Intérêt du RAGE comme biomarqueur circulant : du sRAGE aux autoanticorps anti-sRAGE

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VALUE OF RAGE AS A CIRCULATING BIOMARKER:

FROM sRAGE TO ANTI-sRAGE AUTOANTIBODIES

Thèse dirigée par le Professeur Eric BOULANGER

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Dedication

I dedicate this thesis to my parents Eliseu Lorenzi and Wanda Maria Ledur Lorenzi, who self-deprived and made their best efforts in order to make my life better. It was never in vain. I love you.

"Tant que les gens croient aux absurdités,

ils continueront à commettre des atrocités."

Voltaire

“That which can be asserted without evidence,

can be dismissed without evidence.”

Christopher Hitchens

“Everybody’s looking for the sun. People strain their eyes to see.

But I see you and you see me. Ain’t that wonder?”

Ray Davies

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Abbreviations

ACE	Angiotensin-converting enzyme
ACEi	Inhibitor of angiotensin-converting enzyme
AD	Alzheimer's disease
ADAM	Sheddase a disintegrin and metalloprotease
ADAMTS	A disintegrin and metalloproteinase with a thrombospondin type 1 motif
AGEs	Advanced glycation end-products
Akt	Protein kinase B
ApoE	Apoliprotein E
APP	Amyloid precursor protein
APS	Antiphospholipid syndrome
Aβ	Amyloid beta peptide
BBB	Blood-brain barrier
BMI	Body-mass index
CAD	Coronary artery disease
Ca-IC	Calcium ionophore calcimycin
cDNA	Complementary DNA
CDR	Clinical Dementia Score
CHD	Coronary heart disease
CKD	Chronic kidney disease
CML	Nε-carboxymethyllysine
cRAGE	Cleaved receptor for advanced glycation end-products
CRP	C-reactive protein
CVDs	Cardiovascular diseases
Dia-1	Diaphanous-1

EC	Endothelial cell
esRAGE	Endogenous secretory receptor for advanced glycation end-products
ESRD	End-stage renal disease
FBS	Fetal bovine serum
FcFree-rHu-sRAGE	Fc-free recombinant human sRAGE
FL-RAGE	Full length RAGE
G6P	Glucose-6-phosphate
GFR	Glomerular filtration rate
GLUT	Glucose transporter
GPCRs	G protein-coupled receptors
HbA_{1C}	Glycated hemoglobin
HD	Hemodialysis
HDL	High-density lipoprotein
HMGB1	High-mobility group protein-B1/amphoterin
HOMA-IR	Homeostatic model assessment - insulin resistance
HSA	Human serum albumin
hsCRP	high sensitiviy C-reactive protein
HSP	Heat-shock protein
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IKK	Kappa factor inhibitor kinase
IL	Interleukin
IMT	Intima media thickness
IRAK4	Interleukin-1 receptor-associated kinase 4
JNK	c-Jun N-terminal kinase

LADA	Latent autimmune diabetes of adults
LC–MS/MS	Liquid chromatography coupled to linear ion-trap tandem mass spectrometry
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MMP	Matrix metallopeptidase
MyD88	Myeloid differentiation primary response gene (88)
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC7	Proprotein convertase 7
PKC	Protein kinase C
PMA	Phorbol ester myristate actetate
pNPP	Para-nitrophenylphosphate
PRR	Pattern-recognition receptor
PS	Phosphatidylserine
RA	Rheumatoid arthritis
Rac-1	Ras-related C3 botulinum toxin substrate-1
RAGE	Receptor for advanced glycation end-products
RBANS	Repeatable Battery for the Assessment of Neuropsychological Status
rHu-sRAGE	recombinant sRAGE
ROS	Reactive oxygen species

SAA	Serum amyloid alpha
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cells
SPARC	Secreted protein acidic and rich in cysteine
sRAGE	Soluble receptor for advanced glycation end-products
Src	Sarcoma family of proteins
TACE	Tumor necrosis factor alpha converting enzyme
TGF-β1	Transforming growth factor beta 1
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular-cell adhesion molecule-1
WHR	Waist to hip ratio

Résumé

Les pathologies cardio-vasculaires (CVD) représentent la principale cause de morbidité et de mortalité dans le monde. Le risque de CVD augmente avec l'âge, le tabagisme, le diabète, les dyslipidémies, l'obésité et l'insuffisance rénale. L'incidence et la prévalence des CVD nécessitent le développement de stratégies de prévention et de traitement, et la recherche de nouveaux biomarqueurs. Le récepteur aux produits de glycation avancée (RAGE) est impliqué dans plusieurs pathologies métaboliques ou inflammatoires. L'activation du RAGE par ses multiples ligands, i.e. produits de glycation avancée (AGE), protéines de la famille S100 et amphotérine (HMGB1) induit une cascade pro-inflammatoire. La forme soluble du RAGE (sRAGE) a été proposée comme biomarqueur du risque vasculaire, de la sévérité et du devenir des CVD, particulièrement chez les patients diabétiques ou insuffisants rénaux. Cependant, les données sont contradictoires et des corrélations positives et négatives sont observées pour une même pathologie. L'importance de l'axe ligand-RAGE dans les processus pathologiques et le large éventail de molécules se liant au RAGE (des protéines proinflammatoires aux auto-anticorps), justifient le présent travail.

Au cours du présent travail, dans un premier temps, nous avons d'abord étudié les effets des ligands du RAGE et des auto-anticorps anti-sRAGE récemment décrit, sur la quantification du sRAGE en ELISA. Nous supposons que l'interaction entre le sRAGE et ces molécules pourrait perturber le dosage du sRAGE. Dans un deuxième travail, nous avons évalué les variations du taux de sRAGE et des auto-anticorps anti-sRAGE après chirurgie bariatrique d'une obésité morbide.

Les ligands du RAGE (Nε-carboxyméthyllysine, S100A6, S100A12, S100B, HMGB1 et peptide β-amyloïde) se fixent au sRAGE en différents sites et pourraient potentiellement interférer dans sa quantification par l'intermédiaire d'un masquage d'épitope. Nous avons incubé ces ligands, à des concentrations physiologiques et pathologiques, avec du sRAGE recombinant et du sérum pour évaluer leur effet sur le dosage du sRAGE. Des auto-anticorps anti-sRAGE ont été identifiés et purifiés et leur effet sur le dosage de sRAGE a été évalué. La présence des ligands ou d'auto-anticorps anti-sRAGE ne modifie pas le dosage du sRAGE recombinant ou sérique.

L'obésité favorise les dyslipidémies, les perturbations glycémiques et l'inflammation, conditions au cours desquelles le RAGE pourrait jouer un rôle important. Nous avons étudié les variations des taux sériques du sRAGE et de ses auto-anticorps et leur évolution avec l'amélioration métabolique des sujets obèses après chirurgie bariatrique. Les patients ont été sélectionnés au sein d'une cohorte déjà établie (Patient présentant une obésité morbide et candidat à une chirurgie de bypass gastrique, ABOS, Lille). Les patients présentant des facteurs pouvant modifier les niveaux de sRAGE, tels qu'un traitement par statines, une insuffisance rénale chronique ou une hypertension, ont été exclus. Comparé au groupe contrôle, les taux de sRAGE et d'auto-anticorps étaient significativement plus élevés chez les patients obèses avant la chirurgie. Parallèlement à la baisse de l'indice de masse corporelle, les taux de sRAGE et d'anti-sRAGE ont été significativement diminués un an après la chirurgie. La baisse d'anti-sRAGE a été corrélée à l'augmentation des taux de HDL.

Nous démontrons que les variations des taux de sRAGE constatées dans la littérature ne sont, à priori, pas dues à l'interaction des ligands du RAGE avec le sRAGE. D'autres hypothèses, comme la régulation de la formation et de la clairance du sRAGE, sont discutées. Nous avons, pour la première fois, démontré la présence d'auto-anticorps anti-sRAGE chez les patients obèses, et la diminution du taux de ces auto-anticorps après une chirurgie de bariatrique. Ces résultats suggèrent que l'obésité pourrait être responsable d'une réaction auto-immune contre le sRAGE. Par ailleurs, ces données sont en défaveur de l'utilisation du sRAGE comme biomarqueur mais suggèrent que les auto-anticorps anti-sRAGE pourraient être des bons candidats au suivi du risque métabolique et d'une auto-immunité anti-sRAGE.

Mots-clés : RAGE; sRAGE ; biomarqueur; autoimmunité

Abstract

Cardiovascular diseases (CVDs) are the leading cause of mortality and morbidity in the world. The risk of CVDs increases with age, tobacco, diabetes, dyslipidemia, obesity and kidney dysfunction. The incidence and prevalence of CVDs demand the development of efficient strategies for prevention and treatment, as well as new biomarkers. The receptor for advanced glycation end-products (RAGE) is implicated in several metabolic and inflammatory disorders. RAGE activation by its multiple ligands, i.e. advanced glycation end-products (AGEs), S100 proteins and amphoterin (HMGB1) induces pro-inflammatory events upon RAGE engagement. The soluble circulating form of RAGE (sRAGE) has been proposed as a biomarker of vascular risk, disease severity and outcome, especially in individuals with diabetes or kidney dysfunction. However, data is controversial since positive and negative correlations are observed for a same disease. The importance of the ligand-RAGE axis in pathological processes and the wide range of RAGE-binding molecules (from pro-inflammatory proteins to autoantibodies), appreciates the present study.

In this thesis, we first investigated effects of RAGE ligands and the recently described anti-sRAGE autoantibodies on sRAGE quantification. We hypothesized that interactions between sRAGE and these molecules could impair sRAGE quantification. On the second part, we evaluated the value of sRAGE and anti-sRAGE autoantibodies as biomarkers of metabolic improvement after bariatric surgery for morbid obesity. Patients were selected from the established cohort ABOS (Lille).

RAGE ligands (Nε-carboxymethyllysine, S100A6, S100A12, S100B, HMGB1 and amyloid beta peptide) bind sRAGE at different sites and could potentially impair its quantification through epitope masking. We tested this hypothesis by incubating these ligands, from physiological to pathological concentrations, with recombinant sRAGE and serum to evaluate their effects on sRAGE quantification. Anti-sRAGE autoantibodies were identified and further purified and their effects on sRAGE measurement evaluated. The presence of ligands or anti-sRAGE autoantibodies did not impair recombinant or serum sRAGE quantification.

Obesity is a condition of dyslipidemia, glycemia deregulation and inflammation where RAGE is believed to play an important role. We aimed then to investigate the levels of sRAGE and its autoantibodies according to metabolic improvement in obese subjects submitted to weight loss surgery. Patients were highly selected from a well established cohort (morbidly obese patients eligible for gastric bypass, ABOS, Lille). Patients under statins treatment, with kidney dysfunction or hypertension, factors that could affect sRAGE levels, were excluded. In obese patients, significant higher levels of sRAGE and anti-sRAGE autoantibodies were observed before weight-loss surgery. In parallel to body-mass Index, both sRAGE and anti-sRAGE titers were significantly decreased one year after surgery. The decrease in anti-sRAGE was correlated with the increase in HDL levels.

We demonstrate that the variations of sRAGE levels among the literature are, most likely, not due to an interaction between RAGE ligands and sRAGE. Other hypothesis like the regulation of sRAGE formation and clearance are further discussed. We have, for the first time demonstrated the presence of anti-sRAGE autoantibodies in obese subjects and that their levels decrease after bariatric surgery. Although our data suggest that morbid obese status may lead to an autoimmune reactions against sRAGE. Together, our findings argue against sRAGE as a good biomarker but suggest that anti-sRAGE autoantibodies may have a potential implication to evaluate metabolic risk and autoimmunity associated to RAGE.

Key-words: RAGE; sRAGE; biomarker; autoimmunity

Résumé détaillé

Introduction :

Le RAGE

Le récepteur aux produits de glycation avancée (RAGE, *Receptor for Advanced Glycation End-products*) est une protéine transmembranaire appartenant à la famille des immunoglobulines (Ig). Le fragment extra-cellulaire est constitué de trois domaines semblables aux Ig (*Ig-like*) : un domaine variable (V) et deux domaines constants (C1 et C2). Le RAGE est également composé d'un domaine transmembranaire et d'un domaine intracellulaire. Le gène du RAGE humain est situé dans le chromosome 6, locus 6p21.3, dans la région du complexe majeur d'histocompatibilité III (CMH III). Le RAGE est exprimé de manière constitutive par de nombreux types cellulaires incluant les cellules musculaires lisses, endothéliales, mésangiales, mésothéliales, hépatiques et neuronales... Dans le sang, le RAGE est exprimé par les monocytes/macrophages, les polynucléaires neutrophiles et les plaquettes. Le RAGE est particulièrement exprimé dans les poumons, les muscles squelettiques et le cœur.

Le rôle physiologique du RAGE demeure incertain. Le RAGE est très exprimé par l'embryon mais la diminution de son expression à la naissance suggère qu'il joue un rôle important au cours du développement embryonnaire. Cependant, les souris dont le gène du RAGE a été invalidé ne présentent aucune anomalie de développement, de fertilité ni de longévité. Les souris RAGE-KO ont malgré tout une hypersensibilité auditive et semblent plus agressives que les souris de phénotype sauvage.

Le RAGE a été initialement décrit comme récepteur aux produits de glycation avancée (AGE, *Advanced Glycation End-products*). L'interaction AGE-RAGE est un des mécanismes majeurs impliqués dans les complications vasculaires du diabète. Les globules rouges (GR) de patients diabétiques adhèrent à l'endothélium de manière augmentée par rapport aux GR de sujets sains. Cette adhérence, due à l'interaction des AGE membranaires du GR avec le RAGE endothélial, est suivie par l'induction d'un stress oxydant et l'activation du *Nuclear Factor kappa B* (NF-κB). L'adhérence et les voies de signalisation consécutives peuvent être bloquées par un

anticorps anti-RAGE. De plus, le RAGE est impliqué dans l'hyperperméabilité endothéliale au cours du diabète et dans la surexpression du *Vascular Cell Adhesion Molecule-1* (VCAM-1).

L'activation du RAGE endothélial contribue à l'apparition de la dysfonction endothéliale et de l'athérosclérose, mais le RAGE est également le chef d'orchestre d'une large réponse inflammatoire en raison de son expression par les cellules inflammatoires et de son grand répertoire de ligands. L'adhérence et la migration des leucocytes, étapes essentielles de la réponse inflammatoire et de l'athérosclérose, sont médiées par la liaison des intégrines avec des protéines membranaires. L'intégrine β2 lie le RAGE et potentialise la réponse inflammatoire induite par l'interaction entre le RAGE et la protéine S100B. L'activation du RAGE augmente l'activité du Facteur Tissulaire (FT) des macrophages et des cellules endothéliales via l'activation de la *Nicotinamide Adenine Dinucleotide PHosphate* (NADPH) *oxidase*. La production d'espèces réactives de l'oxygène (ROS, Reactive Oxygen Species) est un évènement majeur des atteintes cardiovasculaires et le RAGE est un médiateur de ce stress. Le RAGE perturbe également l'équilibre redox en diminuant l'expression de la glyoxalase 1 (Glo1).

La surexpression du RAGE chez les souris diabétiques accélère les complications rénales alors que le blocage du RAGE diminue l'albuminurie et la glomérulosclérose. Au cours du diabète, l'activation du RAGE par les AGE participe à la perte des péricytes et au développement de la rétinopathie. En plus de ses effets endothéliaux directs, le RAGE contribue aux pathologies vasculaires en affectant l'hémostase puisqu'il augmente l'expression du FT et du *Plasminogen Activator Inhibitor-1* (PAI1). Les AGE et le peptide β-amyloïde (Aβ), ligands du RAGE, peuvent activer les plaquettes, processus bloqué par l'incubation avec du RAGE soluble (sRAGE).

Bien que très étudié pour son rôle au cours du diabète et de l'athérosclérose, le RAGE est impliqué dans d'autres pathologies telles que la maladie d'Alzheimer, le cancer ou le syndrome métabolique.

L'activation du RAGE par ces ligands induit une réponse inflammatoire et pro-oxydante. La production de ROS, l'activation des *Mitogen Activated Protein Kinases* (MAPK) et la translocation nucléaire du NFκB ont été décrits dans de nombreux types cellulaires en réponse

à l'activation du RAGE. Cette activation passe par l'oligomérisation du RAGE. Une étude de la structure du RAGE aux rayons X a mis en évidence que la région VC1, chargée positivement et capable de lier les ions Zn^{2+}, participe à l'oligomérisation par les acides aminés His180, Glu182 et His158.

Le RAGE, liant une grande variété de ligands de structures différentes, est considéré comme un *Pattern Recognition Receptor* (PRR) capable d'interagir avec des structures tridimensionnelles plutôt que des séquences peptidiques spécifiques. Les différents ligands du RAGE se lient à des sites différents, suggérant diverses réponses cellulaires et la possibilité de liaison avec plusieurs ligands simultanément.

Ligands du RAGE

Les AGE sont des modifications stables des protéines, des lipoprotéines et de l'ADN, initialement décrits comme les produits de la réaction de Maillard dite réaction de brunissement des sucres. La réaction de Maillard consiste en la modification non-enzymatique des groupes amines dans les protéines par un sucre. Des intermédiaires de la réaction de Maillard ou des produits secondaires de la glycolyse, très réactifs, peuvent également générer des AGE. L'interaction des AGE avec le RAGE est exclusivement localisée dans le domaine V du RAGE. Cette interaction est due aux charges négatives des AGE liant les charges positives du RAGE. Parmi les AGE, la Nε-carboxyméthyllysine (CML) a été particulièrement étudiée. La CML est l'AGE ayant la plus forte affinité pour le RAGE. L'interaction AGE-RAGE induit un stress oxydant par la NADPH oxydase, active NF-κB, induit la sécrétion de cytokines telles que l'interleukine-6 (IL6) ou le *Tumor Necrosis Factor-α* (TNFα), et augmente l'expression des molécules d'adhérence et du *Vascular Endothelial Growth Factor* (VEGF).

La HMGB1, ou amphotérine C, est une protéine liant l'ADN. Elle participe à la réparation de l'ADN, sa réplication, sa recombinaison et sa transcription. La HMGB1 est activement sécrétée par les cellules immunocompétentes ou passivement par les cellules nécrotiques et apoptotiques. En liant le RAGE, la HMGB1 stimule la production d'IL6, d'*Intercellular Adhesion Molecule-1* (ICAM-1) et de *Transforming Growth Factor β1* (TGFβ1) et augmente la

perméabilité endothéliale. De plus, l'interaction HMGB1-RAGE stimule la prolifération des cellules tumorales pancréatiques, gastriques et gliales.

Les membres de la famille des S100/calgranulines sont des protéines de bas poids moléculaire possédant deux domaines *EF-hand*. Ils sont impliqués dans l'homéostasie calcique, la prolifération cellulaire et le métabolisme énergétique. Les protéines S100 lient différents domaines du RAGE et activent différentes voies de signalisation. Ainsi, les conséquences de l'activation par les protéines S100 vont de la différenciation neuronale, à l'apoptose, en passant par la sécrétion de cytokines inflammatoires.

Le clivage de la protéine précurseur de l'amyloïde (APP, *Amyloid Precursor Protein*) par la β-sécrétase génère le peptide β-amyloïde (Aβ) dont la taille varie entre 39 et 43 acides aminés. Les dépôts cérébraux d'Aβ sont caractéristiques de la maladie d'Alzheimer. Bien que l'APP soit essentiellement étudié en raison de la formation d'Aβ, son rôle physiologique a été en partie élucidé. L'APP intervient dans la formation et les fonctions des synapses, l'adhérence cellulaire et la maturation des neurones. L'Aβ, sous la forme de fibrilles ou d'agrégats, est un ligand du RAGE. Le RAGE est impliqué dans le transport de l'Aβ à travers la Barrière Hémato-Encéphalique (BHE). L'interaction Aβ-RAGE induit un stress oxydant et peut entraîner l'apoptose des neurones.

En plus de ces ligands, d'autres molécules ont été identifiées comme ligands du RAGE, démontrant sa fonction de PRR dans la réponse inflammatoire et l'infection bactérienne. Ainsi, la *Heat Shock Protein-70* (HSP70), les *Secreted Protein Acidic and Rich in Cysteine* (SPARC), les composants du complément C1q and C3a, le Lipopolysaccharide (LPS), la phosphatidylsérine et les oligonucléotides CpG ont été identifiés comme ligands du RAGE.

RAGE soluble

Des isoformes circulantes du RAGE, dont les domaines transmembranaire et intracellulaire sont tronqués, ont été identifiées. Ces isoformes, formées soit par épissage alternatif soit par clivage du RAGE, sont respectivement appelées *endogenous secretory* RAGE (esRAGE) et *cleaved* RAGE (cRAGE). Ces deux isoformes constituent le pool total de RAGE soluble (sRAGE)

circulant. Le sRAGE agit comme une protéine leurre pour les ligands du RAGE en bloquant l'activation du RAGE cellulaire. Le sRAGE est également capable de former des hétérodimères avec le RAGE membranaire.

Le sRAGE est dosé par technique ELISA dans de nombreuses pathologies afin d'évaluer son rôle biologique et son potentiel en tant que biomarqueur. Dans la majorité des pathologies dans lesquelles le sRAGE a été mesuré (diabète, insuffisance rénale, pathologies cardiovasculaires, maladie d'Alzheimer…), le RAGE est connu pour participer activement à leur développement. Le taux de sRAGE est ainsi interprété comme un reflet de l'expression du RAGE voire un mécanisme anti-RAGE. Les taux sanguins de sRAGE sont augmentés au cours de l'insuffisance rénale.

Controverse

La qualité de sRAGE en tant que biomarqueur est discutée en raison des résultats controversés présents dans la littérature pour une même pathologie. Ainsi, dans le diabète de type 2, des taux élevés ou diminués de sRAGE sont trouvés associés aux complications vasculaires de la maladie. Il nous semble donc hasardeux aujourd'hui de proposer le sRAGE comme biomarqueur du risque vasculaire compte tenu de nombreux biais, comme par exemple la variabilité des paramètres d'inclusion et d'exclusion des patients selon les études. Les facteurs tels que le traitement médicamenteux, l'activité physique ou l'activité des protéases peuvent influencer les taux de sRAGE.

Objectifs

Le sRAGE pouvant lier une grande diversité de ligands, leur effet sur le dosage du sRAGE demeure incertain. La présence d'autoanticorps anti-sRAGE circulants a été décrite notamment au cours la maladie d'Alzheimer (avec ou sans diabète) et de la polyarthrite rhumatoïde, pathologies au cours desquelles le RAGE est surexprimé.

Les objectifs de notre travail étaient :

1/ d'évaluer les effets des ligands du sRAGE et des autoanticorps anti-sRAGE sur le dosage du sRAGE (ELISA) afin d'évaluer le rôle de ces ligands sur les variations des taux de sRAGE observées dans la littérature (1er article) et,

2/ d'évaluer dans la cohorte ABOS (Lille) si les taux de sRAGE et d'autoanticorps anti-sRAGE sont modifiés au cours de l'obésité morbide et si leur taux diminue après chirurgie bariatrique (2e article).

1er article

Dans la première partie de cette thèse, les effets des ligands du sRAGE et des auto-anticorps anti-sRAGE sur le dosage du sRAGE ont été étudiés. Notre hypothèse ici était que leur liaison au sRAGE pouvait perturber le dosage par ELISA par un masquage d'épitope. Nous précisons que, dans la quasi-totalité des études publiées, le dosage du sRAGE est réalisé par technique ELISA à l'aide du Kit Quantikine® sRAGE humain commercialisé par la compagnie R&D Systems.

Nous avons incubé du sRAGE humain recombinant (625, 1250 and 2500 pg/ml) avec de la CML-HSA (10, 100 et 1000 µg/ml), de la S100A6 (10, 100 et 1000 ng/ml), de la S100A12 (10, 100 et 1000 ng/ml), de la S100B (10, 100 et 1000 ng/ml), de la HMGB1 (1, 10 et 100 ng/ml) ou de l'Aβ (1, 10 et 100 ng/ml) pendant une heure à température ambiante. L'incubation avec tous les ligands simultanément et à leur plus forte concentration a été réalisée. Les mêmes incubations ont également été réalisées avec du sérum de sujets sains. Les effets des autoanticorps anti-sRAGE ont été analysés après purification des IgG de patients hémodialysés ayant des taux élevés ou bas d'autoanticorps anti-sRAGE. Ces IgG (0,1 ; 0,5 et 1mg/ml) ont été incubées avec le sRAGE recombinant ou du sérum pendant une heure. Après incubations avec les ligands du sRAGE ou les anticorps anti-sRAGE, les dosages de sRAGE ont été réalisés par le kit ELISA (Quantikine®).

Aucun des ligands du RAGE, quelque soit leur concentration, n'a perturbé le dosage du sRAGE recombinant ou sérique. L'incubation simultanée de tous les ligands n'a pas modifié le dosage

du sRAGE. De façon similaire, les anticorps anti-sRAGE (IgG purifiées) n'ont pas perturbé le dosage du sRAGE.

Nos résultats démontrent donc que les divergences des taux de sRAGE comme biomarqueur du risque vasculaire retrouvées dans la littérature ne sont pas liées à un biais de dosage mais très probablement à d'autres facteurs tels que les critères d'inclusion et les mécanismes de production et de clairance du sRAGE.

2e article

Nos premiers résultats nous ont amenés à mesurer les taux de sRAGE et d'autoanticorps anti-sRAGE dans une cohorte aux critères d'inclusion bien définis. Nous avons ainsi eu accès à la cohorte ABOS composée de patients atteints d'obésité morbide et soumis à une chirurgie bariatrique. Notre objectif initial était d'évaluer l'association entre les taux de sRAGE, les taux en autoanticorps anti-sRAGE et l'obésité morbide et ses paramètres cliniques. A partir des 750 patients initiaux, et afin d'exclure tout biais (connu à ce jour) pouvant influencer les taux de sRAGE, nous avons sélectionné les patients ayant une filtration glomérulaire >90mL/min/1,73m² (MDRD), ne recevant pas de traitements anti-hypertenseurs (inhibiteurs de l'enzyme de conversion, antagonistes du récepteur à l'angiotensine 2) ni de statines et étant non-fumeurs. Après cette sélection, 254 patients demeuraient parmi lesquels 150 ont été choisis afin de former 3 groupes en fonction de leur statut glycémique: normoglycémiques (n=50), intolérants au glucose (n=50) ou diabétiques (n=50). 46 sujets sains ont composé le groupe contrôle (donneurs de sang).

Les taux de sRAGE et d'autoanticorps anti-sRAGE ont été mesurés chez les 150 patients juste avant chirurgie bariatrique et chez 46 sujets sains. La sélection des patients a été poursuivie selon la disponibilité du suivi à un an après la chirurgie bariatrique et selon le type de chirurgie. Seuls les patients ayant subi un *by-pass* gastrique ont été conservés. Les taux de sRAGE et d'autoanticorps anti-sRAGE ont été mesurés un an après chirurgie et comparés aux changements métaboliques ou inflammatoires.

Le jour de la chirurgie, les patients atteints d'obésité morbide avaient des taux de sRAGE et d'autoanticorps anti-sRAGE supérieurs aux sujets sains (1490±513 vs. 1141±458 pg/ml, p<0,0001 et 0,847±0,496 vs. 0,187±0,133 (absorbance à 405nm), p<0,0001, respectivement). Ni les taux de sRAGE, ni les taux d'autoanticorps anti-sRAGE ne différaient entre les patients normoglycémiques, intolérants au glucose et diabétiques. Un an après la chirurgie, les taux de sRAGE et d'autoanticorps anti-sRAGE ont significativement diminué. Les taux de sRAGE chez les patients obèses un an après chirurgie bariatrique ne différaient plus avec le groupe contrôle (1084±344 vs.1141±458 pg/ml) alors que les taux d'autoanticorps restaient supérieurs (0,663±0,460 vs. 0,187±0,133 (absorbance à 405nm), p<0,0001). La chirurgie bariatrique a induit une diminution de l'indice de masse corporelle (IMC) de 31,9% et a amélioré la pression sanguine, les taux de triglycérides, de glucose à jeun, d'insuline et d'hémoglobine glyquée. Cependant, aucun de ces paramètres n'étaient associés à la diminution des taux de sRAGE ou d'autoanticorps anti-sRAGE. Une corrélation est démontrée entre la diminution des taux d'autoanticorps et l'augmentation des taux de HDL-cholestérol (r=0,23, p=0,02).

Bien que nos résultats ne permettent pas d'expliquer le lien entre l'obésité et les taux de sRAGE ou d'autoanticorps anti-sRAGE, nous montrons que l'obésité morbide induit une augmentation des taux de ces deux molécules. Savoir si les taux élevés de sRAGE reflètent une surexpression du RAGE ou traduisent un mécanisme de protection reste à élucider. Néanmoins, l'obésité morbide active une auto-immunité dirigée contre le sRAGE. La corrélation entre les autoanticorps anti-sRAGE et le HDL-cholestérol indique que l'auto-immunité pourrait être liée au risque vasculaire. Des études futures devront être menées pour étudier le rôle physiopathologique des autoanticorps anti-sRAGE et comprendre la signification de leur taux dans les maladies humaines.

Discussion

Ce travail de thèse présente deux articles.

Dans le premier article, notre hypothèse était que les ligands du RAGE, en raison de leur variabilité structurale et de l'hétérogénéité de leur site de liaison, pouvaient altérer le dosage du

sRAGE. Cette hypothèse, si elle était vraie, impliquerait que les taux de sRAGE dans la littérature refléteraient les taux de sRAGE libre (non complexé aux ligands). En incubant le sRAGE avec différentes concentrations de ligands, nous n'avons pas observé de variations de mesure du sRAGE. Dans un deuxième temps, la mise en évidence récente d'autoanticorps anti-sRAGE nous a amené à tester leur effet sur le dosage du sRAGE (les autoanticorps pouvant posséder une affinité pour le RAGE supérieures à celle des ligands). Les autoanticorps anti-sRAGE purifiés n'ont pas modifié le dosage du sRAGE, comme montré dans la première partie de cette thèse.

Dans le deuxième article, nous nous sommes intéressés au sRAGE et aux autoanticorps anti-sRAGE dans la cohorte ABOS (Atlas biologique d'Obésité Sévère) qui inclue des patients éligibles pour une chirurgie bariatrique. Les nombreuses hypothèses concernant les variations des taux de sRAGE nous ont amenés à étudier les taux de sRAGE à partir d'une cohorte nous permettant d'éliminer les facteurs connus comme potentiellement capables de modifier les taux de sRAGE. Par conséquent, nous avons décidé d'étudier les taux de sRAGE et également des autoanticorps anti-sRAGE dans cette cohorte ABOS. Les taux de base (avant chirurgie) du sRAGE étaient plus élevés chez les patients obèses que dans le groupe contrôle. Nous n'avons pas observé de différence entre les patients normoglycemiques, intolérants au glucose et diabétiques. Un an après chirurgie bariatrique, les taux de sRAGE sont normalisés alors que les taux d'autoanticorps anti-sRAGE ont significativement diminué mais restent positifs.

Des nombreuses publications ont étudié les taux de sRAGE au cours de différentes pathologies. Malgré les nombreux travaux ayant étudié sRAGE comme un biomarqueur notamment du risque vasculaire, les taux de sRAGE et leurs associations trouvés dans la littérature sont controversés. Les taux de sRAGE ont été associés chez l'homme aux taux d'AGE circulant, à l'insuffisance rénale et à l'utilisation de statines. Parmi ces facteurs, l'insuffisance rénale est la moins controversée (avec des taux de sRAGE plus élevés) et les taux de sRAGE les plus bas sont associés à une plus forte incidence de maladies cardiovasculaires. Il n'y a à ce jour pas d'explications sur la raison de l'augmentation des taux

de sRAGE chez les patients ayant une atteinte rénale. Cette augmentation pourrait être due à (i) une réponse coordonnée contre le stress oxydant et l'inflammation, (ii) une clairance altérée ou (iii) une conséquence d'une surexpression du RAGE dans les tissus lésés. De plus, d'autres paramètres tels que les polymorphismes ou l'ethnicité sont rarement pris en compte même s'ils sont connus pour influencer les taux de sRAGE. Il en est de même de la prescription des médicaments pouvant influencer les taux de sRAGE. Les inhibiteurs de l'enzyme de conversion, les antagonistes des récepteurs à l'angiotensine 2 et les statines, souvent prescrits chez les patients dits « vasculaires », font rarement partie des critères d'exclusion ou sont rarement référencés dans les études.

Par ailleurs, en plus de ces facteurs mentionnés ci-dessus, d'autres hypothèses pour expliquer la variation des niveaux de sRAGE pourraient être évoquées comme la nécrose cellulaire et la séquestration par les ligands du RAGE ou du RAGE lui-même. Dans le cas de la nécrose, le RAGE membranaire serait libéré, aussi bien que l'esRAGE intracellulaire. Il semble cependant peu probable qu'une nécrose (endothéliale) soit suffisamment importante pour être capable de modifier considérablement les taux systémiques de sRAGE. Dans le cas du sRAGE séquestré, on a montré que le sRAGE forme des dimères avec le RAGE de membrane, un mécanisme par lequel le sRAGE exercerait ses effets bénéfiques. Ici également, il faudrait une expression de RAGE membranaire suffisamment importante pour lier le sRAGE circulant afin d'en affecter ses taux.

Nos résultats concernant le sRAGE chez l'obèse sont contradictoires avec ceux obtenus dans deux autres études. Les taux de sRAGE étaient alors inversement corrélés à l'IMC et diminués chez les patients souffrant d'obésité morbide. De plus, les taux de sRAGE augmentaient deux ans après la chirurgie. Puisque toutes ces études ont utilisé la même technique de dosage du sRAGE, on peut supposer que les différences observées étaient donc dues aux critères d'inclusion et d'exclusion. La controverse entre ces études et la notre pourrait résider dans la régulation à long terme du sRAGE en parallèle avec la perte de poids et l'amélioration métabolique. La perte maximale de poids est observée deux ans après la chirurgie, quelque

soit le type de procédure, et l'amélioration de la résistance à l'insuline survient un an après la chirurgie.

Comme le sRAGE, les taux d'autoanticorps anti-sRAGE étaient augmentés chez les patients obèses, quelque soit le statut glycémique. De plus, la chirurgie a réduit significativement ces taux, mêmes s'ils restaient supérieurs au groupe contrôle. Malgré l'absence de corrélation entre les taux d'autoanticorps anti-sRAGE et l'IMC ou la perte de poids, nous avons observé une faible corrélation entre la diminution des taux d'autoanticorps et l'augmentation du HDL-cholestérol (r^2=0,077, p=0,02). Cette association ne présume pas d'une relation cause-conséquence entre ces deux paramètres, mais pourrait impliquer une association entre les taux d'autoanticorps anti-sRAGE et le risque vasculaire chez les sujets obèses.

Les données biologiques disponibles pour notre étude ne nous permettent pas d'autres spéculations concernant le rôle des autoanticorps anti-sRAGE au cours de l'obésité morbide. Il est possible d'émettre l'hypothèse que le RAGE est surexprimé dans les cellules endothéliales et les adipocytes, sans faire la preuve du rôle de ces types cellulaires dans l'augmentation des taux de sRAGE et d'autoanticorps anti-sRAGE. D'autres marqueurs de dysfonction endothéliale tels que VCAM-1 soluble, l'endothéline et les cytokines inflammatoires (IL1, IL6, IL8) pourraient être dosés afin de mieux comprendre le rôle de l'autoimmunité dirigée contre le sRAGE. Les modèles d'obésité chez la souris invalidée pour le RAGE permettraient d'élucider en partie ces questions.

Dans les études précédentes, les autoanticorps anti-sRAGE ont été mesurés seulement dans la polyarthrite rhumatoïde (PR) et chez les patients atteints de maladie d'Alzheimer (AD), avec dans les deux cas une augmentation par rapport aux groupes contrôles. Chez les patients atteints de PR, les autoanticorps anti-sRAGE ont été observés dans le sérum et également dans le liquide synovial : des niveaux plus élevés d'autoanticorps anti-sRAGE dans le liquide synovial étaient associés à des formes moins érosives de la maladie. Au cours de la maladie d'Alzheimer, le taux des autoanticorps anti-sRAGE sont associés au niveau de démence. Un rôle protecteur des autoanticorps anti-sRAGE a été évoqué avec le développement d'un vaccin composé d'un complexe sRAGE/Aβ, qui augmente la production d'autoanticorps anti-sRAGE.

Les autoanticorps anti-sRAGE vaccin-induits accroissent la viabilité neuronale après exposition à l'Aβ et améliorent les fonctions cognitives chez la souris. Concernant l'auto-immunité chez l'obèse, d'autres études ont démontré l'apparition d'une auto-immunité anti-thyroïde, anti-sperme et anti-cellules β pancréatiques chez les sujets obèses. Néanmoins, la pertinence biologique et clinique de l'auto-immunité liée à l'obésité peut être sous-estimée puisque l'amélioration de la maladie est évaluée par la perte de poids et les changements métaboliques. En effet, des anticorps contre la thyroglobuline, l'hormone stimulant de la thyroïde et l'ADAMTS13 sont augmentés chez les sujets obèses. Cependant, à notre connaissance, il n'y a aucune étude analysant l'incidence des autoanticorps avant et après la chirurgie bariatrique. Nos résultats sont, ainsi, les premiers à montrer une diminution de l'auto-immunité liée à la perte de poids, en dépit du manque de corrélation directe entre les autoanticorps et le BMI ou la perte de poids.

Limitations

Au cours de nos travaux, nous avons mesuré les taux de sRAGE par une technique ELISA qui quantifie aussi bien l'esRAGE que le cRAGE (sRAGE total). En ce qui concerne les sites d'interaction, l'esRAGE et le cRAGE partagent les mêmes domaines de liaison aux ligands et à ce jour, aucun ligand ne semble être en mesure d'interagir avec la séquence spécifique de l'esRAGE. Bien que nos résultats rejettent l'hypothèse initiale (masquage des épitopes par les ligands), la méthodologie testée dans notre étude (ELISA) reste simple pour élucider la question. Une autre limite de notre étude est qu'elle ne fournit pas d'informations supplémentaires quant aux effets des ligands et des autoanticorps anti-sRAGE sur les taux de sRAGE. En effet, ils pourraient augmenter ou diminuer la production ou la clairance de l'esRAGE ou du cRAGE.

Dans la deuxième partie de la thèse, bien que nous ayons choisi notre population d'étude afin éviter les facteurs qui pourraient influencer les taux de sRAGE (c.-à-d. insuffisance rénale, tabagisme, hypertension et médicaments), nous n'avons aucune donnée au sujet de la fréquence des polymorphismes du RAGE parmi les patients. Par ailleurs, nous n'avons pas

étudié l'activité les enzymes responsables pour la formation du cRAGE (ADAM10, MMP-3, MMP-9 et MMP-13) ce qui pourrait fournir des informations utiles concernant les mécanismes régulant la formation de sRAGE. Cependant, une telle recherche exigerait un grand criblage pour évaluer les activités de ces enzymes dans différents tissus. Des approches semblables devraient être encouragées dans d'autres pathologies afin d'élucider les spécificités de chaque pathologie. De plus, nous avons seulement un suivi à un an. L'évolution des taux de sRAGE et des autoanticorps anti-sRAGE devrait être étudiée à plus long terme afin d'analyser leur corrélation avec les changements métaboliques et inflammatoires qui suivent la chirurgie bariatrique.

Conclusions

Nous avons démontré au cours de ce travail que la présence des ligands du RAGE et les autoanticorps anti-sRAGE ne perturbe pas le dosage du sRAGE, suggérant que les variations observées dans les publications ne sont pas liées à une mauvaise fiabilité du dosage. Ces variations dans la littérature pourraient être dues à des différences d'inclusion et d'exclusion des patients et des connaissances limitées des facteurs influençant les taux de sRAGE.

Nous avons aussi démontré que les patients atteints d'obésité morbide ont des taux élevés de sRAGE et d'autoanticorps anti-sRAGE. Dans la cohorte de patients obèses, les taux de ces molécules diminuaient un an après la chirurgie bariatrique. La diminution des autoanticorps anti-sRAGE était corrélée avec l'augmentation du HDL-cholestérol. Ces découvertes montrent une réaction auto-immune dirigée contre le sRAGE au cours de l'obésité et suggèreraient que cette auto-immunité est associée au risque vasculaire.

Chapter One

RAGE and its soluble forms in human diseases

Inflammatory processes favor the formation of modified proteins and lipids, as wells as the release of cytokines, which may interact with the receptor of advanced glycation end-products, RAGE. These interactions take part in deleterious processes that link inflammatory bursts and metabolic disorders to organ dysfunction. Therefore, the regulation of these mechanisms and counteracting molecules represent key therapeutic targets. The biological aspects of RAGE, its binding molecules and soluble forms are discussed in chapter one.

1.1 The Receptor of Advanced Glycation End-products

The RAGE is a transmembrane protein, member of the immunoglobulin (Ig) superfamily of receptors [1]. The extracellular portion is constituted of 3 Ig-like domains: 1 variable (V) and 2 constant (C1 and C2). These domains are followed by a single transmembrane domain and a short cytoplasmic tail [2]. The RAGE gene is localized in the major histocompatibility complex (MHC) class III locus of chromosome 6p21.3 [3]. The MHC is a group of genes involved in the immune response and graft rejection. The class III locus encodes cytokines like the tumor necrosis factor alpha (TNF-α), heat shock proteins (HSPs) and components of the complement [4]. RAGE is expressed constitutively in several human cells like smooth muscle, endothelial, mesangial, hepatic and neuronal cells. In the circulation, RAGE is found in macrophages, neutrophils and platelets [5-7]. Among tissues, RAGE is most abundant in the lungs, skeletal muscle and heart [7].

1.1.1 Pathophysiological roles of RAGE

RAGE was first described as a binding protein for advanced glycation end-products (AGEs) and AGE/RAGE interaction is one of the major mechanisms that contribute to diabetes complications. Erythrocytes from diabetic patients present higher adhesion to endothelial cells than those from healthy subjects and this adhesion is associated with vascular complications [8]. Wautier *et al.* demonstrated that this adhesion increases oxidative stress and activates nuclear factor kappa B (Nf-κB). Both adhesion and further responses are prevented by anti-RAGE antibody [9]. Moreover, RAGE mediates diabetes-associated vascular hyperpermeability [10] and the expression of vascular adhesion molecule-1 (VCAM-1) [11].

RAGE activation in endothelial cells, especially by AGEs, contributes to the development of endothelial dysfunction and atherosclerosis, but RAGE orchestrates a more extensive inflammatory response due to its presence in inflammatory cells and its large ligand repertoire. The adhesion and migration of leukocytes, a major step in vascular inflammation and atherosclerosis, depends on the interaction between integrins and specific cell-surface proteins. The β2-integrin Mac-1 binds RAGE and enhances the proinflammatory responses that follow RAGE/S100B interaction [12]. In endothelial cells and macrophages, AGEs bind RAGE and increase tissue factor activity. In macrophages this higher activity requires activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [13]. Generation of reactive oxygen species (ROS) is a major culprit in cardiovascular complications [14] and RAGE is a key mediator of ROS production. Its activation by AGEs increases cytosolic and mitochondrial ROS production [15], as well as the expression of heme-oxigenase [16]. RAGE also compromises the cellular redox state by downregulating glyoxalase 1 (Glo1) [17], a key enzyme that participates in methylglyoxal detoxification, recycling the major cellular antioxidant, glutathione [18].

RAGE overexpression in diabetic mice accelerates renal impairment [19] while RAGE blockade decreases albuminuria and glomerulosclerosis [20]. RAGE is further implicated in diabetic nephropathy because it is overexpressed in uremic patients and its activation by AGEs, found in peritoneal dialysis fluid, increases the expression of VEGF and VCAM-1 [21-23]. RAGE mediates AGE-toxicity to pericytes, contributing to pericyte loss and the development of retinopathy [24, 25]. Besides vascular damage and dysfunction, RAGE contributes to CVDs by affecting hemostasis. Its activation induces an increase in tissue factor activity and the expression of plasminogen activator inhibitor-1 in endothelial cells [13, 26]. A study showed that amyloid β (Aβ) and AGEs, both RAGE ligands, activate platelets in a process that is inhibited by a soluble form of RAGE (sRAGE) [27]. Nonetheless, the specific participation of RAGE remains to be elucidated, since AGEs and Aβ interact with other receptors.

Ramasamy *et al.* proposed RAGE activation in diabetes as a self-perpetuating axis, where AGE-RAGE engagement triggers inflammation and expression of adhesion molecules. The subsequent recruitment and activation of immune cells increases other RAGE ligands that perpetuate the cycle and lead to vascular perturbation (Figure 1) [28].

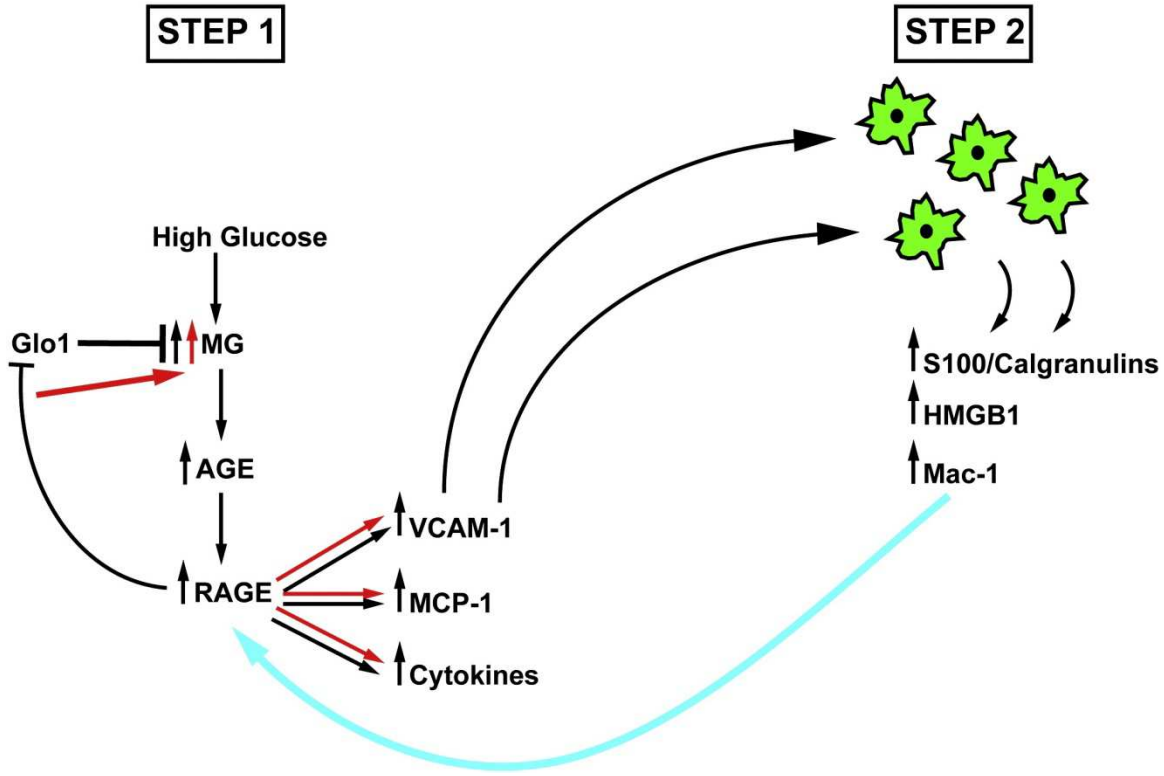

Figure 1. RAGE mediates a perpetual axis of inflammation. First engagement of RAGE by AGEs induces inflammation and recruitment of immune cells. Other RAGE ligands are produced and increase inflammation. MG, methylglyoxal; Glo1, glyoxalase 1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; HMGB1, high-mobility group box protein-1; Mac-1, macrophage-1 antigen [28].

The pathological role of RAGE was further evidenced by its overexpression in the course of many different diseases. This feature was observed in podocytes [29] and vasa vasorum [30] of diabetic patients. In animal models of the same disease, RAGE was found increased in bones [31], kidney [32] and endothelium [33]. Moreover, RAGE is overexpressed in atherosclerotic plaques of diabetic patients, coinciding with activated Nf-κB and ciclooxygenase-2 [34]. RAGE is, with no doubt, a key mediator of diabetes-associated chronic inflammation.

Although RAGE has been most studied for its role in diabetes complications and atherosclerosis, several studies implicate RAGE in other diseases. RAGE mediates the transport of the amyloid beta peptide (Aβ) across the blood-brain barrier (BBB) [35] and

disrupts BBB's thigh junctions [36]. Moreover, Aβ/RAGE engagement in brain endothelial cells increase monocyte adhesion and migration [37]. RAGE is normally expressed in brain endothelial cells, microglia and neurons [38, 39] but it is found overexpressed in neurons and astrocytes of Alzheimer's disease (AD) patients [40].

Carcinogenesis and tumor proliferation may be mediated by RAGE according to the stimuli and cell type. RAGE ligands like S100 proteins and HMGB1 are often overexpressed in tumors and cancer's increased glucose metabolism favors the generation of AGEs. HMGB1 expression is also associated with tumor invasion and metastasis, being co-expressed with RAGE in pancreatic, prostate and colon cancer [41]. S100A6 is overexpressed in breast cancer and colorectal carcinoma, while S100B is overexpressed in melanoma [41]. Also in melanoma, AGE/RAGE interaction induces proliferation and invasion *in vitro* while anti-RAGE therapy improves survival and reduces metastasis *in vivo* [42]. Blockade of RAGE with sRAGE or by genetic deletion reduces the incidence of hepatic tumor in mice [43, 44], while inhibition of RAGE activation decreases cell proliferation and invasion in fibrosarcoma and breast cancer cell lines [45, 46].

RAGE is also believed to play a critical role in metabolic syndrome by mediating adipocyte hypertrophy and insulin resistance [47]. The AGE/RAGE axis activates c-Jun N-terminal kinase (JNK) [48] which plays a crucial role in obesity and insulin resistance by phosphorylating IRS-1 and thus, preventing insulin downstream events [49]. In addition, it was observed that RAGE -/- mice fed an atherogenic diet develop less atherosclerotic plaques and present an attenuated increase of body fat [50].

A lot of evidence comes from studies that apply RAGE knockout (-/-) mice in disease models, corroborating a role for RAGE in the development of sustained inflammation. Table 1 summarizes the effects of RAGE deletion in some disease models.

Table 1. Experimental models using RAGE - /- mice.

Model	References	Findings
Diabetes	[51-53]	RAGE mediates loss of pain perception, activation of NF-κB, atherosclerosis development and pancreatic β cells apoptosis.
Pulmonary fibrosis	[54-56]	Controversial results showing protection and worsening associated with RAGE knockout, although they use 2 different models of fibrosis.
Glomerulosclerosis	[57]	Albuminuria and tubule formation in antibiotic-induced glomerulosclerosis are mediated by RAGE
Sepsis	[58-61]	In the cecal ligation and puncture model, RAGE knockout mice present improved survival and decreased inflammatory cell recruitment. In *Escherichia coli* infection, RAGE has beneficial effects, reducing bacterial dissemination.

The physiological roles of RAGE are still unclear. The constitutive expression in embryonic development and its postnatal downregulation suggest a role for RAGE signaling in development, although mice that do not express RAGE show no developmental or fertility disturbances [7, 62]. Nonetheless, RAGE knockout mice present hyperactivity and higher sensitivity to auditory stimuli than wild-type animals [63]. It is believed that RAGE plays a role in lung homeostasis because of its high expression in that tissue, although the precise importance mechanisms mediated by RAGE in the lung are unknown. RAGE is also important for neuronal development. The interaction between RAGE and amphoterin induces neuronal differentiation and neurite outgrowth [64, 65]. However, there is no evidence of lung or brain malformation in RAGE knockout mice.

As a member of the immunoglobulin superfamily, RAGE has a putative role in the immune response. Indeed, it participates in the macrophage uptake of apoptotic cells, by

interaction with phosphatidylserine (PS) [66]; and immune response through activation by lipopolysaccharide (LPS) [61]. Moreover, RAGE binds the C3a and C1q members of the complement, further implicating RAGE in the immune system [67, 68]. Again, there is no strong evidence showing that the absence of RAGE is immuno-compromising.

As reviewed by Sorci and colleagues, the role of RAGE in chronic inflammation and epithelial cancer is undisputed but there is growing evidence supporting physiological roles for RAGE in a cell-specific fashion [62]. Besides cell-specificity, the concentration of ligands seems to be crucial in determining the downstream pathways that follow RAGE activation. In neuroblastoma cells, low doses of S100B protect against RAGE-mediated amyloid beta toxicity, while higher doses enhance the deleterious effects [69].

While largely accepted as a propagator of inflammation, recent evidence points to an acute anti-inflammatory action of RAGE. In a murine model of tuberculosis, RAGE knockout mice present higher lung inflammation and enhanced mortality [70]. In *Escherichia coli* sepsis, RAGE attenuates inflammatory and pro-coagulant responses [58]. In *Aspergillus fumigates* infection, Toll-like receptor (TLR) 2 activation results in the release of S100B, which paracrinally interacts with RAGE, leading to TLR-2 inhibition and restraint of fungus-induced inflammation [71]. Noteworthy, the anti-inflammatory effect occurs at nanomolar concentrations of S100B. Higher doses enhance inflammation in a RAGE-dependent manner, underscoring the importance of ligand concentration on RAGE biological effects [71]. Interestingly, RAGE is detrimental in pneumococcal and influenza A pneumonia [72, 73], suggesting different roles of RAGE in pathogen-induced inflammation, perhaps depending on the identity and/or concentration of RAGE ligands, the intervening leukocyte population and the amount of expressed RAGE [62].

A role for RAGE in tissue repair has also become evident. Particularly, muscle satellite cells rely on RAGE for their homeostasis. After acute injury, muscle satellite cells have an increase in RAGE expression and its activation by HMGB1 and S100B mediates muscle

regeneration [74, 75]. Secondly, muscle regeneration of RAGE knockout mice was delayed in comparison to wild type animals [74]. It seems however that in the absence of RAGE (knockout mice) other molecules mediate these beneficial effects credited to RAGE [62].

1.2 RAGE downstream signaling

Engagement of RAGE by its ligands is often reported as proinflammatory and pro-oxidative. Increased formation of ROS [15, 76] and activation of mitogen-activated protein kinase (MAPK) signaling cascades, culminating in the translocation of the transcription factor nuclear factor kappa-B (NF-κB) [11, 77, 78] have been documented in different cell types after RAGE activation. RAGE has two putative N-glycosylation sites [2] and the binding of N-glycans to RAGE increases affinity for AGEs [79] and HMGB1 [80]. Furthermore, evidence shows that RAGE is subject to oligomerization in the cell membrane, a process that would be mandatory for RAGE signaling. It was first proposed that RAGE assembly was mediated by the C1-domain [81] but it was later demonstrated by X-ray crystal structure that the VC1 region participates in RAGE oligomerization through His180, Glu182 and His158, with several charged residues and hydrogen bonds with a Zn^{2+} ion at the interface [82]. Later, Xu and colleagues showed that for RAGE oligomerization to occur, heparan sulfate is needed, corroborating their previous study showing that the proteoglycan is mandatory for HMGB1/RAGE signaling [83, 84]. These studies, together with the one from Park *et al*.[85] elucidated the basis of ligand-binding to RAGE, showing that it occurs mainly by the interaction of positive patches on the receptor surface with negatively charged ligands.

RAGE lacks tyrosine kinase activity in its cytoplasmic tail, presupposing the need of adaptor proteins. Indeed, the cytoplasmic portion of RAGE was found associated to Diaphanous-1 (Dia-1) and mediates RAGE/ligand-induced cell migration [86]. Dia-1 is a

formin that regulates endocytosis and modulates the cytoskeleton [87]. By interacting with RAGE, Dia-1 promotes activation of Ras-related C3 botulinum toxin substrate-1 (Rac-1) which in turn activates NADPH oxidase [88]. NADPH oxidase activation has been described as a key step in RAGE signaling [13, 33, 89] (Figure 2A). The recruitment of the sarcoma family of proteins (Src) is necessary for tyrosine kinase activity and downstream signaling [90].

Sakaguchi and colleagues observed that RAGE is phosphorylated at serine 391 by protein kinase C_ζ after ligand-binding, in a kidney cell line and primary endothelial cells. Phosphorylation at Ser391 promotes the binding of the myeloid differentiation primary response gene (88) (MyD88) and toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) to the intracellular domain of RAGE and activates p38, JNK, kappa factor inhibitor kinase (IKK) and NF-κB [91] (Figure 2B).

Downstream signaling to RAGE was recently reviewed by Xie and colleagues [92].The RAGE panorama is a complex network of pathways that may or may not cross-talk, in a cell-specific manner. As presented by the authors, the outcome of RAGE activation may vary among inflammation, apoptosis, autophagy, proliferation, cell mobility and microtubule stabilization [92]. The proinflammatory response of RAGE depends on different MAPK pathways, according to cell type and ligand.

The idea of RAGE-mediated perpetual inflammation is evidenced by the positive feedback on its expression [93]. However, the regulation of RAGE expression seems to be cell and stimuli-specific. In A549 lung cancer cells, retinol-induced oxidative stress activates Nf- κB through p38 MAPK, leading RAGE downregulation [94] .

In 2013, RAGE was found in the mitochondria of pancreatic tumor cells, mediating the increase in mitochondrial function elicited by HMGB1 [95]. In this study, RAGE phosphorylation at serine 377 was mandatory for RAGE mitochondrial location. Since RAGE activation is associated with tumor growth and increased ROS production, one

might infer that other RAGE ligands may regulate the presence of RAGE in the mitochondria and intracellular RAGE-ligand interaction should have a great impact on cell biology. It remains, nevertheless, to be further investigated.

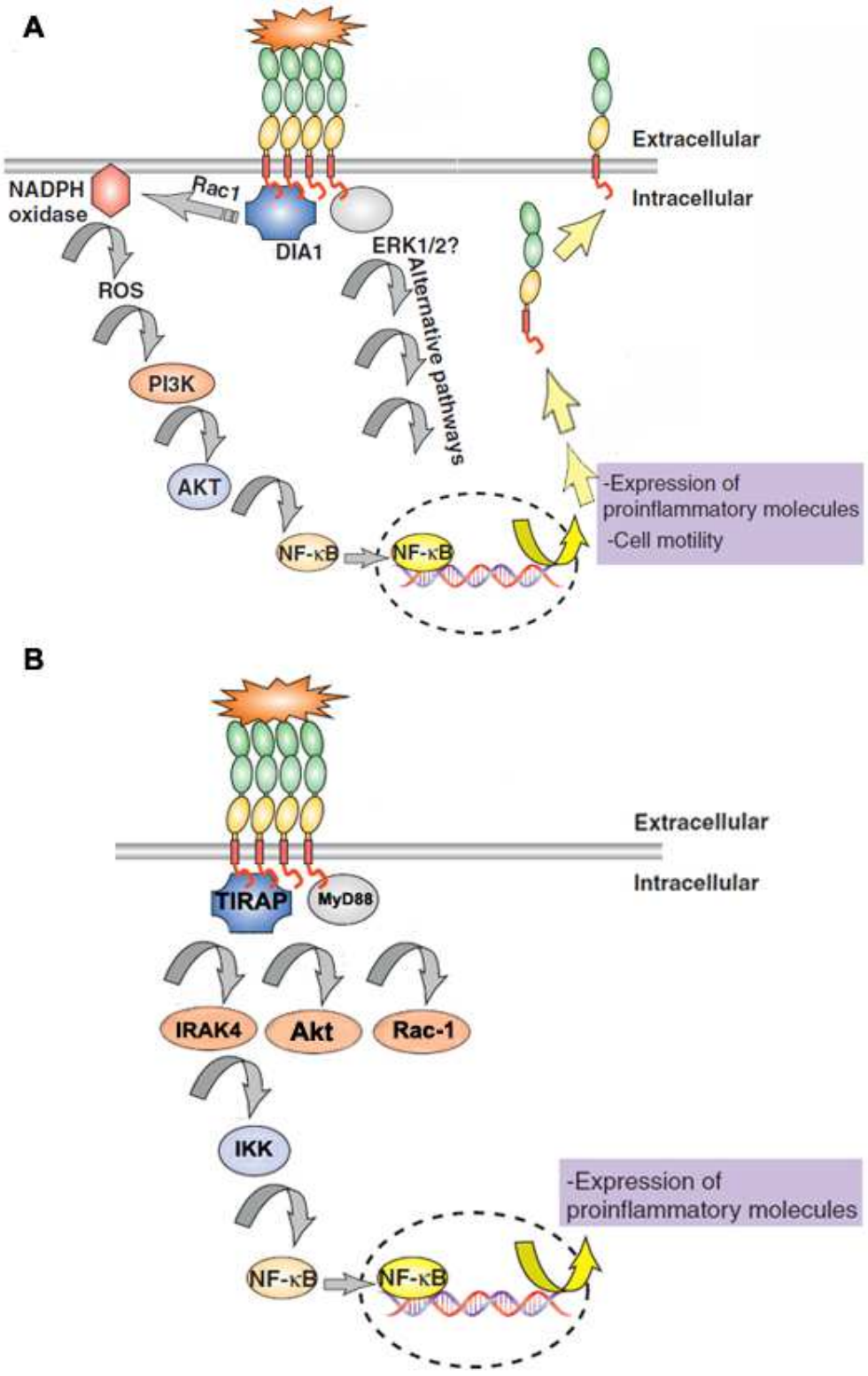

Figure 2. RAGE signaling pathways. In the cytosol, RAGE may be associated with Dia-1, TIRAP or MyD88, which will mediate downstream events that usually translocate NF-κB to the nucleus. TIRAP, toll-interleukin 1 receptor domain containing adaptor protein; Rac-1, Ras-related C3 botulinum toxin substrate-1; Dia-1, diaphanous-1; Akt, protein kinase B; PI3K, phosphatidylinositide 3-kinase; IKK, kappa factor inhibitor kinase; IRAK4, interleukin-1 receptor-associated kinase 4. Adapted from Fritz *et al*. [96]

1.3 RAGE ligands

RAGE binds a variety of ligands that are very different structurally. It has been proposed then that RAGE is a pattern-recognition receptor (PRR) that interacts with three-dimensional structures rather than specific amino acid sequences [97]. Interestingly, different ligands bind RAGE at different sites [98] (Figure 3), suggesting a diversity of cellular responses and the possibility of simultaneous binding.

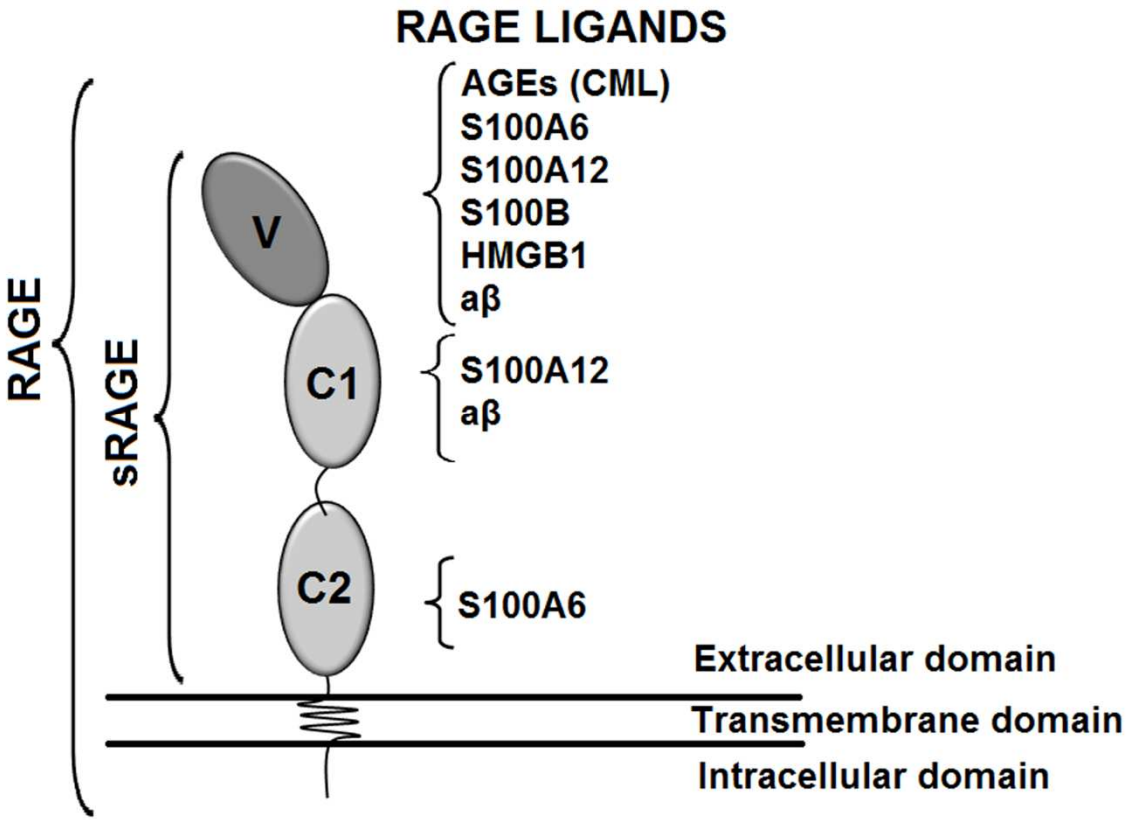

Figure 3. Different RAGE ligands bind RAGE at different sites [98].

1.3.1 Advanced Glycation End-products

Chronic hyperglycemia, a key characteristic of diabetes, is the root cause of diabetes-associated complications (i.e. vasculopathy, nephropathy and neuropathy). Glucose exerts its toxicity through 4 major pathways: the polyol pathway, the hexosamine pathway, the protein kinase C (PKC) pathway and the advanced glycation end-products (AGEs) pathway (Figure 4) [99]. As reviewed by Brownlee, these pathway share the formation of the superoxide anion radical as common link, which inhibits glycolysis and leads to an accumulation of its intermediates and glucose itself [99]. The influx of glucose through the polyol pathway increases the activity aldose reductase and further decreases the cytosolic pool of NADPH. The reduction of oxidized to reduced glutathione needs NADPH as a cofactor and a decrease in this reaction depletes cells of their major antioxidant [100]. The hexosamine pathway is another diversion pathway from glycolysis where fructose-6-phosphate is converted to glucosamine-6-phosphate, which forms UDP-N-acetylglycosamine. N-acetylglycosamine itself may modify proteins such as the endothelial nitric oxide synthase [101]. Hyperglycemia increases diacylglycerol content, which furthers activate the PKC pathway. PKC β and δ isoforms induce the expression of growth factors, activate Nf-κB and decrease nitric oxide synthase activity [99].

AGEs are stable structural modifications in molecules, first described as the non-enzymatic browning or Maillard reaction [102]. The fundament of the Maillard reaction is the non-enzymatic modification of amino groups of proteins and peptides by sugars. In the reaction, the carbonyl group of a reducing sugar forms a Schiff base (group with a carbon-nitrogen double bond, with the latter bound to an alkyl or aryl group) with an amino structure of the biomolecules. The base may undergo further rearrangements to form an Amadori product. The Amadori structure undergoes irreversible cycles of condensations, dehydrations and oxidations to form AGEs [103]. Some AGEs are fluorescent and brown in color. Moreover, intermediates of the glycation reaction and glycolysis may generate reactive molecules that engage further reactions and AGEs formation. Glucose may

oxidize in the presence of transition metals, generating glyoxal and arabinose, while Amadori products decompose to form 3-deoxyglucosone [104]. Methylglyoxal, one of the most relevant glycation agents *in vivo*, derives from triose phosphates [105]. Glycolaldehyde, another reactive dicarbonyl, is also formed through the oxidation of serine by myeloperoxidase [106]. Figure 5 presents a general mechanism of AGE formation.

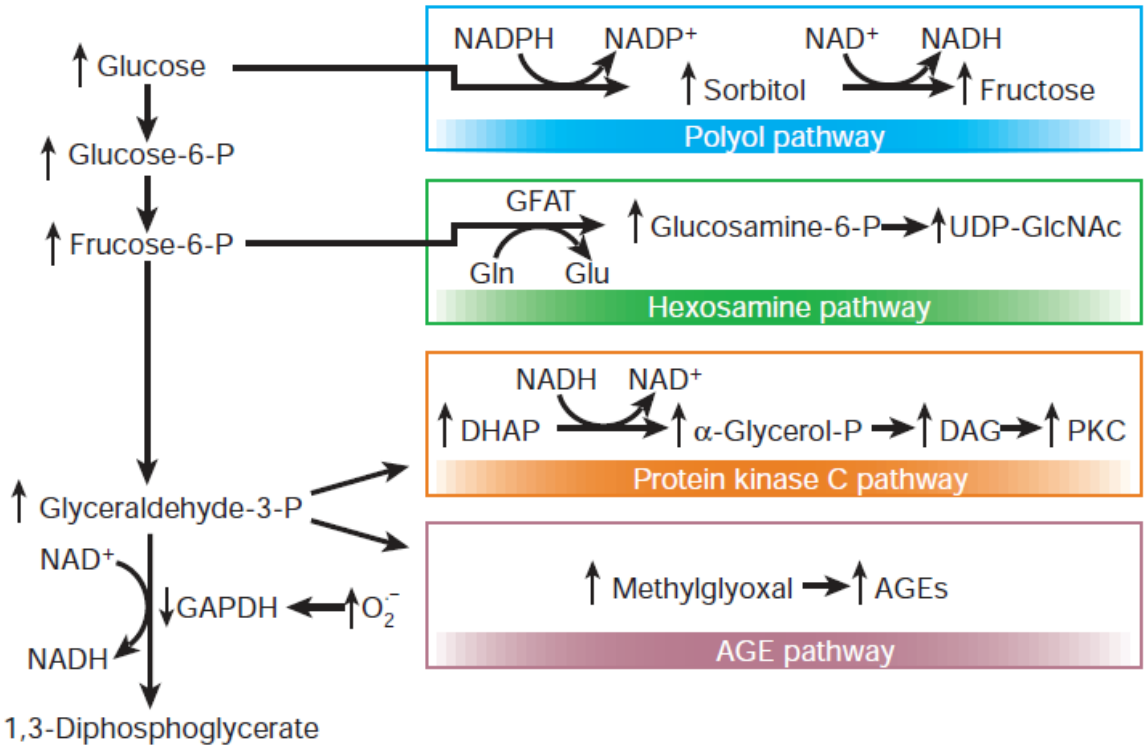

Figure 4. Mechanisms of glucose toxicity [99].

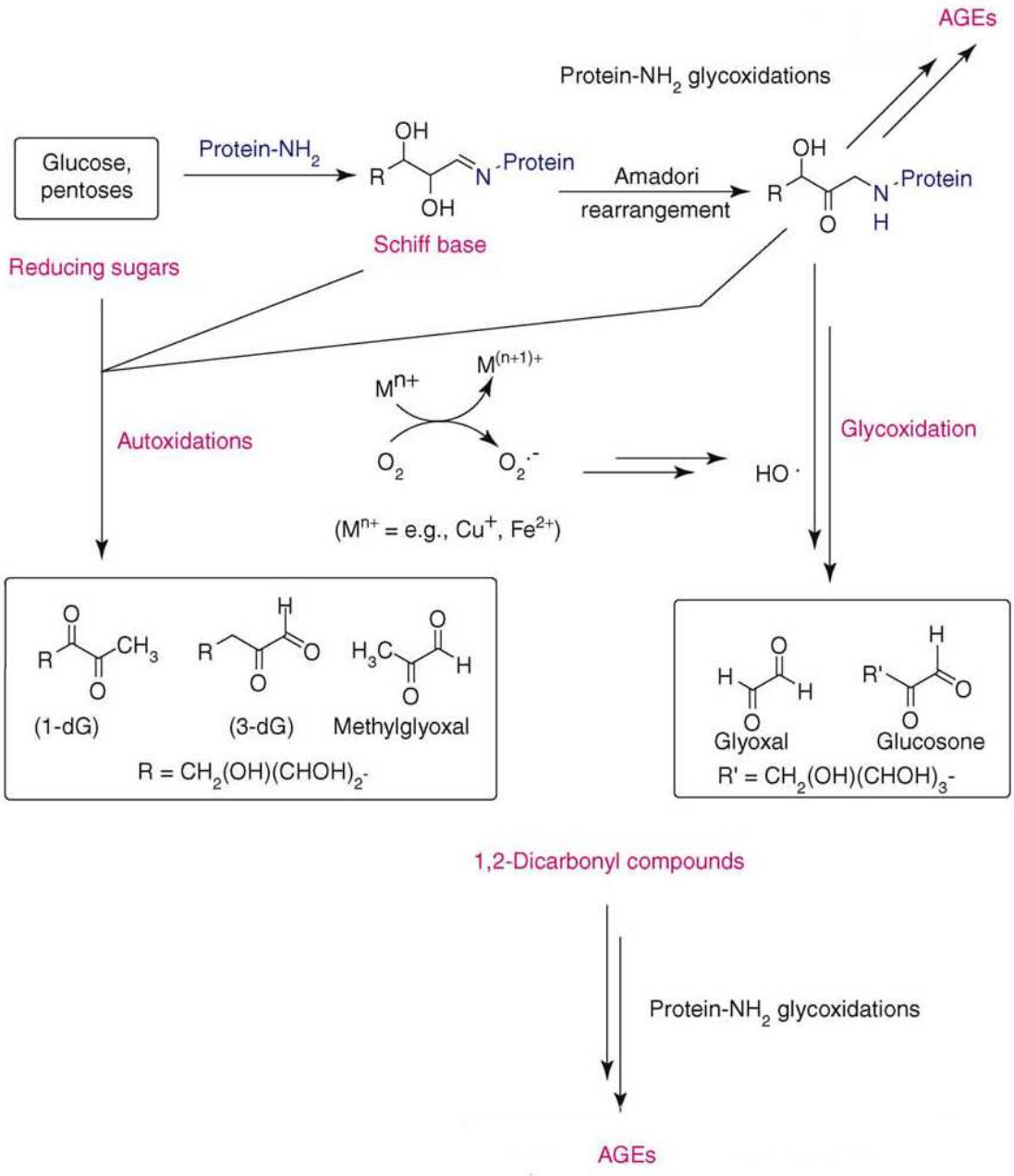

Figure 5. The Maillard reaction and byproducts. The spontaneous reaction between sugars and amino groups forms a Schiff base that undergoes rearrangements to originate an Amadori products

AGEs are more frequently found in proteins with longer half-life and may form intra and intermolecular cross-links, impairing protein function and tissue structure. Superoxide dismutase, an antioxidant enzyme, has its activity impaired by glycation [107, 108]. When glycated, human albumin has a decreased drug-binding capacity [109]. Increased collagen glycation is observed with aging and at accelerated rates in diabetes [110, 111]. Collagen is a major component of the arterial wall and its glycation has substantial consequences to vascular dysfunction and atherosclerosis. Overwhelming evidence demonstrates that collagen glycation, especially by cross-link formation, leads to a more fibrous and less soluble and flexible protein, which contributes to arterial stiffening [112-115]. Moreover, endothelial cells cultured over glycated collagen present premature senescence associated with decreased NO synthesis [116].

The rate and extent of glycation is directly influenced by the temperature, sugar concentration and time of exposure [117-120]. Physiologically, hyperglycemia, the turnover of glycation substrates and the redox nature of the microenvironment are critical to the formation of AGEs [93]. Since ROS participate in the reaction, AGEs are sometimes named advanced glycoxydation end-products [121, 122]. “AGEs” is a general denomination for different molecular structures. The particular molecular formula of each AGE depends on the reacting aldehyde, the target amino acid and the redox status. For example, glucose might generate different AGE structures [109]. In addition, AGEs differ structurally concerning the formation of cross-links, which may be intra or intermolecular. Methylglyoxal-lysine dimer (MOLD), methylglyoxal-derived imidazolium cross-link (MODIC), 3-deoxyglucosone-derived imidazolium cross-link (DOGDIC), 3-deoxyglucosone-lysine dimer (DOLD), glucosepane, pentosidine, glyoxal-lysine amide (GOLA), glyoxal-lysine dimer (GOLD) and glyoxal-derived imidazolium cross-link (GODIC) are cross-link AGEs. On the other hand, carboxyethyllysine (CEL), carboxymethyllysine (CML), methylglyoxal-hydroimidazolone, tetrahydropyrimidine, argpyrimidine, glycolic

acid-lysine amide (GALA) and glyoxal-hydroimidazolone do not form cross-links. Figure 6 depicts the chemical structures of some AGEs.

Besides endogenous formation of AGEs, humans are exposed to exogenous sources of AGEs. Potential glycation agents were found in tobacco smoke and smokers have higher levels of serum AGEs compared to non-smokers [123]. In addition, through high temperature processing, foods commonly present in the western style diet contribute to the daily exposure to AGEs [124-126]. The amount of AGEs in foods depends on cooking temperature, moisture and cooking time. In mice, AGE-rich diet induces insulin resistance [127] and liver inflammation [128].

As reviewed by Wautier and Schmidt [104], AGEs participate in diabetes-associated retinopathy and nephropathy. In vitro, AGEs induce oxidative stress and apoptosis in bovine retinal pericytes [129, 130] and are found accumulated in diabetic retinal vasculature [131]. The reactive glycolaldehyde induces renal oxidative damage *in vivo* [132] and diabetic rats with nephropathy show AGE deposits in the mesangial area and glomerular basement membrane [133].

Interaction between RAGE and AGEs occur exclusively at the V domain [81]. This interaction is due to the negative charge of AGEs that bind to the positively charged structure of RAGE. Moreover, N-glycosylation of RAGE and the G82S polymorphism are known increase AGE-RAGE affinity [79]. It was observed that RAGE oligomerization, which happens through VC1 domain [82], also increases AGE-binding affinity [81]. Among the different AGEs, the most studied is Nε-carboxymethyllysine (CML), which has the greater RAGE-binding affinity [134]. The AGE/RAGE axis is known to increase ROS production via NADPH oxidase, activate NF-κB and induce the secretion of pro-inflammatory cytokines, i.e. IL-6, TNF-α [135, 136], increase the expression of adhesion molecules [11] and vascular endothelial growth factor (VEGF) [21]. These responses, associated to the upregulation of RAGE, are deeply implied in endothelial dysfunction.

The accumulation of AGEs in tissue and in circulation was observed in the course of ageing [137-139] and at higher rates in diabetes [140-142] and other inflammatory conditions such as Alzheimer's disease [143] and rheumatoid arthritis [144]. Serum levels of CML are associated with increased cardiovascular risk [145-147]. Moreover, AGEs deposits are observe atherosclerotic lesions [148, 149] and amyloid deposits from dialysis patients [150].

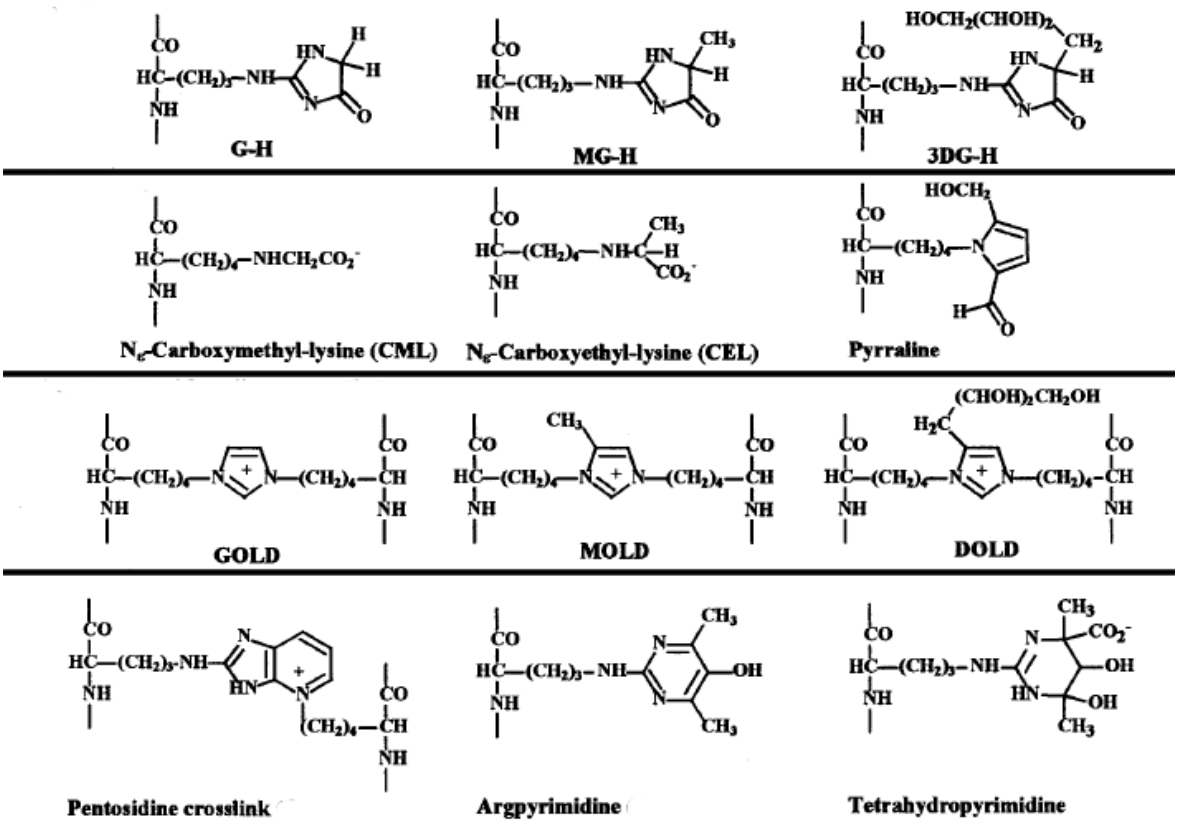

Figure 4. Different reactive molecules generate different AGEs. AGEs are heterogeneous regarding their structure and presence of cross-link (B) [151]. MOLD, methylglyoxal-lysine dimer; DOLD, 3-deoxyglucosone-lysine dimer; GOLD, glyoxal-lysine dimer; CEL, carboxyethyllysine; CML, carboxymethyllysine; MG-H, methylglyoxal-hydroimidazolone; G-H, glyoxal-hydroimidazolone; 3DG-H, 3-deoxyglucosone-hydroimidazolone.

1.3.2 Amphoterin (HMGB1)

High-mobility group box 1 (HMGB1), or amphoterin, is a DNA-binding protein. It participates in DNA reparation, replication, recombination and transcription [152]. HMGB1 may actively secreted by immunocompetent cells or passively, by necrotic and apoptotic cells. It has been show that HMGB1 exerts stronger pro-inflammatory effects when released from necrosis, compared to apoptosis [153, 154].

Besides RAGE [64], HMGB1 binds to toll-like receptors (TLR) 2 and 4 [155]. Amphoterin has a great affinity for RAGE and its binding activity is associated to high molecular weight complexes [156]. By interacting with RAGE, HMGB1 induces IL-6, ICAM-1, TGF-β1 and permeability in human umbilical vein endothelial cells [157, 158]. In pancreatic [95], gastric [159] and glioma [160] tumor cells, HMGB1/RAGE axis promotes growth.

Increased serum levels of HMGB1 are associated with inflammation. In septic patients, it is related to mortality [161], while in both non-diabetic and type 2 diabetic subjects, higher levels of amphoterin are associated with coronary artery disease [162]. Arrigo and colleagues proposed HMGB1 as biomarker of metabolic syndrome since it was increased in obese individuals and associated with insulin resistance and lower levels of adiponectin [163]. In healthy subjects, HMGB1 levels are directly associated with white blood cell count but inversely correlated with sRAGE levels, suggesting a role of sRAGE for HMGB1 clearance [164].

1.3.3 S100 proteins

The members of the S100/calgranulin family are low molecular weight (~11KDa) calcium-binding proteins that possess two helix-loop-helix domains (EF-hand). They are particularly implied in calcium homeostasis, although they play an important role in cell growth and energy metabolism [98, 165]. Today, there are 21 S100 proteins described

and for some of them, the biological role is still unclear. Except for calbindin D_{9K}, all calgranulins are present intracellularly as homodimers. As intracellular dimers, S100 proteins interact with target proteins and regulate mainly the cytoskeleton (microtubules organization and interaction of keratin intermediate filaments) but also regulate enzyme functions (activation of guanilate cyclase and fructose-1,6-biphosphate aldolase, inhibition of glycogen phosphorylase and phospholipase A_2) [165]. As secreted proteins, they exert different actions depending on the target cell and S100 member.

Members of the S100 family bind RAGE differently and activate different downstream pathways. For example, while S100B and S100A12 [98, 166] bind to the VC1 domain, S100A6 has a much higher affinity for the C2 domain [167]. To date, S100A1 [168], S100A2 [98], S100A4 [169], S100A5 [98], S100A6 [167], S100A7 [170], S100A8/A9 [171], S100A11 [172], S100A12 [173], S100A13 [174], S100B [173] and S100P [175] are known to bind RAGE. The activation of NF-κB is observed when RAGE is engaged by S100B, S100A12 and S100A1 [168, 173]. The outcome of this activation varies from neuronal differentiation, apoptosis, and secretion of pro-inflammatory cytokines [165].

S100 proteins are normally increased on inflammatory conditions. Rheumatoid arthritis patients have higher levels of S100A8, S100A9 and S100A12 compared to healthy subjects [176], and so do obese subjects for S100B [177], S100A8 and S100A9 levels [178]. The biology of calgranulins has been particularly studied in tumors and they are indeed increased in the occurrence of cancer [179, 180].

1.3.4 Amyloid beta peptide (Aβ)

The cleavage of the amyloid precursor protein (APP) by a β-secretase gives origin to the short amyloid beta peptide (Aβ) [181]. Its length usually varies from 39 to 43 amino acids. Aβ is the major component of amyloid deposits in AD patients [182, 183]. Although APP is

studied mainly because of Aβ formation, some of its physiological roles have been elucidated. Synapse formation and function [184], cell adhesion [185] and neuronal maturation [186] are attributed, in part, to APP.

Aβ binds RAGE in the form of fibrils and aggregates and both induce apoptosis in neuronal cells [187]. RAGE/Aβ interaction also elicits oxidative stress [188] and the receptor is responsible for the transport of Aβ through the blood-brain barrier [38]. In AD patients, serum Aβ is increased and correlated with serum creatinine [189] and is suspected to be associated with increased cardiovascular risk factors [190].

1.3.5 Other RAGE ligands

Besides the aforementioned ligands, other molecules have been studied, to a lesser extent, by their capacity to bind RAGE. The diverse repertoire of RAGE ligands corroborates its role as a PRR implicated in host response to injury, inflammation and infection. Not only AGE-modified proteins but also hypochlorite-oxidized albumin interacts with RAGE and induces expression of MCP-1 [191].

The role of RAGE in the immune response was further evidenced by the discovery of its interaction with phosphatidylserine [66] and C1q complement component [67], participating in the recognition and elimination of apoptotic cells. LPS, increasing levels of TNF-α, IL-6, HMGB1 and endothelin [61]. In addition, serum Amyloid A (SAA), an acute phase protein that is upregulated in tumors and rheumatoid arthritis, binds RAGE and further activate NF-κB in fibroblasts [192] and induces tissue factor expression in monocytes [193].

Using an ELISA to screen RAGE ligands, Ruan *et al*. [68] increased the repertoire of RAGE binding molecules. Heat-shock protein 70 (HSP70), secreted protein acidic and rich in cysteine (SPARC), C3a complement component and CpG oligonucleotides. RAGE

engagement by C3a and double-strand DNA induces production of interferon alpha (IFN-α) by peripheral blood mononuclear cells (PBMC) [68]. The fusion protein of the human respiratory syncytial virus was, very recently, found to bind RAGE. Nonetheless, RAGE seems to prevent the virus entry mediated by the fusion protein [194].

As novel RAGE ligands are described, a role for RAGE in inflammation becomes more obvious, although the specific mechanisms mediated by these interactions remain unclear, they place RAGE in a complex network that overlaps physiological and pathological pathways.

1.4 Soluble RAGE

In 1999, analysis of complementary DNA (cDNA) revealed a RAGE clone encoding only the soluble extracellular portion of the receptor [195]. Although a synthetic form of soluble RAGE (sRAGE) was already applied to identify and prevent RAGE-dependent responses [10, 196] this was the first evidence of a potentially secreted form of RAGE. Later, Yonekura and colleagues [197] identified other splice variants of RAGE, one of them encoding the then named endogenous secretory RAGE (esRAGE). This form was described as more stable than the first one described in 1999 and was indeed shown to be secreted by human pericytes and endothelial cells.

In 2008, three independent groups demonstrated the cleavage of the full-length RAGE by metalloproteases, a mechanism that liberates cleaved RAGE (cRAGE) in the extracellular milieu [198-200]. All three reported the sheddase a disintegrin and metalloprotease 10 (ADAM10) as the major agent in RAGE cleavage. However, the results of Zhang and colleagues suggested that the matrix metallopeptidase 9 (MMP-9) is also capable of shedding membrane RAGE and that of Yamakawa *et al*. [201], showed that MMP-3 and MMP-13 are responsible for cRAGE release in alveolar epithelial cells. In addition, it was

shown that ADAM17 may also participate in RAGE shedding [202]. Figure 7 presents the amino acid sequence for full-length RAGE, esRAGE and cRAGE.

Figure 7. Amino acid sequences of full-length RAGE, endogenous secretory RAGE and cleaved RAGE.

The cRAGE has a molecular weight around 48 kDa and lacks the unique amino acid sequence of esRAGE. It is possible, thus, to quantify total soluble RAGE (sRAGE) and esRAGE only. It was also observed that esRAGE represents only a small proportion of the total circulating sRAGE [199, 203]. Therefore, most of the literature concerns the levels of sRAGE (cRAGE and esRAGE).

Both esRAGE and cRAGE act as decoy receptors, preventing the activation of membrane RAGE by its ligands. In addition, sRAGE might act by forming heterodimers with membrane RAGE [82]. Seen that RAGE dimerization is essential for its signaling activity

through Dia-1, sRAGE-RAGE assembly would lack one cytoplasmic tail, preventing downstream events.

1.5 sRAGE levels in human diseases

Since its first observation in blood [204], sRAGE has been measured in the course of several human diseases in an attempt to define their biological roles and potential as biomarker. Indeed, in almost 10 years, sRAGE levels have been associated with different pathologies and their severity. Nevertheless, despite several indications of sRAGE and esRAGE as biomarkers, their levels present a huge inter and intra-populations variation and are sometimes controversial. However, there only a few publications presenting both esRAGE and sRAGE levels, the latter being the most studied and most controversial. There is great variation in sample size among publications but without direct association with the journal's impact factor (IF). Moreover, a recent meta-analysis suggested sample size as a potential bias in sRAGE levels associated with coronary artery disease [205]. It is important to notice that, for majority of diseases where sRAGE levels were studied (i.e. diabetes, CVDs, Alzheimer's and renal disease) RAGE plays an important role in their pathophysiology. Thus, serum sRAGE is often interpreted as a direct reflection of RAGE expression or an anti-RAGE mechanism.

1.5.1 sRAGE in diabetes

Diabetes mellitus is the "obvious RAGE disease". High glucose levels and inflammation increase the formation of AGEs, which interact with RAGE. Activation of NF-κB leads to expression of pro-inflammatory cytokines and RAGE itself. Indeed, increased circulating levels of AGEs and cellular expression of RAGE were observed in both type 1 [206-208] and II [209, 210] diabetes.

Total sRAGE and esRAGE have been measured in diabetics, with greater attention given to the former and both increase and decrease in their levels are found. Although most publications showing an increase in sRAGE include patients with kidney dysfunction, this issue is not a consensus. Nevertheless, type 1 diabetics may have increased [211, 212] and decreased [213, 214] sRAGE levels, depending on the study. Cross-sectional studies with specific populations like children [213] and women [214] observed lower sRAGE levels in diabetics, although larger cohort studies, did not find associations with age and sex of the participants [211, 215, 216]. In two longitudinal studies, high baseline sRAGE levels were independently associated with cardiovascular mortality [211, 216].

The same controversy is observed in type 2 diabetics, where high [217-219] and low sRAGE [220, 221] are found associated with complications. Similarly to type 1 diabetes, longitudinal studies with large cohorts show that higher baseline sRAGE levels are associated with CVD risk [219, 222]. Cross-sectional studies often present important differences in their study populations, especially concerning the inclusion of patients under medication and with renal dysfunction. Unfortunately, the evaluated parameters are not always the same, which may lead to misinterpretation. Nonetheless, two studies of the same group failed to show the same associations between sRAGE and clinical parameters, although the sample sizes were similar and inclusion criteria were the same [217, 223]. Moreover, the authors reported that sRAGE positive correlation with AGEs, and soluble VCAM-1 were still significant when analyzed in diabetics without nephropathy only [223].

Although renal dysfunction clearly is associated with high sRAGE levels, it was reported that early stage diabetic nephropathy is not associated with changes in sRAGE [218]. In addition, even after adjustment for renal dysfunction, associations between high sRAGE levels and cardiovascular risk are still found [211, 216, 219, 222]. Separate studies observed that sRAGE levels were correlated with circulating AGEs in both diabetic [224] and non-diabetic subjects [225]. Furthermore, they did not find an association between

sRAGE and kidney function among diabetics. While they conclude that serum sRAGE may reflect cellular RAGE expression, other factors such as polymorphisms and ethnicity may help to explain the differences observed between studies.

Tables 2 and 3 present, respectively, sRAGE and esRAGE levels in type 1 diabetes. Levels of sRAGE and esRAGE in type 2 diabetes are presented in tables 4 and 5 respectively.

Table 2. Serum sRAGE levels and their association with type 1 diabetes.

sRAGE LEVELS	COMMENTS	REFERENCE/ IF
Increased	In diabetics (n=45) compared to non-diabetics (n=35), p<0.01.	[212]/ 7.149
Increased	Diabetic children (n=75) compared to non-diabetic children (n=43), p=0.047. Associated with low-density lipoprotein (LDL, r=0.224) levels and inversely associated with diabetes duration (r=-0.265).	[226]/ 0.747
Increased	Higher in diabetics with cardiovascular diseases (CVD) and albuminuria (n=477), p=0.005.	[215]/ 6.487
Decreased	Lower in diabetic women (n=43) compared to non-diabetics (n=43), p<0.01.	[214]/ 3.317
Increased	Higher baseline levels associated with CVD incidence and mortality (n=169, p=0.001).	[216]/ 7.895
Increased	High baseline levels associated with all-cause and cardiovascular mortality (n=3100, p<0.001).	[211]/ 6.487
Increased	In diabetics (n=42) compared to non-diabetics (n=24),p=0.001.	[227]/ 2.594
Decreased	In both pre-pubertal (n=34) and pubertal (n=30) diabetics, compared to non-diabetics (n=32), p=0.001.	[213]/ 2.939

Table 3. Serum esRAGE levels and their association with type 1 diabetes.

esRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	In diabetics (n=67) compared to controls (n=23), p<0.0001. Inverse correlation with HbA_{1C}.	[228]/ 7.735
Increased	Positively associated with CML (r=0.310) and pentosidine (r=0.536). Inverse association with glomerular filtration rate (r=-0.626, n=70).	[229]/ 6.338
Decreased	Lower in diabetics (n=130) than controls (n=22) (p<0.0001). Inverse correlation with HbA_{1C} (r=-0.361), IMT (r=-0.254), body-mass index (r=-0.28) and total cholesterol (r=-0.2).	[203]/ 2.594
Decreased	Low baseline esRAGE levels are associated with IMT (n=47) (r=-0.432, p=0.0022).	[230]/ 3.706
Decreased	Lower in both pre-pubertal (n=34) and pubertal (n=30) diabetics in comparison to controls (n=32) (p<0.005). Inversely associated with mean kidney volume (β=-0.503).	[213]/ 2.939

Table 4. Serum sRAGE levels and their association with type 2 diabetes.

sRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	In diabetics (n=84) compared to non-diabetics (n=76) (p<0.0001). Inversely associated with HbA_{1C} (r=-0.66)	[220]/ 6.430
Increased	Associated with albuminuria in diabetics (n=110, r=0.18, p<0.05).	[231]/ 4.209
Decreased	In diabetics (n=86) compared to non-diabetics (n=43). Inversely associated with glycated hemoglobin (r=-0.291)	[232]/ 5.271
Increased	In diabetics (n=75) compared to non-diabetics (n=75), p<0.0001. Inversely associated with high-density lipoprotein (HDL, β=-9.951), HbA_{1C} (β=98.51. Positive correlation with LDL (β=3.153), creatinine (β=777.3) and triglycerides (β=0.352).	[217]/ 2.968
Increased	Compared to non-diabetics (n=86, p<0.001). Positively associated with hyperlipidemia, HbA_{1C} and AGEs. Negatively associated with HDL and diastolic blood pressure.	[233]/ 2.397
Decreased	Lower levels in diabetics with renal and retinal complications (n=10) compared to diabetics without such complications (n=20), p=0.028.	[221]/ 2.388
Increased	Positive correlation with serum AGEs (β=0.261) and soluble vascular adhesion molecule 1 (sVCAM-1, β=0.3) (n=82).	[223]/ 2.929

Decreased	Lower in diabetics (n=80) compared to non-diabetics (n=132) without coronary artery disease (CAD), p<0.05. Higher in diabetics with CAD (n=151) compared to non-CAD diabetics (n=80), p=0.002.	[162]/ 3.706
Increased	Positively associated with AGEs (n=180, r=0.22). Inverse correlation with glomerular filtration rate (GFR, r=-0.13).	[218]/ 2.741
Positive and negative associations	Positive correlation with serum AGEs (r=0.24) and negative correlation with high sensitivity C-reactive protein (hs-CRP, r=-0.28) (n=245).	[234]/ 2.741
Increased	Higher levels predict coronary heart disease (CHD, hazard ratio=1.74) (n=718).	[219]/ 7.895
Decreased	In diabetics (n=50) compared to non-diabetics (n=50), p<0.01.	[235]/ 2.079
Increased	Higher in diabetics without retinopathy (n=14, p), compared to controls (n=20), p<0.05. Lower in diabetics with retinopathy (n=23) compared to controls, p<0.05.	[236]/ 2.056
Decreased	In diabetics (n=53) compared to non-diabetics (n=52), p<0.05.	[210]/ 4.859
Increased	Higher in diabetics without diabetic foot (n=30), compared to non-diabetics (n=20), p<0.05. Lower in patients with diabetic foot (n=30), p<0.05.	[237]/ 1.746
Increased	Higher in diabetics with in-stent restenosis (n=35), p=0.003.	[238]/ 1.107
Increased	In diabetics (n=79) compared to non-diabetics (n=220), p=0,025. Also higher in diabetics with heart failure patients (n=125), p<0.001.	[239]/ 5.247
Increased	High sRAGE levels are associated with higher CVD risk (n=276, hazard ratio=1.59).	[222]/ 3.706

Table 5. Serum esRAGE levels and their association with type 2 diabetes.

esRAGE LEVELS	COMMENTS	REFERENCE/ IF
Increased	Higher in patients in dialysis (n=21) compared to diabetics with (n=47) and without nephropathy (n=39), p<0.0001. Correlated with CML (r=0.481) and pentosidine (r=0.502).	[240]/ 2.741
Decreased	Lower in patients with in-stent restenosis (n=86) than those without (n=128), p<0.05.	[241]/ 2.850
Decreased	In diabetics (n=76) compared to non-diabetics (n=78), p=0.005. Inversely correlated with pulse wave velocity, r=-0.296.	[242]/ 5.509

Decreased	In diabetics (n=302) compared to non-diabetics (119), p<0.01. Even lower in diabetics with coronary artery disease (n=357), p<0.01. Inversely correlated with severity (r=-0.169) and extent of disease (r=-0.2).	[243]/ 3.706
Decreased	In diabetics (n=168) compared to non-diabetics (n=434), p<0.001.	[244]/ 2.850
Decreased	Lower in diabetics (n=113) compared to non-diabetics (n=152), p<0.001. Low sRAGE at follow-up is associated with plaque progression (odds ratio=23.477).	[245]/ 2.450
Decreased	In diabetics (n=231) compared to non-diabetics (n=281), p<0.001. Inversely associated with coronary artery disease.	[162]/ 3.706
Decreased	In diabetics (n=79) compared to controls (n=220), p<0.001. Levels are even lower in heart failure diabetics, p=0.001.	[239]/ 5.247
Increased	High levels predict coronary heart disease (n=718), hazard ratio=1.45.	[219]/ 7.895
Decreased	In diabetics (n=53) compared to non-diabetics (n=52), p<0.05.	[210]/ 4.859

1.5.2 Neurological diseases

Low levels of sRAGE are characteristic in Alzheimer's disease (AD). When compared to control [246] and patients with mild cognitive impairment [247] or vascular dementia [248], AD patients present lower circulating levels of sRAGE. Unfortunately, to our knowledge, there is no data concerning esRAGE levels in the plasma of AD patients, although Nozaki and colleagues [249] showed decreased esRAGE content in hippocampal neurons. In the same work they observed and increase in astrocyte esRAGE. Whether such an increase reflects in increased secretion of esRAGE is not known, but a cell-specific-response exists and might be a key process for therapeutic interventions. Although sRAGE levels are globally coherent in neurological disorders, their biological meaning is still uncertain. Larger longitudinal studies with several time points should be performed to understand the

evolution of sRAGE levels in parallel with disease progression. Tables 6 and 7 present the association of neurological disorders with sRAGE and esRAGE, respectively.

Table 6. Serum sRAGE levels and their association with neurological disorders.

sRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	In Alzheimer's disease (n=152) and vascular dementia (n=91), compared to controls (n=161), p<0.05.	[248]/ 6.31
Decreased	In mild cognitive impairment (n=66) and Alzheimer's disease (n=100) in comparison to controls (n=161), p<0.001.	[247]/ 3.052
Decreased	Lower in multiple sclerosis patients (n=37), compared to healthy subjects (n=22), p=0.005.	[250]/ 4.472
Decreased	In amyotrophic lateral sclerosis, compared to controls (n=20), p<0.05.	[251]/ 2.474
Decreased	Lower in Alzheimer's disease patients (n=276) compared to controls (n=254), p<0.0001.	[246]/ 3.052
Decreased	In Alzheimer's disease (n=126), vascular dementia (n=96) and non-Alzheimer's neurodegenerative dementia (n=30), compared to controls (n=98), p<0.001.	[252]/ 1.253

Table 7. Serum esRAGE levels and their association with neurological disorders.

esRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	esRAGE levels are lower in patients with autistic spectrum disorder (n=18) than controls (n=18), p=0.002.	[253]/ 2.026

1.5.3 Cardiovascular diseases

RAGE is over expressed in atherosclerotic lesions and its activation has been implicated in the onset and progress of cardiovascular complications. In ApoE -/- mice, which spontaneously develop atherosclerotic lesions, sRAGE treatment prevents plaque formation [254]. Nevertheless, sRAGE levels in humans do not follow the logical thought

of a protective decoy receptor. In patients with hypertension, sRAGE has been positively associated with inflammation and disease [255, 256], but also found decreased in comparison to normotensive subjects [257]. In non-diabetic patients with coronary artery disease (CAD) sRAGE levels are decreased [258, 259], although Mulder and colleagues did observe a positive correlation between sRAGE and skin autofluorescence [260]. Yan *et al.* [162] found lower esRAGE levels in CAD patients in both type 2 diabetic and non-diabetic populations although total sRAGE levels were increased in diabetics with CAD. In another work, type 2 diabetics with CAD also presented lower levels of esRAGE [243].

The group of Raposeiras-Roubin observed a positive association of sRAGE with chronic heart failure and atrial fibrillation [261-263]. On the other hand, in patients with kidney dysfunction, who usually present increased levels of sRAGE, negative correlations have been observed for atherosclerosis, vascular calcification and ventricular hypertrophy [264-266].

Very recently, Peng and colleagues performed a meta-analysis to investigate the association of RAGE polymorphisms and sRAGE with coronary artery disease [205]. They analyzed 27 articles including prospective and retrospective studies, with and without diabetic patients. The presence of diabetes or renal disease potentiates the statistical association of RAGE polymorphisms with CAD. Moreover, they concluded that, globally, sRAGE levels are not significantly associated with CAD, but a positive association is observed in studies with diabetics. Interestingly, they state that low esRAGE might be a predictor of CAD development in the absence of renal dysfunction. Tables 8 and 9 present, respectively, sRAGE and esRAGE in cardiovascular diseases.

Table 8. Serum sRAGE levels and their association with cardiovascular diseases.

sRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	Lower in CAD (n=328) than in non-CAD (n=328) subjects, p<0.0001.	[258]/ 6.338
Decreased	Lower in hypertensive (n=147) than in normotensive (n=177) subjects, p=0.002.	[257]/ 3.806
Increased	Higher in individuals with cardiac events (n=48) than those without (n=112), p=0.0004.	[267]/ 3.320
Increased	Positive association with skin autofluorescence in CAD patients (n=63, β=0.43).	[260]/ 3.706
Increased	Higher in symptomatic carotid atherosclerosis (n=19) than in asymptomatic (n=10), p=0.009. Positive correlation with CML (r=0.6), CRP (r=0.618) and fibrinogen (r=0.522).	[268]/ 3.365
Decreased	In pre-mature CAD patients (n=100) compared to non-CAD individuals (n=40), p<0.0001.	[259] 3.706
Increased	High levels predict all-cause and CVD mortality in elder women (n= 559), hazards ratio=1.26.	[269]/ 1.006
Decreased	Lower in aortic valve stenosis (n=75) compared no controls (n=39), p<0.01. Inverse correlation with age (r=-0.33), total cholesterol (r=-0.228) and coronary calcium score (r=-0.57).	[270]/ 3.706
Increased	Higher in chronic heart failure with ischemic etiology (n=31), compared to non-ischemic (72), p=0.016. Correlated with heart failure severity (odds ratio=1.18).	[262]/ 5.247
Increased	In acute myocardial infarction (n=54) compared to controls (n=54), p<0.001. Associated with plaque vulnerability (odds ratio=2.47).	[271]/ 3.578
Increased	Higher in patients with cardiac events (n=29) than event-free patients (n=77), p=0.001.	[261]/ 3.209
Increased	Higher in heart failure subjects (n=222) than controls (n=220), p=0.001.	[239]/ 5.247
Increased	Higher in subjects with atrial fibrillation (n=38) than controls (n=59), p=0,001. Associated with atrial area (r=0.536) and volume(r=0.511).	[263]/ 5.509
Decreased	In hypertensive individuals, low sRAGE is associated with albuminuria (p<0,001) and arterial stiffening (p=0.003), n=430.	[272]/ 3.978

Table 9. Serum esRAGE levels and their association with cardiovascular diseases.

esRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	Inversely associated with components of metabolic syndrome (n=337).	[273]/ 6.338
Increased	High levels predict mortality in elder women (n=559), hazards ratio=1.28.	[269]/ 1.006
Decreased	Lower in subjects with coronary artery disease (n=149) than controls (n=132), p<0.001.	[162]/ 3.706
Decreased	In subjects with heart failure (n=222) than controls (n=220), p<0.001.	[239]/ 5.247

1.5.4 Kidney diseases

In general, sRAGE levels are inversely correlated with kidney function. Higher sRAGE levels were observed in patients with increased serum creatinine, albuminuria and low glomerular filtration rate (GFR). The evident question is whether sRAGE formation is increased (through RAGE cleavage and/or esRAGE secretion) or it is just a deficient excretion. However, within patients with kidney dysfunction, some articles demonstrate an inverse association between sRAGE levels and cardiovascular complications [264-266].

If circulating sRAGE is indeed an effective agent against RAGE activation, the lack of effectiveness in cases of renal dysfunction might be a saturation of sRAGE due to the increased levels of RAGE ligands. AGEs, HMGB1, S100A12, were found increased in patients with chronic kidney disease, end-stage renal disease and in peritoneal dialysis [265, 274, 275]. Moreover, sRAGE levels might reflect full length RAGE expression which is increased in renal diseases [276]. Increased RAGE expression and subsequent sRAGE production can be explained by higher levels of ADAM10, although, to our knowledge, there is only one study to date demonstrating its over-expression in kidney disease [277]. The levels of sRAGE and esRAGE in kidney diseases are respectively presented in tables 10 and 11.

Table 10. Serum sRAGE levels and their association with kidney diseases**.**

sRAGE LEVELS	COMMENTS	REFERENCE/ IF
Increased	In chronic kidney disease (CKD) (n=25) and end-stage renal disease (ESRD) (n=25), compared to controls (n=21), p<0.001. Correlated with serum creatinine (r=0.5).	[278]/ 5.294
Increased	Higher in CKD (n=20), compared to healthy subjects (n=20), p<0.001. Correlated with serum AGEs (r=0.842).	[275]/ 3.096
Increased	Higher in CKD patients (n=142) than controls (n=49, p<0.001), but inversely associated with IMT (-0.31) and the number of atherosclerotic plaques (r=-0.24).	[264]/ 7.916
Increased	Associated with proteinuria (r=0.57). Levels decrease after treatment with calcium channel blocker (n=30, p<0.01).	[279]/ 1.834
Increased	In hemodialysis (n=31) and CKD patients (n=46), compared to controls (n=24), p>0.0001.	[280]/ 1.596
Increased	Higher in peritoneal dialysis (n=91) than controls (n=29) (p<0.01), but inversely associated with carotid IMT and calcification score.	[265]/ 3.706
Increased	Higher in CKD patients (n=142) than controls (n=49) (p<0.001), but inversely associated with mean wall thickness (β=-0.17) and left ventricular mass (β=-0.16).	[266]/ 3.978

Table 11. Serum esRAGE levels and their association with kidney diseases**.**

esRAGE LEVELS	COMMENTS	REFERENCE/ IF
Increased	Higher in ESRD patients (p<0.01) but low esRAGE is associated with cardiovascular events (n=206, hazards ratio=0.4).	[281]/ 1.750
Increased	Higher in CKD patients (n=158) than non-CKD (n=858, p<0.0001) and predictor of CKD in the elder (hazards ratio=1.37).	[282]/ 2.623
Decreased	In CKD patients (n=65) compared to controls (n=19), p=0.001). Inversely associated with aortic calcification in CKD patients (r=-0.6).	[283]/ 1.325

1.5.5 Lung diseases

The lungs are the major organs to express membrane RAGE. Thus, it is believed they may contribute considerably to circulating sRAGE. The specific role of RAGE in the lung remains to be elucidated. However, an increase in sRAGE levels seems to be associated with an acute-phase response [284-286], while in chronic lung diseases sRAGE may be found decreased [287]. In lung acute disorders, sRAGE may reflect the degree of inflammation, reflected by the activation of proteases responsible for RAGE cleavage. Indeed, MMP-3, MMP-9 and MMP-13 are increased within 48h of acute lung injury development[288]. The decrease of sRAGE in chronic lung disease may be explained by an enhanced consumption or impaired formation of sRAGE, although these hypothesis are to be investigated. Tables 12 and 13 present sRAGE and esRAGE levels in lung disorders, respectively.

Table 12. Serum sRAGE levels and their association with lung disorders**.**

sRAGE LEVELS	COMMENTS	REFERENCE/ IF
Increased	In lung graft patients with primary graft dysfunction (n=84) compared to those without (n=233), p=0.028. Also elevated in cardiopulmonary bypass (n=109), p<0.005.	[286]/ 11.041
Decreased	Lower in patients who develop acute lung injury (n=9) than those who doesn't (n=8), after sepsis surgery, p<0.05.	[289]/ 1.472
Increased	After cardiopulmonary bypass (n=20), p<0.01.	[290]/ 6.355
Increased	Higher in acute lung injury and acute respiratory distress syndrome (n=33), compared to controls (n=15), p<0.0001. Associated with severity scores (r=0.528).	[284]/ 6.124
Increased	High levels associated with mortality in acute respiratory distress syndrome patients (n=20), β=0.537.	[291]/ 2.450
Decreased	Lower in chronic obstructive pulmonary disease (n=200) than in controls (n=201), p=0.007. Inverse correlation with emphysema severity.	[287]/ 3.642

Positive and negative associations	Positively associated with macrophages (r=0.293) and negatively associated with lymphocytes (r=-0.311) and neutrophils (r=0.306) in bronchoalveolar lavage fluid (n=76).	[292]/ 2.781
Decreased	In asthmatic (n=16) and chronic obstructive pulmonary disease (n=37) patients compared to controls (n=18), p<0.01.	[293]/ 6.355
Increased	Increases after cardiopulmonary bypass and correlates with severity of acute lung injury (n=58, p<0.001).	[294]/ 4.718

Table 13. Serum esRAGE levels and their association with lung disorders**.**

esRAGE LEVELS	COMMENTS	REFERENCE/ IF
Increased	Higher levels in asthmatic patients (n=44) compared to controls (n=15), p<0.001.	[295]/ 2.585

1.5.6 Cancer

Evidence suggests an important role for membrane RAGE in tumor proliferation and invasion [160, 296, 297]. There are only a few publications concerning sRAGE levels in cancer and, although the majority shows a negative association, generalizations are misleading due to the great variability among tumor cells. Low levels of sRAGE are found in patients with lung and pancreatic cancer [298, 299]. Two studies showed lower sRAGE levels in breast cancer patients while Piperis and colleagues found higher levels compared to controls [300-302]. The discrepancies are probably due to different stages of cancer development in the studies. Serum levels of sRAGE and esRAGE in cancer patients are presented respectively in tables 14 and 15.

Table 14. Serum sRAGE levels and their association with cancer**.**

sRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	Lower in breast cancer patients (n=120), compared to controls (n=92), p<0.05.	[302]/ 2.238
Decreased	Lower in breast cancer patients (n=113) than in controls (n=58), p<0.05.	[300]/ 2.238
Decreased	In patients with lung cancer (n=45) compared to controls (n=19), p=0.034.	[298]/ 1.574
Decreased	In patients with pancreas cancer (n=51) compared to controls (n=154), p<0.001. Even lower in patients with impaired glucose tolerance, p<0.05).	[299]/ 2.450
Decreased	Low levels are associated with colorectal cancer risk in male smokers (n=968, relative risk comparing quintiles = 0.65).	[303]/ 4.559
Decreased	Low levels associated with pancreatic cancer in male smokers (n=740, relative risk comparing quintiles = 0.46).	[304]/ 8.650
Increased	Higher in breast cancer patients (n=38), compared to controls (n=38), p<0.05.	[301]/ 1.713
Decreased	Low levels are associated with cases of colorectal adenoma (n=158, odds ratio=0.55)	[305]/ 1.329
Decreased	Inversely associated with liver cancer (n=630, relative risk=0.86).	[306]/ 12.003

Table 15. Serum esRAGE levels and their association with cancer**.**

esRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	Associated with pancreatic cancer incidence at short follow-up (2 years) (n=906, odds ratio=0.46).	[307]/ 4.559

1.5.7 Other disorders

Total sRAGE and esRAGE levels were also investigated in other conditions, especially inflammatory diseases. In septic patients, sRAGE levels increase [308, 309], although mean levels are considerably different between studies (2302±189 vs. 5499±1516 pg/ml).

It decreases in the autoimmune diseases Sjogren's disease and rheumatoid arthritis patients [310, 311], as well as in Charcot arthroneuropathy [312]. Very interestingly, it was shown that sRAGE levels increase within 30 minutes after a severe trauma [313], suggesting that high sRAGE levels for some disorders may be due to blood sampling at an acute phase. In addition, after acute bicycle exercise, CAD patients and healthy subjects show a non-significant increase in sRAGE levels (from 1578 to 1999 and 1824 to 2349 pg/ml, respectively) [314]. In both CAD patients and healthy subjects, exercise induced a significant increase in MMP-9 activity, which may explain the change in sRAGE levels. Despite the lack of statistical difference, this finding raises the question on how precise is sRAGE homeostasis. Moreover, the lack of significance may be due to the small sample size (n=21 for CAD and n=22 for controls). Table 16 shows the associations of sRAGE levels with other diseases not presented above.

TABLE 16. Serum sRAGE levels and their association with other diseases.

sRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	In rheumatoid arthritis patients (n=62), compared to healthy controls (n=45), p<0.0001.	[310]/ 4.302
Increased	Higher in septic patients (n=29) compared to controls (n=8), p<0.05. Even higher in non-survivors, p<0.001.	[308]/ 2.018
Decreased	Lower in Sjogren's syndrome (n=10), compared to anti-nuclear antibody positive patients (n=3), p<0.05.	[311]/ 2.214
Increased	Higher in septic patients (n=20) than controls (n=20), p<0.05.	[315]/ 2.043
Decreased	In non-alcoholic steatohepatitis (n=40) compared to controls (n=14), p<0.01.	[316]/ 2.450
Decreased	Inversely associated with body-mass index and waist-hip ratio (n=176, r=-0.24).	[317]/ 3.978
Increased	sRAGE increases rapidly after severe trauma (n=176), p<0.05.	[313]/ 2.348
Decreased	In patients with knee osteoarthritis (n=36) compared to controls (n=15), p<0.001. Associated with severity, r=-0.65.	[318] 2.450

Decreased	In patients with juvenile idiopathic arthritis (n=121) compared to healthy controls (n=45), p<0.0001.	[319]/ 3.258
Decreased	Negative association, in HIV patients (n=76),, with carotid IMT, r=-0.329, LDL (r=-0.284), body-mass index (r=-0.324), total cholesterol (r=-0.24) and insulin resistance (r=-0.38).	[320]/ 3.706
Decreased	In patients with Charcot neuroarthropathy (n=20) compared to controls (n=30), p<0.01.	[312]/ 7.735
Increased	Higher in septic patients (n=15) than controls (n=15), p<0.01.	[309]/ 2.498
Decreased	Decreased in lupus patients (n=105) compared to controls (n=43), p=0.003.	[321]/ 2.199
Increased	Higher levels in smokers (n=45) than non-smokers (n=53), p=0.002. Associated with the number of cigarettes/day (r=0.6).	[322]/ 2.594

Table 17. Serum esRAGE levels and their association with other diseases**.**

esRAGE LEVELS	COMMENTS	REFERENCE/ IF
Decreased	In patients with rheumatoid arthritis (n=54) compared to healthy subjects (n=20), p<0.0001.	[323]/ 5.271

1.6 Modulation of sRAGE levels

Although the mechanisms of sRAGE (cRAGE and esRAGE) formation are quite well established, very little is known about their regulation. While *in vivo* studies lack the signaling details of this regulation, the few *in vitro* studies are not sufficient to explain or extrapolate an explanation for the high variability of sRAGE levels.

The endogenous secretory RAGE is formed by alternative splicing of RAGE mRNA. While cRAGE is formed at the direct cost of one membrane RAGE, esRAGE is produced with the expense of a potential full-length RAGE mRNA [324]. Unfortunately, very little is known about the regulation of the mRNA splicing that leads to esRAGE formation.

Grossin *et al.* [325] observed that methylglyoxal-modified albumin induces RAGE and esRAGE expression in endothelial cells, while glyoxylic acid-modified albumin increases only the full-length form. Recently, it was shown that insulin increases esRAGE in cultured macrophages [326].

The cleaved form of sRAGE is produced through the action of ADAM10 and other proteases on the extracellular domain of RAGE. Therefore, it is expected that RAGE over-expression associated with activation of these proteases would lead to increased cRAGE. RAGE expression is modulated by several external stimuli. Full-length RAGE may be upregulated by TNF-α, β-estradiol and glycated albumin [327], while it is downregulated by angiotensin-converting enzyme inhibitor (ACEi) [328], retinol (vitamin A) [94], metformin [329] and statins [330-332].

ADAM10 is activated by calcium ionophore calcimycin (Ca-IC) and the phorbol ester myristate actetate (PMA), albeit Galichet *et al.* did not observe an increase in sRAGE formation with PMA-mediated activation [198]. ADAM10 is localized in lipid rafts and has its activity increased by lowering cholesterol levels [333-335] and by retinoic acid receptors [336]. It is possible that cholesterol lowering is one of the mechanism through which statins increase sRAGE levels [337, 338]. Metalloproteases are, in general, activated by cytokines like IL-1β and TNF-α [339-342] and associated with inflammation, tumor proliferation and angiogenesis [343, 344], which are processes where RAGE is implicated. Another issue that renders cRAGE production more complex is that activation of G protein-coupled receptors (GPCRs) also induces RAGE shedding through ADAM10, ADAM17 and MMP9 [202].

Metz and colleagues observed that ligand-induced activation of the V2 vasopressin, oxytocin and pituitary adenylate cyclase-activating polypeptide receptors increase cRAGE production [202]. Since GPCRs are a vast family of receptors, encoded by more than 800 genes [345], implicated in hormone and metabolite signaling [346], these findings extend

the borders of RAGE cleavage regulation and underscore the importance of further studies before clinical conclusions on sRAGE levels. Nevertheless, data on cRAGE formation *in vitro* is scarce. In cultured cells, sRAGE increases after treatment with ACEi [328], H_2O_2 [347] and insulin [326].

The consistency of data concerning sRAGE levels in AD might lie in the regulatory mechanisms of Aβ production. The amyloid precursor protein (APP) is subjected to proteolytic cleavage by secretases [348]. When cleaved by α-secretases (TACE, ADAM10, PC7), it gives origin to α-amyloid peptide, while the γ-secretase produces Aβ. It is not clear if the low sRAGE levels are a result of lower ADAM10 activity, which also favors Aβ formation, or a more complex mechanism of regulation. Regardless, a greater issue might be the intact membrane RAGE that remains fully functional due to this lower activity of ADAM10. Mice knocked down for RAGE present decreased uptake of labeled Aβ in comparison to wild type animals. Furthermore, mice expressing human esRAGE show the same uptake inhibition as the RAGE knock-out group [349].

In vivo, the variation in sRAGE levels may be mostly explained by the differences among study populations. Most of them do not precise information about ethnicity and RAGE polymorphisms, which are known to influence sRAGE levels [219, 350]. Kidney dysfunction, which is clearly associated with higher sRAGE levels, is not well defined among the different studies, a fact that may represent a bias. Nevertheless, after analyzing the biological variations of sRAGE, Brown and colleagues observed that sRAGE has a low index of individuality which is associated with little utility for population-based reference values [351]. In addition, they state that such low index is valuable in monitoring diseases. In a pharmacokinetics study, Renard *et al.* observed that recombinant rat sRAGE has a longer half-life in diabetic rats than non-diabetic animals. The authors suggested that the increased AGEs in diabetes constitute an additional compartment for the distribution of sRAGE [196]. In this case, AGEs in tissues could act

as sequestering agents, decreasing circulating sRAGE. Moreover, it is unknown whether sRAGE-ligand complexes are further cleared from the organism or internalized in tissues.

Different studies present different factors associated with sRAGE levels. Sometimes, probably due to different inclusion criteria and a diverse pathophysiology, opposite associations are observed (Table 18). In addition, only a few studies investigated the association of sRAGE with these factors alone and the high regulatory complexity of sRAGE formation does not allow fairly conclusive interpretations. An increase in soluble RAGE levels is often associated with acute inflammation. After severe trauma, sRAGE increases and is associated with trauma severity (n=176) [313]. This view of an acute increase in sRAGE is supported by other studies where sRAGE increases immediately after surgery [290, 294]. On the other hand, Achouiti *et al*. did not observe changes in sRAGE after experimental human endotoxemia (n=8) or during severe sepsis (n=51) [352].

Another important factor that may underlie sRAGE variations is the genetic background. Hudson and colleagues observed considerable differences in sRAGE mean levels according to ethnicity, with non-Hispanic black subjects having lower sRAGE levels than non-Hispanic white and Hispanic ones [353]. In a study with type 2 diabetics only, afro-Caribbean patients had lower sRAGE than white and south Asian ones [219]. In a recent meta-analysis of 27 studies (7585 CAD patients and 9240 controls), it was observed that ethnicity, study design, matched information and sample size are potential sources of heterogeneity [205]. This study also showed that the contribution of RAGE polymorphisms to CAD incidence depends on ethnicity. However, the authors underscore the coherence of esRAGE as a negative predictor of CAD development.

In conclusion, evidence suggests a very complex regulation of esRAGE and cRAGE formation (Figure 8). Cell-specific mechanisms are very likely to take place and, since the

major source of serum sRAGE remains unknown, the current knowledge of these mechanisms reflects little about the variability of sRAGE levels among publications.

Table 18. Factors associated with sRAGE levels in vivo

FACTOR	ASSOCIATION	REFERENCE
Kidney dysfunction	↑ Higher sRAGE levels in subjects with decreased glomerular filtration rate (1500 pg/ml; n=283) compared to normal kidney function (1200 pg/ml; n=265)	[269]
	↓ Type 2 diabetics with glomerulopathy and retinopathy (1068pg/ml; n=10) have lower sRAGE than those without (1575 pg/ml; n=20)	[221]
	↑ Associated with albuminuria in type 2 diabetes (n=110; β=0.22)	[231]
	↑ Higher sRAGE in non-diabetic chronic kidney disease patients (1244 pg/ml; n=20) than in healthy controls (506.3 pg/ml; n=20)	[275]
	↑ sRAGE is associated with serum creatinine in patients with chronic kidney disease (r=0.5; n=25)	[278]
Smoking	Ø No difference between smokers and non-smokers in COPD (n=200) patients and in controls (n=201)	[287]
	Ø No association with the number of cigarettes/day nor with the years of smoking in male smokers (n=485).	[306]
	↑ Healthy smokers (n=45) have higher sRAGE levels than non-smokers (n=50). sRAGE correlates with the number of cigarettes/day (r^2=0.36).	[322]
	↓ Inversely associated with smoking habit (β=0.223) in acute stroke patients (n=482)	[354]
	Ø No association in elderly hypertensive patients (n=271)	[255]
Obesity/ Overweight	↓ Inversely correlated with waist circumference (r=-0.27) in subjects with metabolic syndrome risks (n=263).	[355]
	↓ Inversely associated with BMI (r=-0.24) and waist-to-hip ratio (r=-0.21) in a general population (n=176)	[317]
	↓ Type 2 diabetics within the lowest BMI tertile have higher sRAGE levels (n=718)	[219]
	↓ Morbidly obese patients (n=85) have lower sRAGE levels than controls (n=40)	[356]
	↓ Inversely associated with BMI in elderly hypertensive patients (n=271; β=-0.181)	[255]
	↓ Inversely associated with BMI in the general population (β=-0.177; n=184)	[225]

Statins	Ø No association in elderly hypertensive patients (n=271)	[255]
	Ø No effects on sRAGE levels after 8-12 months of treatment in acute coronary syndrome patients (n=208)	[357]
	↑ 6 months of atorvastatin treatment slightly increases sRAGE levels (673 to 737 pg/ml) in type 2 diabetics (n=39)	[338]
	↑ Eight-week atorvastatin but not pravastatin increases sRAGE (843 to 1026 pg/ml) in hypercholesterolemic patients (n=10).	[337]
	Ø One-year treatment with atorvastatin does not change sRAGE levels in type 2 diabetics (n=184)	[219]
Ethnicity	Japanese healthy population with mean sRAGE at 450 ± 150 pg/ml (n=184; mean age=66.7 years)	[225]
	Healthy Chinese elderly (mean age=72 years) with median sRAGE 905 pg/ml (n=254)	[246]
	Healthy Japanese elderly (mean age=65 years) with mean sRAGE 1103 pg/ml	[358]
	Non-hispanic black subjects have lower sRAGE levels (757.4 pg/ml; n=197) than hispanic (891.9 pg/ml; n=708) and non-hispanic white individuals (1120.5 pg/ml; n=170)	[353]
	Healthy Brazilian subjects (1472.75 pg/ml; n=24; mean age=32.7 years)	[227]

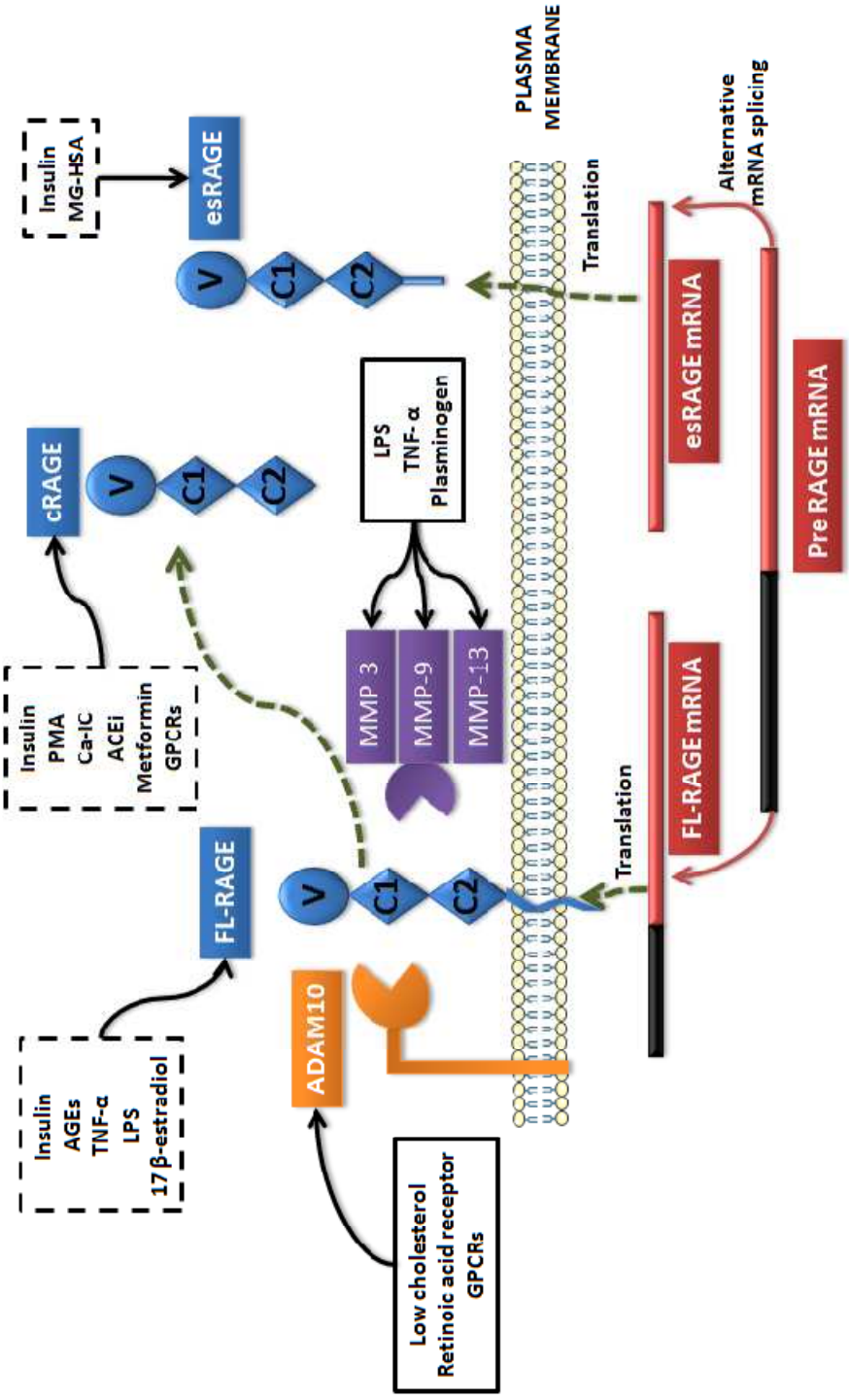

Figure 8. Mechanisms of sRAGE formation. Dashed boxes show the stimuli known to induce each RAGE form. Closed boxes present stimuli that increase the activity of proteases. FL-RAGE, full length RAGE; cRAGE, cleaved RAGE, esRAGE, endogenous secretory RAGE; ACEi, angiotensin-converting enzyme inhibitor; MMP, matrix metallopeptidase; ADAM, a disintegrin and metalloproteinase; TNF, tumor necrosis factor; LPS, lipopolysaccharide; GPCR, G protein-coupled receptor; PMA, phorbol myristate acetate; AGE, advanced glycation end-product; MG-HSA, methylglyoxal-modified human serum albumin; Ca-IC, calcium ionophore calcimycin.

1.7 Soluble RAGE as a therapeutic agent

There is overwhelming evidence supporting the use of sRAGE as a therapeutic molecule. In cultured endothelial cells, recombinant sRAGE blocks AGEs-induced oxidative stress, hyperpermeability and the expression of adhesion molecules [9, 11, 16]. Soluble RAGE also suppresses RAGE, HMGB1 and VCAM-1 overexpression induced by shear-stress [359]. *In vivo*, sRAGE ameliorates diabetes induced hyperpermeability and atherosclerosis [196, 254], and it also accelerates wound healing in diabetic mice [360].

Treatment with sRAGE was shown to improve survival ad reduce levels of proinflammatory cytokines IL-1β, IL-6 and TNF-α in a model of severe sepsis [361]. The use of sRAGE as a therapeutic molecule was further supported by the studies of Renard and co-workers, who showed a relatively long half-life of recombinant sRAGE in rats [196, 362].

Other approaches to block the RAGE axis could be the use of anti-RAGE antibodies, the induction of RAGE cleavage or the use of specific inhibitors. Indeed, PF-04494700, an oral RAGE inhibitor, was already tested in AD patients in a 10-week trial. Although it had no effects on Aβ levels and cognitive outcomes, no deleterious effects were seen either, allowing larger long-term trials [363].

1.8 Anti-sRAGE autoantibodies

Autoimmunity against sRAGE was observed for the first time in mice that were immunized with glycated human brain neurofillament protein [364]. Interestingly, the same animals developed antibodies against Aβ. Anti-Aβ IgGs were already studied in the course of AD with contradictory results. While some works failed to observe an association between anti-Aβ and the disease [365, 366], others suggested a higher toxicity of the Aβ-antibody complexes [367]. In healthy subjects, higher levels of such complexes were observed

compared to the free form of Aβ, but no association with cognitive ability or age was found [368].

In AD patients, anti-sRAGE IgGs are higher compared to healthy seniors [369] and other studies showed an association between anti-sRAGE titers and dementia, assessed by the Clinical Dementia Score (CDR) and the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) [370, 371]. In the same works, higher levels of anti-Aβ were also associated with dementia. Very interestingly, the group that observed these associations investigated a protective role of such autoantibodies by stimulating their production with and oral vaccine [372]. Indeed, this vaccine, constituted of a Aβ/sRAGE complex improves cognitive function in a transgenic mice model of Alzheimer's disease. Moreover, purified anti-sRAGE autoantibodies protect primary rat cortical neurons from Aβ toxicity. Nonetheless, it is unknown if these autoantibodies could neutralize sRAGE decoy effect or even activate RAGE. A double-edged sword effect is very likely and the incidence of these autoantibodies in other diseases where RAGE is implicated needs to be investigated.

Chapter Two

Metabolic and Inflammatory Disorders

RAGE has been particularly studied for its pathological roles in metabolic disorders. Therefore, such disorders were focus of studies on sRAGE levels. Metabolic disorders arise from the impairment of energy processing at tissue and cellular levels. The consequent morbidity and mortality are intimately associated with inflammation and cardiovascular complications. These issues are often originated from dyslipidemia and an exacerbated immune response. Although still unclear, autoimmunity is believed to play an important role in the pathogenesis and complications of these disorders. In this second chapter we summarize the pathological aspects of these diseases in which RAGE is deeply implicated and sRAGE was proposed as a biomarker.

2.1 Cardiovascular Diseases

Cardiovascular diseases (CVDs) are the leading cause of mortality and morbidity in the world. CVDs include hypertension, cerebrovascular disease, heart failure, congenital heart disease, peripheral artery disease, rheumatic heart disease and coronary heart disease (CHD), the latter being responsible for 42% of the mortality associated with CVDs [373]. The major risk factors for the development of CVDs are tobacco, physical inactivity, alcohol consumption and unhealthy diet [373].

CVDs arise mostly from hypertension and atherosclerosis. Essential hypertension, or elevated blood pressure, is a disease of unknown specific cause. Evidence indicate that inflammatory processes play a major role in the expression of adhesion molecules and the

activation of the renin-angiotensin system, which are key mechanisms in the pathogenesis of hypertension [374].

Little is known about RAGE activation in association with hypertension. In an animal model, Bohlender and colleagues observed increased RAGE expression in the glomerulus of hypertensive rats. It was also observed that angiotensin-converting enzyme (ACE) inhibitors (ACEi) reduce the expression of RAGE in diabetic rats [328].

Soluble RAGE was measured in hypertensive patients, with distinctive results. While Geroldi *et al.* [257] showed that hypertension was associated with lower sRAGE levels, while Nakamura and colleagues showed that anti-hypertensive treatment promoted further decrease in sRAGE [256].

Intimately associated with hypertension is atherosclerosis. Atherosclerosis is a chronic inflammatory process, characterized by the arterial wall thickening through accumulation of fatty components (i.e. cholesterol) and macrophages [375]. Atherosclerosis origin is multifactorial, with a major role played by hypertension, hyperlipidemia, diabetes, western diet and smoking [376-378]. It is generally initiated by the expression of adhesion molecules by endothelial cells, in the innermost layer of vessels. Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) play a major role in atherosclerosis, binding T lymphocytes and monocytes [379-381]. The expression of adhesion molecules is induced by pro-inflammatory molecules such as oxidized lipoproteins [382], interleukin 1 (IL-1), interleukin 4 (IL-4) and interferon gamma (IFN-γ) [383].

Once attached to the endothelium, monocytes migrate to the intima layer, due especially to the expression of monocyte chemoattractant protein-1 (MCP-1) [384, 385]. Monocyte migration, also called diapedesis, is clearly dependent on MCP-1 since mice with predisposition to atherosclerosis but lacking MCP-1 or its receptor do not develop lesions [386, 387]. In the intima, monocytes differentiate into macrophages and incorporate modified lipids [388]. Low-density lipoprotein (LDL) may actively or passively pass through endothelial junctions and accumulate [389]. In the subendothelium, LDL is oxidized and cleaved, and these modifications increase its engulfing by macrophages [390].

In contrast to LDL, which increases cholesterol and lipids in extra-hepatic tissues, high-density lipoprotein (HDL) is responsible for removing cholesterol excess. Apolipoprotein A-I is the major protein component of HDL, responsible for cholesterol and lipid binding due to its amphipathic properties. Thus HDL reduces lipid affluence in the vessel walls, further decreasing lipid oxidation and monocyte recruitment [391, 392]. Other mechanisms of HDL action include the inhibition of the expression of vascular adhesion molecules and MCP-1.

Clinically, cardiovascular risk is assessed by factors associated with inflammation and increased cholesterol levels and deposition. Increased plasma LDL and cholesterol are closely related to increased risk of CVDs [393]. On the other hand, HDL plays a protective role by sequestering cholesterol from the endothelium and is, therefore, associated with lower risk of CVDs [393]. In any case, inflammatory and metabolic disorders like diabetes, obesity and kidney dysfunction favor the development of CVDs.

Unfortunately, there are no specific molecules to diagnose CVDs and their severity. The diagnoses are made mostly with imaging techniques or an electrocardiogram. Moreover, inflammatory markers like C-reactive protein (CRP)/high sensitivity (hs) CRP, tumor necrosis factor α (TNF- α), IL-10, IL-8 lack specificity and are more likely to evaluate the associated risk. The imminence and progression of atherosclerosis lesions is easily

assessed by the intima-media thickness (IMT). The IMT, which can be performed by non-invasive ultrasound, provides important information about advancement of lipid deposition and monocyte infiltration.

RAGE actively participates in atherosclerosis progression. Blockade of RAGE with recombinant sRAGE drastically inhibits plaque formation in mice [254]. RAGE activation increases ROS production and the expression of adhesion molecules, which further accelerate atherosclerosis [394].

There are several studies on sRAGE levels and their relation to atherosclerosis. Even though most of them evaluate CVD incidence and risk in the course of other diseases (i.e. diabetes and kidney disease), some studies were made in populations without complications other than CVDs. While sRAGE was found higher in symptomatic atherosclerosis [268] and associated with plaque vulnerability [271], patients with aortic valve stenosis have lower sRAGE levels [270].

2.2 Diabetes

The risk of CVDs increases substantially with diabetes, which doubles the mortality risk compared to peers without the disease. Among diabetics, the leading cause of death are CVDs, which also represent the major co-morbidities associated with the disease [395]. In the world, more than 347 million people are diabetic and this number is expected to double before 2030 [395].

Type 1 diabetes has a strong immune component, responsible for the destruction of pancreatic β cells and it is, in part, genetically determined. It is, sometimes, also called juvenile diabetes because of its early onset (<30 years-old) but the strong environmental component affects drastically the age of onset [396]. Type 2 diabetics, however, is closely associated with obesity and advanced age through the development of insulin resistance

[397]. Insulin resistance is caused by desensitization to insulin due to hyperlipidemia and inflammation. It has been proposed that the increase of fatty acyl CoAs and diacyl-glycerol induce serine/threonine kinases, inhibiting the insulin receptor substrate-1 (IRS-1) and further reducing downstream signaling to insulin action [398]. The decreased anabolic effect of insulin results in hyperglycemia and weight loss.

While the diagnose and progression of diabetes are easily assessed by means of glucose levels and glycated hemoglobin (HbA_{1C}) [399], the risk of associated complications is more difficult to evaluate, due to their complex etiology. However, the clinical value of laboratory tests are under constant debate, for limitations of methodology and the impact of other variables (i.e. ethnicity) should be taken into account [400]. Nonetheless, diabetes complications are often investigated independently of diabetes itself, without neglecting the aggravations of the disease. To date, the major biomarkers diabetes-associated risks are glycemia, HbA_{1C}, lipid profile (LDL, HDL and triglycerides) and urinary albumin excretion [401].

It is widely known that glucose exerts its toxic effects through the non-enzymatic modification of biomolecules. These modified products, which may have their function impaired, are subject to structural modifications that may lead to the formation of AGEs. The reactions that end up with AGE formation might produce highly reactive intermediary products such as methylglyoxal and glyoxal. These dicarbonyl compounds are also capable of modifying biomolecules [402] and spontaneously modify glutathione, impairing the cellular antioxidant pool [18]. The resulting oxidative stress induces damage to proteins, lipids and DNA and increase the rate of glycation, generating a vicious cycle that ultimately promotes cell and tissue dysfunction [403]. As described in the first chapter, AGEs induce oxidation and inflammation through interaction with RAGE.

The first pathological mechanisms involving RAGE were described in diabetes. RAGE activation increases ROS production and endothelial permeability [10]. Moreover, RAGE

plays an important role in diabetes-associated atherosclerosis and decreased insulin secretion [254, 404, 405].

Serum sRAGE has been extensively studied in diabetes and, as presented in the first chapter, there is controversy. Nevertheless, in a nested-case control study, Colhoun *et al*. observed that higher sRAGE levels were associated with increased coronary heart disease incidence in type 2 diabetes [219]. However, the recent findings of insulin regulating RAGE expression and sRAGE production puts a warning in studies concerning sRAGE levels, especially those where patients have impaired insulin signaling [326].

2.3 Obesity

Obesity is considered as a medical condition where the excess of body fat may impair health [406]. In addition, the distribution of accumulated fat is as important as the accumulation rate to determine the risks associated with obesity. For example, abdominal fat, evaluated as waist-to-hip ratio (WHR) and waist circumference, are strongly related to CVD risk in non-obese [407] and obese subjects [408]. The body-mass index (BMI) is the current reference index to diagnose and classify obesity. It is defined as the weight divided by the square of height (kg/m^2). A BMI of 30 or higher characterizes obesity. Table 4 shows the weight classification according to BMI.

Table 19. Weight classification according to body-mass index.

Classification	BMI (kg/m^2)
Underweight	<18.50
Normal weight	18.50 – 24.99
Overweight:	≥ 25.00
Pre-obese	25.00 – 29.99
Obese class I	30.00 – 34.99
Obese class II	35.00 – 39.99
Obese class III	≥ 40.00

Obesity increases the risk of developing diabetes and CVDs. Hubert and colleagues investigated the relationship between obesity and CVD among 5209 subjects in a 26-year follow-up. In both men and women, the Metropolitan Relative Weight (MRW, similar to BMI) was associated with CHD [409]. Particularly in women, MRW was associated with stroke and CVD death. In much larger cohort with 115886 women, the influence of obesity in CHD was evaluated after an 8-year follow-up [410]. Increased body-weight was found associated with hypercholesterolemia, non-fatal and fatal CHD. In the heaviest group (BMI≥29), 70% of coronary events were attributed to obesity. Similarly, Dorn *et al.*, observed an all-cause mortality associated with elevate BMI. However, this association was found only in men younger than 65 years-old. Furthermore, BMI was strongly related to CHD and CVD mortality, in both sexes [411]. Hodgson and colleagues, however, showed a correlation between CHD and WHR, but not BMI [412]. A study in 2003 showed that severe obesity (BMI>45) is responsible for a reduction of life expectancy of 13 and 8 years for men and women, respectively [413]. On the other hand, a recent study of Flegal and colleagues [414] observed an increase in longevity in people with overweight (BMI of 25 – 29.9) even when compared to normal weight subjects (BMI of 18.5 – 24.9).

It is common sense that obesity prevention and treatment are successfully achieved through regular physical activity and lowering of calorie intake. Surgical intervention is particularly considered for patients who have failed previous attempts of losing weight and present a BMI≥40 or 35 with medical conditions other than obesity [415]. One of the most common interventions is the gastric bypass, or *Roux-en-Y*, where a small stomach pouch is created while the rest of the stomach and part of the duodenum are bypassed (Figure 9). The efficacy of this procedure is up to 68% of weight loss [416].

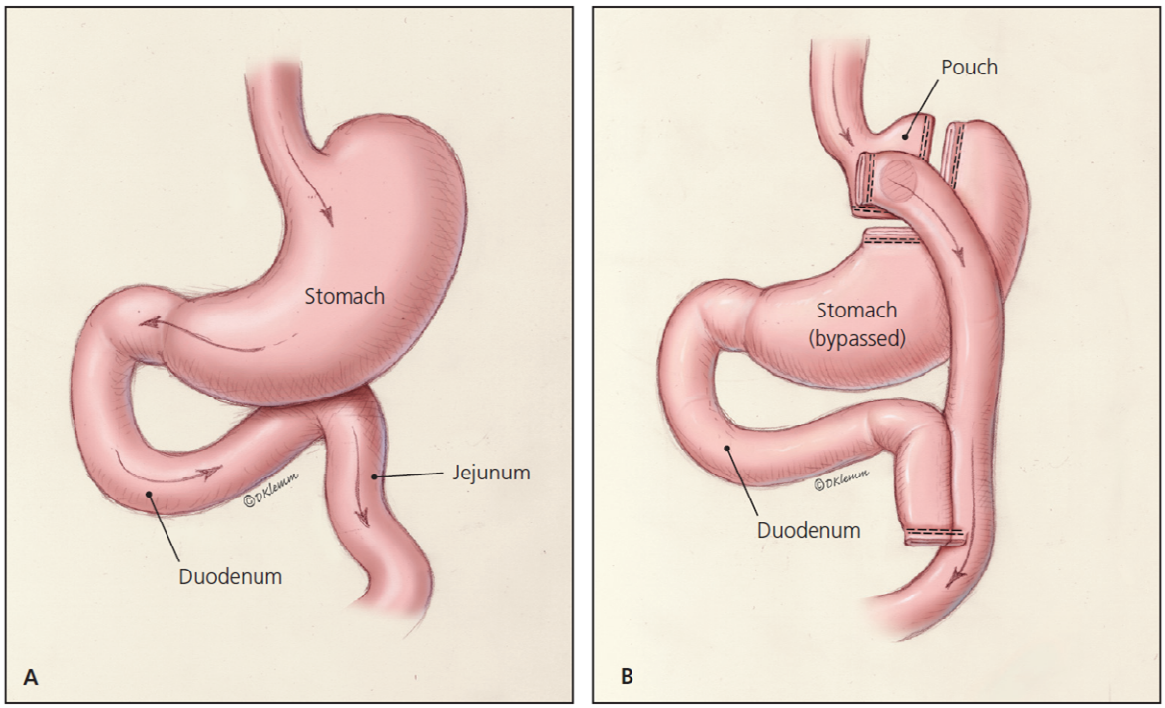

Figure 9. Schematics of a gastric bypass (*Roux-en-Y*). Panel A shows the normal gastric flow. Panel B shows the bypassed stomach and duodenum.

As in diabetes, obesity is considered as an aggravation factor for other complications such as CVDs and renal failure. The impairment of metabolism, which results in increased circulating lipids lead to higher levels of LDL and cholesterol, has a profound impact on inflammation. The increase in adipose tissue is accompanied by a pro-inflammatory state. Adipocytes and infiltrated immune cells release cytokines that, together with the increase in free fatty acids, decrease insulin sensitivity [417]. Several pro-inflammatory cytokines are associated with insulin resistance, like IL-1, IL-6, IL-10, CRP and TNF-α [418]. The subsequent hyperinsulinemia maintain high levels of circulating lipids and Na^{2+}, leading to hypertension [419].Thus, it is clear that while obesity has a substantial behavioral basis, the events following dyslipidemia and adipose tissue hypertrophy impair health condition through inflammation and cardiovascular dysfunction.

The RAGE axis is still to be further investigated in obesity. Different studies found opposite results concerning weight gain and RAGE in animals given a high fat diet. The

first, in 2012, showed that RAGE -/- mice gained weight faster and had higher levels of cholesterol and insulin compared to wild-type animals [420]. The other, in 2013, observed that the presence of RAGE increased weight gain, epididymal fat and adipocyte size [47].

In humans, a few studies have investigated sRAGE levels and their association to body weight. It was first observed that sRAGE levels are inversely correlated with BMI in the general population [317]. More specific was the work of Brix and colleagues, that observed lower sRAGE levels in morbidly obese subjects and an increase in these levels after bariatric surgery [356].

2.4 Autoimmunity

All the above aforementioned diseases have a strong inflammatory component, with a major role played by the immune system. However, an exacerbated inflammation may result in the recognition and destruction of self-antigens, which characterizes autoimmunity. Autoimmune diseases are a heterogeneous group of disorders but some systemic autoimmune conditions like rheumatoid arthritis (RA), antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE) share an increased risk of CVDs [421]. Although genetic and epigenetic factors constitute predisposing factors for autoimmune diseases, trigger factors like diet, smoking and pathogens play an important role [422].

Although autoimmune reactions may arise from infectious agents that mimic some of the host's self-antigens, several cases are reported without apparent infection. Autoimmunity is also attributed to modified biomolecules, such as oxidized LDL [423] and, since the recruitment of inflammatory cells increases the production of ROS, so does tissue damaged [424], amplifying the cycle of autoimmunity (Figure 10).

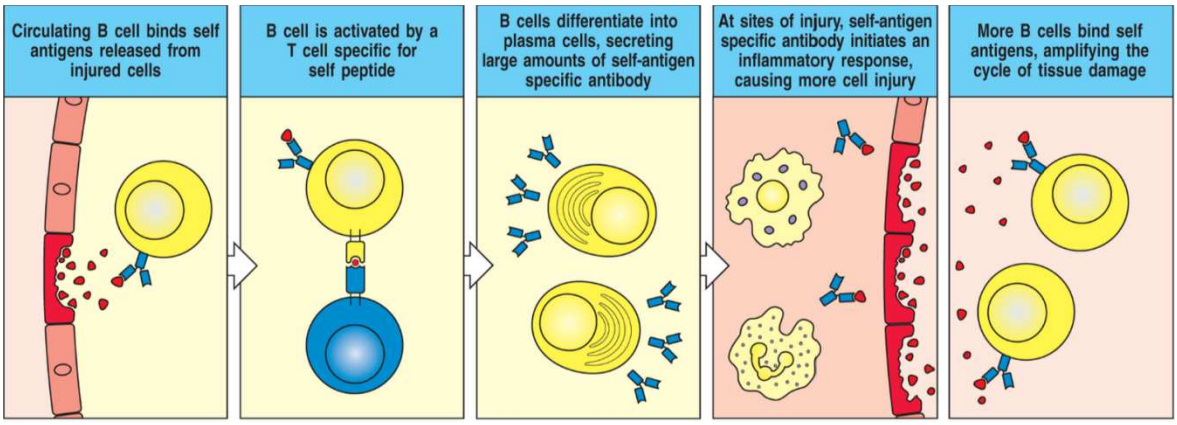

Figure 10. Amplification of autoimmune damage. Once self-antigens promote an autoimmune reaction, further inflammation occurs and tissue damage increases.

The participation of autoimmune reactions in atherosclerosis is still unclear even though cells of the immune system participate actively in atherogenesis. Immunohistological studies show that lymphocytes and macrophages are already present in the intima of early lesions, before clinical symptoms [425, 426]. The presence of vascular antigens in the early events of atherosclerosis has been subject of discussion [427] and the list of candidates is narrow. Oxidized LDL is known to possess immunogenic epitopes [428] and to contribute to atherogenesis [429]. Wick and colleagues have proposed that the origin of atherosclerosis is of inflammatory nature, mainly through the immune response to heat shock protein 60 (HSP60), expressed by endothelial cells [430, 431]. Heat shock proteins (HSPs) are stress-responsive molecules that participate in protein transport, folding and degradation by their chaperone activity [432]. HSP60 is expressed in the mitochondria but may be translocated to the cytosol and further to the cell surface. Its immunogenicity is attributed to the great sequence homology between human and bacterial forms [433]. Damaged endothelial cells increased HSP60 expression and secretion, favoring the antibody production by B lymphocytes (Figure 11). Since the risk of atherosclerosis is increased in virtually all inflammatory and metabolic disorders, this hypothesis places autoimmunity at the root of the major health burden in the world.

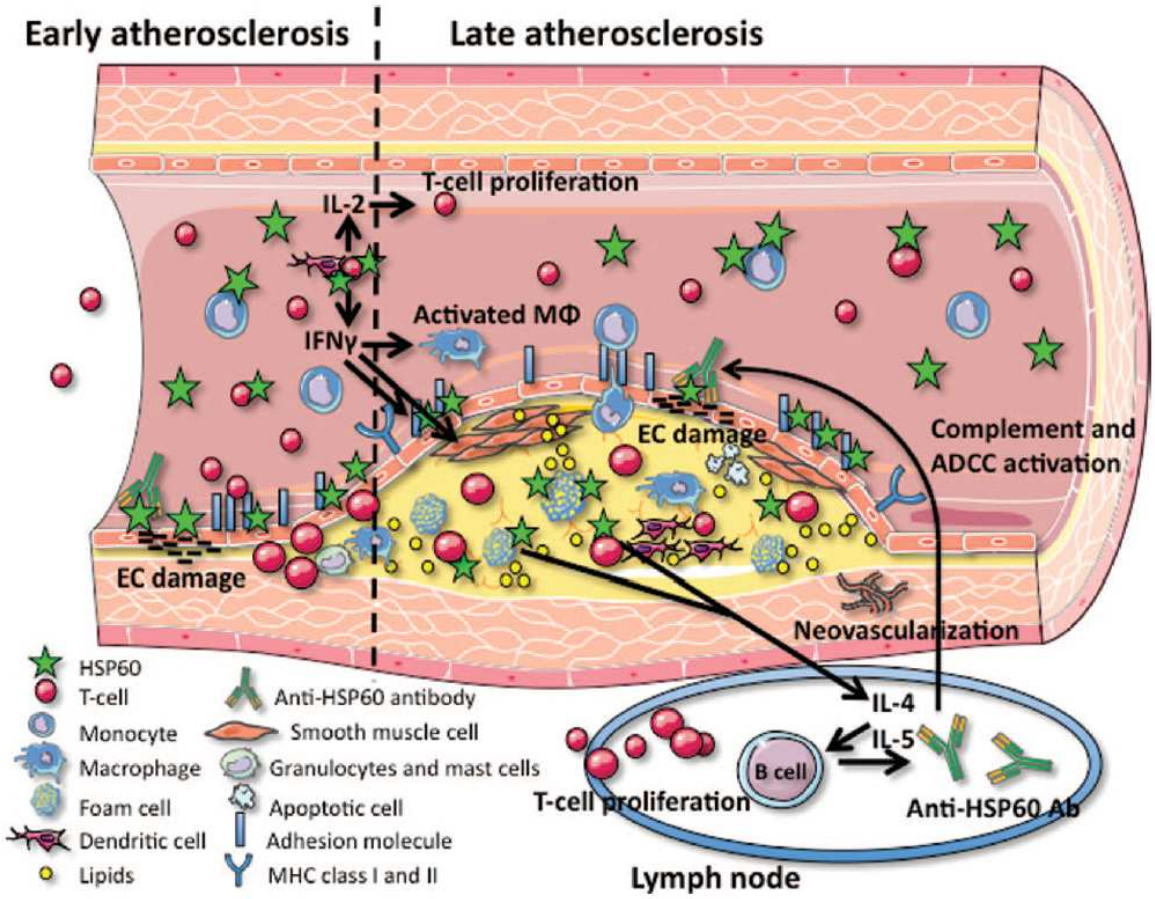

Figure 11. Autoimmune mechanisms of atherosclerosis. Endothelial cells under stress overexpress HSP60, inducing the recruitment and adhesion of T cells and monocytes that will further transmigrate into the intima [434]. EC, endothelial cell; ADCC, antibody-dependent cellular cytotoxicity; IFN, interferon; IL, interleukin

Type 1 diabetes has a well-defined autoimmune component with reactivity against islet cells and insulin. B lymphocytes play a major role by infiltrating the pancreas and producing antibodies against insulinoma-associated antigen-2, glutamic acid decarboxylase 65, insulin and zinc transporter 8 [435]. Very interestingly, Oresic and colleagues proposed that lipid and amino acid metabolism dysregulation precedes autoimmunity in type 1 diabetes [436]. They observed reduced levels of phosphatidylcholine and succinic acid but increased glutamic acid and ketoleucine before seroconversion. Although further investigation is needed, these findings may serve as background for the investigation of autoimmunity in other metabolic disorders. In type 2

diabetes, there is not enough evidence supporting autoimmunity although the immune system plays an important role by mediating the inflammatory events that lead to insulin resistance. Type 1.5 diabetes has been proposed and named latent autoimmune diabetes of adults (LADA). Patients with LADA do not require insulin treatment, but are positive for antibodies against islet cells, just like type 1 diabetics. In addition, as in type 2 diabetes, insulin resistance seems to play an important role, but data is controversial [437].

In the case of obesity, autoimmunity has been mostly associated with the thyroid [438, 439]. Despite the relation between hypothyroidism and obesity, Tamer and co-workers observed an association between anti-thyroglobulin autoantibodies and the levels of triglycerides and cholesterol, independent of thyroid function [440]. On the other hand, Lombardi *et al*. found increased autoantibodies against a disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMST) 13 in obese subjects and these levels decreased after bariatric surgery [441]. Unfortunately, the mechanisms responsible for obesity-associated autoimmunity are unknown and it is unclear whether autoantibodies are just a consequence or play an important role in the origin of obesity-related complications.

RAGE is a member of the immunoglobulin superfamily and has a putative role in immunity. Indeed, binding of phosphatidylserine [66] and complement component C1q [67] to RAGE increases phagocytosis. Furthermore, RAGE knockout mice respond differently to systemic infections, with higher or lower mortality depending on the disease model [58-61]. In autoimmune diseases like Kawasaki disease and Myasthenia gravis, RAGE is over expressed [442, 443].

Soluble RAGE was measured in patients with autoimmune diseases and lower levels were observed in SLE [321], RA [310] , Sjögren's syndrome [311] and Myasthenia gravis [444]. Nevertheless, as for other diseases, it remains unclear whether low sRAGE levels represent reduced protection against RAGE-mediated deleterious effects or just a

fingerprint of such mechanisms. In addition, the recently described anti-sRAGE autoantibodies [364, 369, 445] further implicate RAGE in autoimmunity, though their biological effects in vivo remain unclear.

Chapter Three

Objectives

Soluble RAGE has been extensively studied in human diseases and widely proposed as a biomarker of metabolic and inflammatory complications. However, its levels are controversial and demand a closer examination of the factors that might regulate them.

As a pattern-recognition receptor, sRAGE binds a great diversity of ligands and their effects on sRAGE quantification are unclear. In this context, anti-sRAGE autoantibodies emerge as possible sRAGE-binding molecules and as potential immunological component of metabolic and inflammatory diseases, where RAGE expression is increased. Nevertheless, anti-sRAGE autoantibodies remain to be better studied.

The objectives of this study were:

- ω To investigate the effects of RAGE ligands and anti-RAGE autoantibodies on sRAGE quantification, in order to evaluate a possible role of these molecules in the variation of sRAGE levels presented in the scientific literature. This objective was developed in the first article of this thesis.

- ω To evaluate, in a well-established cohort in Lille (ABOS), if serum levels of sRAGE and anti-sRAGE autoantibodies are associated with morbid obesity, weight loss and metabolic improvement after gastric bypass. This objective was developed in the second article of this thesis.

Chapter Four

Articles

The research developed specifically in this thesis originated two manuscripts that were submitted to peer-reviewed journals. The first manuscript, “Do RAGE ligands or anti-sRAGE autoantibodies interfere with sRAGE quantification?” was accepted for publication in the journal *Annals of Clinical Biochemistry* and is presented herein in its accepted version. The second manuscript, entitled “Anti-sRAGE autoantibody: a new biomarker during obesity” was submitted to the *International Journal of Obesity* and is currently under review. It is presented here in its original version.

4.1 Do RAGE ligands or anti-sRAGE autoantibodies interfere with sRAGE quantification?

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Abstract

Background: The soluble form of the receptor for advanced glycation end-products (sRAGE) has been studied in various diseases. It is not clear why sRAGE levels vary between studies, with controversial results. What also remains to be determined is whether RAGE ligands could affect sRAGE assessment by epitope masking. Recently described anti-sRAGE autoantibodies may play an interfering role. The aim of this study was therefore to investigate the influence of RAGE ligands and anti-sRAGE autoantibodies on sRAGE quantification.

Methods: The RAGE ligands carboxymethyllysine (CML; AGEs with a high affinity to RAGE), S100 proteins, high mobility group protein B1 (HMGB1), and β-amyloid peptide (aβ) were tested by ELISA with recombinant sRAGE (rHu-sRAGE), or serum from healthy controls. Using ELISA, anti-sRAGE autoantibodies (IgGs) were identified in hemodialysis (HD) patients, then purified and incubated with rHu-sRAGE or serum to investigate their effects on sRAGE levels.

Results: RAGE ligands, either alone at 3 different concentrations (CML was also tested at different glycation levels) or a mixture of all these ligands did not affect sRAGE levels when incubated with rHu-sRAGE or control serum. Compared to healthy controls, HD patients have higher levels of sRAGE ($p<0.001$) and anti-sRAGE IgGs ($p<0.05$). However, incubation of rHu-sRAGE with purified IgGs from HD patients had no effect on sRAGE quantification.

Conclusions: RAGE ligands or anti-sRAGE autoantibodies did not interfere with sRAGE quantification. Further studies are required to elucidate the variability in sRAGE levels reported in the literature, and to define the potential of sRAGE for use as a reliable biomarker.

Introduction

Metabolic and inflammatory disorders constitute a major health problem and thus an additional socio-economic burden, and are among the leading causes of mortality and morbidity throughout the world [373, 395, 406, 446, 447]. Although much progress has been made in the fields of prognosis and diagnosis, effective and reliable biomarkers are still needed. Triglycerides and LDL-cholesterol (associated with dyslipidemia, caused by abnormally high levels of lipids and lipoproteins in the blood), glycated hemoglobin and, more recently, C-reactive protein (CRP) are well identified as vascular risk biomarkers [448]. A large number of vascular complications may occur in the course of diseases that do not have specific vascular biomarkers (e.g. renal failure, hypertension, vascular aging). To prevent and more easily manage such risk, new vascular biomarkers need to be found.

RAGE, the receptor for advanced glycation end-products (AGEs) is a multiligand receptor belonging to the immunoglobulin superfamily of cell-surface molecules [1]. It is known to play a major vascular role in metabolic (e.g. diabetes mellitus, renal failure) and inflammatory disorders, and RAGE activation is followed by pro-inflammatory, pro-thrombotic and neoangiogenic cell responses.[21, 22, 449-451] RAGE contains an intracellular domain, a short transmembrane domain and an extracellular domain consisting of 3 immunoglobulin-like regions, i.e. 1 ‘V’ type followed by 2 ‘C’ types. The ‘V’ type is essential for most ligand binding [98].

Soluble RAGE (sRAGE) corresponds to the extracellular domain, and is able to act as a decoy to avoid interaction between RAGE and its pro-inflammatory ligands. Carboxymethyllysine (CML, the AGE with highest affinity to RAGE),[134] S100/calgranulins, high-mobility group protein B1 (HMGB1/amphoterin) and β-amyloid peptide (aβ) are the most widely studied RAGE ligands [28, 452]. These ligands bind to different sites of sRAGE (Fig. 1) [98].

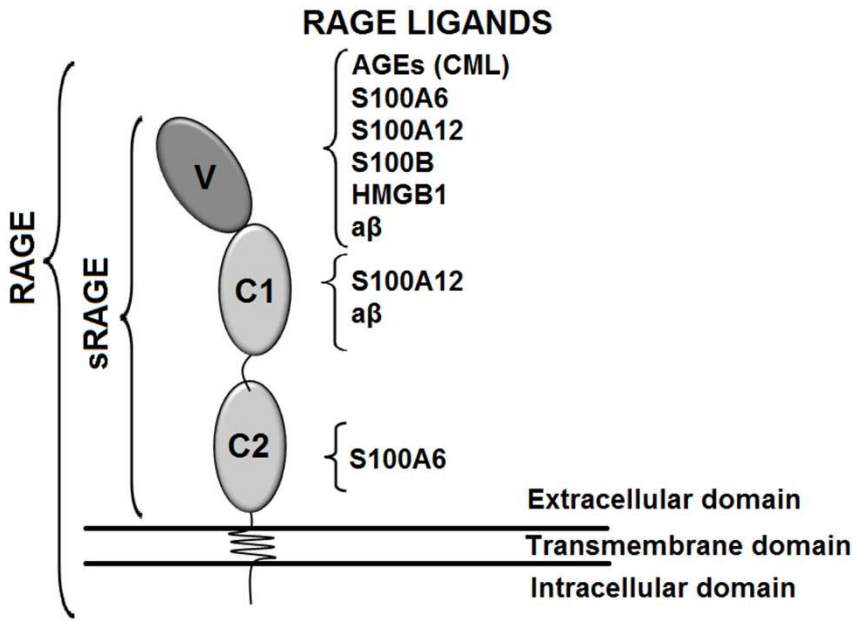

Figure 1. RAGE ligands bind to different sites of sRAGE.
As a pattern-recognition receptor, RAGE has different binding sites depending on which ligand is involved. The preferential binding sites for anti-sRAGE autoantibodies have not yet been determined. CML (carboxymethyllysine), S100A6, S100A12 or S100B, HMGB1 (high-mobility group protein B1), aβ (amyloid beta peptide).

sRAGE has been extensively studied in patients with diabetes or renal failure, and in subjects with certain inflammatory vascular diseases [221, 275, 278]. Although sRAGE may play a potential role as a decoy receptor and vascular biomarker, its quantification remains a subject of controversy. While associations between sRAGE levels and disease severity/complications are often observed, it is surprising to note that for the same disease, the results regarding such associations are frequently conflicting: some may be negative, others positive[212, 217, 220, 221, 224, 258, 260]. Certain factors are also considered to modulate sRAGE levels, including drug intake, renal failure, RAGE polymorphisms and advancing age.

As sRAGE quantification is performed worldwide by ELISA, in this study it was hypothesized that RAGE ligands might impair its quantification by masking its epitopes. In addition, the role of anti-sRAGE autoantibodies was also investigated (their activity has

been described in Alzheimer's disease and in the course of rheumatoid arthritis) [369, 445], thereby increasing the range of RAGE-binding molecules.

The aim of this study was to evaluate the effect of CML, S100 A6, A12 or B, HMGB1 and aβ binding on the 3 domains of sRAGE, and that of anti-sRAGE autoantibodies on sRAGE quantification by ELISA.

Materials and Methods

Chemicals

HMGB1 (ab82100), S100 A6 (ab104645), A12 (ab103393) and B (ab54050) were purchased from Abcam. Anti-CML antibodies (MAB3247) and Fc-free recombinant human sRAGE (FcFree-rHu-sRAGE) (SRG00) were purchased from R&D Systems. Human recombinant sRAGE without the IgG crystallisable fragment (Fc) was used to avoid non-specific antibody interactions in the quantification of anti-sRAGE autoantibodies. The other experiments were performed using Fc-conjugated sRAGE (rHu-sRAGE) (1145-RG-050) from R&D Systems. Anti-human IgG antibodies (A3187), aβ (A1075), glyoxylic acid (G10601), human serum albumin (HSA) (A3782) and sodium cyanoborohydride (156159) were purchased from Sigma–Aldrich. *Para*-nitrophenylphosphate (pNPP) (EU1-2001-100) was purchased from Euromedex. CML-modified human serum albumin (CML–HSA) was obtained as previously described [453]. Briefly, HSA (50 mg/ml) was incubated for 16 h at 37ºC in the presence of glyoxylic acid (60 mM) and sodium cyanoborohydride (20 mM) in phosphate-buffered saline (PBS), pH 7.8. The solution was then extensively dialyzed against PBS, and the level of glycation was analyzed by 2,4,6-trinitrobenzenesulfonic acid (TNBS) free lysine assay [454]. HSA–CML glycation levels were obtained with between 5% to 60% of modified lysine residues.

Effect of RAGE ligands on sRAGE quantification using rHu-sRAGE or control serum

To assess the effect of RAGE ligands on sRAGE quantification, rHu-sRAGE (625, 1250 and 2500 pg/ml) was incubated in triplicate for 1 h at room temperature (RT) with CML-HSA (10, 100 and 1000 µg/ml), S100A6 (10, 100 and 1000 ng/ml), S100A12 (10, 100 and 1000 ng/ml), S100B (10, 100 and 1000 ng/ml), HMGB1 (1, 10 and 100 ng/ml) or aβ (1, 10 and 100 ng/ml) respectively, at concentrations ranging from physiological to supra-physiological levels. Since the extent of glycation affects RAGE binding affinity [81], all the tested incubations of CML–HSA were also performed at 3 levels of glycation (5%, 30% and 60%). One experiment was also carried out with a mixture of all these ligands at their highest concentration, and also at the highest glycation level for CML–HSA. All ligand solutions were prepared in PBS containing 1 mM $CaCl_2$ and 10 µM $ZnCl_2$. To investigate the presence of a possible matrix effect resulting in stronger interactions between sRAGE and its ligands, the effects of the aforementioned RAGE ligands (under the same conditions) on serum sRAGE quantification were analyzed. Sera from 4 healthy control subjects were incubated for 1 h at RT with CML–HSA, S100A6, S100A12 or S100B, HMGB1 or aβ. Each serum sample was also incubated with a mixture of all the selected ligands at their highest concentration.

sRAGE measurement

sRAGE quantification was performed using a Quantikine Human RAGE ELISA kit (R&D Systems) according to the manufacturer's instructions. Briefly, a 50 µL sample (rHu-sRAGE sRAGE + ligand; or serum + ligand) was added to anti-sRAGE-coated wells and incubated at RT for 2 h. The wells were then washed in rinsing buffer, following which 200 µL of peroxidase-conjugated anti-sRAGE solution were added and incubated for 2 h. The wells were washed again and the substrate solution (200 µL) was incubated for 30 minutes, and kept away from the light. The reaction was stopped with 50 µL of stop

solution, and optical density (OD) was measured at 450 nm in a microplate reader (Multiskan Ascent 354, Thermo Scientific).

Study subjects

To assess the effect of anti-sRAGE autoantibodies on sRAGE quantification, their presence was investigated in hemodialysis (HD) patients who are known to have high serum levels of AGEs and to suffer from an inflammatory state [455]. The latter state led the present authors to hypothesize that such patients might be good candidates for producing anti-sRAGE autoantibodies. A total of 36 HD patients were recruited from the Duchenne Hospital (Boulogne-sur-Mer, France). The only exclusion criterion was the presence of diabetes. Table 1 presents the patient characteristics. The control group included 46 healthy blood donors (Etablissement Français du Sang, Lille, France). Recruitment and blood collection were performed according to the Declaration of Helsinki, with the informed consent of all the study subjects.

Anti-sRAGE autoantibody quantification by ELISA

The quantification of anti-sRAGE autoantibodies (IgGs) was adapted from a previously reported protocol [445]. Nunc Maxisorp™ F8 96-well microplates (Thermo Scientific) were coated with FcFree-rHu-sRAGE (0.5 µg/well) in PBS overnight at 4ºC. After coating, the wells were washed and then saturated with 10% fetal bovine serum (FBS) in PBS saturation buffer for 45 min at RT. The buffer was then removed, the wells were washed again and the sera diluted (1:100) in saturation buffer. Samples (100 µL/well) were incubated for 2 h at RT. Following this, the wells were washed four times, then 100 µL alkaline phosphatase-conjugated anti-human IgG were added to each well (1:2000 in saturation buffer) then incubated for 45 min at RT. The wells were washed 5 times in

saturation buffer and 100 µL of 1 mg/ml pNPP were added to each well and incubated for 1 h at 37ºC. Optical density was measured at 405 nm in a microplate reader. Samples were measured in duplicate, and anti-sRAGE IgG titers were calculated by subtracting the blank control sample (wells not coated with FcFree-rHu-sRAGE) from the duplicate mean.

The specificity of the ELISA was confirmed by Western blot using serum from HD patients with low and high anti-sRAGE IgG levels (migration of rHu-sRAGE in SDS–PAGE, transfer to a PVDF membrane, incubation of serum within the membrane, and revelation using peroxidase-conjugated anti-human IgG antibody) (not shown).

To confirm that anti-sRAGE autoantibodies were not just glycated IgGs which could bind to sRAGE as it is a glycated protein receptor, IgG glycation level was assessed by dot–blot (anti-CML antibody) (Fig. 2A) and liquid chromatography coupled to linear ion-trap tandem mass spectrometry (LC–MS/MS), as previously described (Fig. 2B) [456]. Dot–blot and LC–MS/MS were performed with purified IgGs from HD patients with high (n=8) and low (n=8) anti-sRAGE IgG levels.

Effect of purified IgGs on sRAGE quantification using rHu-sRAGE or control serum

The effect of IgGs on sRAGE quantification was assessed by incubating rHu-sRAGE or serum from healthy controls with purified IgGs from HD patients with the lowest (n=8) and highest (n=8) anti-sRAGE IgG titers (Quantikine). IgG fractions were obtained using a MAb-Trap kit (GE Healthcare, Buc, France). Briefly, serum (0.5 mL) was filtered (0.2µm) and diluted 1:1 in binding buffer, applied to a protein G–sepharose column and washed in binding buffer. Bound IgGs were eluted with elution buffer into tubes containing 200 µL/ml neutralizing buffer. The eluted fractions were dialyzed against PBS and the protein content was assayed using a BCA Protein Assay (Thermo Scientific).

Purified IgGs (0.1, 0.5 and 1 mg/ml) from patients with low and high anti-sRAGE autoantibody levels were incubated in triplicate with rHu-sRAGE at 625, 1250 and 2500 pg/ml and also with sera from 4 healthy controls for 1 h at RT (Quantikine).

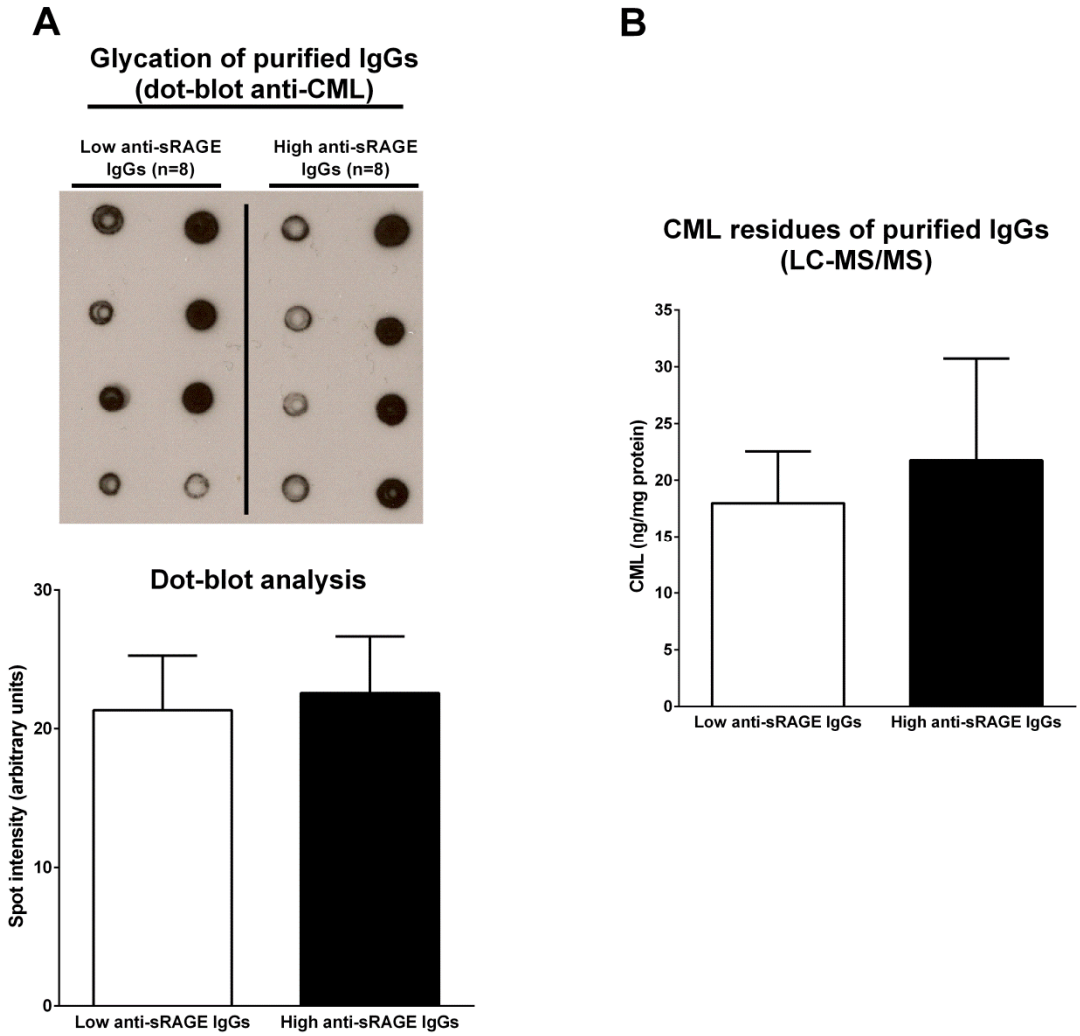

Figure 2. Glycation levels of purified IgGs are independent of anti-sRAGE activity.
Carboxymethyllysine (CML) content was analyzed in purified IgGs from HD patients with low (n=8) and high (n=8) anti-sRAGE IgG levels. Serum IgGs were purified using a protein G–sepharose column. IgG fractions were spotted onto a nitrocellulose membrane and blotted with anti-CML antibody **(A, top)**. Spot intensity was analyzed with ImageJ software **(A, bottom).** The CML content of purified IgGs was also analyzed by LC–MS/MS **(B)**. Mean + SEM (n=8). No statistically significant difference was observed (Student's *t*-test).

Statistical analysis

Anti-sRAGE autoantibodies and sRAGE levels were compared between the control group and HD patients using Student's *t*-test. The effect of RAGE ligands and anti-sRAGE autoantibodies on sRAGE quantification was analyzed by 1-way analysis of variance (ANOVA), followed by Tukey's test. A $p<0.05$ value was considered as being statistically significant.

Table 1. Patient characteristics

Male (n)	15 (42%)
Age (yr)	65±17 [26 – 91]
BMI[a]	25.47±4.95 [19 – 43]
CRP[b] (mg/l)	12.15±21.18 [4 – 126]
Hemoglobin	10.83±1.18 [8.2 – 12.4]
ESRD:[c]	
Urological disease	10 (27.8%)
Vascular nephropathy	9 (25%)
Polycystic kidney disease	8 (22.2%)
Glomerular disease	5 (13.9%)
Interstitial nephropathy	4 (11.1%)

[a]Body mass index; [b] C-reactive protein; [c] End-stage renal disease; [ ] range.

Results

RAGE ligands do not affect sRAGE quantification (using rHu-sRAGE or control serum)

When incubated singly with rHu-sRAGE, CML–HSA (10, 100 and 1000 µg/ml), S100A6 (10, 100 and 1000 ng/ml), S100A12 (10, 100 and 1000 ng/ml), S100B (10, 100 and 1000 ng/ml), HMGB1 (1, 10 and 100 ng/ml) or aβ (1, 10 and 100 ng/ml) or mixed together (at the highest concentration for each above-mentioned RAGE ligand), this had no effect on recombinant sRAGE quantification at 625, 1250 or 2500 pg/ml. Figure 3A shows the

sRAGE levels after incubation with the highest concentrations of these ligands. However, since other serum components could also affect ligand-binding affinity, the influence of the matrix effect was investigated by incubating the same RAGE ligands with sera from healthy control subjects. None of the tested concentrations were found to alter the serum levels of sRAGE as quantified by ELISA (Fig. 3B). In addition, no effect of CML–HSA on sRAGE quantification was observed, whatever the level of glycation (not shown). Figures 3A and 3B show the results obtained for CML–HSA with 60% modified lysine residues.

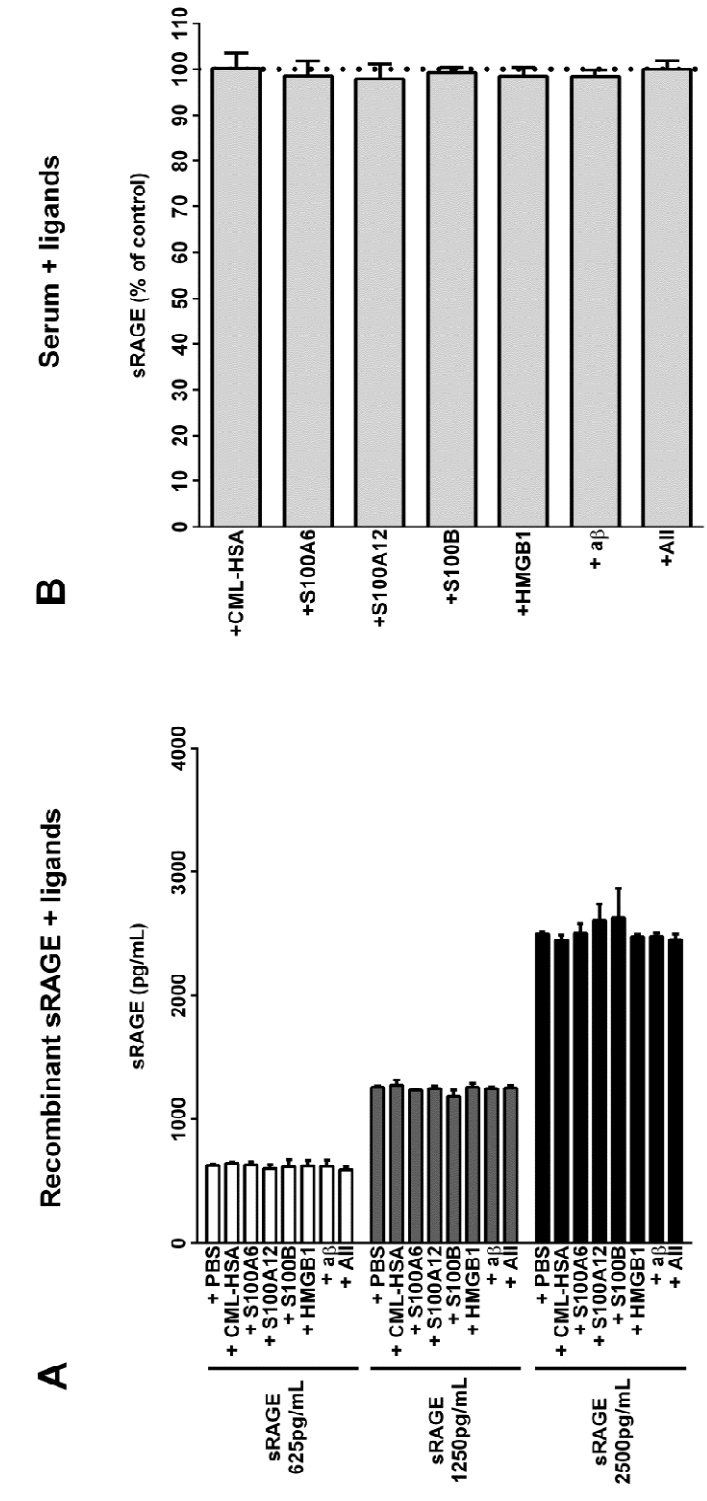

Figure 3. Effect of RAGE ligands on sRAGE quantification.
After incubation of rHu-sRAGE with CML–HSA, S100A6, S100A12, s100B, HMGB1 or aβ alone and then with a mixture of all these ligands combined at their highest concentration (AII), no effects on sRAGE quantification were observed **(A)**. The same RAGE ligands were incubated with sera from healthy control subjects. Serum sRAGE levels were normalized to the normal values of each patient (serum + PBS), represented by the dashed line. No statistically significant effects were observed (p>0.05) **(B)**. Mean + SEM.

sRAGE and anti-sRAGE autoantibody levels are increased in HD patients

Compared to healthy control subjects, HD patients had significantly higher serum sRAGE levels (3449±1752 pg/ml vs 1113±456 pg/ml, $p<0.0001$) (Fig. 4A). In addition, it was possible to identify HD patients with anti-sRAGE autoantibodies; the titers of anti-sRAGE IgGs were significantly higher in HD patients than in controls (OD 0.29±0.26 vs OD 0.19±0.13, $p<0.05$) (Fig. 4B). However, no correlation between sRAGE and anti-sRAGE autoantibody levels was observed (Figs 4C and 4D) either in the control group or in the HD patient group, although the latter presented significantly higher levels of these two biomarkers. The absence of an inverse correlation between sRAGE and anti-sRAGE autoantibody levels, particularly in the HD patient group, was not in favor of the hypothesis initially put forward that anti-sRAGE antibodies might mask sRAGE epitopes and thus affect sRAGE quantification by ELISA ($r^2=0.01$).

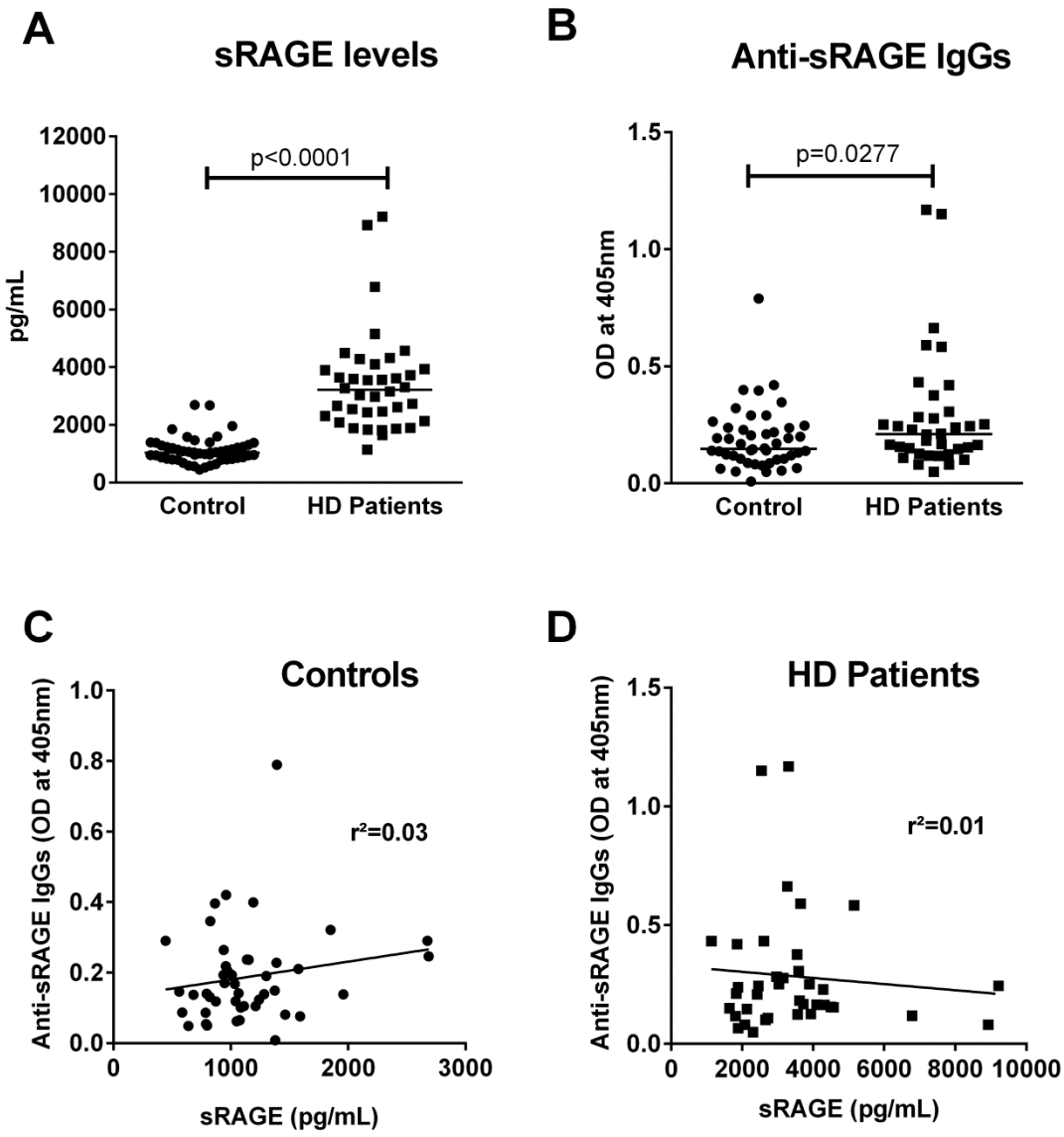

Figure 4. sRAGE and anti-sRAGE antibody levels in HD patients.
Serum sRAGE levels in control subjects (n=46) and HD patients (n=36) **(A)**. These levels were measured twice for each patient. **(B)** Anti-sRAGE autoantibody titers in HD patients and control subjects. No significant association between sRAGE and anti-sRAGE levels was observed in control subjects **(C)** or in HD patients **(D)**. Bars indicate the means in panels **A** and **B**.

Purified IgGs do not affect sRAGE quantification (using rHu-sRAGE or control serum)

The effect of purified IgGs on sRAGE quantification was further analyzed. Purified IgGs (0.1, 0.5 and 1 mg/ml) from HD patients with low (n=8) or high (n=8) anti-sRAGE IgG levels did not affect recombinant sRAGE quantification at 625, 1250 or 2500 pg/ml (Fig. 5A). However, since other serum components could also have an impact on ligand-binding affinity, the possible influence of the matrix effect was investigated by incubating purified IgGs with sera from healthy control subjects (n=4). None of the purified IgGs tested and obtained from patients with low or high anti-sRAGE IgGs was found to affect serum sRAGE levels as quantified by ELISA (Fig. 5B). Figures 5A and 5B show the results following incubation with 1 mg/ml of purified IgGs.

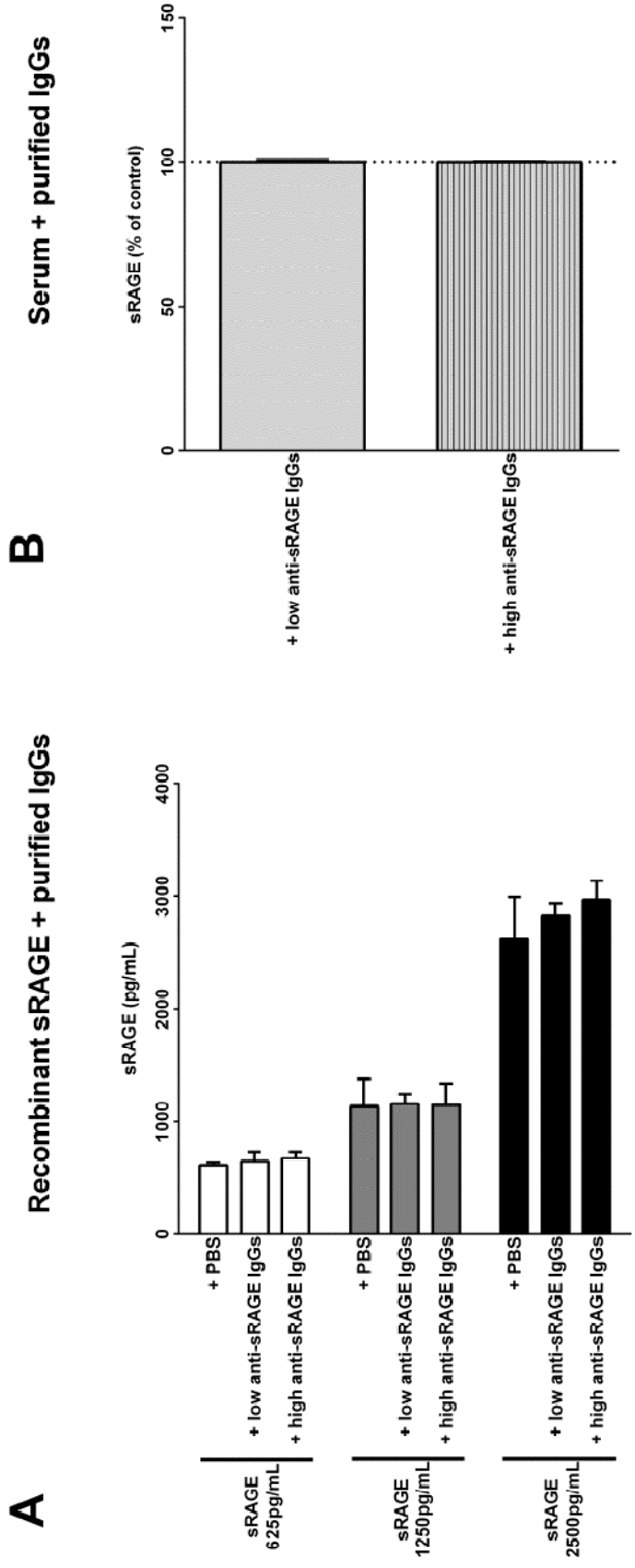

Figure 5. Effect of purified IgGs on sRAGE quantification.
Purified IgGs from HD patients with low and high auto-sRAGE IgG levels were incubated with rHu-sRAGE **(A)** or with serum from healthy control subjects **(B)**. Serum sRAGE levels were normalized to the normal values of each patient (serum + PBS), represented by the dashed line. No statistically significant difference was observed. Mean + SEM.

Discussion

In this study, it has been demonstrated that RAGE ligands CML–HSA, S100A6, S100A12, S100B, HMGB1 and aβ or a mixture of all these ligands at their highest concentration do not affect sRAGE quantification. While not considered as being RAGE ligands, the activity of anti-sRAGE autoantibodies has already been investigated in the literature**,** but their effects on RAGE binding have yet to be elucidated. Herein, HD patients were identified with increased anti-sRAGE titers, and IgGs from their sera were further purified. However, when incubated with rHu-sRAGE or control sera, these IgGs had no effect on sRAGE quantification.

RAGE was first described as a binding protein for AGEs and was later found to be a pattern-recognition receptor that binds to several members of the S100/calgranulin family, HMGB1, and aβ [1]. More recently, lipopolysaccharides (LPS) and phosphatidylserine have also been reported to bind to RAGE [61, 457]. However, in the present work LPS and phosphatidylserine were not tested because their RAGE binding sites have not yet been identified, and these two groups of molecules have not been fully studied as RAGE ligands. As regards RAGE, ligand binding takes place independently of a specific amino-acid sequence [82].

Ligands can bind to RAGE in different domains, suggesting that simultaneous molecular binding is possible. AGEs and HMGB1 mainly bind to the V-domain while S100B [64], S100A6 and S100A12 bind to the VC1 and C1C2 domains, and aβ to the V and C1 domains (Fig. 1) [167]. HMGB1 is the ligand with the highest binding affinity to RAGE, followed by CML. The absence of effect of HMGB1 alone on sRAGE quantification has been previously reported [310]. All the sRAGE domains were investigated in the present study using CML–HSA, S100A6, S100A12, S100B, HMGB1 or aβ. Moreover, epitope masking has been described in the literature as a problematical issue in ELISA protocols, e.g. resulting in lower anti-aβ binding in the presence of aβ fibrils and aggregates [458,

459]. Furthermore, epitope masking via phosphorylation has also been demonstrated for the DO-1 domain of p53 [460], as well as for the binding of cardiolipins to anti-β_2-glycoprotein 1 antibodies [461].

The evaluation of sRAGE blood levels is solely based on ELISA tests. Therefore, it is possible that epitope masking via steric hindrance could interfere with the results of the ELISA test and lead to an under-assessment of serum sRAGE levels. Moreover, the amino-acid sequences recognized by the antibodies of the most commonly used ELISA kits are not available, making it difficult to predict the effect of epitope masking on the quantitative evaluation of sRAGE levels. On the other hand, sRAGE quantification has been shown to be stable through time and to vary minimally between serum and plasma [351]. Wittwer and colleagues also demonstrated that sRAGE levels are comparable between EDTA and heparin sampling, with no loss after repeated freeze-thaw cycles [462]. These previous works, combined with the present results, corroborates the robustness of the assay. Moreover, RAGE–ligand interactions are of electrostatic in nature and antibody affinity maturation could create stronger interactions between sRAGE and commercially produced antibodies [82].

In the present study, after demonstrating for the first time that HD patients produce anti-sRAGE autoantibodies, it was shown that there was no correlation between sRAGE levels and anti-sRAGE autoantibody activity. However, since it is possible that the production of anti-sRAGE autoantibodies could be induced by glycated proteins and sRAGE itself [364], it is not surprising that uremic patients, who have increased AGE and sRAGE levels and thus suffer from a complex inflammatory state, could develop autoantibodies. Secondly, when incubated with recombinant sRAGE or sera, the purified anti-sRAGE IgGs from these patients had no effect on sRAGE quantification as measured by ELISA. In fine, this does not rule out an interaction between anti-sRAGE and sRAGE, although it suggests that the antibodies used in the ELISA method have a higher affinity for, and/or recognize a different epitope. Moreover, purified IgGs from these patients displayed different levels of

glycation which had no association with anti-sRAGE titers (Fig. 2). It is important to point out this aspect, since it could represent a certain bias in the present study methodology. The validity of antigen–antibody binding was also confirmed by Western blot (not shown). The pathophysiology of anti-sRAGE autoantibody production has not been elucidated, and even less the relevance of these autoantibodies as biomarkers. It is suggested here that this may involve a possible autoimmune reaction to vascular dysfunction induced by RAGE ligands that stimulate sRAGE production and subsequent anti-sRAGE autoantibody formation.

The role of sRAGE as a useful vascular biomarker remains uncertain, since a number of factors can influence its serum levels. As mentioned in the Introduction, depending on the authors in question, sRAGE levels have been reported as being either negatively or positively correlated with the severity of the same disease [217, 220, 221, 224, 258, 260]. Thus the findings in this respect are contradictory. Factors capable of modulating sRAGE levels such as angiotensin-converting enzyme (ACE) inhibitors or statins have not met with general consensus in the literature [219, 231, 328]. Among these various factors, age, RAGE polymorphisms, smoking body-mass index and ethnicity could well play an important role in varying sRAGE levels [219, 317, 322, 353]. Furthermore, strong evidence suggests a role of kidney dysfunction in increased sRAGE levels, although the specific mechanisms are unclear [275, 278, 280]. Compared to healthy young individuals, significantly higher sRAGE levels have been reported in healthy centenarians,[463] and a difference of around 500 pg/ml sRAGE has been associated with Gly82Ser polymorphism [350]. However, to date nothing has yet been reported on sRAGE blood levels after a high AGE-containing meal.

The sRAGE assay measures the pool formed by cleaved sRAGE (cRAGE) and endogenous secretory sRAGE (esRAGE) [199]. The secreted form has also been studied as a biomarker for vascular risk. In the present study, we did not specifically investigate

esRAGE since it has the same amino-acid sequence for the ligand-binding domains of cRAGE.

Although the hypothesis that RAGE ligands or anti-sRAGE autoantibodies might negatively affect sRAGE quantification was not confirmed, it can now be underlined that the ELISA technique for sRAGE quantification is an extremely reliable method. The use of sRAGE as a valid biomarker has still to be determined, and in this regard the reports in the literature remain controversial. In the present study, it was demonstrated that anti-sRAGE autoantibodies could be detected in HD patients. Nonetheless, further studies are necessary to more fully assess the role of RAGE ligands and sRAGE on anti-sRAGE autoantibody formation as well as the potential role of such autoantibodies as potential biomarkers for vascular autoimmunity.

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General Conclusions

We have, therefore, demonstrated that the presence of RAGE ligands and anti-sRAGE autoantibodies does not impair sRAGE quantification, suggesting that other variables are responsible for the huge variation in sRAGE levels observed in the literature. In addition, we found anti-sRAGE autoantibodies in serum of HD patients. These observations conducted us to the second part of this thesis, where we investigated a population with well defined inclusion/exclusion criteria, with the aim of evaluating the association of sRAGE and anti-sRAGE autoantibodies with metabolic parameters.

4.2 Anti-sRAGE autoantibody: a new biomarker during obesity

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Abstract

Background/Objectives: Morbid obesity increases risk of cardiovascular diseases (CVDs) and is closely associated with insulin resistance. Usual markers of obesity-associated risk are lipid profile and adipokines. The receptor for advanced glycation end-products (RAGE) is implicated in proinflammatory processes that underlie CVDs and insulin resistance. Its soluble form (sRAGE) has been measured in several human diseases and is proposed as a biomarker of risk, disease severity and outcome. Recently, anti-sRAGE autoantibodies were described and were shown to be increased in diseases where RAGE is overexpressed. In this study we aimed to investigate serum levels of sRAGE and anti-sRAGE autoantibodies in morbidly obese patients. The impact of weight loss on their levels, one year after bariatric surgery, was also evaluated.

Subjects/Methods: From the ABOS cohort (Lille, France) of 750 obese patients, after exclusion criteria, 150 subjects were randomly included in this work. Serum sRAGE and anti-sRAGE autoantibodies were measured after overnight fasting before bariatric surgery. Sixty nine patients were followed up to one year after gastric bypass. Levels of sRAGE and anti-sRAGE autoantibodies were measured. Control group consisted of blood donors.

Results: Baseline levels of sRAGE and anti-sRAGE autoantibodies were significantly higher in obese patients compared to controls ($p<0.001$). One year after gastric bypass, RAGE and anti-sRAGE decreased ($p<0.001$), although the latter remained higher than controls. The decrease in anti-sRAGE autoantibodies was correlated with the increase in high-density lipoprotein (HDL) ($p=0.02$).

Conclusion: Morbid obesity increases sRAGE and anti-sRAGE levels. Weight loss after gastric bypass is followed by a decrease of both titers. The decrease of anti-sRAGE correlates with the increase of HDL. These findings suggest that anti-sRAGE

autoantibodies may be a new biomarker to manage metabolic improvement during obesity.

Introduction

Morbid obesity is a worldwide disease that achieved an epidemic status and grows rapidly in numbers. Obesity is characterized by impaired health due to excess of body fat.[406] In practice, a body-mass index (BMI) ≥30 defines obesity. Obesity is one of the diagnostic components of metabolic syndrome and is associated with insulin resistance and increased vascular risk. Higher coronary heart disease incidence has been related to overweight in different studies.[409-411] To date, gastric bypass is one of the most efficient surgical procedures for weight loss, with up to 68% fat content reduction and improvement of metabolic function.[416]

Due to its complex physiopathology, obesity-associated complications are hard to evaluate and several molecules have been studied as biomarkers. High levels of triglycerides, low-density lipoprotein (LDL) and low levels of high-density lipoprotein (HDL) are characteristic of obesity and often associated with co-morbidities.[464-466] Adipokines, which often have deregulated secretion in obese subjects, are also associated with cardiometabolic risk. The high levels of leptin and leptin resistance observed in the obese are known to contribute to the proinflammatory and pro-thrombotic status of these subjects.[467] On the other hand, adiponectin levels are decreased in obesity and are associated with vascular dysfunction.[468]

The receptor of advanced glycation end-products (RAGE) is a multi-ligand receptor involved in pro-inflammatory and pro-ageing processes. RAGE engagement increases reactive oxygen species (ROS) and induces the expression of adhesion molecules and vascular endothelial growth factor.[21, 22, 89, 191] In adipocytes, RAGE activation

induces an increase in ROS that mediates insulin resistance.[47, 469] The soluble form of RAGE (sRAGE) acts as a decoy receptor, preventing RAGE activation.[10, 254, 470] Soluble RAGE has been extensively studied as a biomarker of vascular risk and metabolic dysfunction. Lower levels of sRAGE were observed in diabetics with vascular complications.[221, 258] Moreover, in the general population, sRAGE levels are inversely correlated with BMI, waist-hip ratio and fasting glucose.[317] Very interestingly, it has been shown that levels of sRAGE were restored after bariatric surgery in morbidly obese patients.[356] In addition, this increase in sRAGE levels was associated with improvement of insulin resistance. Nevertheless, sRAGE levels among publications are controversial, a fact that demands rigorous selection of study populations in order to evaluate the true value of sRAGE as a biomarker.

Recently, autoantibodies directed against sRAGE were found increased in the sera of Alzheimer's disease and rheumatoid arthritis patients.[369, 445] Although they correlate with dementia scores,[369-371, 471] it has been proposed that anti-sRAGE autoantibodies could play a protective role, preventing amyloid β (Aβ) toxicity through RAGE.[372] Our group has observed increased levels of anti-sRAGE autoantibodies in hemodialysis patients,[472] which could be associated to impaired glomerular filtration and increased sRAGE levels.

In this work, we aimed to estimate the value of sRAGE and anti-sRAGE autoantibodies as markers of metabolic homeostasis. To minimize confounding factors, we investigated a well-defined population of morbidly obese patients (ABOS cohort). The association of both titers with metabolic improvement after weight loss surgery was also evaluated.

Materials and Methods

Study population

Patients included in this study were enrolled in ABOS (ClinicalGov NCT01129297). This prospective cohort study is ongoing at the Department of General and Endocrine Surgery, in Lille, France, and currently follows the 750 obese patients who were eligible for or underwent gastric bypass (Roux-en-Y). It aims at studying the effect of bariatric surgery on diabetes and glucose regulation. Details on the ABOS cohort study have been published elsewhere.[473] The exclusion criteria were chosen to avoid any factors that could influence sRAGE levels, as follows: hypertension, glomerular filtration rate <90ml/min/1.73m^2 (Modified of Diet in Renal Disease), angiotensin-converting enzyme inhibitors (ACEi), angiotensin receptor antagonists (ARA2) or satins medication and smokers.

From the whole cohort, 254 obese patients were enrolled. As the main topic of the ABOS study is the glucose regulation in obese patients, these subjects were further subclassified into three groups of patients with diabetes or lesser degrees of impaired glucose regulation. Accordingly, the oral glucose tolerance test criteria for *normoglycemia* are fasting plasma glucose concentration (FPG) < 5.5 mmol/l and 2-h PG < 7.7 mmol/l; *glucose intolerant*: FPG > 5.5 mmol/l and < 6.9 mmol/l or 2-h PG between 7.7 and 10.9 mmol/l; *Type 2 diabetes*: FPG > 6.9 mmol/l or 2-h PG > 11 mmol/l. According to preliminary works of our group (unpublished data), we estimated the number of subjects to include in order to detect a difference in anti-sRAGE antibodies between normoglycemic, glucose intolerant and type 2 diabetes patients. Because three groups were tested, we used the Bonferoni correction providing a significance level of 0.016 (0.05/3). Given a power of 90%, a significance level of 0.016, the expected means and deviations, the number of patients to be included was 50 subjects in each group (46 + 10% security). We therefore randomly included 150 patients in this study, subclassified in

normoglycemics, glucose intolerants and type 2 diabetics (n=50 for each). The control group included 46 healthy blood donors (Etablissement Français du Sang, Lille, France). Recruitment and blood collection were performed according to the Declaration of Helsinki, with the informed consent of all the study subjects. After baseline analysis, we screened patients who underwent a gastric bypass followed by a one year medical check-up and had available frozen serum at one year. The 69 patients who met these further selection criteria were included in the second part of the study on evolution of the anti-sRage concentration. The flow-chart of patients selection is presented in figure 1.

sRAGE quantification by ELISA

Serum sRAGE levels were quantified using the Quantikine ELISA kit by R&D Systems (Minneapolis, MN, USA) according to manufacturer's instructions. Each sample was analyzed in duplicate, incubating 50µl of serum for 2h in 96-wells microplates pre-coated with anti-sRAGE antibody. Wells were washed 4 times with provided washing buffer and peroxidase-conjugated anti-sRAGE was incubated for 2h at room temperature (RT). Plates were washed again and then incubated with substrate for 30 min, protected from light. Color intensity was measured using a microplate reader (450nm) and sRAGE concentrations were calculated using a standard curve.

Anti-sRAGE autoantibodies quantification by ELISA

The quantification of anti-sRAGE autoantibodies (IgGs) was adapted from a previously reported protocol.[445] Nunc Maxisorp™ F8 96-well microplates from Thermo Scientific (Waltham, MA, USA) were coated with Fc fragment-free sRAGE (R&D Systems) (0.5 µg/well) in phosphate buffer saline (PBS) overnight at 4ºC. After coating, the wells were washed and then saturated with 10% fetal bovine serum (FBS) in PBS saturation buffer

for 45 min at RT. The buffer was then removed, the wells were washed again and the sera diluted (1:100) in saturation buffer. Samples (100 µL/well) were incubated for 2h at RT. Following this, the wells were washed four times, then 100 µL alkaline phosphatase-conjugated anti-human IgG (Sigma Aldrich, St Louis, MO, USA) were added to each well (1:2000 in saturation buffer) then incubated for 45 min at RT. The wells were washed 5 times in saturation buffer and 100 µL of 1 mg/ml para-nitrophenylphosphate (Euromedex, Strasbourg, France) were added to each well and incubated for 1h at 37ºC. Optical density was measured at 405 nm in a microplate reader. Samples were measured in duplicate, and anti-sRAGE IgG titers were calculated by subtracting the blank control sample (wells not coated with sRAGE) from the duplicate mean.

Statistical Analysis

Baseline characteristics were presented in terms of mean and standard deviation (SD) for continuous variables, and as frequency and percentage for categorical variables. Comparisons were first done at baseline between the three sub-groups of the whole cohort of 150 obese patients and also with controls subjects. sRAGE levels were compared using one-way analysis of variance (ANOVA), followed by Tukey's test. Levels of anti-sRAGE autoantibodies were compared using Dunn's multiple comparison test.

Comparisons were then made between the characteristics at baseline and one year after bariatric surgery for the 69 obese patients included in the second part of the study. A paired Student t-test was used for all comparisons after verifying for differences in the variance distributions. The percentage of diabetic patients was analyzed by a chi-square test. Lastly, we analyzed the associations between the evolutions in anti-sRAGE antibodies and other characteristics. The Pearson's product moment correlation coefficient was used to test the association between the differences in anti-sRAGE antibodies (baseline value minus value at one year) and the differences in other characteristics

(baseline value minus value at one year). The significance level was 0.05 for all tests performed.

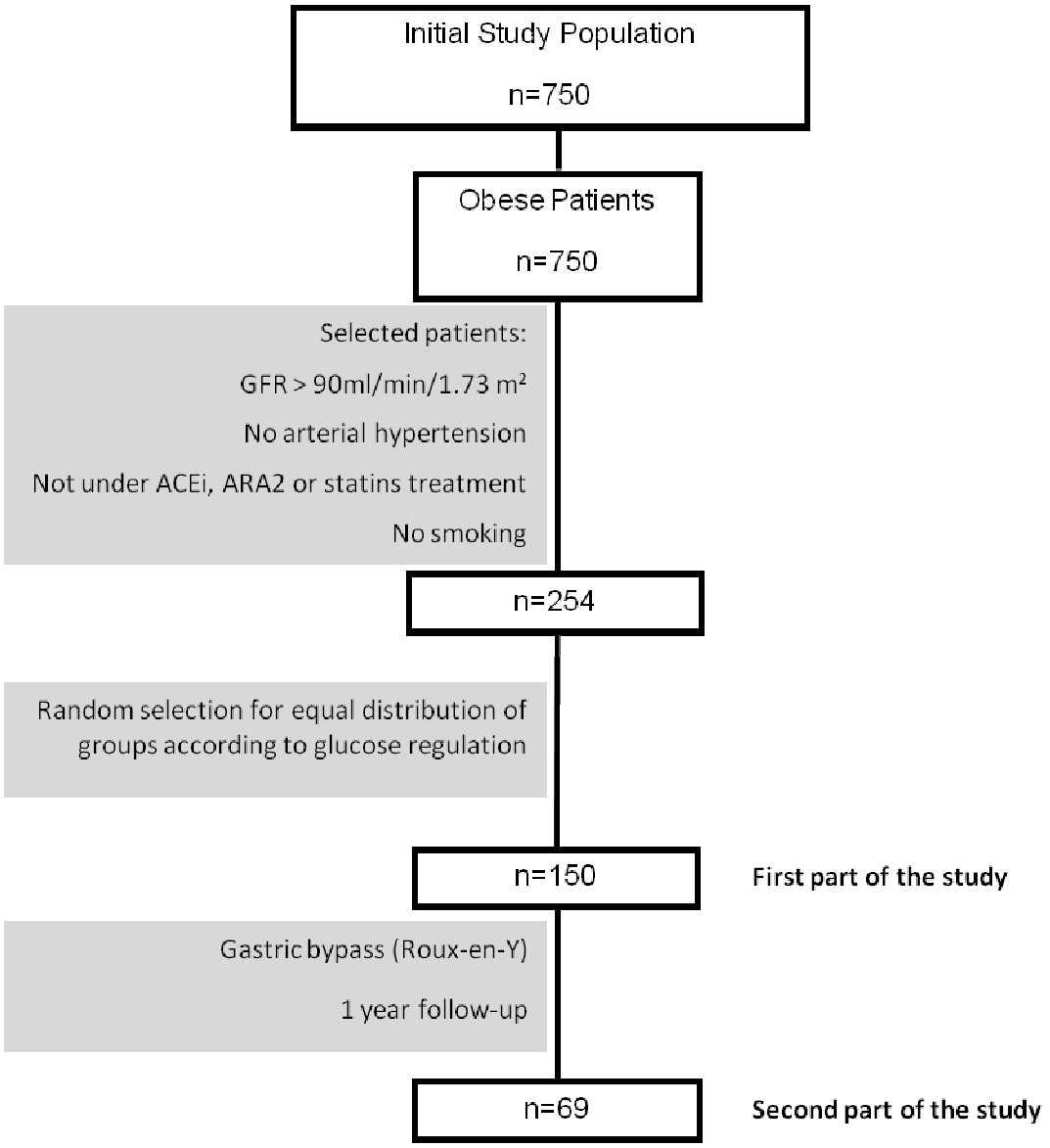

Figure 1. Patients flowchart. From the initial 750 morbidly obese patients of the ABOS cohort, our study population was selected to exclude factors known to influence sRAGE levels.

Results

Baseline levels of sRAGE and anti-sRAGE autoantibodies are higher obese patients

The clinical characteristics of the 150 obese patients of the initial study population are presented in table 1. The 150 patients initially included in this study had higher levels of sRAGE and anti-sRAGE autoantibodies in comparison to the control group ($p<0.0001$ for both) (Figure 2A and 2B). Among the obese patients, neither sRAGE nor anti-sRAGE levels differ between normoglycemic, glucose intolerant and diabetic patients.

Table 1. Clinical data of the initial obese population

	All	NG	GI	DB
n, (men/women)	150 (37/113)	50 (42/8)	50 (37/13)	50 (34/16)
Age, years	35 ± 9	29 ± 8	37 ± 5	40 ± 8
Weight, kg	133.8 ± 24	134 ± 24.4	132 ± 25	135.4 ± 23.3
BMI, kg/m^2	47.1 ± 7.1	46.7 ± 7.2	46.5 ± 6.9	48.2 ± 7.3
SBP, mmHg	130 ± 18.4	130 ± 16.6	128.1 ± 16.9	132 ± 21.4
DBP, mmHg	71.6 ± 14.4	70 ± 16.2	70.2 ± 13.6	74.4 ± 12.9
$HbA1_C$, mmol/mol	45.3 ± 27	35.5 ± 2	41 ± 2	61.7 ± 21
Fasting glucose,mmol/l	6.17 ± 2.5	4.8 ± 0.3	5.3 ± 0.6	8.3 ± 3.4
2h PP glucose, mmol/l	8.25 ± 4.3	5.7 ± 1.2	6.5 ± 1.3	12.5 ± 5.1
sRAGE, pg/ml	1223 ± 354	1301 ± 324	1132 ± 334	1235 ± 388
Anti-sRAGE, absorbance*	0.76 ± 0.5	0.75 ± 0.47	0.83 ± 0.57	0.70 ± 0.44
hs-CRP, mg/l	7.3 ± 2.9	6.9 ± 3.1	7.7 ± 2.5	7.2 ± 3

Data are expressed as mean ± SD. NG, normoglycemic; GI, glucose intolerant; DB, diabetic; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; $HbA1_C$, glycated hemoglobin; PP, postprandial; hs-CRP, high sensitive C-reactive protein.* Absorbance at 405nm.

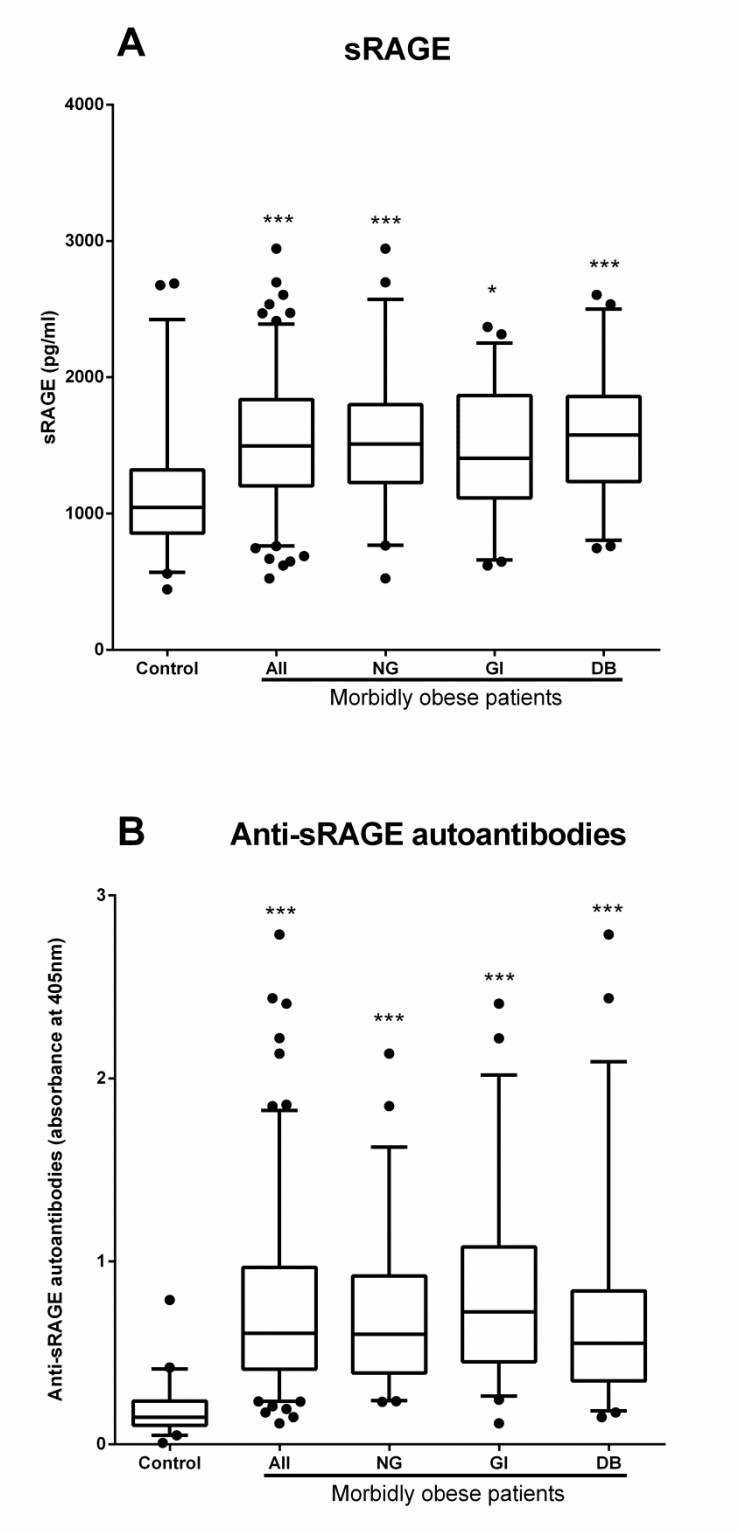

Figure 2. Levels of sRAGE (A) and anti-sRAGE (B) autoantibodies are higher in morbidly obese patients. Regardless of glucose regulation impairment, morbidly obese patients had higher levels of sRAGE and anti-sRAGE autoantibodies compared to controls. *p<0.05, *** p<0.0001, in comparison to control group. NG, normoglycemic; GI, glucose intolerant; DB, diabetics.

Gastric bypass is followed by a decrease in sRAGE and anti-sRAGE autoantibodies

Since sRAGE and anti-sRAGE autoantibodies did not differ according to glucose regulation, we decided to investigate the role of obesity itself. From the original study population, we further selected 69 patients, who were submitted to gastric bypass with a 1 year follow-up. Clinical data of these patients are presented in table 2. One year after weight loss surgery, the percentage of BMI decreased was 31.9% (from 46.7 to 31.8 kg/m^2), along with an improvement in insulin. After gastric bypass, sRAGE and anti-sRAGE autoantibodies levels decreased (Figure.3A and 3B). Serum sRAGE after surgery was different from the levels before surgery only ($p<0.0001$). Anti-sRAGE autoantibodies were significantly lower after surgery ($p<0.0001$) but still higher in comparison to control ($p<0.0001$).

The decrease in anti-sRAGE autoantibodies is associated with the increase in HDL

Univariate analysis showed a correlation between the increase in HDL levels and the decrease of anti-sRAGE autoantibodies after surgery ($r^2=0.077$, $p=0.02$). Figures 4A and 4B represent the correlation between these two parameters and their evolution after surgery, respectively. Other metabolic parameters were not associated with neither anti-sRAGE nor sRAGE levels.

Table 2. Clinical data of obese patients

	Pre-surgery	Post-surgery (1 year)	*P*-value
n, (men/women)	69 (21/48)	69 (21/48)	NA
Age, years	37 ± 9	38 ± 9	NA
Weight, kg	136.1 ± 24	92.8 ± 18.2	<0.0001
BMI, kg/m^2	46.7 ± 9.9	31.8 ± 5.2	<0.0001
Diabetes, n (%)	28 (40%)	5 (7%)	<0.0001
Systolic BP, mmHg	130 ± 22	119 ± 15	<0.0001
Diastolic BP, mmHg	72 ± 16	68 ± 11	0.0045
HbA_{1C}, mmol/mol	47.5 ± 17	37.7 ± 8	<0.0001
Fasting glucose, mmol/l	6.51 ± 2.5	5.01 ± 1.7	<0.0001
2h PP glucose, mmol/l	8.8 ± 4.5	4.7 ± 2.4	<0.0001
Fasting insulin, µU/ml	16.7 ± 9.5	9.8 ± 2.7	<0.0001
2h PP insulin, µU/ml	70.3 ± 56.6	16.2 ± 28.6	<0.0001
HOMA-IR	5 ± 4.4	4.2 ± 20.2	<0.0001
hs-CRP, mg/l	6.8 ± 2.9	2.1 ± 2.6	<0.0001
LDL-C, mmol/l	3.2 ± 0.7	2.6 ± 0.6	<0.0001
HDL-C, mmol/l	1.1 ± 0.3	1.3 ± 0.3	<0.0001
Total cholesterol, mmol/l	5 ± 0.9	4.4 ± 0.9	<0.0001
Triglycerides, g/l	1.6 ± 1	1 ± 0.4	<0.0001

Data are expressed as mean ± SD. BMI, body mass index; BP, blood pressure; HbA_{1C}, glycated hemoglobin; PP, postprandial; HOMA-IR, Homeostasis Model of Assessment - Insulin Resistance; hs-CRP, high sensitive C-reactive protein; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol.

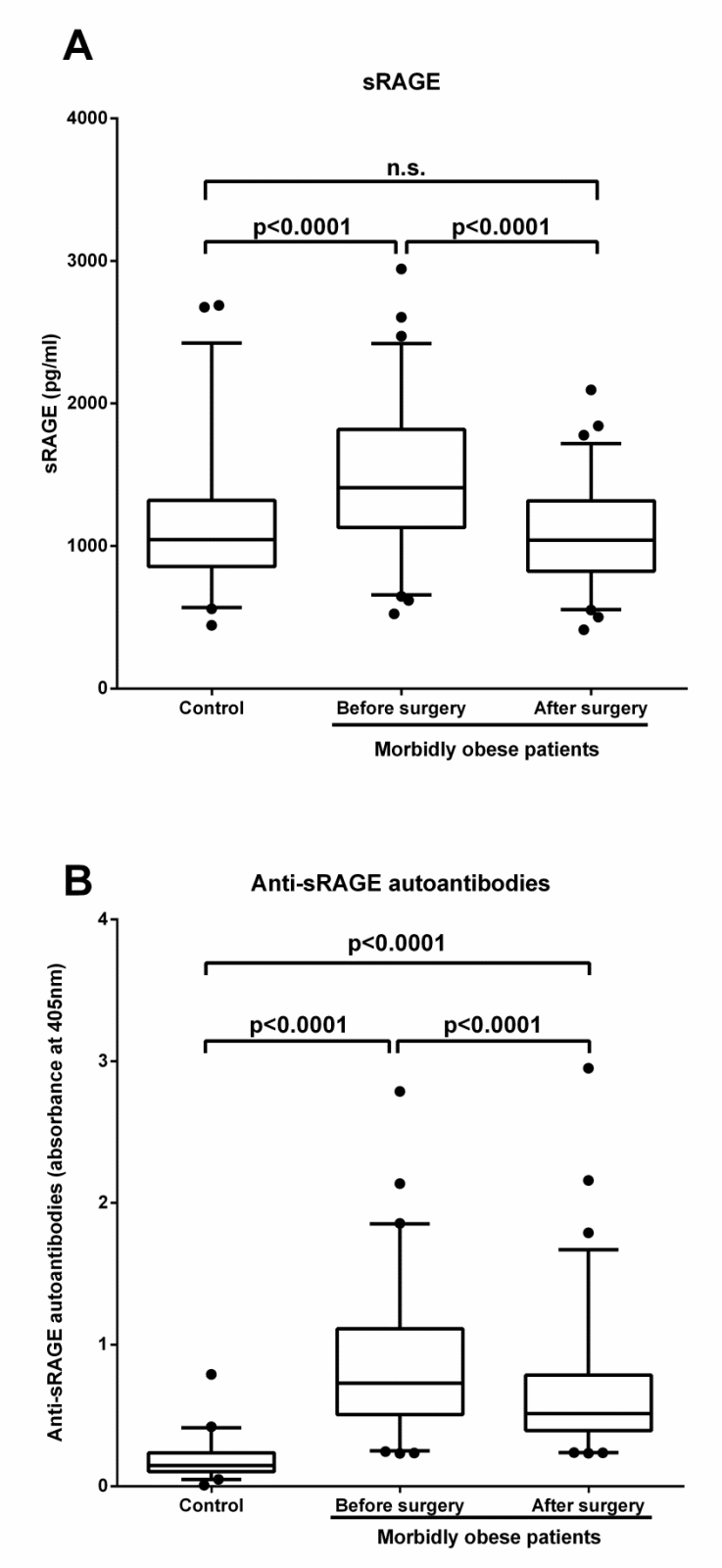

Figure 3. **Serum levels of sRAGE (A) and anti-sRAGE (B) autoantibodies decrease after bariatric surgery**. 69 morbidly obese patients were underwent a gastric bypass. sRAGE and anti-sRAGE levels were measured before and one year after surgery.

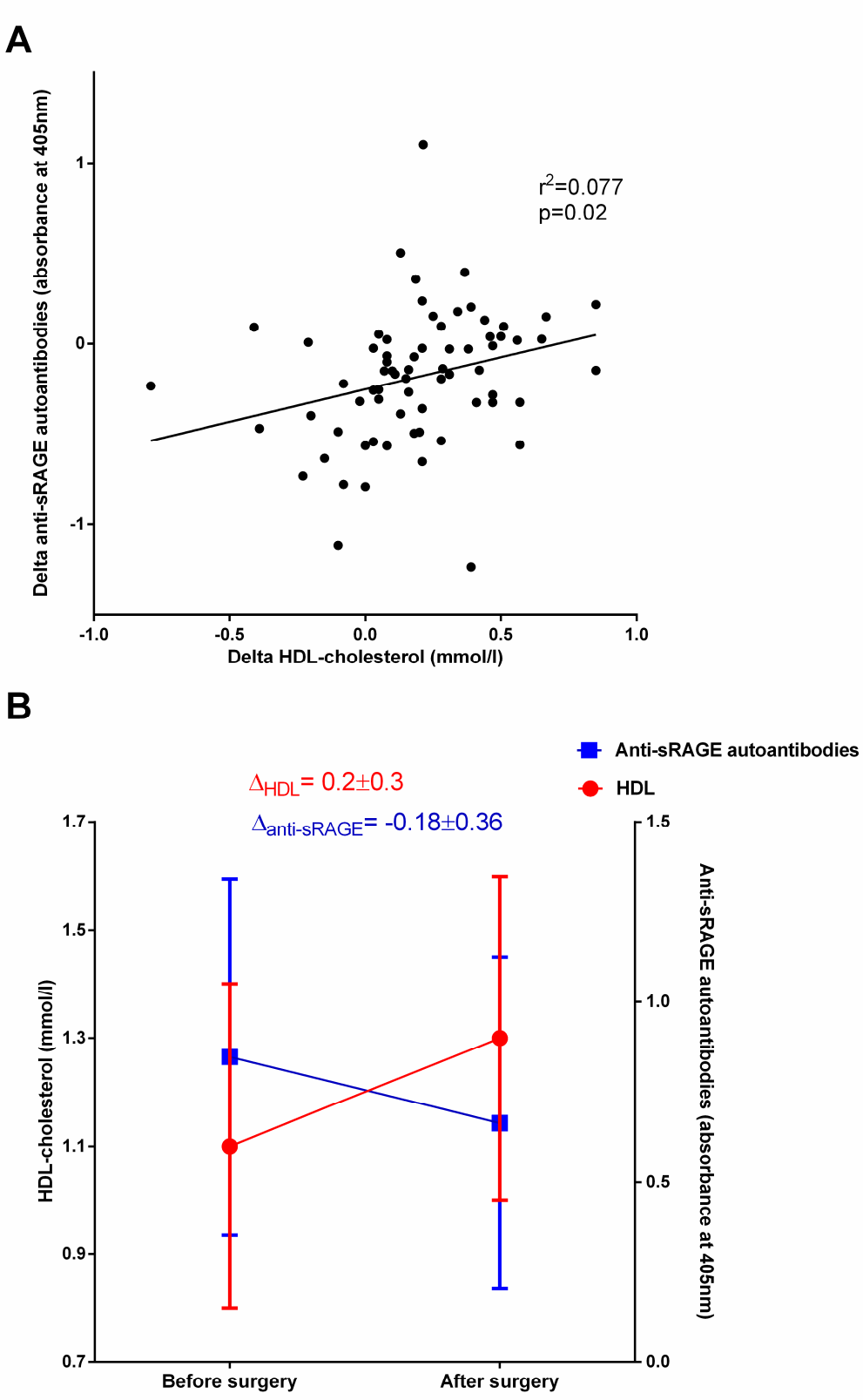

Figure 4. The decrease in anti-sRAGE autoantibodies is correlated with the increase of HDL-cholesterol after gastric bypass. A weak correlation between the variations of HDL-cholesterol and anti-sRAGE was found **(A)**. Both parameters were affected by gastric bypass **(B)**.

Discussion

Herein we demonstrate that morbidly obese subjects, eligible for a weight loss surgery, present higher serum levels of sRAGE and anti-sRAGE autoantibodies. One year after gastric bypass, both titers decrease significantly. While sRAGE levels after surgery were similar to those of control subjects, anti-sRAGE titers remained higher. We also observed that the increase in HDL levels, an usual consequence of weight loss surgery, was associated with the decrease of anti-sRAGE autoantibodies.

Serum sRAGE levels have been measured in patients from several diseases and sRAGE is often proposed as a biomarker of severity or prognosis. Nonetheless, data is quite controversial, with increased and decreased levels observed for a same disease. The regulation of sRAGE expression, either from membrane RAGE cleavage or from mRNA alternative splicing, is not well elucidated. *In vivo*, sRAGE levels seem to be modulated by several factors such as ACEi, statins, BMI, RAGE polymorphisms and kidney dysfunction.[219, 317, 322, 353] The higher levels of sRAGE in our group of obese subjects are in contrast with the work of Brix and colleagues,[356] who observed lower levels compared to controls and an increase after bariatric surgery. Concerning such controversial findings, we hypothesize that it is mainly due to inclusion/exclusion criteria. In our ABOS cohort, we highly selected patients that were not under ACEi or statins, non-smokers and without kidney dysfunction or hypertension. Their study did not include subjects with known diabetes, but we did not observe any influence of diabetes in sRAGE or anti-sRAGE levels. In addition, our study included (one-year follow-up) only patients that were submitted to a gastric bypass (Roux-en-Y). Unfortunately, any possible effects of the surgery type on sRAGE levels were not presented in the other work.

The majority of circulating sRAGE is produced by cleavage of membrane RAGE, which is performed mainly by a disintegrin and metalloproteinase 10 (ADAM10) and matrix metallopeptidase 9 (MMP9).[200] Although it has a putative protective role, studies that

show RAGE blockade use much higher doses of sRAGE than those found in circulation.[474, 475] Furthermore, it remains unknown whether RAGE cleavage follows RAGE activation or it is a simple prevention mechanism. Nevertheless, our findings are in agreement with the hypothesis that sRAGE levels reflect RAGE overexpression.

In rheumatoid arthritis and Alzheimer's disease,[372, 445] anti-sRAGE autoantibodies are believed to play a protective role even though they are associated with dementia scores.[370, 371] An oral vaccine has been developed to increase the production of anti-sRAGE and anti-Aβ. The vaccine improved cognition in a mice model of Alzheimer's and the developed anti-sRAGE autoantibodies prevented Aβ-induced toxicity to neuronal cells *in vitro*.[372] New autoantibody reactivity appearance has been reported in relation to the overexpression of autoantigens, especially in a proinflammatory or proapoptotic context.[476] Such autoreactive specificities could contribute to the regulation of the expression and clearance of overexpressed autoantigens.[477, 478] The high levels of anti-sRAGE antibody observed in our study population may be due to the raise in sRAGE levels associated with the increased inflammation process and endothelial dysfunction. Upregulation of inflammatory cytokines, especially TNF-α, and soluble adhesion molecules like soluble vascular adhesion molecule 1 (sVCAM-1) and soluble intercellular adhesion molecule 1 (sICAM-1) has been reported in obese subjects.[479-481] TNF-α and other pro- inflammatory cytokines are implicated in the pathogenesis of autoimmunity, particularly in type 1 diabetes and rheumatoid arthritis.[482, 483]

One year after gastric bypass, serum levels of sRAGE and anti-sRAGE autoantibodies decreased, in parallel with the decrease of inflammation and CRP levels. Likewise, weight loss surgery improved the lipid profile and insulin sensitivity, with 17.8% of diabetes remission. The increase in HDL levels was correlated with the decrease of anti-sRAGE autoantibodies. Low HDL levels are closely associated with increased vascular risk.[466] In a model of systemic lupus erythematosus, autoantibodies against Apo-I were associated with decreased levels of HDL.[484] Chen and colleagues observed a negative

correlation between sRAGE and HDL in RA patients.[176] Although we did not observe correlations between sRAGE levels and the improvement of lipid profile, the parallel decrease of sRAGE and anti-sRAGE suggests a role of RAGE and autoimmunity in the vascular risk associated with dyslipidemia in obesity. In obesity, the role of autoimmunity is still poorly understood. Some studies have proposed a role for leptin. Obesity favors the development of thyroid autoantibodies and those are associated with leptin levels.[485] Leptin acts directly on T lymphocytes,[486] increasing the release of cytokines by Th2 cells, which participate in the humoral immune system.

Our study is the first to demonstrate the presence of anti-sRAGE autoantibodies in morbidly obese patients. Our findings come from a large cohort with well-defined inclusion/exclusion criteria. Although we did not observe strong correlations between sRAGE or anti-sRAGE and parameters associated with metabolic improvement (i.e. weight loss and HOMA index), the decrease of both titers after surgery suggests that their high levels before weight loss were associated with obesity. Moreover, our data concern a short period after surgery, with a still high BMI. A follow-up of two and five years is intended. Hence, the incidence of anti-sRAGE autoantibodies in pre-obese subjects should be further studied to elucidate the role of increased body fat in the development of autoimmunity against RAGE. Therefore, a role for these molecules and the membrane RAGE in the pathophysiology of obesity is to be investigated.

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Conflict of Interest

The authors declare that there are no competing financial interests.

General Conclusions

In this article we showed, in a well-defined cohort, that morbidly obese patients have higher levels of sRAGE and anti-sRAGE autoantibodies. In addition, no association of these titers with glucose regulation impairment was found. On the other hand, weight-loss surgery promoted a decrease in both markers. Moreover, the decrease in anti-sRAGE autoantibodies was correlated with the increase in HDL levels, suggesting that autoimmunity against sRAGE has an association with increased cardiovascular risk. In conclusion, morbid obesity leads to an increased autoreactivity against sRAGE which is attenuated after surgical intervention. The associated changes in anti-sRAGE and HDL indicate that anti-sRAGE are related to increased cardiovascular risk.

Chapter Five

Discussion

5.1 From sRAGE to anti-sRAGE autoantibodies

Soluble RAGE levels have been measured in the course of several human diseases and often proposed as markers of severity and predictors of outcome. Nonetheless, very little is known about the regulation of sRAGE formation *in vivo* and there is not a consensus on whether sRAGE levels represent a mechanism of protection or just the overexpression of RAGE. In the literature, several factors are known to influence sRAGE levels (i.e. kidney dysfunction, smoking, RAGE polymorphisms and drug treatment). Because of RAGE promiscuity as a receptor, we hypothesized that, besides the aforementioned factors, the presence of RAGE ligands could impair sRAGE measurement by masking epitopes recognized in its quantification.

Therefore, we incubated different RAGE ligands with recombinant sRAGE and control sera to further measure sRAGE concentration. The presence of RAGE ligands did not impair sRAGE quantification. We aimed also to investigate the effects of anti-sRAGE autoantibodies, which were recently described and could compete with the antibodies of the sRAGE ELISA test. We identified hemodialysis (HD) patients with increased anti-sRAGE titers that were further purified and incubated with recombinant sRAGE and control sera. Nonetheless, the presence of anti-sRAGE autoantibodies did not affect sRAGE quantification. Our findings suggested that the variations in sRAGE levels are not due to an impairment of sRAGE measurement.

On a second moment, we intended to further investigate sRAGE in a well established cohort in order to reduce the biases of its measurement. We also aimed to investigate the presence of anti-sRAGE autoantibodies, since they were increased in HD patients and

their pathophysiological role remains unclear. Hence, we studied a population of 150 morbidly obese patients (ABOS, Lille) who were eligible for a bariatric surgery. Both sRAGE and anti-sRAGE levels were higher in morbidly obese patients compared to controls. Although sRAGE levels are modulated in diabetes, we did not observe any association with the degree of glucose regulation impairment. Then, we further analyzed patients from the original population with a 1-year follow-up after gastric bypass. Sixty nine patients were included and we found a decrease in both sRAGE and anti-sRAGE levels after surgery. Moreover, the decrease in anti-sRAGE autoantibodies was correlated with the increase in HDL-cholesterol, suggesting that autoimmunity against sRAGE have an association with vascular risk.

5.2 The sRAGE rollercoaster

In our work we measured sRAGE levels with an ELISA method that quantifies both cRAGE and esRAGE (total sRAGE). Concerning their ligand-binding sites, esRAGE and cRAGE share the same domains and, to date, no ligand was shown to bind the unique amino acid sequence of esRAGE. Although our findings seem to rule out the original hypothesis, they are limited since we use only a simple methodology to assess our question. Preliminary results in our laboratory, using surface plasmon resonance, indicated the presence of high-molecular weight complexes of sRAGE in the serum of diabetic patients. Attempts to quantify free and complexed sRAGE with this technique were proven very hard because of the multifactorial nature of the results (molecular weight, affinity and concentration). An interesting approach would be the use of mass spectrometry to evaluate the ratio of free/complexed sRAGE and value of this information as marker of disease severity and outcome.

It is important to notice that the ELISA method is the only quantitative technique applied to measure sRAGE levels. It has a high specificity because it uses 2 antibodies: a monoclonal antibody that captures sRAGE from sera and a polyclonal antibody that further binds to sRAGE and is measurable due to its conjugated peroxidase. A few in vitro studies measured sRAGE in cell culture media using western blot, but this is a semi-quantitative method that would require pre-treatment of complex samples like serum. The development of new methods, like liquid chromatography coupled to mass spectrometry (LC-MS/MS), would represent an advantage because of its high specificity and sensitivity, as well as the possibility to investigate isoforms and structural modifications (i.e. oxidation and glycation).

Another limitation of these findings is that they do not provide further information on whether RAGE ligands or anti-sRAGE autoantibodies could influence sRAGE levels by increasing/decreasing cRAGE and esRAGE formation or their clearance. Indeed, immune complexes are cleared by specific receptors and HMGB1, the ligand with the highest affinity, is found in the circulation as part of immune complexes with DNA fragments [487]. These high molecular weight complexes represent the major RAGE-binding activity of HMGB1 [156]. As to the induction of esRAGE and cRAGE formation, RAGE cleavage is known to be modulated by calcium [200, 202], a mechanism that could implicate the involvement of S100 proteins, but no evidence of such regulation has been found. On the other hand, methylglyoxal-modified albumin was shown to induce the transcription of esRAGE [325]. It was also hypothesized that sRAGE levels increase at acute phase as a protection mechanism and increased cellular damage, at chronic phase, impairs its formation or consumes most sRAGE by an increase in RAGE ligands [28]. Unfortunately, so far, no studies *in vivo* were performed attempting to elucidate the regulation of sRAGE production and fluctuation of sRAGE levels.

In humans, sRAGE has been associated with circulating AGEs [225], kidney dysfunction [264, 278] and the use of statins [337]. Among these factors, kidney dysfunction, often evidenced by low GFR or increased creatinine is the less controversial. Nevertheless, among patients with renal impairment, those with lower sRAGE seem to be more susceptible to CVDs. Intriguingly, it remains unknown why sRAGE levels increase with the decline of kidney function. It could due to a) a coordinated response against the oxidative and inflammatory stress [488], b) impaired clearance or c) consequence to RAGE overexpression and tissue damage. In any case, kidney dysfunction presents more coherent data concerning sRAGE levels than other diseases. Moreover sRAGE levels are also influenced by RAGE polymorphisms [350] and ethnicity [219], which have been also associated with complications of diabetes. Prevost and colleagues observed and association of the G82S polymorphism with advanced stages of diabetic nephropathy [489], although meta-analyses failed to find this relationship [490, 491]. The G82S polymorphism is associated with higher ligand-binding affinity and with enhanced proinflammatory responses [492] and it was shown to be associated with lower sRAGE levels [350]. However, the mechanisms underlying this association remain unknown. Hence, this multifactorial regulation of sRAGE levels jeopardizes studies in terms of inclusion/exclusion criteria and sample size.

Besides these factors mentioned above, we hypothesized that RAGE ligands, because of their structural variability and binding-site heterogeneity, could impair sRAGE quantification. This hypothesis, if true, would imply that sRAGE levels among publications only reflect free sRAGE and not ligand-bound (complexed) sRAGE. Using different ligands at different concentrations we saw no effect on sRAGE measurement. The recent discovery of anti-sRAGE autoantibodies led us to investigate their effects on sRAGE quantification as well, especially because antibodies may have increased affinity through processes of affinity maturation [493]. Nonetheless, in the first article of this thesis, we

showed that the presence of RAGE ligands or anti-sRAGE autoantibodies could not impair sRAGE quantification.

Other hypotheses to explain the variation of sRAGE levels are necrosis and sequestering by full-length RAGE or RAGE ligands. In the case of necrosis, membrane RAGE would be released, as well as the pool of intracellular esRAGE. It seems, however, very unlikely to reach such an extensive necrosis capable of considerably changing systemic sRAGE levels. In the case of sRAGE sequestering, it was shown that sRAGE forms dimers with membrane RAGE, a mechanism through which sRAGE would exert its beneficial effects [494]. Again, whether RAGE expression is important enough to capture circulating sRAGE and affect its measured levels, remains speculative. Moreover, tissues overexpressing RAGE ligands, especially AGEs, could act as additional compartments to interact with sRAGE [196].

The many hypothesis concerning sRAGE levels led us to further investigate them in a study designed to eliminate most of the factors known to influence sRAGE levels. Therefore, we opted to study sRAGE and anti-sRAGE autoantibodies in a cohort of morbidly obese patients.

5.3 The interest of the ABOS cohort

ABOS (Biological Atlas of Severe Obesity) is a study cohort that includes patients eligible for a weight loss surgery (bariatric surgery) with a BMI ≥ 35 kg/m^2, regardless of the degree of glucose regulation impairment. Because of its prospective nature and considerably well-defined patients' phenotypes, it is a cohort of great value to study molecules like sRAGE, of which the regulatory mechanism are still poorly understood.

For our study we selected 254 patients from the 750 individuals of the cohort. These patients met the inclusion criteria that were defined to eliminate factors that could

influence sRAGE levels. We only included patients with GFR >90ml/min/1.73m2, without arterial hypertension, who were not under ACEi, ARA2 or statins treatment and who were not smokers. Patients were then randomly selected to be equally distributed according to glucose regulation: normoglycemic, glucose intolerant and diabetic (n=50 each).

Baseline (before surgery) levels of sRAGE were higher in morbidly obese patients than in the control group. No difference between normoglycemic, glucose intolerant and diabetics was observed, further supporting that this increase was due to obesity. Sixty nine patients submitted to a gastric bypass and with a 1-year follow-up were included in the second part of the study. Serum sRAGE levels decreased to control values 1 year after surgery.

If we consider sRAGE as marker of RAGE overexpression and inflammation, an increase in its levels seems logical. To date, there is no data concerning the expression of RAGE in obese patients. In rats, high-fat diet increases RAGE expression in the retina of diabetic rats [495]; Zucker fatty rats have higher RAGE mRNA expression in the aorta than Zucker lean animals [496, 497]. Again, there is no data concerning the contribution of each tissue to the circulating pool of sRAGE. If RAGE expression increases in adipocytes of obese individuals, the infiltration of inflammatory cells may be responsible for sRAGE increase. Macrophage-conditioned medium induces expression of MMP-9 and MMP-3 in adipocytes [498]. Both metalloproteinases can cleave membrane RAGE to form sRAGE [201]. In our study, however, we did not investigate the activity of these enzymes.

Our findings concerning sRAGE levels in the obese contrast with those from 2 previous works where sRAGE was found inversely correlated with BMI [317] and lower in morbidly obese patients [356].Since all three studies used the same methodology to quantify sRAGE, we can only infer that the controversy is due to different inclusion/exclusion criteria. Brix *et al*. observed an increase in sRAGE levels 2 years after bariatric surgery while we had a 1-year follow-up. The controversy between their findings and ours may reside in a long-term regulation of sRAGE levels in parallel with weight loss and metabolic

improvement. The maximal loss of excess weight is achieved within 2 years after surgery, regardless of the procedure [499], and insulin resistance improvement is achieved within 1 year [500]. If, at first glance, both studies could seem contradictory, the complex regulation of sRAGE formation may explain such differences and this question should be addressed with *in vitro* and *in vivo* models.

Similarly to sRAGE, anti-sRAGE autoantibodies were increased in morbidly obese patients, regardless of glucose regulation impairment. In addition, gastric bypass led to a decrease in anti-sRAGE titers 1 year after intervention, although levels remained higher than control group. Although no correlation between anti-sRAGE and BMI or weight loss was observed, we found a weak association between the decrease in anti-sRAGE titers and the increase in HDL-cholesterol (r^2=0.077, p=0.02). This association does not support a cause-consequence relationship between both parameters, but may imply an association of anti-sRAGE autoantibodies and the increased vascular risk of morbidly obese patients.

The biological data available for our study population do not allow further speculation about the meaning of anti-sRAGE autoantibodies in morbid obesity. In addition, we can only hypothesize that RAGE is overexpressed in endothelial cells and adipocytes, without any evidence of one's contribution to the levels of circulating sRAGE and anti-sRAGE autoantibodies. Other markers of vascular dysfunction like sVCAM-1, endothelin and inflammatory cytokines (i.e. IL-6, IL-8 and IL-1) should be measured to better identify the scenario associated with autoimmunity against sRAGE. Also, we should further investigate the expression of RAGE, its ligands and sheddases responsible for sRAGE formation in the adipose tissue of these patients. Thereby, we would achieve an insightful view of adipocyte inflammation surrounding RAGE. Evidently, animal models of obesity in RAGE -/- background are an overwhelming tool in the pursuit to explain these phenomena.

5.4 Autoimmunity against sRAGE

In previous works anti-sRAGE autoantibodies were measured only in rheumatoid arthritis (RA) and Alzheimer's disease (AD) patients, both with increased levels compared to healthy controls. In RA patients they were observed in serum and synovial fluid, with higher levels in the latter being associated with the less erosive form of disease [445]. In AD, anti-sRAGE are associated with dementia scores [369, 371, 471] and were further attributed a protective role, with the development of a vaccine, consisted of a sRAGE/Aβ complex, that increases their production [372]. Vaccine-induced anti-sRAGE autoantibodies enhance neuronal viability after exposure to Aβ and improve cognitive function in AD mice [372].

In this work we observed increased levels of sRAGE in HD patients and morbidly obese subjects eligible for weight-loss surgery. Both study populations present a pro-inflammatory status and sRAGE was increased in comparison to controls. Serum levels of Aβ are increased with obesity and decrease after bariatric surgery [501]. If Aβ is increased in our obese patients, its association with high sRAGE levels could form the highly-immunogenic complex that could induce anti-sRAGE production [372].

Oxidative stress plays an important role in autoimmunity-associated complications and the role of ROS in the pathogenesis of autoimmune diseases is also subject of debate. Gerling suggests that antigens of autoimmune diseases are actually oxidatively-modified proteins, a hypothesis that would exempt true self antigens of a pathological role [502]. It would also imply antioxidants as potential therapeutic agents for these diseases. In the same line, Stark proposes that thiol oxidation impairs protein degradation, which will further lead to the generation of unfamiliar immunogenic peptides [503]. These hypotheses linking oxidative stress to autoimmunity pathogenesis have a profound impact on RAGE biology since its activation by different ligands increases ROS production.

Should RAGE be a major culprit in the pathogenesis of autoimmunity or just a “victim” of other sources of oxidative stress, it remains to be investigated.

There is evidence suggesting that RAGE is implicated in insulin resistance and adipocyte hypertrophy. In adipocytes and muscle cells, AGEs increase ROS production and inhibit glucose uptake in a RAGE-dependent manner [469, 504]. In adipocytes, the AGE-RAGE axis increases the expression of MCP-1, which is involved in atherosclerosis and insulin resistance. Two similar works presented controversial results concerning the role of RAGE in weight gain. The one of Monden and colleagues observed the RAGE activation mediates adipocyte hypertrophy *in vitro* and mice fed a high-fat diet present reduced weight gain, epididymal fat weight and higher insulin sensitivity in the absence of RAGE [47]. However, in a previous study, it was observed that RAGE -/- mice have a faster weight gain, higher cholesterol and insulin resistance [420]. Although the composition of the high fat diet differed between both studies, it remains intriguingly unclear why the outcomes were almost opposite. In addition, RAGE activation by AGEs was shown to promote adipogenesis of senescent preadipocytes, further implicating RAGE in adipocyte biology and obesity [505].

Although autoimmunity is frequently seen as deleterious, growing evidence indicates a role for autoimmune reactions in tissue repair. The group of Michael Schwartz demonstrated that autoimmune T lymphocytes mediate recovery after optical nerve injury or contusion of the spinal cord [506-509] and the induction of anti-sRAGE autoantibodies was protective in a mice model of AD [372]. Nonetheless, we cannot infer a protective role for anti-sRAGE autoantibodies in our study. Other autoantibodies should be investigated in the future to specify the nature of such autoreactivity.

Other studies have demonstrated the occurrence of autoimmune reaction in obese subjects, especially against β cell antigens, thyroid and sperm [510]. Nonetheless, the biological and clinical relevance of obesity-associated autoimmunity may be underrated

since improvement of the disease is likely to be evaluated by weight loss and metabolic changes. Indeed, antibodies against thryroglobulin, thyroid-stimulating hormone and ADAMTS13 are increased in obese subjects, compared to lean ones [441, 485]. However, to our knowledge, there are no studies analyzing the incidence of autoantibodies before and after weight-loss surgery. Our findings, thus, are the first to show a decrease in autoimmunity associated with weight loss, despite the lack of direct correlation between autoantibodies and BMI or weight loss.

One year after bariatric surgery both sRAGE and anti-sRAGE autoantibodies decreased, suggesting an association with obesity. Even though no correlations with weight loss or inflammatory markers like CRP were found, our data are in agreement with other works where sRAGE is increased in inflammatory diseases. In addition, the decrease in anti-sRAGE was correlated with the increase in HDL-cholesterol levels, implicating autoimmunity against sRAGE in the increased cardiovascular risk of obesity. Improved endothelial function was observed after bariatric surgery, associated with an increase in adiponectine and a decrease in leptin levels [511]. Our study is, however, limited because we did not investigate other markers of vascular dysfunction, neither adipokines like leptin and adiponectin.

5.5 Limitations

Our work has several limitations. In the first part, we only show that the presence of ligands and anti-sRAGE autoantibodies does not impair sRAGE quantification. We do not present the effects of RAGE ligands and anti-sRAGE autoantibodies in the production or clearance of sRAGE. In addition, as proposed by Renard and colleagues [196], AGEs could represent an additional compartment for sRAGE distribution. Therefore, AGEs-bearing cells would capture sRAGE, reducing its measurable levels in plasma or serum. Another limitation of the first study is that we do not use techniques other than ELISA to

quantify sRAGE. Nonetheless, the ELISA method is the standard technique used to measure sRAGE. Furthermore, we only measured total sRAGE levels, with no discrimination between esRAGE and cRAGE. To date there is no method to quantify cRAGE only and both cRAGE and esRAGE share the same ligand-binding domains, which should not represent a bias to our study.

In the second part of the thesis, although we selected our study population to avoid factors that could influence sRAGE levels (i.e. renal dysfunction, smoking, hypertension and statins), we have no data concerning the distribution of RAGE polymorphisms among our patients. Moreover, we did not investigate the activity of RAGE-cleavage enzymes (ADAM10, MMP-3, MMP-9 and MMP-13), which would provide useful information about the mechanisms regulating sRAGE formation. However, such an investigation would demand a large screening to evaluate the activities of these enzymes in different tissues. Similar approaches should be encouraged in other diseases to elucidate the specificities of each pathology. Finally, we have only a short-term follow-up. The evolution of sRAGE and anti-sRAGE levels should be further investigated in order to better associate them with the metabolic and inflammatory changes that follow bariatric surgery.

5.6 Conclusion

We have thus demonstrated that the presence of RAGE ligands and anti-sRAGE autoantibodies does not impair sRAGE quantification, suggesting that the differences in sRAGE levels among publications is not due to a lack of efficacy in the measurement. After a review of the literature, we indicate that inconsistencies may lie in differences of inclusion/exclusion criteria and insufficient knowledge of the potential factors that influence sRAGE levels.

We also demonstrated that hemodialysis and morbidly obese patients have higher levels of sRAGE and anti-sRAGE autoantibodies. In our cohort of morbidly obese patients, with well-defined inclusion criteria, sRAGE and anti-sRAGE titers are elevated and decrease 1 year after bariatric surgery. The decrease in anti-sRAGE is weakly correlated with the increase in HDL-cholesterol. These findings show an autoimmune reactivity against sRAGE associated with obesity and, although fairly conclusive, suggest that this autoimmunity is associated with increased vascular risk.

Chapter Six

Perspectives

In this work we have shown that the variability of sRAGE levels among publications is not due to the impairment of its quantification by RAGE ligands or autoantibodies. We have further demonstrated that individuals with morbid obesity, eligible for a gastric bypass, present increased levels of anti-sRAGE autoantibodies and these levels decrease after weight-loss surgery. Together, our results raise some questions that should be subject of further investigation:

a) Do RAGE ligands or autoantibodies affect RAGE cleavage?

   By studying several cell types overexpressing RAGE, different ligands and autoantibodies would be incubated. RAGE cleavage would be investigated by the activity of proteases and the quantification of sRAGE and esRAGE in the culture media.

b) Do RAGE ligands or autoantibodies affect sRAGE clearance?

   This question should be addressed by the injection of labeled sRAGE and different ligands in animals to evaluate any changes in clearance times. In addition, this investigation could be done in different disease models to better understand the impact of each pathology on the pool of sRAGE.

c) Do anti-sRAGE autoantibodies activate RAGE?

   In cultured cells overexpressing RAGE, purified autoantibodies would be added and RAGE activation would be assessed by the classical pathway that leads to NADPH activation and NF-κB translocation, for example.

d) Do anti-sRAGE autoantibodies block sRAGE?

With simple experiments applying sRAGE blocking effects, sRAGE would be pre-incubated with anti-sRAGE autoantibodies and further added to cultured cells in order to evaluate its RAGE-blocking activity.

e) Are anti-sRAGE autoantibodies biomarkers of RAGE-overexpressing diseases?

The occurrence of anti-sRAGE would be assessed in different disease cohorts or animal models. In animals, the effect of RAGE expression on anti-sRAGE formation would be assessed by regulating RAGE expression with interference RNA.

f) Is there a role for RAGE autoimmunity in obesity-related complications?

To answer this question, animal models of obesity, like the ob/ob mice, should be used. In this model, we would, at first, investigate the occurrence of anti-sRAGE in obese animals. The occurrence of anti-sRAGE should be verified at different stages of obesity and their production would be induced by the oral vaccine previously described. Immunosuppressant therapy would be applied to elucidate the participation of autoimmunity in obese-associated insulin resistance, inflammation and vascular dysfunction. Further studies in humans, with obese and pre-obese patients should be performed to elucidate the evolution of anti-sRAGE autoantibodies in association with the development of obese-related complications.

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