls are progressively reduced in number while their volume increases, resulting in a global hypertrophy<sup>279,280</sup>. In addition, both the myocardium and large vessels stiffen, the valves thicken, leading to a reduced diastole<sup>281–284</sup>. Associated with a decline in maximal heart rate, due a depressed pacemaker activity<sup>285</sup>, heart function is greatly diminished with age. Similarly, there is a stiffening of central arteries associated with a reduced relaxation leading to an increasing systolic pressure<sup>283,286–289</sup>. On the other hand, diastolic blood pressure is reduced<sup>290</sup> and is an independent predictor of cardiovascular mortality<sup>291,292</sup>.

### **Respiratory system**

Ageing of the respiratory system is mainly characterized by changes in pulmonary mechanics, ventilatory control, respiratory muscle strength and gas exchanges. Chest wall rigidity is increased (decreasing compliance)<sup>293</sup>, while respiratory muscle strength is reduced<sup>294,295</sup>, resulting in increased residual volume and closing capacity and an augmented forced expiratory volume<sup>296,297</sup>. Increasing thickening of alveolar basement membrane limit gas exchanges leading to a decreased diffusing capacity with age<sup>298,299</sup>. Finally, the response to hypercapnia and hypoxia is reduced in the elderly<sup>300,301</sup>.

### **Gastrointestinal system**

The gastrointestinal tract is affected by neuromuscular changes, particularly at the level of the oropharynx and of the oesophagus, changes in its structure, most notable in the colon, and potential changes in absorptive and secretory functions. Neuromuscular dysfunction can notably be involved in problems of aspiration, in dysphagia or in a deficient response to normal oesophageal peristalsis associated with a weakened and slower contraction<sup>302–304</sup>. Mucosa thickness and composition changes as its regeneration is impaired<sup>305–307</sup>. It is, however, unclear whether there are age-related changes in the height and/or density of microvilli in humans<sup>308,309</sup>. There is, though, a confirmed thickening of the muscular layers in the colon, mostly due to changes in elastin content<sup>310–312</sup>. While age-related changes in gastric acids are potentially caused by *Helicobacter pylori* infection, pepsin secretion is independently reduced with ageing<sup>313</sup>. Finally, gut permeability might be increased with age<sup>314</sup>. It should also be noted that gut microbiota diversity significantly diminishes and changes with age<sup>314–316</sup>.

### **Hepatobiliary system**

The ageing of the hepatobiliary system is either poorly studied or deemed to be of little impact, but there are some clues regarding both anatomical and functional changes. Firstly, there is a 20-40% reduction in liver size with ageing (though the exact amount is debated<sup>317</sup>), related to diminished blood flow and associated with a diminution in cell numbers that is nevertheless partly compensated by an increased cell volume<sup>318-320</sup>. The uptake function of the liver is diminished in the elderly<sup>321</sup>, and while there are some discrepancies regarding phase I drug metabolism, overall the results show that this function is also increasingly compromised<sup>322,323</sup>. Similarly, hepatobiliary functions appear impaired as bile flow and bile acid secretion decline while serum bilirubin, cholesterol and phospholipids levels increase<sup>322</sup>. Finally, the regeneration rate and capacity of the liver is also reduced<sup>321,322,324,325</sup>.

### **Nervous system**

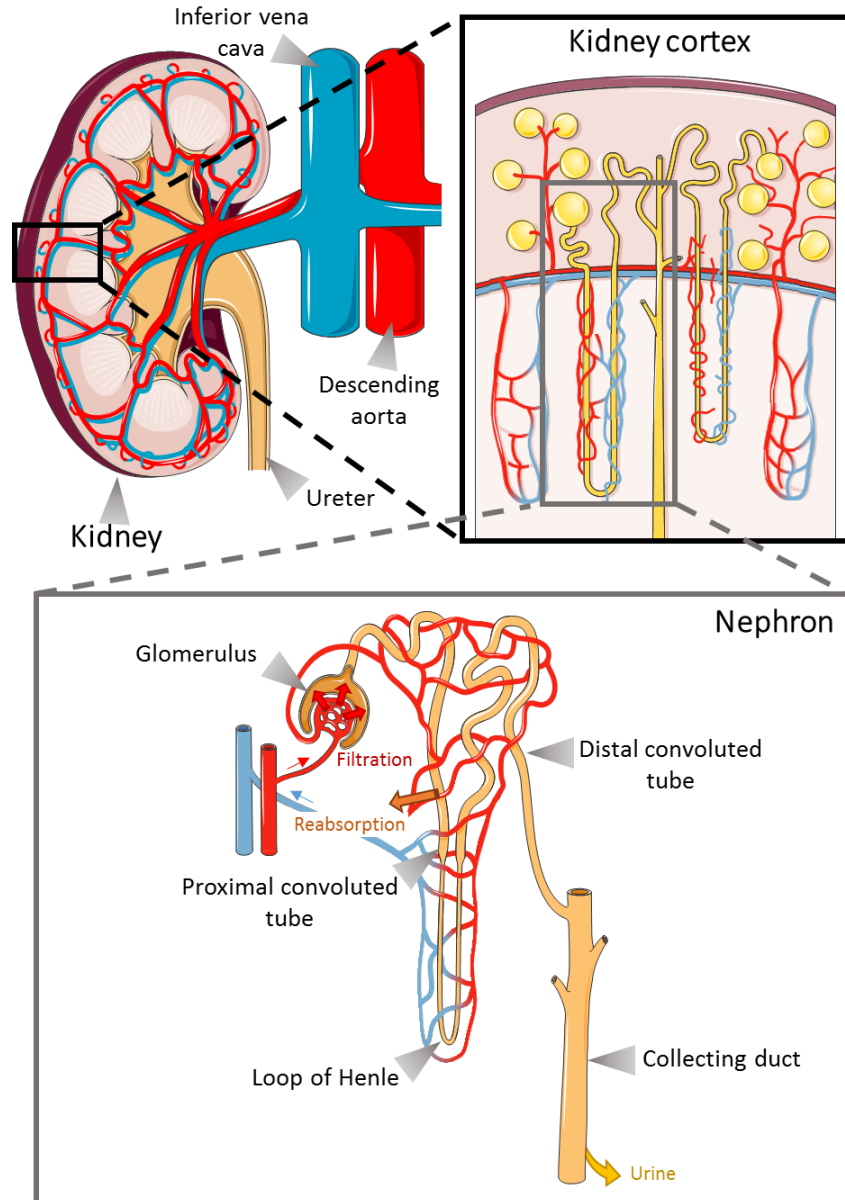
Contrary to long-held beliefs, there is no to little cortical neuron loss during normal ageing<sup>326</sup>. There is, however, a global cortical atrophy while ventricles' size is increased with age<sup>327</sup>. Cerebral blood flow and metabolic rate for glucose and oxygen both decrease with age, independently of cerebral atrophy<sup>328,329</sup>. In addition, a number of functions relying on afferent neurons are affected. For example, retina receptors' density decreases with age<sup>330,331</sup>, there is a degeneration of cochlear cells<sup>332,333</sup>, mechanoreceptors' morphology changes while density and sensitivity are decreased, associated with a loss of myelinated sensory fibres<sup>334-336</sup>. Autonomic neural responses are affected as well, such as the vasoconstriction in response to cold<sup>337</sup> or the ability to precisely respond to changes in blood pressure *via* the baroreflex<sup>338</sup>. Overall, while basal activity of the sympathetic nervous system is increased with age, its reactivity and the activity of the parasympathetic system are both reduced, with consequences on functions such as cardiovascular or urinary functions<sup>339,340</sup>.

### **Immune system**

The immune system is affected by what is called immunosenescence. The immune system becomes less responsive to pathogen infections<sup>341</sup>, there is a reduced efficacy of vaccination with age<sup>342</sup> and a delayed wound healing<sup>343</sup>. This is the consequence of major dysregulations in both innate and adaptive immunity<sup>344</sup>. Haematopoietic stem cells' differentiation is altered<sup>345</sup>, and while the number of most innate immune cells seems stable, many dysfunctions have been reported<sup>346-350</sup>. Naïve T cell production by the thymus is decreased with age but as the thymus contributes only poorly to the maintenance of the T cell repertoire, this is instead considered to be "immunoremodeling"<sup>247,261</sup>. Other changes on precursors of adaptive immunity were briefly

described in the “The hallmarks of ageing – Stem cell exhaustion” section, above, and inflammaging is also known to impair adaptive immune response<sup>261</sup>.

#### 1.4.2 | Kidney physiological ageing



**Figure 10. Kidney structure.** Overview of sagittal kidney section (top left), close view of the kidney cortex (top right) and nephron structure and its main functions (bottom)

Kidney ageing is characterised by many structural changes that significantly affect its function. The main kidney function is to filter blood, thus leading to the clearance of noxious molecules while precisely regulating the concentrations of various essential molecules and adjusting blood pressure. This function is ensured by the presence of hundreds of thousands of nephrons



throughout the kidney. A nephron is composed of a glomerulus, a proximal convoluted tube, followed by the loop of Henle, a distal convoluted tube and finally a collecting duct (**Fig. 10**).

The glomerulus contains a network of capillaries, called tuft, and the blood from these capillaries is filtered to glomerular space, delimited by the Bowman's capsule, therefore allowing the filtration of small molecules and constituting the primary urine. Importantly, the tuft is associated with a basement membrane, podocytes and mesangial cells that have both structural and functional properties. Thereafter, multiple transporters ensure both passive and active reabsorption of molecules from the tubules to the circulation, precisely adjusting the osmotic pressure. Urine finally reaches the collecting duct, which is common to several nephrons.

The glomerular filtration rate (GFR) is known to decrease with age, with a reduction of 0.4 to 2.6 mL/min/1.73 m<sup>2</sup> each year<sup>351</sup>, starting with a GFR at ~100 mL/min/1.73 m<sup>2</sup> at 40 years old decreasing to ~60 mL/min/1.73 m<sup>2</sup> at 80 years old. Thus, elderly people are very susceptible to the development of chronic kidney disease<sup>352</sup>, defined by a GFR between 90 and 60 mL/min/1.73 m<sup>2</sup>. A GFR below this range is considered an indicator of chronic kidney insufficiency<sup>353</sup>. Kidney ageing is highly heterogeneous in the general population and while about a third of old people maintain a normal kidney function<sup>351,354–356</sup>, a non-negligible part of the population develop chronic kidney disease or insufficiency, with incidence influenced by sex<sup>357</sup>, genetic polymorphism<sup>358–361</sup>, behaviour<sup>362–364</sup> and kidney injuries<sup>365</sup>. This function can further be evaluated measuring markers of impaired GFR including urine albuminuria, or proteinuria, serum cystatin C, blood urea nitrogen, neutrophil gelatinase-associated lipocalin (NGAL) or kidney injury marker 1 (KIM-1)<sup>366–370</sup>. Interestingly, there is no direct correlation between kidney function (GFR) and nephrosclerosis in healthy adults. However, nephrosclerosis is notably associated with hypertension<sup>371</sup>. It suggests that there is probably a threshold in nephrosclerosis that determines kidney function, rather than a progression of global glomerular dysfunction with nephrosclerosis.

Reabsorption and endocrine function of the kidney are also impaired with age. Sodium reabsorption and excretion are altered and predispose elderly people to acute kidney injury, volume depletion, salt retention hypertension and cardiovascular congestion<sup>351,372–375</sup>. The elderly are also at high risk of developing hyperkalaemia<sup>376</sup>. The activity and responsiveness of the renin-angiotensin system are modified with age and increase the susceptibility of elderly to imbalances in electrolytes and fluids, and to kidney diseases more generally<sup>374,377,378</sup>. These

changes are associated with an impaired ability to concentrate or dilute urine among the elderly, potentially because of reduced abundance of aquaporins, urea and vasopressin receptors<sup>379–382</sup>, associated with increased nocturia or hyponatremia with age<sup>383–385</sup>.

These losses of function are associated with a set of renal lesions that influence each other. First, there is a slight increase in kidney volume followed by a shrinkage in older adults<sup>386</sup>. Thereafter, all kidney structures are affected and age-related lesions in glomeruli, tubules, arterioles and in the interstitium are designated as glomerulosclerosis (global and focal, but not segmental), tubular atrophy and diverticula, arteriosclerosis, or arteriolar hyalinosis, and interstitial fibrosis, respectively<sup>386,387</sup>. Such lesions lead to a gradual loss of nephrons with age<sup>388,389</sup>.

Glomerulosclerosis is characterised by the thickening of the basement membrane, a wrinkling (until complete collapse) of the tuft, and the filling of the bowman's space with a matrix-like hyaline material and an increased mesangial volume<sup>390,391</sup>. Glomerular hypertrophy precedes complete atrophy, which is the final step of glomerulosclerosis<sup>386,392,393</sup>. While the number of cortical glomeruli decreases by 30 to 50 % by 70 years old, juxtamedullary glomeruli were better preserved<sup>394–396</sup>. Tubular atrophy is defined by an attrition of the brush border of the tubular epithelium, associated with a reduced tubular size and a thickening of the tubular basement membrane<sup>351</sup>. Arteriolar hyalinosis is characterised by the thickening of the arterioles' walls and is evidenced by a pink staining using periodic acid Schiff. Finally, interstitial fibrosis is characterised by accumulation of collagen, and related molecules, in the interstitium<sup>397</sup>. It has been suggested that interstitial fibrosis precedes the development of glomerulosclerosis or tubular atrophy<sup>398,399</sup>.

### 1.4.3 | Age-related disorders

#### Accelerated ageing

Contrary to the previously presented genes whose knock-out extends lifespan, it has been demonstrated that the knock-out of some genes causes the induction of an accelerated ageing referred to as progeroid syndrome. A number of progeroid syndromes have been identified in humans including the Hutchinson-Gilford progeria syndrome, the Werner Syndrome, the Bloom syndrome or the Rothmund-Thomson syndrome<sup>268</sup>. Interestingly, all of these progeroid syndromes are associated with mutations involved in the maintenance of DNA integrity. As an example, the Werner syndrome is caused by mutations on the *WRN* gene, belonging to the RecQ subfamily, which codes for a protein having helicase and exonuclease properties. Its absence leads to the collapse of replication forks, Holliday junction formation and subsequent DNA

damage<sup>400–402</sup>. The Bloom syndrome and Rothmund-Thomson syndrome are also related to genes belonging to the RecQ subfamily<sup>403–405</sup>. The Hutchinson-Gilford progeria syndrome is caused by a mutation on the *LMNA* gene, coding for lamin A which is normally involved in the production of the nuclear lamina, in nuclear stability and chromatin structure. Mutations involved in the Hutchinson-Gilford progeria syndrome prevent post-translational modifications required for the function of this protein<sup>406</sup>.

These conditions show to what extent DNA integrity is important in ageing, supporting the theory that genomic instability is a prime mechanism of ageing. However, as demonstrated by the IIS pathway, metabolic routes can also have important effects upon longevity. KLOTHO is a protein with pleiotropic activities, notably involved in phosphate excretion, synthesis of vitamin D or suppression of growth factor signalling<sup>407–409</sup>. While under expression of KLOTHO leads to ageing-like features, its overexpression promotes longevity, potentially by inhibiting IGF-1 signalling<sup>410,411</sup>. Similarly, the sirtuin SIRT6 is also involved in metabolism, and while its conditional disruption leads to severe metabolic disorders<sup>412,413</sup> or even perinatal lethality when fully ablated<sup>414</sup>, its knock down has been shown to cause premature senescence associated with increased DNA mutations<sup>415–417</sup>. On the contrary, SIRT6 overexpression increases healthspan and lifespan, at least in male mice, and is associated with increased IGFBP-1 and reduced IGF-1 expression<sup>207,418</sup>.

### **Conditions increased with ageing**

Many age-related diseases have been reported. They can affect most organs and have serious consequences on the body's function<sup>419,420</sup>.

The most common age-related diseases are probably cardiovascular diseases<sup>278,421–424</sup>. This includes chronic ischemic heart disease<sup>425,426</sup>, left ventricular hypertrophy<sup>279,427,428</sup>, heart failure<sup>429,430</sup> and arrhythmia<sup>431,432</sup>, notably caused by tissue remodelling and fibrosis<sup>433</sup>. On the level of vessels, we can also mention arteriosclerosis and the subsequent atherosclerosis which can lead to highly incapacitating or deadly myocardial infarctions and cerebrovascular accidents<sup>434,435</sup>. Central hypertension gradually increases with age and can cause or aggravate the above-mentioned diseases<sup>286,436,437</sup>.

Cardiovascular events can have major consequences on cognitive functions. Minor events could also explain noticeable age-related loss of some functions<sup>438–440</sup>. Major age-related cognitive diseases are also widely reported but their link with the cardiovascular system, although sometimes suspected, is not established<sup>441–443</sup>. Neurodegenerative diseases such as Alzheimer's

disease (AD) or Parkinson's disease (PD) are very well described pathologies although their exact cause is not fully understood<sup>441,444-447</sup>. AD is characterised by accumulation of peptide amyloid  $\beta$  (A $\beta$ ) plaques, protein tau hyperphosphorylation and entanglement and neurodegeneration, leading to shrinkage of the cortex, hippocampus, enlargement of ventricles and memory loss<sup>444</sup>. PD is characterised by the presence of Lewy bodies (protein aggregates) in the substantia nigra (although this has been contested<sup>448</sup>) and nigral cell loss, notably leading to motor functions alteration<sup>447</sup>.

Multiple age-related bone or bone-related diseases have been characterised as well, such as osteoarthritis<sup>425,449</sup> or rheumatoid arthritis<sup>450,451</sup> linked to periodontitis<sup>452,453</sup> (which are inflammatory diseases), and osteoporosis which leads to loss of bone density<sup>454,455</sup>. The causes of these conditions are multifactorial, albeit they may have converging points that will be highlighted in this manuscript. Sarcopenia, as described earlier, might seem physiological but its extent can significantly differ between individuals<sup>456-458</sup>. Thus, bone diseases and sarcopenia jointly contribute to a reduction of mobility.

Type-2 diabetes is defined as a chronic hyperglycaemia caused by an insulin resistance. The consideration of this disease as an age-related disease is debatable since its onset can occur quite early in life. However, as glucose intolerance and insulin resistance naturally increase with age<sup>459,460</sup>, the ageing of the population will mean disease is becoming more common and in addition, its complications are closely related to ageing<sup>266,267</sup>. Diabetic complications are often listed as follows: diabetic nephropathy, retinopathy, neuropathy, cardiomyopathy and angiopathy<sup>461-463</sup>.

Finally, ageing is associated with a number of cancers, which is the second leading cause of death in elderly, even though its incidence varies at very old age<sup>278</sup>. Cancer onset and proliferation are inextricably bound to the very mechanisms of ageing detailed earlier<sup>102,464-467</sup>. Importantly, while senescence was initially thought to be a cancer-preventing mechanism, it actually promotes its onset and growth through multiple stresses induced by the SASP and inflammaging<sup>468-470</sup>.

Ageing in humans is therefore associated with multiple chronic conditions and a geriatric patient is almost always polypathologic. The resultant simultaneous treatment of several pathologies with medication means that iatrogeny is increasingly considered problematic in these patients as the accumulation of several treatments can become noxious<sup>471</sup>.

## 1.5 | Interventions that have an impact on lifespan and healthspan

So far, all the main characteristics of ageing have been described including its demography, evolutionary origin, its mechanisms and finally the consequences of ageing. Some of the most important works that have led to an improvement of health and/or lifespan will now be examined.

### 1.5.1 | Modulation of cells

We have seen how reproduction and lifespan are connected. In accordance with this connection, the impact of germline removal has been investigated. It has been shown in *C. elegans* that laser ablation of the germline, but not the somatic gonad, led to a ~60% increase in lifespan<sup>472-474</sup>. However, such an extension was not seen in work undertaken in *D. melanogaster*, excluding the cost of egg production and/or laying as explanatory to this phenomenon, but suggesting that this pattern has not been conserved during evolution<sup>475</sup>. More recent work in *D. melanogaster*, using a different model, suggested on the contrary that germline ablation can indeed induce a lifespan extension but that more parameters have to be taken into consideration<sup>476</sup>.

In mice, transplantation of young bone marrow to old mice ameliorated their recovery from vascular injury, suggesting a form of rejuvenation<sup>477</sup>. Similar findings were made regarding the preservation of cognitive function using young bone marrow<sup>478</sup>. Along the same line, aged haematopoietic stem cells were rejuvenated when interacting with microvesicles from young mesenchymal stromal cells, possibly related to the AKT pathway<sup>479</sup>. Very similar results were also found regarding hypothalamic stem cells. Their ablation led to reduced cognitive and motor functions and a decreased lifespan. However, injection of these cells rejuvenated old mice, ameliorating all of these parameters, and was completely mimicked by hypothalamic stem cells exosomes<sup>480</sup>. These results are strongly supported by the finding that, in a parabiosis experiment, old stem cells' activity was recovered in the presence of a young systemic environment<sup>243,257</sup>.

Innovative work has shown that mitochondria removal from senescent cells alleviated pro-ageing features of senescence. It is also suggested that these cells survive using glycolytic pathways as a source of energy<sup>481</sup>.

On the contrary, several studies have demonstrated that the senescent cells' SASP was indeed responsible for ageing and the onset of senescence. Indeed, transplantation of senescent cells into young mice promotes physical dysfunctions in these mice<sup>240,482</sup>.

### 1.5.2 | Genetic models

Different genetic models have indicated very solid possible interventions that could have a significant impact on healthspan and lifespan.

One of the most striking results was the use of genetic models that were able to clear senescent cells. p16<sup>Ink4a</sup>, a cyclin-dependent kinase inhibitor, is expressed in most senescent cells, increases with ageing and is proposed to be a very good (although imperfect) marker of senescent cells<sup>483,484</sup>. Accordingly, various models designed to suppress cells expressing p16<sup>Ink4a</sup> have demonstrated that the removal of senescent cells by these means led to a significant improvement in overall healthspan and in lifespan<sup>485–488</sup>.

Another key study focussed on the engineering of inducible telomerase in mice. A knock-in was built in mice producing a telomerase reverse transcriptase-oestrogen receptor (TERT-ER) construct inducible by 4-hydroxytamoxifen (4-OHT). Thus, the telomerase gene was constitutively repressed without 4-OHT, leading to an accelerated ageing in these mice, associated with short, dysfunctional telomeres and DNA damage signalling. Reactivation of telomerase by 4-OHT in adult mice successfully improved many parameters of ageing, including neural stem cell function, brain size and olfactory function<sup>144</sup>. It should be noted, however, that telomerase reactivation must be carefully monitored as it is a key part in cancer outgrowth and reactivation of telomerase in cancer cells enables full malignancy<sup>489</sup>.

### 1.5.3 | Pharmacologic interventions

Among the pharmacologic interventions that positively modify healthspan or lifespan, mTOR targeting represents perhaps the most promising and feasible strategy. Countless studies have shown that mTOR inhibition with rapamycin was able to extend lifespan in *C. elegans*, *D. melanogaster*, *S. cerevisiae* and even in elderly<sup>490,491</sup>. However, inhibition of mTOR must be finely regulated and needs to be specific to avoid the accumulation of undesirable side-effects. Because of these concerns, rapalogues and second generation mTOR inhibitors have been developed, which can be specific to mTORC1 or mTORC2 or else target them both<sup>492</sup>. Thus acute, short-term use of AZD8055, a pan-mTORC inhibitor, reversed the senescence phenotype in fibroblasts<sup>493</sup>. Interestingly, inhibition of the RNA polymerase III, which notably generates tRNAs, reproduced the effects of mTORC1 inhibition, suggesting that lifespan extension could be bound to protein translation<sup>494</sup>.

Another emerging pharmacologic intervention is the use of senolytics, molecules that selectively trigger death in senescent cells. Again, an mTORC1 inhibitor, Torin1, has recently

been described as a senolytic<sup>495</sup>. But the most promising approaches rest in more specific treatments. A recent study showed that nanoparticles with a galacto-oligosaccharide coating, loaded with doxorubicin (a non-specific cytotoxic drug) or navitoclax (a BCL-2 family inhibitor senolytic), were able to greatly improve lung function<sup>496</sup>. Another strategy was the use of a forkhead box O (FoxO) 4 peptide, preventing FoxO4 and p53 interaction and promoting cell death in senescent cells, which ameliorated overall fitness in mice<sup>497</sup>. Simple drug treatments have been undertaken, using navitoclax, dasatinib, quercetin or fisetin, and while the results are promising, a lack of specificity may induce undesirable side-effects<sup>482,496,498,499</sup>.

Supplementation in nicotinamide adenine dinucleotide (NAD<sup>+</sup>) or its precursors might be a promising treatment as well as it has been shown to improve healthspan, although it failed to increase lifespan in mice<sup>500-502</sup>.

#### 1.5.4 | Behaviour modulation

One widely reported behaviour modulation that affects healthspan and lifespan is calorie restriction. Countless studies have shown that varying degrees of dietary restriction (though above starvation) could extend lifespan in multiple animal models, including yeasts, worms, flies and mice<sup>503</sup>. It is suggested that this increase is due to a modulation of the IIS and sirtuins. However, the effects of dietary restriction in higher mammals is less obvious. Indeed, two major studies conducted in rhesus monkeys yielded conflicting views on the effects of calorie restriction. While one study suggested that there was an improved health and survival in these monkeys<sup>504,505</sup>, the other noted few changes in health and no increase in survival<sup>506</sup>. As a consequence, this effect is still a matter of debate, at least in monkeys. This debate also exists in murine studies as a paper reported that the effects of dietary were highly strain-specific, even leading to a shortened lifespan in most strains<sup>507</sup>. However, multiple factors should be considered, and other parameters might explain such discrepancies<sup>508</sup>.

**Box 2 – Ageing**

- Ageing is a gradual loss of organ function resulting in an increased probability of death with time
- There is an extreme variation in longevity between species, from days to thousands of years. In humans, life expectancy varies between countries, is higher in women and is steadily increasing worldwide, independent of infant mortality
- Ageing is partly bound to reproduction capabilities, and while evolution could not primarily select “ageing genes”, there are however genes whose functions modulate ageing because of antagonistic pleiotropy
- Ageing is caused by primary hallmarks, bound to genome and proteome integrity, and influencing a set of cell functions which further aggravate the course of ageing
- Ageing is not immutable and a number of interventions may successfully slow or reverse the consequences of ageing

Ageing is a very complex and multifactorial process. But, while the description of the hallmarks of ageing provides a conceptual framework to investigate and explain ageing, in all likelihood many of these hallmarks are more or less affected by the glycation reaction, by virtue of it being able to alter every component of a cell. We describe this reaction below and thereafter the extent of its direct or indirect consequences.



## 2 | Glycation and advanced glycation end-products

### 2.1 | Definition and formation

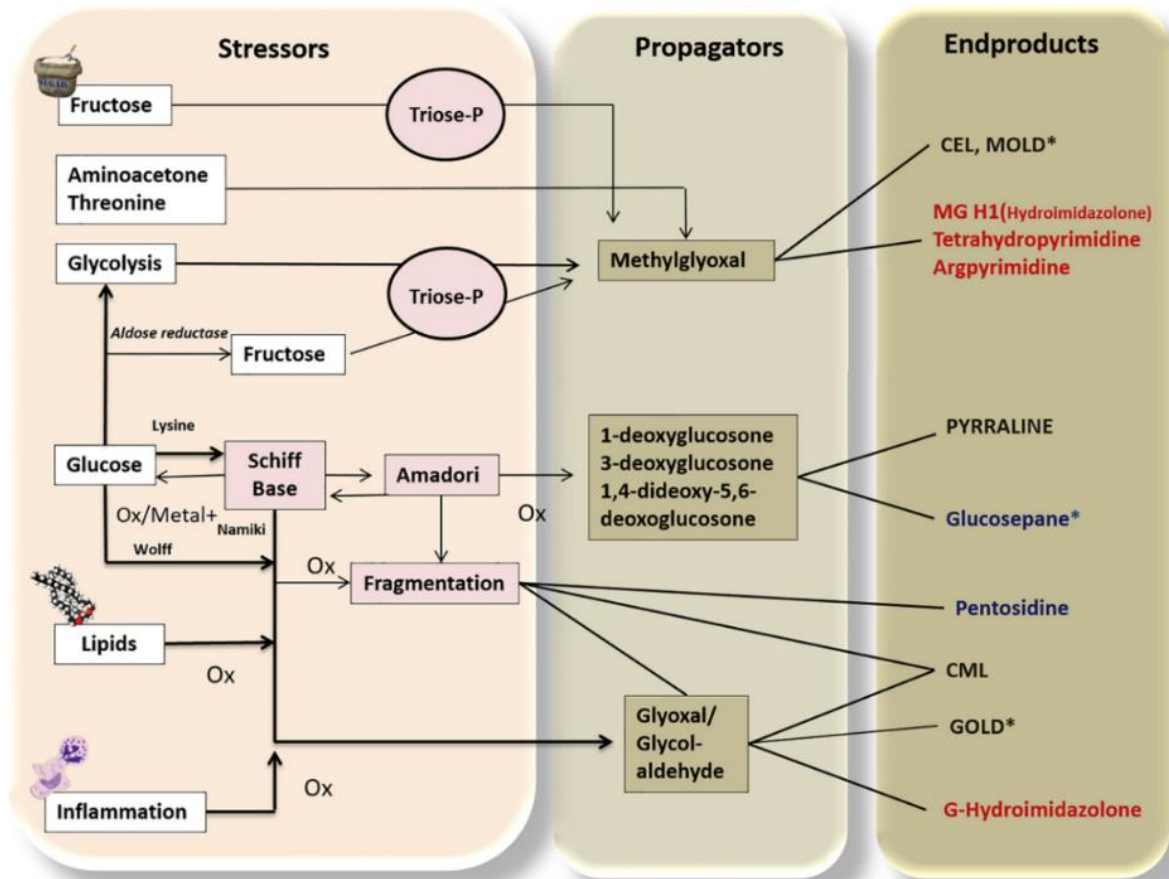
#### Box 3 – Glycation and AGEs

- What are glycation and glycation products?
- How are they modulated?
- What are their molecular and biological consequences?

Advanced Glycation End-products (AGEs) are the result of glycation, a process discovered in 1912 by Louis Camille Maillard and that was initially named the Maillard reaction<sup>509</sup>. Glycation is the non-enzymatic binding between carbonyl compounds and nucleophiles. Among the carbonyl compounds we commonly find reducing sugars but also dicarbonyls, such as glyoxal and methylglyoxal, or lipid peroxidation products. They are mainly described for their

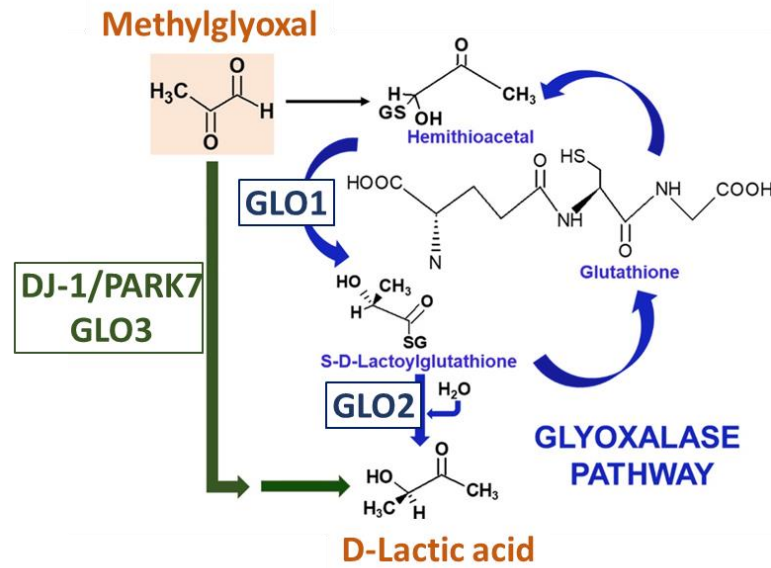
glycation of amino acids (mainly lysines and arginines) at their amino group, or else at the protein amino-terminus – but nucleic acids, and to a lesser extent some lipids, can also be glycated<sup>510-513</sup>. The reactivity of carbonyl compounds with their target is highly dependent on their structure and it has been shown that glucose is almost ten-fold less reactive than ribose, while both are much less reactive than dicarbonyls, thus showing that shorter carbonyl compounds are more reactive<sup>514-517</sup>.

Glycation is a multiple-step reaction which involves many possible pathways where intermediate species are normally reversible while the end-products, AGEs, are considered irreversible (**Fig. 11**)<sup>510</sup>. The most commonly described pathway is the interaction of glucose with lysine. This reversible reaction will lead to a Schiff base which in turn, after rearrangements, will produce a less reversible Amadori product, fructosamine. Finally, after various modifications such as fragmentation, the Amadori products will give rise to an irreversible AGE. Oxidation can also intervene and, through different pathways, will eventually favour the production of AGEs, some of which are also the result of glycation *via* reducing sugars. Oses and other sources can also directly produce dicarbonyls such as methylglyoxal which will lead to different AGEs. Of note, modified adducts can subsequently interact with other residues, thus forming cross-links within or between proteins.



**Figure 11. Glycation.** The principal glycation pathways with the main carbonyl compounds sources (left) in absence or presence of catalysers such as oxidation (Ox) or metals, leading to very potent propagators (middle) and finally advanced glycation end-products (right). In right column: black – lysine modifications; red – arginine modifications; blue – lysine-arginine modifications. \* indicates cross-linked products. Modified after Chaudhuri et al., *Cell metabolism*, 2018

Glycation is also greatly affected by temperature, moisture, pH and the turnover of the target<sup>518-521</sup>. Thus, the accumulation of AGEs *in vivo* can take months to years whereas it can only take a few minutes *in vitro*, notably in cooking processes requiring high temperatures<sup>521-525</sup>. Consequently, unique *in vitro* conditions can lead to glycation products that do not exist *in vivo* such as acrylamide, pyrraline or hydroxymethylfurfural<sup>526-530</sup>. AGEs produced *in vivo* are therefore generally referred to as endogenous AGEs while AGEs produced *in vitro* are often called exogenous or dietary AGEs, independent of the specific AGE molecule under discussion.



**Figure 12. The glyoxalase system.** Methylglyoxal can be converted into D-Lactic acid by a glutathione GSH-dependent (GLO1 and GLO2 – blue) pathway or a glutathione-independent pathway (DJ-1/PARK7, GLO3 – green). Modified after Chaudhuri et al., *Cell metabolism*, 2018

*In vivo*, these reactions can be prevented, slowed down or reversed by the presence of glyoxalases which can notably detoxify the very reactive dicarbonyls (**Fig. 12**)<sup>531,532</sup>. Glyoxalase 1 (GLO1) and GLO2 both intervene in a cycle requiring glutathione GSH to produce D-lactic acid from methylglyoxal. Glyoxal is similarly detoxified by this glyoxalase system, although the intermediates differ<sup>533</sup>. Other glyoxalase systems, GLO3 and protein deglycase (DJ-1) (or Parkinsonism associated deglycase (PARK7)), can also convert dicarbonyls to D-lactic acid, although independently of glutathione. Importantly, DJ-1 has recently been described as a deglycase, restoring amino acids modified by dicarbonyls<sup>534,535</sup>. Widely conserved aldo-keto reductases (AKRs) can also detoxify dicarbonyls by first producing reactive acetol that is then converted into innocuous 1,2-propanediol by glycerol dehydrogenase<sup>536–538</sup>.

Fructosamines can also be reverted to amino acids thanks to fructosamine-3-kinase (FN-3K). FN-3K is able to phosphorylate fructosamines, forming fructosamine 3-phosphates which are unstable and spontaneously release an inorganic phosphate and 3-deoxyglucosone, thus providing an unglycated amino acid<sup>539–541</sup>.

Once formed, it nevertheless remains possible to remove AGEs. Exogenous AGEs are digested and cleaved to give dipeptides or free AGE adducts. Following this cleavage, these small molecules can be absorbed in the circulation<sup>542–544</sup> and join other circulating, cleaved

endogenous AGEs<sup>545–547</sup>. These circulating AGEs can then be excreted through the renal system<sup>547,548</sup>. There is however a reabsorption of most peptides at the level of the proximal tubules, while free adducts are excreted<sup>546,549</sup>. AGE sequestration by the lysozyme increases their renal excretion<sup>550</sup> and renal clearance is (obviously) negatively affected by renal failure, leading to an accumulation of AGEs<sup>551,552</sup>.

Protein glycation is also in direct competition with another non-enzymatic reaction, carbamylation, characterised by the binding of isocyanic acid, derived from urea, to amino acids of proteins. Therefore, carbamylation of lysine, giving rise to homocitrulline, is competing with its glycation. Similar to glycation, this reaction increases with chronic kidney diseases and has been described as a hallmark of ageing<sup>155,553,554</sup>.

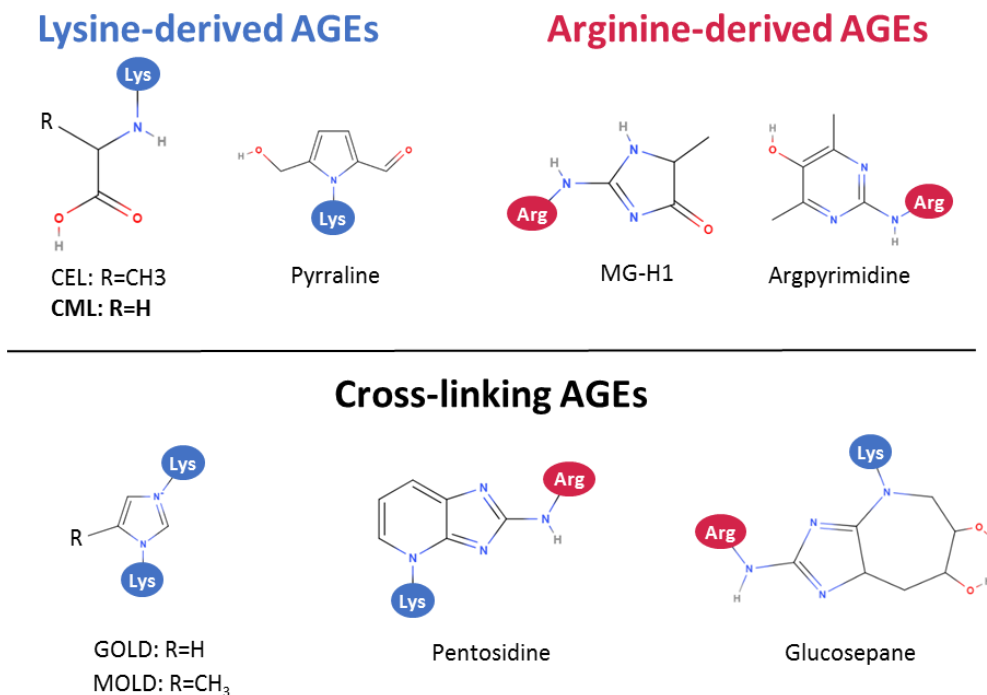
## 2.2 | Advanced glycation end-products

### 2.2.1 | The structures of AGEs

As evoked in the previous section, glycation can lead to a wide variety of AGEs. We focus here on the most common and most-studied AGEs. We classify here AGEs into two distinct groups: modified amino acids and cross-linked peptides (**Fig. 13**). Although they exist, we will not describe here nucleic acid AGEs<sup>511,555</sup> or lipid AGEs<sup>556</sup>. Free amino groups are the first targets of carbonyl compounds: as a consequence, lysines and arginines can produce vastly-modified adducts by the addition of various compounds and the subsequent rearrangements.

N<sup>ε</sup>-carboxymethyllysine (CML) and N<sup>ε</sup>-carboxyethyllysine (CEL), some of the most abundant AGEs, are both formed after the binding of glyoxal and methylglyoxal, respectively, to a lysine. CML can also be the result of the fragmentation of a fructoselysine. Pyrraline is more complex as it is formed by the addition of deoxyglucosones, which are longer carbonyl compounds than dicarbonyls, leading to the formation of a stable ring after rearrangements. Arginine-derived AGEs look more complex as carbonyl compounds can react with both available amino groups. Thus, both methylglyoxal-derived hydroimidazolone 1 (MG-H1) and argpyrimidine (or argpyrimidine) possess a ring in their structure. Finally, cross-linked AGEs, which require more time to form, are even more complex in structure as they involve two different amino acids. Glyoxal lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD) are nevertheless quite simple structures as they only involve one ring between two lysines. The difference between GOLD and MOLD is defined by the nature (C or H) of the radical bound to the ring. Pentosidine and glucosepane are more complex again as they are formed by two rings between a lysine and an arginine. Of note, AGEs that have an aromatic ring can be detected through fluorescence.

Indeed, this is how AGEs such as pentosidine were first discovered and the AGE reader is therefore based on this principle<sup>557</sup>. However, one of the most reliable, quantitative and specific methods to detect such molecules is high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)<sup>558</sup>.

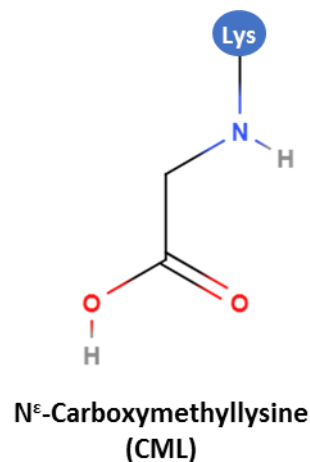


**Figure 13. Examples of major AGEs.** Main described AGEs with lysine-derived AGEs, *N*<sup>ε</sup>-carboxymethyllysine (CML), *N*<sup>ε</sup>-carboxyethyllysine (CEL) and pyrrolaline, arginine-derived AGEs methylglyoxal-derived hydroimidazolone 1 (MG-H1) and argpyrimidine and cross-links involving lysine and/or arginine, glyoxal lysine dimer (GOLD), methylglyoxal-lysine dimer (MOLD), pentosidine and glucosepane

### 2.2.2 | Carboxymethyllysine

The CML (Fig. 14) was the first AGE to be described and was historically characterised in skin<sup>559</sup>, lenses<sup>560,561</sup> and urine<sup>562</sup>. It has now been found in all human tissues and also in food<sup>563</sup>. This AGE can be found in two forms, the protein- or peptide-bound form and the free form, devoid of any amino acid linked to the lysine. Thus, protein-bound CML and free CML have very different overall structures which suggests that their properties may also be very different.

CML is one of the most abundant AGEs, has both endogenous and exogenous origins and can bind to the receptor for

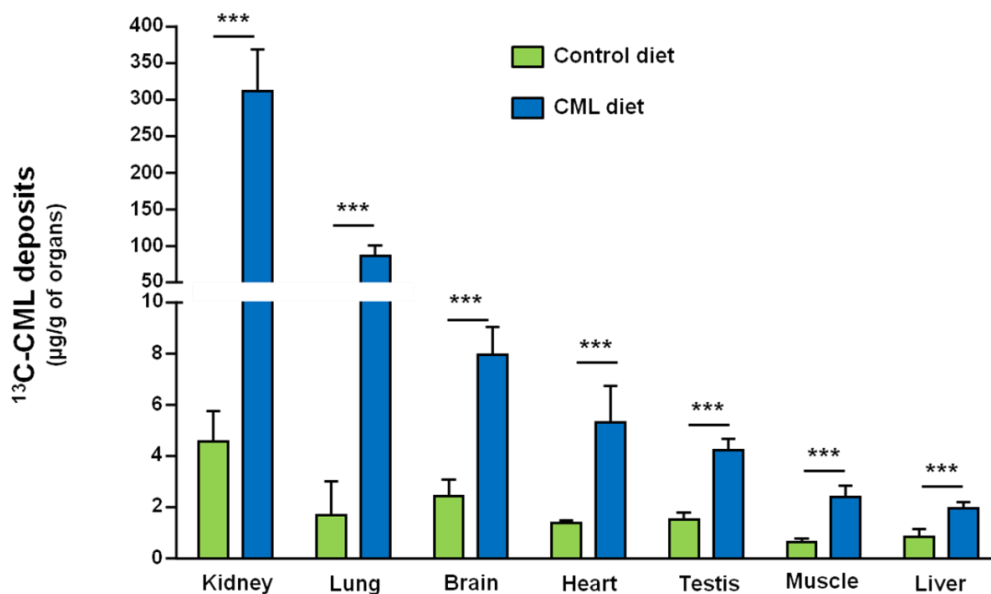


**Figure 14.** *N*<sup>ε</sup>-Carboxymethyllysine

advanced glycation end-products (RAGE – see section 3.). *In vitro*, CML can be produced very easily: bovine serum albumin (BSA) incubated with glyoxylic acid and sodium cyanoborohydride at 37°C can modify up to 40% of BSA lysines within 16 hours<sup>288</sup>. BSA having 59 lysines, this reaction produces 17 to 23 CML/BSA. This rate, however, is non-physiological as the rate of lysines glycation is many times lower *in vivo*<sup>564,565</sup>. In mice, BSA-CML can be used to mimic a CML-rich diet, as opposed to BSA-AGE whose AGE composition often remains uncharacterised and uncontrolled after simply incubating BSA with glucose.

Both models, BSA-CML and BSA-AGE, have their limits and are complementary. While the BSA-CML diet is more controlled and provides specific information about one category of AGEs, BSA-AGE is more representative of the real-life diversity of AGEs found in the diet and *in vivo*. Indeed, given major structure differences between AGEs, it would be very unlikely that their effects are identical.

It has been shown by our research group that dietary, <sup>13</sup>C<sub>2</sub>-labeled CML did not accumulate equally in murine tissues and while some organs barely accumulated any <sup>13</sup>C-labeled CML others become very exposed<sup>566</sup>. As such, kidney, then ileum, colon (not shown) and lung are the major organs of dietary CML accumulation (**Fig. 15**). Of important note, the diet did not affect food consumption, indicating that accumulation of dietary CML is only dependent on the diet<sup>288</sup>. Thus, there is a possibility that CML consumption preferentially alters the kidneys.



**Figure 15. Organ distribution of dietary <sup>13</sup>C<sub>2</sub>-labeled CML.** 6-month-old mice were fed ad libitum a BSA-<sup>13</sup>C<sub>2</sub>-labeled CML diet or a BSA control diet for 30 days and free <sup>13</sup>C<sub>2</sub>-labeled CML was subsequently measured in kidney, lung, brain, heart, testis, muscle and liver. \*\*\* p < 0.001. Modified after Tessier et al., *Mol. Nutr. Food. Res.*, 2016

### 2.3 | Deleterious effects of AGEs

The principal effect of AGEs is the inclusion of uncontrolled, or at least poorly-controlled, post-translational modifications into the peptide chain of a protein. The primary consequence arising from this change is the possible degradation of the glycated protein by degradation systems such as lysosomal degradation involving cathepsins<sup>546,567,568</sup>, the proteasome or metalloproteinases for bulky proteins<sup>569</sup>. Interestingly, glycation has been shown to prevent proteasomal degradation and only small proteins or peptides can be degraded by this mechanism. It is thought that this is the consequence of large protein structure modifications that impair proteasome handling of these modified proteins, and of changes in proteasome activity as well<sup>570,571</sup>. AGEs also stimulate transcription of matrix metalloproteinases (MMPs), leading to collagen degradation<sup>572,573</sup>. Thus, we can surmise that MMPs could be acting in response to glycated collagen to degrade it.

These events therefore lead to the degradation of glycated proteins in response to structural change, and even if this suggests the loss of important long-lived proteins, this might nevertheless be the best-case scenario.

In the case where AGEs are not degraded, these post-translational modifications can greatly change the proteins' structure, thereby altering their function. The first outcomes can be a decrease in, or a loss of activity and functionality as has been reported for albumin<sup>574</sup>. Thereafter, due to misfolding, glycated proteins can form detrimental, toxic aggregates as seen in Lewy bodies in PD<sup>575-577</sup>. Similar observations have been made regarding A $\beta$  peptides and tau aggregation in AD. Thus, there is a chance that protein glycation might intervene in the initiation of amyloidosis of  $\beta$ -sheet fibrils<sup>578-580</sup>.

Cross-links can further impair proteins' function as these modifications provoke even more important structural changes. As already evoked, cross-links can occur within a single protein but also between different proteins. This last is of particular interest. Indeed, long-lived proteins that constitute the extracellular matrix such as collagen or crystallin in the lens accumulate such modifications which can in turn tighten up their interaction and rigidify the surrounding tissue<sup>581-583</sup>.

Finally, AGEs, including this time both endogenous and exogenous AGEs, are also able to interact with various receptors, inducing many responses (and notably inflammatory responses)

that are involved in inflammatory diseases<sup>584</sup>. This will be detailed in the next section (3. RAGE).

Overall, these properties of glycation strongly suggest that they have a central role in what we described earlier as the “Loss of proteostasis”. Glycation may thus have a key role in various mechanisms of ageing. Furthermore, not only is proteostasis altered but also genomic stability<sup>511,555</sup> and possibly lipid quality<sup>268,269,513</sup>, since both nucleic acids and lipids are glycated.

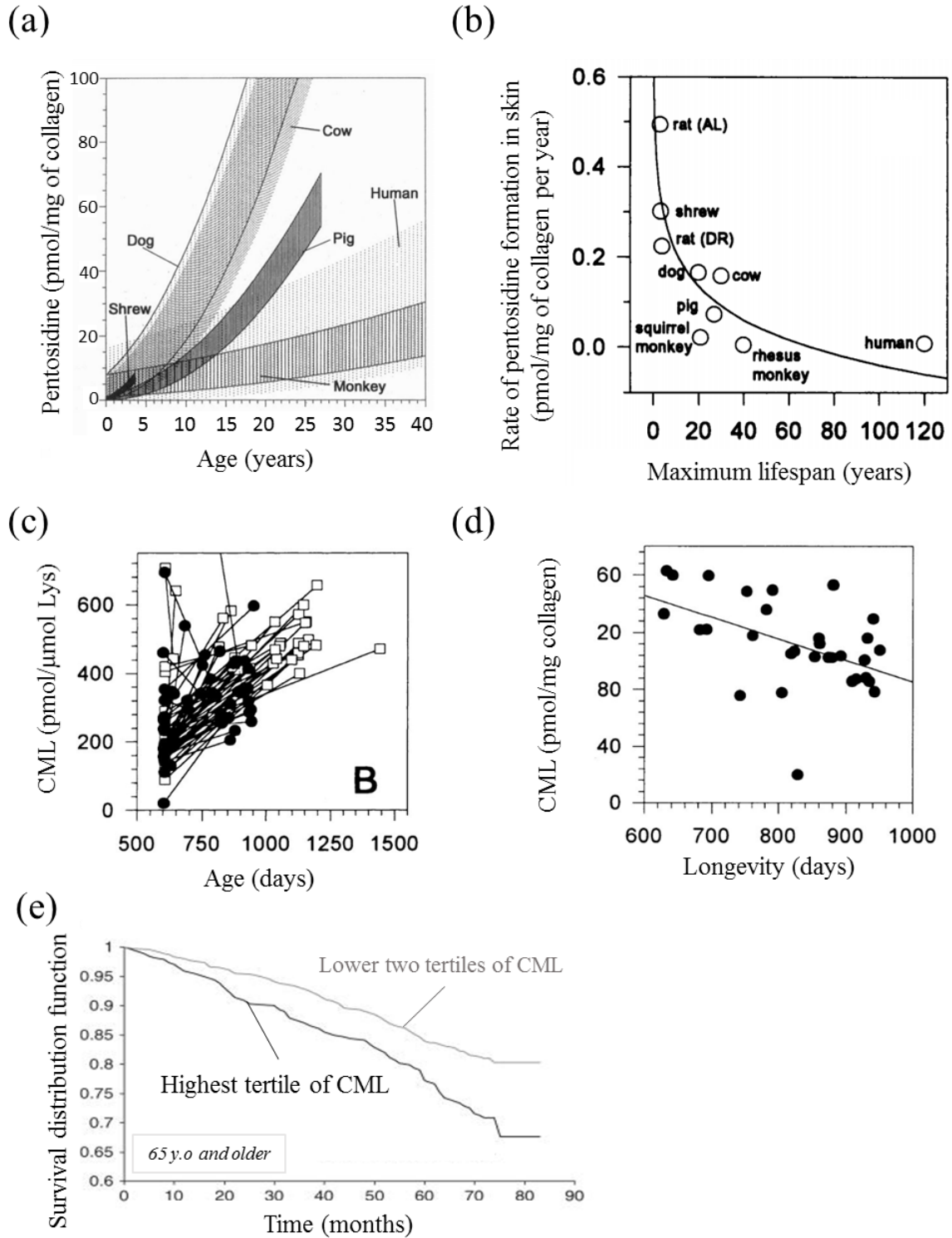
## 2.4 | AGEs during ageing

The first major study to show a clear link between ageing and AGEs was probably the results published by Sell *et al.* in 1996 where they showed that skin pentosidine accumulated much faster in short-lived organisms such as rats, shrews or dogs, than in longer-lived organisms such as primates, including humans (**Fig. 16. a & b**)<sup>154</sup>. Subsequent results regarding skin CML accumulation in mice showed similar results (**Fig. 16. c & d**), CML levels increase with age and there is a correlation between CML level at 605 days and mouse longevity ( $r = -0.50$ ,  $p = 0.005$ )<sup>585</sup>. Similar results have been reported in humans (**Fig. 16. e**) as the highest tertile of serum CML concentrations, at 65 years old and older, was associated with greater all-cause mortality<sup>586</sup>. In senescent fibroblasts, while the level of mitochondrial DNA glycation decreased, glycation of genomic DNA and of some proteins increased<sup>587</sup>. This increase of AGEs with age might be favoured by the decrease of *GLO1* expression at old age<sup>588,589</sup>.

Therefore, it is highly possible that, while perhaps not causal, there is at least a strong association between the levels of AGEs and longevity.

In addition, more specific changes, related to tissue ageing, have been reported. As already mentioned, some of the most striking observations concern tissue containing long-lived proteins. In the ageing lens, multiple AGEs exhibit elevated concentrations and have been correlated with cataract severity and occurrence. Interesting experiments have shown that *in vitro* glycation of lens proteins lead to a similar phenotype as that seen in cataracts, hence it is very likely that AGEs are indeed responsible for ageing of this tissue<sup>561,582,590,591</sup>.





**Figure 16. Association of AGEs and longevity.** Accumulation of pentosidine over time in mammals' skin (a). Correlation of the rate of pentosidine formation in skin and maximum lifespan in mammals (b). Accumulation of CML with time in skin of mice fed ad libitum (filled circles) or calorie restricted (open squares) (c). Correlation of mouse longevity and the level of skin CML at 605 days (d). Human survival beyond 65 years-of-age for all-cause mortality as a function of plasma CML concentrations (e). Modified after Sell et al., *PNAS*, 1996; Sell et al., *FASEB* 2000; Semba et al., *J. Am. Geriatr. Soc.*, 2009

As already mentioned, the accumulation of AGEs occurs in multiple tissues including skin<sup>559,582,583</sup>, dentine<sup>592–594</sup>, intervertebral disks<sup>595</sup>, cartilage<sup>596</sup>, tendons<sup>597,598</sup>, skeletal muscles<sup>599</sup>, lungs<sup>600</sup>, the cardiovascular system<sup>601–603</sup> and kidneys<sup>602,604</sup> and it is thought that it induces an overall stiffening and diminished organ efficacy<sup>605</sup>. Importantly, glycation of arteries has whole-body consequences since arterial stiffness causes hypertension potentially because of a loss of aorta elasticity<sup>283,606–608</sup>. These parameters increasing with age, glycation could therefore be a major driver of arterial ageing and the ageing of many other tissues.

These effects are strongly linked to the glycation of long-lived proteins, the turnover of which can be very slow. On the other hand, glycation of proteins with a rapid turnover can be imperceptible because of this turnover rate, but may also lead to an increased stability which, as suggested above, can lead to amyloidosis<sup>609</sup>, a common feature of ageing<sup>610</sup>. Finally, circulating AGEs, which include glycated circulating proteins and cleaved glycated endogenous or exogenous proteins, could also play a role in ageing. The question of whether their simple accumulation is detrimental remains open but it has been shown that the presence of receptors for these AGEs is essential for many of the associated deleterious effects to become apparent. Notably, it has been shown by our research group that a CML-enriched diet lead to multiple features of ageing in mice such as a decreased aortic endothelium-dependent relaxation, and an increase in CML content of vascular walls and the surface of aortic walls, elastin disruption and pulse wave velocity<sup>288</sup>. Other authors have suggested that increased pulse wave velocity in response to high plasma AGEs in humans was due to a pressure-independent role, which we demonstrated to be correct as the deletion of the receptor for AGEs (RAGE) completely protected against all of these effects in mice<sup>288,611</sup>.

## 2.5 | AGEs and age-related diseases

Given the nature of glycation, it is hard to differentiate physiological ageing from disease, as exemplified by the role of glycation in the cataract onset. Indeed, the consideration of glycation itself as a physiological process is questionable. Glycation is a widespread process but, contrary to oxidation, no study has yet shown the participation of glycation to be important for the functioning of cellular mechanisms. Rather, we should consider that protein glycation is to proteins as DNA damage is to DNA. Therefore, glycation is a *normal* process, but also a *non-physiological* process. A brief description of the role of glycation in *non-normal* processes, contrary to the ageing processes evoked in the previous section, is therefore required.

Owing to the chronic hyperglycaemia characteristic of diabetes, glycation is one of its major consequences and AGEs are highly elevated in type 2 diabetes patients<sup>612</sup>. A number of diabetes complications result from glycation, including nephropathies, neuropathies, retinopathies and cardiomyopathies<sup>461,510,574</sup>. There is not only an association between these complications and AGEs, but knock-down of *glo-1* leads to similar features while its overexpression mostly prevents them<sup>613–616</sup>. Results suggest that AGEs are the source of inflammation, oxidative stress and carbonyl stress which participate in AGE-associated damage<sup>510,574</sup>. As a consequence, diabetes can be considered as an ageing-accelerating condition whose outcomes are driven, at least in part, by glycation.

AGEs have also been found at elevated concentrations in a number of age-related diseases. It has been found that in the brains of AD patients, AGEs were associated with A $\beta$  plaques or tau aggregation and glycation could be responsible for these aggregations<sup>578–580,617</sup>. Accordingly, AGE levels in the cerebrospinal fluid and serum of these patients is increased<sup>618,619</sup>. Similar findings have been made regarding PD<sup>575,620,621</sup>. Furthermore, DJ-1 might be important to prevent the onset of this disease as a low level or activity of DJ-1 both favour PD while its overexpression is neuroprotective<sup>531,622–624</sup>.

Further, AGEs have been implicated in age-related diseases of the bone such as osteoporosis, associated with a fracture risk independent of bone density<sup>625,626</sup>, osteoarthritis<sup>627,628</sup> or rheumatoid arthritis<sup>629</sup> which is associated with periodontitis, also influenced by AGEs<sup>452,630</sup>. As already mentioned, AGEs are increased in skeletal muscle with age and are also associated with sarcopenia<sup>599,631</sup>. Finally, AGEs are also often associated with cancer<sup>632–634</sup>.

**Box 4 – Glycation and AGEs**

- Glycation leads to the production of irreversible protein modifications by carbonyl compounds: AGEs
- *In vivo*, AGE accumulation is favoured by chronic hyperglycaemia and ageing while various enzymatic systems, protein turnover and renal excretion limit it
- *In vitro*, dietary AGEs are favoured by high temperature, low moisture and are modulated by the pH
- Protein glycation can lead to their degradation or dysfunction, to the formation of cross-links or their interaction with receptors
- AGEs play an important role in ageing and longevity, in a number of inflammatory diseases and in complications of diabetes

Glycation has many consequences through the alteration of proteins, nucleic acids and lipids and may be a major driver of ageing. However, while it is in itself detrimental, a large part, if not most, of their noxious properties are linked to the signalling cascades of receptors of AGEs. In the next section, there follows a description of the most-studied receptor for AGEs, RAGE, whose properties explain many of the consequences of AGEs that have been detailed so far. The existence of this receptor also suggests that dietary AGEs may have detrimental effects.

### 3 | The Receptor for Advanced Glycation End-products

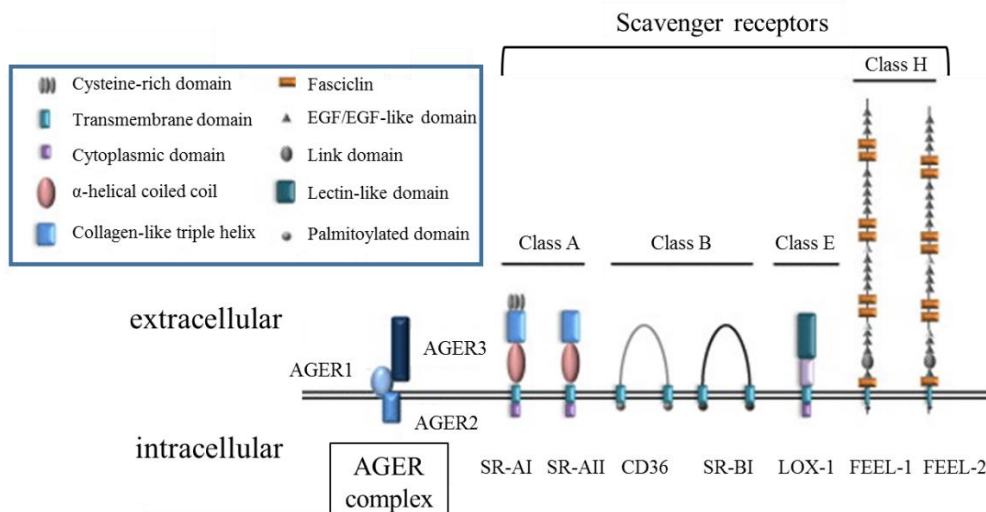
#### 3.1 | The receptors for AGEs

##### Box 5 – RAGE

- What is RAGE and how are its expression and stimulation modulated?
- What is the physiological role of RAGE?
- What is its role in pathologies?

Thus far the effects of AGE accumulation have been described, but an important mode of action for these compounds resides in their ability to bind to receptors. Various receptors for AGEs have been reported, including RAGE, but also the AGE receptor 1 (AGER1, or oligosaccharyltransferase 48 (OST-48)), AGER2 (or 80K-H) and AGER3 (or Galectin-3), which together form a complex, as well as scavenger receptors (SR) such as macrophage SR types I and II (class A scavengers), cluster of differentiation (CD) 36 and SR-B1

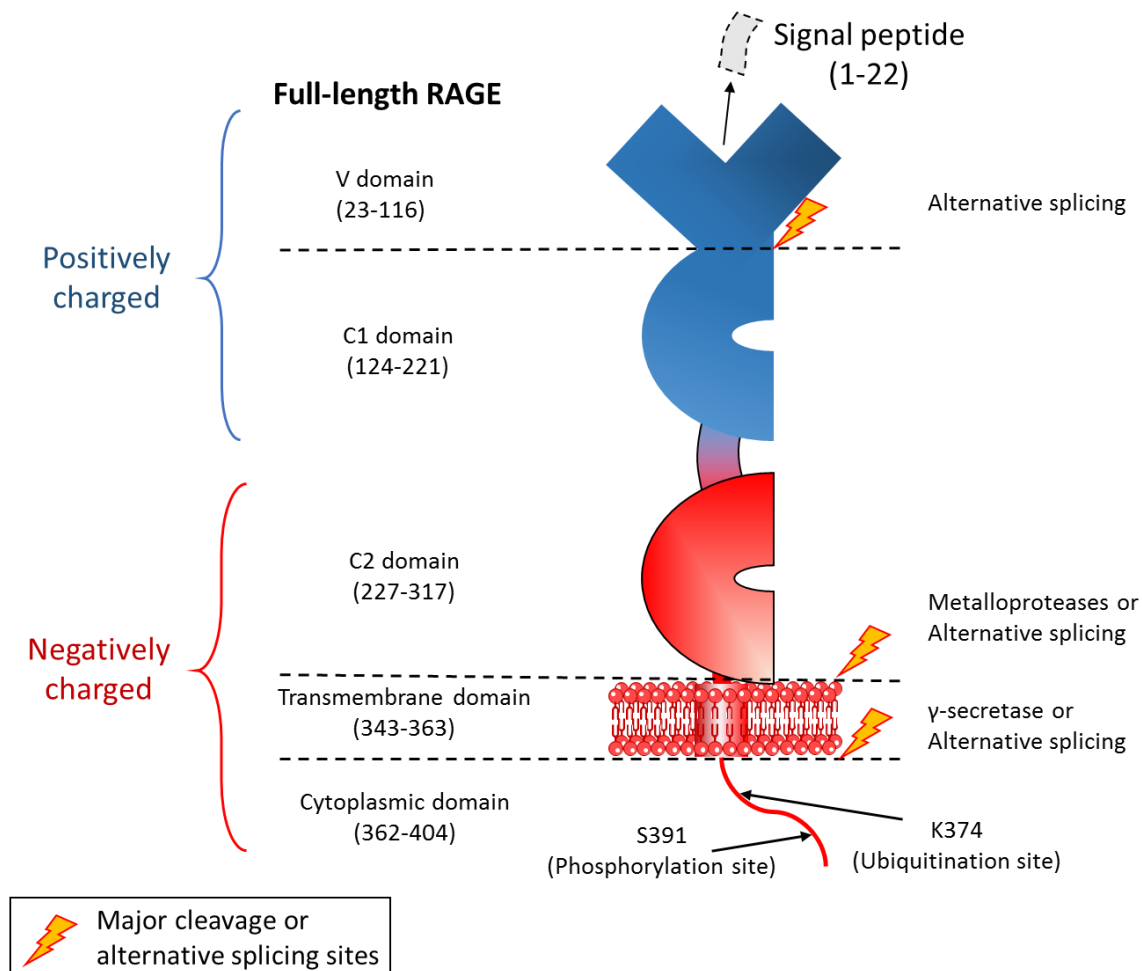
(class B scavengers), lectin-like oxidized LDL receptor-1 (LOX-1) (class E scavenger) and fasciclin, EGF-like-, laminin-type EGF-like, and link domain-containing scavenger receptor-1 (FEEL-1) and FEEL-2 (class H scavengers) (**Fig. 17**)<sup>635</sup>. None of these receptors is specific for AGEs and while some are often considered beneficial, such as AGER1<sup>636–639</sup> (which is nevertheless debated<sup>640</sup>), the study of RAGE has provided an increasing body of data describing its role in detrimental mechanisms. Given the link of AGEs with ageing and the growing evidence for the negative effects of RAGE signalling, it is highly possible that this receptor plays a key role in AGE-induced ageing.



**Figure 17. Non-RAGE receptors for AGEs.** The receptors for AGEs other than RAGE are described here with AGER1, AGER2 and AGER3 forming a complex at the membrane, and scavenger receptors from class A, B, E and H scavenger receptors, also localised at the membrane. Modified after Ott et al., *Redox Biol.*, 2014

### 3.2 | RAGE structure

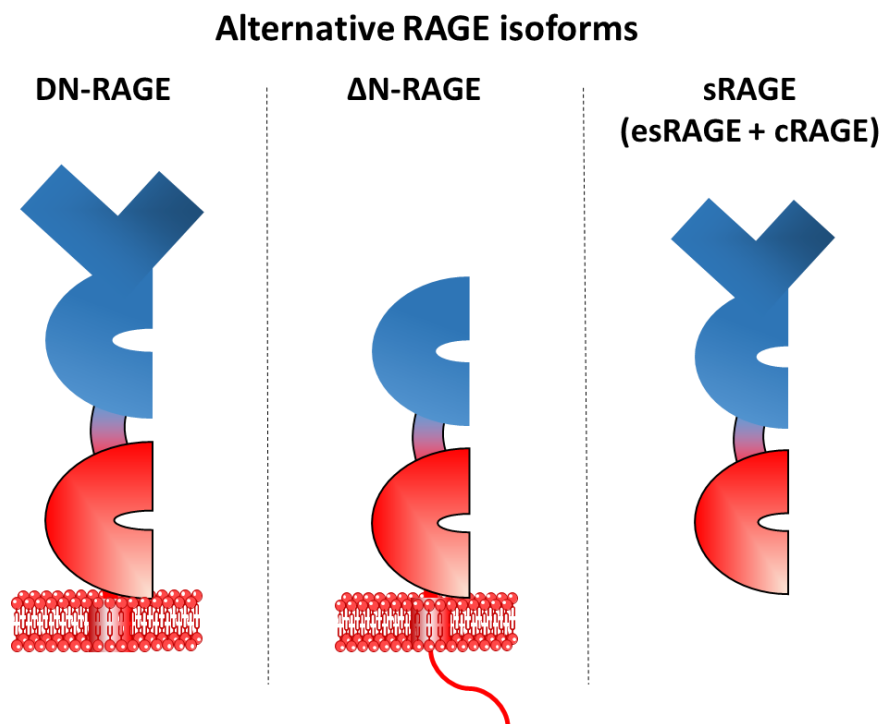
RAGE is a transmembrane receptor comprised of an extracellular domain, a transmembrane helix and an intracellular domain. Its extracellular domain is divided in three sub-domains: a V domain is directly associated to a C1 domain and a flexible linker then connects the C1 domain to a C2 domain<sup>641-644</sup> (**Fig. 18**). The V and C1 domains are positively charged while the C2 domain, the transmembrane domain and the cytoplasmic domain are negatively charged. There are, however, some negatively charged areas in the VC1 domain that are necessary for interaction with ligands<sup>644-646</sup>.



**Figure 18. Full-length RAGE structure.** RAGE comprises V, C1 and C2 extracellular domains, a transmembrane domain and an intracellular domain. Amino acid number range is indicated in brackets. The signal peptide is cleaved before RAGE is localised at membrane. The positively charged areas are in blue while the negatively charged areas are in red. Dotted lines represent major cleavage or alternative splicing sites leading to various RAGE isoforms

There exist different RAGE isoforms that are derived from the above structure (**Fig. 19**). This first isoform is commonly named full-length RAGE (FL-RAGE) or simply RAGE. Other

isoforms are either the result of alternative splicing or cleavage of FL-RAGE. Other important isoforms are the dominant-negative RAGE (DN-RAGE) whose intracellular domain is lacking as a result of cleavage by  $\gamma$ -secretase or alternative splicing<sup>647,648</sup>, N-truncated RAGE ( $\Delta$ N-RAGE), lacking the V domain because of alternative splicing<sup>648,649</sup>, and soluble RAGE (sRAGE) which comprises the endogenous soluble RAGE (esRAGE), resulting from alternative splicing<sup>650</sup>, and the cleaved RAGE (cRAGE), resulting from FL-RAGE constitutive and inducible cleavage of the extracellular domain by metalloproteinases (including A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) and multiple MMPs, varying following organs and conditions)<sup>651–653</sup>. The remaining intracellular domain attached to the membrane can further be released by  $\gamma$ -secretase<sup>651,654</sup>. Other isoforms have been reported, such as isoforms specific to the brain, but they are much less expressed or studied<sup>648</sup>. Finally, other splice variants are also described but their significance is unknown as no associated protein has been reported so far<sup>650</sup>.



**Figure 19. RAGE isoforms.** Major RAGE isoforms produced after alternative splicing or cleavage of the full-length RAGE: dominant negative RAGE (DN-RAGE), without the intracellular domain; N-truncated RAGE ( $\Delta$ N-RAGE), without the V domain; soluble RAGE (sRAGE), gathering the endogenous soluble RAGE (esRAGE) and the cleaved RAGE (cRAGE), without the transmembrane and the intracellular domain. Positively charged areas are in blue while the negatively charged areas are in red

### 3.3 | RAGE expression

The gene coding for RAGE belongs to a group of genes coding for cell adhesion molecule (CAM) proteins, suggesting a common origin and properties. While *RAGE* first appeared in mammals, the locus to which it belongs probably emerged in earlier metazoans. This locus belongs to the major histocompatibility complex Class III region and is located on chromosome 6p21.31 in humans, together with genes involved in immunity and inflammation<sup>655–657</sup>. These characteristics, together with other properties that will be described in the next sections, such as its ability to bind to many structurally different ligands (section 3.4) or its pro-inflammatory response to stimulation (section 3.5), strongly suggest that RAGE could be considered a pattern recognition receptor (PRR)<sup>658,659</sup>.

*RAGE* possesses 11 exons leading to a ~55 kDa protein when completely transcribed and translated. However, multiple mRNA variants exist and to date at least 20 variants have been reported<sup>647,650,660</sup>. As stated earlier, many of these variants lack a corresponding protein<sup>650</sup>. Some major variants are reported however, such as RAGE\_v1 (esRAGE), RAGE\_v2 ( $\Delta$ N-RAGE) and RAGE\_v20 (DN-RAGE) (**Fig. 20. a**). It should be noted that multiple alternative splicings can produce an esRAGE and RAGE\_v1 is just one of them<sup>660</sup>. RAGE\_v1 is transcribed when intron 9 is included while exon 10 and intron 10 are excluded. RAGE\_v2 is expressed when intron 1 is included. This results in the selection of an early stop codon that will allow the use of a new start codon located in exon 3, hence the absence of the V domain. RAGE\_v20 is expressed by adding a few bases during the splicing of intron 10. It results in a frameshift and in the selection of an early stop codon, preventing the translation of 16 amino acids of the intracellular domain, including the phosphorylation site. At least RAGE\_v1 and RAGE\_v2 are conserved between humans and mice<sup>660</sup>.

Three binding sites for NF- $\kappa$ B and two binding sites for specificity protein 1 (SP1) have been localized in the *RAGE* promoter, which seems to be a TATA-less promoter. Two of the three binding sites for NF- $\kappa$ B and SP-1 binding sites were shown to be necessary for the upregulation of *RAGE* mRNA transcription<sup>661,662</sup>, thus RAGE expression is notably induced by pro-inflammatory events. Other transcription factor binding sites have been reported, notably apurinic/apyrimidinic-2 (AP-2),  $\gamma$ -interferon response element ( $\gamma$ -IRE) and a nuclear factor for interleukin-6 expression (NF-IL6) binding sites, but their role has either not been thoroughly investigated or is minor.



A major transgenic *RAGE*<sup>-/-</sup> murine model was produced by Constien *et al*<sup>663</sup>. These mice are completely unable to produce any RAGE isoform while continually expressing enhanced green fluorescent protein (EGFP) thanks to a thymidine kinase promoter (**Fig. 20. b**).



**Figure 20. RAGE gene.** RAGE gene and main variants. RAGE is composed of 11 exons (orange boxes). *NF-κB* and *SP1* can both activate RAGE transcription via binding sites located in its promoter. Alternative splicing and intron inclusion can lead to different variants of RAGE mRNA and conditions to produce major RAGE isoforms are represented in yellow for N-truncated RAGE ( $\Delta$ N-RAGE), in blue for endogenous soluble RAGE (*esRAGE*) and in green for dominant negative RAGE (*DN-RAGE*) (a). Construction of the *Rage* knock-out in mice by Constien *et al.*, *Genesis*, 2001. Transgenic mice were produced removing *Rage* sequence from intron 1 to intron 7 while *Egfp* gene, with a thymidine kinase (*tk*) promoter, was inserted in the opposite direction (b)

RAGE expression varies significantly according to tissue type. First reports of RAGE expression were made by Brett *et al.*, using human and bovine adult tissues<sup>664</sup>. Enzyme-linked immunosorbent assay (ELISA) showed that RAGE was highly expressed in skeletal muscles and lung (~5 000 pg/mL), then heart (~2 700 pg/mL), liver (~1 500 pg/mL), kidney and uterus (~500 pg/mL). It was only poorly expressed in the brain. However, there were serious discrepancies with mRNA level as this was massively expressed in the lung, but much lower in heart and skeletal muscle and even less in other organs. This discrepancy can be explained by translational regulation or simply by a lack of specificity of the anti-RAGE antibody. There is now a general consensus that RAGE is indeed highly expressed in lungs, especially in alveolar epithelial cells, and increases from neonatal development to adulthood<sup>665</sup>; its expression in other adult tissues, although wide spread, is much lower at homeostasis<sup>666-668</sup>. RAGE expression has also been described in most immune cells as well<sup>669-675</sup>.

Literature about RAGE expression during embryogenesis is scarce. It is expressed after a few days in early blastocysts<sup>635,676</sup> and its expression has been associated with embryonic vessels, neurites and, more importantly, lung organogenesis<sup>665,677,678</sup>. However its depletion in mice is not lethal and does not involve major developmental defects<sup>663</sup>. On the contrary, overexpression of RAGE before alveolarisation notably leads to pulmonary dysplasia<sup>679,680</sup>.

RAGE expression during ageing has not been thoroughly investigated either. While its expression remains high in the older lung<sup>681</sup>, it increases in the heart, possibly due to a reduced degradation<sup>682,683</sup>. sRAGE, however, is reduced in plasma with ageing, but its significance and its use as a biomarker remain controversial<sup>684,685</sup>.

Most of the other RAGE variants are significantly less expressed in most tissues in humans, mice and other species. However, the  $\Delta$ N-RAGE mRNA is often highly expressed, sometimes even higher than the canonical RAGE<sup>650,660,686</sup>. Of particular note, sRAGE is highly expressed in the human hippocampus, much more so than other variants, and is thought to be released from the cytoplasm to the extracellular medium in response to stimuli such as A $\beta$ -peptides or AGEs<sup>648,649,666</sup>.

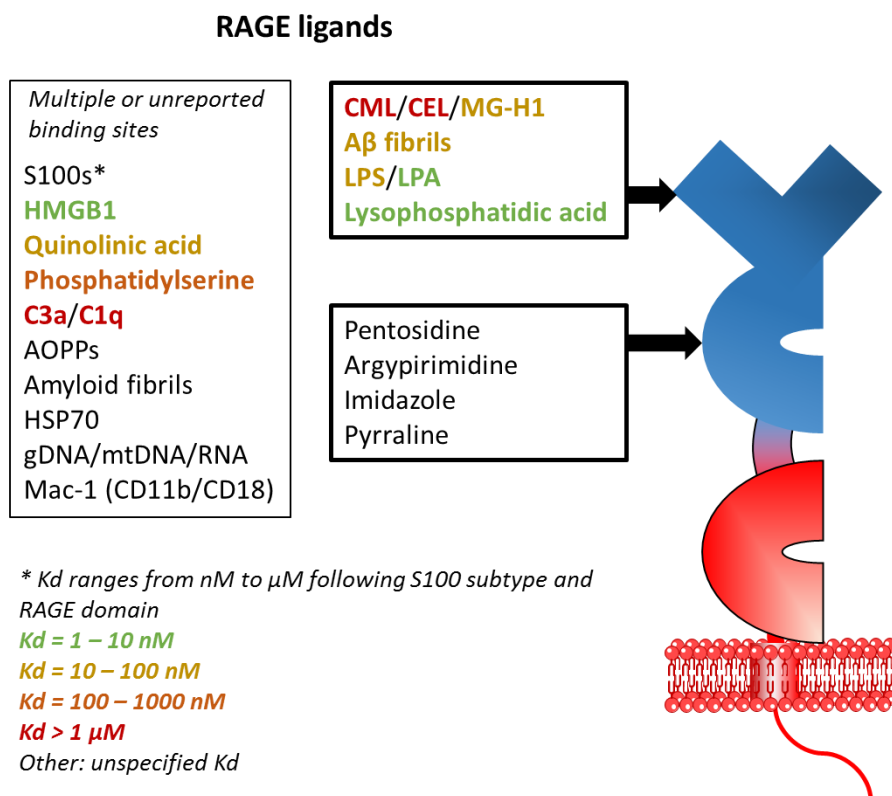
RAGE has also been reported to be able to relocate to the nucleus in lung cells where it could enhance DNA repair activities<sup>687</sup>. However, to date only one study supports this result and more work is needed to confirm this finding.

Finally, genetic polymorphisms in *RAGE* have been described, resulting in potential changes regarding the above-mentioned expression profiles which can be associated with different conditions. As an example, the G82S polymorphism has been associated with rheumatoid arthritis, ulcerative colitis, diabetes and complications of diabetes<sup>688-691</sup>. Accordingly, this polymorphism has been associated with an amplified inflammatory response upon ligands binding to RAGE<sup>692</sup>. Other polymorphisms have been detected in the *RAGE* promoter and were also associated with diabetic complications<sup>693,694</sup>, but also with several other diseases<sup>695-697</sup>.

### 3.4 | RAGE ligands

One of the main characteristics of RAGE is the number and variety of its ligands (**Fig. 20**). To date, they number more than 20, all of which have very different origins, roles and structures. A number of them are chemically modified proteins, peptides or amino acids, such as AGEs (including CML, CEL and MG-H1), advanced oxidation protein products (AOPPs) or phosphatidylserine, while amyloid fibrils are structurally modified proteins. Proteins involved in inflammatory processes can also bind to RAGE (high-mobility group box 1 (HMGB1), S100 proteins). We also find as RAGE ligands the components of bacterial walls, lipopolysaccharides (LPS), smaller molecules such as quinolinic acid or lysophosphatidic acid, and various kinds of nucleic acids. Many of these ligands can be categorised as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) – typical PRR ligands.

The best-characterised RAGE ligands are described in the following section, with regard to their binding to this receptor.



**Figure 21. RAGE ligands.** Most described RAGE ligands bind to the V domain of RAGE. But some ligands, such as S100s, can bind to multiple sites. It is predicted that the AGEs pentosidine, argypirimidine, imidazole and pyrrolidine can bind to the C1 domain. Estimated  $K_{ds}$  range from  $\sim 1$  nM to over  $1$   $\mu$ M. Positively charged areas are in blue while the negatively charged areas are in red. Abbreviations are available in List of abbreviations. Modified after Teissier et Boulanger, *Biogerontology*, 2019

### 3.4.1 | Advanced glycation end-products

AGEs are the first ligands of RAGE to be described, hence its name. However, owing to the heterogeneity of AGEs described earlier, there is no guarantee that they can all bind to RAGE. Indeed, AGE binding to RAGE was first described using uncharacterised AGEs, such as BSA-AGE where BSA has been incubated with glucose for a set time and temperature but without any other control of the type of glycation nor the AGEs produced<sup>642,698</sup>. Subsequent work has provided more details on the binding affinity of various AGEs to RAGE. Nuclear magnetic resonance (NMR) and fluorescence titration experiments have shown that Methylglyoxal-derived hydroimidazolone-1 (MG-H1) was able to bind to the V domain of human RAGE with a  $K_d = 40 \text{ nM}$ <sup>699</sup>. Similar experiments showed that peptide- and protein-bound CML and CEL were also able to bind to RAGE with a  $K_d = 100 \text{ }\mu\text{M}$ <sup>643,645</sup>. However, available data from binding and competition assays suggest that there is an absence of binding of free CML and CEL to RAGE<sup>645,700</sup>. *In silico* docking analysis predicts that pyrroline, imidazole, argyrimidine and pentosidine are all able to bind to the C1 domain of RAGE<sup>701</sup>.

### 3.4.2 | HMGB1

HMGB1, or amphoterin, is considered to be the most abundant non-histone, DNA-binding protein. It is mainly localized in the nucleus but can transit to the cytoplasm *via* nuclear pores when acetylated, thereby regulating its function. By interacting with proteins involved in autophagy, cytosolic HMGB1 potentially regulates associated apoptotic processes<sup>702</sup>. HMGB1 is involved in nucleosome formation and can modulate transcription by interacting with transcription factors and histones<sup>703,704</sup>. Its role in DNA repair remains elusive and a subject of debate<sup>705</sup>. Its overexpression in breast cancer sensitizes cancer cells to cisplatin, an antitumor drug<sup>706</sup>, and its depletion enhances the excision repair mechanism following cisplatin treatment<sup>707</sup>, but it has also been shown to play a role in genomic and mitochondrial DNA repair<sup>708,709</sup>. HMGB1 can also be released in the extracellular medium by active or passive processes. Active release generally involves a severe stress, such as inflammation or ischemia, which can inhibit HMGB1 deacetylation and lead to its sequestration into the cytoplasm until it is finally secreted<sup>710-713</sup>. Passive release of HMGB1 occurs in conditions such as necrosis<sup>714</sup>. HMGB1 is increased with age in mouse kidney and liver and is also associated with macrophage infiltration<sup>715</sup>. Circulating HMGB1 is associated with a set of pro-inflammatory processes, senescence and several pathologies<sup>716,717</sup>. Among the RAGE ligands, HMGB1 has one of the highest reported affinities with a  $K_d = 6 - 10 \text{ nM}$  as determined by an *in vitro* saturation binding assay<sup>678</sup>.

### 3.4.3 | Calgranulins

Another group of molecules, calgranulins, has also been reported to interact with RAGE. Calgranulins are a family of S100, EF-hand, calcium-binding proteins which are considered to be sensors of intracellular  $\text{Ca}^{2+}$  involved in different cellular processes such as cell migration, cell proliferation or cell differentiation<sup>718</sup>. Dysregulated levels of S100 have been associated with cancer, inflammatory response and cardiomyopathies<sup>719–721</sup>. S100B is increased with age in mouse kidneys and liver and is also associated with increased infiltration of activated macrophage<sup>715</sup>. The majority of the 21 members of the S100 family have been reported to interact with the V, C1 and even C2 domain of RAGE<sup>641,722</sup>. Some work suggests that S100B interacts with positively-charged areas of the V domain<sup>645</sup>, while others suggest an hydrophobic  $\text{Ca}^{2+}$ -dependent interaction in the C'D loop of the V domain<sup>646</sup>. The calculated  $K_d$  ranged from  $\mu\text{M}$  to  $\text{nM}$  and were positively affected by S100 oligomerization<sup>723,724</sup>.

### 3.4.4 | Amyloid fibrils

Amyloid fibrils have also been shown to interact with RAGE. Amyloid fibrils are defined by  $\beta$ -sheet-rich, super-coiled, insoluble structures forming fibrils. These fibrils can be formed by the nucleation of soluble proteins to form protofilaments, which in turn give rise to amyloid fibrils. Many proteins such as the  $\text{A}\beta$  peptide, serum amyloid A (SAA), Ig light chains or even insulin have been reported to be responsible for different kind of amyloidosis<sup>609,725</sup>. While amyloidosis due to proteins such as apolipoprotein A-II (apoA-II) is very rare in humans, it is common in other species like mice mainly because of species-specific differences in methionine content and dimerization, which is also why amyloidosis is specific to some proteins<sup>726–729</sup>. Amyloidosis has been linked to a number of pathologies including AD where  $\text{A}\beta$  plaques are increased<sup>730–732</sup>, scrapie or bovine spongiform encephalopathy caused by prion proteins (PrP)<sup>733,734</sup>, or primary systemic amyloidosis characterized by the accumulation of amyloid light (AL) and heavy (AH) chains in multiple organs<sup>725,735</sup>. Among the amyloid fibril RAGE ligands,  $\text{A}\beta$  is the best characterized and interacts with the V domain with a  $K_d = 70 - 80 \text{ nM}$ . This interaction plays a role in  $\text{A}\beta$  neurotoxicity and circulating  $\text{A}\beta$  influx into the brain<sup>736,737</sup>. Other amyloid fibrils of SAA, prion-derived peptides and amylin have also been reported as ligands of RAGE. Notably, these same proteins presented in a random conformation, as well as an all- $\beta$  sheet protein erabutoxin B, were unable to interact with RAGE, suggesting a specificity of sequence and conformation is important for this interaction<sup>738,739</sup>. sRAGE was, however, reported to interact with both soluble and fibrillar transthyretin with equal affinity ( $K_d = 120 \text{ nM}$ )<sup>740</sup>.

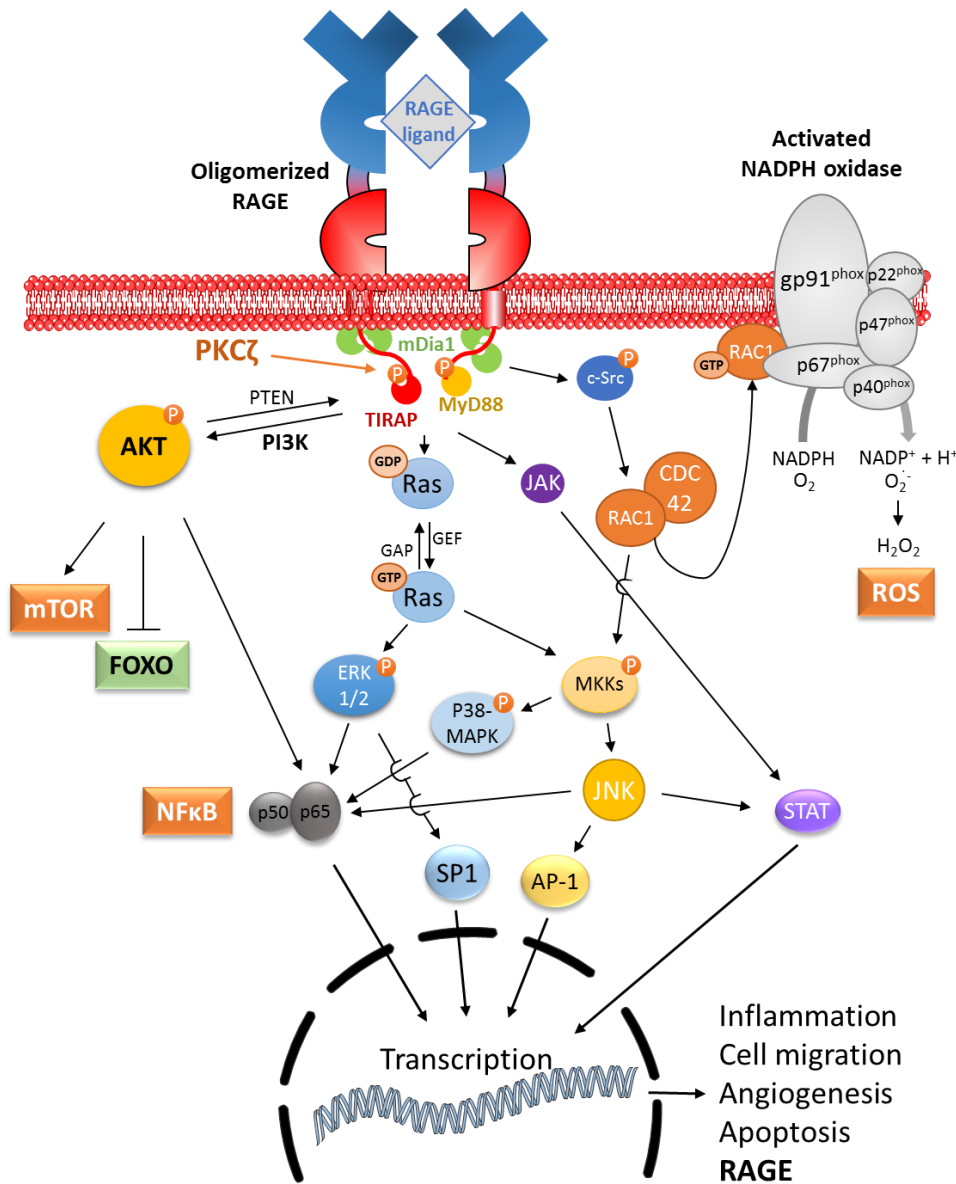
### 3.4.5 | Complement components

The complement system is a major component of immunity and is characterized by multiples cleavage products. Importantly, it participates in pathogen lysis through the formation of a membrane attack complex (MAC) at their surface, allowing free diffusion of molecules through this pore and engendering cell death. It is also involved in a process called “antigen opsonisation”, by which addition of complement molecules to pathogens’ membrane facilitates recognition by immune cells. Chemotaxis of innate immune cells may also be enhanced by complement components. The complement component C3a, resulting from C3 cleavage, is an anaphylatoxin which is possibly involved in systemic lupus erythematosus<sup>741,742</sup>. C3a was able to bind to RAGE in an ELISA assay and formed a ternary complex with oligonucleotides<sup>743</sup>. Another complement component, C1q is able to initiate complement activation and antibody-independent opsonisation and its absence is also associated with systemic lupus erythematosus<sup>744</sup>. Interaction of monomeric C1q with RAGE exhibited weak affinity ( $K_d = 5.6 \mu\text{M}$ ) in surface plasmon resonance (SPR) analysis, but multivalent bonds may enhance this binding. This interaction is suspected to enhance monocyte phagocytosis<sup>745</sup>, but while few studies have investigated the interaction between components of the complement and RAGE, it plays a key role in immunity and it thus merits further characterization in the context of inflammaging.

### 3.4.6 | Other RAGE ligands

Other ligands of RAGE, though relatively poorly studied, have also been reported. LPS and its lipid component A both interact with the V domain, potentially at the KGAPKKPPQRLEWKLN site, with a  $K_d = 35 \text{ nM}$  and  $K_d = 2 \text{ nM}$ , respectively<sup>746</sup>. RAGE was also able to interact with CpG oligonucleotides, double strand DNA and double strand RNA<sup>646,743</sup>. The leukocyte cell surface molecule Mac-1 (CD11b/CD18) interacts with RAGE with an undefined  $K_d$ <sup>658</sup>. Phosphatidylserine binds to sRAGE with a  $K_d = 0.563 \mu\text{M}$ <sup>747</sup>. AOPPs have been reported to interact with RAGE in an ELISA assay<sup>748</sup>, as was extracellular heat-shock protein 70<sup>749</sup>. Quinolinic acid, an undesirable and excitotoxic tryptophan metabolite, interacts with RAGE at multiple sites in the VC1 domain with a mean  $K_d = 43 \text{ nM}$ <sup>750</sup>. Lysophosphatidic acid, an endogenous phospholipid with roles in homeostasis and diverse pathologies, also binds to the V domain of RAGE with a  $K_d = 9 \text{ nM}$ <sup>751</sup>.

### 3.5 | RAGE and its inflammatory signalling



**Figure 22. RAGE signalling.** Major identified signalling pathways upon RAGE stimulation by its ligands. Oligomerization of RAGE upon ligand binding provokes its phosphorylation on Ser391 by PKC $\zeta$ , thus recruiting TIRAP and MyD88, and activation of mDIA1 by other mDIA1. The PI3K/AKT/mTOR pathway is activated while FOXO is inhibited. Multiple MAPK pathways are also triggered, activating ERK 1/2, p38-MAPK and JNK. The JAK/STAT is also activated upon RAGE stimulation. Finally, c-Src is also phosphorylated, activating RAC1/CDC42, which can activate MAPK kinases (MKKs) and more importantly the NADPH oxidase, producing NADP<sup>+</sup> and ROS. Important transcription factors are involved, including NF- $\kappa$ B, SP1, AP-1 and STATs, promoting inflammation, cell migration, angiogenesis, apoptosis and RAGE expression. Oranges boxes: major pro-ageing and pro-inflammatory molecules; green box: pro-longevity molecule

Although different ligands can induce different responses, varying in intensity and the pathways involved, the available literature is insufficiently precise for such distinctions be confidently made. Therefore, what follows hereafter is a description of the most common signalling pathways activated upon RAGE stimulation, irrespective of the ligand (**Fig. 22**).

Upon interaction with its ligands, the intracellular domain of RAGE is phosphorylated by protein kinase C  $\zeta$  (PKC $\zeta$ ) at serine 391 (Ser391). Adaptor proteins toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP) and myeloid differentiation primary response 88 (MyD88) consequently bind to the phosphorylated RAGE to transduce signalling, similar to Toll-like receptors<sup>752</sup>. Mammalian diaphanous 1 (mDia1) is also able to interact *via* its formin homology (FH1) domain to an  $\alpha$ -turn of the intracellular domain of RAGE, very close to the membrane. It is thought that upon binding with its ligands, RAGE forms a cluster (homodimerization, oligomerization<sup>644,645,753</sup> or possibly heterodimerization<sup>753,754</sup>), hereby increasing mDia1 concentration at the membrane. mDia1 is auto-inhibited and interaction between different mDia1 proteins can remove this inhibition. Thus, increasing local mDia1 concentration at the membrane activates mDia1 which can then transduce the signal<sup>755-757</sup>. Of note, while sRAGE function is usually interpreted as a RAGE ligand decoy, it is possible that it could also directly interact with RAGE and prevent its oligomerization<sup>753</sup>.

Diverse pathways are activated in consequence to the interaction of these adaptor proteins to RAGE. Notably, mDia1 is able to activate c-Src and downstream effectors cell division control protein 42 homolog (CDC42) and Ras-related C3 botulinum toxin substrate 1 (RAC1)<sup>755,758,759</sup>. This pathway can notably induce nicotinamide adenine dinucleotide phosphate (NADPH) oxygenase 1 (NOX1) activation and associated ROS production, cell migration and activation of various transcription factors<sup>758</sup>. Other major signalling pathways can be activated upon recruitment of different adaptors and include: the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, c-Jun N-terminal kinase (JNK), the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, and the mitogen-activated protein kinases (MAPK) pathway with extracellular-signal-regulated kinases (ERK) 1/2 or p38-MAPK<sup>755,760-767</sup>. A set of transcription factors, such as NF- $\kappa$ B, SP1, activator protein 1 (AP-1) or STATs, are then activated and can translocate into the nucleus<sup>768-772</sup>. Among the many targets of these transcription factors, the transcription of those genes with pro-inflammatory consequences are up-regulated, notably through NF- $\kappa$ B. Of particular interest is that both NF- $\kappa$ B and SP1 have been described as being able to activate *RAGE* transcription<sup>661,662</sup>. There is therefore a positive feedback loop established upon RAGE stimulation.



Finally, RAGE signalling can be stopped through lysosomal degradation after ubiquitination on lysine 374 (**Fig. 18**), thus also limiting its expression level<sup>773</sup>.

More indirectly, RAGE can participate in inflammation through mitochondrial dysfunction, inflammasome activation and ER stress<sup>659</sup>. GLO1 expression is increased in RAGE<sup>-/-</sup> mice, suggesting that RAGE activity may, either indirectly or directly, downregulate *Glo1*. On the other hand, inhibition of GLO1 leads to accumulation of AGEs and RAGE stimulation<sup>774</sup>.

Regarding other RAGE isoforms, although signalling is obviously lacking with DN-RAGE, there is a chance that some ligands can bind to the  $\Delta$ N-RAGE and induce a similar or modified signalling. However, the absence of the V domain might significantly alter cell-cell or cell-matrix interactions. In addition, the intracellular domain cleavage by  $\gamma$ -secretase results in the production of a short peptide that displays signalling and transcriptional functions<sup>651,654</sup>.

### 3.6 | Physiological role of RAGE

As suggested earlier, RAGE possesses all the characteristics of a PRR: its gene is in a locus comprising many genes of immunity; the variety of its ligands suggest a pattern recognition, possibly governed by ionic interactions; its main ligands are almost all DAMPs (HMGB1, S100s, DNA, RNA) or PAMPs (LPS, DNA, RNA), or else are molecules involved in non-physiological conditions which require resolution (AGEs, amyloid fibrils, complement, Mac-1/CD11b, phosphatidylserine), and engender a pro-inflammatory response. As described earlier, by virtue of being an ubiquitous protein, RAGE is also expressed on innate immunity cells, similar to other PRRs, and its activation mainly triggers PRR-like pro-inflammatory responses, notably through NF- $\kappa$ B signalling<sup>762,764,765,775</sup>.

RAGE has a role in innate immunity and sepsis and RAGE<sup>-/-</sup> mice are protected against lethal septic shock and exhibit significantly reduced NF- $\kappa$ B activity in the lung<sup>776</sup>. According to its suspected functions and its genomic proximity to other immune system components in the major histocompatibility complex (MHC) class III locus<sup>656,777</sup>, RAGE has been labelled a “noncanonical Toll”<sup>778</sup>. The role of RAGE in innate immunity is not straightforward, however. When infected by *S. pneumonia*, RAGE<sup>-/-</sup> mice exhibited better survival, a lower pulmonary bacterial load and a decreased dissemination to both blood and the spleen, suggesting a detrimental role of RAGE in host response<sup>779</sup>. Conversely, *M. tuberculosis* challenge of RAGE<sup>-/-</sup> mice was associated with increased inflammation, pulmonary bacterial load and loss of body weight, while survival decreased<sup>780</sup>.

Despite this evidence, the precise role of RAGE remains elusive and other functions of this receptor have been investigated. Since RAGE expression is significantly greater in the lung, we might expect its function to be more important and obvious. However, its contribution to lung function remains unclear, under physiological or pathological conditions<sup>781</sup>. We have already mentioned that RAGE was expressed during embryonic stages and possibly participated in alveolarisation<sup>677</sup>. However, no major respiratory dysfunctions have been reported in RAGE<sup>-/-</sup> mice. Nonetheless, there is a diminished elastance and an augmented compliance in the lungs of RAGE<sup>-/-</sup> mice, but these same mice were protected against cigarette smoke-induced lung pathology, probably due to a reduced infiltration of macrophages<sup>782</sup>. Other workers have reviewed the negative implications of RAGE in many inflammatory lung diseases<sup>783</sup>. Of note is that, while RAGE is mainly overexpressed in most cancers<sup>669,784,785</sup>, it is on the contrary downregulated in lung cancer<sup>786</sup>.

RAGE's role in lung structure or function remains poorly understood: it could have a physiological role in inflammatory response but, so far, it has mainly been linked to inflammatory diseases and its role in infectious diseases can be detrimental as well as beneficial, depending on the conditions. In line with antagonistic pleiotropy, RAGE's function might be important early in life and become detrimental with time and, as suggested earlier, such properties can be missed under laboratory conditions despite being important in the real world., The precise physiological role(s) of RAGE remain(s) to be fully elucidated, therefore.

The story of RAGE is reminiscent of the Duffy antigen and human susceptibility to a parasite, *Plasmodium vivax*. Deadly malaria is caused by the *Plasmodium falciparum* parasite, but another parasite of the same genus, *P. vivax*, is responsible for a non-lethal but seriously incapacitating form of the disease, notably involving very high fevers (up to 41°C). Population evolution in some African regions has led to a complete protection against this parasite or the disease. It was later shown that individuals protected against *P. vivax* were all Duffy-negative<sup>787</sup>. Indeed, additional work showed that *P. vivax* uses the Duffy antigen as a receptor to invade erythrocytes<sup>788,789</sup>. But despite the absence of this antigen, no major defects have been reported in this population. The role of this protein thus remained elusive for years. It was only later that mounting evidence suggested a physiological, yet not indispensable, role for this protein<sup>790</sup>. Of particular interest, if the parasite were to disappear, the *raison d'être* of the Duffy-negative allele would become meaningless<sup>787</sup>. And this is maybe what happened to *RAGE* or other genes whose function is so hard to grasp: perhaps the main physiological purpose of these genes has disappeared with the passing of time and the many events that govern evolution and life history.

### 3.7 | RAGE-associated pathologies

#### 3.7.1 | Non age-related diseases

RAGE is involved in a number of inflammatory diseases unrelated to ageing, which include inflammatory bowel disease, sepsis and acute respiratory failure<sup>791,792</sup>.

Several *RAGE* polymorphisms were associated with inflammatory bowel diseases, including Crohn's disease and ulcerative colitis<sup>696,793</sup>. Inflammatory bowel diseases are also associated with reduced plasma sRAGE levels<sup>794,795</sup>. In parallel, murine models of colitis and enteritis showed that deletion of RAGE or its inhibition efficiently reduced inflammation development<sup>792</sup>.

Similarly, there are also *RAGE* polymorphisms associated with the risk of the development of sepsis, suggesting a detrimental role of RAGE<sup>697</sup>. However, while most studies suggest that RAGE aggravates the outcomes of sepsis, there are discrepancies in the literature and it is not clear whether RAGE blocking is beneficial<sup>791,796</sup>. It might be dependent on the pathogens responsible for sepsis.

Despite the high basal level of RAGE expression in lungs, it has also been associated with various pulmonary diseases. Various models of acute lung injury have shown an increased expression of RAGE<sup>797</sup> while *Rage* knock-out limited the effects of hyperoxia-induced lung injury<sup>798</sup> and improved alveolar fluid clearance while limiting pulmonary vascular albumin leakage<sup>799</sup>. In addition, RAGE is proposed to be a marker of alveolar type I cells' acute lung injury<sup>800</sup>; it is also central in the pathogenesis of chronic lung diseases such as asthma<sup>801</sup>.

While the role of RAGE has been extensively studied in diabetes<sup>688,802</sup>, whose complications are mostly age-related and will be presented in the next subsection, it has also been shown that RAGE has an important role in foetal dysmorphogenesis during pregnancy of diabetic mice<sup>803</sup>.

#### 3.7.2 | Age-related diseases

Unsurprisingly, RAGE is associated with all the age-related diseases associated with AGEs (Section 2.5). More interestingly, accumulating data show that RAGE is the missing piece of the puzzle that connects AGEs to age-related diseases. For example: the blockade of RAGE prevents bone loss associated to periodontitis in diabetic mice<sup>804</sup>; it has already been mentioned that the G82S polymorphism was associated with rheumatoid arthritis<sup>692</sup>, which is greatly

limited when blocking RAGE<sup>805</sup>; osteoarthritis is similarly affected by RAGE signalling but RAGE<sup>-/-</sup> mice are protected against it<sup>689,806,807</sup>, while osteoporosis is equally affected<sup>625,626,808,809</sup>.

RAGE is also central in all kinds of micro- and macroangiopathies<sup>810-813</sup>, many other aspects of cardiovascular disease such as left ventricular hypertrophy, arrhythmia<sup>814</sup> or accelerated atherosclerosis<sup>693,815-819</sup>, as well as in normal vascular ageing<sup>288,811,820,821</sup>. On the other hand, high plasma sRAGE levels are associated with a slower development of cardiovascular ageing features<sup>822</sup>. RAGE inhibition successfully limited vascular ageing and aorta inflammation, mimicking the effect of exercise<sup>823</sup>. Thus, it is accordingly involved in all kinds of complications of diabetes such as retinopathies<sup>824</sup>, neuropathies<sup>825-827</sup> or nephropathies<sup>828,829</sup>. It is therefore not surprising that RAGE co-localises with AGEs in diabetes<sup>830</sup> and it has been proposed that elevated sRAGE in type-2 diabetes patients is aimed at limiting RAGE signalling<sup>612</sup>.

Along the same lines, similar to AGEs, RAGE has been implicated in AD where its pro-inflammatory signalling could increase inflammation in AD brains, critical for the formation of A $\beta$  plaques and prevented when blocking RAGE<sup>831,832</sup>. In addition, RAGE is also important in A $\beta$  peptides flux across the brain blood barrier<sup>736,833</sup>. Accordingly, a RAGE antagonist, Azeliragon (TTP488) recently underwent clinical trials, which terminated at phase 3 owing to a lack of efficacy in the selected cohort. Importantly, however, no significant undesirable effects were reported or were responsible for the cessation of the trial, and it is suggested that the lack of efficacy might be explained by late initiation of treatment: RAGE blocking remains of interest, therefore, as a therapeutic approach in the early stages of cognitive impairment<sup>834</sup>. PD, though to lesser extent, has also been associated with RAGE signalling<sup>835</sup>.

Finally, RAGE has been extensively associated with cancer. RAGE expression is elevated in almost every type of cancer<sup>669,784,785</sup>, except in lung cancer where it is decreased<sup>786</sup>, contrary to its high basal expression in this tissue. Interestingly, it is suspected that RAGE is determinant in the increased occurrence of cancer in diabetes<sup>836</sup>.

**Box 6 – RAGE**

- RAGE, although first described as the main representative of AGE receptors, is a multiligand transmembrane receptor, represented by four main isoforms: sRAGE, DN-RAGE,  $\Delta$ N-RAGE and the fully functioning FL-RAGE
- RAGE (or FL-RAGE) is the dominant isoform, is expressed in most cell types at low level but is highly expressed in the lungs. Its expression is up-regulated upon a pro-inflammatory signalling and through its own stimulation
- RAGE signalling is controlled by many various ligands, often DAMPs or PAMPs, thereby suggesting that RAGE acts as a PRR. Ligand binding promotes RAGE's oligomerization, activation of intracellular effectors and notably the stimulation of pro-inflammatory and pro-oxidant pathways
- Physiological role of RAGE remains elusive although a role in lung development and protection against *some* pathogens is suspected
- RAGE is involved in a number of age- and non-age related pathologies, often caused by inflammatory processes, and seems to be essential in AGEs-related defects, including complications of diabetes

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# Aims

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There is a significant body of literature demonstrating that glycation, through the accumulation of AGEs with age in tissues, is a motor of ageing. Diabetes, in which patients accumulate AGEs because of chronic hyperglycaemia, is an accelerated-ageing condition as it leads to the early onset of ageing-like organs dysfunction. As an example, cataracts are directly caused by accumulating AGEs within the lens. Different means explaining glycation's noxious effects have been described such as the degradation, loss of function, aggregation or the cross-linking of glycated proteins. However, many of the deleterious properties of AGEs are driven by their interaction with the AGE receptor RAGE. While the role of endogenous AGEs in ageing and diverse pathologies is widely studied, the impact of dietary AGEs remains poorly understood. In contrast with endogenous AGEs, dietary AGEs are processed through digestion and absorption, and therefore have different properties from endogenous protein-bound AGEs as their action is restricted to their interaction with AGE receptors.

Our research group has shown that dietary CML was able to induce an accelerated vascular ageing in middle-aged WT mice and it was subsequently shown that the kidney is the organ that predominantly accumulates dietary CML, though no exclusively age-related lesions were found in kidneys at this age.

Therefore, **the first aim of this thesis was to determine the impact of a long-term CML-enriched diet on murine kidney ageing.**

RAGE is involved in a number of pathological age-related disorders, notably through inflammatory processes, and its ligands tend to accumulate with age. In addition, RAGE knock-out completely prevented dietary CML-induced vascular ageing in middle-aged mice but age-related kidney lesions were similar between all groups at this age.

Hence, **the second aim of this thesis was to assess the role of RAGE expression in kidney ageing of old mice fed with either a control or a CML-enriched diet.**

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# Results

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## **Knock-out of Receptor for advanced glycation end-products (RAGE) attenuates age-related renal lesions**

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Thibault Teissier\*<sup>1</sup>, Valentine Quersin\*<sup>1,2</sup>, Viviane Gnemmi<sup>3</sup>, Maité Daroux<sup>1</sup>, Mike Howsam<sup>1</sup>, Florian Delguste<sup>1</sup>, Cécile Lemoine<sup>1</sup>, Chantal Fradin<sup>1</sup>, Ann-Marie Schmidt<sup>4</sup>, Christelle Cauffiez<sup>5</sup>, Thierry Brousseau<sup>6</sup>, François Glowacki<sup>2,5</sup>, Frédéric J Tessier<sup>1</sup>, Eric Boulanger<sup>#1,7</sup> and Marie Frimat<sup>#1,2</sup>

<sup>1</sup> University of Lille, INSERM, CHU Lille, U995 - Lille Inflammation Research International Center, F-59000 Lille, France

<sup>2</sup> CHU Lille, department of nephrology, F-59000 Lille, France

<sup>3</sup> University of Lille, INSERM, CHU Lille, Department of pathology, U1172 - Jean-Pierre Aubert Research Center, F-59000 Lille, France

<sup>4</sup> Diabetes Research Center, Department of Medicine, NYU Langone Medical Center, New York, NY, USA

<sup>5</sup> University of Lille, EA4483 IMPECS-IMPact of Environmental ChemicalS on Human Health, CHU Lille, F-59000 Lille, France

<sup>6</sup> CHU Lille, service Biochimie automatisée Protéines, F-59000 Lille, France

<sup>7</sup> CHU Lille, department of geriatrics, F-59000 Lille, France

\* *T.T. and V.Q. contributed equally to this study.* # *M.F. and E.B. co-directed this study*

## Summary

The pro-aging effects of endogenous advanced glycation end-products (AGEs), particularly through their binding to RAGE (the main AGE receptor) which promotes inflammation and fibrosis, are widely reported. The predominantly renal accumulation of dietary carboxymethyllysine (CML) suggests the kidneys may be particularly affected, though the precise role of dietary AGEs in aging remains ill-defined. We present a study of the impact of RAGE invalidation and a CML-enriched diet on renal aging.

Two-month-old male, wild-type (WT) and RAGE<sup>-/-</sup> C57Bl/6 mice were fed a control or a CML-enriched diet (200µg CML/g<sub>food</sub>) for 18 months. Compared to controls, we observed higher CML levels in the kidneys of both CML WT and CML RAGE<sup>-/-</sup> mice, with a predominantly tubular localization. The CML-rich diet had no significant impact on the studied renal parameters, whereby only a trend to worsening glomerular sclerosis was detected. Irrespective of diet, RAGE<sup>-/-</sup> mice were significantly protected against nephrosclerosis lesions (hyalinosis, tubular atrophy, fibrosis and glomerular sclerosis) and renal senile apolipoprotein A-II (ApoA-II) amyloidosis ( $p < 0.001$ ). A positive linear correlation between sclerosis score and ApoA-II amyloidosis score ( $r=0.92$ ) was observed. Compared with old WT mice, old RAGE<sup>-/-</sup> mice exhibited lower expression of inflammation markers and activation of AKT, and greater expression of *Sod2* and SIRT1.

Overall, nephrosclerosis lesions and senile amyloidosis was significantly reduced in RAGE<sup>-/-</sup> mice, indicating a protective effect of RAGE deletion with respect to renal aging. This could be due to reduced inflammation and oxidative stress in RAGE<sup>-/-</sup> mice, suggesting RAGE is an important receptor in so-called inflamm-aging.

**Key Words:** Advanced glycation end-products (AGEs), amyloidosis, chronic kidney disease, nephrosclerosis, receptor for AGEs (RAGE), renal aging.

## 1 | Introduction

Kidney function declines with age (Bolignano, Mattace-Raso, Sijbrands, & Zoccali, 2014; Glassock, Warnock, & Delanaye, 2017), resulting in elderly people being particularly susceptible to developing chronic kidney disease (CKD). The prevalence of CKD – defined as a glomerular filtration rate (GFR) inferior to  $60\text{ml}/\text{min}/1.73\text{m}^2$  – was thus more than 30% in a European cohort of patients over 70 years old (Ebert et al., 2016; Glassock et al., 2017). Non-specific morphologic changes are associated with aging kidneys and designated by the generic term ‘nephrosclerosis’. Histologically this is characterized by nephron loss, global glomerulosclerosis (GS, >10%), arteriosclerosis, tubular atrophy and interstitial fibrosis (>5%) (Denic, Glassock, & Rule, 2016; O’Sullivan, Hughes, & Ferenbach, 2017).

Consequent to the rapid growth in the proportion of elderly people throughout the world (“European Commission: The 2015 ageing report. Eur Econ 3: 424,” 2015; GA, Atlanta, 2013), an increase in the number of CKD diagnoses would appear to be inevitable. However, more than a third of people exhibit no change in GFR with age (Bolignano et al., 2014; Lindeman, Tobin, & Shock, 1985), suggesting that age-related renal impairment could be preventable. Gender, ethnicity, genetic factors and comorbidities are known to influence renal outcomes (Choudhury & Levi, 2011; Smyth, Duffy, Maxwell, & McKnight, 2014), but others remain to be clarified.

The impact of advanced glycation end products (AGEs) on CKD, especially through binding to their main receptor RAGE (receptor for AGEs), has received significant research attention (Clarke, Dordevic, Tan, Ryan, & Coughlan, 2016; Stinghen, Massy, Vlassara, Striker, & Boullier, 2016). The AGEs form a heterogeneous group of molecules resulting from permanent binding of reducing sugars to a range of amino-compounds. Their endogenous formation occurs under various conditions such as hyperglycaemia and oxidative stress, but also aging. Their presence is moreover clearly identified in foods: daily intake of *N* $\epsilon$ -carboxymethyllysine (CML, the most studied AGE) can be as high as  $252\ \mu\text{g}/\text{kg}$  body weight in a typical European diet (Tessier & Birlouez-Aragon, 2012). Evidence has recently accumulated incriminating the endogenous AGE/RAGE axis in age-related diseases. RAGE is a multiligand, transmembrane receptor activating major pro-inflammatory and pro-oxidative signalling pathways. Its expression in numerous cell types increases with aging and pathological conditions such as diabetes, but a role for this receptor has been postulated in the premature dysfunction of several organs, even in the absence of diabetes (Frimat et al., 2017; Ramasamy, Shekhtman, & Schmidt,

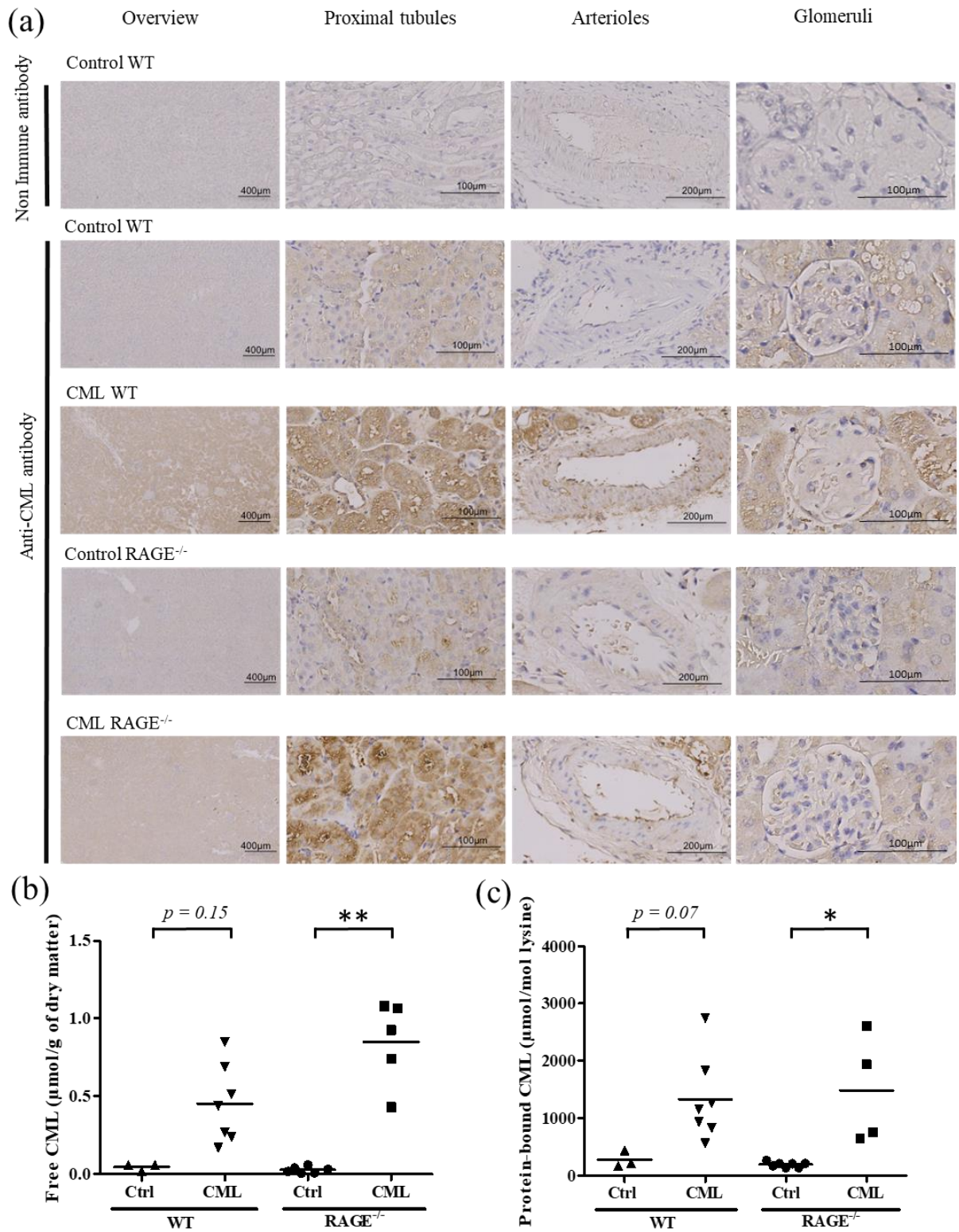
2016). The impact of chronic exposure to dietary AGEs on aging remains poorly studied, however.

Considering the preferential accumulation of CML in the kidneys under a CML-enriched diet (Li et al., 2015; Tessier et al., 2016) and studies linking dietary AGEs and kidney damage (Feng et al., 2007; Zheng et al., 2002), we hypothesised that kidneys are target organs for accelerated aging induced by AGE/RAGE interactions. In order to study this question, histologic markers of renal aging were analysed in 2-month-old, male wild-type (WT) and RAGE<sup>-/-</sup> C57Bl/6 mice fed a control or a CML-enriched diet over 18 months.

## 2 | Results

### 2.1 | Prolonged intake of a CML-enriched diet results in renal accumulation of glycation products

A diffuse antibody-mediated CML staining was observed in the kidneys of all WT mice but was more intense in mice fed a CML-enriched diet compared with control mice. CML staining predominated in the proximal tubular cells and along the internal wall of arteries. We found similar results in the kidneys of RAGE<sup>-/-</sup> mice suggesting that CML accumulation from the CML-enriched diet is governed by RAGE-independent mechanisms (Fig. 1A). These results were confirmed using liquid chromatography with tandem mass spectrometric detection in kidney samples. The mean levels of free CML were higher among the mice fed the CML-enriched diet, both in WT (0.05 ±0.02 and 0.45 ±0.2 μmol/g of dry matter for control and CML diet, respectively: *p* =0.15) and RAGE<sup>-/-</sup> mice (0.03 ±0.02 and 0.85 ±0.3 μmol/g of dry matter for control and CML diet, respectively: *p* <0.01). These differences between groups were also observed for concentrations of protein-bound CML (275 ±145 and 1331 ± 740 μmol/mol lysine for control and CML WT mice, respectively: *p* =0.07; 190 ±47 and 1486 ±947 μmol/mol lysine for control and CML RAGE<sup>-/-</sup> mice, respectively: *p* <0.05), thereby suggesting a RAGE-independent mechanism of CML formation (Fig. 1B-C).



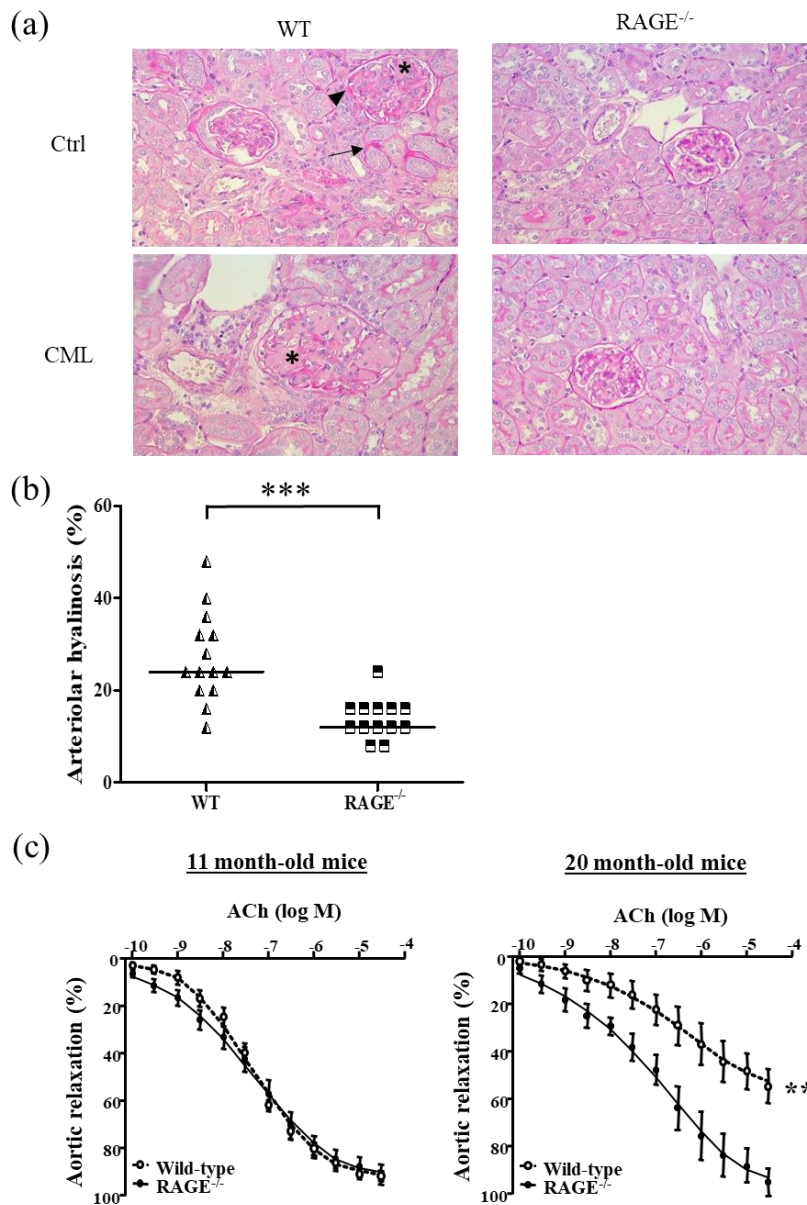
**Figure 1. CML accumulation in the kidneys of WT and RAGE<sup>-/-</sup> mice was diet-dependant.** (a) Representative localization of protein-bound CML studied by IHC on kidney sections showed that mice fed a CML-enriched diet exhibited predominantly tubular staining. From left to right: low magnification, high magnification on proximal tubules, arterioles and glomeruli. (b-c) Quantification by HPLC-MS/MS of (b) free and (c) protein-bound CML in kidneys. \**p* < 0.05, \*\**p* < 0.01, Kruskal-Wallis test

## 2.2 | Prolonged intake of a CML-enriched diet does not significantly affect renal aging

Prior to assessing the effects of a CML-enriched diet and RAGE invalidation on renal aging, we first ensured that weight, glycaemia, cholesterolemia and triglyceridemia were comparable between mice fed the control and CML-enriched diets. None of the WT or RAGE<sup>-/-</sup> mice became diabetic, dyslipidemic or obese under the influence of a CML-enriched diet (supplementary table 1). Renal parameters were then studied, and lesions of arteriolar hyalinosis, tubular atrophy, interstitial fibrosis and GS (histological markers of nephrosclerosis, Fig. 2A) were all assessed 'blind'.

Renal weight, plasma creatinine and urea were not statistically different between control and CML groups, in either WT or RAGE<sup>-/-</sup> mice (data not shown). We analysed the expression of *Kim-1* and *Ngal* as sensitive markers of kidney injury associated with CKD progression in humans (Alderson et al., 2016): the expression of these genes was also unaffected by the CML-rich diet (supplementary Fig. 1A-B).

The assessment of nephrosclerosis markers at 20 months of age gave similar results: the CML-rich diet had no significant effect on arteriolar hyalinosis, tubular atrophy or fibrosis score (supplementary Fig. 1C-E). There was a light worsening of GS under the influence of the CML-rich diet in WT mice (supplementary Fig. 1F-H): only 6.5% glomeruli lacked any sign of sclerosis in CML WT mice against 31, 61 and 67% in control WT, control RAGE<sup>-/-</sup> and CML RAGE<sup>-/-</sup>, respectively; the median GS score and percentage of global GS – the end-stage of glomerular lesion – also followed this trend (CML WT > control WT > control and CML RAGE<sup>-/-</sup>). These differences were not significant however, in contrast to the results observed when we compared WT with RAGE<sup>-/-</sup> mice. In order to analyse the impact of the RAGE invalidation on renal aging, we thus focused on differences between WT and RAGE<sup>-/-</sup> mice, irrespective of diet.



**Figure 2. Vascular aging of renal arterioles was reduced in RAGE<sup>-/-</sup> mice.**

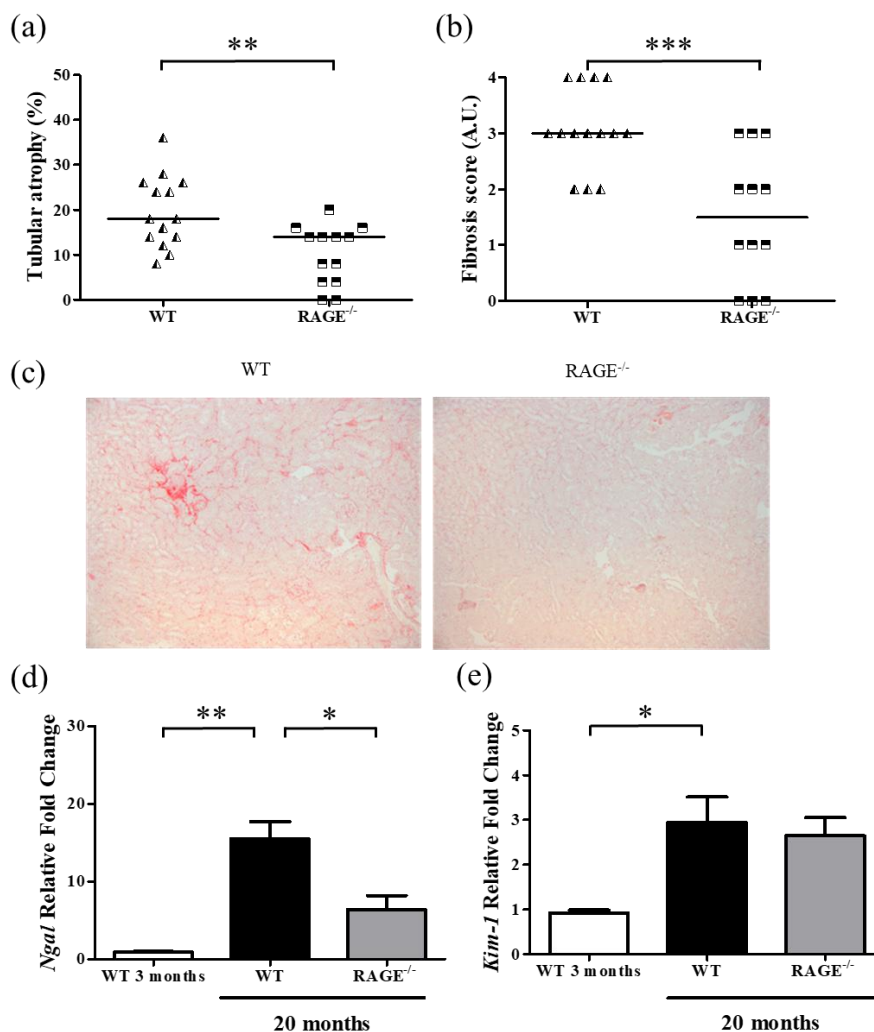
(a) Representative PAS staining of paraffin-embedded kidney sections of WT (left panels) and RAGE<sup>-/-</sup> mice (right panels) fed a control (upper panels) or CML-enriched diet (lower panels) revealed the presence of hyalinosis (arrowheads), tubules undergoing tubular atrophy (arrows) and glomerulosclerosis (asterisks) (x400). (b) Kidney arteriolar hyalinoses determined by PAS staining in WT and RAGE<sup>-/-</sup> mice and (c) alteration in endothelium-dependent relaxation in 11-month-old and 20-month-old WT and RAGE<sup>-/-</sup> mice fed a control diet. Aortic relaxation is expressed as mean ± SEM (n = 5–6 mice in duplicate). \*\*p < 0.01, \*\*\*p < 0.001, unpaired t test on area under the curve

### 2.3 | RAGE knock-out mice are protected against nephrosclerosis lesions

All mice had hyaline deposits on the arteriole walls (10 to 48% of studied vessels), but the median percentage of vessels with hyaline deposits was higher in WT than in RAGE<sup>-/-</sup> mice (24% vs. 12%, respectively, p < 0.001) (Fig. 2B). In order to study the evolution of endothelial function with age, we measured the percentage of aortic relaxation mediated by different concentrations of acetylcholine (ACh) in middle-aged (11-month-old) or old (20-month-old) WT and RAGE<sup>-/-</sup> mice. No difference was observed between 11-month-old WT and RAGE<sup>-/-</sup> mice (92 ± 7 and 91 ± 2% of max relaxation, respectively). At 20 months old, however, WT mice exhibited reduced responsiveness to ACh (55 ± 2% of max. relaxation vs. 11-month-old WT

mice,  $p < 0.001$ ) in contrast to  $RAGE^{-/-}$  mice with conserved endothelial function ( $95 \pm 1\%$  of max. relaxation) (Fig. 2C).

Both tubular atrophy and interstitial fibrosis lesions were also more present in WT than in  $RAGE^{-/-}$  mice. The median percentage of tubular atrophy was 18 and 14% ( $p < 0.01$ ) and the median fibrosis scores were 3 and 1 A.U. ( $p < 0.001$ ) in WT and  $RAGE^{-/-}$  mice, respectively (Fig. 3A-B). Fibrosis was identified in renal interstitium of all WT mice, but was often mild to absent in  $RAGE^{-/-}$  mice (Fig. 3C). The *Ngal* expression, more specifically associated with tubulointerstitial damage (Rysz, Gluba-Brzózka, Franczyk, Jabłonowski, & Ciałkowska-Rysz, 2017), was significantly higher in WT mice ( $\sim 15.5 \pm 6.6$ ) than  $RAGE^{-/-}$  mice ( $\sim 6.4 \pm 5.6$ ,  $p < 0.05$ ). Comparing old WT with old  $RAGE^{-/-}$  mice, there was no difference in the *Kim-1* expression (Fig. 3D-E).



**Figure 3.  $RAGE^{-/-}$  mice were protected against tubulointerstitial aging.**

Quantification of (a) tubular atrophy was determined by PAS staining and (b) interstitial fibrosis by Sirius red staining. (c) Representative Sirius red staining of control and CML WT and  $RAGE^{-/-}$  mice at low magnification (x 100). (d- e) Expression of kidney injury markers *Ngal* (d) and *Kim-1* (e) in renal tissue from 3- or 20- month- old WT and  $RAGE^{-/-}$  mice (mean  $\pm$ SEM,  $n = 3$  for 3- month- old mice and  $n = 10$  for 20- month- old mice). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , unpaired  $t$  test or Kruskal-Wallis test for multiple comparisons

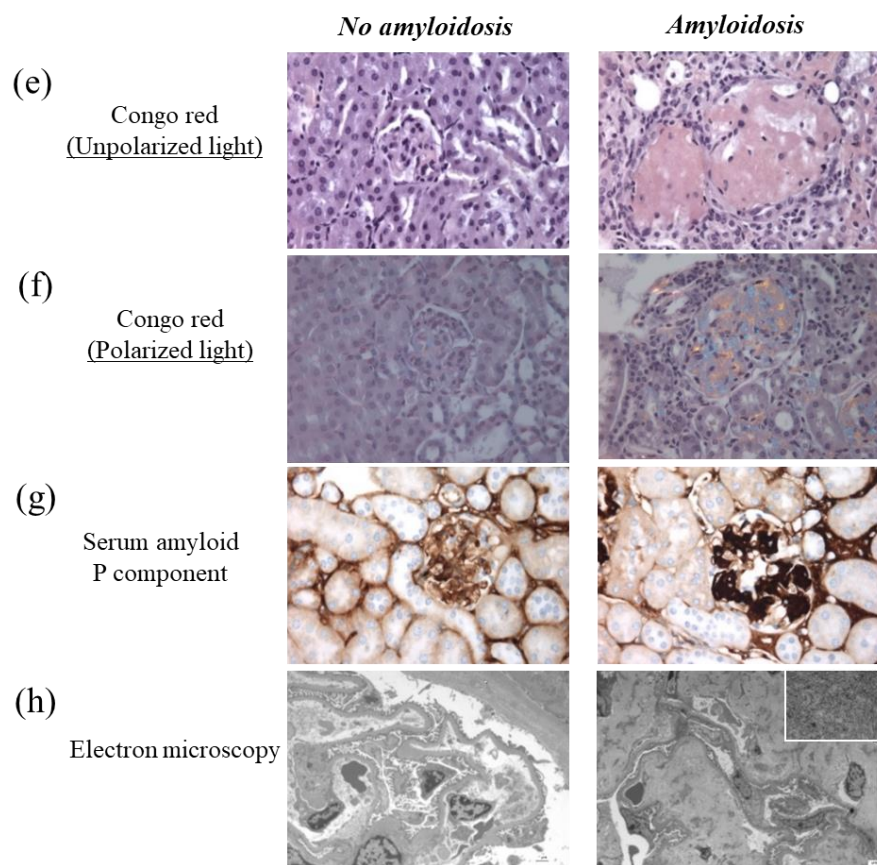
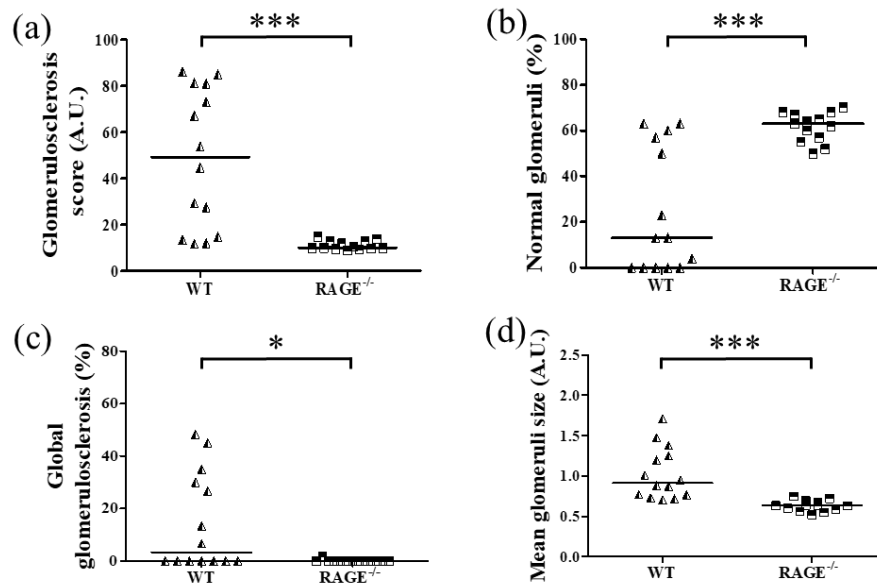


The median GS score at 20 months of age, calculated after histological evaluation of sclerosis intensity of 60 glomeruli per mouse, was higher in WT mice (49.3 A.U.) than in RAGE<sup>-/-</sup> mice (10 A.U.,  $p < 0.001$ ). Only 13% of the glomeruli lacked any sign of sclerosis in WT mice against 63% in RAGE<sup>-/-</sup> mice ( $p < 0.001$ ). The mean percentage of global GS (glomeruli with a GS score of 100) was 14.6% in WT mice, while no global GS was observed in RAGE<sup>-/-</sup> mice (Fig. 4A-C).

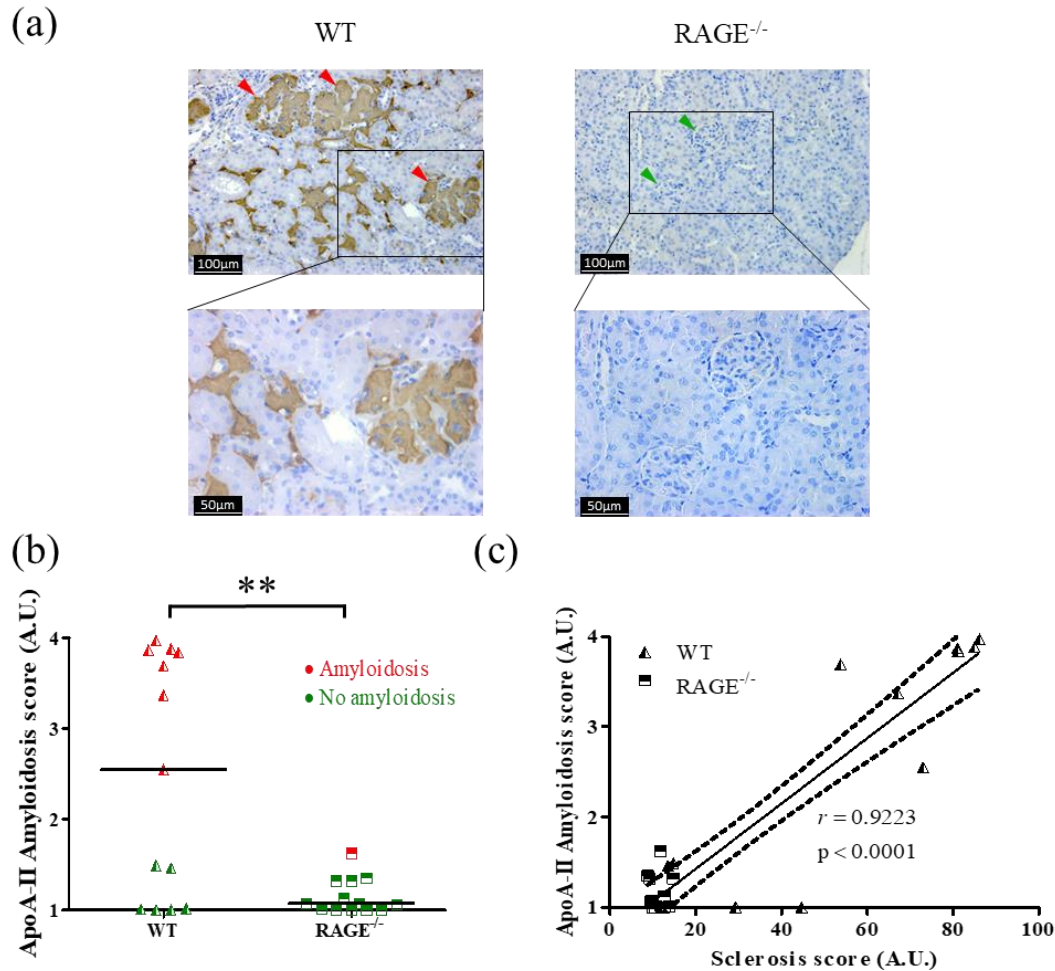
## 2.4 | RAGE knock-out prevents senile amyloidosis

The GS appeared nodular with developing hypertrophic glomeruli. The mean size of the glomeruli was significantly larger in WT than in RAGE<sup>-/-</sup> mice ( $1.0 \pm 0.3$  and  $0.6 \pm 0.1$  A.U., respectively;  $p < 0.001$ , Fig. 4D). Birefringence under polarized light, obtained after Congo Red staining, suggested that this nodular sclerosis was at least partly caused by amyloidosis. This was confirmed by the presence of serum amyloid protein (SAP) in immunohistochemistry assays, and the identification of 5-10nm fibrils by electron microscopy (Fig. 4E-H). Thus, 8 of the 14 (57%) WT mice were amyloidosis positive against only 1 of the 13 (8%) RAGE<sup>-/-</sup> mice (7 of the 9 amyloid mice were fed the CML-rich diet, Fig. S2A).

A positive ApoA-II protein staining occurred in the same locations as the amyloid deposits, suggesting an ApoA-II amyloidosis (AApoA-II) thought to be related to aging processes in mice (Kitagawa et al., 2003). The ApoA-II deposition was mainly localized in glomeruli, with various intensities according to the glomeruli and individual mice (Fig. 5A). Interstitial AApoA-II was also observed when the vast majority of glomeruli showed extensive deposits, suggesting a more advanced stage of amyloidosis. Other proteins known to be amyloidogenic were tested (serum amyloid A, involved in chronic inflammatory diseases, and peptides  $\beta 1-40$  and  $\beta 1-42$ , responsible for amyloid plaques in Alzheimer's disease), but none were correlated with the identification of amyloidosis (data not shown). The WT mice had a median AApoA-II score significantly higher than RAGE<sup>-/-</sup> mice which had no or very few deposits ( $2.5 \pm 1.3$  vs.  $1.1 \pm 0.2$  A.U., respectively;  $p < 0.01$ ; Fig. 5B). A trend also suggested greater AApoA-II deposition in CML WT than in control WT mice (1.2 A.U.) but this trend was not observed when RAGE<sup>-/-</sup> groups were compared (Fig. S2B). The GS intensity was correlated with AApoA-II score ( $r=0.92$ ,  $p < 0.0001$ , Fig. 5C).



**Figure 4. Amyloidosis- linked glomerulosclerosis was largely prevented in RAGE<sup>-/-</sup> mice.** Quantification of GS in paraffin- embedded kidney sections indicated (a) the GS score (60 glomeruli/ mouse) which was used to determine (b) the percentage of normal glomeruli (glomeruli with GS score of 0) and of (c) global GS (glomeruli with a GS score of 100). (d) Mean glomeruli size (30 glomeruli/ mouse). Determination of amyloidosis- positive lesions in paraffin- embedded kidney sections after (e) Congo red staining under non- polarized light (x 400) and (f) polarized light showing birefringence (x400), (g) Immunohistochemistry of Serum Amyloid P component (SAP, x 400) and (h) electron microscopy showing the absence in control WT mouse (*left panel*, x 1,000) and the presence in CML WT mouse (*right panel*, x 1670 low magnification, x 21,560 high magnification in the frame) of amyloid fibrils. \**p* < 0.05, \*\*\**p* < 0.001, Mann–Whitney test, representative images



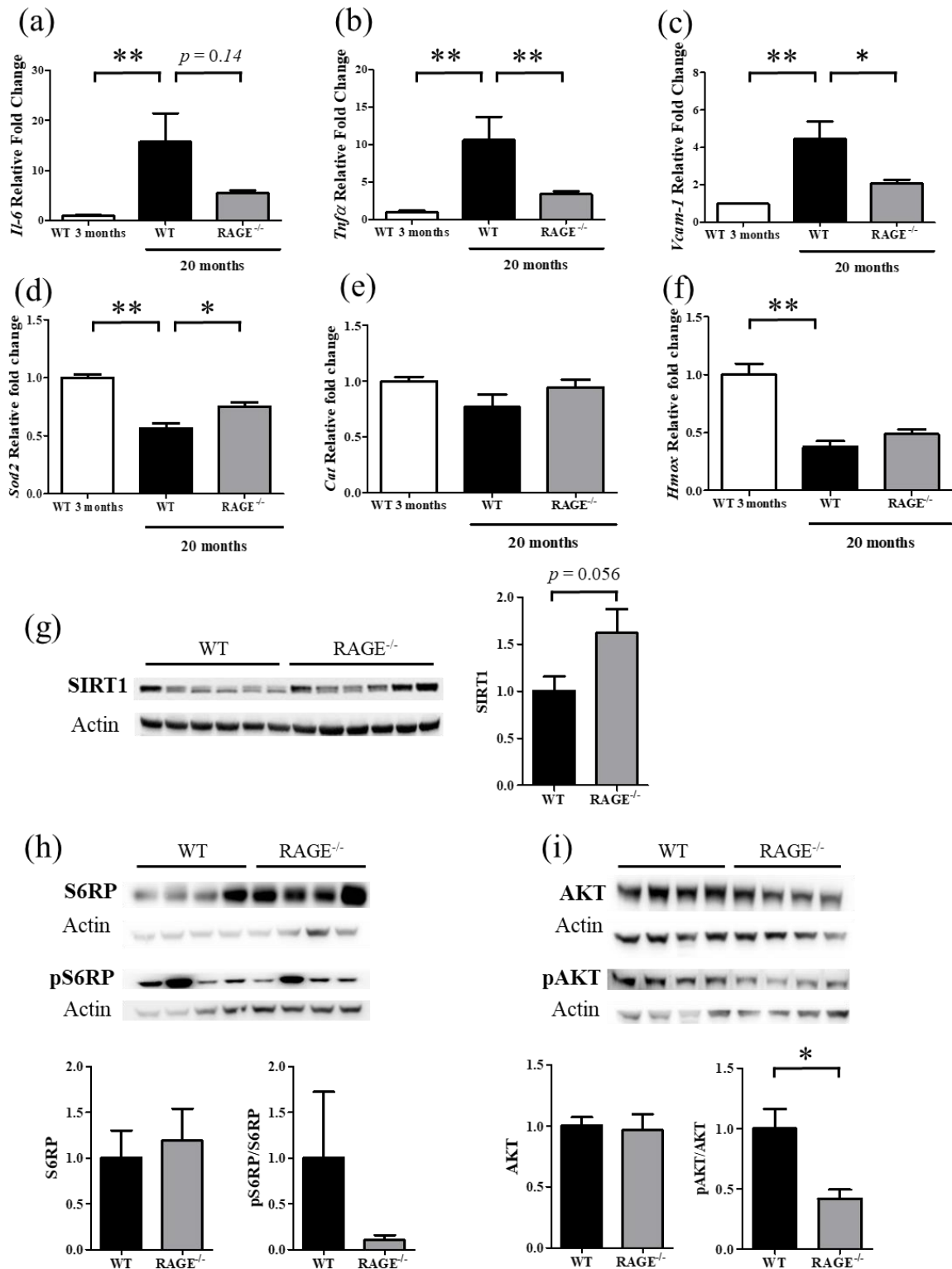
**Figure 5. AApoA- II was largely absent in RAGE<sup>-/-</sup> mice.** AApoA- II in kidney tissue sections from 20-month-old WT mice (left panel) and RAGE<sup>-/-</sup> mice (right panel) were immunostained using an anti-ApoA- II antibody (a) green arrowheads, no deposits; red arrowheads, extensive deposits (representative image). (b) Scoring of ApoA- II amyloid deposits obtained from IHC and calculated from the grading of 100 glomeruli per mouse, where 1 = no deposit, 2 = small, 3 = moderate and 4 = extensive deposits. Red dots, amyloidosis-positive mice; green dots, amyloidosis-negative mice. (c) Linear regression analysis between AApoA- II and sclerosis scores obtained for all four conditions,  $y = 0.036x + 0.70$ ,  $r = 0.9223$ , dotted line: 95% confidence intervals (C). \*\* $p < 0.01$ , unpaired  $t$  test

## 2.5 | RAGE knock-out mice exhibited less inflammation and were better protected against oxidative stress

Compared to 3-month-old WT mice, the mRNA levels of *Il-6*, *Tnf- $\alpha$*  and *Vcam-1* increased respectively ~15.8-, 10.6- and 4.5-fold in 20-month WT mice and ~5.6-, 3.4- and 2.1-fold in RAGE<sup>-/-</sup> mice (Fig. 6A-C). The increase of these markers was significant in old WT mice but not in old RAGE<sup>-/-</sup> mice, suggesting a similar pro-inflammatory profile in young WT and old RAGE<sup>-/-</sup> mice. Expression of the antioxidant genes *Sod2* (a mitochondrial manganese-

dependant superoxide dismutase), *Cat* (a peroxide hydrogen detoxifier) and *Hmox* (known to increase under influence of oxidative stress and recently described as ameliorating the oxidative stress-induced endothelial senescence (W. Luo et al., 2018)), were also analysed. While *Cat* expression did not change with age or genotype, *Sod2* expression was significantly lower in old WT mice when compared with young WT ( $0.56 \pm 0.1$ ,  $p < 0.01$ ) and old  $RAGE^{-/-}$  mice ( $0.75 \pm 0.1$ ,  $p < 0.05$ ). An important decrease of *Hmox* was also observed in both old WT ( $0.38 \pm 0.2$ ) and  $RAGE^{-/-}$  ( $0.49 \pm 0.1$ ) mice but was only significant in the former ( $p < 0.01$ ) (Fig. 6D-F).

As a first step to elucidate the mechanisms underlying this potential protective effect of RAGE knock-out, and in light of our results on inflammation and oxidation markers, we focused on SIRT1 (associated with longevity and known to decrease with age (Yuan et al., 2016)) and the AKT/mTor pathway (involved in kidney disease (Kajiwara & Masuda, 2016)). SIRT1 was higher in  $RAGE^{-/-}$  than WT mice (1.6 fold  $\pm 0.8$ ,  $p = 0.056$ , Fig. 6G). Conversely, the S6 subunit of the 40S ribosome (pS6RP or S235/236), a mTORC1 activity indicator and, more significantly, pAkt (S473, a mTORC2 activity indicator) were lower in old  $RAGE^{-/-}$  than WT mice ( $0.1 \pm 0.11$  and  $0.42 \pm 0.15$ , respectively,  $p < 0.05$ ) (Fig. 6H-I).



**Figure 6. Inflammation and oxidation markers were decreased in RAGE<sup>-/-</sup> mice.** Inflammation markers (a) *Il-6*, (b) *Tnfa* and (c) *Vcam-1* and oxidation markers (d) *Sod2*, (e) *Cat* and (f) *Hmox* mRNA expression were measured in renal tissue from 3- or 20- month- old WT and RAGE<sup>-/-</sup> mice (mean  $\pm$ SEM,  $n = 3$  for 3- month- old mice and  $n = 10$  for 20- month- old mice). Representative western blot and quantification of protein levels of (g) SIRT1 (mean  $\pm$ SEM,  $n = 10$ ), (h) pS6RP (Ser235/236)/S6RP and (i) pAKT (Ser473)/AKT in kidney extracts of 22- to 26- month- old mice (mean  $\pm$ SEM,  $n = 4$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , Mann-Whitney test or Kruskal-Wallis test for multiple comparisons

### 3 | Discussion

Although the small sample sizes necessitate caution in interpreting our results, we here report that RAGE knock-out mice developed significantly less nephrosclerosis and senile amyloid lesions than WT mice, suggesting a key role for this receptor in the renal aging process. Prolonged exposure to a CML-enriched diet was associated with CML accumulation in kidneys but there was no evident relationship between these deposits and renal injuries; CML accumulation was RAGE-independent and thus could not explain the differences between WT and RAGE<sup>-/-</sup> mice in kidney function/structure. We found lower levels of inflammation and oxidative stress in RAGE<sup>-/-</sup> mice, which may contribute to our proposed protective effect of RAGE invalidation through modulation of SIRT1 expression and the AKT/mTOR pathway.

In accordance with other studies, CML accumulated in kidneys of animals fed a CML-enriched diet (Li et al., 2015; Tessier et al., 2016). We confirmed the RAGE-independent nature of this accumulation (Tessier et al., 2016), and also separately quantified both free/dipeptide CML and protein-bound CML. Since dietary CML cannot enter the circulation in protein-bound form (Alamir et al., 2013), protein-bound CML must result from endogenous formation. This could be the consequence of oxidative stress, a condition thought to favour CML production from dicarbonyl compounds and known to occur in aging and CKD (Roca, Grossin, Chassagne, Puisieux, & Boulanger, 2014; Stinghen et al., 2016). Thus, ingested CML could activate other, RAGE-independent oxidative stress pathways, such as the epidermal growth factor receptor pathway (Chen et al., 2010), and/or participate in protein synthesis as described with non-canonical amino acids in artificial systems (Calve, Witten, Ocken, & Kinzer-Ursem, 2016). Surprisingly, we did not observe a significant negative effect of a CML-enriched diet on nephrosclerosis lesions in comparison with the significant positive impact of RAGE invalidation. Only GS and ApoAII amyloid deposits were higher (but not significantly so) in the CML WT *vs.* control WT mice. While there is no published study establishing a direct link between AGE accumulation and renal aging, these results nevertheless seem to run counter to a body of related evidence. A potential deleterious effect of AGE accumulation can be inferred from diabetic nephropathy (Choudhury & Levi, 2011; Vlassara et al., 1994), in which CML deposits are, however, more pronounced. Moreover, an AGE-rich diet has been associated with proteinuria and structural changes in the kidneys of rodent models, whereas an AGE-poor diet induced fewer histological lesions and conserved renal function (Feng et al., 2007; Zheng et al., 2002). We hypothesize that any effect of dietary AGEs could be obscured by the relatively

advanced age of our mice. Indeed, the majority of old control WT mice presented significant lesions of all renal structures and a third exhibited AApoA-II, suggesting that aging is important in this regard.

Among the age-related renal lesions that we reported, this observation of a link between nodular GS and AApoA-II is interesting. While AApoA-II is very uncommon in humans, it is described as a senile renal marker in mice and its prevalence varies according to genotype (Kitagawa et al., 2003). This age-related susceptibility to AApoA-II in mice may be explained by two characteristics of this protein. Firstly, human ApoA-II is dimerized while the murine ApoA-II is monomeric and hence less stable. Secondly, ApoA-II has a higher methionine content in mice than humans and methionine is an amino acid known to be prone to oxidation (Gursky, 2014). It has been shown that the lipid peroxidation of high density lipoproteins (HDLs, of which ApoA-II is a major constituent) induced oxidation of methionine components (Anantharamaiah et al., 1988); consequently, the 3D structure of ApoA-II could change and alter its affinity for HDLs. With subsequent dissociation from HDLs, ApoA-II could unfold until its aggregation and subsequent nucleation, the first step in amyloid fibrils formation (Wong, Binger, Howlett, & Griffin, 2010). Promoting oxidation and lipid peroxidation, aging and RAGE expression together could thus favour structural changes and the deposition of ApoA-II in the kidneys, causing amyloidosis followed by GS. In a susceptible model such as C57BL6 mice, AApoA-II appears to be a useful marker of aging that is highly reduced when RAGE is knocked-out, and would further appear to be accelerated by prolonged intake of a CML-enriched diet.

Not only AApoA-II, but all the nephrosclerosis lesions assessed here were reduced in RAGE<sup>-/-</sup> mice. The involvement of this receptor has already been reported in different kidney diseases, especially diabetic nephropathy. Indeed, genetic deletion or pharmacologic blockage of RAGE were associated with a decrease of renal lesions in different animal models of diabetes (Kaida et al., 2013; Myint et al., 2006; Vlassara et al., 1994). Independent of the underlying etiology, RAGE has been also implicated in CKD and its attendant cardiovascular complications through the formation of AGEs and the promotion of oxidative stress induced by renal failure itself (Stinghen et al., 2016). Our work here has illustrated that, even in the absence of prior kidney disease, RAGE could be involved in “normal” renal aging. The pro-aging mechanisms induced by RAGE are multiple and especially include the perpetuation of a pro-inflammatory phenotype and ROS generation; in accordance with this, RAGE<sup>-/-</sup> mice here presented less inflammation and were better protected against oxidative stress. Previous studies have shown that a deficiency in SOD2 results in oxidative stress, interstitial inflammation and accelerated renal senescence

in a murine model (Rodriguez-Iturbe et al., 2007). Thus, the smaller decrease of *Sod2* expression in RAGE<sup>-/-</sup> mice with age could suggest enhanced protection against renal aging. Concurrently, and in accordance with previous studies showing that knock-out or inhibition of RAGE prevented CML- or LPS-induced reduction of SIRT1 expression (Grossin et al., 2015; Huang et al., 2015), SIRT1 expression was here down-regulated in old WT compared with old RAGE<sup>-/-</sup> mice. Interestingly, reduced renal inflammation and fibrosis have been reported in genetic or pharmacological boosting of SIRT1 models (Hong et al., 2018; Morigi, Perico, & Benigni, 2018); on the contrary, its deletion was associated with an aggravation of kidney injuries such as GS (Chuang et al., 2017). Together, these results suggest a potentially major role for SIRT1 in renal aging. Moreover, our observation of decreased levels of phosphorylated AKT and S6 ribosomal protein (S6RP) in RAGE<sup>-/-</sup> mice could indicate a role for RAGE invalidation in a pro-longevity phenotype and renoprotection. Indeed, mTOR inhibition is the only therapeutic intervention that leads to enhanced health and lifespan in all studied models (Johnson, Rabinovitch, & Kaeberlein, 2013; Weichhart, 2018), and downregulation or inhibition of mTORC1 prevents GS, glomerular hypertrophy macrophage infiltration and other lesions in murine models of diabetic nephropathy (Kajiwara & Masuda, 2016). We thus suggest that the greater inflammation we observed in old WT mice compared with old RAGE<sup>-/-</sup> mice could be the result of AKT- and/or SIRT1-dependant NF-κB pathway activation (Kane, Shapiro, Stokoe, & Weiss, 1999; Kauppinen, Suuronen, Ojala, Kaarniranta, & Salminen, 2013).

Taken together, our results point to a role for the RAGE receptor and its attendant signalling pathways in regulating inflammation, elevation of which is a recognized hallmark of aging (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013) and central to so-called inflamm-aging (Franceschi et al., 2000). They also suggest that RAGE<sup>-/-</sup> mice present a pro-longevity phenotype, as evidenced by their apparently attenuated renal aging. Further studies, in particular using tissue culture techniques, will be helpful not only in determining the mechanisms governing the pathways involved in this phenotype, but also in determining the potential utility of RAGE inhibitors in reducing renal aging in particular, and perhaps other age-related phenomena.



## 4 | Experimental procedures

### 4.1 | Animal experimentation

All animals were housed in the experimental research department of Lille University (Agreement number: C59-35010). They were maintained at constant temperature and humidity in a room with a 12h light cycle. All experiments were conducted in accordance with institutional guidelines and French Ministry of Agriculture recommendations for the care and use of laboratory animals, and with the approval of the Charles Darwin Ethics Committee for animal experimentation (number #00822.01). The male WT (Janvier Laboratories, Le Genest-St-Isle, France) and RAGE<sup>-/-</sup> (from Pr. A.M. Schmidt, New York University) C57Bl/6 mice (6–10/group) received either a control diet incorporating unglycated bovine serum albumin (control-BSA), or an isocaloric diet enriched with glycated BSA rich in CML (CML-BSA). Mice were fed *ad libitum* for 18 months (2 months old at beginning of protocol). Three or 11-month-old mice fed control-BSA were used as controls to analyse baseline renal and endothelial function. Old 22 to 26-months-old WT and RAGE<sup>-/-</sup> mice fed a standard diet were used for western blot analysis.

CML-BSA was prepared as previously (Grossin et al., 2015). After glycation (16h, 37°C), 37–40% of the lysine moieties were found to be modified, 80–99% of which (on a molar basis) had been modified to CML. The control-BSA was prepared under the same conditions but glyoxylic acid was omitted. The control-BSA and CML-BSA preparations (diluted in water) were added to mouse powdered standard diet (A04, Safe, Augy, France) at 200µg CML/g<sub>food</sub>. The concentration of protein, fatty acids, fibre and carbohydrate did not differ between control-BSA and CML-BSA diets. A basal concentration of 17.5 ±0.7µg CML/g<sub>food</sub> was determined in control-BSA diet. Diets were renewed every 7 days and remaining food was weighed to assess mean daily CML intake. Mice were weighed every 3 months. At 20 months, surviving mice (6 control WT, 8 CML WT, 8 control RAGE<sup>-/-</sup> and 5 CML RAGE<sup>-/-</sup>) were culled and blood samples collected by inferior vena cava puncture. Kidneys were halved longitudinally and immediately frozen for biochemical and chemical analyses, or preserved in FAA (Formaldehyde, Alcohol, Acetic acid), RNA later and glutaraldehyde for electron microscopic analyses.

## 4.2 | Immunohistochemistry (IHC)

Four micrometre kidneys sections were deparaffinized and rehydrated by successive 3 min. washes in xylene then ethanol (100% and 95%, respectively). Standard procedures were followed for antigen retrieval (Target Retrieval Solution, Dako) and blockage steps (kit Vectastain ABC Elite Peroxydase), except for ApoA-II. Antigen retrieval was performed as previously (Kai et al., 2012): briefly, sections were first treated with proteinase K for 3 min. (Dako, ready-to-use) followed by an ethylenediaminetetraacetic acid (EDTA) bath (0.01M, pH6 at 121°C for 10 min.) then a formic acid bath (70% for 5 min. at room temp.). Non-specific fixation sites were blocked with rabbit or goat serum 5% (Sigma, R1933, G9023), while for ApoA-II assays endogenous peroxydases were blocked using a Dual Endogenous Enzyme Block (Dako, S200380) and endogenous biotins with an Endogenous Avidin/Biotin Blocking Kit (Abcam, ab64212).

Primary antibodies (18h, 4°C) were: anti-CML (rabbit Ig; 1:1000; Abcam ab27684), anti-SAP (rabbit Ig, Biocare PP132AA), anti-ApoA-II (goat, 2µg/mL, Santa Cruz sc-23609). Corresponding nonimmune Igs served as negative controls. After incubation (1h, room temp.) with Streptavidin HRP Conjugate (ImmunoReagents, Ba-103-HRPX), sections were stained using DAB Substrate (Cell Signaling, Kit #8059P) and counterstained using hematoxylin solution (Sigma Aldrich, GHS316) before mounting under coverslips.

## 4.3 | HPLC-MS/MS

A high-pressure liquid chromatograph (HPLC)-triple quadrupole mass spectrometer (MS/MS) was used to quantify CML and lysine by isotope dilution in kidney extracts (Vantage LC-MS/MS, ThermoFisher Scientific). Frozen kidneys were lyophilized (Alpha 1-2 LD plus, Bioblock scientific) for 24h at -40°C and -0.1mbar. Twenty mg of kidney was suspended in 10% trichloroacetic acid (Sigma Aldrich) for 30 min. on ice. After centrifugation at 21 000 G for 10 min. at 4°C, the supernatant (free CML) and the pellet (protein-bound CML) were separately incubated in NaBH<sub>4</sub> (83mM) for 2h at room temp. The reaction was stopped by adding HCl 37% (1:1). The samples were then incubated at 110°C for 21h and a 200µL aliquot evaporated using a SpeedVac Concentrator (Thermo Scientific). Isotope-labelled internal standards were added to the final volume of 200µL of aqueous nonafluoropentanoic acid (NFPA, 5mM, Sodipro), as follows: CML-d<sub>2</sub> 0.05mM (Polypeptide Group) or Lysine-<sup>15</sup>N<sub>2</sub>

50mM (Cortecnet). The samples were then filtered through 0.45µm nylon filters (Sodipro). Separation was performed on a Hypercarb column (100 x 2.1mm, 5µm particle size; ThermoFisher Scientific) equipped with a guard column (10 x 2.1mm, 5µm particle size). Injection volume was 10µL, column temp. 10°C, and compounds were eluted with a binary mobile phase at 0.2mL/min: mobile phase A was a 10mM aqueous solution of the ion-pairing agent NFPA, while mobile phase B was LC-MS grade acetonitrile (Sigma Aldrich). The elution gradient was: 0-9 min. 100-75% A; 9-11 min. 40% A; 11-13 min. 40-100% A; 13-21 min. 100% A. The heated electrospray interface was operated in positive ionisation mode (3 500 V, 250°C, sheath and auxiliary gas flows at 50 and 20 arbitrary units, respectively). The MS/MS transitions monitored were:  $m/z$  205→130 and  $m/z$  207→130 for CML and CML-d<sub>2</sub>, and  $m/z$  147→130 and  $m/z$  149→131 for lysine and Lysine-<sup>15</sup>N<sub>2</sub>, respectively. Quantification was performed using the ratio of responses for the unlabelled molecules and their respective isotope-labelled internal standards in a 9-point calibration covering the concentration ranges 0-0.2mM (CML) and 0-20mM (Lysine).

#### 4.4 | Histological analyses

Two sections were stained (3 or 5µm according to purpose, at an interval of 200µm) from the halves of paraffin-embedded kidneys retained for histological analyses: by Periodic Acid Schiff (PAS) to assess GS, tubular atrophy and arteriolar hyalinosis; by Sirius red to evaluate interstitial fibrosis under polarized light; and by Congo red to detect amyloid deposits under optical and polarized light. Samples were labelled without reference to treatment group and all parameters (defined below) were analyzed ‘blind’ by a qualified anatomopathologist and nephrologist using an optical microscope (Leica DM5500 B).

##### 4.4.1 | Definitions

*Arteriolar hyalinosis* appeared as a pink hyaline material in arteriolar walls after PAS staining. For each mouse, 25 arterioles (10-15/section) were analyzed and described as positive or negative.

*Tubular atrophy* was defined by an attrition of the brush border of the tubular epithelium, a reduced tubular size and a thickening of the tubular basement membrane. For each mouse, 50 tubules (25/section) were analyzed and described as positive or negative.

*Interstitial fibrosis* was defined by a red-signal in Sirius red staining in the cortex (excepting the physiological staining in the border of the vessels). For each mouse, this parameter was graded thus: grade 0, no fibrosis; grade 1, at least 1 focal fibrosis lesion; grade 2, at least 2 focal or fibrosis lesions. The scores attributed by the two examiners were summed.

*Glomerulosclerosis (GS)*, identified as an increased mesangial matrix, was assessed on 60 superficial and juxtamedullary glomeruli (30/section) for each mouse. This parameter was scored between 0 to 100% (intervals of 10%) for each glomerulus. A GS score was expressed as the mean for each mouse. Normal glomeruli yielded a GS score of 0 while global GS was defined by the maximum possible score of 100, indicating glomeruli were completely sclerotic.

To assess the size of glomeruli, 6 fields/section were studied for each mouse. The area of each glomerulus was measured using ImageJ.

*Amyloidosis* was defined by positive Congo red staining. Amyloid deposits in glomeruli were graded thus: grade 1, no deposit; grade 2, small amount; grade 3, moderate amount; grade 4, extensive deposits. An amyloidosis score was adapted from the literature and represented the mean score of a section for 100 glomeruli analyzed (H. Luo et al., 2015).

#### **4.5 | Endothelial dysfunction: aortic reactivity**

Aortic ring relaxation was investigated in 11- and 20-month-old WT and RAGE<sup>-/-</sup> mice fed the control diet. Aortic ring responsiveness was tested as previously (Grossin et al., 2015) in an automated dual small-vessel myograph (ADInstruments, Chalgrove, UK). Briefly, aortas were contracted by adding  $3 \times 10^{-6}$  M phenylephrine (Sigma) to standard Krebs solution in an organ bath (37°C, 5% CO<sub>2</sub> in O<sub>2</sub>). Once the contraction phase had stabilized, the endothelium-dependent relaxation (EDR) was tested by the cumulative addition of acetylcholine (ACh,  $10^{-10} \times 10^{-4}$  M; Sigma). Relaxation responses were expressed as a percentage of the maximal phenylephrine-induced contraction.

#### **4.6 | RT-qPCR**

RNA extraction from half-kidneys was performed with a commercial kit (Qiagen). ARN was quantified by spectrophotometry at 260 nm (Biotech Spec-nano, Shimadzu Biotech) and quality analyzed by RNA Standard Sens kit (BIO-RAD). For RT-qPCR, amplification of cDNA was

performed with the following probe mixes (ThermoFisher): *Kim-1* (Mm00506686), *Ngal* (Mm01324470), *Il-6* (Mm00446190\_m1), *Tnfa* (Mm00443258\_m1), *Vcam-1* (Mm01320970\_m1), *Sod2* (Mm01313000\_m1), *Cat* (Mm00437992\_m1) and *Hmox* (Mm00516005\_m1). The mean cycle threshold (CT) values for both the target and internal control (*Ppia*, Mm02342430\_g1) were determined for each sample. The fold change in the target gene, normalized to *Ppia* and relative to the expression of WT mice fed the control diet, was calculated as  $2^{-\Delta\Delta CT}$ .

#### 4.7 | Western Blot

Kidney tissues were homogenized in RIPA buffer in presence of protease and phosphatase inhibitors in 1.5%  $\beta$ -mercaptoethanol using a precllys homogenizer. After centrifugation (19,000g for 10 min at 4°C), supernatants were collected for protein analysis. Protein concentration was determined by the Bradford method (Bio-Rad protein assay dye, Bio-Rad): 20  $\mu$ g of protein were separated using bolt 4-12% Bis-Tris plus (Invitrogen) gels, or NuPAGE 7% Tris-Acetate gels (ThermoFisher) for larger molecular weights, and were transferred to a polyvinylidene difluoride membrane (Bio-Rad). Nonspecific binding sites were blocked for 1h at 20°C with 5% milk or BSA, according to the antibody, in Tris-buffered Saline with 0.05% Tween 20 (TBS-T). Membranes were incubated with anti-SIRT1 (ab7343, Abcam, 1 : 900), anti-FoxO3A (12829, Cell Signaling, 1 : 1000), anti-pFoxO3A (Ser253, Ab47287, Abcam, 1 : 750), anti-S6RP (2317, Cell Signaling, 1 : 1000), anti-pS6RP (Ser235/236, 4858, Cell Signaling, 1 : 2000), anti-Akt (4691, Cell Signaling, 1 : 1000), anti-pAkt (Ser473, 9271, Cell Signaling 1 : 1000) or anti-Actin (A2066, Sigma Aldrich, 1 : 2000) overnight at 4°C followed by washing and incubation with horseradish peroxidase- (HRP-) conjugated anti-rabbit (7074, Cell Signaling, 1 : 5000) or anti-mouse (7076, Cell Signaling, 1 : 2000). After washing, chemiluminescent reaction was induced using Clarity Western ECL Substrate (Bio-Rad) and detection performed using a Fusion FX Spectra imager (Vilber). Bands were normalized to Actin levels using ImageJ.

#### 4.8 | Biologic parameters

Cholesterol, triglycerides, urea and fasting glucose serum levels were measured with a biochemistry analyzer VetTest® (IDEXX laboratory) according to manufacturer's instructions.

Serum creatinine levels were measured by an enzymatic colorimetric assay with an autoanalyzer (COBAS 8000 modular analyzer series; kits #CREP2-05168589190).

#### **4.9 | Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5.0 (La Jolla, CA, USA). A Kruskal-Wallis test followed by a Dunn's multiple comparison test was applied for multiple comparisons; otherwise a two-tailed, unpaired t-test was performed for samples with  $n \sim 10$  and a Mann-Whitney test was performed for smaller samples. A  $p$ -value  $< 0.05$  was considered as significant in either case ( $\alpha = 0.05$ ).

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#### **Conflict of interest**

The authors declare no conflict of interest

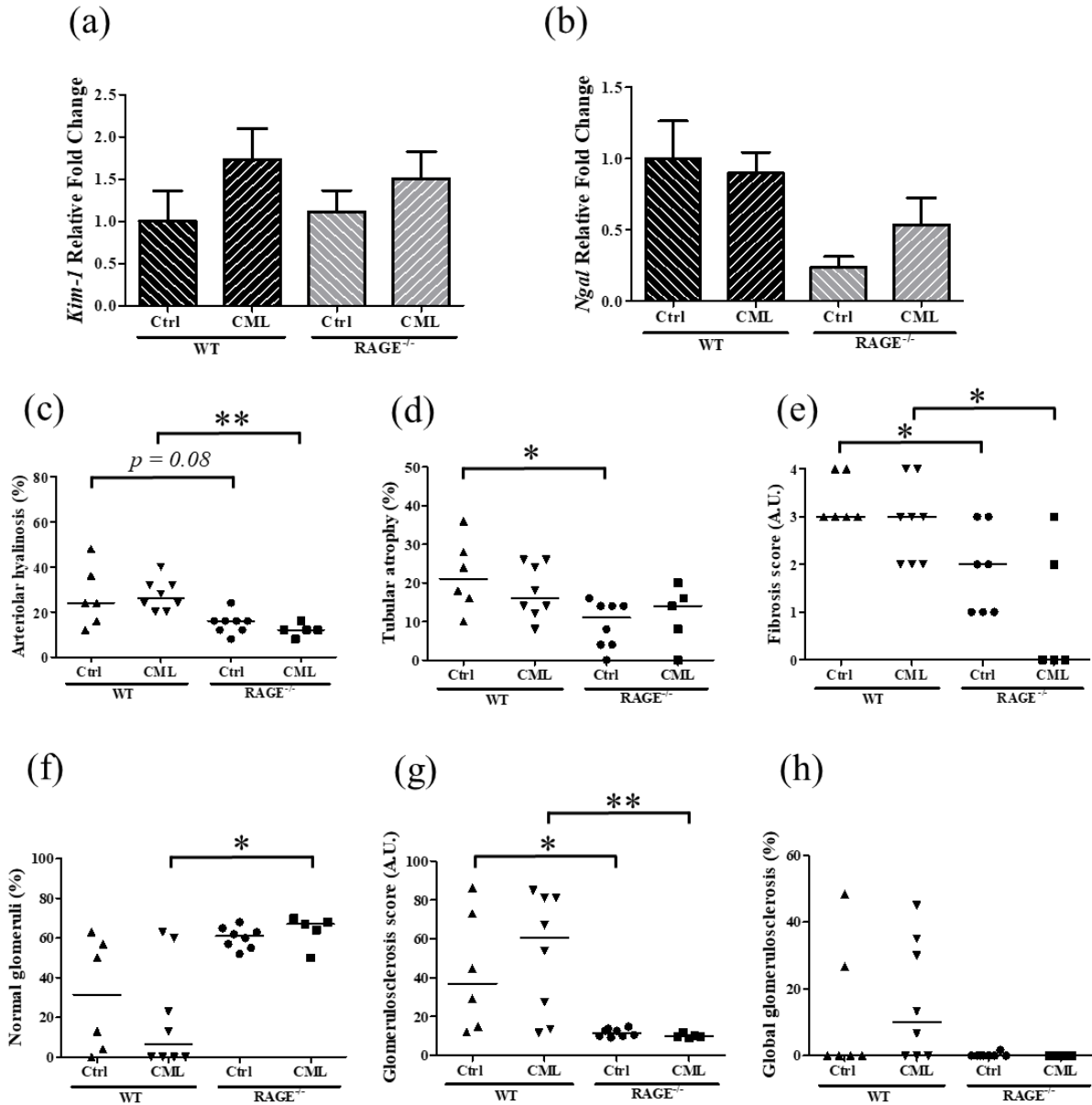
#### **Author contributions**

Study design: MF and EB. Provided RAGE-KO mice: AMS. Performed research: TT, VQ, MD, MH, FD, CL and TB. Histological data analyses: VQ, VG and MF. Statistical analyses: TT and FG. MH provided English proofreading assistance. All authors discussed the data and approved the submission.

## Supplementary figures

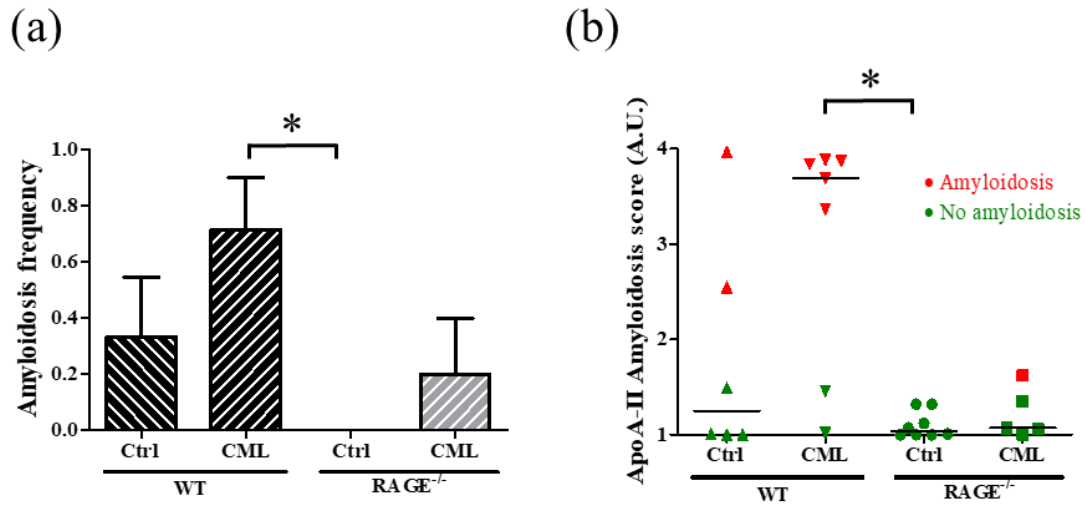
|                             | WT           |              | RAGE <sup>-/-</sup> |              |
|-----------------------------|--------------|--------------|---------------------|--------------|
|                             | Ctrl         | CML          | Ctrl                | CML          |
| Fasting blood glucose (g/L) | 1.11 ± 0.30  | 1.02 ± 0.11  | 1.07 ± 0.14         | 1.03 ± 0.10  |
| Serum cholesterol (mg/L)    | 0.55 ± 0.16  | 0.74 ± 0.19  | 0.81 ± 0.22         | 0.63 ± 0.22  |
| Triglyceridemia (mg/L)      | 0.93 ± 0.32  | 0.72 ± 0.31  | 0.83 ± 0.31         | 0.58 ± 0.14  |
| Mice weight (g)             | 33.70 ± 1.87 | 33.74 ± 2.47 | 33.15 ± 1.28        | 33.32 ± 2.05 |
| Kidneys weight (mg)         | 223 ± 32     | 227 ± 27     | 204 ± 21            | 217 ± 37     |

**Supplementary Table 1. Control and CML WT and RAGE<sup>-/-</sup> mice have similar metabolic conditions.** Values of serum glycaemia (g/L), cholesterolemia (mg/L), triglyceridemia (mg/L) and mice weight (g) at last follow-up showed no signs of obesity or diabetes. Kidney weights (g) measured before longitudinal cut (n=4-5). Kruskal-Wallis test



**Supplementary figure 1. CML has a limited impact on renal suffering markers and renal histological lesions.** (a-b) Expression of kidney injury markers Kim-1 (a) and Ngal (b) in renal tissue from 20-month-old control and CML WT and RAGE<sup>-/-</sup> mice (mean ± SEM, n=3 for 3-month-old mice and n=5 for 20-month-old mice). Histological lesions in paraffin-embedded kidney sections such as (c) arteriolar hyalinosis and (d) tubular atrophy were determined by PAS staining and (e) fibrosis by Sirius red staining. Quantification of glomerulosclerosis (60 glomeruli/mouse) gave (f) the normal glomeruli percentage (glomeruli with GS score of 0), (g) the GS score and (h) the global GS percentage (glomeruli with a GS score of 100). \**p* < 0.05, \*\**p* < 0.01, Kruskal-Wallis test





**Supplementary figure 2. RAGE<sup>-/-</sup> mice are protected against AApoA-II.** (a) Frequency of renal amyloidosis in each group. (b) Scoring of ApoA-II amyloid deposits obtained from IHC in Control and CML WT and in Control and CML RAGE<sup>-/-</sup> mice (n=5-8). \**p* <0.05, Kruskal-Wallis test.

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## 2 | Unpublished results

### 2.1 | Introduction

In this section are presented those data that have not yet been published, either because they are preliminary data, unsuitable for publication but suggesting some routes to follow for future studies, or because their interpretation requires further investigations to fully understand the processes at work.

Hereafter are described some of the studies that are complementary to the publication in *Aging Cell*, above. These works notably investigated how autophagy is regulated in the kidneys of old  $RAGE^{-/-}$  mice, using protein and mRNA markers. As autophagy is often involved in ageing processes and the activity of mTORCs, contributory to autophagy, are diminished in old  $RAGE^{-/-820}$ , it was investigated whether autophagy could be modulated in these mice as well. The renal expression of AGE receptors such as *AGER1*, a receptor usually considered as having consequences opposite to *RAGE*, was also investigated in old WT and  $RAGE^{-/-}$  and *Rage* in young and old WT mice. Finally, the phosphorylation of FoxO3a was studied in these same mice. FoxO3a activity is widely reported to be involved in pro-longevity processes and is notably phosphorylated by *AKT*<sup>837</sup>, hence its activity may be modulated in  $RAGE^{-/-}$  mice.

Subsequently, complementary to our study of kidney function, the motor functions in old WT and  $RAGE^{-/-}$  mice were investigated, as was whether our findings regarding kidney function were similar in female mice.

Finally, in order to further investigate the signalling of *RAGE* and the effects of various *RAGE* antagonists, or of its knock-out, on inflammatory responses and senescence, a protocol to isolate mesangial cells from mouse kidneys was established. These cells were chosen for their structural and functional roles in glomeruli function, hence relevant regarding our previous results on renal ageing. The isolation and characterisation of these cells is described herein.



## 2.2 | Methods

### 2.2.1 | Animal experimentation

The mice used in Fig. U1 are the same as those presented in the previous section (section 1 | Instrumental role of RAGE in ageing): n = 6 WT and n = 6 RAGE<sup>-/-</sup> mice.

Other mice had the same genetic background, were raised under the same conditions but with a standard diet, and were 22 to 26 months old. Organs harvested for histological analyses were conserved in 4 % paraformaldehyde at 4°C prior to analysis; kidneys collected for cell isolation were immediately placed in 4°C Hanks' Balanced Salt solution (HBSS (-Ca<sup>2+</sup>)), fungizone 1 %; organs harvested for protein analyses were immediately snap-frozen in liquid nitrogen and conserved at -80°C; organs harvested for RNA analyses were conserved in RNA later, similarly snap-frozen in liquid nitrogen and conserved at -80°C.

### 2.2.2 | Motor function analysis

Evaluation of the effects of ageing on motor function was made according to published behavioural tests<sup>838,839</sup>. n = 8 WT and n = 5 RAGE<sup>-/-</sup> mice.

#### **Endurance test**

The endurance test was performed using a 50-cm-long, 2-mm-wide tightrope attached to a platform at each side. The tightrope was suspended 20 cm above a soft bedding. The mouse was suspended by its forepaws in the centre of the tightrope. The score was calculated as follows: if the mouse fell within 60 seconds, the time was recorded and taken as the score; if the mouse held for 60 seconds or more, the score was recorded as 60; if the mouse did not fall and reached one of the platforms within 60 seconds, the score was equal to 120 minus the time to reach the platform. The final score was finally corrected to mice weight so that lighter mice were not at an advantage. There were two sessions, with a one-week interval, and in each session two trials were timed.

#### **Rearing counts**

Rearing counts were calculated using a 12-cm-wide and at least 14-cm-high glass cylinder. A mouse was placed in the cylinder for 3 minutes and the rearing number counted. A rear was defined as total lifting of both forepaws and was completed when the forepaws returned to the floor of the cylinder. Between each mouse, the cylinder walls were rinsed using distilled water to avoid any interference. There were two sessions, with a one-week interval.

### **Scurry speed**

Scurry speed was calculated using an 80-cm-long, 3.5-cm-large track with 15-cm-high walls, leading to a 15-cm-long, 10-cm-large and 15-cm-high dark box. A camera was placed above the track to record the runs which were analysed using Kinovea. A run was started as soon as a mouse entered the track and stopped when their nose tip reached the entrance of the dark box. First, the mouse was acclimatised for 1 minute in the dark box, which was completely closed. Then, the mouse was placed in a cylinder, right at the entrance of the track, while very slightly pulling its tail to provoke an escape reaction. The run started when releasing the mouse, associated with a mild puff of air to further encourage the mouse to run away. The dark box was kept closed for 30 seconds once the mouse reached it, to allow it to rest. This was repeated 5 times in total for each mouse but only the 3 last runs were timed. There were two sessions, with a one-week interval.

#### **2.2.3 | Biologic parameters**

Urine was collected over 24 hours in the week before sacrifice, using mouse-specific metabolism cages. Serum creatinine levels were measured by an enzymatic colorimetric assay with an autoanalyzer (COBAS 8000 modular analyzer series; kits #CREP2-05168589190). Only female mice were used here, with  $n = 4$  WT mice and  $n = 2$  RAGE<sup>-/-</sup> mice.

#### **2.2.4 | Histological analyses**

Histological analyses were performed as described earlier and analysed following the same criteria (section 1 | Instrumental role of RAGE in ageing). Only female mice were used here, with  $n = 4$  WT mice and  $n = 2$  RAGE<sup>-/-</sup> mice.

#### **2.2.5 | Western Blot**

Western blots were performed as previously described (section 1 | Instrumental role of RAGE in ageing). Membranes were incubated overnight at 4°C with anti-microtubule-associated proteins 1A/1B light chain 3B (LC3B; ab51520, Abcam, 1 : 3000), anti-FoxO3A (12829, Cell Signaling, 1 : 1000), anti-pFoxO3A (Ser253, Ab47287, Abcam, 1 : 750), anti- S6 Ribosomal Protein (S6RP; 2317, Cell Signaling, 1 : 1000), anti-AGER1 (sc-25558, Santa Cruz, 1 : 500), anti-GAPDH (sc-32233, Santa Cruz, 1 : 500) or anti-Actin (A2066, Sigma Aldrich, 1 : 2000), followed by washing and incubation with horseradish peroxidase- (HRP-) conjugated anti-rabbit (7074, Cell Signaling, 1 : 5000) or anti-mouse (7076, Cell Signaling, 1 : 2000). After washing, the chemiluminescent reaction was induced using Clarity Western ECL Substrate

(Bio-Rad) and detection performed using a Fusion FX Spectra imager (Vilber). Bands were normalized to Actin or GAPDH levels using ImageJ.

### 2.2.6 | RT-qPCR

RNA extraction from half-kidneys was performed with a commercial kit (Qiagen). RNA was quantified by spectrophotometry at 260 nm (Biotech Spec-nano, Shimadzu Biotech) and quality analyzed by RNA Standard Sens kit (BIO-RAD). For real-time quantitative polymerase chain reaction (RT-qPCR), amplification of cDNA was performed with the following probe mixes (ThermoFisher): *Ulk1* (Mm00437238\_m1), *Bnip3* (Mm01275600\_g1), *Atg5* (Mm01187303\_m1), *Rage* (Mm01134785\_g1). The mean cycle threshold (CT) values for both the target and internal control (*Ppia*, Mm02342430\_g1) were determined for each sample. The fold change in the target gene, normalized to *Ppia* and relative to the expression of WT mice fed the control diet, was calculated as  $2^{-\Delta\Delta CT}$ .

### 2.2.7 | Mesangial cells isolation

Fat, adrenals and renal capsules were removed from freshly collected kidneys. Each kidney was subsequently minced in 100  $\mu$ L of collagenase IV 1 % and 900  $\mu$ L of HBSS (+Ca<sup>2+</sup>) were added before incubation for 30 minutes at 37°C under agitation. Lysates were then filtered through a 200  $\mu$ m, a 100  $\mu$ m and a 40  $\mu$ m mesh filter using HBSS (-Ca<sup>2+</sup>) and the 100-40  $\mu$ m fraction was centrifuged for 5 minutes at 800 G. Pellets were resuspended in 1 mL of 0.6 % collagenase in HBSS (-Ca<sup>2+</sup>) and incubated for 30 minutes at 37°C under agitation. Digestion was stopped by adding 1 mL 37°C medium (M199, Zell Shield (1 %), glutamine 2 mM, foetal bovine serum (FBS) 20 %). Lysates were centrifuged for 5 minutes at 800 G and the pellets were resuspended in medium before seeding in 0.1 % gelatine-coated flasks. Cells were incubated at 37°C with 5 % CO<sub>2</sub>. The medium was first changed on day 2, then every 4 days. Uncoated flasks were used after the first passage. Cells were passaged before reaching confluence.

### 2.2.8 | Immunocytofluorescence

For immunocytofluorescence analysis, cells were seeded at 12 000 cell/cm<sup>2</sup> the day before analysis. Cells were fixed with 4 % paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature, followed by 3 washes in PBS for 5 minutes each. When permeabilized was required, cells were incubated with 0.3 % triton X-100 for 10 minutes then washed 3 times in PBS for 5 minutes each. Non-specific sites were blocked using 1 % BSA, 22.52 mg/mL glycine in PBS-tween (PBS-T, PBS + 0.1 % tween 20) for 30 minutes. Cells were then incubated with a primary antibody in 1 % BSA (PBS-T) for 1 hour at room temperature

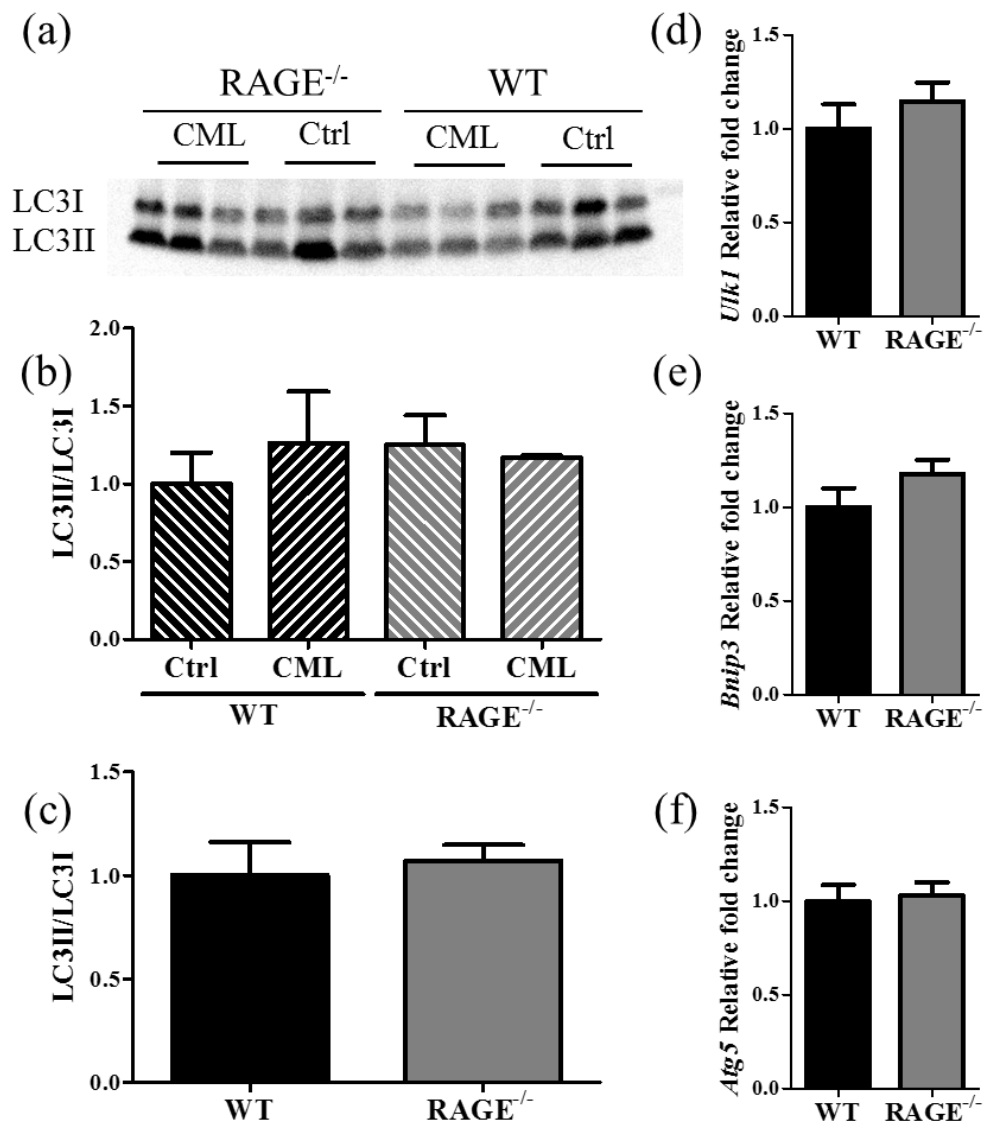
followed by 3 washes in PBS for 5 minutes each. Cells were then incubated with the corresponding secondary antibody in 1 % BSA (PBS-T) for 1 hour at room temperature and again followed by 3 washes. For actin cytoskeleton staining, cells were incubated with phalloidin (1/40 in PBS) for 20 at room temperature, followed by 3 washes. For nuclei staining, cells were incubated with 4',6-diamidino-2-phenylindole (DAPI, 1/2 000 in PBS) for 5 minutes at room temperature, followed by 2 washes. Cells were analysed with a Leica ASMDW microscope magnified 250 to 400 times. Primary antibodies used were anti- $\alpha$ SMA (1 : 500, ThermoFisher, MA5-11547), anti-CD31 (1 : 100, ThermoFisher, MA3105), anti-WT-1 (1 : 100, ThermoFisher, MA1-46028) and anti-RAGE (1 : 20, R&D, AF1145).

### 2.2.9 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (La Jolla, CA, USA). A Kruskal-Wallis test followed by a Dunn's multiple comparison test was applied for multiple comparisons; otherwise a Mann-Whitney test was performed for comparisons of two groups and a t test with Welch's correction was performed for very small samples. A  $p$ -value  $< 0.05$  was considered as significant in either case ( $\alpha = 0.05$ ).

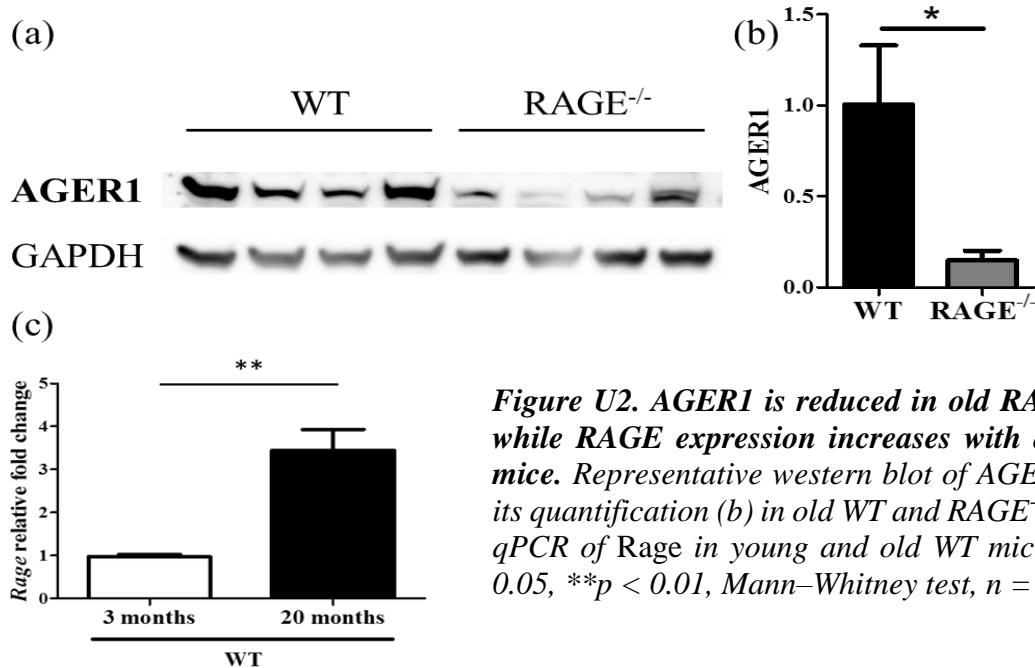
### 2.3 | Complementary results

Markers of autophagy (**Fig. 7**) were measured in kidney extracts to assess autophagy activity following the diet and the genotype. Western blot analysis of LC3B and the LC3II/LC3I ratio did not reveal any influence of a CML-rich diet or of *Rage* deletion in old mice (**Fig. U1. a-c**). Similarly, *Ulk1*, *Bnip3* and *Atg5* expression did not change when comparing old WT and  $RAGE^{-/-}$  mice (**Fig. U1. d-f**).



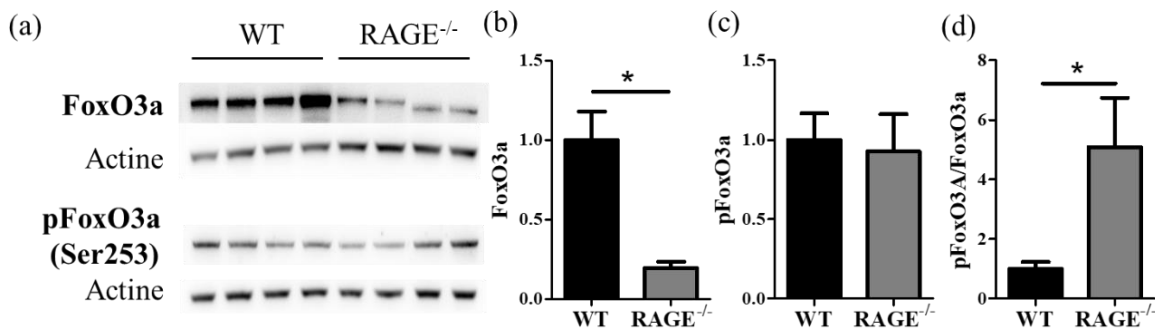
**Figure U1. Autophagy is not modified in old  $RAGE^{-/-}$  mice.** Representative western blot of LC3I and LC3II in kidney extracts of old  $RAGE^{-/-}$  and WT mice fed with a CML-rich or a control diet (a), quantification of the LC3II/LC3I ratio considering the (b) and neglecting the effect of diet (c). RT-qPCR of markers of autophagy *Ulk1*, *Bnip3* and *Atg5* in kidney extracts of old  $RAGE^{-/-}$  and WT mice. ANOVA 1 and Mann-Whitney tests,  $n = 6$

Expression of the AGE receptor *AGER1* was measured in the kidneys of old WT and *RAGE*<sup>-/-</sup> mice, and renal *Rage* expression in both young and old WT mice. The renal expression of *AGER1* was assessed by western blot and a 5-fold reduction ( $p < 0.05$ ) was observed in old *RAGE*<sup>-/-</sup> when compared with old WT mice (**Fig. U2. a-b**). Subsequently, we demonstrated a ~3-fold increase ( $p < 0.01$ ) in *Rage* expression in kidneys of WT mice with age (**Fig. U2. c**).



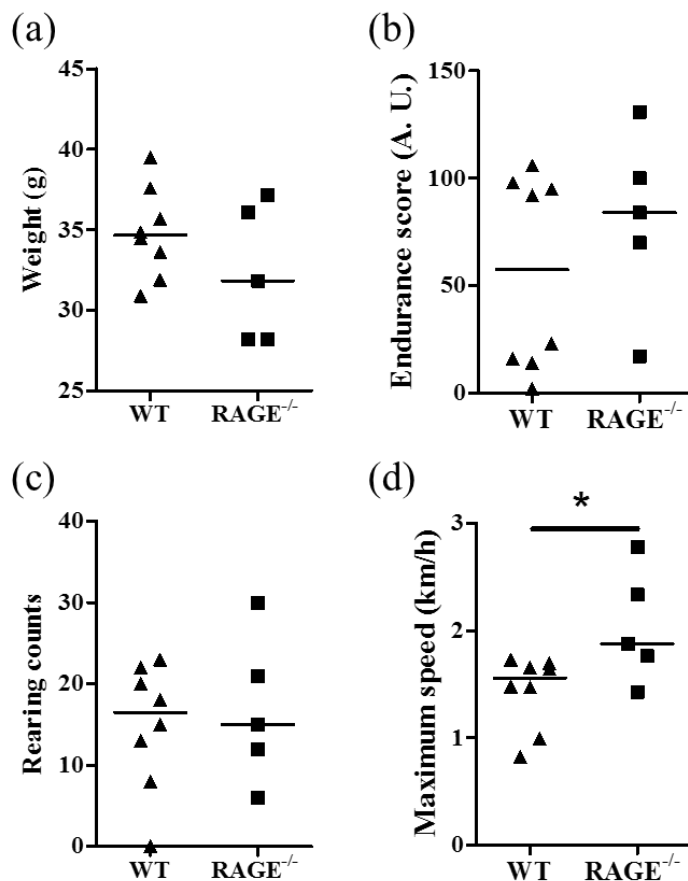
**Figure U2. *AGER1* is reduced in old *RAGE*<sup>-/-</sup> mice while *RAGE* expression increases with age in WT mice.** Representative western blot of *AGER1* (a) and its quantification (b) in old WT and *RAGE*<sup>-/-</sup> mice. RT-qPCR of *Rage* in young and old WT mice (c). \* $p < 0.05$ , \*\* $p < 0.01$ , Mann-Whitney test,  $n = 4$

FoxO3a expression and its phosphorylation on Ser253 were also assessed in kidney extracts of old WT and *RAGE*<sup>-/-</sup> mice. The expression level of FoxO3a was greatly decreased in old *RAGE*<sup>-/-</sup> mice (~5-fold reduction,  $p < 0.05$ ) but the amount of pFoxO3a (Ser253) remained stable. As a consequence, the pFoxO3a/FoxO3a ratio was ~5-fold higher in old *RAGE*<sup>-/-</sup> mice than in WT mice (**Fig. U3. a-d**).



**Figure U3. FoxO3a expression is strongly reduced in old *RAGE*<sup>-/-</sup> mice.** Representative western blots of FoxO3a and pFoxO3a (Ser253) in old WT and *RAGE*<sup>-/-</sup> mice (a), respective quantification (b-c) and pFoxO3a/FoxO3a ratio (d). \* $p < 0.05$ , Mann-Whitney test,  $n = 4$

Subsequent to the analysis of molecular markers various motor functions in WT and RAGE<sup>-/-</sup> mice were assessed (**Fig. U4**). We first weighed the mice to control for this potential bias in performance. Although there was a small trend, there was no significant difference in weight between old WT and RAGE<sup>-/-</sup> mice (**Fig. U4. a**). Endurance scores were calculated after a tight rope test and further corrected with mice weight. No significant difference was observed between old WT and RAGE<sup>-/-</sup> mice although there was a trend toward a better score in RAGE<sup>-/-</sup> mice (**Fig. U4. b**). Rearing counts, notably reflecting coordination, was not affected by genotype (**Fig. U4. c**). Maximum speed, calculated on an 80-cm-long track, was significantly higher in old RAGE<sup>-/-</sup> mice than in WT mice (~18.8 km/h and ~15.5 km/h, respectively,  $p < 0.05$ ) (**Fig. U4. d**).

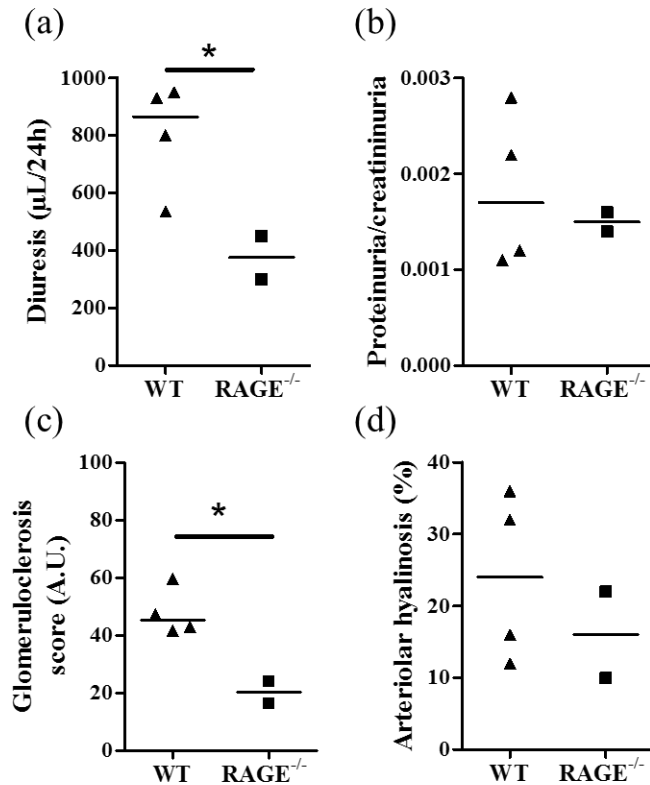


**Figure U4. Fast execution motions are improved in old RAGE<sup>-/-</sup> mice.**

Mice weight (a), endurance score calculated with a tight rope test (b), rearing counts in a cylinder in 180-second sessions (c) and scurry speed calculated from an 80-cm-long course (d) in old WT and RAGE<sup>-/-</sup> mice. \* $p < 0.05$ , Mann–Whitney test

Finally, while these data remain preliminary, kidney function was assessed in old female WT and RAGE<sup>-/-</sup> mice as they were previously assessed in male mice (**Fig. U5**). For the most part, the data presented earlier were validated. Diuresis, calculated from 24h-urine, was significantly higher in WT than in RAGE<sup>-/-</sup> mice ( $p < 0.05$ ) (**Fig. U5. a**). Proteinuria may be decreased in RAGE<sup>-/-</sup> mice as well but medians for WT and RAGE<sup>-/-</sup> mice were very close (~0.0015) (**Fig. U5. b**). Similar to diuresis, glomerulosclerosis was significantly decreased in old RAGE<sup>-/-</sup> mice

when compared with WT mice (~20 A.U. and ~45 A.U., respectively,  $p < 0.05$ ) (**Fig. U5. c**). Although not significant, arteriolar hyalinosis was slightly diminished in  $RAGE^{-/-}$  mice (~16 %) when compared with WT mice (~24 %) (**Fig. U5. d**).



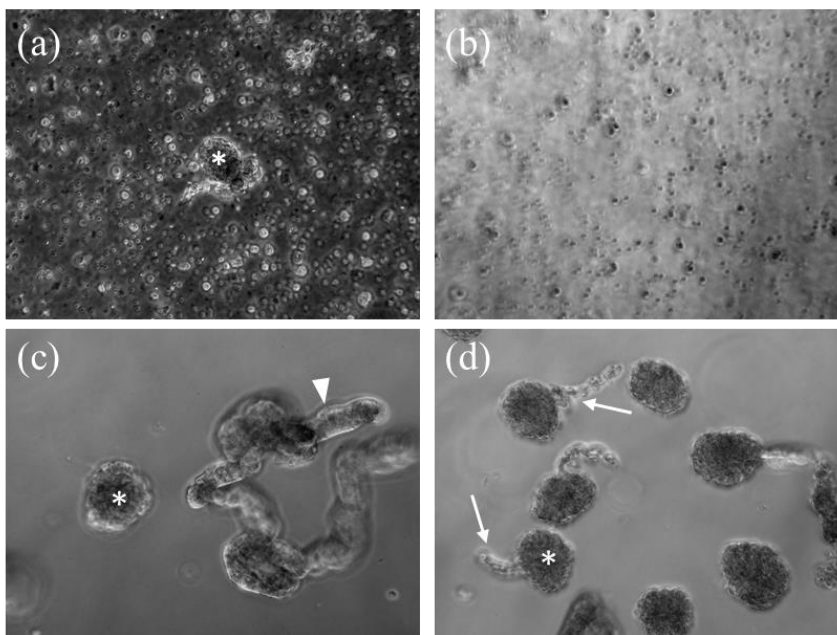
**Figure U5. Kidney protection against physiological ageing is conserved in female  $RAGE^{-/-}$  mice.**

24-hour diuresis ( $\mu\text{L}$ ) in metabolism cage (a), kidney function evaluated by the proteinuria/creatininuria index (b), quantification of GS score in paraffin-embedded kidney sections (30 glomeruli/mouse) (c) and kidney arteriolar hyalinosis determined by PAS staining (25 arterioles/mouse) (d) in old WT and  $RAGE^{-/-}$  mice. \* $p < 0.05$ , *t* test with Welch's correction



## 2.4 | Isolation and characterisation of mesangial cells

Kidneys were dissected from mice using sterilised tools and immediately placed in HBSS (fungizone 1 %) and without  $\text{Ca}^{2+}$  to prevent any contamination and prepare the kidney for dissociation. The organs were immediately processed for isolation of glomeruli. The first dissociation step consisted of a mechanical tissue dissociation followed by enzymatic digest with collagenase IV 0.1 %, in the presence of calcium, at 37°C for 30 minutes. The lysate product was then filtered using multiple filters to divide different fractions and isolate the glomeruli fraction (**Fig. U6**). Three filters were used: a 200  $\mu\text{m}$ , a 100  $\mu\text{m}$  and a 40  $\mu\text{m}$  filter. Prior to filtration, the lysate was composed of undissociated tissues, singles cells, tubules and glomeruli (**Fig. U6. a**). After filtration, the sub-40  $\mu\text{m}$  fraction contained only single cells (**Fig. U6. b**), the fraction above 200  $\mu\text{m}$  was composed of undissociated tissues (not shown), the fraction between 200 and 100  $\mu\text{m}$  was mainly composed of tubules but also of some glomeruli (**Fig. U6. c**), while the fraction situated between 100 and 40  $\mu\text{m}$  was composed of glomeruli with some remnants of arterioles (**Fig. U6. d**).

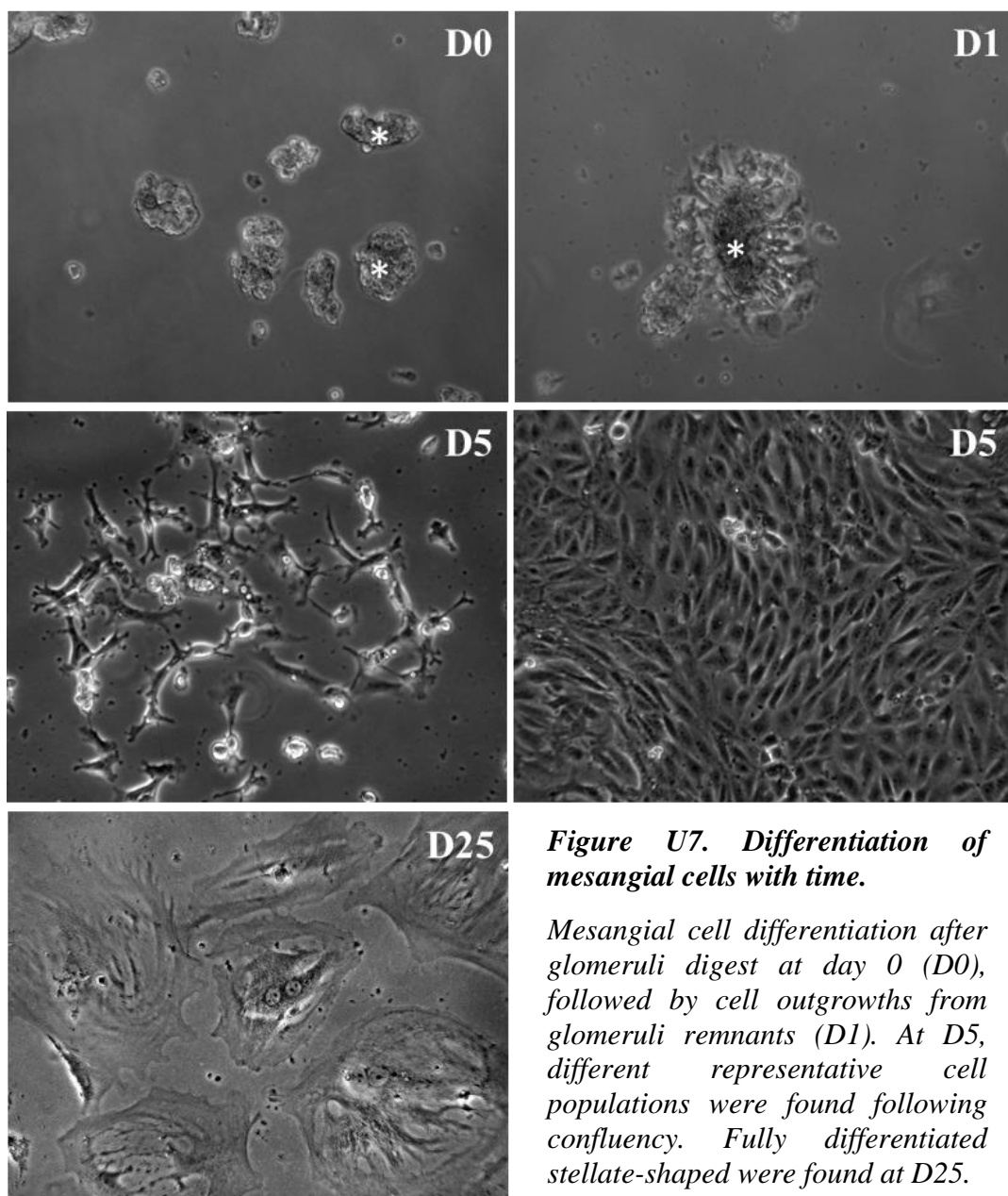


**Figure U6. Isolation of glomeruli.**

*Kidney lysate before filtration (a). Sub-40  $\mu\text{m}$  (b), 200-100  $\mu\text{m}$  (c) and 100-40  $\mu\text{m}$  filtration fractions (d). Asterisk – glomerulus; arrowhead – tubule; arrow – arteriole*

Glomeruli were further dissociated in 0.6 % collagenase IV, in the presence of calcium, at 37°C for 30 minutes in order to allow outgrowth from dissociated glomeruli. The digested glomeruli were then incubated in coated flasks (0.1 % gelatine) at 37°C in M199 medium supplemented with antibiotics, glutamine 2 mM and FBS 20 %. The coating was removed after the first passage.

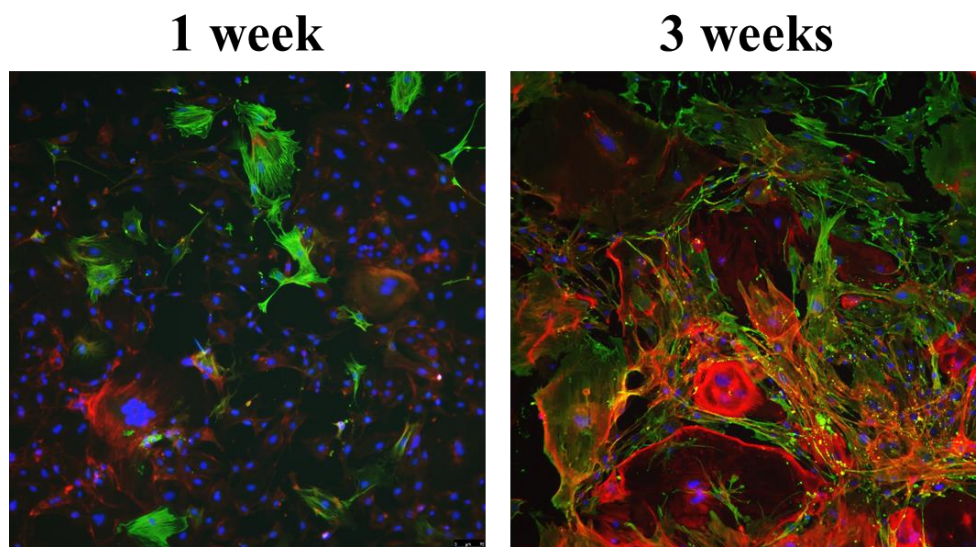
At day 0 (D0), fragments of glomeruli and some single cells were found in culture. As soon as D1, outgrowth emerged from these fragments and quickly spread throughout the flask. By D5, following the confluency, different morphological populations were detectable, mostly having a fibroblast-like or epithelial-like phenotype that may gather in multi-layered mesangial cell hillocks. However, by D25 mesangial cells completely outcompeted other cell types in this medium and were fully differentiated. They exhibited a typical mesangial cell phenotype: large cells with a stellate appearance and able to grow in multiple layers (Fig. U7). Despite this advanced differentiation, these cells could be maintained in culture through several passages, suggesting the existence of progenitors and an ability to proliferate.



**Figure U7. Differentiation of mesangial cells with time.**

*Mesangial cell differentiation after glomeruli digest at day 0 (D0), followed by cell outgrowths from glomeruli remnants (D1). At D5, different representative cell populations were found following confluency. Fully differentiated stellate-shaped were found at D25.*

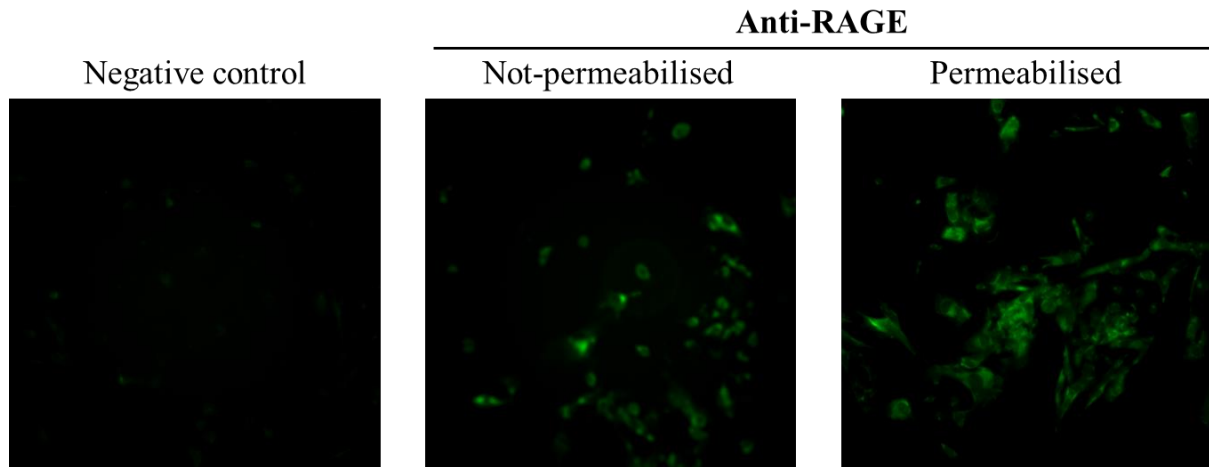
Cell cultures were further characterised by immunocytofluorescence using an anti- $\alpha$  smooth muscle actin ( $\alpha$ -SMA) antibody. There is no known specific marker of mesangial cells but  $\alpha$ -SMA is not expressed in other glomerular cell types such as visceral epithelial cells (podocytes), parietal epithelial cells or endothelial cells<sup>840</sup>. While it is expressed in fibroblasts, the observed morphology and multi-layered proliferation allowed this cell type to be excluded as a potential interference. There was a progressive differentiation towards mesangial cells as  $\alpha$ -SMA was only expressed in some cells one week after isolation, but became more widely expressed after 3 weeks (**Fig. U8**). Immuno-labelling for WT-1 (visceral and parietal epithelial cells), podocin (podocytes) and CD31 (endothelial cells) were all negative (not shown).



**Figure U8.** *Markers of mesangial cells are not expressed before 3 weeks. Immuno-labelling of  $\alpha$ -SMA by immunocytofluorescence one week after cell isolation (left) and 3 weeks after isolation (right). Green –  $\alpha$ -SMA; Red – phalloidin (actin); Blue – DAPI (nuclei)*

Thus, the isolation of mesangial cells was successfully performed using these culture methods within 3 weeks. However, for subsequent experiments it was necessary to confirm that this cell type expresses RAGE.

The immuno-labelling of RAGE showed that it was expressed in mesangial cells (**Fig. U9**). Interestingly, permeabilization of membranes with triton showed a more intense and wide-spread fluorescence, suggesting that RAGE is expressed both at the membrane and in the cytoplasm. However, the antibody (R&D AF1145) used is potentially able to recognize different RAGE isoforms, and it is therefore impossible to conclude on the nature of RAGE isoforms identified here.



**Figure U9. RAGE is expressed in mesangial cells.** Immunocytofluorescence of RAGE in mesangial cell. Non-immune antibody (left), RAGE in unpermeabilized cells (middle) and in permeabilized cells with 0.3 % triton (right)

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# Discussion

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## 1 | Summary of the results

The work in this thesis has shown a clear involvement of RAGE in the renal ageing of mice, although long-term dietary CML exposure did not significantly affect the parameters analysed here.

Immunohistochemistry revealed accumulation of protein-bound CML in the tubules of WT and RAGE<sup>-/-</sup> mice fed a CML-enriched diet but not in control diet. This result was further confirmed by HPLC-MS/MS and both free- and protein bound-CML were significantly elevated in kidneys following diet but no differences were found according to the genotype.

In addition to previous results from our research group regarding vascular ageing in 11-month-old RAGE<sup>-/-</sup> mice<sup>288</sup>, the work presented herein has shown that 20-month-old RAGE<sup>-/-</sup> mice were completely protected against age-related loss of aortic relaxation while arteriolar hyalinosis was also significantly reduced in these mice. Subsequently, it was found that the increased level with age of the marker of chronic kidney injury, *Ngal*, was greatly reduced in old RAGE<sup>-/-</sup> mice when compared with old WT mice. *Kim-1* expression did not change between these two groups, although it increased with age. This was accompanied by reduced tubular atrophy, interstitial fibrosis and more importantly, glomerulosclerosis. Not only was the glomerulosclerosis score homogeneously and strongly reduced in RAGE<sup>-/-</sup> mice, but also ~60 % of their glomeruli remained completely normal by 20 months old and they did not exhibit any global glomerulosclerosis. In WT mice, glomerulosclerosis was accompanied by glomerular hypertrophy, which can precede complete collapse of the nephron or be a compensatory mechanism of nephrons loss<sup>841,842</sup>, but this was not observed in RAGE<sup>-/-</sup> mice whose glomeruli size remained stable. Glomerulosclerosis was also closely associated with apoA-II senile amyloidosis ( $r = 0.9223$ ) and was consequently almost absent in RAGE<sup>-/-</sup> while most WT mice had medium to very severe apoA-II amyloidosis in glomeruli. In the most severe cases, amyloidosis was also found in the interstitium of WT animals.

The expression of markers associated with ageing, including markers involved in inflammation, oxidation and in metabolic routes was also investigated. The expression of inflammation markers *Il-6*, *Tnfa* and *Vcam-1* were all significantly and greatly increased with age in WT mice but their levels were 2 to 3 times lower in old RAGE<sup>-/-</sup> mice. Expression of the antioxidants *Sod2* and *Hmox*, but not *Cat*, were significantly decreased with age in WT mice but were better maintained in RAGE<sup>-/-</sup> mice, especially *Sod2*. SIRT1 was higher (though not significantly so)

in old RAGE<sup>-/-</sup> than in old WT mice ( $p = 0.056$ ) and phosphorylations of S6RP (Ser235/236) and AKT (Ser473), representing activity of mTORC1 and mTORC2, respectively, were greatly reduced in old RAGE<sup>-/-</sup> mice.

Complementary to these results it was also shown, however, that autophagy is identical between WT and RAGE<sup>-/-</sup> mice, suggesting that various pathways may regulate autophagy despite differences in mTORCs activities. These results are associated with an intriguing significant and large reduction of the expression of FoxO3a in old RAGE<sup>-/-</sup> mice when compared with old WT mice. The overall level of its phosphorylation (Ser253) was nevertheless very similar between the two groups, leading to a pFoxO3a/FoxO3a ratio in favour of RAGE<sup>-/-</sup> mice. Analysis of expression of receptors for AGEs revealed that, as expected, *Rage* expression was increased with age; contrary to expectations, AGER1 expression was decreased in RAGE<sup>-/-</sup> mice.

To further characterise healthspan in RAGE<sup>-/-</sup> mice, we analysed their motor functions. While endurance and rearing ability were similar between old WT and RAGE<sup>-/-</sup> mice, RAGE<sup>-/-</sup> mice's top speed was significantly higher. Finally, preliminary results are presented suggesting that the protection against renal lesions afforded by RAGE knockout in males was also found in RAGE<sup>-/-</sup> female mice, although the sample size was very small and should be interpreted with caution.

## 2 | Discussion

### 2.1 | Effects of dietary CML are outcompeted at old age

The absence of obvious dietary CML effects on kidney ageing at 20-months-old contrasts with previous reports from this research group of CML-accelerated vascular ageing in 11-month-old mice<sup>288</sup>. Importantly, RAGE expression did not affect dietary CML accumulation or localisation in organs<sup>566</sup> and did not induce changes in food or calorie intake<sup>288</sup>. Pro-ageing effects of dietary CML on vessels were observed at 11 months old but major lesions in kidneys were not detectable at this age, including arteriolar hyalinosis (not shown), suggesting that: 1) kidney ageing is detectable later than vascular ageing, and might be partly caused by vascular ageing, and; 2) in old age, the effects of dietary CML become negligible. Current literature suggests that free-CML is not able to bind to RAGE<sup>645</sup>, thus the effects of free-CML from dietary CML digestion on vascular ageing are not directly caused by interaction with RAGE in vessels. There is a possibility that free-CML interacts with other molecules or receptors and

that RAGE is an essential downstream effector of a complex signalling pathway. Another possibility is that undigested dietary CML in intestine interacts *in situ* with RAGE to further provoke inflammation which promotes vascular ageing seen in 11-month-old mice. This hypothesis is supported by the findings that inflammation in inflammatory diseases is associated with decreased endothelial function and increased arterial stiffness<sup>843</sup> and is reduced when RAGE is blocked or deleted<sup>792</sup>. In addition, intestinal permeability is increased in inflammatory diseases and with ageing<sup>314,844,845</sup>, suggesting a possible leakage of protein-bound CML in those conditions. On the other hand, the absence of an effect of dietary CML in old age might be explained by an age-related increase in competition with other RAGE ligands. It has been shown that RAGE ligands such as AGEs<sup>154,585</sup>, HMGB1<sup>715</sup>, S100s<sup>715,846</sup> increase with age in serum and/or in some tissues, including kidneys. LPS exposure because of leaky gut might increase as well. As a consequence, with ageing dietary CML becomes negligible when competing with other RAGE ligands that have much higher affinities for RAGE and there is a chance that physiological ageing catches up with, and eventually eclipses, dietary CML-accelerated ageing seen in 11-month-old mice.

This chain of events may also explain why there is increased protein-bound CML following diet. Protein-bound CML could be the result of a stress induced by dietary CML, hereby promoting oxidation and glycation of proteins. However, our results do not show such an induction with a CML-enriched diet at old age. The leaky gut at old age could allow the absorption of protein-bound CML, which thereafter accumulates in kidneys. Finally, it is also possible that technical limits prevent a clear distinction between protein-bound and free-CML. The antibody used in the immunohistochemistry assays here was able to bind to protein-bound but not to free-CML in a dot blot assay (not shown), suggesting it only recognises protein-bound CML. However, this absence of binding could be explained by a lack of attachment of free-CML to the membrane. Although HPLC-MS/MS is highly specific, there is also a possibility that free CML was insufficiently separated from the protein-bound fraction, thus leading to an overestimation of the level of protein-bound CML via contamination by free-CML.



## 2.2 | ApoA-II amyloidosis is a relevant marker and cause of ageing

Although senile apoA-II amyloidosis is extremely rare in humans, it is a common marker of murine ageing<sup>728</sup>. ApoA-II is notably less stable in mice because of its monomeric structure and the presence of amyloidosis hot-spots, rich in methionines that are oxidation targets<sup>726</sup>. Of note in mice, a genetic polymorphism of *Apoa2* has been described, including *Apoa2<sup>a</sup>*, *Apoa2<sup>b</sup>* and *Apoa2<sup>c</sup>*. *Apoa2<sup>c</sup>* is very amyloidogenic and *Apoa2<sup>a</sup>* is mildly amyloidogenic, while *Apoa2<sup>b</sup>* is protective<sup>727,728</sup>. The genetic background of our mice was C57Bl/6, which has the mildly amyloidogenic *Apoa2<sup>al</sup>* allele. It has been demonstrated that this polymorphism alters the interaction between apoA-II and high-density lipoproteins (HDLs), and changes in HDLs' structure favours the formation of apoA-II amyloidosis<sup>847,848</sup>. Lipid peroxidation and apolipoprotein oxidation also destabilise HDLs while glycation of proteins is one factor promoting amyloidogenesis<sup>609,726</sup>. Thus, it would be interesting to analyse the structure and number of HDLs, modifications of apoA-II and liver ageing in old WT and RAGE<sup>-/-</sup> mice. In humans as well, amyloidosis can increase with age, as seen in A $\beta$  amyloidosis<sup>849</sup> or transthyretin amyloidosis (ATTR)<sup>850,851</sup>, and many kinds of amyloidosis (including ATTR) involve deposits in the kidneys<sup>852</sup>. Furthermore, in patients positive for amyloidosis, glomeruli are the most commonly and severely affected compartment of the kidney and severe amyloidosis is an indicator of poor prognosis<sup>853</sup>. In murine kidney and liver, ApoA-II amyloidosis was shown to induce ER stress, promoting the unfolding protein response (UPR) and leading to DNA damage and apoptosis<sup>854</sup>. *In vitro*, TTR fibrils were able to bind to RAGE and induce inflammation *via* NF- $\kappa$ B<sup>740</sup>. However, while ATTR was co-localised with RAGE and AGEs in humans, RAGE-mediated cytotoxicity was distinct from NF- $\kappa$ B signalling<sup>855,856</sup> suggesting it might rather involve ER stress, similar to apoA-II amyloidosis. It would therefore be very interesting to assess the cytotoxicity of apoA-II fibrils through RAGE signalling. This also suggests that, although apoA-II amyloidosis is rare in humans, there might be some similar kidney ageing routes between mice and humans in this regard.

The presence of apoA-II amyloidosis in the interstitium only occurred when all glomeruli had the highest grade of amyloidosis, suggesting an advanced stage of amyloidosis severity and of kidney damage. This was never observed in RAGE<sup>-/-</sup> mice kidneys. Although it has been mentioned that glycation could help initiate amyloidosis<sup>609,857</sup>, there is also a chance that this glycation of amyloid fibrils could enhance their interaction with RAGE<sup>619</sup> or could even be a determinant factor in this recognition. Finally, taking into consideration the strong correlation between apoA-II amyloidosis and glomerulosclerosis, and the negative impact of apoA-II

amyloid fibrils and RAGE on surrounding tissues<sup>854</sup>, it seems reasonable to suggest that apoA-II amyloidosis might trigger the glomerulosclerosis observed in mice.

There is also a potential interplay between apoA-II, apoE, A $\beta$ <sub>1-42</sub> and RAGE. In humans, the *APOE- $\epsilon$ 4* allele is a widely-described risk factor for Alzheimer's disease, which also affects normal brain function<sup>858,859</sup>, while the *APOE- $\epsilon$ 2* allele is considered protective<sup>860</sup>. Interestingly, apoE is able to bind to A $\beta$  and prevent its cytotoxicity and this binding affinity is greatly enhanced when apoE is complexed with apoA-II. However this increased binding only occurs with apoE- $\epsilon$ 2 (and apoE- $\epsilon$ 3) but not with apoE- $\epsilon$ 4<sup>861</sup>. In addition, gene delivery of human *APOE- $\epsilon$ 2* in murine models of AD reduced the hippocampal level of insoluble A $\beta$ <sub>1-42</sub>, while delivery of *APOE- $\epsilon$ 4* increased it<sup>862</sup>. RAGE mediates A $\beta$  transport to the brain through the blood-brain barrier, and its internalisation in neurones and neurotoxicity<sup>737,863-865</sup>. There is therefore a chance that this complexation limits A $\beta$  deposition and nucleation and its interaction with RAGE while the *APOE- $\epsilon$ 4* allele facilitates it. This association between AD and RAGE, hinted at by other observations such as the increased level of AGEs in AD patients' brain, led to a clinical trial using the RAGE inhibitor Azeliragon. Although it was terminated due to a lack of efficiency during phase III trials, a post-hoc analysis revealed positive effects in patient with AD and diabetes, both pathologies that have been associated with RAGE<sup>866</sup>. Moreover, this result underlines the potential of a link between these two pathologies<sup>867</sup>.

### 2.3 | Metabolism is partially altered by RAGE

As well as the above, metabolism is also altered in RAGE<sup>-/-</sup> mice with ageing. The diminution of the phosphorylation of AKT (Ser473) reported in this thesis is supported by the literature as stimulation of RAGE is known to activate the PI3K/AKT pathway<sup>868,869</sup>. However, it should be noted that AKT is not only phosphorylated on Ser473 by mTORC2 and is not simply a marker of mTORC2 activity<sup>493,870,871</sup>. Similar findings have been reported regarding S6RP phosphorylation on Ser235/236<sup>872</sup>. However, these accumulating data suggest that activity of mTORC1 and mTORC2 are both greatly reduced in old RAGE<sup>-/-</sup> mice. This is in line with the pro-ageing effects of this pathway that were described in the introduction. In addition, it has also been shown that AGE/RAGE interaction was able to phosphorylate AKT through transactivation of IGF-R1<sup>873</sup>. This result adds further complexity to the ensemble of RAGE signalling, but also suggests a very interesting link between RAGE and the insulin/insulin-like growth factor 1 signalling (IIS) pathway, that is still very poorly studied. SIRT1, a pro-longevity

molecule also involved in nutrient sensing, is increased in old RAGE<sup>-/-</sup> mice, according to previous results in middle-aged mice<sup>288</sup>. However, beyond the protein level of SIRT1, it would even more interesting to determine its activity by analysing its phosphorylation on Thr522<sup>874</sup>, its oligomerization<sup>873</sup>, or the level of acetylation of its targets such as PGC-1 $\alpha$ , NF- $\kappa$ B or p53<sup>875,876</sup>. However, despite these results strongly suggesting changes in metabolism, no change in markers of autophagy were seen in the work presented herein. This is contrary to expectations since the literature on RAGE signalling and autophagy is rich<sup>877-879</sup>, even showing increased RAGE expression with a lowered LC3II/I ratio in aged kidney tissues<sup>880</sup>, and mTORC1 is well described as preventing autophagy<sup>91</sup>: an increased autophagy in old RAGE<sup>-/-</sup> mice at homeostasis was thus expected. But autophagy is a very complex mechanism which can be activated by multiple stressors<sup>881</sup> and can be beneficial or detrimental, depending on the context, its intensity and duration<sup>882-887</sup>. It is therefore necessary to further investigate this mechanism in more controlled conditions.

FoxO3a activity is regulated by its phosphorylation and acetylation status. AKT is able to phosphorylate FoxO3a on Thr32, Ser253 and Ser315 in humans, which sequesters it in the cytoplasm and prevents its transcriptional activities; phosphorylation on other multiple sites by ERK 1/2 and p38-MAPK leads to its inhibition and degradation, while phosphorylation of another set of serines by AMPK or JNK activates or transactivates it<sup>837</sup>. Acetylation of FoxO3a by CREB-binding protein (CBP)/p300 coactivator attenuates its transcriptional activity, limiting its DNA-binding activity<sup>888,889</sup> and promoting its degradation<sup>890,891</sup>, while deacetylation by SIRT1 increases transcriptional activity<sup>889,892,893</sup>. However, under some conditions, deacetylation decreases FoxO3a activity<sup>894,895</sup>. This discrepancy might be explained by more complex associations between the phosphorylation and acetylation status. FoxO3a activation is clearly associated with pro-longevity pathways, transcribing genes involved in survival, ROS detoxification or DNA repair, but can also promote cell death when the nuclear form is continuously expressed<sup>896,897</sup>. Thus, the results presented here regarding AKT activity predicted an increased activity of FoxO3a in RAGE<sup>-/-</sup> mice, characterised by a diminished phosphorylation of Ser253, in line with a global pro-longevity phenotype. Not were these expected differences in pFoxO3a (Ser253) not observed but the total protein was greatly reduced. Therefore, a better characterisation of its acetylation and phosphorylation status is urgently needed to understand this striking result, and to determine the pathways which regulate the degradation or expression of FoxO3a in old RAGE<sup>-/-</sup> mice.

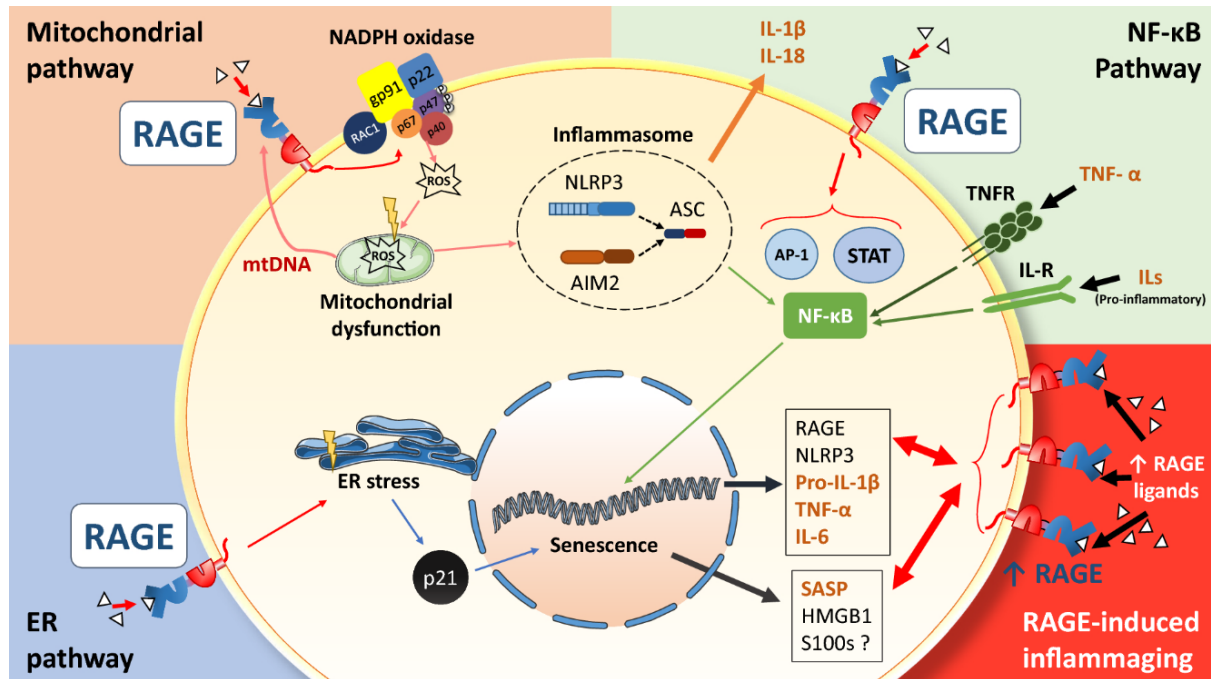
## 2.4 | RAGE induced-oxidation is arguably determinant in ageing

Expression of antioxidants is modestly changed by *Rage* deletion. The work presented here shows that *Hmox* expression decreases in both old WT and RAGE<sup>-/-</sup> mice, but the lack of published data on this marker in the context of ageing precludes meaningful comparison with the literature. However, the literature suggests that catalase expression is reduced in models of ageing and premature ageing, but increased in extended longevity models<sup>212,898,899</sup>. Our results do not show such variations although there was a suggestion of a trend towards a diminution of *Cat* expression in old WT mice. Finally, a decrease of *Sod2* expression in WT mice, that is less important in old RAGE<sup>-/-</sup> mice, is also reported herein. Similar findings were made in young mice where WT mice already had lower levels of SOD2 expression and activity<sup>900</sup>. Importantly, glycation inhibits the activity of these enzymes and their expression is reduced with high levels of AGEs<sup>901,902</sup>. However, RAGE stimulation promotes ROS production, including mitochondrial superoxide<sup>903</sup>. There is a good chance that this promotes SOD2 expression and activity in the short term which is not guaranteed to persist in the longer term. The results presented here only represent mRNA levels of these enzymes: it would naturally be interesting to investigate their protein levels and activity as well. Similarly, it would also be of interest to investigate the level of oxidation products such as carbonyl compounds, 8-oxo-2'-deoxyguanosine or malondialdehyde. It should be remembered, however, that the role of ROS in ageing remains elusive and is not as detrimental as initially thought<sup>102,214,215</sup>.

## 2.5 | RAGE has a pivotal role in ageing through inflammaging

The observed decrease in inflammation in old RAGE<sup>-/-</sup> mice, represented by *Il-6*, *TNF-α* and *Vcam-1* expression, was according to our expectations. Although already very interesting, this work would benefit from the addition of data on protein levels of pro- and anti-inflammatory molecules, and the characterisation of infiltration of immune cells like macrophages or neutrophils, since RAGE is also involved in this process<sup>782,904,905</sup>. Indeed, RAGE is primarily characterised by its pro-inflammatory properties and the data reported here supports a whole body of literature suggesting that RAGE participates in a pro-inflammatory phenotype. As such, different routes were investigated during this thesis that are widely supported in the literature and propose a role for RAGE in inflammaging (**Fig 23**)<sup>659</sup>. Inflammaging is described as a sterile, chronic and low-grade inflammation, notably characterised by accumulation of pro-inflammatory molecules (the SASP), that both increase with and actively participate in

ageing<sup>233–235</sup>. Thus, RAGE is involved in a mitochondrial pathway, through mitochondrial dysfunction that converges *via* inflammasome activation to a NF- $\kappa$ B pathway, which is also engaged by direct RAGE activation and activation of other PRRs by their ligands. A third pathway is described in this thesis, although less-often described in the context of RAGE activation, that involves senescence induced by the ER stress. These pathways all favour senescence, SASP sustainment, production of RAGE ligands and RAGE expression, engendering a vicious circle<sup>659</sup>.



**Figure 22. RAGE in inflammaging.** RAGE-induced inflammaging pathways. Three major pathways have been identified leading to inflammaging and induced via RAGE activation by its ligands. The mitochondrial pathway involves NADPH oxidase activation by RAGE. Subsequent production of ROS impairs mitochondrial function and mitochondria accumulate damage and ROS: the mitochondrial products released can either reactivate RAGE or the inflammasome, thereafter responsible for release of ILs and NF- $\kappa$ B activation. NF- $\kappa$ B can also be activated directly by RAGE or by pro-inflammatory cytokine activation of TNFR or IL-R. NF- $\kappa$ B and other transcription factors, such as AP-1 or STATs, will thereafter promote the transcription of RAGE, NLRP3 and pro-inflammatory cytokines, all of which participate in inflammaging. RAGE activation can also provoke sustained ER stress, inducing a p21-dependant senescence. Subsequently, the SASP will fuel inflammaging and senescent cells will release RAGE ligands such as HMGB1 and S100s. Ultimately, RAGE expression increases with age, as do concentrations of a number of its ligands, thus engaging a vicious circle of RAGE-induced inflammaging with advancing age. The pro-inflammatory components responsible for inflammaging are highlighted in orange. Abbreviations are available in List of abbreviations. After Teissier and Boulanger, *Biogerontology*, 2019

The results presented here strongly support this hypothesis since not only is chronic inflammation reduced in old RAGE<sup>-/-</sup> mice, but *Rage* expression is also increased with age. The

decrease of AGER1 expression in old RAGE<sup>-/-</sup> mice compared with WT mice was, however, unexpected as different works have described AGER1 as functioning in opposition to RAGE. AGER1 also binds AGEs<sup>906,907</sup> and is involved in their removal<sup>637,908,909</sup>: it is down-regulated in diabetes and with diabetic complications<sup>910,911</sup>, although it might be increased in the early stages<sup>912</sup>, and is negatively correlated with serum AGE levels<sup>913</sup>. Furthermore, its overexpression limits AGEs-induced activation of the NADPH oxidase<sup>914</sup>, FoxO3a inhibition and SOD2 downregulation<sup>915</sup>, NF-κB and ERK 1/2 phosphorylation through RAGE signalling, and also prevents RAGE up-regulation<sup>637</sup>. Finally, while AGEs decrease NAD<sup>+</sup> levels and the expression of AGER1 and SIRT1, their respective level was restored when overexpressing AGER1 or restricting AGEs while AGER1 silencing increased these effects<sup>636,638,916</sup>. These data support a strong role for AGER1 in promoting longevity or healthspan by limiting the influence of AGEs and RAGE, however, more recent work by another group identified a detrimental role for AGER1 in AGEs-induced hepatic injury and there is a chance that it does not protect against diabetic kidney disease where it could actually promote podocyte dysfunction<sup>640,917</sup>. Therefore, this downregulation of AGER1 in our old RAGE<sup>-/-</sup> mice deserves more investigation to provide a satisfying mechanistic explanation of its role in ageing.

In addition, to further validate the model presented above, it would also be very interesting to assess the concentration of various circulating and tissue RAGE ligands in our mice, including HMGB1, S100s and other AGEs such as pentosidine, glucosepane or MG-H1.

## 2.6 | RAGE signalling potentially accelerates sarcopenia

Motor function in old RAGE<sup>-/-</sup> mice was not significantly changed except for scurry speed that was higher than in old WT mice. This result is in accord with current knowledge about sarcopenia. Indeed, major changes involve a depletion in type 2 muscle fibres that are fibres dedicated to fast-twitch<sup>273,918</sup>. This difference might be explained by reduced inflammaging in RAGE<sup>-/-</sup> mice, which has a negative impact on muscle performance<sup>919-922</sup>, a possible preferential accumulation of AGEs<sup>596-599</sup> and other RAGE ligands<sup>715</sup> in structures related to movement in WT mice which can further provoke dysfunction through RAGE. Mitochondrial function and dynamics are essential in muscle function<sup>923,924</sup> and the Ligand/RAGE axis has notably been involved in myocardial<sup>900,925-927</sup> and skeletal<sup>928,929</sup> contractile and mitochondrial dysfunctions. However, these data should not be over interpreted. Even though protocols used here were validated in the literature, the conditions were not optimal and the tests should be

performed again, in a larger cohort and with complementary analyses including the grip strength test, horizontal and vertical movements in an open-field or the rotarod test, measuring balance and motor coordination. As an aside, muscle mass reduction with age affects the rate of creatinine production, thus, evaluation of kidney function measuring serum creatinine might not be a good marker to compare old WT and RAGE<sup>-/-</sup> mice<sup>930</sup>.

## 2.7 | RAGE physiological role remains imperceptible and might be compensable

In the bigger picture, the precise physiological function(s) of RAGE remain(s) elusive as no deleterious side effect of its deletion was detected. As detailed in the introduction, there is no gene for ageing but there is the possibility that, in line with inflammaging, RAGE is an example of antagonistic pleiotropy. Although the role of RAGE is uncertain, some positive roles have nevertheless been described in the introduction, including a degree of protection against some pathogens; however important this function may be, the drawbacks of RAGE expression appear to outweigh its absence under laboratory conditions. It is indeed important to consider the context of research on RAGE in animal models and its knock-out could reveal more obvious functions under “real-life” conditions. Despite this statement, results in humans remain valid and, although it is different from a complete knock-out, the use of a RAGE antagonist in a clinical trial did not reveal any negative side effect<sup>834</sup>. There is also the possibility that RAGE’s original purpose has been lost through evolution and the existence of unnecessary genes is not that rare since gene loss, including the *DUFFY* gene evoked in the introduction, is a driver of evolution<sup>931–934</sup>. It is also possible that there is a redundancy in genes’ functions and that in the absence of *RAGE*, other genes ensure its function while limiting inflammation resulting from redundant systems. Indeed, gene duplication is another important force of evolution and could explain such overlapping functions<sup>657,935</sup>. As such, Toll-like receptors (TLRs), especially TLR4, share many common features with RAGE. They are very well described PRRs, are multi-ligand receptors, including RAGE ligands such as HMGB1, S100s, LPS and nucleic acids<sup>936</sup>, have similar intracellular effectors TIRAP and MyD88<sup>752</sup>, are strongly associated with inflammation and inflammaging<sup>716,937–941</sup>, and can be involved in pathologies such as atherosclerosis<sup>942–945</sup>. But it should be noted that their involvement in age-related diseases seems much less prominent and that they are perhaps less pleiotropic than RAGE. On top of these properties, several studies suggest that at least TLR4 and TLR9 can heterodimerize with RAGE<sup>753,754,946</sup>. Thus, it is

sensible to think that in the absence of RAGE, some of its functions could be ensured by other receptors, such as TLRs, and that its presence might duplicate already functioning inflammatory systems.

### 3 | Future directions

This work opens prospective avenues for improving our understanding of the role of RAGE in ageing by raising at least as many questions as it answers. Hopefully, however, it has also provided tools that will facilitate future studies.

The isolation of mesangial cells is one such tool, and will be potentially very useful as it will allow the initiation of primary cell cultures of WT and RAGE<sup>-/-</sup> mice, either young or old, to assess the impact of RAGE ligands, or RAGE blocking, in the many signalling pathways that have described so far, to determine the role of the Ligand/RAGE axis in the onset of senescence and the SASP, and study the trafficking of RAGE which is still poorly understood. In line with this, our research group has also developed cell line models of human embryonic kidney (HEK) 293 overexpressing FL-RAGE, DN-RAGE and ΔN-RAGE, while RAGE remained undetectable in the control line. This will enable the screening of the effects of many RAGE ligands depending upon the structure of RAGE.

A large collection has also been established of many different organs from old and middle-aged WT and RAGE<sup>-/-</sup> mice, including kidneys, lungs, liver, gut, heart, aorta, brain, gastrocnemius muscles, femur, skin, and eyes. They were collected in order to perform protein, RNA or histologic analyses, following the relevance of each organ. In addition, 24-hour urine and faeces, together with serum and circulating cells, were also collected during the work described in this thesis. These sample archives will provide useful means to investigate the role of RAGE in the ageing of different organs and perhaps establish links between them, as already suggested in this discussion regarding ageing of the liver or gut.

Finally, a longevity study will be initiated to determine the lifespan of RAGE<sup>-/-</sup> mice, which has never been reported so far. This study will also be the opportunity to longitudinally evaluate ageing in these mice using non-invasive experiments such as the evaluation of motor function together with behavioural and memory tests. Further, the use *in vivo* of RAGE antagonists,



previously selected through screening in cell culture, will permit the evaluation of their impact on WT mice ageing and on inflammaging.

The literature shows that many deleterious effects of AGEs are bound to RAGE signalling and their accumulation appears much less harmful when RAGE is absent<sup>288</sup>. There exists means to limit their accumulation by controlling glycaemia, targeting dicarbonyl compounds with aminoguanidine, pyridoxamine or benfotiamine<sup>947-951</sup>, or enhancing detoxifying systems with flavonoids<sup>952,953</sup>. But however interesting it may be, and without discussing specificity issues, these treatments only target one category of the many ligands of RAGE and, as shown by the loss of effects of dietary CML at old age, the effects of AGEs are increasingly competing with the accumulation of other RAGE ligands. While glycation needs to be prevented to limit RAGE-independent effects, in the context of RAGE signalling it is virtually impossible to simultaneously target every single RAGE ligand. Therefore, it is perhaps much more relevant to directly target RAGE, the focal point of these many ligands.

Many strategies have been considered and new strategies and compounds are still being developed in order to allow an efficient blocking of RAGE signalling. RAGE blocking may give significantly different results from RAGE knock-out. Indeed, while RAGE knock-out may induce some developmental changes<sup>782</sup> and is immutable, a pharmacologic treatment would not involve developmental alterations and is reversible should it become necessary to stop<sup>780</sup>.

The first anti-RAGE therapies to have been proposed include several examples of anti-RAGE antibodies. While the reported outcomes on renal diabetic complications<sup>954</sup>, acute respiratory distress syndrome<sup>955</sup>, crush injury consequences<sup>956</sup>, ischemia recovery<sup>957</sup> or sepsis<sup>958,959</sup> are positive, they are accompanied with issues bound to the use of antibodies as a treatment: they are expensive, difficult to produce and purify, and they are large molecules which may hinder efficient delivery to tissues – in addition, while they can be sensitive, their specificity is not always guaranteed<sup>960,961</sup>.

Although sRAGE possesses some of the disadvantages of antibodies, owing primarily to its size, it has the major advantage of being able to target all RAGE ligands, including many DAMPs and PAMPs that activate pro-inflammatory signalling *via* other PRRs, and it can also directly bind RAGE to prevent its oligomerization or binding to ligands<sup>962,963</sup>. sRAGE has also been administered in multiple pre-clinical studies aimed at limiting atherosclerosis<sup>964-966</sup>, renal

complications of diabetes including glomerulosclerosis<sup>967,968</sup>, diabetes-induced loss of pain<sup>969</sup>, amyotrophic lateral sclerosis development<sup>970</sup>, asthma<sup>971</sup> or the consequences of enterocolitis<sup>775</sup>. Other therapeutic strategies related to sRAGE have been proposed. Indeed, instead of administering sRAGE, different molecules such as angiotensin-converting enzyme (ACE)<sup>972</sup>, statins<sup>973,974</sup>, thiazolidinediones<sup>975</sup>, methotrexate<sup>976</sup> and possibly vitamin D<sup>977,978</sup> have been shown to indirectly increase sRAGE levels. But side effects should be considered when working with molecules that have not been designed primarily to increase the concentrations of sRAGE. Finally, an interesting strategy to increase sRAGE levels would be to increase its shedding from FL-RAGE, providing cRAGE (or DN-RAGE depending upon the target). Although this is possible *in vitro*<sup>979</sup>, this opportunity has yet to be further investigated *in vivo*. The recent production of a mouse model overexpressing sRAGE offers a real possibility to comprehensively investigate its role in ageing, as well as its potential as a target for therapeutic intervention<sup>980</sup>.

A still poorly-explored possibility is acting directly upon RAGE translation. A few studies have shown that the use of small interfering RNAs (siRNAs), which target *RAGE* mRNA, successfully prevented myocardial consequences of acute ischemia and reperfusion<sup>814,981,982</sup> and improved hepatic function in a hepatic fibrosis model<sup>983,984</sup>. siRNAs are highly specific and significantly reduce mRNA and protein levels of their target. They can also be designed to specifically target selected RAGE variants and could therefore allow the production of sRAGE while preventing RAGE translation. However, some of the main constraints of *in vivo* use of siRNAs are their stability, their delivery to tissues and their cellular uptake: thus, while local response is achievable, it is much harder to provide a whole-organism knock-down profile. Current work aims to overcome these limitations, notably by producing more efficient delivery systems<sup>985-987</sup>.

Finally, much current research is focussed on the use of small molecules targeting RAGE and some of them have been subjected to, or are currently undergoing, clinical trials. RAGE-aptamers were reported as being able to protect mouse kidneys against diabetic complications<sup>988,989</sup> and deoxycorticosterone acetate/salt-induced injury<sup>990</sup>, and also suppressed the proliferation and metastasis of hepatic malignant melanoma<sup>991</sup>. Two major families of small molecules inhibiting RAGE have been described, namely theazole derivatives and tertiary amides. Members of each family have common pharmacophores but differ in their functional moieties, notably their hydrophobic and electron-rich or -poor groups<sup>641,736</sup>. Azeliragon (also called TTP488 or FP-04494700) is anazole derivative and is currently undergoing a phase 3

clinical trial for the assessment of AD limitation in diabetic patients after it failed to show efficacy among a broader spectrum of AD patients<sup>834,866</sup>. Although the primary outcome was not fulfilled in this recently terminated trial, it nevertheless confirmed that RAGE inhibition is safe in humans, which is highly important. It has already been mentioned that this lack of efficacy may be due to treatment being initiated too late; interestingly, sRAGE is a major isoform in the hippocampus<sup>648,649,666</sup>, therefore there is also the possibility that Azeliragon alters sRAGE function in the brain. FPS-ZM1, a tertiary amide, holds great promise as it efficiently inhibits A $\beta$ /RAGE interaction, has a very good brain uptake and protects against A $\beta$ -mediated disorders in a murine model of AD<sup>736,992</sup>. It has also been shown to prevent vascular<sup>993-995</sup>, pulmonary<sup>996,997</sup>, renal<sup>998,999</sup> and gut<sup>792</sup> injuries in multiple models. Despite these promising outcomes, no clinical trial has been conducted to date. A novel strategy is also underway aiming to inhibit the interaction of RAGE's cytoplasmic tail with intracellular effectors, notably mDia1<sup>641,756</sup>. This strategy permits the interactions of RAGE with other receptors, matrix or its ligands whilst preventing its signalling (at least through mDia1). This kind of molecule has the unique advantage of not interfering with sRAGE or DN-RAGE, thereby preserving their anti-RAGE signalling properties, but it must be specific enough to prevent any alteration of other pathways requiring mDia1.

The ensemble of results presented herein, and the strategies for future research which build upon the work contained in this thesis, underline the need to provide optimal molecules for RAGE inhibition. In addition, the results presented here provide a proof of principle that RAGE actively participates in inflammaging and, further, that inflammaging is indeed an important driver of ageing. This work is not just about RAGE signalling but provides an insight into how preventing inflammaging is important for the prevention of age-related physiological and non-physiological disorders and hence potentially improving healthspan and possibly lifespan. As a result, work is continuing in our research group to select, from among a library of available molecules, suitable candidates able to bind to RAGE. The aim is to identify a number of new molecules with a higher affinity for RAGE, able to inhibit or activate this receptor, that can bind to different RAGE domains and for which the uptake into different tissues can be modulated. Indeed, while the focus of this thesis has been on RAGE inhibition, there is no known RAGE-specific ligand and so in every study of RAGE stimulation, other receptors were involved in the process. The identification of specific RAGE ligands would be highly valuable to study its signalling *in vitro* or even in animal models.

Beyond these fundamental studies, the hope is to be able to provide molecules and guidelines for the prevention or attenuation of RAGE signalling in a pathological context such as diabetic nephropathy where, despite various reports, no rigorous clinical trial has yet investigated the end result of Ligand/RAGE blockage<sup>1000</sup>. Another aim is to apply these tools in studies of *physiological* conditions such as normal ageing where, in the context of a steadily-increasing human lifespan, the hope is to be able to intervene in a major driver of ageing and age-related disorders: inflammaging.

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# Valorisation

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## Articles

- 1 **Teissier T.**, Quersin V., Gnemmi V., Daroux M., Howsam M., Delguste F., Lemoine C., Fradin C., Schmidt A.-M., Cauffiez C., Brousseau T., Glowacki F., J. Tessier F., Boulanger E. and Frimat M.  
Knockout of receptor for advanced glycation end- products attenuates age-related renal lesions  
**Ageing Cell.** 2019 Apr;18(2):e12850. doi: 10.1111/accel.12850.  
Original article
- 2 **Teissier T.** and Boulanger É.  
The receptor for advanced glycation end-products (RAGE) is an important pattern recognition receptor (PRR) for inflammaging.  
**Biogerontology.** 2019 Apr 9. doi: 10.1007/s10522-019-09808-3.  
Review
- 3 **Teissier T.** Litke R., Taront-Dezitter S., Lancel S., Fradin C. and Boulanger É.  
The Receptor RAGE in Vascular and Cerebral Dysfunctions. Chapter 3: Involvement of the RAGE-Ligand axis in aging  
**Cambridge Scholars Publishing.** ISBN (13): 978-1-5275-2692-1. Apr. 1. 2019.  
Review
- 4 **Teissier T.**, Howsam M., Boulanger É. and Tessier F.  
Mechanisms and effects of DNA glycation  
**IMARS Highlights**, Volume 13, Number 5, Dec. 25, 2018.  
Review

## Presentations

- National **Ageing and glycation: involvement of RAGE**  
Interregional and cross-border gerontology congress - 2017  
Lille, France
- International **Knock-out of receptor for advanced glycation end-products (RAGE) attenuates age-related renal lesions in mice.**  
The 13th international symposium on the Maillard reaction (ISMR) – 2018  
Montreal, Canada
- Attenuation of age-related renal lesions in a model of receptor for advanced glycation end-products (RAGE) knock-out**  
British Society for Research on Ageing, 68th Annual Scientific Meeting - 2018  
University of Oxford, Oxford, UK
- Knock-out of receptor for advanced glycation end-products (RAGE) attenuates AGE-related renal lesions**  
3rd Young AGErs symposium on Maillard reaction in food and in vivo – 2018  
Wageningen University & Research, Wageningen, Netherlands

## Awards

|                                           |                                                                                    |
|-------------------------------------------|------------------------------------------------------------------------------------|
| My thesis in 180 seconds –<br>Third prize | <b>Oct. 2018</b> , Pasteur Institute of Lille<br>Fund granted: 100 €               |
| Young scientist travel award              | <b>Sept. 2018</b> , International Maillard Reaction Society<br>Fund granted: 670 € |
| Fund raising                              | <b>Feb. 2017</b> , Flandres business club<br>Fund granted: 3 000 €                 |
| Ph. D. scholarship                        | <b>Oct. 2016</b> , DigestScience foundation<br>Fund granted: 258 000 €             |
| Fund raising                              | <b>May 2016</b> , GEFLUC federation<br>Fund granted: 20 000 €                      |
| <b>Total: 281 770 €</b>                   |                                                                                    |

## Training

|                                                                |                                                                                       |
|----------------------------------------------------------------|---------------------------------------------------------------------------------------|
| Workshop - Publication                                         | <b>Jun. 2019</b> , Bioaddoct, University of Lille, Lille, France                      |
| Conference/debate on scientific<br>integrity                   | <b>Mar. 2019</b> , H. Maisonneuve, University of Lille, Lille,<br>France              |
| Collaboration -<br>Oral presentation                           | <b>Sept. 2018</b> , A.-M. Schmidt's laboratory, New York<br>University, New York, USA |
| Small and big animals<br>experimentation training              | <b>Dec. 2017</b> , University of Lille, Lille, France                                 |
| 2nd International Scientific<br>Symposium on Healthy Ageing    | <b>Dec. 2017</b> , Pasteur Institute of Lille, Lille, France                          |
| International Cell Senescence<br>Association (ICSA) Conference | <b>May 2017</b> , ICSA, Paris, France                                                 |
| First International Symposium<br>Research on Healthy Ageing    | <b>Dec. 2016</b> , Pasteur Institute of Lille, Lille, France                          |
| <b>ECTS: 60/60</b>                                             |                                                                                       |

## Teaching

Cell and molecular biology      **May – Jun. 2018**, Inserm UMR 995, University of Lille, France  
Supervising an undergraduate student in laboratory: Setting up experimental procedure, teaching of cell culture, RT-qPCR and data analysis and supervising report writing.

## Memberships

2018 - present      International Maillard Reaction Society  
2018 - present      British Society for Research on Ageing  
2017 - present      Francophone Maillard Reaction Society



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# Annexes

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## The role of DNA glycation in aging

Thibault Teissier<sup>1</sup>, Michael Howsam<sup>1</sup>, Éric Boulanger<sup>1,2</sup>, Frédéric Tessier<sup>1</sup>

1. Univ. Lille, Inserm, CHU Lille, U995 - LIRIC - Lille Inflammation Research International Center, F-59000 Lille, France
2. Department of Geriatrics and Ageing Biology, School of Medicine, Lille University, France

### Aging and glycation

Age is one of the main risk factors for a number of pathologies. Among them we find brain disorders such as Alzheimer's disease or Parkinson's disease, cardiovascular diseases such as atherosclerosis, left ventricular hypertrophy, vascular stiffening and hypertension, as well as diseases such as arthritis, osteoporosis, sarcopenia, cataracts, type-2 diabetes and some cancers. This diverse range of pathologies has come to be thought of collectively as age-related disease.

As a result of efforts to tackle these age-related diseases but also to improve healthspan (or indeed lifespan) by delaying aging itself, researchers have proposed different theories of aging that have shaped what are widely known as "The Hallmarks of Aging", as reviewed by López-Otín *et al.* (López-Otín *et al.*, 2013). Nine hallmarks are thus described: loss of proteostasis, epigenetic alterations, altered intercellular communication, cellular senescence, mitochondrial dysfunction, stem cell exhaustion, deregulated nutrient-sensing, telomere attrition and genomic instability.

Glycation leads to uncontrolled, post-translational modifications that are characterized by loss of protein function, protein misfolding and aggregation if not degraded by autophagy or proteolysis. It was first proposed in 1981 by Monnier and Cerami that glycation could play a potential role in aging as suggested by links found between glycation of long-lived proteins and age (Monnier and Cerami, 1981). This idea was further supported by results showing a correlation between accumulation rate of advanced glycation end-products (AGEs) and lifespan

of different species (Sell et al., 1996). Histones, just like any other protein, can also be glycosylated as first described over 30 years ago (De Bellis and Horowitz, 1987). Similar to the methylation and acetylation of histones that alter genes' expression, glycation could also be considered an almost irreversible, non-heritable epigenetic alteration. However despite this obvious parallel, and the widely-reported direct links between glycation and aging, glycation was not cited as a potent inducer of the hallmarks of aging described by López-Otín *et al.* in their review. If perhaps not pertinent to all 9 hallmarks, glycation is nevertheless potentially directly involved in at least two: the loss of proteostasis and in epigenetic alterations. The dual purpose of this review is to highlight the potential roles of glycation in aging, and to encourage researchers to better integrate glycation in studies of aging.

### **Glycation beyond protein modification**

In addition to the deleterious effects intrinsic to glycation itself, a growing body of work has shown that the resultant AGEs can have negative impacts through interaction with their main receptor, the receptor for AGEs (RAGE). Thus, glycation could be important to other hallmarks of aging such as altered intercellular communication, cellular senescence or mitochondrial dysfunction owing to the pro-inflammatory and pro-oxidant signaling they induce *via* RAGE – factors that are often considered as causative in aging.

There is another aspect of glycation that is still largely overlooked by both the aging and the glycation research communities. As professor Monika Pischetsrieder reminded us in her introduction “Beyond glucose and lysine: the relevance of less common Maillard reactions”, glycation is not only about glucose and lysine: different carbonyl compounds can be responsible for glycation and different nucleophiles can be glycosylated. Among the nucleophiles DNA and, perhaps more importantly, its nitrogenous bases, is of great importance for both glycation and aging. Intriguingly, DNA glycation has received little attention while extensive research has been carried out on protein glycation.

### **Mechanisms and effects of DNA glycation**

The first report of glycation of nucleic acids probably dates back to 1959 when Matthys Staehelin demonstrated that incubation of RNA from a tobacco virus with carbonyl compounds such as glyoxal, methylglyoxal or formaldehyde could reduce the virus's activity and infectiousness as a result of reactions with its guanine derivatives (Staehelin, 1959). It was later shown that

glyoxal could react to a lesser extent with other nucleic acid bases (Nakaya et al., 1968; Shapiro et al., 1970).

In 1973, Noberto Krymkiewicz showed that  $^{14}\text{C}$ -labelled methylglyoxal could bind very quickly to denatured DNA, then to tRNA, and more slowly with native DNA. This reaction was shown to occur differently with different nucleotides: while poly-guanine had a very high affinity with  $^{14}\text{C}$ -methylglyoxal, poly-adenine and poly-cytosine reacted much less and poly-uridine did not react at all under these conditions (Krymkiewicz, 1973). Brambilla *et al.* subsequently showed that methylglyoxal can induce protein-DNA crosslinks, thereby describing a novel kind of glycation (Brambilla et al., 1985). These DNA modifications have been shown to lead to various defects that are not merely the result of changes in DNA structure from the simple addition of carbonyl adducts to nucleic acid nitrogenous bases.

Deamination of nitrogenous bases is one of the possible results of DNA glycation. Kasai *et al.*, have shown that glyoxal can not only lead to a large production of cyclic glyoxal-deoxyguanosine (dG) adducts, but also to the deamination of deoxycytidine (dC) resulting in the production of deoxyuridine (dU) and, to a lesser extent, 5-hydroxyacetyl-dC. In addition, some crosslinks between different nucleosides were found (Kasai et al., 1998). Another model of deamination, NO-induced deamination, has also demonstrated that deamination of guanine and cytosine could respectively lead to the formation of xanthine and uracil – similar to Krymkiewicz's findings, double-strand DNA was 10-fold less reactive than single-strand DNA in this regard. The authors finally suggested that guanine deamination could potentially lead to its depurination, forming an abasic site (Caulfield et al., 1998). More recent work has gone further and demonstrated a C:G to T:A mutation due to deamination, accompanied by double strand breaks and replication fork collapse (Bhagwat et al., 2016; Langston and O'Donnell, 2006).

Concerning depurination, *in vitro* glycation of guanosine by D-glucose or dihydroxyacetone has been shown to form N2-carboxyethyldeoxyguanosine (CEdG), believed to decrease the N-glycosidic bond stability and favor CEdG hydrolysis to CEguanine: the subsequent release of CEguanine gives rise to an abasic site and loss of purine (Seidel and Pischetsrieder, 1998). Some years earlier, it had already been shown that depurination by other processes could lead to deletions and frameshifts in human cells which could be the cause of some major genetic defects (Klinedinst and Drinkwater, 1992). Indeed, recent work has proposed that depurination

induced by estrogen could be linked to carcinogenesis as an initiator of some cancers (Cavalieri and Rogan, 2010; Cavalieri et al., 2012).

Interestingly, CEdG was shown to be significantly increased in kidneys and serum from patients with diabetes and/or undergoing hemodialysis (Li et al., 2006). It was subsequently shown that auto-antibodies from diabetic patients serums were able to react with glycated human DNA, suggesting a possible autoimmune response in this context (Ahmad et al., 2014). In addition, DNA glycation has been linked to increased cytotoxicity and mutation frequencies (Murata-Kamiya et al., 1997; Pischetsrieder et al., 1999) that may be responsible for a diminution in protein expression and function, as suggested by decreased expression and activity of luciferase upon glycation of its coding gene (Breyer et al., 2008).

### Conclusion

Taken together these results highlight the exciting potential of DNA glycation as a field of research, actually relatively poorly studied, and with many outstanding questions such as the effect of processes like deamination or depurination on gene expression and mutations. As Baynes suggests in his 2002 review “The Maillard Hypothesis on Aging: Time to Focus on DNA”, DNA glycation could explain a part of a species lifespan, not only by altering DNA structure, but also by provoking DNA mutations that may be prominent in short lived organisms with less efficient DNA repair systems (Baynes, 2002; Cortopassi and Wang, 1996). Much remains to be studied but recent technological advances and the increased accessibility of key analytical tools (e.g. NMR and mass spectrometry) have already added to our understanding of DNA glycation. It thus seems timely to widen our horizons, to look beyond proteins and include DNA glycation in studies of aging.

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## CHAPTER THREE

### INVOLVEMENT OF THE RAGE-LIGAND AXIS IN AGEING

THIBAUT TEISSIER<sup>1</sup>, RACHEL LITKE<sup>1,2</sup>, SOLENNE  
TARONT-DEZITTER<sup>1</sup>, STEVE LANCEL<sup>3</sup>, CHANTAL FRADIN<sup>1</sup>  
AND ERIC BOULANGER<sup>1,2</sup>

1. "Glycation: from Inflammation to Ageing" Lille Inflammation Research International Center (LIRIC-INSERM.U995) Lille, France.
2. Department of Geriatrics and Ageing Biology, School of Medicine, Lille University, France
3. Departments of Physiology and Cell Biology, School of Medicine, Lille University, France

#### Introduction

Ageing presents multiple and complex phenotypes influenced by genetics, disease and environmental modulators such as psychosocial factors and lifestyle, including diet and physical activity. Age represents the main risk factor for diseases such as cancer and physical, neurological, cardiovascular and metabolic disorders. Ageing thus corresponds to a progressive deterioration of physiological functions in multiple organ systems, and is a multifaceted process characterized by accumulation of complex deleterious changes in tissues and cells (López-Otín et al., 2013).

Glycation, a post-translational modification of proteins, comprises a series of complex chemical reactions between amino compounds and reducing sugars leading to an amino-sugar condensation (Maillard, 1912). advanced glycation end-products (AGEs) are a heterogeneous group of macromolecules formed by the glycation of proteins, lipids, and nucleic acids. Another pathway leading to the formation of AGEs has been described, namely glycoxydation by highly reactive dicarbonyl compounds, notably methylglyoxal or glyoxal, which can bind amino groups to form glycated proteins (Miyata et al., 2000; Rabbani et al., 2007). Humans are exposed to two main sources of AGEs: exogenous AGEs that are ingested in food and endogenous AGEs that are formed in the body. The Western diet is rich in AGEs which are formed when food is processed at elevated temperatures such as during deep-frying, broiling, roasting, grilling and pasteurization. Hence, high-temperature processing of many processed foods such as pasteurized dairy products, cheeses, sausages, processed meats and commercial breakfast cereals means they may be rich in AGEs (Guilbaud et al., 2016; Tessier et al., 2016). Endogenous AGEs are generated and accumulate at higher rates during diabetes, renal failure, inflammation and physiological ageing. AGEs exert their toxicity through three mechanisms: *in situ* glycation, AGE accumulation, and interaction with receptors, notably RAGE (the key receptor for AGEs) (Daroux et al., 2010).

RAGE is a transmembrane protein and member of the immunoglobulin (Ig) superfamily of receptors. RAGE activation induces a pro-inflammatory, pro-thrombotic and pro-angiogenic cell response (Schmidt et al., 1992; Wautier et al., 1996) and it also orchestrates a more extensive inflammatory response due to its presence in inflammatory cells and numerous ligands. RAGE activation in diabetes is proposed as a self-perpetuating axis

whereby AGE-RAGE engagement triggers inflammation, oxidation (glycoxidation) and expression of adhesion molecules (Wautier and Wautier, 2013). The subsequent recruitment and activation of immune cells increases concentrations of other RAGE ligands (such as HMGB1, S100 proteins) that perpetuate the cycle and lead to cellular dysfunction (Ramasamy et al., 2012).

Since glycation increases during ageing, and because AGE-RAGE gives rise to a multidirectional cell response, we have focused this chapter on the implication of the RAGE-Ligand axis in ageing. Eight key hallmarks of ageing have structured our review (López-Otín et al., 2013).

## **Altered intercellular communication**

During ageing, modifications occur at the intercellular communication level. These modifications involve endocrine, paracrine and autocrine messaging, but also cell-cell adhesion changes *via* cell adhesion molecules. The age-dependent changes in intercellular communication are in fact the combined effects of all the other hallmarks of ageing. Senescent cells participate in low grade inflammation which can furthermore have deleterious effects on ageing organs, tissues and cells.

RAGE may be involved in blood cell-vessel cellular interactions. The interaction of red blood cells bearing AGEs with endothelial RAGE alters endothelium function through vascular hyperpermeability and inflammatory reactions including NADPH oxidase stimulation, generation of reactive oxygen species (ROS) and cytokine production (Wautier et al., 1994, 2001; Zhou et al., 2018). Cell adhesion molecule expression is modified after RAGE activation by AGEs or other ligands. It has been shown that RAGE engagement by CML–albumin, a well-defined AGE ligand of RAGE, is followed by an endothelial or mesothelial overexpression of vascular cellular adhesion molecule-1 (VCAM-1). This cellular response is accompanied by enhanced leukocyte adhesion and activation, indicating that this mechanism may participate in inflammatory processes (Boulanger et al., 2002). The endothelial intercellular adhesion molecule-1 (ICAM-1) and RAGE act together to mediate leukocyte adhesion (Frommhold et al., 2010). RAGE activation by CML stimulates vascular endothelial growth factor (VEGF) production, participating in local neovascularization (Boulanger et al., 2007; Roca et al., 2014). During ageing endothelial VCAM-1 overexpression, induced by dietary CML, is prevented by RAGE invalidation (Grossin et al., 2015).

In other tissues, it has recently been demonstrated that allergen and lung cytokine-induced VCAM-1 expression is RAGE dependent and contributes to type-2 innate lymphoid cell (ILC2) accumulation and downstream eosinophilic inflammation, mucus metaplasia, and type 2 inflammatory responses (Perkins et al., 2018). The amyloid- $\beta$  (A $\beta$ ) peptide–RAGE interaction produces neuronal expression of macrophage-colony stimulating factor (M-CSF), which corresponds to a known pro-inflammatory pathway in Alzheimer's disease (Du Yan et al., 1997). Further, the interaction of A $\beta$  with RAGE on microglia stimulates chemotaxis and expression of cytokines (Yan et al., 1996), while RAGE invalidation is associated with an attenuated increase of renal VCAM-1 expression during ageing.

Cells are continuously communicating with each other, simultaneously functioning as “sender and receiver”. This equilibrium varies from cell to cell and depends upon the situation (inflammation, oxidation, fibrosis, cancer...) – and ageing is also a determining factor. Inflammaging results from multiple origins, such as pro-inflammatory tissue damage accumulation, failure of the immune system to control and kill pathogens, a predisposition of senescent cells to produce pro-inflammatory cytokines, improved stimulation of the nuclear factor-kappa B (NF- $\kappa$ B) transcription factor, or a defective autophagic response. Senescent cells in particular are known to produce tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin 6 (IL-6) (Franceschi et al., 2018).

RAGE, especially *via* autocrine and paracrine mechanisms, could play an important role in inflammaging. Many studies report that RAGE activation is followed by a pro-inflammatory, profibrotic, prothrombotic and neoangiogenic cell responses. The inflammatory response is accompanied by an overproduction of RAGE ligands such as S100 proteins and high-mobility group box 1 (HMGB1). This reaction could amplify RAGE activation, but also activates a RAGE independent inflammatory cell response (Schmidt et al., 1994). Senescent cells producing TNF $\alpha$  and IL-6 can induce low grade inflammation, but also oxidation leading to AGE production *via* glycooxidation (Grossin et al., 2010). AGEs can subsequently activate RAGE. Beside AGEs, S100 proteins and HMGB1 are also involved in low grade inflammation and probably in inflammaging. (Cardoso et al., 2018). We should note that TNF $\alpha$  and IL-6 are also overproduced after RAGE activation and thereafter participate in a RAGE-independent inflammation reaction with production of S100 proteins and HMGB1 by other cells. All this corresponds to a RAGE rollercoaster of inflammaging (Fig.3.1). We have demonstrated that twenty-month-old RAGE null mice were protected with respect to physiological renal ageing, exhibiting a lower glomerulosclerosis index and senile amyloidosis. RAGE null mice had a reduced age related inflammation and oxidation. RAGE invalidation also prevented the decrease of SIRT-1 expression and the phosphorylation of AKT and pS6RP (mTOR).

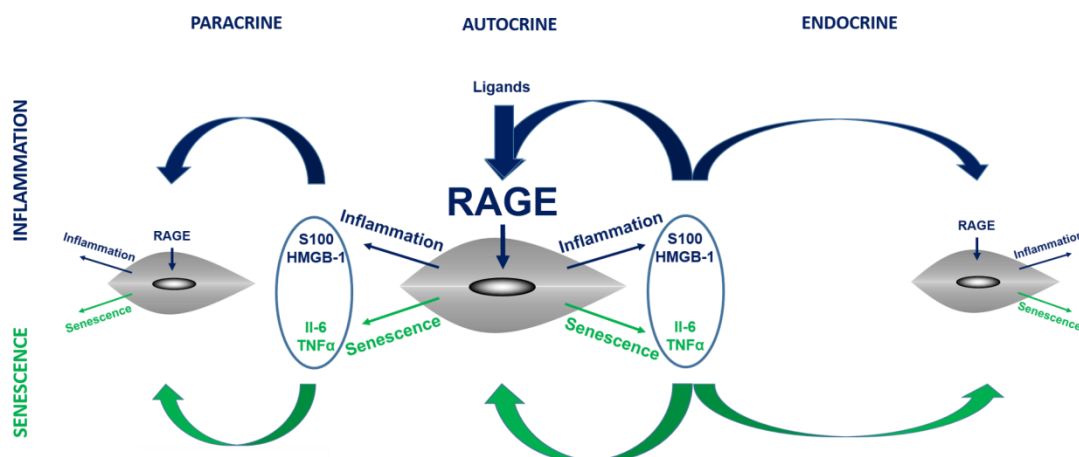


Figure 3.1: RAGEING: the roller-coaster of RAGE in inflammaging. Cell senescence is accompanied by a secretion of inflammatory molecules that can stimulate cells to produce RAGE ligands.

S100 (calgranulins), HMGB1 (HighMobility Group Box-1), IL-6 (Interleukin-6), TNF $\alpha$  (Tumor Necrosis Factor  $\alpha$ ).

## **Deregulated nutrient-sensing**

Nutrient homeostasis is of vital importance for all living organisms. The ability to respond to fluctuations in access to nutrient is essential for life. Nutrient shortage has operated as a selection pressure for efficient nutrient sensing mechanisms which are conserved among species. Nutrient sensing pathways undertake anabolic measures and storage in times of food plenty, whereas food insufficiency drives catabolism and mobilization of internal stores through mechanisms such as autophagy. The key role that metabolites play in all biological processes is emerging, particularly in physiological and pathological ageing.

Several studies summarized by Fontana et al. confirm the hypothesis that anabolic signaling accelerates ageing while reduced nutrient signaling increases longevity (Fontana et al., 2010). Moreover, many human genes linked to metabolism, for example forkhead box O3 (*FOXO3*) or genes involved in PI3K/AKT signaling, are prevalent in centenarians in particular variant forms (Broer and van Duijn, 2015).

In the course of ageing and in the case of metabolic disorders, signaling pathways involved in the identification and response to fluctuations in nutrient levels are commonly deregulated (Efeyan et al., 2015). These pathways are linked to ensure coordinated regulation of cellular metabolic responses in line with cellular energy status and nutrient availability input. Feedback loops operate within the pathways to regulate signal intensity and duration.

RAGE has been shown to have a role in inflammation associated with obesity, diabetes and high-fat feeding, all states known to accelerate ageing (Song et al., 2014). RAGE interacts with some of the best-known nutrient sensing pathways which have themselves been shown to play a role in ageing. Direct evidence of the role of RAGE in nutrient sensing is rare, though many elements are assembled in favor of interactions between RAGE and the different nutrient sensing pathways. We here discuss the available evidence of RAGE's interaction with the main nutrient sensing regulation pathways, in particular RAGE-insulin and IGF1 signaling, RAGE-sirtuins and RAGE-AKT/mTOR.

Insulin and IGF1 signaling are known as the 'insulin and IGF1 signaling' (IIS) pathway as they activate the same intracellular signaling pathway which participates in glucose sensing. Strikingly, the IIS pathway is the most conserved ageing-control pathway in evolution. The FOXO family of transcription factors and the mTOR complexes are examples of targets for the IIS pathway, mentioned earlier as being involved in ageing (Kenyon, 2010; Tatar et al., 2003). Reduction of signaling strength of the IIS pathway by a multitude of genetic manipulations consistently extends the lifespan of worms, flies and rodents (López-Otín et al., 2013). The two main pathways of IIS emanating from the insulin receptor and insulin-like growth factor receptor 1 (IR/IGF-1R) are the phosphatidylinositol 3-kinase (PI3K, a lipid kinase)/AKT pathway (Cantley, 2002) and the Raf/Ras/MEK/MAPK (mitogen activated protein kinase, also known as ERK or extracellular signal regulated kinase) pathway. The PI3K pathway is responsible for most metabolic effects of insulin, and is connected exclusively through IRS. RAGE cannot be directly activated by insulin, IGF1 or glucose sensing signals, but it is known that RAGE modulates the PI3K/AKT pathway by inhibiting AKT signaling and therefore countering the IIS pathway (Hou et al., 2014; Yu et al., 2017) and potentially modulating ageing.

Sirtuins (silent mating type information regulation homologs) are NAD-dependent deacetylases that are highly conserved from bacteria to humans. They couple the deacetylation of lysine to the hydrolysis of NAD<sup>+</sup> by transferring the acetyl group to the ADP-ribose to form O-acetyl-ADP-ribose, releasing free nicotinamide. Because NAD<sup>+</sup> is required for this reaction and the NAD<sup>+</sup>/NADH is determined by the nutritional state of the cell, sirtuins directly link cellular metabolic signaling to the state of protein post-translational modifications, sensing low energy states by detecting high NAD<sup>+</sup> levels (Houtkooper et al., 2012). Sirtuins tend to be downregulated with ageing while the pharmacological up-regulation of sirtuins is thought to increase longevity. We and other groups (Cai et al., 2012; Grossin et al., 2015; Teissier et al., 2019) have found that activation of the AGE/RAGE axis could decrease expression of SIRT1, which has been shown to modify cellular energetics during the ageing process. In addition, chronic inflammation, in which the RAGE-ligand axis participates, is implicated in mechanisms accounting for the loss of NAD<sup>+</sup>, the main cofactor of SIRT1 (Verdin, 2015), thereby reducing SIRT1 signaling.

The mammalian target of rapamycin (mTOR) is a highly conserved kinase. It is part of two multiprotein complexes, mTORC1 and mTORC2. mTORC1 integrates energy, nutrients (glucose, amino acids and fatty acids), stress and growth factors and, in response to these stimuli, drives the growth of cells, organs and whole organisms. mTORC2, which is activated by growth factors, promotes cell proliferation and survival. mTOR signaling enhances energy storage and consumption. Genetic down-regulation of mTORC1 activity in yeast, worms and flies extends longevity. Treatment of mice with rapamycin, an inhibitor of mTOR pathway, extends longevity, as does downregulation of mTORC1 or a deficiency in S6K1, a main mTORC1 substrate, in genetically-modified mice (Lamming et al., 2012; Selman et al., 2009). The downregulation of mTORC1/S6K1 is the critical mediator of longevity in relation to mTOR, a major regulator of the complex autophagic process. Evidence for a role of RAGE in deregulated nutrient sensing involves the mTOR signaling pathway and its role in autophagy regulation. It has been shown in some studies that AGEs may induce autophagic modifications in rat neonate cardiomyocytes *via* inhibition of the PI3K/AKT/ mTOR pathway in a RAGE-dependent manner (Hou et al., 2014; Hu et al., 2015). On the other hand, activation of the AGE/RAGE axis has also been shown to trigger phosphorylation of mTOR (Peres et al., 2017), which may lead to autophagy inhibition. Further studies are needed to explore the complex links between AGE/RAGE and the PI3K/AKT/mTOR pathway that modulate autophagy. A recent study demonstrates that components of the uremic state potentiate vascular smooth muscle cell (VSMC) calcification by generating continuous and exaggerated activity of mTORC1 (Panda et al., 2018).

### **Mitochondrial dysfunction**

A decrease in mitochondrial membrane potential, along with reductions in mitochondrial respiration and ATP production, have been consistently reported in various cell types (*i.e.* chondrocytes, osteoblasts, neuron cells, lens epithelial cells, cardiomyocytes, mast cells and pancreatic beta cells) treated with RAGE ligands such as HMGB1, methylglyoxal, CML or other AGEs. These defects were mediated by sRAGE, anti-RAGE antibodies, RAGE silencing or gene deletion. Underlying mechanisms implicate mitochondrial superoxide production by complex I of the respiratory chain (Coughlan et al., 2009) as mitochondrial ROS scavengers such as mitoQ (Mao et al., 2018) or mitoTEMPO (Y. Song et al. 2017) improve mitochondrial function. Nevertheless, NADPH oxidase activation

and subsequent cytosolic ROS generation appear to be upstream of mitochondrial ROS since apocynin reduced these mitochondrial alterations (Q. Wu et al. 2016; Daffu et al. 2013). Another possible pathway leading to mitochondrial ROS formation is ceramide synthesis. Myriocin, an inhibitor of the serine palmitoyltransferase, prevented CML-induced impairment of mitochondrial respiration (Nelson et al., 2015). A vicious cycle is therefore apparent whereby ROS promote mitochondrial DNA mutations and deletions (Lo et al., 2015) which are known to further alter mitochondrial function and promote mitochondrial oxidative stress.

Mitochondrial function is also regulated by several quality control pathways regulating mitochondrial networks, degradation, and synthesis. Far from being isolated and static organelles, mitochondria constitute a living web constantly remodeled by fission and fusion processes. Fragmentation, mainly controlled by Drp1 and Fis1 proteins, is usually associated with impaired mitochondrial function while elongated networks, regulated by outer membrane-located mitofusins (Mfn1, Mfn2) and the inner membrane protein Opa1, are considered a mark of healthy and functional organelles. Associated with reduced mitochondrial length and a fragmented network (Lo et al., 2015; Mao et al., 2018), activation of RAGE-ligand axis favors expression of Fis1 (Mao et al., 2018) and Drp1 (Lo et al., 2015) while decreasing the ratio of long functional Opa1 isoforms (Opa1L) to short Opa1 isoforms (Opa1S) (Lo et al., 2015; Mao et al., 2018) - hence mitochondrial fission is promoted and Mfn1 and Mfn2 expression decrease (Lo et al., 2015) or are unchanged (Mao et al., 2018). Some reports have also suggest that the NF- $\kappa$ B pathway, known to be activated upon RAGE stimulation, could regulate mitochondrial networks (Laforge et al., 2016). Mitophagy, a process that removes damaged organelles, also controls mitochondrial quality. Elevation of the autophagic/mitophagic activity, detected by LC3 staining, higher Pink1 expression and more mitochondria-localized Parkin, have also been reported upon RAGE activation (Lo et al., 2015). This elevated activity was also associated with mTOR inhibition, hence beclin1-phosphatidylinositol 3 kinase complex formation and autophagy/mitophagy induction (Kang et al., 2011). Depending on the organ and the inducer of RAGE activation, genetic deletion of RAGE has also been reported to activate autophagy (Yu et al., 2017). Reduction in mitochondrial density has also been described with RAGE deletion (Mao et al., 2018), however further studies are required to determine whether this is related to changes in the mitochondrial biogenesis program, mainly controlled by Peroxisome proliferator-activated receptor Gamma Coactivator 1-alpha (PGC-1 $\alpha$ ). Several studies report that AGE treatment is associated with a reduction in sirtuin 1 (SIRT1) activity (Neviere et al., 2016), the NAD<sup>+</sup>-deacetylase required for PGC-1 $\alpha$  activation.

Changes in mitochondrial functions and quality may lead to apoptosis and inflammation. In the presence of RAGE ligands and when RAGE is inhibited or invalidated, cytochrome c release, mitochondrial calcium overload and swelling, caspase-9 and -3 activation, as well as DNA fragmentation, are all reduced (Coughlan et al., 2009; Mao et al., 2018; Wu et al., 2016; Yoshimaru et al., 2008). These phenomena are partly mediated by the mitochondrial permeability transition pore (MPTP) opening, since cyclosporin A and bongkreikic acid blocked RAGE-induced apoptosis (Mao et al., 2018; Yoshimaru et al., 2008). Higher susceptibility to MPTP opening upon RAGE activation may be a consequence of increased oxidative stress, c-Jun N-terminal kinase (JNK) and glycogen synthase kinase 3 (GSK3) activation by RAGE (Shang et al., 2010; Tanno et al., 2014). RAGE activation could also promote apoptosis through changes in the expression of pro- and anti-apoptotic Bcl2-related proteins (Mao et al., 2018). The underlying mechanism could involve the unfolded protein response (UPR) and the expression of

the pro-apoptotic transcription factor C/EBP homologous protein (CHOP) (Wu et al., 2016). Beside apoptosis, a part of RAGE-dependent inflammation may rely upon mitochondrial defects. Mitochondrial damages that are RAGE-dependent may also activate the inflammasome. Inflammasome is a multiprotein complex gathering, among others, the NOD-like receptor family pyrin domain containing 3 (NLRP3), the adaptor protein apoptosis-associated speck-like (ASC) and the immature caspase-1. This NLRP3 inflammasome complex, essential for the innate immune response, is activated by a two-step mechanism: priming (induced by pathogen-associated molecular patterns (PAMPs)) and assembling (triggered by danger-associated molecular patterns (DAMPs)). A recent study indicates that AGE-induced inflammasome activation, thus promoting caspase-1 activation and maturation of pro-interleukin-1 $\beta$  (pro-IL-1 $\beta$ ) into IL-1 $\beta$ , could depend on the RAGE/NF- $\kappa$ B pathway and the mitochondrial alterations (Song et al., 2017). Different studies also indicate that the release of mtDNA fragments and the mitochondrial transcription factor A (TFAM) to the cytoplasm due to unprocessed degrading mitochondria (Ward et al., 2013; Yao et al., 2015) promote, in a RAGE-dependent manner, TLR9-mediated inflammatory signaling resulting in NF- $\kappa$ B/ASC-Caspase-1 activation.

In non-cancerous cells and organs, the RAGE-ligand axis is responsible for mitochondrial ROS production, membrane collapsing, calcium overload, network fragmentation and cytochrome c release. These alterations trigger energy crisis, inflammation, autophagy and apoptosis activation at the cellular level (Fig.3.2), while RAGE inhibition, silencing or deletion is protective of various cellular and organ dysfunctions. Another study explored the consequences of RAGE activation by HMGB1 on mitochondria in pancreatic tumors (Kang et al., 2014). HMGB1 promotes cell growth, cell migration and NF- $\kappa$ B activation in pancreatic tumor cell lines but improves ATP production by mitochondria. Silencing of RAGE resulted in lower complex I activity and ATP production and, in turn, reduced cell proliferation induced by HMGB1. Indeed, it seems that HMGB1, in an ERK-dependent manner, induced complex I phosphorylation as well as RAGE phosphorylation on S377, driving its translocation to mitochondria and increasing ATP production. Thus, in this context inhibition of RAGE may again be beneficial as it reduces tumor growth by reducing mitochondrial function.

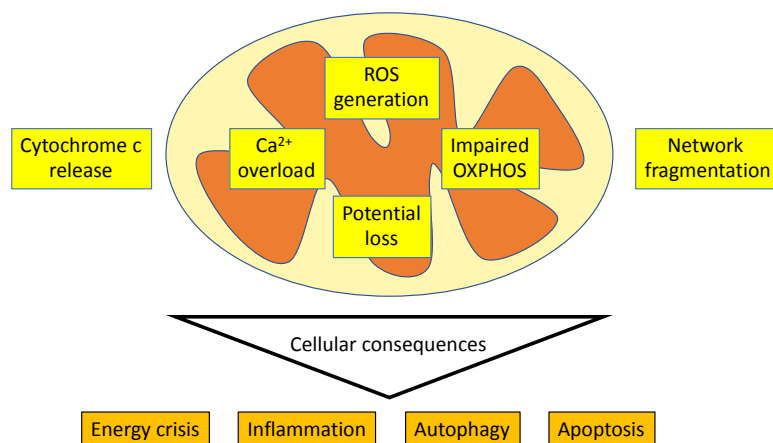


Figure.3.2: Mitochondrial and subsequent cellular consequences of RAGE activation.  
ROS: reactive oxygen species, OXPHOS: oxidative phosphorylation.



## **Unfolded Protein Response (UPR) and loss of proteostasis**

Maintenance of protein homeostasis, or proteostasis, is ensured by different mechanisms that control several protein processes including synthesis, folding, conformational maintenance and degradation. Any imbalance between these can result in loss of proteostasis. Ageing and age-related diseases are accompanied by a dysregulation of one or more of these systems and the equilibria among them. Several reports have highlighted the control of protein folding and degradation by RAGE-dependent mechanisms.

Protein misfolding and aggregation are more frequent during ageing and several pathological conditions. For example, oxidation of proteins results in both reversible and irreversible modifications that can have a strong impact on protein folding and function. These modifications generally occur during oxidative stress and are associated with inflammation. Levels of oxidized proteins are higher in subjects presenting high grade inflammation, possibly *via* RAGE, compared with subjects displaying low grade inflammation (Rodríguez-Ayala et al., 2005). Oxidative stress is a key mechanism involved in RAGE-dependent complications of disease. The interaction of RAGE with its various ligands in many cell types, tissues and conditions can generate cytoplasmic ROS production through activation of NADPH oxidase (Chen et al., 2010; Lee et al., 2010; Vincent et al., 2007; Wautier et al., 2001; Yan et al., 1994). Blockade or genetic deletion of RAGE or NADPH oxidase reverses the adverse effects of the RAGE-ligand interaction and tends to be protective (Hong et al., 2016; Soro-Paavonen et al., 2008; Wautier et al., 2001). Two mechanisms have been suggested for the activation of NADPH oxidase *via* the RAGE-ligand axis: 1) diaphanous1 (mDia1) binding to the cytoplasmic tail of RAGE and signaling through the Rho GTPase Rac1, which is a component of activated NADPH oxidase (Hudson et al., 2008; Young and Copeland, 2010); 2) protein kinase C (PKC) activation *via* the MAP kinases MAPK1/ERK2 and MAPK3/ERK1 (Cao et al., 2014). Other mechanism(s) might be involved, as NADPH oxidase activation *via* RAGE is certainly dependent upon RAGE ligands, cell type and condition.

In hyperglycemic conditions, RAGE-dependent oxidative stress is amplified through the induction of mitochondrial ROS (Coughlan et al., 2009). RAGE ligands can be formed during oxidative status, worsening the loss of proteostasis and further activating RAGE-dependent pathways including oxidation and inflammation (Kuhla et al., 2010). Enhanced steady-state level of ROS induces oxidative catabolism of lipids, amino acids and carbohydrates, resulting in the formation of reactive carbonyl species such as glyoxal, methylglyoxal and glucosone (Uchida, 2000). These reactive aldehydes can react with free amino groups to generate AGEs. Endogenous glycation of proteins can lead to their misfolding and loss of function but can also increase their stability when crosslinks are formed, contributing to protein aggregates and then protein toxicity (Kuhla et al., 2010; Vicente Miranda et al., 2017). Additional RAGE ligands thus produced bind to their receptor and can further extend RAGE-dependent loss of proteostasis. Some ligands, such as protein S100B, even need to be oxidized to bind to RAGE and induce expression of the angiogenic factor VEGF, which potentially stimulates neovascular age-related macular disease (Ma et al., 2007). Advanced oxidation protein products (AOPPs), which are dityrosine-containing and crosslinking plasma protein products, are markers of oxidative protein damage. These products bind to RAGE on vascular endothelial cells and stimulate ROS production, activate NADPH oxidase, MAPK 1/3 and p38, and induce nuclear translocation of NF- $\kappa$ B (Guo et al., 2008).

RAGE-dependent oxidation exhausts the potent antioxidant glyoxalase pathway (elimination of carbonyl compounds) and repair mechanisms (mainly disulfide reductases and MeSOX reductase). Decline of these different defense mechanisms with ageing worsens the disruption of proteostasis, weakening the elderly. Low grade inflammation might indeed be sufficient in these subjects to alter proteostasis. Two effective systems are needed to eliminate damaged proteins: the “ubiquitin proteasomal system” (UPS) and the “autophagy-lysosomal system”. The UPS is the main proteolytic machinery that plays a key role in proteostasis, whereas turnover of organelles and large protein aggregates will be eliminated through autophagy. Unlike UPS, autophagy can be directly controlled by the RAGE-ligand axis as described in the preceding section on mitochondrial dysfunction.

Organelle-specific responses may regulate both UPS and autophagy, contributing to efficient repair of misfolded proteins. The endoplasmic reticulum (ER) is a multifunctional organelle where protein folding occurs. Disruption of ER homeostasis induces accumulation of misfolded proteins generating a pathological state termed ER stress. An adaptive coordinated response, the unfolded protein response (UPR), is developed by the cell to limit the accumulation of unfolded proteins in the ER. Prolonged ER stress leads to inflammation, pro-apoptotic signaling and cell death. There is increasing evidence linking activation of ER stress response pathways and pathological conditions such as metabolic syndrome, diabetes and neurodegenerative diseases. AGEs induce ER stress signals in a time- and concentration-dependent manner, as well as apoptosis which is mediated by the UPR pathway protein kinase RNA-like endoplasmic reticulum kinase (PERK) and the mediator CCAAT/enhancer-binding protein homologous protein (CHOP) (Chiang et al., 2016). The interaction of AGE with RAGE induces a prolonged ER stress that mediates premature senescence of proximal tubular epithelial cells (Liu et al., 2014). This phenotype is accompanied by elevated expression of the ER stress marker glucose-regulated protein 78 (GRP78) and the cell-cycle regulator p21, correlating ER stress and premature senescence, both induced by the AGE-RAGE axis. Other RAGE ligands such as AOPPs can trigger ER stress that mediates apoptosis of podocytes through the different UPR pathways (PERK, ATF6 and IRE1) and the mediators CHOP and caspase 12 (Rong et al., 2015). Prolonged ER stress induced by ligand-RAGE interaction is coupled to a negative regulation of an efficient UPR since NF-E2 p45-related factor 2 (Nrf2), one of the transcription factors activated through the UPR, is inhibited by RAGE (Tang and Chen, 2014). Activation of the RAGE-ligand axis thus directly affects oxidative stress because Nrf2 is a key transcription factor controlling antioxidant defense. ER stress could be further induced by RAGE *via* disruption of calcium homeostasis in the ER: S100 proteins such as S100A8 and S100A9 are indeed essential to regulate intracellular calcium homeostasis (Donato, 1999). Binding of S100 proteins to RAGE could alter calcium homeostasis inside the cell and its organelles, including the ER.

Different mechanisms dependent on the RAGE-ligand axis can thus affect proteostasis. Most of these mechanisms favor a loss of proteostasis, that triggers complications common in age-related diseases and is one of the key hallmarks of ageing.

### **Cellular senescence and telomere attrition**

Senescence is commonly defined as an irreversible arrest in cell growth, accompanied by cell resistance to apoptosis and aberrant gene expression patterns, leading to the senescence-associated secretory phenotype (SASP) which involves the uncontrolled expression and secretion of multiples factors. Somatic cells have been shown to

have a limited replicative capacity, the so-called Hayflick's limit, leading to replicative senescence (RS) (Hayflick, 1965) owing to shortened telomeres that are eventually recognized as double-strand DNA breaks (Arnoult and Karlseder, 2015; d'Adda di Fagagna, 2008). This event induces a DNA-damage response, promoting the ATM/ATR pathways and leading to p53 (Lossaint et al., 2011; Roake and Artandi, 2017; Wang et al., 2011) then p21 activation, responsible for the cell cycle arrest (Beauséjour et al., 2003; Xiong et al., 1993). The telomere shortening can be prevented by telomerase activity, but while this enzyme(?) is widely expressed in embryonic and adult stem cells and confers immortality to cell lines *in vitro*, it is also one of the key proteins that allows cancer cells to divide indefinitely (Jafri et al., 2016).

Recent advances have shown that senescence can also occur *via* other processes allowing scientists to develop *in vitro* models that mimic RS in the absence of telomere shortening, such as oncogene-induced senescence (Serrano et al., 1997) or oxidative stress-induced senescence (Brandl et al., 2011; Chen et al., 2007; Toussaint et al., 2002).

Aside from cell growth arrest, the SASP is one of the main features of senescent cells. It is characterized by the secretion of different factors such as interleukins (e.g. IL-1, IL-6), chemokines (e.g. IL-8, MCP-1), growth factors or matrix metalloproteinases (Coppé et al., 2010; Malaquin et al., 2016). It has been shown that the SASP itself can promote cancer progression (Krtolica et al., 2001; Liu and Hornsby, 2007; Malaquin et al., 2013) and even features of ageing such as osteoarthritis when senescent cells are transplanted into or near joints (Jeon et al., 2018; Xu et al., 2017). Conversely, *in vivo* clearance of senescent cells by genetic manipulation (Baker et al., 2011, 2016; Jeon et al., 2017), selective apoptosis (Muñoz-Espín et al., 2018) or targeting senescent cell viability (Baar et al., 2017), lead to a seemingly improved health span and attenuated ageing, suggesting a rejuvenation of several tissues. Other strategies, such as the effective and selective inhibition of the mechanistic target of rapamycin (mTOR), have been reported to delay or even reverse senescence and have a positive impact on ageing (Walters and Cox, 2018). On the other hand AKT activation, which is strongly associated with mTOR activity, leads to increased telomerase activity and is associated with gastric cancer (Sasaki et al., 2014). Taken together, these studies show that tackling senescence is one of the keystones to promoting better health span and potentially extending longevity – determining the role of RAGE in senescence, given that it is a well-characterized contributor to chronic inflammation, would thus seem to be of the utmost importance.

Several studies have already linked prolonged, elevated glucose concentrations and hyperglycemia to senescence and telomerase activity (Blazer et al., 2002; Salpea et al., 2010; Sibbitt et al., 1989) and high levels of AGEs to senescence and telomere attrition (Sell et al., 1996; Sellier et al., 2015), but there is very little evidence to date showing a direct link between RAGE and cellular senescence.

Liu *et al.* (Liu et al., 2014), have shown that in proximal tubular epithelial cells (PTECs) of patients with diabetic nephropathy, senescence-associated  $\beta$ -galactosidase (SA  $\beta$ -gal) expression was positively correlated with p21, glucose-regulated protein 78 (GRP78) and RAGE. They further showed in PTECs from C57BL/6 mice that AGE-BSA induced p21-dependant senescence through ER stress after the activation of RAGE by AGE-BSA. Lastly, an anti-RAGE antibody successfully prevented cell cycle arrest, senescence-associated heterochromatin foci (SAHF, a typical feature of senescent cells) and SA  $\beta$ -gal staining.

The role of RAGE has also been studied in senescent-prone model, the senescence accelerated mouse-prone 8 (SAMP8), or senescent-resistant senescence accelerated mouse-resistant 1 (SAMR1), with or without acute liver injury induced by a galactosamine/lipopolysaccharide (G/L) treatment (Kuhla et al., 2013). Levels of plasma malondialdehyde (MDA), a marker of lipid peroxidation, both plasma and hepatic AGEs and hepatic RAGE were compared between 2 and 6-months old SAMP8 and SAMR1 mice. AGE and RAGE was increased in SAMP8 mice as early as 2 months-old and MDA, alongside AGE and RAGE, was significantly increased at 6 months-old. Necrosis and other markers of liver injury increased significantly in both SAMP8 and SAMR1 mice upon G/L injection, more so in the senescent-prone SAMP8 animals. RAGE expression was also increased and an anti-RAGE antibody significantly prevented these lesions in SAMP8 mice. Surprisingly, all of these markers were aggravated in the SAMR1 model by the addition of an anti-RAGE antibody. The authors suggest that anti-RAGE antibodies could allow RAGE homodimerization and signaling in a context of low AGE concentrations, hence a counter-intuitive worsening of the phenotype in SAMR1 mice that had low AGE concentrations.

Conversely, Chen *et al.* (Chen et al., 2012) suggest a beneficial role of the AGE-RAGE axis in senescent preadipocytes in cell culture. They first demonstrated RAGE overexpression with age and obesity in mice fed with a low- or high-fat diet. In parallel, *in vitro* results indicate that while adipogenesis in 3T3-L1 preadipocytes is impaired with RS, addition of methylglyoxal- or glyceraldehyde-glycated BSA to senescent 3T3-L1, but not to pre-senescent 3T3-L1, restored their capacity to accumulate lipids. This restoration was inhibited by anti-RAGE antibodies, while RAGE down-regulation additionally induced p21 and p53 overexpression. However, in the same cell type the AGE-RAGE axis has also been reported to promote the AKT pathway (Yang et al., 2013) that is involved in senescence as mTOR is a target of choice for limiting senescence (Astle et al., 2012; Walters and Cox, 2018).

Finally, a study in mice has also shown that different ligands of RAGE such as AGEs, S100 $\beta$  and HMGB1 are all increased with age in kidneys and liver but not in skeletal muscle. The increase of these ligands are correlated with *in situ* macrophage infiltration and glyoxalase-1 (GLO-1) downregulation (Son et al., 2017). In humans, the nuclear protein HMGB1 can be secreted in response to tissue damage but also by senescent cells. The binding of HMGB1 to RAGE and the toll-like receptor 4 (TLR4) initiate IL-6 secretion by senescent cells (Davalos et al., 2013). IL-6 and HMGB1 are features of the SASP, thus RAGE may hold a central role in SASP initiation or maintenance (fig.3.3).

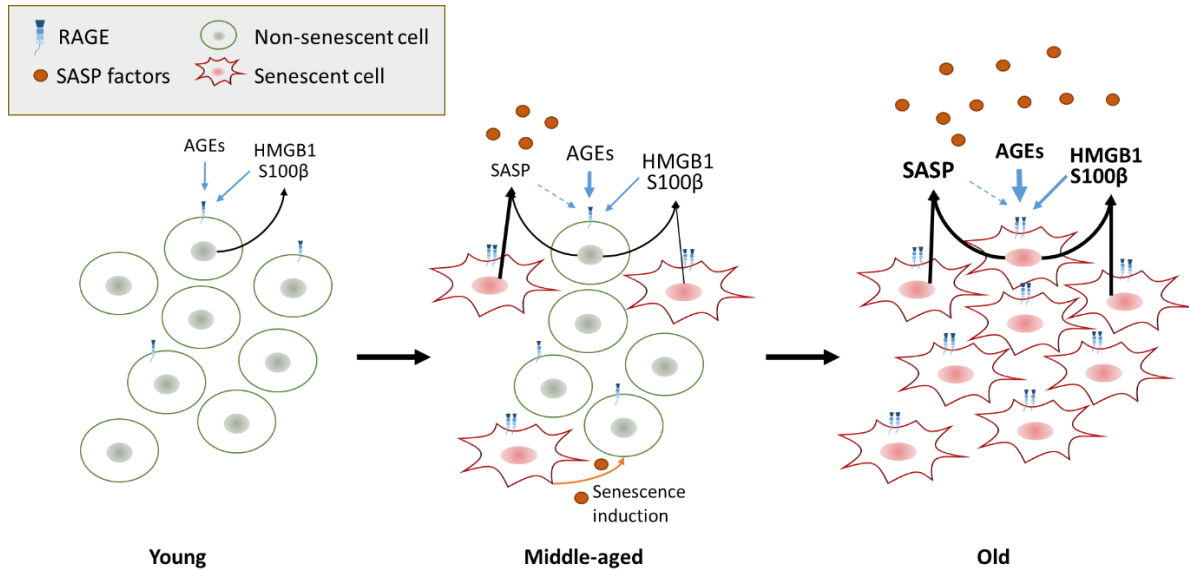


Figure 3.3: RAGE is a potential initiator and contributor to senescence and the SASP.

In young cells, expression of the receptor for advanced glycation end-products (RAGE) is low and so are its ligands and the pro-inflammatory signaling that results from activation of the RAGE-ligand axis. With age, RAGE and RAGE ligands expression is increased promoting pro-inflammatory conditions and contributing to the senescence-associated secretory phenotype (SASP). Some SASP factors can also activate RAGE and induce senescence, thus a vicious circle of RAGE activation and accelerated senescence is established.

### Genomic instability and epigenetic alterations

Ageing is characterized by a time-dependent decline in different functions and the integrity of cells. Age-related diseases could appear following the deregulation of gene expression (modification of the methylation status of genes, for example) or because of mutations in gene sequences.

Hudson and collaborators assessed four known polymorphisms located in the RAGE promoter (-429T/C, -374 T/A, a 63 bp deletion and +20 A/T). They demonstrated that the C allele of the -429 polymorphism was related to diabetic retinopathy in type 2 diabetic subjects (Hudson et al., 2001). Other polymorphisms in the RAGE gene could affect the occurrence of age-related macular degeneration (AMD). Banevicius and colleagues (Banevicius et al., 2018) assessed the association of the rs1800624 and rs1800625 polymorphisms in the RAGE gene with the susceptibility of early and exudative AMD. They demonstrated that the T allele at rs1800624 seems to be protective against AMD development, while the G allele at rs1800625 could be considered as a marker of poor prognosis in AMD development. Ageing and human disease states, including cancer, are accompanied by changes in the transcriptional profile of cells (Busuttill et al., 2007) and by the alteration of the patterns of epigenetic marks (Fraga et al., 2007).

Epigenetic marks such as DNA methylation and post-translational modifications (PTMs) of nucleosomal histones (histone H3 lysine acetylation (H3KAc) and H3K methylation (H3Kme)) regulate chromatin structure and function (Kouzarides, 2007; Portela and Esteller, 2010). The deregulation of epigenetic mechanisms has been

implicated in various diseases including diabetes and its complications (Ling and Groop, 2009; Pirola et al., 2010; Reddy and Natarajan, 2011; Villeneuve and Natarajan, 2010). Some studies have demonstrated a decreased number of repressive marks in the RAGE gene with age. Tohgi et al. analyzed the number of methylcytosines in the RAGE promoter and showed a decrease with age in the brains of subjects free from neurological disease, particularly in the cortex. The authors suggested that these modifications may activate the transcription and consequently increase expression of RAGE and the susceptibility of neurons to AGEs (Tohgi et al., 1999). Similarly, Kan and co-workers detected a decreased methylation of the RAGE gene promoter in PBMCs from type 2 diabetes retinopathy patients compared with healthy subjects, which may increase RAGE expression (Kan et al., 2018). During type 2 diabetes and diabetic nephropathy in *db/db* mice, Reddy and colleagues showed an increased glomerular expression of RAGE versus control *db/+* mice. This result was associated with RNA polymerase II recruitment and permissive histone marks in the RAGE gene and decreased repressive histone marks. Losartan, an Angiotensin II type 1 Receptor blocker, attenuated increased permissive histone marks on the *ager* gene promoter in mesangial cells cultured under diabetic conditions. (Reddy et al., 2014).

In summary, polymorphisms or epigenetic modifications in the RAGE gene sequence leading to the increased expression of RAGE seem to be important factors in predisposition for age-related diseases. But, in contrast with genetic changes to the DNA sequence, epigenetic changes are reversible and therefore represent a potential point of intervention for attenuating ageing processes.

### **RAGE implication in ageing of vessels, kidney, heart and brain**

The deleterious effects of endogenously produced AGEs on arterial function have been widely described in diabetes mellitus, uremia, and during ageing (Goldin et al., 2006; Grossin and Wautier, 2007). We have further demonstrated that chronic dietary CML exposure induced endothelial dysfunction and aortic stiffness with functional and structural changes. The functional alterations in aortic reactivity were correlated with structural modifications associated with lower SIRT-1 expression. The progression of endothelial dysfunction was related to CML accumulation and to overexpression of RAGE and VCAM-1. The arterial stiffness was associated with higher pulse wave velocity, a marker of arterial ageing (Grossin et al., 2015). Measuring endothelial dependent aortic relaxation (Mulvany chamber), we recently demonstrated that RAGE invalidation prevented physiological arterial stiffness and ageing in 20 month-old mice (Teissier et al., 2018). Other studies have demonstrated the vasculature-protecting effects of exercise, especially during ageing (Guitar et al., 2018). It has been demonstrated that the RAGE inhibitor FPS-ZM1, in addition to exercise, reduced formation of malondialdehyde, 3-nitrotyrosin and ROS, increased the GSH/GSSG ratio, suppressed activation of NF- $\kappa$ B and reduced levels of IL-6 in rats. FPS-ZM1 attenuated aortic stiffening with age and was associated with reduced collagen levels, increased elastin levels and reduced pulse wave velocity (PWV); it also prevented ageing-related endothelial dysfunction as shown by restored endothelium-mediated vascular relaxation (Gu et al., 2014).

Using a radio marker ( $^{13}\text{C}$ ) to produce  $^{13}\text{C}$ CML added to food, it has been shown that this oral AGE preferentially accumulates in the kidneys, then in the gut and lungs (Tessier et al., 2016). In the kidney, dietary CML accumulates primarily in the proximal tubes (Nakamura et al., 2010) where RAGE (expression?) is also concentrated, suggesting a potentially important role for the RAGE-AGE axis in the kidney. RAGE implication in nephropathies

such as diabetic nephropathy is well-known and considered to play a key role in the onset or evolution of diabetic complications (Daroux et al., 2010). It has been proposed that its expression during diabetes contributes to increased VEGF and a pro-inflammatory environment in glomeruli, favoring mesangial activation and TGF- $\beta$  production which lead to glomerulosclerosis and albuminuria (Wendt et al., 2003). Conversely, RAGE deletion or blockage prevents diabetic complications such as nephropathy (Matsui et al., 2017; Reiniger et al., 2010). The implication of RAGE during physiological kidney ageing has yet to be completely described. A recent study (Teissier et al., 2018) has shown that the deletion of RAGE in a mouse model significantly reduced typical age-associated kidney injuries such as vascular hyalinosis or tubular atrophy, and substantially prevented glomerulosclerosis and glomerular hypertrophy. Glomerular apolipoprotein A-II senile amyloidosis was similarly prevented in this model. This protection was accompanied by a significantly reduced inflammation and mTOR activity while SIRT1 and some antioxidants were better maintained in RAGE<sup>-/-</sup> mice during ageing. It is proposed that low inflammation seen in RAGE<sup>-/-</sup> mice may play an important role in these observations, as both acute and chronic inflammation are associated with chronic kidney disease (Akchurin and Kaskel, 2015; Silverstein, 2009) and are central in the SASP, one the key hallmarks of ageing (López-Otín et al., 2013). Additionally, decreased mTOR activity associated with a pro-longevity phenotype is consistent with numerous studies suggesting mTOR inhibition as a target for improved health span and extended lifespan (Walters and Cox, 2018). Thus, targeting RAGE during nephropathy or physiological renal ageing could have the added benefit of reducing chronic low-grade inflammation caused by the SASP and mTOR activity, since both contribute to ageing and age-related disorders (Fig.3.4).

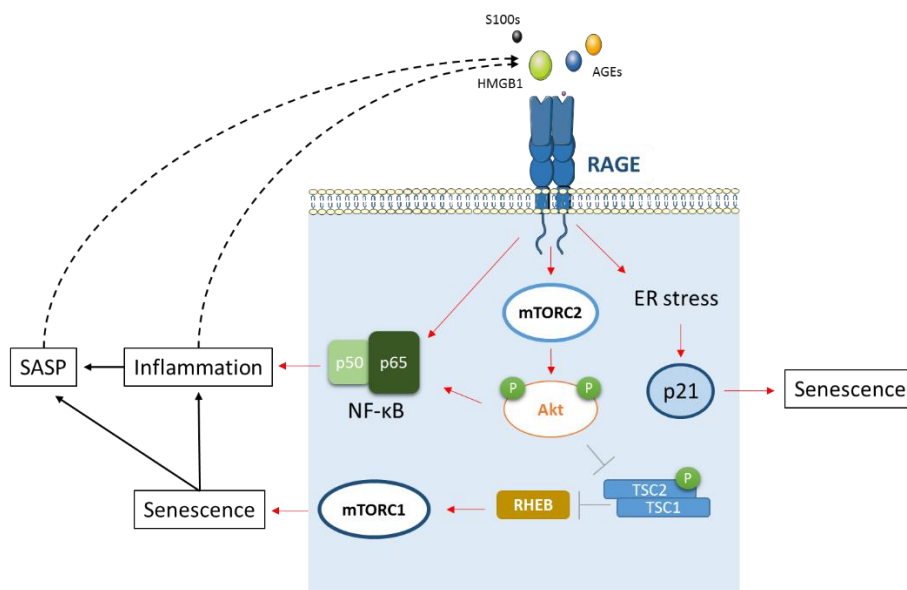


Figure 3.4: RAGE contribution to senescence may involve mTOR and ER stress.

Interaction between receptor for advanced glycation end-products (RAGE) and its ligands leads to favored inflammation and senescence through NF- $\kappa$ B and mechanistic targets of rapamycin (mTORC) 1 and 2 pathways. The endoplasmic reticulum (ER) stress is also a potent pathway from RAGE to senescence *via* the induction of p21.

Muscle mass begins to decrease from as early as 30 years of age. This age-related mass loss, sarcopenia, can reach up to 5% per decade in inactive people, reducing their lifespan and quality of life. An evaluation of the

accumulation of AGEs and their binding to RAGE did not show significant changes between young and old mice (Son et al., 2017). Nevertheless, increased muscle CML staining and RAGE expression was associated with oxidative injury and inflammation in old patients, suggesting a possible role of RAGE activation in sarcopenia (de la Maza et al., 2008). Underlying mechanisms remain to be elucidated, but could involve impaired proliferation and differentiation of muscle stem cells, a part of this impairment being RAGE-dependent (Beccafico et al., 2011). In the human heart, higher RAGE expression was found in elderly compared with young subjects (Simm et al., 2004). A significant inverse correlation between RAGE and cardiac index was observed, indicating that RAGE protein expression could participate in the reduced cardiac ejection fraction observed with age (Simm et al., 2004). Interestingly, comparison between the ageing heart and diabetic heart in the understanding of the pathogenesis of cardiovascular diseases indicates that RAGE activation in coronary vessels and cardiomyocytes is a shared feature, leading to pro-inflammatory responses, ROS production, mitochondrial dysfunction, calcium mishandling, autophagy and apoptosis induction, all of which can lead to contractile dysfunction and heart failure (Frimat et al., 2017; Yan et al., 2007).

More than 20 years ago *clinicians and scientists* drew attention to the possible implication of carbohydrates in the pathophysiology of brain ageing during dementia, especially in vascular dementia and Alzheimer's disease (Smith et al., 1994). Cerebrospinal fluid concentrations of CML or pentosidine, as well as reactive dicarbonyl compounds, are increased during vascular dementia and Alzheimer's disease (Shuvaev et al., 2001). Numerous molecular processes have recently been suggested as linking glycation products and neurodegenerative disorders, among them oxidative stress, inflammation, mitochondrial dysfunction and amyloidogenesis as seen in Alzheimer's disease (Yang and Song, 2013). It is now well known that Alzheimer's disease risk is increased in a diabetic population. AGEs are able to stimulate Tau overexpression and Tau phosphorylation. Amyloid  $\beta$ -peptide is a RAGE ligand and brain endothelial and microglial RAGE activation by A $\beta$ -peptide is followed by secretion of pro-inflammatory proteins (Fang et al., 2010). The AGE-RAGE interaction could thus contribute to Alzheimer's disease by promoting vascular permeability and A $\beta$ -peptide influx through the brain blood barrier, and by mediating A $\beta$ -peptide-induced oxidative stress and the AGE-related hyperphosphorylation of tau (Candela et al., 2010). A multimodal, RAGE-specific inhibitor was able to reduce A $\beta$ -mediated brain disorder in a mouse model of Alzheimer disease (Deane et al., 2012). Disappointingly, the recent "STEADFAST" clinical trial of an oral RAGE inhibitor (Azeliragon) failed to halt the progression of mild cognitive impairment (MCI) (Burstein et al., 2018). This failure could be related to late commencement of the RAGE inhibitor treatment, because MCI is diagnosed after the disease has already been in development for around ten years (perhaps longer). Beside the recent focus on RAGE implication during Alzheimer disease, it remains important to not forget the role of glycation in the amyloid cascade and plaque formation.

### **Conclusion: RAGE in inflammaging**

Glycation, as well as low-grade inflammation, is increased during ageing. AGEs and inflammatory RAGE ligands participate, *via* the RAGE-ligand axis as well as in a RAGE-independent manner, in inflammation, oxidation and glycooxidation pathways leading to inflammaging. Like endogenous glycation products, dietary AGEs also accelerate arterial ageing while vascular and renal physiological ageing are prevented by RAGE inactivation. The increase of SIRT1 expression and the decreased of S6RP and AKT phosphorylation in RAGE<sup>-/-</sup>



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mice suggest that RAGE could be an important mediator implicated in ageing pathways. Since RAGE invalidation in old mice also reduces the expression of Il-6 and TNF- $\alpha$ , two inflammation markers implicated in senescence and ageing, we postulate that RAGE could be a key receptor of inflammaging.

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# The Receptor for Advanced Glycation End-Products (RAGE) is an important Pattern Recognition Receptor (PRR) for inflammaging

Thibault Teissier<sup>1#</sup> and Éric Boulanger<sup>1,2,3</sup>

1. “Glycation: from Inflammation to Ageing” Lille Inflammation Research International Center (LIRIC-INSERM.U995) Lille, France.

2. Department of Geriatrics and Ageing Biology, School of Medicine, Lille University, France

3. Department of Geriatrics, Lille University Hospital, France

# Corresponding author: thibault.teissier.tt@gmail.com

## Abstract

The receptor for advanced glycation end-products (RAGE) was initially characterized and named for its ability to bind to advanced glycation end-products (AGEs) that form upon the irreversible and non-enzymatic interaction between nucleophiles, such as lysine, and carbonyl compounds, such as reducing sugars. The concentrations of AGEs are known to increase in conditions such as diabetes, as well as during ageing. However, it is now widely accepted that RAGE binds with numerous ligands, many of which can be defined as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The interaction between RAGE and its ligands mainly results in a pro-inflammatory response, and can lead to stress events often favouring mitochondrial dysfunction or cellular senescence. Thus, RAGE should be considered as a pattern recognition receptor (PRR), similar to those that regulate innate immunity. Innate immunity itself plays a central role in inflammaging, the chronic low-grade and sterile inflammation that increases with age and is a potentially important contributory factor in ageing. Consequently, and in addition to the age-related accumulation of PAMPs and DAMPs and increases in pro-inflammatory cytokines from senescent cells and damaged cells, PRRs are therefore important in inflammaging. We suggest here that, through its interconnection with immunity, senescence, mitochondrial dysfunction and inflammasome activation, RAGE is a key contributor to inflammaging and that the pro-



longevity effects seen upon blocking RAGE, or upon its deletion, are thus the result of reduced inflammaging.

**Keywords:** Ageing, Inflammaging, Receptor for advanced glycation end-products (RAGE), Pattern recognition receptor

## Introduction

The receptor for advanced glycation end-products (RAGE) expressed in mammals is a 45 kDa transmembrane receptor from the immunoglobulin superfamily (Ig) which also serves as a cell adhesion molecule (Sessa et al., 2014). While it is very strongly expressed in the lung, its basal expression is low in most tissues. While its precise physiological function remains unclear, there is good evidence that it could be involved in immunity due to its pro-inflammatory signalling. Nonetheless, *Rage* deletion in mice is viable and has shown to be protective against a number of pathologies, including age-related diseases, such as cardiovascular diseases or Alzheimer's disease (Cai et al., 2016; Park et al., 2011), and even features of physiological ageing (Grossin et al., 2015; Teissier et al., 2019). RAGE interaction with its ligands results primarily in increased inflammation and oxidation. Given the properties of RAGE, its role in age-related diseases and its association with some features of ageing, we here suggest that it could also play a key role in physiological ageing.

Ageing is characterized by a number of events that lead to reduced efficacy of organ functions, to an increased probability of developing so-called age-related diseases and to an increased risk of death with time. Hallmarks or pillars of ageing that cause or result from ageing have recently been described and reviewed (Kennedy et al., 2014a; López-Otín et al., 2013). Chronic inflammation, or rather inflammaging, is considered one of the most important processes of ageing that both explains and is explained by different hallmarks of ageing. We here suggest that RAGE signalling might be an important contributor to inflammaging with respect to its inherent pro-inflammation properties and its suspected role in ageing.

In this review, we summarize the current knowledge of RAGE expression and structure and its interaction with downstream effectors and extracellular ligands. Consequently, we describe inflammaging and its links with immunosenescence, damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and the senescence-associated

secretory phenotype (SASP), thereby showing that RAGE is classifiable as a pattern recognition receptor (PRR) and how it is involved in inflammaging and ageing.

## **RAGE and its ligands**

### **RAGE expression**

RAGE is a multiligand protein found in mammals, belonging to the immunoglobulin superfamily and to the family of cell adhesion molecules (Sessa et al., 2014). It is widely expressed across all organs, notably in skeletal muscle, lung, heart, liver and kidney (Brett et al., 1993) and is also expressed in circulating adaptive and innate immune cells (Akirav et al., 2012; Chen et al., 2008; Manfredi et al., 2008; Moser et al., 2007; Narumi et al., 2015; Schmidt, 2017) and is involved in their recruitment *via* vascular cell adhesion molecule 1 (VCAM-1) (Boulanger et al., 2002, 2007). While its expression is relatively low in most tissues under basal conditions, it is more strongly expressed in the lung, notably in alveolar epithelial cells (Cheng et al., 2005; Demling et al., 2006; Shirasawa et al., 2004). At least 20 splice variants have been described so far but only a few of them have a fully reported corresponding protein. While some of these variants are shared between mice and human, others are exclusive to one or other species and mice potentially express less soluble RAGE (sRAGE) through alternative splicing than human. Nonetheless, despite these differences, the main reported isoforms of RAGE are conserved across species and the mRNA coding for full-length RAGE is the most prevalent variant across organs (Hudson et al., 2008a; Kalea et al., 2009). There exists some exceptions and sRAGE is dominant in the human hippocampus (Ding and Keller, 2005a). With age, RAGE expression is increased, at least in the human heart, and its role might therefore become more important in ageing people (Simm et al., 2004). Mainly described at the plasma membrane, it is also often found in the cytoplasm. So far, only one study has indicated that it could also be found in the nucleus, having a role in DNA-damage response (DDR) and double-strand break repair, but more work is required to confirm this (Kumar et al., 2017).

### **RAGE structure**

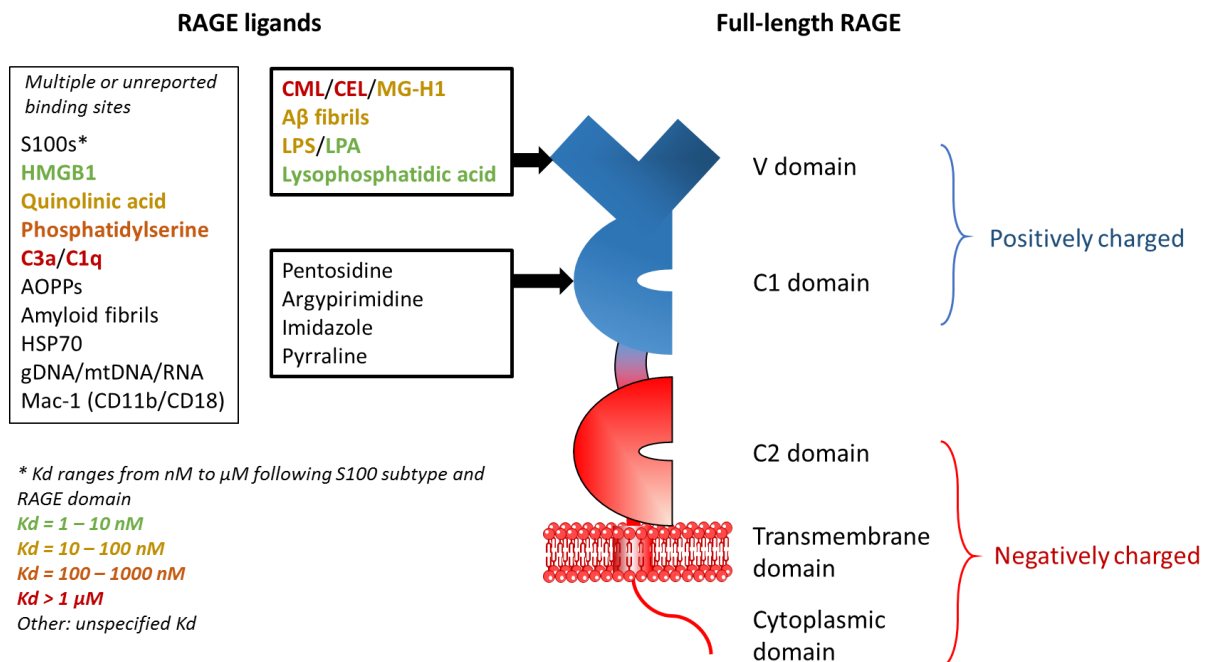
Full-length RAGE is comprised of three Ig domains that form its extracellular part, including one V-type Ig domain (V domain) linked *via* a short linker to a C-type Ig domain (C1 domain) and then to a second C-type Ig domain (C2 domain). These extracellular domains are then followed by one transmembrane and one intracellular domain which transduces the signalling (Fig. 1) (Bongarzone et al., 2017; Nepper et al., 1992; Xue et al., 2011; Yatime and Andersen,

2013). Naturally-occurring isoforms are also found. Two soluble forms (sRAGE) have been widely reported: the endogenous secretory RAGE (esRAGE), which results from alternative splicing of *Rage* mRNA (Hudson et al., 2008a; Jules et al., 2013), and the cleaved RAGE (cRAGE) which is the result of the extracellular RAGE domain cleaved by metalloproteases such as a disintegrin and metalloproteinase 10 (ADAM10) or matrix metalloproteinase-9 (MMP9) (Raucci et al., 2008; Zhang et al., 2008). Interestingly, sRAGE is a possible biomarker of chronic inflammatory diseases (Maillard-Lefebvre et al., 2009). Two additional forms of RAGE have also been reported: the dominant-negative RAGE (DN-RAGE), lacking any intracellular domain, and a form lacking the V domain ( $\Delta$ N-RAGE) (Ding and Keller, 2005b; Hudson et al., 2008a). Other splice variants have been reported, but the expected proteins are either unknown or untranslated (Hudson et al., 2008a). However, we will focus hereafter upon the full-length RAGE.

Upon interaction with its ligands, RAGE may be phosphorylated by PKC $\zeta$  and/or recruit various adaptor proteins or effectors including toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP), MyD88, similar to Toll-like receptors (TLRs) (Sakaguchi et al., 2011), or mammalian diaphanous-1 (mDia1) (Hudson et al., 2008b; Manigrasso et al., 2016; Rai et al., 2012a). In parallel, RAGE can undergo homodimerization, oligomerization (Xie et al., 2008; Yatime and Andersen, 2013; Zong et al., 2010) and possibly heterodimerization (Tian et al., 2007; Zong et al., 2010), to engage its signalling pathways. These pathways are diverse and complex, and include the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, c-Jun N-terminal kinase (JNK), the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, and the mitogen-activated protein kinases (MAPK) pathway with extracellular-signal-regulated kinases (ERK) 1/2 (Huang et al., 2001; Hudson et al., 2008b; Li et al., 2015; Liu et al., 2010; Ray et al., 2016; Wautier et al., 2001; Yeh et al., 2001). Its activation mainly results in the activation of transcription factors activator protein 1 (AP-1), STATs and more importantly NF- $\kappa$ B, leading to pro-oxidant and pro-inflammatory responses (Bianchi et al., 2010; Bierhaus et al., 2006; Kierdorf and Fritz, 2013; Yan et al., 2009b). Interestingly, adaptor proteins such as TIRAP and MyD88, both of which lead to a pro-inflammatory response upon activation of TLRs and RAGE (at least via the NF- $\kappa$ B signalling pathway), suggest similar functions and a potential crosstalk between the two receptor families. Thus, regulating these adaptors might have an important impact on inflammation by modulating the activity of TLRs and RAGE. On the other hand, up- or down-regulation of one of these receptors may alter the activity of other receptors owing to

decreased or elevated availability of MyD88 and TIRAP to transduce their signalling. On the contrary, however, RAGE activation can temper NF- $\kappa$ B activation in some cases, as shown in the liver (Zeng et al., 2012). A more detailed description of RAGE downstream signalling pathways is proposed elsewhere (Sorci et al., 2013; Xie et al., 2013).

While RAGE was first described as a receptor for advanced glycation end-products (AGEs), hence its name (Neeper et al., 1992; Schmidt et al., 1992), it is now widely known that RAGE is in fact a multi-ligand receptor. Proteins and molecules from various origins interact with RAGE and induce a transduction signal. To better understand how RAGE functions and in what conditions its contribution is involved, it is important to fully describe the ligands that trigger RAGE signalling.



**Fig. 1 RAGE structure** Full-length RAGE is comprised of the V, C1 and C2 extracellular domains, of a transmembrane domain and a cytoplasmic domain. While the V and C1 domains are closely bound together, a flexible linker is present between the C1 and C2 domains. The V and C1 domains (in blue) are positively charged with some localized negatively-charged sites, while the C2, transmembrane and cytoplasmic domains (in red) are negatively charged. Most RAGE ligands interact with the VC1 domain

Abbreviations: A $\beta$  – Amyloid  $\beta$ ; AOPPs – Advanced oxidation protein products; CEL – Carboxyethyllysine; CML – Carboxymethyllysine; gDNA – genomic DNA; HMGB1 – High-mobility group box 1; HSP70: Heat shock protein 70; LPA – Lipopolysaccharide lipid component A; LPS – Lipopolysaccharide; Mac-1 – Macrophage-1 antigen; MG-H1 – Methylglyoxal-derived hydroimidazolone-1; mtDNA – mitochondrial DNA

Adapted from Kierdorf et al. (Kierdorf and Fritz, 2013)

## **RAGE ligands**

### *Advanced glycation end-products*

Advanced glycation end-products (AGEs) are a family of molecules issuing from the reaction between a nucleophile, such as the amino group of a lysine and a carbonyl compound, such as reducing sugars. Upon this interaction, successive reversible rearrangements produce the early glycation products known as Schiff bases and Amadori products which eventually lead to a final reaction forming AGEs, so far considered as irreversible. This reaction can be influenced by pH and temperature. While it takes months or years to form AGEs *in vivo* at 37°C, they can be produced in minutes or seconds in cooking processes utilising high temperatures. Their concentration *in vivo* is elevated in pathologies such as diabetes (Thornalley et al., 2003), Alzheimer's Disease (AD) (Shuvaev et al., 2001) and cataracts (Monnier and Cerami, 1981), as well as with age (Sell et al., 1996). Tissues composed of proteins with a long half-life are more likely to accumulate AGEs and become altered by this accumulation, as exemplified by crystallin in the lens and collagen in the extracellular matrix (Masters et al., 1977; Monnier and Cerami, 1981; Verzijl et al., 2000). Thus, many different AGEs are formed either from endogenous or exogenous origin and, despite RAGE being named as the receptor for AGEs, owing to their heterogeneity it is not guaranteed that all AGEs can interact with this receptor. Methylglyoxal-derived hydroimidazolone-1 (MG-H1) has been reported to interact with the V domain of human RAGE with a  $K_d = 40$  nM in a nuclear magnetic resonance (NMR) and fluorescence titration experiment (Xue et al., 2014). Other NMR and fluorescence titration studies have demonstrated that peptide- and protein-bound carboxymethyllysine (CML) and carboxyethyllysine (CEL) were also able to interact with RAGE V domain with a  $K_d = 100$   $\mu$ M (Xie et al., 2008; Xue et al., 2011). However free CML and CEL, modified lysine not bound to other amino acid residues, do not seem to be able to interact with RAGE (Xie et al., 2008). This was supported by the observation that excess free CML was unable to inhibit CML-bovine serum albumin (CML-BSA) interaction with  $^{125}$ I-sRAGE (Kislinger et al., 1999). *In silico* docking analysis predicts that pentosidine, argyrimidine, imidazole and pyrroline are able to interact with the C1 domain of RAGE (Fatchiyah et al., 2015).

### *Calgranulins*

Another group of molecules, calgranulins, has also been reported to interact with RAGE. Calgranulins are a family of S100, EF-hand, calcium-binding proteins which are considered as a sensor of intracellular  $Ca^{2+}$  and are involved in different cellular processes such as cell migration, cell proliferation or cell differentiation (van Hout et al., 2016). Dysregulated levels

of S100 have been associated with cancer, inflammatory response and cardiomyopathies (Bresnick et al., 2015; Most et al., 2006; Oliveira et al., 2018). S100B is increased with age in mouse kidneys and liver and is also associated with increased infiltration of activated macrophage (Son et al., 2017). The majority of the 21 members of the S100 family has been reported to interact with the V, C1 and even C2 domain of RAGE (Bongarzone et al., 2017; Leclerc et al., 2009). Some work suggests that S100B interacts with positively-charged areas of the V domain (Xie et al., 2008), while others suggest an hydrophobic  $\text{Ca}^{2+}$ -dependent interaction in the C'D loop of the V domain (Park et al., 2010). The calculated  $K_d$  ranged from  $\mu\text{M}$  to  $\text{nM}$  and were positively affected by S100 oligomerization (Ostendorp et al., 2007; Xie et al., 2007).

### *HMGB1*

High-mobility group box 1 (HMGB1), or amphoterin, is considered to be the most abundant non-histone, DNA-binding protein. It is mainly localized in the nucleus but can transit to the cytoplasm *via* nuclear pores when acetylated, thus regulating its function. HMGB1 is involved in nucleosome formation and can modulate transcription by interacting with transcription factors and histones (Bianchi and Agresti, 2005; Harris and Andersson, 2004). Its role in DNA repair remains elusive and the subject of debate (Lange and Vasquez, 2009). Its overexpression in breast cancer sensitizes cancer cells to cisplatin, an antitumor drug (He et al., 2000), and its depletion enhances the excision repair mechanism following cisplatin treatment (Li et al., 1997), but it has also been shown to play a role in genomic and mitochondrial DNA repair (Ito et al., 2015; Lange et al., 2008). HMGB1 can also be released in the extracellular medium by active or passive processes. Active release generally involves a severe stress, such as inflammation or ischemia, which can inhibit HMGB1 deacetylation and lead to its sequestration in the cytoplasm until it is finally secreted (Evankovich et al., 2010; Gardella et al., 2002; Tsung et al., 2014; Venereau et al., 2013). Passive release of HMGB1 occurs in conditions such as necrosis (Scaffidi et al., 2002). HMGB1 is increased with age in mouse kidney and liver and is also associated with macrophage infiltration (Son et al., 2017). Circulating HMGB1 is associated with a set of pro-inflammatory processes, senescence and several pathologies (Davalos et al., 2013; Magna and Pisetsky, 2014). Among all RAGE ligands, HMGB1 has one of the highest reported affinities with a  $K_d = 6 - 10 \text{ nM}$  as determined by an *in vitro* saturation binding assay (Hori et al., 1995).

### *Amyloid fibrils*

Amyloid fibrils have also been shown to interact with RAGE. Amyloid fibrils are defined by  $\beta$ -sheet-rich, super-coiled, insoluble structures forming fibrils. These fibrils can be formed by the nucleation of soluble proteins to form protofilaments, which in turn give rise to amyloid fibrils. Many proteins such as the amyloid  $\beta$  ( $A\beta$ ) peptide, serum amyloid A (SAA), Ig light chains or even insulin have been reported to be responsible for different kind of amyloidosis (Iannuzzi et al., 2014; Rambaran and Serpell, 2008). While amyloidosis due to proteins such as apolipoprotein A-II (apoA-II) is very rare in humans, it is common in other species like mice mainly because of species-specific differences in methionine content and dimerization, which is also why amyloidosis is specific to some proteins (Gursky, 2014; Higuchi et al., 1991; Kitagawa et al., 2003; Morizane et al., 2011). Amyloidosis has been linked to a number of pathologies including AD where  $A\beta$  plaques are increased (Glennner and Wong, 1984; Masters et al., 1985a, 1985b), scrapie or bovine spongiform encephalopathy caused by prion proteins (PrPc) (Prusiner, 1982, 1991), or primary systemic amyloidosis characterized by accumulation of amyloid light (AL) and heavy (AH) chains in multiple organs (Comenzo, 2000; Rambaran and Serpell, 2008). Among amyloid fibril RAGE ligands,  $A\beta$  is the best characterized and interacts with the V domain with a  $K_d = 70 - 80$  nM. This interaction plays a role in  $A\beta$  neurotoxicity and circulating  $A\beta$  influx into the brain (Deane et al., 2012; Yan et al., 1996). Other amyloid fibrils of SAA, prion-derived peptides and amylin have also been reported as ligands of RAGE. Notably, these same proteins presented in a random conformation, as well as an all- $\beta$  sheet protein erabutoxin B, were unable to interact with RAGE, suggesting a specificity of sequence and conformation is important for this interaction (Abedini et al., 2018; Yan et al., 2000). sRAGE was, however, reported to interact with both soluble and fibrillar transthyretin with equal affinity ( $K_d = 120$  nM) (Sousa et al., 2000).

### *Complement components*

The complement system is a major component of immunity and is characterized by multiples cleavage products. Importantly, it participates in pathogen lysis through the formation of a membrane attack complex (MAC) at their surface, allowing free diffusion of molecules through this pore and engendering cell death. It is also involved in a process called “antigen opsonisation”, by which addition of complement molecules to pathogens’ membrane facilitates recognition by immune cells. Chemotaxis of innate immune cells may also be enhanced by complement components. The complement component C3a, resulting from C3 cleavage, is an anaphylatoxin which is possibly involved in systemic lupus erythematosus (Peng et al., 2009;

Porcel et al., 1995). C3a was able to bind to RAGE in an ELISA assay and formed a ternary complex with oligonucleotides (Ruan et al., 2010). Another complement component, C1q is able to initiate complement activation and antibody-independent opsonisation and its absence is also associated with systemic lupus erythematosus (Teh et al., 2011). Interaction of monomeric C1q with RAGE exhibited weak affinity ( $K_d = 5.6 \mu\text{M}$ ) in SPR analysis, but multivalent bonds may enhance this binding. This interaction is suspected to enhance monocyte phagocytosis (Ma et al., 2012), but while few studies have investigated the interaction between components of the complement and RAGE, it plays a key role in immunity and it thus merits further characterization in the context of inflammaging.

#### *Other RAGE ligands*

Other ligands of RAGE, though relatively poorly studied, have also been reported. Lipopolysaccharide (LPS) and its lipid component A both interact with the V domain, potentially at the KGAPKKPPQRLEWKLN site, with a  $K_d = 35 \text{ nM}$  and  $K_d = 2 \text{ nM}$ , respectively (Yamamoto et al., 2011). RAGE was also able to interact with CpG oligonucleotides, double strand DNA and double strand RNA (Park et al., 2010; Ruan et al., 2010). The leukocyte cell surface molecule Mac-1 (CD11b/CD18) interacts with RAGE with an undefined  $K_d$  (Chavakis et al., 2003). Phosphatidylserine binds to sRAGE with a  $K_d = 0.563 \mu\text{M}$  (He et al., 2011). Advanced oxidation protein products have been reported to interact with RAGE in an ELISA assay (Guo et al., 2008). Extracellular heat-shock protein 70 was also able to interact with RAGE in an ELISA assay (Somensi et al., 2017). Quinolinic acid, an undesirable and excitotoxic tryptophan metabolite, interacts with RAGE at multiple sites in the VC1 domain with a mean  $K_d = 43 \text{ nM}$  (Serratos et al., 2015). Lysophosphatidic acid, an endogenous phospholipid with roles in homeostasis and diverse pathologies, also binds to the V domain of RAGE with a  $K_d = 9 \text{ nM}$  (Rai et al., 2012b).

As reviewed elsewhere with respect to HMGB1 and S100 proteins binding to RAGE, different RAGE ligands might initiate different signalling pathways (Sparvero et al., 2009). To our knowledge, currently available data are insufficient to clearly depict which ligand activates which pathways and the literature lacks studies designed to exclude non-specific interactions or unequivocally identify the different pathways involved, but some work has provided interesting tools to address issues such as compounds specifically inhibiting interaction between RAGE and adaptor proteins such as mDia1 (Manigrasso et al., 2016). It is therefore necessary to study RAGE role and signalling in more detail and in a greater variety of contexts in order to fully elucidate its properties.



Unlike ligands that bind to the V domain of RAGE (CML, LPS, ...), those binding to the C1 or C2 domain can still bind to the  $\Delta$ N-RAGE and presumably induce a pro-inflammatory response. Binding affinities should therefore be considered in order to anticipate competition between various ligands – low concentrations of high affinity ligands such as HMGB1 or LPS could compete even with high concentrations of lower affinity ligands such as CML and other AGEs. In events leading to significant release of HMGB1 or LPS into the circulation, these ligands would thus have a proportionally greater effect upon RAGE-mediated inflammation.

The vast heterogeneity of RAGE ligands suggests that these interactions are probably not dependent upon sequence recognition: rather, it is thought that RAGE acts as a PRR whose interaction with ligands is mostly governed by ionic interactions. While it was first described as a PRR in 2003 (Chavakis et al., 2003), its involvement in immunity has been poorly studied in comparison with other PRRs such as toll-like receptors (TLRs) and also, in a more general sense, compared to the work that has been undertaken on AGEs and diabetes. However, as presented below, many studies indicate that RAGE has a key place in inflammaging or its parameters.

## **RAGE and inflammaging**

### **Inflammaging**

Inflammaging was first used as a term in 2000 by Franceschi *et al.* (Franceschi et al., 2000) following their counterintuitive observation that, upon stimulation by a mitogen, mononuclear cells from healthy elderly donors (~80 years old) produced more cytokines such as interleukine-6 (IL-6), IL-1 $\beta$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), than those from younger donors (~27 years old) (Fagiolo et al., 1993). Later, it was shown that in plasma from centenarians the overall load of pro-inflammatory cytokines was significantly increased but was not associated with an enhanced immune response (Morrisette-Thomas et al., 2014). Thus, inflammaging was defined as chronic, low-grade and sterile inflammation associated with age, and it is now considered to be a pillar of ageing since inflammation status is one of the best predictors of longevity and successful ageing (Arai et al., 2015; Franceschi et al., 2000; Kennedy et al., 2014b).

The characterization of inflammaging has evolved to include a number of pro-inflammatory cytokines and other pro-inflammatory molecules such as IL-1 $\beta$ , IL-6, the already mentioned TNF- $\alpha$ , but also IL-18, C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell-expressed and secreted (RANTES) protein and

macrophage inflammatory protein 1  $\alpha$  (MIP-1 $\alpha$ ), all of which increase with age in peripheral blood mononuclear cells (PBMCs) or serum (Dinarello, 2006; Fagiolo et al., 1993; Ferrucci et al., 2005a, 2005b; Gerli et al., 2000; de Gonzalo-Calvo et al., 2010; Mariani et al., 2006; Morrisette-Thomas et al., 2014; Seidler et al., 2010). Similarly, anti-inflammatory molecules IL-1 receptor antagonist (IL-1ra), soluble TNF-receptor I (sTNF-RI) and sTNF-RII, or markers of anti-inflammation such as soluble CD30 (sCD30), are also increased with age and inflammatory activation (Gerli et al., 2000; Morrisette-Thomas et al., 2014). Overall, there exists a weight of evidence to indicate development of a global imbalance with increasing age favouring secretion of pro-inflammatory molecules and a concurrent increase in circulating anti-inflammatory molecules in response. These markers have been more comprehensively reviewed elsewhere (Minciullo et al., 2016).

Inflammaging is closely linked to immunosenescence, or the ageing of the immune system. With time, both innate and adaptive immunity become dysregulated, the number and efficacy of various cell types are greatly affected in such a way that the immune system becomes less responsive to pathogens (Nguyen et al., 2018), vaccine efficacy is reduced with age (Zhang et al., 2016) and propensity to develop cancers may increase (Pawelec, 2017). Among the many changes in innate immunity over time, key changes are that the most pro-inflammatory of the monocytes appear to be increased with age and may contribute to inflammaging (Hearps et al., 2012), while the inflammasome is over-activated by age-related damage-associated molecular patterns (DAMPs), increasing TNF- $\alpha$  level or mitochondrial dysfunction.

Nonetheless, the relationship between immunosenescence and inflammaging is not straightforward and inflammaging is not simply, nor indeed exclusively, induced by immunosenescence. One recent model proposes an interaction where immunosenescence and inflammaging mutually induce each other: on the one hand, inflammaging can decrease the strength of the adaptive immune response and lead to immunosenescence; on the other hand, a decrease of the adaptive immune response leads to increased innate immunity and inflammaging (Fulop et al., 2018). However current understanding of inflammaging tends to put innate rather than adaptive immunity at the centre of the process, since the former becomes mildly hyperactive with age. PRRs are key element of innate immunity that are able to recognize pattern-associated molecular patterns (PAMPs) and DAMPs and induce inflammation in response, notably through the involvement of NF- $\kappa$ B, AP-1, interferon regulatory factors (IRFs) and of a cytosolic multiprotein complex, the inflammasome (Franchi et al., 2012; Kaufmann and Dorhoi, 2016; Man and Kanneganti, 2015; Rivera et al., 2016; Wang

et al., 2014). With the accumulation of PAMPs and DAMPs with age, such as cytoplasmic or cell-free DNA (Crow and Manel, 2015; Dou et al., 2017; Mankan et al., 2014; Shen et al., 2015; Shimada et al., 2012), a “Garb-aging” theory has been proposed where excess production or a lack of clearance of molecular “garbage” might play a central role in the increase of chronic inflammation with age (Franceschi et al., 2017).

Finally, senescence might be another key contributor to inflammaging. Senescent cells are not quiescent cells, they accumulate multiple damages and modifications and exert an aberrant secretory phenotype – the SASP. The SASP is mainly characterized by increased secretion of various interleukins (IL-1 $\beta$ , IL-6), chemokins (MCP-1), growth factors and metalloproteinases which can be secreted by a wide range of cell types (Coppé et al., 2010; Malaquin et al., 2016). In addition, cytoplasmic chromatin fragments (CCFs) are a common feature of senescent cells and they are able to induce inflammation through their interaction with the cyclic GMP-AMP synthase (cGAS)/ stimulator for interferon genes (STING) pathway (Dou et al., 2017; Ivanov et al., 2013). As such, senescence is able to promote a pro-inflammatory microenvironment which can induce age-related inflammatory diseases such as osteoarthritis (Jeon et al., 2018; Xu et al., 2017). Interestingly, chronic inflammation itself is able to induce telomere dysfunction and engage the DNA damage response involved in senescence and ageing (Jurk et al., 2014). It thus seems sensible to consider senescence and the SASP as contributors to inflammaging. Accordingly, *in vitro* (Franceschi and Campisi, 2014) and *in vivo* (Xu et al., 2018) studies have shown a causative role between senescent cells and a pro-inflammatory environment, suggesting a strong interconnection and mutual influence between senescence and inflammaging. Of note, senescent cells are normally recognized and removed by the immune system; immunosenescence may impede clearance of senescent cells, however, and accelerate their accumulation with age (Burton and Stolzing, 2018), suggesting another interesting link between immunosenescence and inflammaging.

### **RAGE, mitochondrial dysfunction and inflammasome**

Mitochondrial dysfunction is a prominent attribute of ageing and rejuvenation upon removal of mitochondria shows that it is better for a cell to rely upon a different source of energy (*i.e.* glycolysis) than to continue with harmful mitochondria (Correia-Melo et al., 2016). The detrimental effects of RAGE on mitochondria are two-fold, namely mitochondrial damage and the inflammatory responses induced by mitochondrial dysfunction.

Firstly, studies of the AGE-RAGE axis have shown that it is able to disrupt the mitochondria membrane potential by generating ROS, perhaps by activating the NADPH oxidase (Coughlan et al., 2009; Neviere et al., 2016; Tan et al., 2009; Yu et al., 2017). As soon as the membrane potential is perturbed, generation of ROS increases, first because of electron leakage from the respiratory chain (Jastroch et al., 2010; Suski et al., 2012), and then because of accumulating mtDNA mutations. Malondialdehyde, for example, resulting from lipid peroxidation, is highly genotoxic and promotes mutations in the highly accessible mtDNA (Del Rio et al., 2005). Finally, mtDNA mutations have been associated with further increases in ROS generation, mitochondrial dysfunction and chronic inflammatory diseases, probably due to altered mitochondrial protein functions (Kim et al., 2015; Tuppen et al., 2010). RAGE activation by AGEs was responsible for increased mitophagy (*i.e.* mitochondria renewal) and mitochondrial dysfunction. But it is not clear whether mitophagy is increased only upon RAGE activation or by the resultant dysfunctional mitochondria (Lo et al., 2015). Even though RAGE signalling is able to activate autophagy/mitophagy, it has been associated with mitochondrial-dependent cell death, potentially due to overextended mechanisms (Lo et al., 2015; Mao et al., 2018). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), an important protein involved in mitochondrial biogenesis and ROS homeostasis, is notably controlled by acetylation/deacetylation processes dependent on sirtuine 1 (SIRT1) activity (Aquilano et al., 2010; Austin and St-Pierre, 2012). Interestingly, the AGE/RAGE axis and RAGE expression have been associated with decreased SIRT1 activity and protein levels, suggesting further impaired mitochondrial biogenesis (Grossin et al., 2015; Neviere et al., 2016; Teissier et al., 2019). Accordingly, the presence of AGEs or RAGE overexpression decreased mitochondrial density and aggravated mitochondrial abnormalities and dysfunction (Mao et al., 2018).

Secondly, it has also been demonstrated that both mitochondrial transcription factor A (TFAM) and mtDNA released upon mitochondrial dysfunction induce a RAGE-dependent inflammatory response, notably activation of NF- $\kappa$ B and the inflammasome component ASC, mediated by TLR9 (Ward et al., 2013; Yao et al., 2015). This crosstalk between RAGE and TLR9 might be explained by the TLR9-HMGB1-RAGE complex found elsewhere (Tian et al., 2007). The noncanonical NF- $\kappa$ B pathway has been related to mitochondrial dynamics, and it would be interesting to know whether RAGE is involved here as well (Laforge et al., 2016).

Other relationships between RAGE and inflammasome activation have been investigated which might also be linked to mitochondrial dysfunction. Recent work shows that both mitochondrial damage and the AGE/RAGE axis are able to activate the NOD-like receptor family, pyrin

domain containing 3 (NLRP3) inflammasome and IL-1 $\beta$  production (Song et al., 2017). Additionally, NLRP3 expression itself is upregulated by AGEs while an antagonist peptide or 4'-Methoxyresveratrol, both targeting RAGE, prevented this upregulation and subsequent inflammation, suggesting that RAGE activation promotes NLRP3 expression (Chen et al., 2017; Shahzad et al., 2015; Yu et al., 2018). In a model of acute pancreatitis, RAGE also actively participates in AIM2 inflammasome activation, notably more so than TLR2 or TLR4, and its knockout significantly reduced the associated damage and systemic inflammation (Kang et al., 2016). Unsurprisingly, another ligand of RAGE, HMGB1, is also responsible for NLRP3 inflammasome activation and RAGE downregulation significantly attenuated this inflammatory response. However, downregulation of TLR2 and TLR4 provided similar results, suggesting common pathways (Kim et al., 2018; Thankam et al., 2018).

Thus, as RAGE ligands accumulate with age (notably AGEs), mitochondrial dysfunction increases over time, and both RAGE activation and inflammasome activation subsequent to mitochondrial dysfunction and/or RAGE signalling are also augmented with age, RAGE represents a potentially important centre of activity in the promulgation of inflammaging.

### **RAGE and senescence**

Interestingly, RAGE expression is increased by its own ligands (Howes et al., 2004) and several important ligands of RAGE increase or accumulate with age, such as HMGB1, S100s or AGEs (Sell et al., 1996; Son et al., 2017). It has been shown that RAGE expression increased with age in diverse organs such as rats' aorta (Hallam et al., 2010), rat and human hearts (Gao et al., 2008; Simm et al., 2004) and mouse spleen (Cai et al., 2007). We can therefore hypothesize that increasing amounts of RAGE ligands with age contribute to its upregulation and augment its stimulation, thereby enhancing its pro-inflammatory role and favouring increased low-grade, sterile inflammation with age.

HMGB1 itself has not only been described as a promoter of the SASP, but also as a SASP factor which is secreted by senescent cells (Malaquin et al., 2016). In a study focusing on TLR4, rather than RAGE, HMGB1 initiated IL-6 secretion by senescent cells (Davalos et al., 2013). TNF- $\alpha$ , another SASP factor, has also been reported to upregulate RAGE expression through NF- $\kappa$ B signalling and the existence of an NF- $\kappa$ B-binding site on the *Rage* promoter (Li and Schmidt, 1997; Tanaka et al., 2000). Thus, the factors of the SASP might activate RAGE, producing even more SASP factors which favour both senescence and RAGE, thereby forming a vicious circle. Because of the SASP, widespread senescence is one of the main sources of cytokine-sustained

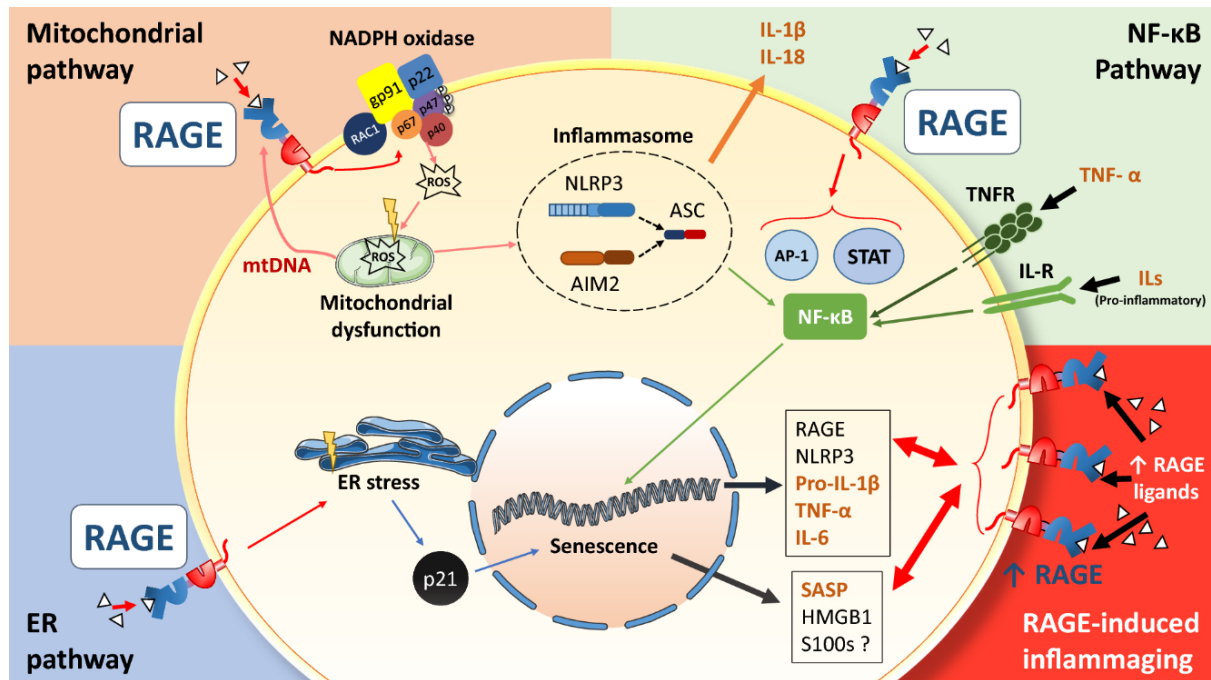
inflammaging. While several studies have already linked the RAGE ligands AGEs to senescence and telomere attrition (Navarrete Santos et al., 2017; Sell et al., 1996; Sellier et al., 2015), only a few studies have directly linked senescence to RAGE.

In a study of the murine model of accelerated ageing, senescence-accelerated mouse-prone 8 (SAMP8), and its senescence-resistant counterpart, senescence accelerated mouse-resistant 1 (SAMR1), Kuhla *et al.* showed that plasma and/or hepatic AGEs, malondialdehyde (a marker of lipid peroxidation and oxidative stress) and RAGE expression were all increased in SAMP8 compared with SAMR1 mice. Acute liver injury induced by a galactosamine/lipopolysaccharide (G/L) challenge induced MAPK signalling associated with elevated apoptosis, necrosis and liver injury markers which were all greater in SAMP8 mice. Blocking the receptor with an anti-RAGE antibody significantly diminished these markers in SAMR8, but paradoxically also increased these markers in SAMR1. The authors suggest this apparent contradiction could be explained by the antibody dimerizing and thereby activating RAGE when RAGE ligands (*i.e.* AGEs) are at low concentrations (Kuhla et al., 2013). These results nevertheless show that RAGE signalling might be increased during senescence and that inhibiting its activation might prevent its deleterious effects in an ageing context. A study with the same model of inflammatory liver injury, but in young C57Bl/6 mice, showed that mitochondrial dysfunction significantly increased oxidative stress, AGEs and RAGE. Blockage of RAGE significantly reduced both G/L- and mitochondrial dysfunction-associated damage (Kuhla et al., 2010). Mitochondrial dysfunction being an important part of inflammaging, it is interesting to note that the deleterious effects of dysfunctional mitochondria involve RAGE signalling.

In another study in diabetic nephropathy patients, a correlation was found in proximal tubular epithelial cells (PTECs) between senescence-associated  $\beta$ -galactosidase (SA  $\beta$ -gal – a marker of senescence), p21 (a marker of cell cycle arrest), glucose-regulated protein 78 (GRP78 – a marker of endoplasmic reticulum (ER) stress) and RAGE. The authors further confirmed in murine PTECs that activation of RAGE by AGEs induced a p21-dependant senescence involving ER stress. Markers of senescence were successfully prevented by blocking the receptor with an anti-RAGE antibody (Liu et al., 2014).

Finally, we have recently shown that RAGE deletion prevents important features of mouse renal ageing such as glomerulosclerosis or apolipoprotein A-II amyloidosis, and that this was

associated with significant limitation of the age-related increase in the inflammation markers, IL-6, TNF- $\alpha$  and VCAM-1 (Teissier et al., 2019).



**Fig. 2 RAGE-induced inflammaging pathways** Three major pathways have been identified leading to inflammaging and induced *via* RAGE activation by its ligands. The mitochondrial pathway involves NADPH oxidase activation by RAGE. Subsequent production of ROS impairs mitochondrial function and mitochondria accumulate damage and ROS: the mitochondrial products released can either reactivate RAGE or the inflammasome, thereafter responsible for release of ILs and NF- $\kappa$ B activation. NF- $\kappa$ B can also be activated directly by RAGE or by pro-inflammatory cytokine activation of TNFR or IL-R. NF- $\kappa$ B and other transcription factors, such as AP-1 or STATs, will thereafter promote the transcription of RAGE, NLRP3 and pro-inflammatory cytokines, all of which participate in inflammaging. RAGE activation can also provoke sustained ER stress, inducing a p21-dependant senescence. Subsequently, the SASP will fuel inflammaging and senescent cells will release RAGE ligands such as HMGB1 and S100s. Ultimately, RAGE expression increases with age, as do concentrations of a number of its ligands, thus engaging a vicious circle of RAGE-induced inflammaging with advancing age. The pro-inflammatory components responsible for inflammaging are highlighted in orange

Abbreviations: AIM2 – absent in melanoma 2; AP-1 – activator protein 1; ASC – apoptosis-associated speck-like protein containing CARD; ER – Endoplasmic reticulum; IL – Interleukin; IL-R – Interleukin receptor; mtDNA – mitochondrial DNA; NADPH – nicotinamide adenine dinucleotide phosphate; NLRP3 – NLR pyrin domain-containing 3; RAGE – Receptor for advanced glycation end-products; ROS – Reactive oxygen species; SASP – Senescence associated secretory pathway; STAT – signal transducers and activators of transcription; TNF- $\alpha$  – tumor necrosis factor- $\alpha$ ; TNFR – TNF receptor

PRRs are important actors in inflammaging. They represent the point of convergence of all DAMPs and PAMPs, to which exposure increases significantly with age, and will lead to a

number of pro-inflammatory signalling pathways fuelling the inflammatory repertoire of inflammaging. As RAGE is able to recognize multiple ligands, often DAMPs or PAMPs, is expressed on multiple cell types including immunity cells, triggers a pro-inflammatory response similar to other PRRs and has a significant role in ageing as presented above, we here suggest that RAGE is a key PRR in inflammaging (Fig. 2) whose role in this feature of ageing is currently underestimated.

Collectively, these results show that RAGE is strongly involved in both physiological ageing and inflammaging. As presented below, and in accordance with these data, there is increasing evidence in the literature that RAGE expression and activation are linked to a number of age-related diseases and despite the growing literature, its physiological role remains elusive.

## **RAGE and pathologies**

### **Physiological role of RAGE**

RAGE possesses all the characteristics of a PRR: its main ligands are almost all DAMPs (HMGB1, S100s, DNA, RNA) or PAMPs (LPS, DNA, RNA), or else are molecules involved in non-physiological conditions which require resolution (AGEs, amyloid fibrils, complement, Mac-1/CD11b, phosphatidylserine), and engender a pro-inflammatory response. As described earlier, by virtue of being an ubiquitous protein, RAGE is also expressed on innate immunity cells, similar to other PRRs, and its activation mainly triggers PRR-like pro-inflammatory responses, notably through NF- $\kappa$ B signalling (Hofmann et al., 1999; Li et al., 2015; Liu et al., 2010; Yeh et al., 2001).

Similar to TLRs, RAGE has a role in innate immunity and sepsis and *Rage*<sup>-/-</sup> mice are protected against lethal septic shock and exhibit significantly reduced NF- $\kappa$ B activity in the lung (Liliensiek et al., 2004). According to its suspected functions and its genomic proximity to other immune system components in the MHC class III locus (Hauptmann and Bahram, 2004; Sugaya et al., 1994), RAGE has been labelled a “noncanonical Toll” (Lin, 2006). The role of RAGE in innate immunity is not straightforward, however. When infected by *S. pneumonia*, *Rage*<sup>-/-</sup> mice exhibited better survival, a lower pulmonary bacterial load and a decreased dissemination to both blood and the spleen, suggesting a detrimental role of RAGE in host response (van Zoelen et al., 2009). Conversely, *M. tuberculosis* challenge of *Rage*<sup>-/-</sup> mice was associated with



increased inflammation, pulmonary bacterial load and loss of body weight, while survival decreased (van Zoelen et al., 2012).

Despite this evidence, the precise role of RAGE remains elusive and other functions of this receptor have been investigated. Since RAGE expression is significantly greater in the lung, we might expect its function to be more important and obvious. However, as reviewed elsewhere, its contribution to lung function remains unclear, under physiological or pathological conditions (Buckley and Ehrhardt, 2010). Recent work has shown a diminished elastance and an augmented compliance in the lungs of *Rage*<sup>-/-</sup> mice, but these same mice were protected against cigarette smoke-induced lung pathology, probably due to a reduced infiltration of macrophages (Wolf et al., 2017). Other workers have reviewed the negative implications of RAGE in many inflammatory lung diseases (Oczypok et al., 2017). Of note is that, while RAGE is mainly overexpressed in most cancers (Leclerc and Vetter, 2015; Shahab et al., 2018; Sparvero et al., 2009), it is on the contrary downregulated in lung cancer (Bartling et al., 2005).

RAGE's role in lung structure or function remains poorly understood, but its inherent properties and those of its ligands, its signalling pathways and its widespread expression, all seem to suggest it plays a significant role in ageing and inflammaging. A growing body of literature demonstrates RAGE's involvement in age-related pathologies.

### **RAGE and age-related diseases**

Similar to inflammaging, RAGE is not only involved in a number of pathologies, including retinopathy (Barile and Schmidt, 2007), neuropathy (Misur et al., 2004; Sugimoto et al., 2008; Wada and Yagihashi, 2005), nephropathies (Forbes et al., 2003; Yamamoto et al., 2007), type 2 diabetes (Kang et al., 2012; Keri et al., 2018) or cancer (Pelucchi et al., 2006; Turner, 2015; Yamagishi et al., 2015), but also in more specific age-related diseases such as osteoarthritis (Larkin et al., 2013; Loeser et al., 2005; Sun et al., 2016; Yang et al., 2015), cardiovascular diseases (Barlovic et al., 2010; Fukami et al., 2014; Park et al., 2011; Pettersson-Fernholm et al., 2003) or AD (Batkulwar et al., 2018; Cai et al., 2016; Yan et al., 2009a). With this weight of accumulating evidence, we here suggest that the deleterious effects of RAGE in ageing are closely linked to its pro-inflammatory properties and its contribution to inflammaging.

In a diabetic context, RAGE has been shown to be involved in microvascular and macrovascular complications (Goldin et al., 2006). However, there is also evidence that even in a “healthy” context, the impact of RAGE signalling on vessels is greater than expected, and might even be

a driver of arterial ageing. In a study of exposure to CML (an important RAGE ligand), it has been shown that a CML-enriched diet induced accelerated vascular ageing in middle-aged WT mice, characterized by increased vascular stiffening and elastin disruption, reduced aortic relaxation and increased VCAM-1 and RAGE expression, while SIRT1 expression was diminished. However, in *Rage*<sup>-/-</sup> mice exposed to the same amount of CML, all of these parameters were completely absent and *Rage*<sup>-/-</sup> mice fed a CML-enriched diet exhibited similar vascular ageing to control WT and control *Rage*<sup>-/-</sup> mice (Grossin et al., 2015). In another study, aortic relaxation was also measured during physiological ageing and was significantly reduced when old mice were compared with middle-aged mice; strikingly, *Rage*<sup>-/-</sup> mice did not exhibit any reduced aortic relaxation at all and were similar to the middle-aged mice in this regard (Teissier et al., 2019). Accordingly, FPS-ZM1, a commercially available RAGE antagonist, was able to efficiently mimic the positive effect of exercise training in old rats by significantly limiting vascular ageing and aorta inflammation (Gu et al., 2014). Finally, RAGE, as much as TLRs, has been proposed as an important target for treatments against atherosclerosis since inflammation is strongly involved in this process (Lin et al., 2009; Olejarz et al., 2018; Senatus and Schmidt, 2017).

Dietary CML (dCML) has been shown to preferentially accumulate in kidneys and this accumulation was independent of RAGE (Tessier et al., 2016). However, dCML had little impact on kidney ageing in old mice perhaps due to an increase in competing ligands with age, thus limiting any effect of dCML, or an absence of any dCML effect, or too low a dCML concentration being studied. On the other hand, RAGE deletion significantly reduced features of kidney ageing such as fibrosis, tubular atrophy, arteriolar hyalinosis and, more importantly, glomerulosclerosis and glomerular apolipoprotein A-II amyloidosis. Kidney inflammation, characterized by *Il-6*, *Tnf- $\alpha$*  and *Vcam-1* levels, was also significantly reduced (Teissier et al., 2019). These findings are in line with other results in diabetic nephropathy where RAGE signalling contributes to a pro-inflammatory environment leading to glomerulosclerosis and proteinuria (Daroux et al., 2010; Wendt et al., 2003). Unsurprisingly, chronic inflammation is an important contributor to chronic kidney disease and RAGE could therefore play a role in the occurrence and development of this disease (Akchurin and Kaskel, 2015; Silverstein, 2009).

Initial studies of RAGE in a murine model of AD showed that RAGE signalling was responsible for inflammation in microglia, critical for the formation of the A $\beta$  amyloid plaques characteristic of the disease (Fang et al., 2010). RAGE activation by AGEs participated in NLRP3 upregulation and in neuroinflammation by activating pro-inflammatory macrophages

– this activation was cancelled by the RAGE inhibitor FSP-ZM1 (Chen et al., 2017). Furthermore, RAGE has also been implicated in other features of AD onset independent of inflammation. Its interaction with A $\beta$  peptides is notably thought to have an important role in the transport of A $\beta$  peptides across the blood-brain barrier, favouring the subsequent formation of amyloid plaques (Candela et al., 2010). The use of FPS-ZM1 in a murine model of AD successfully limited A $\beta$  transport as well as associated inflammation, and normalized cognitive functions (Deane et al., 2012). Following these results, a clinical trial was undertaken with another RAGE antagonist, Azeliragon (TTP488); the trial reached phase 3 but was recently terminated owing to a lack of efficacy in the selected cohort. Importantly, however, no significant undesirable effects were reported or were responsible for the cessation of the trial, and it is suggested that the lack of efficacy might be explained by treatment being initiated too late: RAGE blocking remains of interest, therefore, as a therapeutic approach in the early stages of cognitive impairment (Burstein et al., 2018).

Gut ageing is notably associated with changes in microbiota. On the one hand, the diversity of gut microbiota is decreased, while on the other hand pro-inflammatory microbiota such as pathobionts are increased (Biagi et al., 2010; Rampelli et al., 2013) at the expense of anti-inflammatory microbiota such as bifidobacteria, *Faecalibacterium prausnitzii* or *Clostridium* cluster IV (Biagi et al., 2016; Hopkins and Macfarlane, 2002; Hopkins et al., 2001; Lopetuso et al., 2013; Walton et al., 2012; Zwieler et al., 2009). It has been demonstrated in mice and non-human primates that such a dysbiosis could lead to increased gut permeability and that gut permeability increases with age (Thevaranjan et al., 2017; Tran and Greenwood-Van Meerveld, 2013). As a consequence, altered gut permeability has been linked to leakage to the circulation of bacterial components including the pro-inflammatory LPS (Thevaranjan et al., 2017; Tulkens et al., 2018). While LPS has been described as a ligand of RAGE, little work has been undertaken to clearly describe and understand the importance of this interaction. There is nonetheless a potential role for RAGE where increased LPS leakage from the gut with age could activate RAGE and other LPS receptors to promote inflammaging. Indeed, this hypothesis is strongly supported by a recent study where microbiota from old mice was transferred to young, germ-free mice and promoted several parameters of inflammaging (Fransen et al., 2017). Conversely, older germ free mice had significantly lower plasma IL-6, more functional macrophages and improved survival (Thevaranjan et al., 2017). In addition, in humans, obesity is associated with increased gut permeability, inflammasome activity and RAGE expression (Rainone et al., 2016) and it would be interesting to determine whether this chain of events is

also increased in physiological ageing. RAGE signalling is also involved in inflammatory bowel disease in mice and human (Body-Malapel et al., 2019; Wang et al., 2016), suggesting another potential role in gut dysfunction and ageing.

Taken together, then, these findings clearly demonstrate that RAGE is significantly involved in increased chronic inflammation with age, and that this inflammation is closely linked to age-related diseases and perhaps to physiological ageing. Additional, very promising results on the relationship between RAGE, inflammaging and ageing could soon come from a newly generated sRAGE<sup>high</sup> murine model overexpressing circulating sRAGE which is able to block RAGE signalling and/or serves as a decoy for RAGE ligands (Peng et al., 2019).

## Conclusion

Historically, RAGE was first isolated from lungs thanks to its binding affinity with AGEs, hence it was first described as the receptor for advanced glycation end-products (Neeper et al., 1992; Schmidt et al., 1992). However, this discovery and the subsequent name and role attributed to RAGE may have diverted attention from its wider physiological role(s) and limited research beyond the effects of RAGE linked to AGEs. Besides, the binding affinity between RAGE and several AGEs is relatively weak in comparison with other ligands such as HMGB1. While an increasing large body of work has proposed different functions for RAGE, much remains to be done before the full extent of RAGE's physiological functions are clearly defined. Its high expression in the lung gives clear hints as to a possible role for RAGE and indeed, as stated above, its expression positively correlates with protection against some pulmonary pathogens (van Zoelen et al., 2012). Thus, close attention should be paid to susceptibility to pathogens when considering the use of RAGE antagonists – however, the recent clinical trial with Azeliragon did not report increased infection rates or mortality associated with this antagonist. Despite the suspected beneficial roles of RAGE, no major defects have been reported upon *Rage*<sup>-/-</sup> deletion in mice and, on the contrary, they seem to be protected against a number of physiological and non-physiological conditions.

While full-length RAGE was at the centre of this review, it should be noted that sRAGE, resulting from alternative splicing or RAGE cleavage, is of equivalent importance despite being less expressed in most tissues. Contrary to full-length RAGE, sRAGE is thought to be beneficial by acting as a decoy for RAGE ligands and/or by interacting with RAGE at the membrane, thus

preventing further interactions, oligomerization and RAGE signalling. Accordingly, lower levels of circulating sRAGE were found in patients with mild cognitive impairment or AD compared with control subjects (Emanuele et al., 2005; Ghidoni et al., 2008) while sRAGE is the most expressed isoform in the hippocampus (Ding and Keller, 2005a), suggesting a protective role of sRAGE against age-related cognitive impairment. Additionally, sRAGE was significantly diminished in precocious patients with acute myocardial infarction compared with young healthy controls while it has been shown to be increased in healthy centenarians (Geroldi et al., 2006). We could surmise that greater sRAGE prevents RAGE signalling and inflammaging and limits the impact of its numerous ligands, particularly if sRAGE remains high with advancing age. This could mirror a paradigm proposed to discriminate successful from unsuccessful ageing: “optimization”, where inflammaging is increased but counteracted by immuno-remodeling and anti-inflammaging against “deterioration”, where inflammaging is increased but immune-remodeling and anti-inflammaging are insufficient to provide an effective counterbalance (Fulop et al., 2018). Thus, sRAGE could serve as a counterbalance to RAGE and its ligands which increase with age.

Despite its name, RAGE is much more than a receptor for AGEs and attention should not be limited to AGEs or diabetes when studying RAGE and its involvement in different conditions. The variety of its ligands covers a number of molecules involved in many stress events, all leading to a pro-inflammatory response. Thus RAGE should be considered, more than anything else, as a PRR similar to TLRs, NLRs, RLRs or CLR. As proposed by the literature presented herein, innate immunity is essential in the onset and maintenance of inflammaging and PRRs are important contributor to this innate response. As demonstrated by different studies, the deletion of *Rage* in mice leads to a pro-longevity phenotype where features of ageing are significantly diminished in vessels or kidneys (Grossin et al., 2015; Teissier et al., 2019), and is associated with a decreased overall inflammatory burden. Evidence is accumulating, therefore, that RAGE plays an important role in inflammaging and that its deletion or blockage has beneficial effects on longevity. In addition, we here suggest that studies on *Rage*<sup>-/-</sup> mice or RAGE antagonists provide a proof of concept that PRR blocking is indeed a good target to limit the progression of inflammaging, which seems to be key component of ageing.

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## **Compliance with ethical standards**

## **Conflict of interest**

The authors declare that they have no conflict of interest.

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