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Modifications post-traductionnelles des lipoprotéines de haute densité (HDL) et risque cardio-vasculaire dans l'insuffisance rénale chronique

Nans Florens

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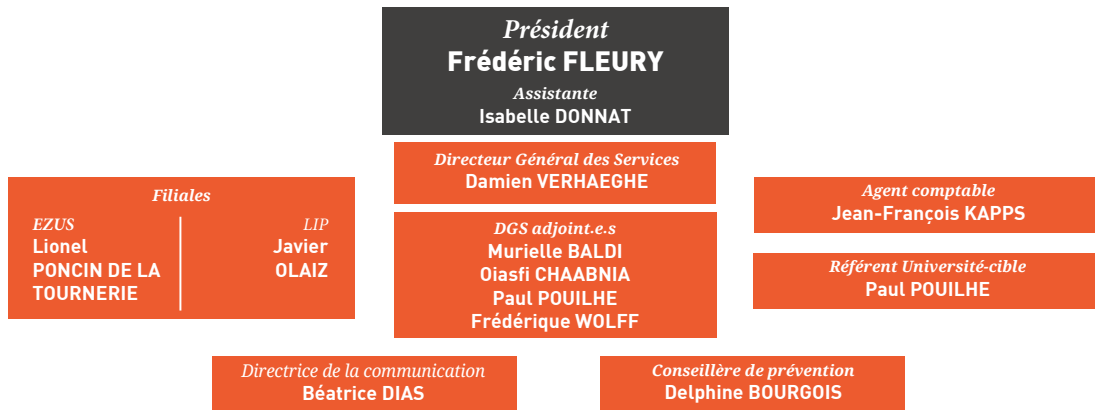
Modifications post-traductionnelles des lipoprotéines de haute densité (HDL) et risque cardio-vasculaire dans l'insuffisance rénale chronique

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* administrateur provisoire

*Pour l'enfant, amoureux de cartes et d'estampes,
L'univers est égal à son vaste appétit.
Ah ! que le monde est grand à la clarté des lampes !
Aux yeux du souvenir que le monde est petit !*

Charles Baudelaire ; Le voyage, *Les Fleurs du Mal*, 1857

Je dédie ce manuscrit à ma femme Caroline et à mes enfants Elisabeth et Arthur.

Puisse la vie vous apporter tout le bonheur que vous méritez.

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Abréviations

4-HHE : 4-hydroxy-2-hexenal

4-HNE : 4-hydroxy-2-nonenal

ADMA : asymmetrical dimethylarginine

AGEs : advanced glucose end products ou produits avancés de la glycation

ALEs : advanced lipid end products ou produits avancés de la peroxydation lipidique

AMBP : alpha-1-microglobuline

ApoA1 : apolipoprotéine A1

B2M : beta-2-microglobuline

CETP : cholesterol ester transfer protein

CD36 : cluster of differentiation 36

CFD : complement factor D

CML : carboxy-méthyl-lysine

CO₂ : dioxyde de carbone

Da : dalton

DFG : débit de filtration glomérulaire

DOLD : dimère désoxyglucosone-lysine

EPO : érythropoïétine

GFR : glomerular filtration rate

GOLD : dimère glyoxal-lysine

HD : hémodialyse

HDL : high density lipoprotein

HTA : hypertension artérielle

IAA : indole-3-acetic acid

IRC : insuffisance rénale chronique

JNK : Jun-kinase

KDIGO : kidney disease improving global outcomes

LDL : low density lipoprotein

LOX-1 : Lectin-type oxidized LDL receptor 1

MDA : malondialdehyde

MOLD : dimère methylglyoxal-lysine

MRC : maladie rénale chronique

NO : monoxyde d'azote

NO₂ : dioxyde d'azote

PON-1 : paraoxonase-1

ROS : reactive oxygen species

SDMA : symmetrical dimethylarginine

SFTPb : Pulmonary surfactant-associated protein

SR-B1 : scavenger receptor B1

SRC : Sarc

TG : tryglycérides

TMAO : triméthylamine-N-oxide

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1. Introduction

1.1. La maladie rénale chronique

La maladie rénale chronique (MRC) associe une baisse du débit de filtration glomérulaire (DFG) en dessous de 90 mL/min/1.73m² et des marqueurs d'atteinte rénales représentés par une protéinurie (ou micro-albuminurie) et/ou des anomalies du sédiment urinaire (hématurie, leucocyturie). Elle se décline en 5 stades définis par les recommandations internationales K.D.I.G.O.¹ (**Figure 1**). Si ces anomalies persistent plus de trois mois on parle alors de MRC.

Renal dysfunction > 3 months



The diagram shows three kidneys arranged vertically, illustrating the progression of kidney damage. The top kidney is a healthy, dark red kidney. The middle kidney is smaller and lighter in color, indicating moderate damage. The bottom kidney is significantly smaller and more fragmented, representing end-stage renal disease.

Stages of Chronic Kidney Disease		
Stage	Qualitative Description	Renal function (mL/min/1.73m ²)
1	<i>Kidney damage - Normal GFR</i>	≥ 90
2	<i>Kidney damage - Mild ↓ GFR</i>	60 - 89
3	<i>Moderate - ↓ GFR</i>	30 - 59
4	<i>Severe ↓ GFR</i>	15 - 29
5	<i>End-stage renal disease</i>	< 15 (or dialysis)

Kidney damage: hematuria, proteinuria, leucocyturia

Figure 1 – Stades de la maladie rénale chronique selon les recommandations KDIGO¹

A mesure que le rein dysfonctionne, les grandes fonctions biologiques qu'il assure sont perturbées (**Figure 2**). Ainsi, l'insuffisance rénale chronique (IRC) que cela entraîne est responsable d'un certain nombre de complications qui en découlent directement (anémie, troubles phospho-calciques, surcharge hydro-sodée, hypertension, troubles hydro-électrolytiques) mais aussi d'autres, qui sont la conséquence de plusieurs de ces dysfonctions, comme le sur-risque cardio-vasculaire.

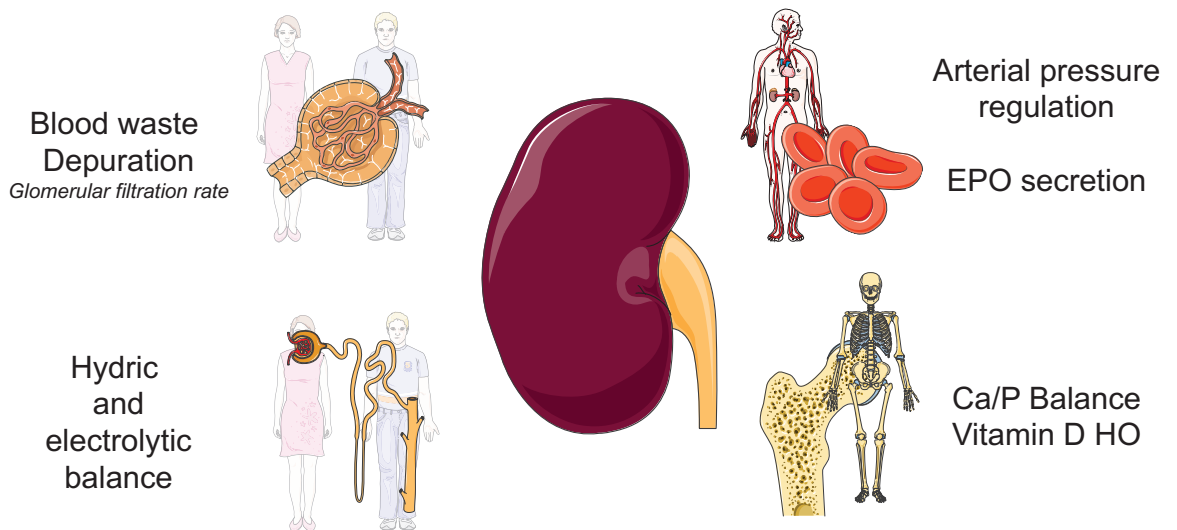


Figure 2 – Illustration des grandes fonctions biologiques du rein.

Le rein assure l'épuration des déchets métaboliques non volatiles et hydrosolubles. La capacité de filtration du rein est définie par le débit de filtration glomérulaire ou DFG. Au niveau tubulaire, le rein va permettre d'ajuster la balance hydro-électrolytique et de concert avec la sécrétion hormonale de rénine, la régulation de la pression artérielle. La sécrétion d'érythropoïétine va permettre la synthèse des globules rouges. Le rein intervient aussi dans l'hydroxylation de la vitamine D et joue ainsi un rôle majeur dans la régulation du métabolisme phospho-calcique.

1.2. Le risque cardio-vasculaire dans la maladie rénale chronique

L'insuffisance rénale chronique (IRC) s'accompagne d'un sur-risque cardiovasculaire responsable d'une morbi-mortalité importante (**Figure 3**).² Ce sur-risque s'intensifie à mesure que l'insuffisance rénale progresse avec un maximum atteint chez les patients en IRC terminale hémodialysés.³ Les déterminants de ce sur-risque, bien que largement étudiés, restent encore assez mal compris. Chez les patients atteints d'IRC, les facteurs traditionnels de risques cardio-vasculaires tels l'âge, le sexe, le diabète, l'HTA ou encore le tabagisme et les dyslipidémies sont souvent présents. Cependant, de nombreux autres facteurs non traditionnels viennent se surajouter comme l'accumulation de toxines urémiques, l'inflammation, les désordres phospho-calciques ou le stress oxydant.⁴

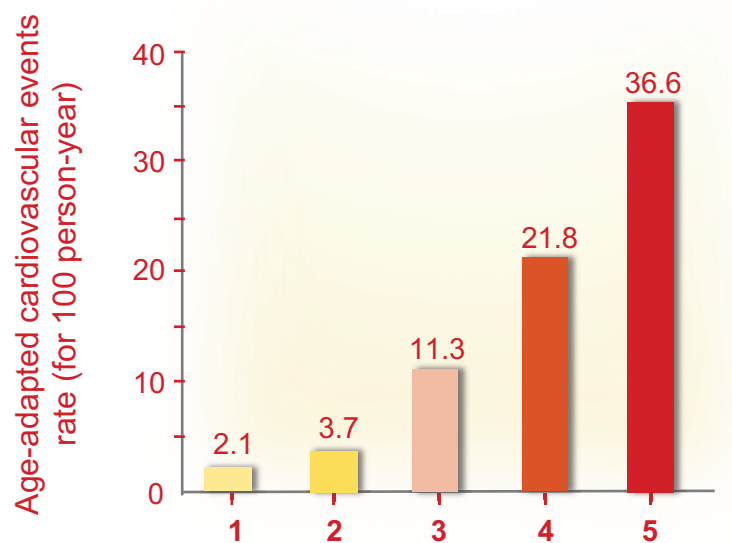


Figure 3 – Incidence des évènements cardio-vasculaires selon les stades d’insuffisance rénale chronique. Adapté de Go et al, NEJM 2004.²

L’IRC per se est associée à un sur-risque cardio-vasculaire. La baisse du DFG est associée à un sur-risque d’évènements cardio-vasculaires après ajustement sur les facteurs de risques traditionnels. Ces données sont issues de l’analyse d’une cohorte de plus d’un million de patients.

1.3. Le rôle des lipoprotéines dans le risque cardio-vasculaire lié à l’IRC

Le caractère unique de l’IRC dans ce domaine est aussi souligné par l’échec des stratégies de prévention du risque cardio-vasculaire pourtant démontrées dans d’autres populations à risque. Au premier plan, nous pouvons citer l’inefficacité des statines à réduire le risque cardiovasculaire chez le patient hémodialysé malgré une baisse réelle du LDL cholestérol.^{5,6} D’autres indices comme la meilleure santé cardiovasculaire des patients hémodialysés présentant un rapport TG/HDL-cholestérol élevé,⁷ alors que ce rapport est associé à plus de morbi-mortalité cardiovasculaire dans la population générale, questionne aussi sur la place centrale des lipoprotéines et de leur métabolisme dans la genèse de la maladie cardiovasculaire de l’IRC.

La perturbation du métabolisme des lipoprotéines aboutissant à ce phénotype particulier pourrait être en grande partie expliquée par les modifications oxydatives et non-oxydatives qu'elles subissent tout particulièrement dans les situations d'IRC avancées.

1.4. Place des travaux de thèse d'université de ce manuscrit

Le présent travail s'intéressera donc plus spécifiquement aux modifications oxydatives et post-traductionnelles des lipoprotéines de haute densité (HDL) dans l'IRC ainsi qu'à leur impact leurs propriétés biologiques et plus particulièrement sur leurs effet anti-agrégant plaquettaire .

2. La toxicité urémique

Une toxine urémique est définie comme un composé 1) normalement excrété dans les urines et qui donc s'accumule chez l'insuffisant rénal du fait d'un défaut de clairance rénale ; et 2) qui a des effets biologiques délétères.⁸ Le groupe de travail européen EuTox (European Uremic Toxin Workgroup, www.uremic-toxins.org) a référencé près d'une centaine de solutés répondant à ces caractéristiques et cette liste n'est pas encore exhaustive.

Les toxines urémiques sont traditionnellement classées en trois groupes (**Table 1**):

- Les composés de faible poids moléculaire (MW < 500 Da) dont le chef de file est l'urée
- Les moyennes et larges molécules (MW 500Da - 60kDa) dont le chef de file est la β 2-microglobuline.
- Les toxines urémiques liées aux protéines dont la particularité est d'être fortement liées aux protéines plasmatiques circulantes. Leur chef de file est l'indoxyl-sulfate.

Ainsi, l'accumulation de toxines urémiques dans l'IRC est responsable de la perturbation de nombreuses fonctions biologiques essentielles. Parmi les nombreux composés recensés par l'EuTox,⁸ certaines ont un tropisme toxique particulièrement intense sur le système cardiovasculaire comme l'indoxyl-sulfate,^{9,10} l'acide indole-3-acétique¹¹ et la Triméthylamine-

N-oxyde.¹² Cet environnement urémique contribue sans doute largement au sur-risque cardiovasculaire mais ne peut à lui seul pas l'expliquer complètement. En effet, un certain nombre de ces toxines n'ont pas fait la preuve directe de leur toxicité cardio-vasculaire.¹³ D'autre part, les concentrations plasmatiques de ces toxines sont souvent moins élevées chez les patients dialysés que chez les patients IRC de stade terminal non-dialysés⁸ alors que leur risque cardio-vasculaire et leurs complications en général sont plus importantes.

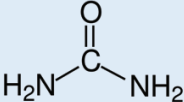
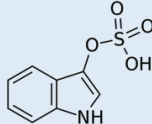
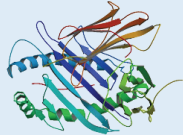
	Petites molécules hydrosolubles (<500 Da)	Toxines liées aux protéines	Moyennes et larges molécules (> 500 Da)
Exemples de molécules	Urée, triméthylamine-N-oxyde (TMAO), asymmetrical and symmetrical dimethylarginine (ADMA et SDMA)	Indoxyl sulfate, p-cresyl sulfate, indole-3-acetic acid (IAA), acide hippurique	Leptine, Complement Factor D (CFD), β2-microglobuline
Chef de file	Urée 	Indoxyl sulfate 	β2-microglobuline 

Table 1 – Les trois groupes de toxines urémiques tels que définis par le groupe EuTox.⁸

3. Le stress oxydant et les modifications post-traductionnelles

La concentration de certaines toxines urémiques tend cependant à être plus basse dans la population hémodialysée que dans la population insuffisante rénale pré-terminale et terminale non dialysée alors que celle-ci présente le plus haut risque de complications cardiovasculaires.³ L'impact du stress oxydant et des modifications post-traductionnelles sur différents composants du système cardiovasculaire joue un rôle majeur dans l'apparition et l'aggravation des lésions d'athérosclérose.¹⁴

3.1. Le stress oxydant

Le stress oxydant résulte d'un déséquilibre de la balance pro-oxydante et anti-oxydante de l'organisme, responsable d'une surproduction d'espèces réactives de l'oxygène appelées reactive oxygen species ou ROS. Ces composés ont une demi-vie très courte et vont rapidement

réagir dans les matrices biologiques avec d'autres molécules comme les ions métalliques (Fe^{2+} et Cu^{2+}), les protéines, les lipides et les acides nucléiques. Les modifications oxydatives peuvent alors être directes, par oxydation des biomolécules, ou indirectes par des modifications stables et peu réversibles des acides nucléiques et des protéines appelées adduits covalents. Pour ces dernières, on parle alors de modifications post-traductionnelles.

3.2. Les modifications post-traductionnelles

Ce terme regroupe plusieurs classes de modifications qui ne sont pas toutes en lien avec les résultats d'un excès de stress oxydant. On peut donc distinguer plusieurs mécanismes détaillés tels la carbonylation, la glycation ou la carbamoylation. La plupart de ces réactions d'adduction sont non-enzymatiques et ont lieu sur les résidus lysine, cystéine et histidine des protéines. D'autres réactions peuvent être catalysées par des enzymes comme les myéloperoxydases dont l'activité a été montrée comme étant augmentée dans l'IRC.¹⁵⁻¹⁷

3.2.1. La carbonylation

La carbonylation résulte de l'adduction sur les protéines d'aldéhydes réactifs issus de la peroxydation des acides gras polyinsaturés. Parmi ces aldéhydes, on peut citer le malondialdéhyde (MDA), le 4-hydroxy-2-nonenal (4-HNE) et le 4-hydroxy-2-hexenal (4-HHE) (**Figure 4**). Le 4-HNE et le 4-HHE sont issus respectivement de l'oxydation des acides gras polyinsaturés des familles ω -6 et ω -3. Ces aldéhydes peuvent former des adduits covalents sur les résidus lysine, cystéine et histidine des protéines par la réaction de Michael¹⁸ (i.e. production d'adduits de Michael non réversibles, voir **Figure 5**), non catalysée par un processus enzymatique, et ainsi former des produits avancés de la peroxydation lipidique (ALEs*) ou

* ALEs, Advanced lipoxidation end-products. Les ALEs sont l'équivalent pour les aldéhydes issus de lipides des AGEs (Advanced glycation end-products) pour les aldéhydes issus du glucose tel le méthylglyoxal.

encore nommés carbonyles. Ces résidus sont notamment impliqués dans la perturbation de la fonction endothéliale^{19,20} et la prolifération des cellules musculaires lisses dans la paroi artérielle.²¹

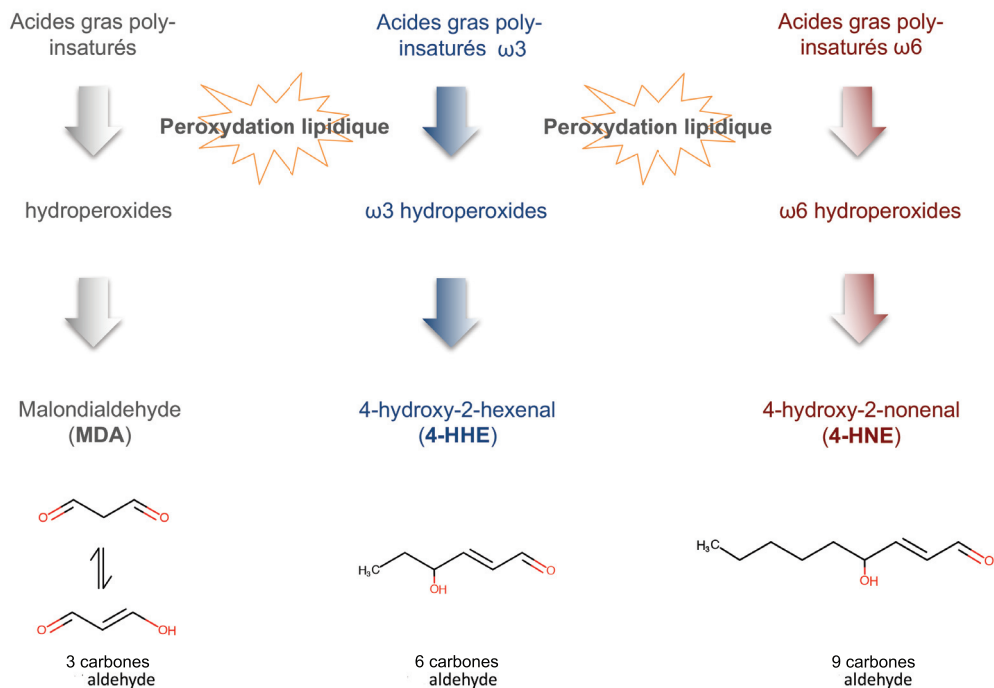


Figure 4 — Principaux aldéhydes issus de la peroxydation lipidique (adapté d’après Florens et al, 2016)²²

La peroxydation lipidique entraîne la formation d’hydroperoxydes issues des différentes sous-classes d’acides gras polyinsaturés. Ces derniers vont, selon la classe incriminée, donner du malondialdéhyde (MDA), du 4-hydroxy-2-hexenal (4-HHE) ou du 4-hydroxy-2-nonenal (4-HNE), respectivement pour les acides gras polyinsaturés (quel que soit leur nombre de double liaison), les acides gras des familles oméga 3 ou oméga 6.

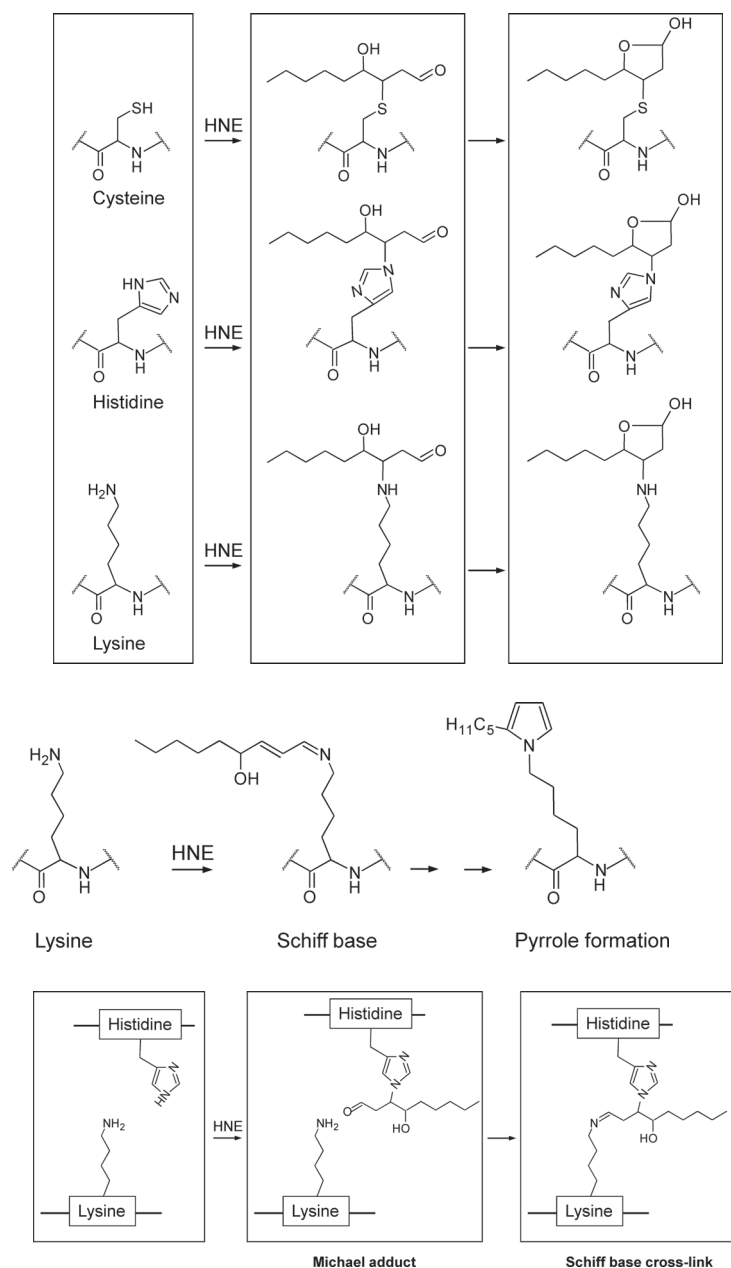


Figure 5 — Exemple de formation des adduits de Michael sur la cystéine, l’histidine et la lysine par le 4-hydroxy-2-nonenal (HNE).

Le HNE se lie de façon covalente sur la fonction thiol de la Cystéine, le groupe ε-amino de la Lysine ou encore le noyau imidazole de l’Histidine. L’adduit peut alors se stabiliser sous forme de noyau hémiacétal par cyclisation de la terminaison aldéhyde et le carbone-4 de la molécule de 4-HNE¹⁸.

On peut aussi noter la formation de noyau Pyrrole lorsque l’adduit se forme sur la Lysine et la formation de pont d’adduit 4-HNE entre une Lysine et une Histidine.

Source de la figure : http://users2.unimi.it/HNECLUB/?page_id=502

3.2.2. *La glycation non enzymatique*

La glycation des protéines se fait aussi selon un mécanisme non enzymatique appelé réaction de Maillard.²³ Celle-ci est le fruit de la liaison du groupe carbonyle d'un sucre réducteur sur les résidus amines des acides aminés. Cette liaison va former un produit de glycation précoce instable qui va rapidement se transformer en céto-amine (réaction dite d'Amadori) ou réagir avec certains aldéhydes issus de la peroxydation lipidique. Les produits de Maillard ainsi formés sont aussi appelés produits avancés de la glycation ou AGEs. Parmi ces derniers, on peut citer la N-carboxyméthyllysine, la pyrroline, le glyoxal (**Figure 6**). Des dimérisations avec la lysine peuvent aussi émerger de ces réactions comme la pentosidine, le MOLD (dimère méthylglyoxal-lysine), le GOLD (dimère glyoxal-lysine) ou le DOLD (dimère désoxyglucosone-lysine).^{24,25}

La glycation des protéines est très bien documentée et ses effets délétères sur de nombreuses fonctions biologiques sont reconnus. Il est à noter que le phénomène de glycation affecte tout particulièrement les lipoprotéines circulantes. La glycation non enzymatique des LDL accélère la formation et l'accumulation de cellules spumeuses, constituants importants de la plaque d'athérome.²⁶ Cette glycation est particulièrement marquée chez le patient diabétique. Cependant, elle a aussi été décrite chez les patients IRC et notamment les patients hémodialysés chroniques.^{27,28} Dans cette population, la glycation de l'albumine a été reliée à de nombreux effets délétères comme la génération de ROS par activation des polynucléaires neutrophiles.²⁹

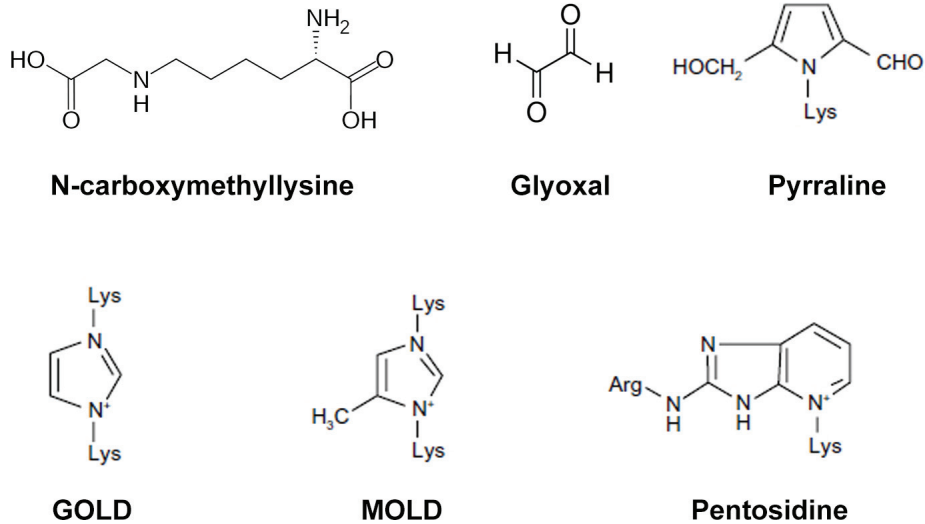


Figure 6 — Principaux produits avancés de glycation (AGEs, advanced glycation end-products) issus de la réaction de Maillard

3.2.3. La carbamoylation

Parmi les autres modifications post-traductionnelles, la carbamoylation joue un rôle particulièrement important dans l'insuffisance rénale. Cette réaction résulte de la fixation d'acide isocyanique sur les groupements amines des acides aminés, en position α (formation de protéine α -carbamoylée) ou en position ϵ sur la lysine formant alors un dérivé connu sous le nom de ϵ -carbamoyl-lysine. L'acide isocyanique est issu du cyanate (dont il est le principal tautomère[†]) par trois voies métaboliques principales (**Figure 7**) :

- la dégradation non-enzymatique de l'urée par transformation spontanée en cyanate d'ammonium
- la transformation, catalysée par les myéloperoxydases, du thiocyanate en acide isocyanique et en cyanate par l'adjonction de peroxyde d'hydrogène

[†] Un tautomère est un isomère de constitution issue de la réaction chimique de tautomérisation. Celle-ci est caractérisée par la migration d'un atome d'hydrogène avec changement de localisation de la double liaison.

- la tautomérisation du cyanate issu d'un apport exogène comme par exemple la fumée du tabac

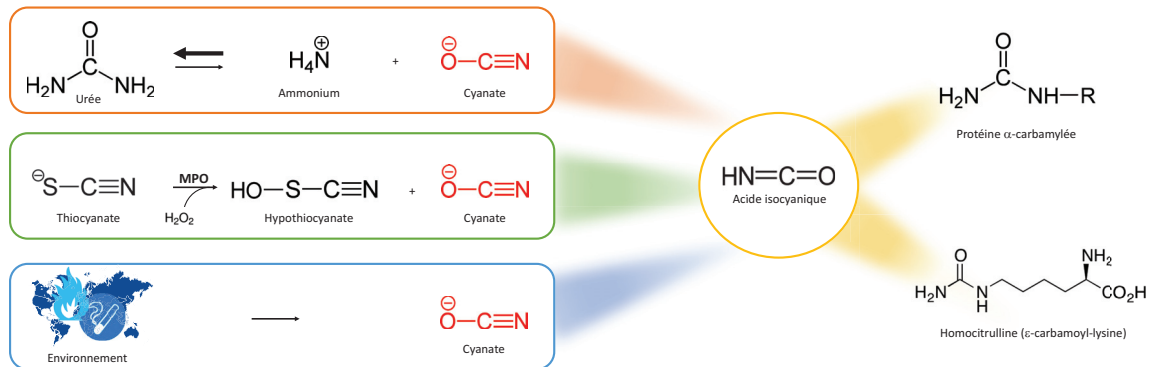


Figure 7 — Voies de carbamoylation des protéines

Le cyanate est issu de la dégradation spontanée de l'urée, de la métabolisation du thiocyanate par les myéloperoxidases (MPO) et/ou d'un apport exogène issue de la combustion de la biomasse et de la fumée de cigarettes. Son isomérisation en acide isocyanique le rend particulièrement réactif vis à vis des groupements amines des acides aminés.

La carbamoylation est de façon abusive nommée carbamylation dans de nombreux articles. Cependant, la carbamylation correspond stricto sensu à l'ajout d'une molécule de CO_2 et non d'isocyanate. La carbamoylation est généralement reconnue comme étant l'une des plus fréquentes chez les patients IRC. Ainsi, la circulation en grande quantité des formes carbamoylées de l'albumine sont reliées à une importante morbi-mortalité cardiovasculaire.³⁰ L'évolution de la concentration en albumine carbamoylée après le début de la dialyse semble être un marqueur pronostique fort concernant la mortalité et la morbidité cardio-vasculaire.³¹ De plus, les formes carbamoylées de l'albumine semblent jouer un rôle pro-fibrosant au niveau du parenchyme rénal.³² Cette carbamoylation affecte également les lipoprotéines circulantes. De nombreux travaux ont démontré l'implication de la carbamoylation des lipoprotéines circulantes dans l'avènement de complications cardio-vasculaires particulièrement quand elle

concerne les LDL. Ces derniers, par le biais de leur fixation à des récepteurs scavengers‡, entraînent l'accumulation de cholestérol dans les macrophages, qui par la suite se logent dans la paroi vasculaire³³ et favorisent la transformation des macrophages en cellules spumeuses.³⁴ Les LDL vont aussi acquérir des propriétés pro-agrégantes par le biais de la fixation sur le récepteur scavenger LOX-1.³⁵

Cette carbamoylation des protéines semble donc être un déterminant majeur dans la morbi-mortalité cardiovasculaire en général³⁶ et dans l'IRC.³⁷ Elle est ainsi pour beaucoup, considérée comme la modification post-traductionnelle dominante chez le patient IRC.

3.2.4. Autres modifications post-traductionnelles

D'autres modifications post-traductionnelles peuvent apparaître sur les protéines. On peut notamment citer la nitration (adduction de NO), la nitrosylation (adduction de NO₂) et la chlorination (impliquant l'acide hypochloreux ou HClO). Ces modifications touchent particulièrement la tyrosine et sont catalysées par les myéloperoxydases. Alors que la chlorination joue potentiellement un rôle délétère sur les apolipoprotéines,³⁸ le rôle spécifique de la nitration et de la nitrosylation reste mal défini.

La guanidinylation correspond à l'adduction d'une molécule de guanidine sur la fonction amines des acides aminés. Cette modification est bien décrite dans la chimie des polymères car elle permet d'améliorer la biocompatibilité de certains matériaux comme le chitosan³⁹ ainsi que leurs propriétés anti-bactériennes.⁴⁰ Sur les lysines, l'adduction de guanidine forme l'homoarginine, un composé qui, lorsqu'il est libre, voit sa concentration inversement corrélé à la mortalité des patients IRC terminaux non-dialysés.⁴¹ Cet acide aminé

‡ Les récepteurs scavengers (« éboueurs » en français) représentent une famille de récepteurs membranaires de natures extrêmement variées qui reconnaissent essentiellement les LDL modifiées ou oxydées afin de permettre leur élimination. Ils sont situés sur les macrophages, les cellules musculaires lisses artérielles, les cellules endothéliales et les plaquettes. Leur rôle est central dans la survenue des pathologies cardiovasculaires. On peut citer dans cette famille les récepteurs CD36, LOX-1, SR-A1 et SR-B1.

est aussi le précurseur de l'oxyde nitrique. Sa toxicité en tant que modification post-traductionnelle est cependant peu documentée. La guanidinylation de l'albumine a cependant été décrite comme réduisant ses capacités à lier des composés hydrophobes et notamment certaines toxines comme l'indoxyl-sulfate.⁴²

L'ensemble des modifications oxydatives et d'adduction sur les lipoprotéines va mener à une modification profonde de leurs fonctions biologiques et sans doute contribuer au phénotype dyslipidémique unique associé à l'IRC.

4. Notions d'agrégation plaquettaire

L'agrégation plaquettaire est un maillon essentiel de l'hémostase primaire en cas de lésion vasculaire. La première étape est l'adhésion plaquettaire au sous-endothélium via la reconnaissance par la glycoprotéine Ib du facteur von Willebrand. Parallèlement, les intégrines $\beta 1$ et la glycoprotéines VI vont reconnaître les fibres de collagène exposées. S'en suit un changement conformationnel de la forme plaquettaire qui va donc s'activer. L'activation se fait donc d'une part, pour les plaquettes liées au sous-endothélium par la transduction du signal intraplaquettaire en réponse à la fixation des glycoprotéines Ib et VI. Cette fixation va aussi entraîner le relargage des granules intra-plaquettaires qui contiennent des médiateurs de l'activation plaquettaire permettant le signal d'amplification de l'activation :

- L'ADP, qui se fixe sur les récepteurs P2Y1 et P2Y12 et inhibe la production intracellulaire d'AMPc intracellulaire. Cette inhibition va donc favoriser la dégranulation plaquettaire.
- Le thromboxane A2 qui va venir se lier aux récepteurs PAR, TP et P2Y12.
- La thrombine va aussi activer la plaquette via sa fixation sur les récepteurs PAR1 et 4
- Le facteur Von Willebrand soluble va lui aussi pouvoir activer la plaquette.

Cette activation va entraîner in fine, d'une part le relargage des granules pour l'amplification de la réponse plaquettaire et aussi le changement de conformation de la glycoprotéine GpIIb/IIIa qui va, en fixant le fibrinogène entraîner la liaison des plaquettes entre elles et donc la formation du clou plaquettaire (**Figure 8A**). Il existe néanmoins de nombreux autres récepteurs présents à la surface plaquettaire pouvant être impliqués dans la régulation de son activation, comme les récepteurs scavenger SR-B1 (plutôt inhibiteur⁴³), LOX-1³⁵ et CD36⁴⁴ (plutôt activateurs). Ces récepteurs ont comme ligands les lipoprotéines et particulièrement leur forme modifiées (oxydées ou présentant des modifications post-traductionnelles).

Les anti-agrégants plaquettaires vont agir à différent endroit de l'activation des plaquettes. L'aspirine inhibe la fonction de la cyclo-oxygénase 1 et donc limite la génération du thromboxane A2. Le clopidogrel, prasugrel et ticagrélor sont des inhibiteurs des récepteurs P2Y1 et P2Y12. L'abciximab quant à lui va empêcher la fixation du fibrinogène sur la glycoprotéine IIb/IIIa.

Dans l'insuffisance rénale chronique, il existe à la fois des arguments pour un état pro-thrombotique et pro-hémorragique, en lien avec tantôt une hyporéactivité ou une hyperréactivité plaquettaire.⁴⁵ En effet, l'indoxyl-sulfate a été relié à un état pro-thrombotique par sa capacité à activer les plaquettes.⁴⁶ Cependant, il existe aussi des arguments pour étayer l'hypothèse d'une hyporéactivité plaquettaire dans l'IRC. En effet, il existe une baisse de l'expression et une dysfonction des glycoprotéines Ib et IIb/IIIa⁴⁷ et une augmentation des prostaglandines anti-agrégantes.^{48,49}

Concernant le rôle des HDL, elles exercent une activité plutôt anti-agrégante par le biais de la fixation de l'apoA1 sur le récepteur scavenger SR-B1. Il existe aussi une autre voie d'inhibition plaquettaire par le biais de la fixation de l'apoE sur le récepteur ApoER2. Ce dernier exerce aussi une activité pro-activatrice par la phosphorylation de la p38-MAP kinase (**Figure 8B**).^{43,50}

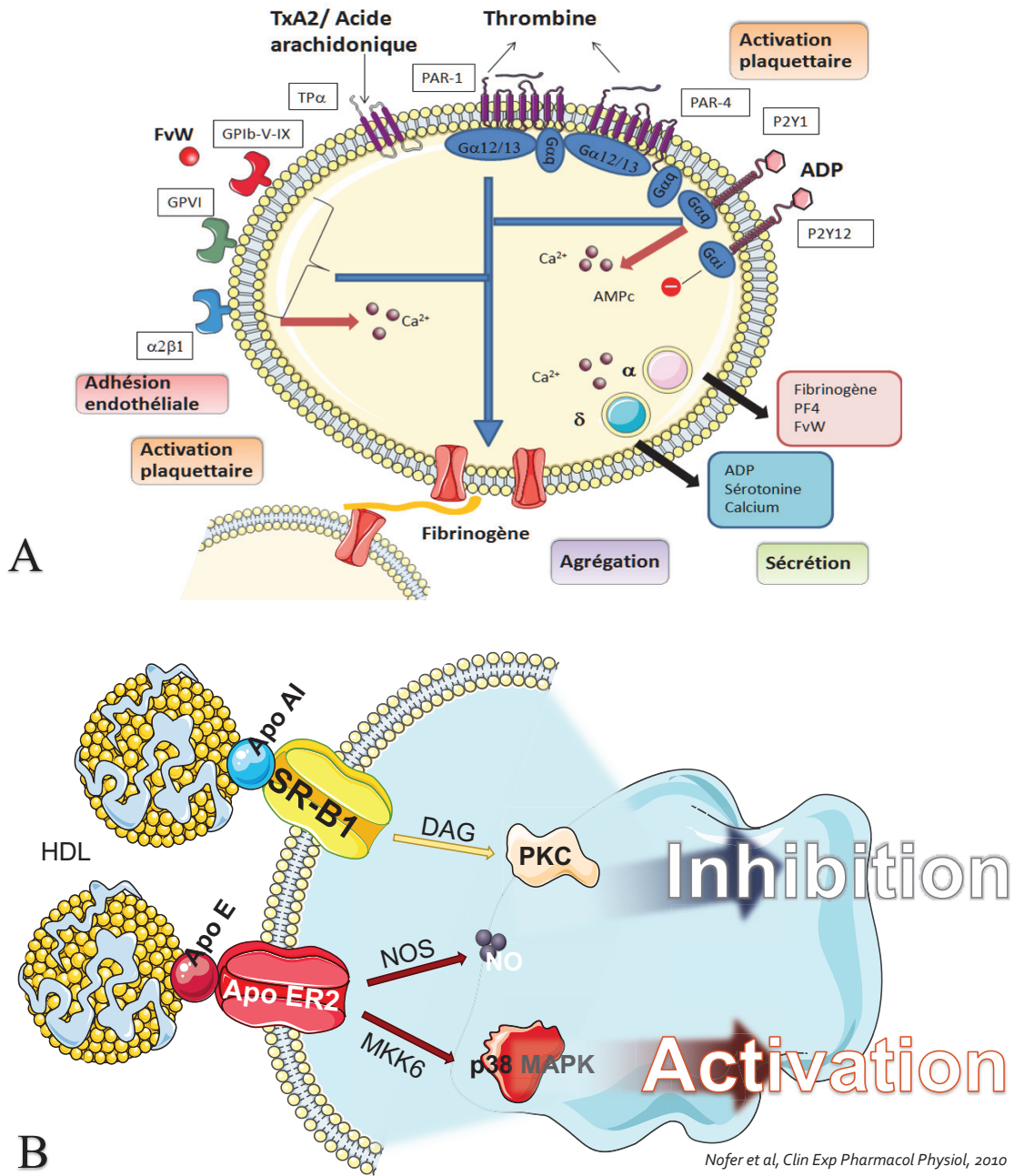


Figure 8 – Voies d'activation plaquettaires

A : Résumé des différents récepteurs impliqués dans l'activation et l'agrégation plaquettaire. Figure adaptée de Perez P. Fonction plaquettaire et altération de l'hémostase après hémorragie sous arachnoïdienne. Médecine humaine et pathologie. 2017.

B : Voies physiologiques impliquées dans les propriétés anti-agrégantes des HDL. DAG : di-acyl-glycérol ; NOS : NO synthase ; MKK6 : Mitogen activated protein kinase kinase 6 ; PKC : protéine kinase C. Figure adaptée de Nofer et al.⁴³

5. Lipides et lipoprotéines oxydés dans l'IRC :

Modified Lipids and Lipoproteins in Chronic Kidney Disease: A New Class of Uremic Toxins.

N. Florens, C. Calzada, E. Lyasko, L. Juillard, C. O. Soulage.

Toxin (Basel). 2016. 8(12): 376. Special issue on Uremic toxicity.

La première partie de ce travail est une revue de la littérature qui discute le bien-fondé de considérer lipides et les lipoprotéines modifiés par des processus oxydatifs ou non-oxydatifs comme de réelles toxines urémiques. En effet, la définition d'une toxine urémique proposée par l'EuTox, est définie comme un composé normalement excrété dans les urines et qui s'accumule chez l'insuffisance rénale du fait d'un défaut de clairance rénale ; et qui a des effets biologiques délétères sur l'organisme.⁸ De ce fait, les lipides et lipoprotéines oxydés peuvent être considérés comme tels. Les lipides ne sont toutefois pas tous *stricto sensu* excrétés par le rein et à cet égard les considérer comme toxines urémiques reste questionnable.

D'une part, l'accumulation dans l'insuffisance rénale chronique de lipides oxydés est désormais bien démontrée. C'est le cas des aldéhydes issus de la peroxydation des acides gras insaturés : le malondialdéhyde (MDA)⁵¹ et les aldéhydes lipidiques (tels les hydroxyalkenals) avec au premier plan, les 4-HHE et 4-HNE.⁵² Les mêmes constatations peuvent être faites avec l'accumulation des F2-isoprostanes issus de l'oxydation de l'acide arachidonique,⁵³ des phospholipides oxydés⁵⁴ et des oxystérols⁵⁵ issus de l'oxydation du cholestérol (voir le **Tableau 1** de l'article).

D'autre part, ces modifications entraînent des altérations importantes des propriétés biologiques jusqu'à pour certains, leur conférer un caractère toxique. En effet, ces résidus oxydés présentent une toxicité particulièrement marquée sur le système cardiovasculaire avec

un tropisme particulier sur les cellules endothéliales et les cellules musculaires lisses vasculaires (voir la **Figure 3** de l'article).

Ainsi, la combinaison de la toxicité et de l'accumulation dans l'IRC rend cohérent le reclassement de certains de ces résidus en toxines urémiques. Pour aller plus loin, si l'on considère ces mêmes critères (toxicités et accumulation), certains effecteurs biologiques essentiels peuvent aussi, dans une certaine mesure, être considérés comme toxines urémiques.

C'est notamment le cas des lipoprotéines circulantes avec un intérêt particulier pour les LDL et les HDL. Ces lipoprotéines vont pouvoir se charger en lipides oxydés et, pour les HDL en assurer leur clairance. Les modifications post-traductionnelles précédemment définies peuvent en outre massivement perturber le fonctionnement normal de ces lipoprotéines et aboutir à un phénotype dyslipidémique unique. D'un côté les LDL, dont les modifications sur l'apolipoprotéine B100 vont empêcher leur fixation et leur régulation par le LDL récepteur et accélérer leur clairance par des voies médiées par des récepteurs scavengers échappant à tout rétrocontrôle.⁵⁶ De l'autre, les HDL, lipoprotéines indispensables dans le métabolisme des triglycérides et du cholestérol dans l'organisme, verront leurs fonctions biologiques modifiées et ainsi aboutir à une profonde perturbation du métabolisme des lipoprotéines expliquant en partie le phénotype unique de la dyslipidémie dans l'IRC. Celui-ci rassemblant une hypertriglycéridémie, une élévation du LDL cholestérol, une accumulation des lipoprotéines contenant les apolipoprotéines B et une diminution des concentrations de HDL cholestérol.^{57,58} Les HDL vont également perdre des fonctions directement reconnues comme athéroprotectrices (voir la **Figure 3** de l'article) :

- La capacité à induire l'efflux de cholestérol, fonctionnalité majeure des HDL au niveau périphérique et reconnue comme directement liée à l'athérosclérose lorsqu'elle est perturbée.^{59,60}

- La protection endothéliale avec la perte d'un phénotype plutôt vasodilatateur par induction de sécrétion de NO et de l'effet anti-apoptotique sur les cellules endothéliales.⁶¹
- L'effet anti-inflammatoire avec l'induction de sécrétion de chemokines, de protéines d'adhésion cellulaire et de cytokines pro-inflammatoires.⁶¹

Cette revue s'intéresse donc au bien-fondé de considérer certains résidus lipidiques et par extension certaines lipoprotéines comme **des acteurs essentiels de la toxicité urémique.**

Review

Modified Lipids and Lipoproteins in Chronic Kidney Disease: A New Class of Uremic Toxins

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Abstract: Chronic kidney disease (CKD) is associated with an enhanced oxidative stress and deep modifications in lipid and lipoprotein metabolism. First, many oxidized lipids accumulate in CKD and were shown to exert toxic effects on cells and tissues. These lipids are known to interfere with many cell functions and to be pro-apoptotic and pro-inflammatory, especially in the cardiovascular system. Some, like F2-isoprostanes, are directly correlated with CKD progression. Their accumulation, added to their noxious effects, rendered their nomination as uremic toxins credible. Similarly, lipoproteins are deeply altered by CKD modifications, either in their metabolism or composition. These impairments lead to impaired effects of HDL on their normal effectors and may strongly participate in accelerated atherosclerosis and failure of statins in end-stage renal disease patients. This review describes the impact of oxidized lipids and other modifications in the natural history of CKD and its complications. Moreover, this review focuses on the modifications of lipoproteins and their impact on the emergence of cardiovascular diseases in CKD as well as the appropriateness of considering them as actual mediators of uremic toxicity.

Keywords: uremic toxin; oxidative stress; lipid; lipoprotein

1. Introduction

Chronic kidney disease (CKD) is associated with an increased risk of cardiovascular disease as these patients develop accelerated atherosclerosis [1–7]. The main mechanisms underlying this increased CV risk in this population are oxidative stress [8], accumulation of uremic toxins [9], dyslipidemia and phosphocalcic metabolism disorders. Enhanced oxidative stress and uremic environment can strongly modify circulating lipids and lipoproteins leading to profound alterations of their biological properties. Indeed, lipid peroxidation by-products such as malondialdehyde (MDA) are increased and are negatively correlated with the glomerular filtration rate in CKD. Large amounts of oxidized lipids, such as F2 α -isoprostanes are associated with CKD progression [10].

Renal dysfunction is also associated with many perturbations in lipoprotein metabolism leading to dyslipidemia and accumulation of atherogenic particles [11]. Lipoprotein metabolism is complex and is associated with multisite regulations (involving liver, colon, plasma, macrophages and endothelial cells) that can be individually affected by CKD. Post-translational modifications such as carbamylation, glycation or oxidation particularly affect circulating lipoproteins (both on their protidic or lipidic fraction) leading to altered behaviors in the cardiovascular system. These particular modifications of lipid metabolism in CKD are a novel way of explaining the failure of statins in the prevention of cardiovascular diseases in hemodialysis patients [12,13].

According to the European Uremic Toxin Work Group (EuTox, <http://www.uremic-toxins.org/>), uremic toxins are defined as accumulated solutes, normally excreted by the kidneys, that interact negatively with biological functions [9]. Even if some lipids and lipoproteins are neither excreted by the kidneys in normal conditions nor accumulated in CKD, their modifications and altered metabolism unambiguously change their interactions with biological functions and especially cardiovascular physiology. This review will explain why in CKD, some lipids and lipoproteins can be considered as uremic toxins.

2. Uremic Lipoproteins, Evidences of Toxicity

2.1. Dyslipidemia in CKD, A Unique Phenotype

CKD is associated with dyslipidemia associating hypertriglyceridemia, elevated LDL cholesterol, an accumulation of ApoB containing lipoproteins, increased concentrations of lipoprotein(a) particles and low HDL levels [14,15]. Many recent reviews analyzed this dyslipidemia in detail [11,14,16–18]. Dyslipidemia in CKD is unique for many reasons. First, cardiovascular (CV) diseases are the leading cause of mortality in CKD patients. Number of cardiovascular events has been strongly correlated with GFR decline [1] and despite constant improvement of renal suppletion therapies, such as hemodialysis, this cardiovascular mortality remains at the forefront [19]. Traditional strategies for cardiovascular prevention, including the prescription of statins, failed in some CKD populations. Even if post hoc analysis of large prospective studies sketched a potential benefit in early stages of CKD [20–22], this positive effect is diminished in advanced stages (4 and 5), either on intima/media thickness [23] or cardiovascular mortality and related events, as shown by 4D [12] and AURORA [13] studies. Recent meta-analysis from the Cochrane Collaboration confirmed this observation in dialysis patients [24] but also suggested its interest for CKD patients who did not require hemodialysis [25] or transplant recipients [26]. However, beyond its effects on CV mortality, statins exhibited beneficial effects for impeding renal failure progression [27,28]. Indeed, statins can modulate intracellular pathways of inflammatory and fibrogenic responses and inhibit the proliferation of mesangial and renal tubular epithelial cells [27,29]. Moreover, recent data corroborate their importance in lipid control to prevent the progression of CKD. The increase of one standard deviation of TG level and TG/HDL-cholesterol ratio was correlated with an increased risk of developing CKD. Additionally, increases of HDL-cholesterol level, LDL-cholesterol/ApoB and HDL-cholesterol/ApoAI ratios seemed to be protective [30].

2.2. Very Low Density, Intermediate Density Lipoproteins (VLDL, IDL) and Chylomicrons

An earlier report suggested that triglyceride-rich lipoproteins (TGRL), including chylomicrons, VLDL and their remnants, accumulate in CKD [31]. Okubo et al. found that ApoB48 levels, composed of chylomicrons and their remnants, are inversely correlated with GFR levels and increased proteinuria [32]. ApoB48 levels were also found elevated in ESRD diabetic patients [33]. In transplantation, ApoB gene polymorphism was associated with poor cardiovascular outcomes in patients presenting deletion of a part of ApoB signal peptide [34].

TGRL, chylomicrons and VLDL, deliver lipids to peripheral cells. After delivering their triglycerides cargos, these lipoproteins are converted into IDL and LDL or are removed by the liver. Chylomicrons and nascent VLDL need apolipoprotein C and E (ApoC, E) for their maturation. These proteins are delivered by HDL-2. In CKD, HDL metabolism is impaired and HDL-3 are not matured into HDL-2 due to a lecithin-cholesterol acyl-transferase (LCAT) deficiency [35–37]. ApoE and ApoC are necessary for binding and activation of lipoprotein lipase (LPL) respectively and such defect leads to a reduced release of triglycerides in peripheral tissues and leads to an accumulation of TGRL. Moreover, significant evidence showed that peripheral LPL is lacking in CKD [38–40].

In normal conditions, VLDL and chylomicrons are transformed into IDL and chylomicrons remnants after lipolysis in peripheral tissue. Then, part of IDL and remnants are removed by the liver via LDL receptor protein (LRP) that has been found to be downregulated in CKD [41]. The other part of IDL is transformed into LDL by the removal of their triglycerides by the hepatic lipase and enrichment

in cholesteryl esters from HDL-2 by cholesteryl-ester transfer protein (CETP). As previously described, lack of HDL-2 impedes this phenomenon and leads to the accumulation of highly pro-atherogenic IDLs [42]. Moreover, there is a downregulation of hepatic lipase expression in CKD [43–45]. A part of VLDL is removed by their binding on VLDL-receptor in myocytes and adipocytes. The expression of this receptor is also down-regulated in CKD [46,47] (Figure 1).

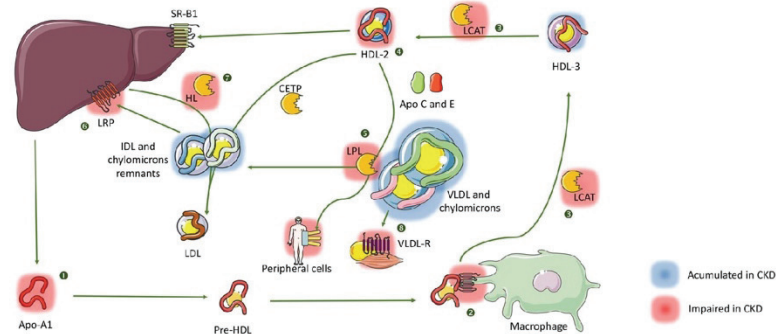


Figure 1. Main modifications of lipoprotein metabolism induced by chronic kidney disease (CKD). CKD induces a deep modification in lipoprotein metabolism resulting in the accumulation of pro-atherogenic particles such as intermediary density lipoprotein (IDL) and triglyceride-rich lipoproteins (TGRL). Main modifications are listed below: In CKD, ApoA1 and A2 levels are decreased resulting in low level of circulating high density lipoprotein (HDL) (1). In CKD, modifications of ApoA1 decrease HDL binding to macrophages and participate in the observed impaired cholesterol efflux (2). Nascent HDL are transformed into discoid HDL-3 and then spherical HDL-2 enriched in cholesterol by the action of lecithin-cholesterol acyltransferase (LCAT). In CKD, LCAT level and activity are impaired (3), leading to the accumulation of HDL-3 and reduced level of HDL-2 (4). Thus, low HDL-2 concentration result in less transfer of triglycerides from TGRL to HDL-2 by cholesteryl-ester transfer protein (CETP). Moreover, HDL-2 fail to enrich very-low density lipoprotein (VLDL) and chylomicrons with ApoC and E, essential for the binding and activation of lipoprotein lipase (LPL) respectively and such defect, associated with evidence of peripheral LPL lacking in CKD, leads to a reduced release of triglycerides into peripheral tissues and leads to an accumulation of TGRL (5). IDL and remnants accumulate in CKD because of a down-regulation of LDL receptor protein (LRP) (6), the lower level of CETP (3) and the down-regulation of hepatic lipase (HL) expression (4). A part of VLDL accumulates because of the down-regulation of the VLDL-receptor (VLDL-R) in myocytes and adipocytes (5). Abbreviations: refer to abbreviation section.

2.3. Low Density Lipoproteins (LDL)

As LDL remains the main target of cardiovascular prevention strategies, their metabolism in CKD was the focus of numerous works. Patients in early stages of CKD commonly exhibit elevated LDL-cholesterol level [48]. Uncommonly, ESRD patients and particularly HD patients have normal or reduced LDL cholesterol and total cholesterol levels and interestingly, an inverted association has been found between cholesterol level and mortality in those patients [49–51]. Beyond LDL-cholesterol level itself, CKD leads to various structural modifications of lipids and proteins that make up LDL particles.

Oxidized-LDLs (oxLDL) result from the action of enzymatic and non-enzymatic pathways of oxidative stress. Several levels of oxidation from electronegative (minimally modified) to extensively oxidized LDL can coexist in the bloodstream and lead to the activation of several pathways involved in atherosclerosis through their binding to scavenger receptors [52]. In CKD, there is substantial evidence that those oxLDL accumulate, especially in HD patients [53–56]. oxLDL concentration was shown to increase after an HD session [56]. Moreover, oxLDL are correlated with left ventricular hypertrophy in pediatric HD patients [57] and with the intensity of peripheral arterial disease [58].

Oxidized epitopes of LDL can activate immunity and then lead to the formation of antibodies directed against oxLDL. OxLDL/antibodies against oxLDL ratio was also correlated with carotid atherosclerosis and cardiovascular events in HD patients [59].

As stated above, CKD is associated with an enhanced MPO activity that plays a substantial role in the generation of post translational modification derived products (PTMDPs). Indeed, serum MPO levels have been correlated with mortality in an HD cohort [60] and with oxLDL levels [61]. MPO can promote LDL modifications through several mechanisms. First, reaction between HOCl generated by MPO and tyrosine residues of ApoB100 creates 3-chlorotyrosine, found in atherosclerotic lesions [62] and well-known for their pro-atherogenic properties through their binding with lectin-like oxidized LDL receptor 1 (LOX-1) [52,63]. Interestingly, 3-chlorotyrosine levels were found to be higher in HD patients compared to healthy volunteers [64]. Secondly, MPO can also catalyze the generation of reactive nitrogen species (RNS) and create pro-atherogenic nitrosilated-LDL [65]. Thirdly, MPO catalyzed the addition of thiocyanate to the LDL (derived from the decomposition of urea) and leads to the formation of carbamylated-LDL (cLDL) [66,67]. These cLDL have potent pro-atherogenic effects such as the transformation of macrophages into foam cells [68] through their binding to the pro-atherogenic CD36 receptor [69] (upregulated in CKD [70]) and associated with endothelial toxicity [71,72] and platelet aggregation [73] through LOX-1 [74]. cLDL levels are raised by chronic uremia and were linked with atherosclerosis in CKD [75,76].

2.4. High Density Lipoproteins (HDL)

Accelerated atherosclerosis in CKD patients and relative failure of statins in advanced stages of CKD demonstrate that traditional cardiovascular risk factors are not at the forefront. A recent focus on HDL quality rather than quantity revealed a potential role of HDL dysfunction in the set-up of cardiovascular disease in CKD.

HDL metabolism is impaired in CKD. In normal physiological conditions, ApoA1 and A2 are released into the circulation by the liver. These proteins are loaded with cholesterol and phospholipids to form nascent HDL. In CKD, ApoA1 and A2 levels are decreased [77,78]. Then, nascent HDL binds to the ABCA-1 receptor on circulating macrophages and activates cholesterol ester hydrolase allowing their loading with cholesterol. AcetylCoA acyl transferase (ACAT) limits this reverse efflux of cholesterol from macrophages by catalyzing the esterification of intracellular cholesterol. In CKD, modifications of ApoA1 limit HDL binding on macrophages [79] and upregulation of hepatic ACAT-2 in CKD [37,80] participates in the observed impaired cholesterol efflux. After its uptake, free cholesterol is esterified into cholesteryl esters by lecithin cholesterol acyl transferase (LCAT), which is transferred to the core of the lipoprotein. Nascent HDL are then transformed into discoid HDL-3 and then into spherical HDL-2 enriched in cholesterol. In CKD, LCAT level and activity are impaired [35–37], leading to the accumulation of HDL-3 and reduced level of HDL-2, especially in HD patients [81]. The latter, in normal conditions, enrich VLDL and chylomicrons with ApoC and E, essential for their normal metabolism (see above). Moreover, cholesterol ester transfer protein (CETP) transfers triglycerides in exchange for cholesterol from TGRL to HDL and LDL resulting in TG-enriched HDL-2 and LDL species. There is no evidence of deficit of CETP in CKD but as HDL-2 level is lowered, its activity may suffer. Then, HDL is cleared from their cholesterol content by their binding with hepatic SR-B1 receptor and the cycle starts again (Figure 1).

Beyond a dysfunctional metabolism, HDL is also a major target for oxidative stress and post-translational modifications. As stated above, CKD leads to a modification of ApoA1, impairing its binding on ABCA-1 [79]. As MPO activity is enhanced in CKD, MPO-modified ApoA1 results in decreased reverse cholesterol efflux and a reduced binding with ABCA-1 receptor [82,83]. Moreover, MPO leads to the increased formation of 3-chlorotyrosine, an oxidation product of MPO, in HDL and impairs LCAT and paraoxonase activities and then anti-inflammatory proprieties of HDL [84]. MPO-modified HDL are also potentially involved in the generation of foam cells in atherosclerotic lesions through the activation of SR-B1 in macrophages [85] and increase of pro-inflammatory secretion

activity and adhesion molecules expression in endothelial cells [86]. These MPO-modified HDL exhibit impaired anti-apoptotic properties in endothelial cells [86]. Interestingly, high levels of oxidized HDL are correlated to increased cardiovascular mortality in HD patients [87] as well as to HDL anti-inflammatory index elaborated by Kalantar-Zadeh et al. [88]. Recent analysis of HDL proteome showed an association of acute phase protein serum amyloid A with CKD-HDL that may participate in impaired biological functions [89,90].

Normal HDL are known to be anti-atherogenic thanks to several properties [17]. First, HDL induces a reverse cholesterol transport from circulating macrophages. This property is well known to be atheroprotective [91]. In CKD, this function is dramatically impaired as CKD-HDL have reduced capacities of inducing this efflux [81,89,92–94]. Even in a pediatric CKD cohort, the deeper was CKD, the lower was the cholesterol efflux [95]. Moreover, restoration of renal function by transplantation is associated with an enhancement of these capacities without retrieving normal-HDL levels [92]. Nevertheless, Kopecky et al. sowed the seeds of doubt by showing that cholesterol efflux levels capacity in diabetic HD patients is not a prognostic marker of cardiovascular events [96].

Secondly, normal HDL increases the production of nitric oxide through the activation of eNOS in endothelial cells resulting in a vasorelaxant phenotype. In CKD, evidences show that HDL from CKD children lose their protective effect as the production of NO by endothelial cells is significantly reduced with HDL [95]. CKD-HDL can probably induce the uncoupling of eNOS from endothelial cells as the superoxide production in endothelial cells is significantly enhanced with CKD-HDL [95].

Thirdly, normal HDL inhibits the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), which prevent the attachment of circulating monocytes to endothelial cells. In CKD, HDL promotes an enhanced expression of VCAM-1 and ICAM-1 on endothelial cells [93,95]. Moreover, CKD-HDL upregulates the expression of pro-inflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1), interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF- α) [93,94].

Normal HDL exhibit anti-apoptotic effects on endothelial cells through the downregulation of caspase-3 activity [97]. CKD-HDL inhibits endothelial cell proliferation [93]. Finally, normal HDL has anti-oxidative properties thanks to PON1 and GPX enzymes on its surface. PON1 was firstly recognized as a hydrolytic enzyme for various toxic organophosphates. Mainly expressed in the liver and the kidney, this enzyme exhibited anti-oxidant properties against lipid peroxidation as it binds to HDL and in a minor part to VLDL [98]. Thus, this enzyme is considered as the main anti-oxidant enzyme bound to HDL. PON1 activity is lowered in CKD [99–101]. GPX is another important anti-oxidant enzyme at HDL surface. Its activity and expression on HDL are lowered in CKD [35].

2.5. Lipoprotein A (Lp(a))

Lp(a) is composed of a LDL-like particle bound with an apolipoprotein(a) (Apo(a)) on lysine residues of ApoB100. Apo(a) is secreted by the liver and contains a repetition of kringle-IV units. Genetic variants of Lp(a) and its concentration have been deeply correlated with coronary heart disease and cardiovascular morbidity [102–105]. As its concentration is closely associated with OxPL/ApoB, Lp(a) may play a role in OxPL clearance even though its role(s) still remain unclear. Lp(a) levels were found to be risk predictors of all-cause mortality in HD patients [106]. Lp(a) accelerates atherosclerosis in a mouse model of CKD [107]. Moreover, Lp(a) clearance is partly done by the kidney explaining why its clearance is lowered in hemodialysis patients even if its generation does not seem to be higher than in healthy subjects [108]. However, in Tzanatos et al. study, Lp(a) levels seems to be increased after an HD session [109] while no change was found in Bossola et al. study [56]. As HD seems to be ineffective for Lp(a) clearance, it can explain higher levels in ESRD patients as its generation is not increased in these patients. Unlike hemodialysis, nephrotic syndrome exhibits enhanced secretion of Lp(a) by the liver [110]. In a nutshell, CKD exhibits higher levels of Lp(a) than in healthy subjects (Table 1) and it accumulates with CKD [111] severity while it decreases with renal transplantation [112], Lp(a) is a prototype candidate to be classified as a uremic toxin.

Table 1. Concentrations of plasma oxidized lipids and lipoproteins in control and CKD patients.

	Normal	CKD	Clearance	HD Behavior	References
Oxysterols (Total)					
7-ketocholesterol, nM	32.3 ± 16.7	42.2 ± 30.1 ^δ	Liver metabolism	Generated during HD session	[113,114]
7β-OH-cholesterol, nM	14.4 ± 7.7	42.6 ± 24.1 ^δ			
Oxidized Phospholipids					
OxPL/ApoB ratio, AU	0.068 ± 0.07	0.138* ± 0.170*	Enzymatic detoxification	Reduced after HD session	[56,103,115]
PUFAs Aldehydes					
Malondialdehyde (MDA), µg/L	257.7 ± 81.7	388.8 ± 21.6 ^δ	Enzymatic detoxification Renal excretion	Controversial (decrease, no change and increase)	[7,9,23,116–118] [9,116,119–121]
4-hydroxy-decenal, µg/L	10.3 ± 7.1	36.6 ± 22.3 ^δ			
4-hydroxy-2-hexenal (4-HHE), µg/L	25.1 ± 9.9	63.8 ± 25.3 ^δ			
4-hydroxy-2-nonenal (4-HNE), µg/L	16.4 ± 9.0	117.3 ± 47.7 ^δ	Enzymatic detoxification, renal excretion	4-HNE: Reduced after HD session	
4-hydroxy-octenal, µg/L	10.7 ± 3.6	27.8 ± 13.8 ^δ			
Arachidonic Acid By-Products of Lipid Peroxidation					
Total F ₂ -isoprostanes, pg/mL*	162 ± 73	270 ± 10 ^δ	Renal excretion, Enzymatic detoxification	No change	[119,122–128]
Unesterified F ₂ -isoprostanes, pg/mL	37.6 ± 17.2	96.2 ± 48.8 ^δ			
Esterified F ₂ -isoprostanes, pg/mL	146.8 ± 58.4	220.4 ± 154.8 ^δ			
Lipoprotein Products					
ApoB ₄₈ level, mg/L	3.7 ± 2.3	19.3 ± 13.9 ^δ			[33]
Oxidized LDL, mg/L	0.22 ± 0.05	1.92 ± 0.29 ^δ	Accumulation in atherosclerotic lesions	Increased after HD session	[53–56]
3-chlorotyrosine, µmol/mol of tyrosine	<0.3	3.5 ± 0.5 ^δ	-	-	[64]
Lp(a) level, mg/dL	18.4 ± 22.8	23.4 ± 34.6 ^δ	Renal and hepatic clearance	No changes or increased after HD session	[56,109–111]

Data are expressed as means ± SD. * computed from the data available in the original article, ^δ $p < 0.05$ vs. control; Lp(a): lipoprotein A, PUFAs: polyunsaturated fatty acids.

3. Oxidative Stress/Non-Oxidative Modifications of Lipids and Lipoproteins in CKD

3.1. Oxidative Stress, Lipid Peroxidation and Antioxidant Defenses

Oxidative stress is defined as a lopsided balance of the pro/anti-oxidant state in favor of the pro-oxidant [129]. The origin of this stress is the formation of reactive oxygen species (ROS) like superoxide anion $O_2^{\bullet-}$, hydroxyl radical $\bullet OH$ or hydrogen peroxide H_2O_2 . Major part of reactive oxygen species in our organism is produced by the mitochondrial respiratory chain [130], the NADPH oxidase [131,132] and 5-lipoxygenase enzyme [133]. Other enzymes such as xanthine oxidase or NO synthase [134] can also provide ROS in pathological conditions. ROS, by many intertwined reactions, produce free radicals in the presence of transition metal ions (Fe^{2+} , Cu^{2+}) or carbon-composed molecules like proteins, nucleic acids or lipids. Nitric oxide (NO) can also yield free radicals often referred to as reactive nitrogen species (RNS). NO is generated by NO synthases and plays many roles in the regulation of vascular tone, permeability and platelet adhesion. NO can rapidly react with $O_2^{\bullet-}$ to generate a more oxidized form of a nitric product: the peroxinitric ion ($ONOO^-$). The latter can easily react with proteins, lipids or nucleic acids, resulting in oxidized or nitrosylated forms.

As previously described, lipids can be affected by oxidative stress. First step of lipid peroxidation is the reaction of a free radical with a poly-unsaturated fatty acid (L). This reaction results in the formation of a lipid radical L^\bullet . This radical can react with oxygen and create lipid peroxy radicals (LOO^\bullet). From this point, LOO^\bullet can react with other lipids and create new lipid radicals and lipid hydroperoxide ($LOOH$). The degradation of lipid hydroperoxide provides new lipid radicals (LO^\bullet , LOO^\bullet) and aldehydes as stable end-products of lipid peroxidation process (malondialdehyde or MDA, 4-OH-2,3-alkenals). $F_2\alpha$ -isoprostanes are end-products from the oxidation of arachidonic acid (i.e., 20:4 (n-6)). These three end-products are routinely used for in vivo evaluation of lipid peroxidation level [135,136]. However, there are other products of lipid peroxidation such as oxysterols and oxidized phospholipids that play a substantial role in the onset and progression of atherosclerosis and lipid dysmetabolism [137].

Under physiologic conditions, there is a basal rate of production of ROS as well as a limited production of oxidized molecules [138]. Nevertheless, this production of oxidants is balanced by a complex pattern of antioxidant mechanisms that protect the cells and tissues from oxidative damages. The protection from the ROS damage is permitted by antioxidant enzymes such as superoxide dismutase (SOD) which catalyses the dismutation of $O_2^{\bullet-}$ into H_2O_2 , glutathione peroxidase (GPX) or catalase, which detoxifies H_2O_2 and other hydroperoxide containing molecules. Non-enzymatic antioxidants include reduced glutathione (GSH), which allows the scavenging of $\bullet OH$ and acts as a substrate for GPX as well as ascorbic and uric acids that are scavengers of $\bullet OH$, singlet O_2 and peroxy radicals. Ferritin, ceruleoplasmin, transferrin, lactoferrin and metallothionein can also be regarded as antioxidant proteins as they trap transition metal ions and prevent ROS formation from Fenton reaction [133]. To prevent lipid peroxidation, aforementioned antioxidant molecules act together with several liposoluble antioxidants such as tocopherols, ubiquinol, flavonoids and carotenoids. Moreover, the protein paraoxonase-1 (PON1) from HDL is a major anti-oxidant preventing lipoproteins from oxidation although its exact mechanism remains unclear [98]. Some molecules such as tocopherols or curcumin, are considered as chain-breaking antioxidants, corresponding with their ability to intercept intermediary radicals during the lipid peroxidation process and then break the oxidative chain. Finally, albumin can be considered as a major antioxidant protein of the plasma. In fact, serum albumin can bind various ligands such as copper, iron, long chains fatty acids (LCFA), poly-unsaturated fatty acids (PUFAs) and cholesterol and prevent them from oxidative modifications [139]. It can also bind bilirubin [140,141] and inhibit lipid peroxidation as well as prevent damages of α -tocopherol [142] and bind homocysteine. Albumin also contains a reduced cysteine residue (Cys34) which can scavenge hydroxyl radicals [143]. Due to the large amount of albumin in the plasma, it represents the largest amount of thiols available in the circulation [144]. Albumin also scavenges hypochlorous acid (HOCl) responsible for chlorination of proteins mediated by myeloperoxidase.

3.2. Oxidative Stress in CKD

Substantial literature is available about the enhanced oxidative stress in CKD [145–154]. The unbalanced pro-oxidative state appears almost at the onset of CKD and increases as the glomerular filtration rate (GFR) declines [8]. There are multiple reasons for this enhanced oxidative stress and they are often intertwined.

As we will discuss later, antioxidant defenses are lowered in CKD leading to a higher sensitivity to oxidative stress induced by classical cardiovascular risk factors (hypertension, advanced age, diabetes and obesity [155]). Indeed, hypertension is well known to be a major state of oxidative stress [156,157]. Upregulation of NADPH oxidase via the activation of renin-angiotensin system (RAS) is well-recognized as a major provider of ROS in hypertension [157–159]. In CKD, RAS has been directly linked with enhanced oxidative stress and CKD progression through the up-regulation of pro-oxidative pathways (NF- κ B, NADPH oxidase, cyclooxygenase 2, 12-lipoxygenase) by angiotensin II and its binding to angiotensin-1 receptor [159].

Most antioxidant defenses are lowered in CKD. However, some controversies still exist as many studies produced conflicting results. Total antioxidant status is decreased in CKD [160,161]. Nevertheless, the large range of techniques used for this determination and the numerous confounding factors in CKD make it difficult to interpret [8,162]. The determination of Superoxide dismutase (SOD) activity is prototypical. Some studies found a decreased SOD activity in CKD patient [163,164] while others reported a normal level of activity [165]. Surprisingly, SOD activity was even found to have increased in CKD [166]. As there are several isoforms of SOD (cytosolic, mitochondrial and extra-cellular), assays are difficult to extrapolate between all the different studies. As a matter of fact, SOD activity seems to be correlated with CKD stage and its activity is restored by several interventions in CKD patients (erythropoietin, vitamin E supplementation and kidney transplantation) [167,168]. Similar contradictory observations were reported for catalase activity (e.g., either lowered [169], normal [165,170] or increased [160]). Glutathione activity and concentration are lowered in CKD and are correlated with uremic toxins' concentrations [171,172] and glomerular filtration rate (GFR). PON1 activity is lowered in CKD [98–100]; however, further studies are needed to fully understand its implication on lipid dysmetabolism and lipoprotein dysfunction [97,173] associated with CKD.

3.3. Post-Translational Modification Derived Products (PTMDPs) of Lipoprotein and Covalent Modifications of Lipids

Proteins and lipids are especially prone to oxidation and their irreversible oxidative modifications lead to a profound alteration of their biological functions. Carbonylation is the addition of compounds made from glycation and lipid peroxidation onto proteins. These residues can react with lysine and arginine residues and then create advanced glycation end products [174] (AGEs) and advanced lipoxidation end products [175] (ALEs). Proteins can also be carbonyled by direct oxidation by ROS [176]. 3-desoxyglucosone, D-arabinose, glyoxal can react with proteins and create AGEs as pentosidine and carboxy-methyl-lysine. Lipid peroxides of polyunsaturated fatty acids (PUFAs) such as 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxy-2-hexenal (4-HHE) can also react with lysine, cysteine and histidine residues of proteins and create ALEs (Figure 2).

AGEs and ALEs have several biological effects involved in atherosclerosis [177]. AGEs have significant effects on lipids as they can make LDL more prone to oxidative modifications [178], increase glycated-LDL uptake from macrophages by scavenger-receptors and accelerate the formation of foam cells [179]. ALEs also exhibit several pro-inflammatory effects and are involved in the progression of atherosclerosis [180]. Both AGEs and ALEs were reported to accumulate in CKD [181–185].

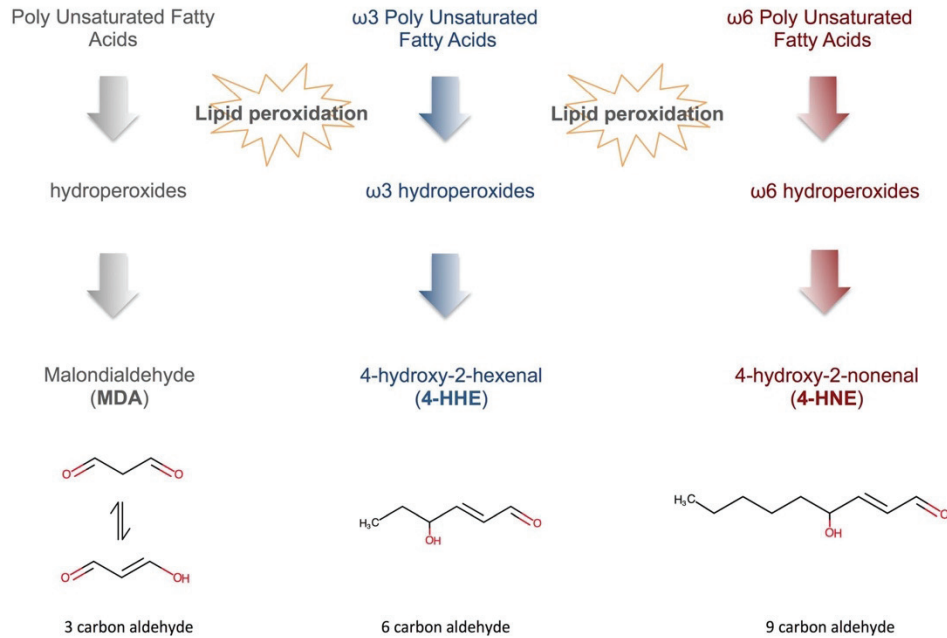


Figure 2. Major reactive lipid aldehydes derived from poly unsaturated fatty acids (PUFAs) oxidation. Malondialdehyde (MDA) results from the oxidation of various polyunsaturated fatty acids containing more than two double bonds. MDA binds with nucleic acids or lysine amino-groups and creates toxic adducts called advanced peroxidation lipid end products (ALEs). On ApoB, these adducts were associated with atherosclerosis. 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxy-2-hexenal (4-HHE) result respectively from the oxidation of ω6 PUFAs and ω3 PUFAs. They can react with proteins by a Michael addition mechanism and create ALEs. These ALEs disrupt several biological functions and lead to the formation of atherosclerosis and foam cells. Abbreviations: refer to abbreviation section.

Carbamylation is the fixation of isocyanic acid, derived from the decomposition of urea on amine groups of proteins. Protein carbamylation is associated with cardiovascular disease [186], mortality in CKD [187,188] and can also affect lipoproteins [75] and promote atherosclerotic complications [68,189] (Figure 3).

Myeloperoxidase (MPO) activity contributes to the formation of chlorinated [64] and nitrosilated [65] proteins or lipids that are correlated with poor cardiovascular outcomes [62,190]. Its activity can also lead to the oxidation and modification of lipoproteins and especially the adduction of thiocyanate produced from the decomposition of urea [67]. In CKD and especially in hemodialysis, this activity is enhanced [60,61,191,192]. Wada et al. showed that MPO expression is associated with aortic stenosis in hemodialysis (HD) patients [193].

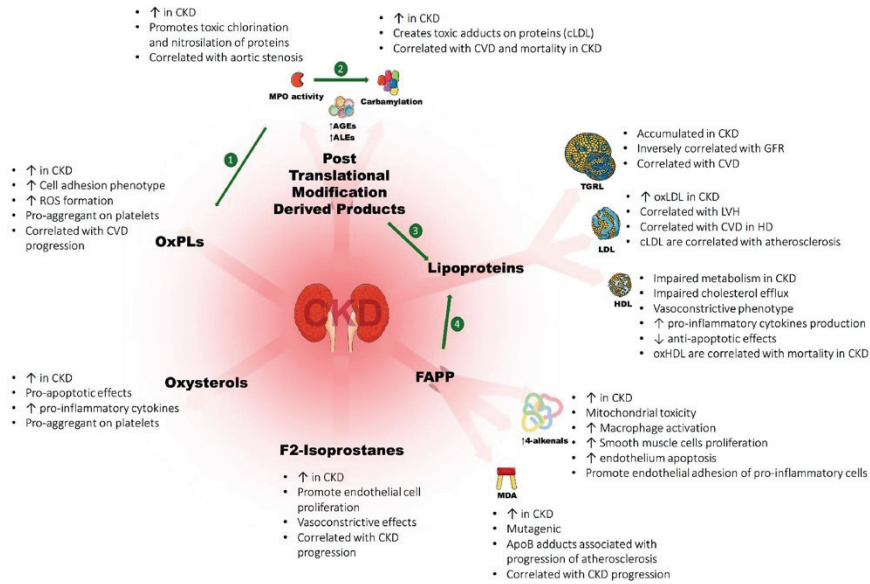


Figure 3. Main effects of oxidized lipids and lipoproteins in chronic kidney disease (CKD). CKD is associated with increased oxidative stress, which promotes covalent modifications of lipids and lipoproteins. Lipid products of this unbalanced metabolism are oxidized phospholipids (oxPLs), fatty acid peroxidation products (FAPPs), oxysterols and F2-isoprostanes. Posttranslational modification derived products (PTMDPs) are the result of an enhanced myeloperoxidase (MPO) activity in CKD, an increased carbamylation and a massive production of advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs). ALEs are derived from lipid aldehydes issued from peroxidation of fatty acids (FAPPs). MPO catalyzes the nitrosilation on phospholipids to create oxPLs (1). MPO are also involved in carbamylation process by the addition of thiocyanate on proteic residues (2). Lipoproteins are also modified in CKD. First, triglyceride-rich lipoproteins (TGRL) have an impaired metabolism leading to their accumulation. Low-density lipoproteins (LDL) exhibit large amount of toxic oxidized (oxLDL) and carbamylated (cLDL) forms in CKD. These modifications lead to impaired functions and promote the progression of cardiovascular disease (CVD) especially in hemodialysis (HD) patients. High density lipoproteins (HDL) are also modified in CKD. Their whole metabolism is impaired and this dysregulation leads to many pro-atherosclerotic effects. MPO and carbamylation are greatly responsible for lipoproteins’ modifications and dysfunctions (3) so are FAPP products that generate ALEs, especially on apolipoproteins A and B (ApoB) (4). Abbreviations: refer to abbreviation section.

4. Oxidized Lipids in CKD: Evidences of Toxicity

4.1. Cholesterol and Oxysterols

Cholesterol is a major component of cell membranes. Its presence in almost all of the cell membranes makes it a perfect target for ROS. There are four major products of ROS-mediated cholesterol oxidation: 7 α , β -OOH-Cholesterol, 7 α , β -OH-Cholesterol, 7-oxo-Cholesterol and 5,6-epoxy-Cholesterol. However, there are many other minor forms of oxysterols produced by non-radical pathways (5- α -OOH-Cholesterol, 6 α , β -OOH-Cholesterol with singlet oxygen; 5,6 β -epoxy-Cholesterol and ozone; 5,6-dichlorocholestane and HOCl from neutrophils) [194].

Oxysterols play a role in the set-up of atherosclerosis as several studies found them in fatty streaks, aortic or atherosclerotic plaques [195–197]. Oxysterols exhibited a pro-apoptotic effect on monocytes [198,199], vascular cells (smooth muscle cells [198,200], endothelial cells [198,201]) and

hepatocytes [202]. Particularly, 7-oxo-cholesterol and 7 α , β -OH-Cholesterol triggered the major toxic effect [198] but other products such as 5,6-ep-oxy-Cholesterol also showed a potential pro-apoptotic effect [201,203]. Other deleterious effects have been shown from various oxysterols including death of macrophages in latter stages of atherosclerosis [204], production of pro-inflammatory cytokines [205,206], LDL oxidation [207] and platelet aggregation [208]. Nevertheless, their role in the modification of cholesterol metabolism still remains unclear [194,209].

Oxysterols are metabolized by the liver and excreted as bile acids. In healthy subjects, oxysterols are found at very low plasma concentrations [210] (Table 1). These concentrations are significantly increased in CKD patients compared to a control population and especially in end-stage renal disease (ESRD) patients undergoing hemodialysis [113,114]. Moreover, Siems et al. reported an increase of their concentration after an HD session [114].

4.2. Oxidized Phospholipids

Phospholipids (PLs) are the main components of cell membranes and, as cholesterol, are more exposed to oxidative stress and ROS. They also make up the external layer of lipoproteins and are a preferential site of oxidative and covalent modifications. Oxidized phospholipids can be produced through several pathways. Peroxyl radicals are derived from the free-radical-dependent oxidation of PUFAs esterified into PLs. [211]. Nitration can happen on PLs and generate nitrated-PUFAs and PLs [212]. Halogenation by a direct action of HOCl or HOBr released by neutrophilic and eosinophilic cells can also modify PLs by the addition of halogenide and hydroxyl groups leading to halohydrins (chlorhydrins [213] and bromohydrins [214]) residues on PLs. Indeed, MPO and eosinophilic peroxidase activities are increased in CKD [60,61,191,192]. Enzymatic pathways of oxidation are almost always associated with the activity of 12- and 15-lipoxygenases, leading to the formation of hydroperoxides residues on PLs either in cell membranes [215] or lipoproteins [216,217].

These oxidized PLs (Ox-PLs) exhibit several biological effects [218]. First, they play a role in enhanced cell expression of adhesion molecules. Ox-PLs are involved in the activation of β 1-integrin/fibronectin [219,220] and P-selectin [221,222] pathways of adhesion. Ox-PLs can also increase the generation of ROS by the elevation of the activity of NADPH oxidase [223]. As PLs are key activators of blood coagulation and platelet activation, Ox-PLs can also modulate this phenomenon. Effects on blood coagulation are mitigated as Ox-PLs can exert opposite effects on several steps of the coagulation cascade even if a pro-coagulation tendency raises from these studies [224–226]. Ox-PLs have shown pro-aggregate properties on platelets and vascular cells [227–230]. Many others effects of Ox-PLs are described in the literature (smooth muscle cells, bone, pro-angiogenic properties . . .) [211,231].

Part of Ox-PLs biological activity results from their binding to CD-36 receptor (a class B scavenger receptor) [232,233]. This receptor is well-known for its implication in atherosclerosis [69] and its expression is increased in CKD patients [70].

Moreover, high OxPL/ApoB ratio has been related to severe coronary artery disease [102] in patients without CKD (mean level of about 0.20 and 0.15 for respectively <60 years and >60 years population). This ratio has been correlated with the presence and progression of carotid, femoral atherosclerosis and cardiovascular disease for a ratio value higher than 0.088 [103]. Interestingly, in ESRD patients undergoing HD, this mean ratio was found to be at 0.13 [56] (Table 1). Nevertheless, this ratio was not found to be associated with cardiovascular disease in hemodialysis patients [56,115]. Indeed, their concentration decreases after an HD session [56]. This decrease may partially explain the absence of association in HD patients. This ratio has been also associated with renal progression in systemic lupus erythematosus patients [234].

4.3. Fatty Acid Peroxidation Products (FAPP)

Fatty acids represent an important source of energy in human body physiology. Most of the time, they are derived from triglycerides or phospholipids and can be either saturated or unsaturated. Poly unsaturated fatty acids (PUFAs) are more prone to oxidation, and especially in CKD.

Malondialdehyde (MDA) is the result of polyunsaturated fatty acid oxidation containing more than two double bonds. More than a simple marker of lipid peroxidation, it covalently binds to proteins and nucleic acids, interfering with their normal biological functions. Indeed, MDA binding with nucleic acids can form several toxic adducts [235] and induce frameshift mutations and base-pair substitutions [236] (Figures 2 and 3). Furthermore, MDA can react with lysine amino group and generate lysine-lysine bounds [237]. On ApoB, these bounds were associated with atherosclerosis [238]. MDA can be assayed itself or detected as a derived product of its reaction with thiobarbituric acid (TBA) that produces thiobarbituric acid reactive species (TBARS) [239]. Several studies have shown that MDA is elevated in CKD and represents a good marker of increased oxidative stress [116,165,166,240–244]. Moreover, MDA (or TBARS) levels are correlated with GFR [241], creatinine levels [245] and intensity of renal damages [166]. Surprisingly, data on HD behavior of MDA concentration are controversial. In Kuchta et al. study, it was not affected by HD procedure [162]. In other reports, HD session triggered a significant decrease of the MDA concentration [116,117] but was found to increase in a Nigerian cohort [118]. As an evidence of toxicity and as a significant part of MDA is excreted in the urine [246], EuTox group has already classified MDA as an uremic toxin [9].

Polyunsaturated fatty acids can also generate other reactive lipid aldehydes such as acrolein and 4-hydroxy-2-alkenals [237]. The latter, and particularly, 4-hydroxy-2-nonenal (4-HNE) (a by-product of the peroxidation of n-6 PUFAs) and 4-hydroxy-2-hexenal (4-HHE) (a by-product of the peroxidation of n-3 PUFAs) can react with proteins by a Michael addition mechanism (Figure 2). These adducts can especially bind to histidine, cysteine and lysine residues [247]. These ALEs disrupt several biological functions such as Na⁺/K⁺ ATPases [248], mitochondrial functions (potential role in permeability transition [249] and membrane fluidity [250]). These adducts are also found in large amounts in human atherosclerotic lesions [251]. Furthermore, HNE-modified LDL can activate macrophages and increase the up-regulation of class A scavenger receptors involved in the transformation of these cells into foam cells [252]. 4-HNE adducts induce smooth muscle cell proliferation in aortas of rats by the up-regulation of ERK1 and ERK2 pathways [253]. 4-HNE can also alter vascular permeability and trigger apoptosis of endothelial cells [254] and promote the adhesion of pro-inflammatory cells to the endothelium [255]. There is some evidence that 4-HNE accumulates in CKD and especially in ESRD patients [256]. In a study by Sommerburg et al., mean level of 4-HNE was 3 fold higher in HD patients compared to healthy controls [257]. Alhamdani et al. showed that alkenals, alkenals and 4-hydroxy-alkenals concentrations are increased in HD patients compared to controls [258] (Table 1). Interestingly, HD procedure tends to reduce the 4-HNE concentration in several reports [116,119,120]. However, as the major part of 4-HNE reacts with proteins and creates stable adducts (ALEs), this observed reduction can over-estimate the decrease of the overall pool of 4-HNE in an HD session. As a major proof of toxicity and as part of alkenals are excreted in the urine [121], EuTox group has already classified 4-hydroxy-2-alkenals as a uremic toxins [9]. To the best of our knowledge, there is only limited data in the literature regarding the levels of 4-HHE in patients although its cytotoxic effect on proximal tubular cells was demonstrated in vitro [259].

4.4. F2-Isoprostanes

Arachidonic acid peroxidation generates F2-isoprostanes by a cyclo-oxygenase independent pathway [260–262]. Part of these isoprostanes is unesterified but a great majority remains esterified. 8-epi-PGF_{2α} acts as an agonist/antagonist on platelet aggregation via Thromboxane A2 receptor (TxA2-R). Added at high concentrations, it directly induced platelet aggregation via TxA2-R whereas at low concentrations, it inhibited platelet aggregation. Thus, low amount of 8-epi-PGF_{2α} acts as an antagonist of this receptor and inhibits platelet aggregation [263] induced by a TxA2 agonist. F2-isoprostanes promote endothelial cell proliferation and endothelin-1 secretion [264]. Moreover, these compounds have potential vasoconstrictive effects on smooth muscle cells [265]. Intra-arterial infusion of F2-isoprostanes led to a decrease in GFR and these effects were counteracted with TxA2-R antagonists [266]. Additionally, high containing-vitamin E diet in aged rats increased the GFR by 50%

and reduced glomerular sclerosis concurrently with a reduction of F2-isoprostanes concentration [267]. Therefore, F2-Isoprostane accumulation may play a role in CKD progression [10], especially since its concentration is closely correlated with GFR [8,268]. In addition, F2-isoprostane concentrations are elevated in HD patients in several studies [119,122–125] compared with intra-studies control groups and data from the literature [269] (Table 1) making them suitable for classification as uremic toxins and particularly because their concentrations decrease after kidney transplantation [270,271]. Part of these F2-isoprostanes are cleared by the kidney [126,127] but an HD session seems to have no effect on their concentration [119,128].

5. Conclusions

CKD is associated with deep modifications in oxidative stress balance, lipid metabolism and turnover, which is responsible for an accumulation of various toxic forms of lipids and lipoproteins. EUtox defined a uremic toxin as an accumulated solute, normally excreted by the kidneys, that interacts negatively with biological functions. As these molecules accumulate in ESRD and exhibit many noxious effects on cell metabolism, CKD progression, cardiovascular system they can be regarded as uremic toxins even if they are not always excreted by the kidneys. Future efforts need to be concentrated on the enhancement of the removal of these lipids and the avoidance of their generation.

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Abbreviations

ABCA-1	ATP-binding cassette 1
ACAT	acetyl-CoA acetyltransferase-1
AGEs	advanced glycation end-products
ALEs	advanced lipoxidation end-products
ApoA/B/C/E	apolipoprotein A/B/C/E
CETP	cholesterol-ester transfer protein
CKD	chronic kidney disease
cLDL	carbamyated low-density lipoprotein
CV	cardiovascular
CVD	cardiovascular disease
ESRD	end-stage renal disease
EuTox	European uremic toxin work group
FAPPs	fatty acid peroxidation products
GFR	glomerular filtration rate
GPX	glutathione peroxidase
HD	hemodialysis
HDL	high density lipoproteins
4-HNE	4-hydroxy-2-nonenal
4-HHE	4-hydroxy-2-hexenal
HOCL	hypochlorous acid
ICAM-1	intercellular adhesion molecule 1
IL-1 β	interleukine 1 β
LCAT	lecithin-cholesterol acyltransferase
LCFA	long chain fatty acids
LDL	low-density lipoproteins
LOX-1	lectin-like oxidized low density receptor 1
Lp(a)	lipoprotein a
LRP	LDL receptor protein
LVH	left ventricle hypertrophy
MCP-1	monocyte chemoattractant protein 1
MDA	malondialdehyde
MPO	myeloperoxidase
NO	nitric oxide

oxPLs	oxidized phospholipids
oxLDL	oxidized low-density lipoproteins
PLs	phospholipids
PON1	paraoxonase 1
PTMDPs	post translational modification derived products
PUFAs	polyunsaturated fatty acids
RAS	renin-angiotensin system
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
SR-B1	scavenger receptor class B member 1
TBARS	thiobarbituric acid reactive species
TGRL	triglyceride-rich lipoproteins
TNF- α	tumor necrosis factor α
TxA2-R	thromboxane A2 receptor
VCAM-1	vascular cell adhesion molecule 1

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6. Développement d'un modèle animal non chirurgical d'insuffisance rénale chronique chez le lapin :

Adenine Rich Diet Is Not a Surrogate of 5/6 Nephrectomy in Rabbits

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Le deuxième point s'attache au développement d'un modèle animal d'insuffisance rénale chronique pertinent en lipidologie. En effet, notre intérêt était d'observer l'effet propre de l'IRC sur les modifications que nous avons précédemment discuté dans notre revue. De ce fait, le recours à l'expérimentation animale permet de s'affranchir en partie des effets imputables aux autres facteurs de risques cardio-vasculaires classiques que l'on retrouve chez l'homme. Les modèles d'IRC classiquement implémentés au laboratoire (i.e. néphrectomie subtotale des 5/6 ou néphrectomie chimique à l'adénine) utilisent comme modèle animal des rongeurs, principalement des rats et des souris. Le métabolisme lipidique du lapin étant plus proche de celui de l'homme que celui du rat ou de la souris, particulièrement car ce dernier exprime la Cholesterol Ester Transfer Protein (CETP), cet animal nous a semblé être le plus pertinent pour étudier les modifications des lipoprotéines circulantes et particulièrement sur les LDL et les HDL.

Les « 3Rs », introduites en 1959 par Russel & Butch, sont des recommandations qui régissent les bonnes pratiques de l'expérimentation animale avec trois grands principes :

- Le remplacement : éviter d'utiliser les animaux lorsqu'une autre alternative peut être aussi efficiente (ex : culture cellulaire).
- La réduction : développer des techniques permettant de réduire le nombre d'animaux nécessaires.

- Le « raffinement » : développer des techniques permettant de réduire ou de faire disparaître la douleur et le stress et/ou améliorer le bien-être et la convalescence des animaux.

De ce fait, le lapin pose trois problèmes majeurs qui ont justifié notre tentative de développement d'une nouvelle technique non chirurgicale d'induction de l'IRC chez le lapin:

1. Les modèles « lapin » d'IRC disponibles dans la littérature sont anciens et mal documentés.
2. Le lapin est un animal fragile pour lequel la gestion péri-opératoire est un frein important initial à la pratique chirurgicale. En effet, l'anesthésie est souvent périlleuse chez le lapin (du fait d'une sensibilité accrue aux drogues hypnotiques et analgésiques) et la ventilation invasive compliquée du fait d'un tractus respiratoire d'abord difficile.
3. Les complications post-opératoires sont fréquentes. La grande sensibilité aux infections nécessite une pratique chirurgicale rigoureuse et une asepsie parfaite. De plus, l'arsenal thérapeutique antibiotique est très restreint du fait d'une intolérance digestive majeure et létale chez ce mammifère cœcotrophe.

Le régime riche en adénine a montré sa fiabilité, sa simplicité et sa reproductibilité chez le rat et chez la souris et semblait être une parfaite alternative à la chirurgie de néphroréduction chez le lapin. C'est donc dans cette optique que nous avons tenté d'induire l'IRC avec ce type de régime chez des lapins.

Du fait d'une physiologie urinaire particulière et d'un mode de délivrance de l'adénine inadapté, nous n'avons pas constaté d'induction d'IRC significative chez les lapins nourris par un régime enrichi en adénine à 0.75% (w/w). Le protocole ainsi que les raisons précises de cet échec sont discutés dans l'article intitulé : « *Adenine Rich Diet Is Not a Surrogate of 5/6 Nephrectomy in Rabbits* », publié dans le journal *Nephron* en 2017.

Du fait de l'échec de la néphrectomie chimique, nous avons donc mis en place un modèle plus classique de réduction néphronique par néphrectomie subtotale des 5/6 s'inspirant du modèle décrit par Gotloib et al.⁶² L'induction de l'IRC était donc réalisée par une première chirurgie d'électrocoagulation au bistouri électrique de 2/3 du cortex rénal gauche (**Figure 9**),

suivie une semaine plus tard d'une néphrectomie totale à droite. On note lors de la nécropsie une hypertrophie du parenchyme rénal résiduel associé à une involution fibrotique des pôles électrocoagulés (**Figure 10**).

Les caractéristiques de l'IRC ainsi induite sont détaillées dans la partie du travail intitulée : « Chronic kidney disease increases carbonylation of HDL and is associated with impaired antiaggregant proprieties. »

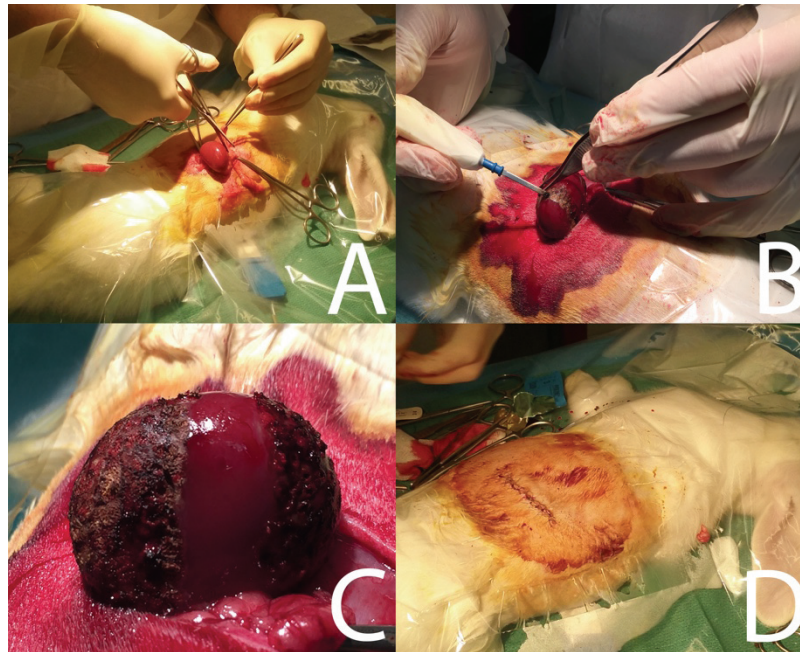


Figure 9 — Étapes de la néphrectomie partielle gauche

A : Le rein est externalisé avec soin afin de ne pas léser ou tordre le pédicule rénal.

B : Les deux pôles du cortex rénal (2/3) sont électro-coagulés par une application d'une seconde avec un bistouri électrique.

C : Aspect macroscopique du rein gauche immédiatement après l'électrocoagulation.

D : Le rein est replacé dans la cavité rétro péritonéale en faisant attention à ne pas faire un volvulus du pédicule. Les muscles et la peau sont refermés séparément plan par plan.

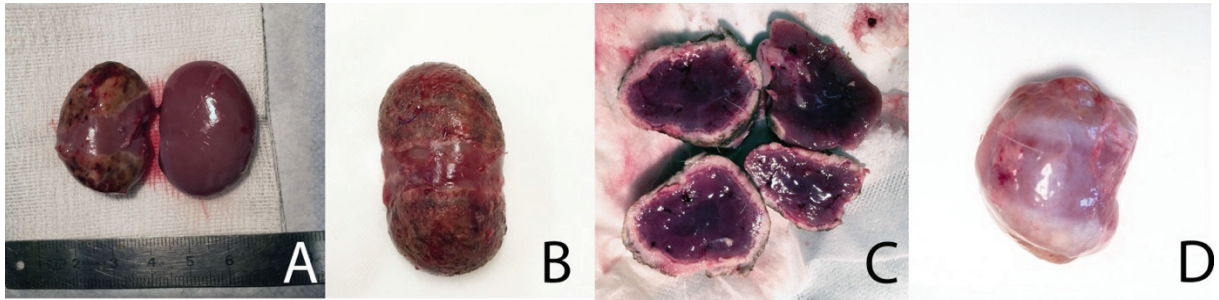


Figure 10 — Aspect macroscopique du rein gauche électrocoagulé

A : Rein droit normal et rein gauche électrocoagulé (J+1)

B : Notez la limite entre la zone thermocoagulée et le cortex sain situé à proximité du pédicule.

C : Vue en coupe de l'aspect à J+7 du cortex coagulé. On note une involution fibrotique comparée au cortex sain situé en haut à droite.

D : Aspect du rein gauche après trois semaines de néphrectomie subtotale. On note une hypertrophie du parenchyme sain avec une involution majeure des deux pôles.

Adenine Rich Diet Is Not a Surrogate of 5/6 Nephrectomy in Rabbits

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Keywords

Adenine · Chemical nephrectomy · Animal model · Rabbit · Chronic kidney disease

Abstract

Background: Animal models are important tools needed to understand the mechanisms underlying the progression of renal disease and to implement new therapeutic approaches. A non-surgical model of chronic kidney disease (CKD) developed by chemical nephrectomy using an adenine-enriched diet has been shown to be a robust model to induce kidney failure in mice and rats. The purpose of this study was to implement an adenine diet to induce CKD in rabbits. **Methods:** Male New Zealand rabbits were fed for 4 weeks with a diet containing 0.75% (w/w) adenine, and renal function was assessed by measuring plasma urea and creatinine concentrations. The glomerular filtration rate (GFR) was measured using the plasmatic clearance of iohexol. Kidney histology was performed with haematoxylin erythrosine saffron and Sirius red staining. **Results:** In contrast to what was observed in rodents, adenine diet failed to induce kidney failure in rabbits as is evident in the plasma concentrations of creatinine and urea and the direct measurement of GFR or histopathological studies. **Conclusion:** Adenine diet

is not a surrogate of subtotal nephrectomy to induce kidney failure in rabbits. Several interspecies differences in metabolism and renal physiology could account for this observation.

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Introduction

An increasing trend in the incidence of chronic kidney disease (CKD) is being observed worldwide, thereby imposing an important economic burden. Animal models are vital to understand the mechanisms underlying the progression of renal disease and to implement new therapeutic approaches. Several animal models of kidney failure were proposed to study the pathophysiology and complications associated with CKD [1–5]. The most common animal model of CKD is the so-called 5/6 subtotal nephrectomy model (also referred to as the “remnant kidney” model) in which there is a surgical reduction of the renal mass and nephrons number. The reduction of the kidney mass is usually achieved in 2 steps, an infarction (i.e., a ligation of branches of the renal artery) or the

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surgical excision of both poles of one kidney, and one week later, the removal of the contralateral kidney [6]. Although this method is the preferred technique for inducing CKD, it however requires technical abilities for surgery and postoperative care. Moreover, this technique needs to be ethically approved, as the morbidity and discomfort inflicted to the animals are quite high. A non-surgical model of CKD by chemical nephrectomy using an adenine-enriched diet has been shown to be a robust model of CKD in mice and rats. Adenine diet induces a substantive kidney failure [7, 8] depending on the duration and the concentration of adenine in the diet [9]. Furthermore, the reproducibility of CKD is greater than that in surgical models. Supplementation with adenine in the diet induces kidney fibrosis, increased blood urea nitrogen and plasma creatinine [8] associated with an enhanced oxidative stress [10]. Adenine is metabolized to 2,8-dihydroxyadenine, which precipitates and forms tubular crystals that damage the kidney tissues [11]. Adenine can also be metabolized by xanthine oxidase into uric acid that can also precipitate in renal tubules. Mice and rats are the most extensively used animals to study kidney failure models due to the existence of a large variety of KO models and the easiness of manipulation and stall. Rabbits are less commonly used than mice and rats as a model of CKD. This could be because of the relative costs and the highly frail nature of rabbits when used as a surgical model. Nevertheless, this animal remains of great interest for research on cardiovascular diseases (e.g., atherosclerosis) and lipid metabolism. Indeed, laboratory species greatly differ in the proportion of the plasma cholesterol-containing lipoprotein, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) [12, 13]. On the one hand, rodents (such as mouse and rat) carry a majority of cholesterol in HDL rather than LDL, making them questionable models of dyslipidemia. On the other hand, rabbits transport most of the cholesterol in LDL, as do humans, making it a more suitable model for cardiovascular research [14]. Furthermore, the rabbit has a larger blood volume (~56 mL/kg BW) [15], thereby making it more suitable for repeated blood sampling and research on extracorporeal devices (e.g., hemodialysis or peritoneal dialysis) [16, 17]. To the best of our knowledge, only a limited number of studies implemented 5/6 subtotal nephrectomy to induce CKD in rabbits [18–27].

The “three Rs” (3Rs) are guidelines proposed to promote a more ethical use of animals in research. They were first introduced by Russell and Burch in 1959 [28]. The “3Rs” stand for replace (i.e., to develop alternative methods that avoid the use of animals), reduce (i.e., to imple-

ment new methods to limit the numbers of animals needed for research) and refine (i.e., to develop methods to prevent or minimize potential pain, distress, and enhance animal welfare). The definition of refinement has evolved from that provided by Russell and Burch. The refinement is now commonly accepted to be “any approach which avoids or minimizes the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved, and which enhances their wellbeing” [29]. Refinement techniques may especially include the use of noninvasive (i.e., non-surgical) methods [30]. In this context, chemical nephrectomy, using adenine, could be a valuable strategy to refine the induction of CKD in rabbits. Chemical nephrectomy with adenine is easy to perform with no mortality and less morbidity compared to surgery. Wang et al. [31] demonstrated that chronic renal failure can be generated in rabbits by daily oral gavage with high doses of adenine (350 mg/kg) for 10 weeks. Repeated oral gavage is, however, a stressful procedure and can sometimes lead to complications. To the best of our knowledge, there is no available model of adenine-enriched diet in rabbits. In order to refine the protocol for inducing renal failure in rabbits, the aim of this study was to test the ability of an adenine-enriched diet to induce CKD in rabbits. To this end, rabbits were fed for 4 weeks with a custom diet containing 0.75% (w/w) of adenine, and the kidney function was explored using standards tests.

Methods

Animal

All experiments were performed under the authorization No. 69-266-0501 and were in agreement with the guidelines laid down by the French Ministry of Agriculture (No. 2013-118) and the European Union Council Directive for the protection of animals used for scientific purposes of September 22, 2010 (2010/63UE). The number of animals included ($n = 5$ per group) in this study was calculated using the R software (<https://www.r-project.org/>) and package EpiR 0.9–30 to detect a decrease of glomerular filtration rate (GFR) of at least 25% with a statistical power of 0.9. Ten adult male White New Zealand rabbits were housed in individual cages at a constant ambient temperature (21–23°C) and humidity (45–50%) with a 12 h light cycle. All animals had free access to tap water and their randomized diet after a 7-day period of acclimation in the animal facility. Ten rabbits were randomly divided into 2 groups: group 1 (control, $n = 5$) was fed with control diet (ref 110, 11.4 kJ/g, SAFE, Augy, France) and group 2 (adenine, $n = 5$) was fed with a custom diet (SAFE, Augy, France) containing 0.75% (w/w) of adenine (ref A8626, Sigma Aldrich, France) for 4 weeks. Compositions of diets are shown in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000454990). The food and water intake of the animals

were measured twice weekly. At the end of the protocol, the 24-h urines were collected, weighed to calculate the volume, and an aliquot centrifuged 10 min at 3,500 g. Supernatant was collected and frozen at -20°C for proteinuria measurement.

Measurement of GFR

The GFR was determined by measuring the plasma clearance of a single intravenous injection of a contrast medium (iohexol, Omnipaque 300[®], GE Healthcare), calculated from plasma sampling at multiple time points using a 2-compartment mathematical model for the plasma disappearance curve. Rabbits were anesthetized with an intravenous injection of 27.5 mg/kg of sodium pentobarbital (CEVA Santé Animale, Centravet, Lapalisse, France). The neck was shaved and a median incision was performed after local anesthesia with a subcutaneous infiltration of 1 mL of xylocaine (Aguettant, France). A PE-50 catheter (Phymep, Paris, France) filled with heparinized saline (10 mg/L) was disposed in the left jugular vein for the injections and a heparinized PE-60 catheter (Phymep) filled with heparinized saline (10 mg/L) was disposed in the left carotid artery for blood sampling. Rabbits were given hourly intravenous injections of sodium heparin (2 mg/kg/h) to prevent blood clotting in catheters. A bolus of 1 mL of iohexol was administered and the timer was started after flushing the residue left in the jugular catheter with heparinized saline. The syringe was weighed to the nearest milligram before and after the injection to precisely calculate the exact amount of iohexol delivered to the rabbit. Blood was sampled at 5, 15, 30, 45, 60, 120, and 180 min on lithium-heparin coated tubes to construct a plasmatic concentration decay curve. The plasma was aliquoted after a centrifugation at 5,976 g for 2 min and stored at -20°C until analysis. The serum iohexol concentration was measured by HPLC as previously described [32]. The plasma clearance of iohexol was calculated from the dose and the area under the curve, using a bi-compartmental model with GraphPad Prism (GraphPad software, La Jolla, CA, USA) and was expressed in mL/min/kg.

Sacrifice and Necropsy

At the end of the GFR measurement, rabbits were deeply anesthetized with an overdose of sodium pentobarbital (70 mg/kg i.v.). Blood was removed by cardiac puncture and put in EDTA-coated and lithium heparinate-coated tubes. After a centrifugation at 3,500 g for 10 min, plasma was sampled and stored at -80°C . Kidney was dissected out, weighted, and stored in buffered formaline 10% (w/v) for histological examination.

Renal Histology

Kidneys were dehydrated using ethanol and included in paraffin. Hematoxylin Erythrosine Saffron (HES) and Sirius Red staining were performed. Renal fibrosis was quantitated in Sirius red staining using ImageJ software (<https://imagej.nih.gov/ij/>). The surface area of glomeruli was measured with Image J on HES staining and the mean glomerular diameter was computed from the area of the equivalent disk. Measurements were routinely performed on 50–75 glomeruli per animal.

Biochemistry

Serum creatinine measurement was performed with the Siemens enzymatic method (Vista 1500; Siemens Healthcare, Erlangen, Germany) traceable to National Institute of Standards and Technology Creatinine Standard Reference Materials 914 (verified with

National Institute of Standards and Technology SRM 967) with calibration certified by isotope dilution mass spectrometry. Urea was measured with ureasetest (Vista 1500; Siemens Healthcare, Erlangen, Germany). Cholesterol and triacylglycerols levels were measured with enzymatic kits (Biomerieux, Marcy l'Etoile, France). Proteinuria was measured with Bradford protein assay (BioRad, Marne-la-Coquette, France) using bovine serum albumin as a standard.

Statistical Analyses

Data were expressed as mean \pm SEM. All analyses were performed using GraphPad Prism version 6.0. Normality was assessed using the D'agostino and Pearson test. Simple comparisons were performed using the Mann-Whitney U test. Differences were considered significant at the $p < 0.05$ level.

Results

Food Intake and Body Growth

To induce kidney failure, rabbits were fed with an adenine-rich diet (0.75% w/w) for 4 weeks. Body weight, energy intake, and water intake were monitored throughout the study (Fig. 1a–c). Note that no difference in cumulative food intake, water intake, or body weight was observed between the 2 groups throughout the study period. The total food intake was 5.0 ± 0.3 and 4.5 ± 0.1 kg (-9% , $p = 0.127$) for the control and the adenine group, respectively. The total water intake was 9.9 ± 0.7 and 9.4 ± 0.6 L (-5% , $p = 0.658$) for the control and the adenine groups respectively. The daily and total adenine intake calculated from the daily food intake was 1.06 ± 0.03 g/24 h and 33.9 ± 0.8 g respectively. The diet was well tolerated and no difference in body weight gain or body weight was noticed at the end of the protocol (Table 1).

Renal Function

Plasma urea and creatinine were measured longitudinally during the 4 weeks of adenine diet (Fig. 2). Creatinine and urea were not different at the beginning of the study ($p = \text{ns}$). Rabbits fed with an adenine diet exhibited only a transient increase in plasma creatinine after 15 days (control vs. adenine, 53 vs. 65 μM , $+23\%$, $p < 0.005$), while no change was noticed in urea. After 4 weeks of adenine diet, plasma urea and creatinine were not different ($p = \text{ns}$) within the normal range for rabbits, excluding the development of kidney failure. We measured GFR using a reference method of iohexol clearance that showed no difference between the 2 groups at the end of the diet (Table 1), excluding the development of adenine-induced kidney failure. No difference in 24-h diuresis or proteinuria was noticed between the 2 groups. The kidney weights were not altered by 4 weeks of adenine diet (Table 1). We

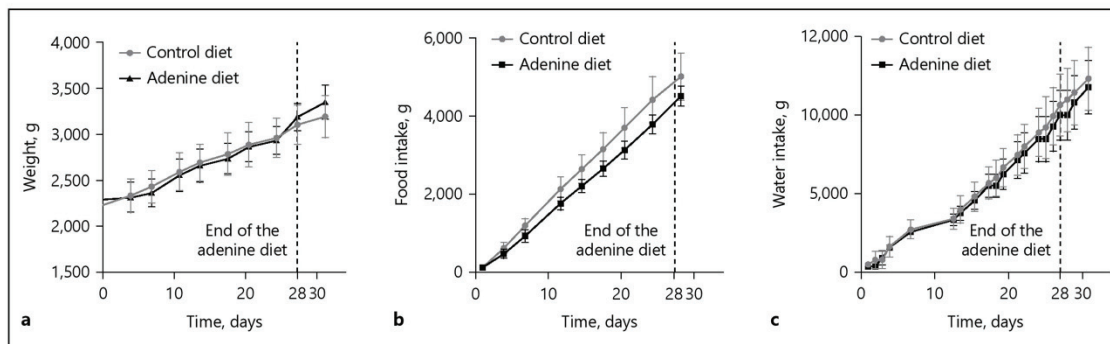


Fig. 1. Evolution of body weight and food and water intake of the rabbits fed with adenine or control diet. **a** Evolution of body weight during the whole experiment. Note that at the end of the protocol, body weights were not significantly different. **b** Cumulative food intake during the whole experiment. Note that food intake was

slightly lower in adenine diet, but this difference was not significant. **c** Cumulative water intake along the protocol. Data are mean \pm SEM for $n = 5$ in each group. No difference was found to be significant at the $p < 0.05$ level.

found no major difference in renal histology (Fig. 3). In Sirius red staining, no significant area of fibrosis was observed in the kidneys from rabbits fed with adenine diet (1.5 ± 0.2 vs. $1.3 \pm 0.2\%$, for control and adenine rabbits respectively, $p = 0.944$). We failed to find any tubular necrosis injury or glomerulomegaly as evidenced by the mean glomerular radius (22.3 ± 0.6 vs. $22.6 \pm 0.4 \mu\text{m}$ for control and adenine rabbits respectively, $p = 0.667$).

Metabolic Profile

At the end of the protocol, plasma lipids (total cholesterol and triacylglycerols) were within the normal range for both groups (Table 1) excluding the development of metabolic syndrome.

Discussion

This study was designed to implement a non-surgical model of CKD in rabbit. Chemical nephrectomy using an adenine-enriched diet has been shown to be a robust and well-tolerated model of CKD in mice and especially rats [7–9]. The aim of this study was, therefore, to test the ability of an adenine-enriched diet to induce CKD in rabbits. Then, rabbits were fed for 4 weeks with a custom diet containing 0.75% (w/w) of adenine and kidney function was monitored. As evidenced by the plasma concentrations of creatinine and urea, adenine-enriched diet failed to induce kidney failure in rabbits in contrast to what was observed in mice and rats [7, 8]. As small variations of cre-

Table 1. General characteristics of rabbits fed with a standard or adenine-rich diet (0.75% w/w) for 4 weeks

	Control diet	Adenine diet	<i>p</i> value
<i>n</i>	5	5	
Biometry			
Body weight, kg	3.37 \pm 0.25	3.32 \pm 0.06	0.310
Body weight gain, kg	1.05 \pm 0.08	0.97 \pm 0.03	0.341
Kidney weight, g/kg	6.24 \pm 0.99	6.63 \pm 0.25	0.397
Plasma analysis			
Urea, mM	6.98 \pm 0.88	6.73 \pm 0.74	0.898
Creatinine, μM	82.80 \pm 5.44	76.00 \pm 8.61	0.446
Glucose, mM	5.78 \pm 0.55	6.04 \pm 0.29	0.657
Total cholesterol, mM	1.43 \pm 0.34	2.18 \pm 0.48	0.310
Triacylglycerols, mM	4.73 \pm 1.47	3.10 \pm 0.62	0.486
Renal function			
mGFR, mL/min/kg	4.17 \pm 0.23	3.98 \pm 0.40	0.635
24 h-diuresis, mL	73 \pm 5	127 \pm 44	0.944
Proteinuria, mg/24 h	39 \pm 17	37 \pm 12	0.667

Data are expressed as means \pm SEM. Measured glomerular filtration rate (mGFR) was measured by iohexol clearance as described in the Methods section.

atinine can be associated with significant modification of GFR, we also performed the direct measurement of GFR by iohexol clearance. We, however, failed to observe any difference in GFR between the 2 groups excluding the development of kidney failure since GFR at the end of the study corresponding to normal rabbit GFR. Indeed, the GFR measured in this study using “golden standard”

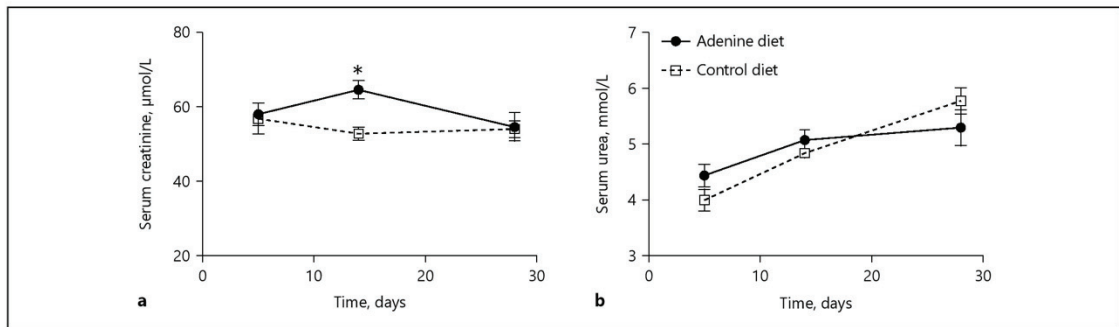


Fig. 2. Evolution of serum urea and creatinine concentrations in rabbits fed with adenine or control diet. Serum creatinine concentration (a) and serum urea concentration (b) were measured as an index of renal function. Data are mean \pm SEM for $n = 5$ in each group. * Indicates a significant difference between groups at the $p < 0.05$ level.

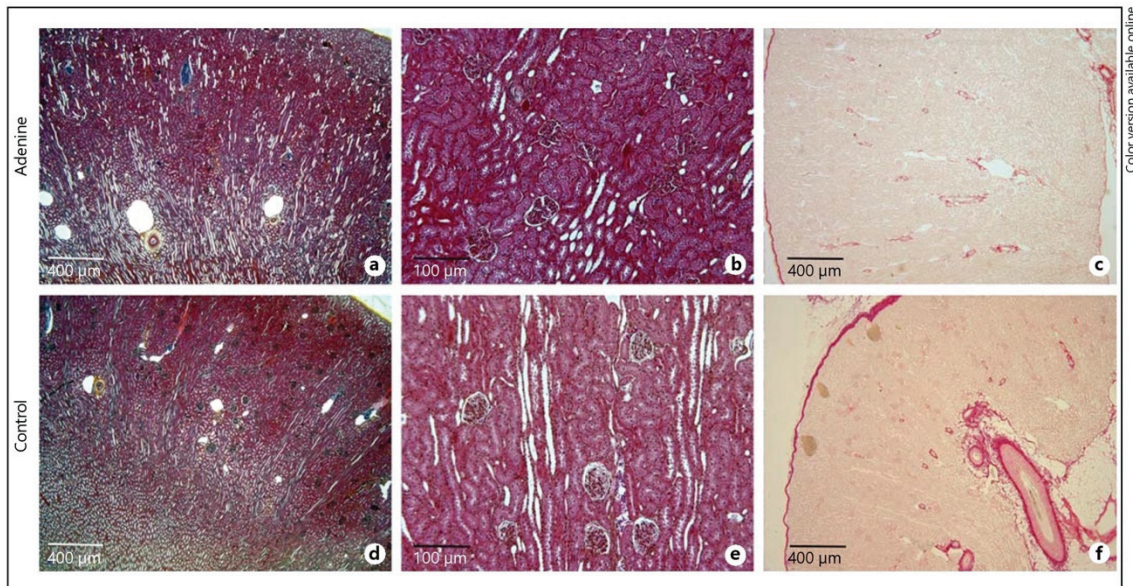


Fig. 3. Histological examinations of rabbit kidneys in HES and Sirius red stainings. Kidneys from control animals are shown in the upper panel (a–c), while kidneys from the adenine fed rabbits are shown in the lower panel (d–f). a, d Haematoxylin Erythrosine Saffron (HES) staining. Renal parenchymas were normal with fibrosis restricted to perivascular areas (magnification $\times 2.5$). HES staining. b, e Glomeruli were normal in size and cellularity (magnification $\times 10$). c, f Sirius red was used to stain collagen. No evidence of fibrosis was found in Sirius red staining (magnification $\times 2.5$).

methods (i.e., ~ 4 mL/min/kg BW) was in perfect agreement with that measured in the previous study in rabbits [33, 34] (online suppl. Fig. 1). Moreover, a histological study confirmed the absence of fibrosis and renal lesion classically associated with adenine diet in rats (Fig. 3).

In contrast with these data, Wang et al. [31] recently reported that daily oral gavage with a high dose of adenine (350 mg/kg/day for 10 weeks) induced chronic renal failure in rabbit. Several hypotheses could account for these differences and the inability of adenine diet to induce re-

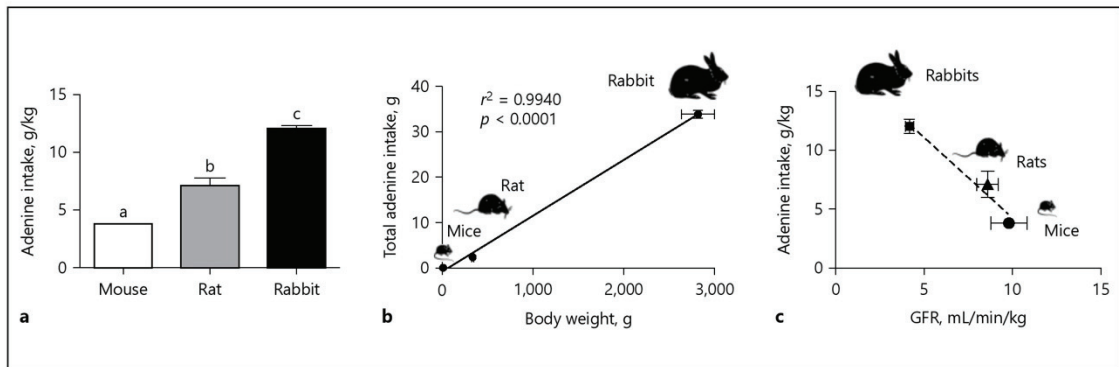


Fig. 4. Comparison of adenine intake in mouse, rat, and rabbit. **a** Total adenine intake after 4-weeks of adenine diet in mice (adenine 0.25% w/w), rats and rabbits (adenine 0.75% w/w). Different letters indicate a significant difference at the $p < 0.05$ level. Total adenine intake was calculated from the daily food intake. **b** Relationship between body weight and total adenine intake ($r^2 = 0.994$, $p < 0.0001$). **c** Relationship between mean adenine dose and GFR. GFR from rabbit were measured using iohexol clearance.

GFR in rat and mice were issued from literature [40, 41]. Adenine intake in mice and rats was obtained from ongoing studies in the lab. In these studies, mice were fed with a 0.25% (w/w) enriched adenine diet for 4 weeks and rats with a 0.75% (w/w) enriched adenine diet for 3 weeks. In both experiments, renal failure was evidenced by plasma urea and creatinine levels and histological observation of the kidneys.

renal failure in rabbits. First, the relative dose of adenine and the regimen used in this study could be insufficient to induce renal lesion. Indeed, 0.75% (w/w) corresponds to the same concentration used to induce renal failure in rats. The concentration of adenine in the diet used in mouse is 0.25% (w/w), while it ranges from 0.25 to 0.75% (but usually 0.75%) in studies with rats. The level and the reversibility of kidney failure were demonstrated to be dose- and time-dependent in rats [9]. Indeed, 2 weeks of feeding with a diet containing 0.75% (w/w) adenine induced an acute kidney injury (that heals in one month), while 4 weeks induced an irreversible kidney disease. Of importance is the observation that 6 weeks of feeding with adenine induced an irreversible kidney disease but with a significant mortality. Rabbits are bigger (i.e., 10-fold) than rats in terms of size and thus one can expect that an increase in the concentration of adenine in the diet would produce kidney failure. However, the total adenine intake during the protocol was higher in rabbits than in rats (roughly 2-fold; Fig. 4). Total adenine intake as reported in the study by Wang et al. [31] was 24.5 g/kg. In our study, adenine was included in the food (0.75%, w/w) for 4 weeks and the total adenine intake was 10.5 g/kg. This study, however, differs from the present in terms of the dosage, the administration mode, the duration, and the pattern of intake. After 4 weeks of oral gavage with adenine (total adenine intake 12.25 g/kg), rabbits from Wang

et al. [31] exhibited a significant increase of creatinine and urea plasma levels and a significant proteinuria. The total adenine intake after 4 weeks was very similar in the 2 studies (i.e., 10.5 vs. 12.25 g/kg); however, some difference in adenine disposition could be involved. Direct gavage with adenine could increase the bioavailability of adenine and thus increase its mean plasma concentration and promote the formation of crystals in the tubule leading to kidney failure. Adenine, delivered as a bolus leading to a massive urinary excretion, could then have a significant effect on the kidneys in contrast to the continuous delivery when adenine was supplemented in the food. We computed the daily adenine intake from the daily food intake during the 30 days of our protocol (online suppl. Fig. 2). The daily adenine intake was not constant during the whole protocol, ranging from 650 mg/kg/day (week 1) to 140 mg/kg/day (week 4) due to body weight gain. Indeed, the rabbits grew while daily food intake remained unchanged leading to a relative decrease in adenine intake. However, if a more concentrated diet can be tested, it could not easily be used as a routine model. There are indeed 2 major limitations of increasing the concentration of adenine in the diet. (1) Adenine strongly alters the palatability of the food thus leading to decreased food intake and thus reduced adenine intake. (2) As adenine is expensive, it is not cost effective when compared to the surgical model.

The renal toxicity of adenine in rabbits deserves some comments. The renal toxicity of orally absorbed adenine is due to the formation of crystals that damage the nephrons. Indeed, adenine is metabolized to 2,8-dihydroxyadenine, which precipitates and forms tubular crystals that damage the renal tubules [11]. It is well known that 2,8-dihydroxyadenine exhibits a pH-dependent solubility in aqueous medium [35]. In human urine, the 2,8-dihydroxyadenine solubility is 2.68 mg/L at pH 5.0, while it rises to 4.97 mg/L at pH 7.8 (i.e., +85%) [35]. Adenine can also be metabolized by xanthine oxidase to produce uric acid that can also precipitate in renal tubules. Three factors account for uric acid precipitation: the amount of uric acid excreted, the volume of urine as it affects the urinary concentration of uric acid, and the urinary pH. The most important factor for uric acid stone formation is a low (i.e., acidic) urinary pH. Uric acid solubility in the urine falls from approximately 200 mg/dL at a pH 7 to 15 mg/dL at pH of 5 [36, 37]. Of note, rabbits are herbivores and as such, their urine is physiologically very alkaline. The urinary pH in rabbits is therefore naturally higher than in most laboratory species, namely, between 7.6 and 8.8. Moreover, very large amounts of urine can be produced daily (due to a limited ability to concentrate urine) and thus, 24-h diuresis can reach 300 mL/kg. Second, rabbit renal metabolism is quite different from that of mice and rats. Rabbits are not as efficient as other mammals for bicarbonates reabsorption and this leads to a higher urinary pH; their elimination of urates greatly differs from that of humans. Indeed, 267% of the filtered load is excreted in rabbits [38], whereas only 5–10% is excreted in humans [39]. As adenine may induce CKD by uric acid and 2,8-dihydroxyadenine precipitation in renal tubules, this phenomenon could be strongly attenuated by the alkaline environment and the particular physiology of a rabbit kidney.

Adenine diet was described in mice and rats to have a poor palatability and thus to entail a significant decrease in food intake, resulting in a severe loss in body mass. In contrast, rabbits did not exhibit any change in food intake as evidenced by the record of cumulative food intake (Fig. 1). The main limitation of this study is that we failed to keep adenine intake constant during the protocol. Indeed, rabbits were fed during the whole protocol with the same diet containing 0.75% (w/w) of adenine. However, while food intake basically remained constant, body weight increased. As adenine toxicity is reversible in rats after a 2-week diet, the variation of adenine intake could explain why the rabbits exhibited a

significant rise of creatinine after 2 weeks of the diet, while no kidney failure was noticed at the end of the study.

Conclusion

The “3R” guidelines, as a step toward refinement, urged scientists to find non-surgical strategies to induce kidney disease in rabbits. However, adenine diets, which have extensively proved to be efficient to induce kidney failure in rodents (rats and mice), is not an option in rabbits. As the adenine intake needs to be maintained at a high level throughout the protocol, oral gavage with a constant dose of adenine, although more stressful and demanding, seems to remain the best option to non-surgically induce renal failure in rabbits.

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Author Contributions

N.F. and C.O.S. conceived and designed the study. N.F., S.L., C.C.P., and C.O.S. performed experiments. M.R. performed the histological studies. N.F. and C.O.S. performed statistical analysis, interpreted results, wrote and revised the manuscript. L.J. edited and revised the manuscript. C.O.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Statement of Ethics

The experiments were performed under the authorization No 69-266-0501 and were in agreement with the guidelines laid down by the French Ministry of Agriculture (No. 2013-118) and the European Union Council Directive for the protection of animals used for scientific purposes of September 22, 2010 (2010/63UE). This study was submitted to the French Ministry of Research (MENRT) for formal approval by the ethic committee.

Disclosure Statement

The authors have no conflict of interest to disclose.

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7. Effet du stress oxydant dans l'IRC sur les HDL circulantes :

Chronic kidney disease increases carbonylation of HDL and is associated with impaired antiaggregant properties.

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La troisième partie de ce travail est centrée sur l'analyse des modifications oxydatives et notamment de la carbonylation des HDL par le 4-hydroxy-2-nonenal dans l'insuffisance rénale chronique. Grâce au modèle animal, ce travail permet la description de la part imputable à l'IRC indépendamment des autres facteurs de risques cardio-vasculaires. Ces données expérimentales sont complétées par une étude clinique sur des lipoprotéines isolées de patients IRC dialysés.

Premièrement, nous avons observé une oxydation plus importante des HDL chez les animaux IRC. Cette oxydation semble surtout plus intense sur les acides gras polyinsaturés car l'augmentation est surtout marquée sur les concentrations de MDA et d'adduits de 4-HNE. Par contre, la concentration de F2-isoprostanes issus de l'oxydation de l'acide arachidonique n'est pas augmenté. Cependant ils ne sont pas en concentration importante dans les lipoprotéines. Il en est de même pour les adduits de 4-HHE qui ne sont pas retrouvés de façon plus importante dans les HDL des lapins IRC. L'augmentation des résidus d'oxydation dans les HDL peut être expliqué par deux mécanismes :

- 1) La plus grande propension à l'oxydabilité des HDL dans l'IRC retrouvée dans notre travail les rend plus vulnérables à l'oxydation.

2) La clairance des lipoprotéines oxydés est probablement perturbée car la liaison avec le récepteur SR-B1 au niveau hépatique est impactée par la diminution de la population HDL-2 dans l'IRC. Cela augmenterait donc la population d'HDL-3 circulants avec ces résidus. En effet, comme nous l'avons décrit plus haut, le métabolisme des HDL est profondément perturbé dans l'IRC. Ces modifications sont déjà observées dans des stades précoces d'IRC chez des enfants.⁶¹ Notre modèle est assez proche de ce que l'on observe chez les enfants IRC, à savoir une insuffisance rénale assez « pure » sans autres facteurs de risques cardio-vasculaires. Les travaux de Drozd et al⁶³ renforcent cette similarité entre notre modèle et l'IRC observée chez les enfants. En effet, dans leur travail, ils ne retrouvent pas de différences de concentration concernant les oxLDL entre les différents stades d'IRC alors que parallèlement les HDL dysfonctionnent déjà.⁶¹

D'autre part, nos travaux confirment l'effet délétère de l'IRC sur le phénotype anti-agrégant des HDL. En effet, cette fonction ne semble pas échapper aux précédentes observations sur les propriétés biologiques perturbées des HDL. L'incubation de plaquettes humaines avec des HDL issues de lapins IRC montre un phénotype anti-agrégant profondément altéré sans être pour autant inversé. Du fait de l'augmentation des adduits de 4-HNE sur les résidus protéiques des HDL d'IRC, nous avons voulu tester l'effet de la modification in-vitro d'HDL issus de lapins contrôles spécifiquement avec cet adduit. Pour ce faire des HDL de lapins contrôle ont été incubé avec 100 μ M de 4-HNE afin de générer des adduits covalents dont l'existence a été mise en évidence par immunoblotting. Le résultat est cohérent avec observations réalisées sur les HDL de lapins IRC car les HDL-HNE présentent une moindre capacité à inhiber l'agrégation plaquettaire. Nous avons aussi testé l'effet de toutes les lipoprotéines circulantes dans leur proportion d'origine sur l'agrégation plaquettaire. L'IRC induit un phénotype limite pro-agrégant alors que dans la population contrôle la tendance globale est plutôt à l'anti-agrégation.

Pour confirmer cette tendance, nous avons regardé s'il existait adduits de carbonylation en excès sur les HDL issus de patients en hémodialyse (groupe HD). Nous avons fait appel à des patients non-diabétique car cette pathologie est pourvoyeuse de modifications post-traductionnelles (e.g. des phénomènes de glycation non enzymatique) pouvant potentialiser l'effet observé chez les patients. Les patients contrôle non IRC étaient quant à eux des donneurs potentiels de rein (i.e. des patients en particulièrement bonne santé). L'immunoblotting confirme la présence importante de ces adduits sur les protéines composant les HDL des patients HD. Pour aller plus loin dans la caractérisation, nous avons fait appel à des techniques plus spécifiques de spectrométrie de masse (protocole complet dans les Supplementary data de l'article joint) afin d'identifier précisément les protéines modifiées et la place exacte de ces adduits dans la séquence peptidique. Cette analyse nous a conduit à plusieurs constatations :

- Il existe effectivement un nombre d'adduits de 4-HNE présent en quantité significative chez les patients hémodialysés par rapport aux contrôles.
- Ces adduits sont majoritairement situés sur l'apolipoprotéine A1 qui est probablement la plus importante pour le métabolisme des HDL et leurs fonctions athéroprotectrices.
- Les adduits ont été retrouvés sur des acides aminés situés dans des portions clés de la protéine.

Concernant les propriétés d'anti-agrégation chez les patients dialysés, nous avons observé une sur-activation plaquettaire induite par le collagène dans la population hémodialysée. Il existait par contre une grande hétérogénéité au sein de la cohorte. Afin de limiter la part imputable à la technique d'hémodialyse, nous avons inclus des patients non-diabétiques en dialyse péritonéale (groupe PD). Ceux-ci présentaient aussi une sur-activation plaquettaire en réponse au collagène après l'incubation avec les HDL. De la même façon que chez le lapin, l'incubation d'HDL contrôle avec du 4-HNE entraînait une sur-activation plaquettaire. Du fait de la grande hétérogénéité des résultats des groupes de patients en hémodialyse et en dialyse péritonéale, nous avons testé plusieurs concentrations de 4-HNE et seule la plus élevée (100 μ M) a affecté

les propriétés anti-agrégantes des HDL. Ces constatations confirment donc que la carbonylation par le 4-HNE des HDL dans l'IRC contribue à l'augmentation du risque thrombotique.

L'implication de la voie du récepteur scavenger CD-36, des SRC-kinases et de Jun Kinase (JNK) semblent participer à ces propriétés délétères comme nous avons pu le démontrer en utilisant un anticorps bloquant anti-CD36 et un inhibiteur pharmacologique des SRC-kinases. Ce récepteur (CD36) a déjà prouvé son implication dans le phénotype pro-agrégant des oxLDL⁴⁴ avec la phosphorylation de la MAP-kinase JNK. Le blocage de ce récepteur par un anticorps spécifique permet de rétablir *ad integrum* le phénotype anti-agrégant des HDL dans le groupe HD et PD et dans les HDL modifiés *in vitro* par l'adjonction de 4-HNE. Le blocage des SRC-kinases a montré le même effet d'accentuation du phénotype anti-agrégant des HDL. La phosphorylation de JNK est aussi retrouvée diminuée par le blocage du CD36 et des SRC-kinases (**Figure 10**).

Comme le récepteur SRB1 est quant à lui impliqué dans les propriétés anti-agrégantes des HDL par le biais de la fixation de l'ApoA1,⁴³ nous avons voulu observer les effets de son blocage sur le phénotype d'agrégation. Nous confirmons son rôle pivot dans les propriétés anti-agrégantes car son blocage entraîne une majoration très significative de l'activation plaquettaire et de la phosphorylation de JNK. Ce récepteur semble ainsi exercer son potentiel anti-agrégant d'un côté par la phosphorylation de la protéine Kinase C⁴³, mais aussi potentiellement par l'inhibition de la phosphorylation de JNK (**Figure 11**).

Ainsi, nous décrivons ici pour la première fois la modification du phénotype anti-agrégant des HDL dans l'IRC. De précédents travaux ont permis de confirmer l'effet pro-agrégant des LDL dans l'IRC avec notamment l'implication des résidus carbamoylés via la voie du récepteur LOX-1³⁵. Notre travail renforce ainsi l'hypothèse du rôle central des HDL dans la survenue de complications cardiovasculaires associées à l'IRC. Des travaux complémentaires

sont toutefois nécessaires pour préciser cette implication et proposer des solutions thérapeutiques.

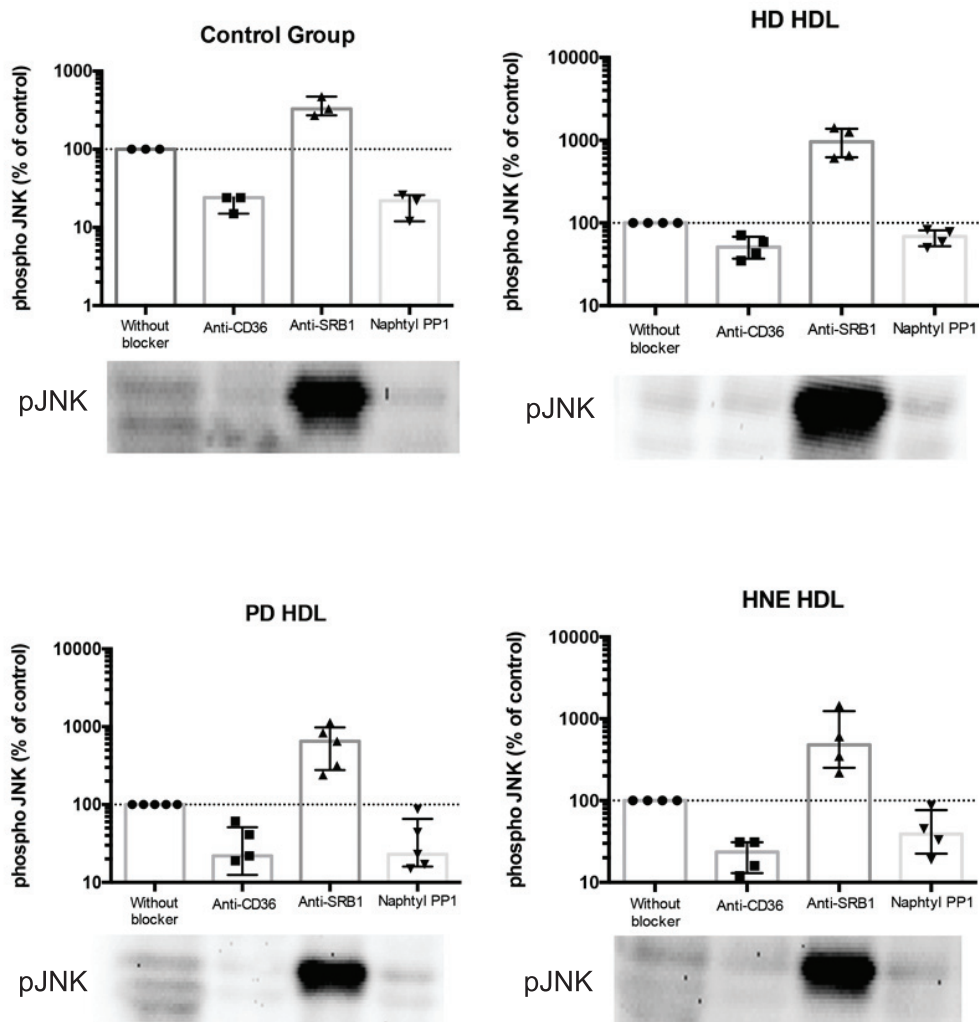


Figure 11 – Niveau d’expression de la forme phosphorylée de la Jun-kinase (JNK) selon les différentes modalités d’agrégation

Le blocage du récepteur CD-36 et des SRC-kinases réduisaient significativement la phosphorylation de JNK alors que le blocage de SR-B1 était plutôt responsable d’une augmentation importante de celle-ci. (sans bloqueurs=without blocker, avec les anticops anti-CD36 et SR-B1= anti-CD36 et anti-SRB1 et avec l’inhibiteur pharmacologique des SRC-kinases= naphtyl PP1)

Chronic kidney disease increases carbonylation of HDL and is associated with impaired antiaggregant properties.

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Significance Statement

Chronic kidney disease (CKD) is associated with an increased risk of cardiovascular disease. The failure of statins in hemodialysis patients shed light on the unique phenotype of dyslipidemia in dialysis patients. HDL atheroprotective properties (cholesterol efflux, anti-inflammatory and vasorelaxation) in CKD have been found to be impaired. Nothing is known about the anti-thrombotic properties of HDL in CKD. The present study describes the impaired anti-aggregant properties of HDL induced by CKD, and the putative role of carbonylation by 4-HNE adduction on these properties. This study provides important insights into the potential implication of HDL modifications in atherothrombosis and cardiovascular morbidity and mortality among dialysis patients.

Abstract

BACKGROUND: Cardiovascular disease remains the major cause of death in chronic kidney disease (CKD). As oxidative stress is raised in CKD we aimed to explore the specific role of oxidative modifications of HDL in CKD and their impact on the anti-aggregant properties of HDL.

METHODS: Blood samples were taken from 15 healthy volunteers 25 hemodialysis (HD) and 20 peritoneal dialysis (PD) patients at the Lyon university hospital. Eight 5/6-nephrectomized rabbits (CKD) were compared with 9 control rabbits (CR). Malondialdehyde (MDA), 4-hydroxy-nonenal (HNE), and 4-hydroxy-2-hexenal (HHE) protein adduct levels were analyzed. Platelet aggregation and activation was assessed in an aggregometer or TxB2 and FACS. HDL from controls were modified by an incubation overnight at 37°C with 100µM of HNE.

RESULTS: HNE-adducts were increased in HDL from CKD rabbits and HD patients. Percentage of platelet aggregation/activation induced by collagen was significantly higher when were incubated with HDL from CKD rabbit and HD groups than with HDL from the control group. Platelet aggregation/activation in presence of HNE-modified HDL was also higher than control group either in rabbit or humans ($p < 0.05$ compared to CR or Control patients). Incubation with a blocking antibody directed against CD36 and a pharmacological inhibitor of SRC-kinases restored the anti-aggregative phenotype in CKD rabbit, HD and PD patients and HNE-modified HDL.

CONCLUSIONS: HDL from CKD rabbits and HD patients exhibited an impaired ability to inhibit platelet aggregation suggesting that altered HDL properties could contribute to the increased cardiovascular risk in this population.

Introduction

Chronic kidney disease (CKD) is recognized as a major cardiovascular risk factor.¹⁻³ It is involved in the onset of cardiovascular events such as myocardial infarction, peripheral arterial disease and cerebral ischemia¹, and cardiovascular mortality remains the major cause of death in hemodialysis (HD) and peritoneal dialysis (PD) patients despite the improvement of renal replacement therapies.⁴ CKD is associated with an increased oxidative stress that is correlated with the occurrence of cardiovascular events.⁵⁻⁷ This stress is associated with the accumulation of many uremic toxins^{6,8}, some of which have recognized cardiovascular effects.⁹⁻¹² Modifications induced by increased oxidative stress particularly affect circulating lipoproteins such as HDL that exhibit anti-atheromatous and anti-thrombotic properties *in vitro*.

There are many mechanisms that lead to lipoprotein modification, both enzymatic and non-enzymatic, and these affect different lipoprotein sites.¹³ Oxidized and carbamylated LDL are present at higher concentrations in CKD compared to healthy patients^{5,14} and play a major role in the onset and aggravation of atherosclerotic lesions.^{14,15} The concentration of oxidized and carbamylated LDL are, however, lowered by statins.¹⁶⁻¹⁸ HDL is considered to be antiatherogenic as a result of its antiaggregant, anti-inflammatory and antiapoptotic properties¹⁹ as well as its ability to induce cholesterol efflux from macrophages²⁰, a mechanism known as atheroprotective.²¹ HDL is not affected by conventional hypolipidemic strategies, and functional studies in CKD have found impaired biological effects, including decreased capacity of macrophage cholesterol efflux²²⁻²⁴ and anti-oxidation,²⁴ as well as impaired endothelial protection.²⁵ These disorders become more significant with CKD progression.²⁵ In parallel, HDL oxidation has been related to the onset of cardiovascular events in CKD patients.²⁶ These functional modifications could partly explain the failure of statins to reduce cardiovascular risks associated with haemodialysis.^{27,28}

Platelets are the target of anti-aggregant treatments which have been shown to be beneficial in reducing cardiovascular events in at-risk populations. Oxidized LDL of obese and diabetic patients has shown some pro-aggregating properties.^{29,30} In CKD, carbamylated LDL exhibits pro-aggregant properties through binding with lectin-like oxidized low density lipoprotein receptor 1 (LOX-1)³¹, and oxidized LDL through binding to the CD36 receptor.³² Oxidation of HDL leads to a loss of antiaggregation properties in some populations,³³ although this observation was not found in type 2 diabetes³⁴ in whom oxidized HDL has shown antiaggregant properties through a SR-B1 -mediated pathway.³⁵ To the best of our knowledge, there is, however, no published data regarding the impact of CKD on the antiaggregant properties of HDL.

In the present study, we therefore aimed to explore the specific role of CKD in the oxidation profile of HDL and the effects of HDL modification on platelet aggregation in a rabbit model of CKD. We then aimed to assess, *ex vivo*, the antiaggregant properties of HDL isolated from HD and PD patients.

Concise Methods

Subjects and ethics statement

Patients were sampled at the Lyon teaching hospitals. Control patients were healthy volunteers for a living kidney donation, hospitalized for their pre-donation laboratory and clinical work-up. HD patients were sampled in the hemodialysis unit of the Edouard Herriot hospital (Lyon) before the mid-week session. PD patients were sampled in the peritoneal dialysis unit of the Association pour l'Utilisation du Rein Artificiel (AURAL, Lyon, France). Inclusion criteria were age ≥ 18 years, undergoing HD or PD for more than 6 months. Exclusion criteria were diabetes mellitus, ongoing inflammatory disease, liver cirrhosis, recent cardiovascular event (< 3 months; myocardial infarction, stroke, acute peripheral artery occlusion), uncontrolled anemia, coagulopathy, and BMI greater than $35 \text{ kg}\cdot\text{m}^{-2}$. The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional review board (*CPP Lyon Sud Est IV*, ref: L16-57). A written informed consent was obtained from all subjects. Blood samples were obtained by venipuncture on EDTA coated tubes. Blood samples were centrifuged at $3500 \times g$ for 10 min to isolate plasma which were stored at -80°C until use.

Animal procedures

All experiments were performed under the authorization no. n°69-266-0501 and agreed with the guidelines laid down by the French Ministry of Agriculture (n° 2013-118) and the European Union Council Directive for the protection of animals used for scientific purposes of September 22, 2010 (2010/63UE). Adult male White New Zealand rabbits (CEGAVssc, Saint Mars d'Egrenne, France) were housed in individual cages at constant ambient temperature ($21\text{-}23^{\circ}\text{C}$) and humidity (45-50%) with a 12h light cycle. All animals had free access to tap water. After a 7-day period of acclimation, rabbits were randomized to either the 5/6 nephrectomy group or in the control group. Nephrectomy was performed as described by Gotloib *et al.*³⁶

Measurement of glomerular filtration rate

The glomerular filtration rate (GFR) in rabbits was measured through the kinetics of decrease of plasma concentration of iohexol as described in Florens *et al.*³⁷ The serum iohexol concentration was measured by HPLC.³⁸ GFR was calculated using a bi-compartmental model equation.

Sacrifice and necropsy

At the end of the GFR measurement, rabbits were deeply anesthetized with an overdose of sodium pentobarbital (70 mg/kg iv). Blood was removed by cardiac puncture and placed in EDTA-coated tubes. After a centrifugation at $1250 \times g$ for 10 min, plasma was aliquoted and stored at -80°C . Urine was obtained via a direct bladder puncture and stored at -20°C . Kidneys were removed, weighed and stored in buffered formalin 10% (w/v) for histological examination.

Isolation of lipoproteins from the plasma

Lipoproteins were separated from plasma by stepwise potassium bromide (KBr) density gradient ultracentrifugation as described by Havel *et al.*³⁹ For the isolation of total lipoproteins, a single ultracentrifugation was performed at $54,000 \times g$ for 6 h 30 min at 4°C after an adjustment of plasma density to $1.21 \text{ g}\cdot\text{mL}^{-1}$ with KBr. For platelet aggregation and copper-induced HDL oxidation, lipoproteins were extensively dialyzed against phosphate-buffered saline (PBS) with 1mM EDTA for 3 h, twice at room temperature and then overnight at 4°C . A last dialysis without EDTA was performed just before the platelet aggregation and copper-induced oxidation.

Biochemistry

Serum creatinine, urea and lipids were assayed using standard laboratory methods. Malondialdehyde (MDA) levels were measured using HPLC-UV as described by Grotto *et al.*⁴⁰ Antioxidant activity (AOA) of the plasma was measured as described by Koracevic *et al.*⁴¹ Proteinuria was measured using the Bradford protein assay (BioRad, Marne-la-Coquette, France) using bovine serum albumin (BSA) as a standard.

Lipoprotein assays

MDA concentration in HDL was determined by HPLC according to the method described by Therasse and Lemonnier.⁴² Anti-4-hydroxy-nonenal (HNE)-Michael adduct (ref 393207) and anti-4-hydroxy-2-hexenal (HHE)-Michael adduct (ref NOF-N213730-EX) antibodies were obtained from Calbiochem (San Diego, CA, USA) and Cosmobio (Tokyo, Japan), respectively. Fifty micrograms of HDL were loaded directly on to a nitrocellulose membrane using the Bio-Dot apparatus (BioRad, Marne-la-Coquette, France). Following saturation with 5% BSA, membranes were probed overnight with primary antibodies; anti-HHE-Michael adducts, or anti-HNE-Michael adducts. After incubation with HRP-coupled secondary antibodies, membranes were processed for chemiluminescence (ECL plus, GE Healthcare, Chicago, IL, USA) and quantitated by densitometry using Image J software (NIH, Bethesda, MD, USA). 8-isoprostane was measured using an immunoassay (Bertin Pharma, Montigny Le Bretonneux, France). 4-HNE was added to HDL solutions to final concentrations of 1, 10, 50 or 100 μ M. After a 16 h (overnight) incubation at 37°C in a water bath, HDL were dialyzed three times against PBS to remove the free fraction of 4-HNE.

Platelet aggregation, activation and intra-cellular pathways assays

Blood was collected at the regional blood center from healthy volunteers who had not ingested any aspirin or other non-steroidal anti-inflammatory drug in the previous 10 days. Platelets were

prepared for the assays as described by Lagarde *et al.*⁴³ Platelet function test was carried out according to the Born turbidimetric method.⁴⁴ Platelet aggregation was measured in isolated platelets in a dual-channel aggregometer (Chrono-log; Coulter, Margency, France). Platelet suspensions were pre-incubated for 5 min at 37°C in the presence or absence of lipoproteins (0.025 mg of protein/mL for rabbit, 0.050 mg/mL for human) and stimulated with threshold concentrations of collagen (75 ± 9 ng/ml) while being continuously stirred at 1,000 rpm. The threshold concentration of collagen was defined as the concentration of collagen that induced a 50% increase in light transmission. The extent of platelet aggregation was expressed in terms of percentage of change in light transmission 4 min after the addition of collagen. Blocking of CD36 or SRB1 receptor was achieved by the pre-incubation with 10 μ L of an anti-CD36 (Ab-CD36) or anti-SRB1 (Ab-SRB1) antibodies (dilution 1:500, Abcam, Paris, France) for 10 min at 37°C before the incubation with HDL. Blocking of SRC-kinases was achieved by the pre-incubation with Naphtyl PP1 (final concentration 1 μ M, Santa Cruz Biothechnologies, Dallas, TE, USA) for 10 min at 37°C before the incubation with HDL. Aggregation values from lipoprotein assays were expressed as a percentage of the maximum aggregation induced by the collagen alone (considered as 100%). Levels of thromboxane B2 (TxB2) was measured using an immunoassay expressed as a percentage of the maximum level induced by the collagen alone (considered as 100%, Cayman Chemical, Ann Arbor, MI, USA). After pre-incubation with HDL and antibodies or pharmacological inhibitor, platelets were activated with collagen then fixed in paraformaldehyde 5% and stained with anti-CD62/P-Selectin (Thermo Fisher Scientific, San Jose, CA, USA) according to the manufacturer protocol and analyzed by flow cytometry.⁴⁵

Copper-induced HDL oxidation

Aliquots of freshly dialyzed HDL (50 μ g protein) were oxidized in the presence of 2.5 μ M of CuSO₄ within the day following the last dialysis. The oxidation was monitored by measuring the formation of conjugated dienes at 234 nm.⁴⁷

Mass spectrometry

Samples were reduced, alkylated and digested with trypsin at 37°C overnight. They were desalted with spin column C18, dried and then analyzed in triplicate using an Ultimate 3000 nano-RSLC (Thermo Fisher Scientific) coupled on line with a Q-Orbitrap (Q Exactive HF, Thermo Fisher Scientific). Briefly, peptides were separated on a C18 nano-column with a linear gradient of acetonitrile and analyzed in a Top 20 HCD (Higher Collision Dissociation) data-dependent mass spectrometry. Data were processed by database searching using SequestHT (Thermo Fisher Scientific) with Proteome Discoverer 2.2 software (Thermo Fisher Scientific) against a human Swissprot database and quantified with a label free quantitation approach. Precursor and fragment mass tolerance were set at 10 ppm and 0.02 Da respectively. Trypsin was set as enzyme, and up to 2 missed cleavages were allowed. Oxidation (M), acetylation (Protein N-terminus) and HNE (+ 156.115 Da on K or H) were set as variable modification as fixed modification. Peptides were filtered with false discovery rate (FDR) at 1%.

Molecular modelling

To study the accessibility of residues, the structure of truncated human apolipoprotein A-I (pdb code 1av1) was downloaded from the PDB database⁴⁸. All observations were performed using PyMOL as software by representing the α -helices and histidine and lysine residues (colored in blue) in CPK to facilitate the study. The same method was achieved with the HDL model which was download from the PDB database.⁴⁹

Statistical analysis

Data were expressed as median \pm interquartile range (IR). All analyses were performed using GraphPad Prism version 6.0 (GraphPad software, La Jolla, CA, USA). Simple comparisons were made using Mann-Whitney U-test. Multiple comparisons were made with Kruskal-Wallis test and whenever appropriated Dunn's tests. Differences were considered as significant at the $P < 0.05$ level.

Results

General characteristics of the CKD rabbit model

Rabbits developed a significant renal insufficiency in the CKD group; there was a significant difference ($P < 0.001$) in the median GFR between the control group and the CKD group (-58%, **Table 1**). CKD rabbits exhibited increased levels of lipid peroxidation in the plasma as evidenced by the 10-fold significant increase in MDA concentration ($P < 0.001$, **Table 1**). Median antioxidant activity of the plasma was significantly ($P < 0.05$) lower in the CKD group compared with control (-15%, **Table 1**). Histological examination found a diffuse fibrosis in the parenchyma in the CKD group as well as a glomerulomegaly (**Supplementary Figure 1**).

HDL oxidation level and oxidizability in CKD rabbits

HDL from CKD rabbits had significantly higher levels of HNE-Michael adducts on proteins ($p < 0.05$, **Figure 1A**). By contrast, HHE-adducts on proteins were not significantly higher in the HDL of the CKD group (**Figure 1A**). MDA concentration was significantly higher in the HDL of the CKD group (**Figure 1B**) while the 8-isoprostane concentration was not significantly different (**Figure 1C**). Despite a non-significant difference in the concentration of tocopherols (**Figure 1D**), HDL lipids from CKD rabbits were more prone to oxidize than HDL from control animals; there was a significant difference from 70 min until the end of the experiment ($p < 0.05$, **Figure 1E**).

Platelet aggregation induced by HDL from rabbits

When human platelets were incubated with HDL from the CKD group, the median aggregation was 75% as compared to collagen alone; when incubated with HDL from the

control group it was 30% ($p < 0.05$ as compared to when incubated with HDL from the CKD group, **Figure 2A**). As HNE-adducts were increased in CKD HDL, we incubated control rabbit HDL with $100\mu\text{M}$ HNE; median platelet aggregation in the presence of HNE-modified HDL was 85% (**Figure 2A**, $p < 0.05$ as compared to when incubated with HDL from the control group). Pre-incubation of platelets with an anti-CD36 antibody restored the antiaggregant effect of the HDL from CKD rabbits and HNE-modified HDL (median: 25 and 22%, respectively, **Figure 2A, C**). Pre-incubation of platelets with Ab-CD36 did not change significantly the aggregation induced by collagen (median: 95% vs. 100%, NS).

To investigate the effect of total lipoprotein (HDL, LDL, and triglyceride-rich lipoproteins; LPP) of CKD animals, platelets were incubated with a lipoprotein mix resulting from a single ultracentrifugation. The median aggregation in the CKD LPP group was 98% vs. 21% in the control group ($p < 0.05$, **Figure 2B**). Platelet aggregation in the presence of HNE-modified LPP was 99% ($p < 0.05$ compared to control LPP group). Pre-incubation of platelets with an anti-CD36 antibody restored the antiaggregant effect of the LPP from CKD rabbits and HNE-modified LPP (median: 23 and 11%, respectively, **Figure 2B**). HNE-adduct levels in HNE-HDL and LPP were higher than in control using an immuno-blot (**Figure 2D**).

Carbonylation of HDL from HD patients

Nine controls were compared to 9 non-diabetic HD, the main characteristics of whom are presented in **Supplementary Table 1**. HD patients had significantly lower total cholesterol, LDL-cholesterol, and HDL cholesterol than the controls ($p < 0.05$). There was a higher level of HNE-adducts in HDL from HD patients than in controls (**Figure 3A**). To map sites on proteins susceptible to carbonylation, HDL from control and HD patients were digested with trypsin; digested peptides were separated by liquid chromatography (LC), and peptides were analyzed for carbonylation with tandem mass spectrometry (MS/MS). We then expressed a ratio of

amount of the 4-hydroxy-2-nonenal into the HD patient sample versus the mean amount of control patients. An higher ratio (>1) of 4-HNE Michael adduct was detected onto 48 amino-acids (Lysine or Histidine) from 8 constitutive proteins of HDL from HD patients (**Table 3**). The majority was located onto apolipoprotein A1 and concerned various important functional sites of the protein (**Figure 3B**). Lys 250, located in the C terminal part of apolipoprotein A1, was the most frequently modified site in HD patients (5.3-fold increase compared to controls, $p < 0.01$, **Figure 3C**).

In silico modelling of 4-HNE adduction onto apolipoprotein A1

In order to gain insights into the accessibility of histidine and lysine residues of apolipoprotein A1, a molecular modeling study was achieved with the lipid-free structure of the truncated protein (**Figure 4A**).⁴⁸ Histidine and lysine residues mentioned in the primary sequence of amino-acids namely K83, K130, H179, H186, H216, H223, K226, K250 and K262 were examined and were found to be readily accessible and susceptible to react with 4-HNE (not available for H20, K47 and K64 residues in the truncated protein). Therefore, the modification of these residues may occur in a random event on free apolipoprotein A1. Careful examination of the model of HDL proposed by Wu et al (**Figure 4B**)⁴⁹ led to the following observations: some histidine and lysine residues are not involved in interactions with lipids and are located at the external part of HDL. These residues which can react with 4-HNE may not affect the binding of apolipoprotein A-I with lipids but with other proteins involved in HDL metabolism (see **Figure 3B**, residues K47, K83, H216, K250 and K262); in contrary, some histidine and lysine residues (residues K64, K130, H179, H186 and H223) interact tightly with lipids in the internal side of the complex. In this case, it can be proposed that these modifications may alter the ability of apolipoprotein A-I to bind lipids. Overall, this molecular modeling study shows that the reaction of lysine and histidine residues leading to structural modifications may occur

randomly but may be critical for lipids and protein binding depending on the residue which is modified.

Platelet activation and aggregation phenotype of HDL from HD and PD patients and effects of carbonylation

As HD procedure *per se* can lead to an enhanced oxidative and carbonylated stress, we included PD patients to avoid any effects resulting from bio-incompatibility of HD devices. Fifteen healthy controls were compared to 25 non-diabetic HD and 20 PD patients, the main characteristics of whom are presented in **Table 2**. HD patients had significantly lower total cholesterol, LDL-cholesterol, and HDL cholesterol than the controls ($p < 0.05$). Platelet activation was measured with the production of thromboxane B2 and expressed as a percentage of the amount found in platelet activated with collagen. When human platelets were incubated with HDL from the HD group, the median activation was 147% as compared to collagen alone; when incubated with HDL from the control group it was 75% ($p < 0.05$ as compared to when incubated with HDL from the HD group, **Figure 5A**). When human platelets were incubated with HDL from the PD group, the median activation was 221% as compared to collagen alone ($p < 0.05$ as compared to when incubated with HDL from the control group, $p = \text{NS}$ as compared to when incubated with HDL from the HD group, **Figure 5A**). As HNE-adducts were increased in HD HDL, we incubated 5 healthy donors HDL with $100\mu\text{M}$ HNE; median platelet activation in the presence of HNE-modified HDL was 123% as compared to collagen alone; when incubated with HDL from the control group it was 29% (**Figure 5B**, $p < 0.05$ as compared to when incubated with HDL from the healthy donor group).

As we performed aggregation assays in rabbits, we chose 9 control and 9 HD patients (characteristics in **Supplementary Table 1**) and performed platelet aggregation assays. When

human platelets were incubated with HDL from the HD group, the median aggregation was 50% as compared to collagen alone; when incubated with HDL from the control group it was 19% ($p < 0.05$ as compared to when incubated with HDL from the HD group, **Figure 5C**). We found a correlation between percentage of activation with TxB2 assay and percentage of aggregation ($r^2 = 0.3894$, $p < 0.05$, **Supplementary Figure 2**). As HNE adducts were increased in HD HDL, we incubated healthy donors HDL with $100 \mu\text{M}$ HNE; median platelet aggregation in the presence of HNE-modified HDL was 170% (**Figure 5C**, $p < 0.05$ as compared to when incubated with HDL from the control group).

As there was an interindividual variability of platelet response to HD-HDL, we tested several concentrations of HNE for the modification of control HDL from healthy donors (1, 10, 50, and $100 \mu\text{M}$). A threshold effect was found as only the $100 \mu\text{M}$ solution of HNE led to a significant alteration of the anti-aggregant properties of HDL (median: 5% at $100 \mu\text{M}$ vs. 98, 99, and 99% at 1, 10, and $50 \mu\text{M}$, respectively, $p < 0.05$, **Figure 5D**). Immunoblotting found a dose-dependent increase of HNE-adducts in HDL incubated with 4-HNE (1- $100 \mu\text{M}$; **Supplementary Figure 3**).

Activation is mediated by the phosphorylation of JNK through a CD36 and SRC kinases pathway.

To get an insight in the potential pathways involved in the effects described herein, the effect of CD36, SRB1 blockade and SRC kinase pharmacologic inhibitor were tested. Pre-incubation of platelets with an anti-CD36 antibody significantly decreased their activation measured by TxB2 levels when incubated with HDL from control, HD, PD and HNE-modified HDL (median: 39%, 41%, 22% and 49% respectively, $p < 0.05$, as compared to when incubated without the antibody, **Figure 6A**). P-selectin expression was also lowered by the pre-incubation of platelets with an anti-CD36 antibody for HDL from control, HD, PD and HNE-modified

HDL (median: -43%, -30%, -42% and -27% respectively, $p < 0.05$, as compared to when incubated without the antibody, **Figure 6B**). Pre-incubation of platelets with an anti-CD36 antibody restored the antiaggregant effect of the HDL from HNE-modified HDL (median: 12%, $p < 0.05$, as compared to when incubated without the antibody, **Supplementary Figure 4**). Pre-incubation of platelets with Ab-CD36 did not change significantly the aggregation induced by collagen (median: 107% vs. 100%, NS, **Supplementary Figure 4**).

Pre-incubation of platelets with an anti-SRB1 antibody significantly increased their activation measured by TxB2 levels when incubated with HDL from control, HD, PD and HNE-modified HDL (median: 385%, 178%, 288% and 758% respectively, $p < 0.05$, as compared to when incubated without the antibody **Figure 6A**). P-selectin expression was also increased by the pre-incubation of platelets with an anti-SRB1 antibody for HDL from control, HD, PD and HNE-modified HDL (median: +35%, +93%, +54% and +88% respectively, $p < 0.05$, as compared to when incubated without the antibody, **Figure 6B**).

Pre-incubation of platelets with Naphtyl PP1, a potent pharmacological inhibitor of SRC-kinases, decreased their activation measured by TxB2 levels when incubated with HDL from control, HD, PD and HNE-modified HDL (median: 22%, 46%, 24% and 22% respectively, $p < 0.05$, as compared to when incubated without the inhibitor **Figure 6A**). P-selectin expression was also decreased by the pre-incubation of platelets with Naphtyl PP1 for HDL from control, HD, PD and HNE-modified HDL (median: -48%, -48%, -53% and -14% respectively, $p < 0.05$, as compared to when incubated without the inhibitor, **Figure 6B**).

We confirmed that intra-platelet phosphorylation of JNK was reduced by CD36 and SRC Kinases blocking while SRB1 blocking increased its phosphorylation (data not shown).

Discussion

The present study confirms that HDL isolated from patients with CKD exhibits impaired biological functions that may participate in the onset of cardiovascular disease in this population. The work emphasizes the effect of carbonylation from long-chain n-6 fatty acids (4-HNE adducts) on the antiaggregative properties of HDL. This adduction onto the protein component of the HDL particle was responsible for a deep and significant alteration of the antiaggregant properties of HDL in CKD. Combined with all the others impaired functions of HDL, this can be a potent contributor to the increased cardiovascular morbidity and mortality in CKD³ and the failure of statins in HD patients.^{27,28} Moreover, a significant part of this pathological phenotype was mediated by a CD-36 dependent pathway as its blockade restored normal anti-aggregant properties. Furthermore, CKD *per se* was responsible for a large part of this carbonylation as these adducts were obtained in a rabbit CKD model without other cardiovascular risk factors.

The rabbit model is of interest for cardiovascular research (e.g. atherosclerosis, lipid metabolism) since these animals have a lipoprotein metabolism much closer to humans than mice or rats due to the expression of the cholesterylester transfer protein (CETP) gene. The CKD rabbits, despite having a lower body weight, developed a metabolic disturbance as their plasma cholesterol and glucose levels were significantly higher. The median measured GFR reduction in CKD animals corresponded approximately to stage 3 CKD in humans, and results of oxidative stress assays (MDA and AOA) were consistent with values observed in a human CKD population,⁵ which supports the appropriateness of the model. However, a natural history of 3-weeks is unlikely to lead to the same modifications as several years of interaction between

cardiovascular risk factors and uremia in humans. Nevertheless, we found an increase in 4-HNE adducts and the impaired antiaggregant properties of HDL in HD and PD patients, rendering the animal findings relevant.

The results of the present study suggest that CKD *per se* may trigger a strong oxidation of HDL particles. Interestingly, 4-HNE adducts were both increased in the HDL of CKD rabbits and HD and PD patients. This can be due to a loss of antioxidant capacity of HDL particles in CKD as HDL from CKD rabbits were more sensitive to copper-induced oxidation. This is in accordance with data from Holzer *et al.* who reported in PD and HD patients a profound modification of HDL structure in particular a lower concentration of paraoxonase.²⁴ Moreover, the short natural history of CKD in the model used herein suggests that these modifications occur during the early stages of the disease. These results are consistent with that reported by Shroff *et al.*²⁵ and Kaseda *et al.*⁵⁰ in pediatric CKD patients, who can be considered as close to the model used because of a short natural history of CKD and a lower number of CKD-associated comorbidities.

The results regarding platelet activation and aggregation are consistent with the altered properties of HDL in CKD; we observed a significant increase in the activation of platelets incubated with HDL from HD and PD patients and a 2-fold reduction in anti-aggregation with HDL from HD patients and CKD rabbits suggesting a blunted anti-thrombotic profile of uremic HDL. To exclude that the blunted anti-aggregant properties in HD patients did not only result from HD procedure *per se*, we confirmed this profile in PD patients. This strengthened the role of CKD and especially ESRD in the pathological properties of HDL. This is consistent with the increase in the incidence of thrombotic events in CKD, especially in HD patients^{27,28,51,52} and with a higher platelet reactivity in CKD.⁵³⁻⁵⁶ The pathophysiological pathways of the platelet

aggregation profile in CKD has only been studied for LDL; Holy *et al.* found a pro-aggregant profile of LDL in CKD that was mostly explained by carbamoylation of these proteins.³¹ Moreover, this study highlighted the role of the LOX-1 receptor in the onset of this profile; a receptor mainly involved in LDL-mediated pathophysiological pathways. The pro-aggregant profile of oxidized LDL has been described by Chen *et al.* to be mediated by CD-36³² and this receptor is also known to bind heavily modified-HDL.^{57,58} The results of the present study suggest a strong implication in the blunted anti-aggregant profile of CD-36-mediated pathway as its blocking restored the antiaggregant properties of CKD and HNE-modified HDLs. Furthermore, this study suggests the involvement of SRC-kinases mediated pathways in the platelet activation process in response with CKD and HNE-modified HDLs as the use of a specific pharmacological inhibitor (Naphthyl PP1) also restored the antiaggregant properties of these HDLs. Finally, we confirmed that the binding of HDL onto the CD36 receptor resulted in a significant increase in JNK phosphorylation as demonstrated by Chen *et al.* for oxidized LDL.³² Schematic summary of these pathways is summed up in **Figure 7**.

The study also suggests that there is a threshold effect of 4-HNE adduction on aggregative properties of HDL as the incubation of control HDL with 4-HNE in concentrations lower than 100 μ M did not modify the anti-aggregant profile of HDL. This could be explained by the multiple receptors that are involved in the aggregant properties of HDL. Indeed, native and mild oxidized HDL binds ApoER2 and SR-B1 scavenger receptor, inducing an intracellular down regulation of platelet activation pathways. Conversely, heavily oxidized HDL binds CD-36 and platelet activation is upregulated.^{57,59} Thus, we can hypothesize that the adduction induced by the incubation with 4-HNE at a concentration lower than 100 μ M caused light or mild modifications of the protein component of HDL insufficient to bind CD-36. 4-HNE adducts to the protein component of HDL particles were associated with pro-aggregative properties of HDL. Interestingly, mass spectrometry assay specifically identified such 4-HNE

adducts on lysines and histidines from 8 constitutive proteins of HDL and especially onto Apolipoprotein A1 of HD patients. This apolipoprotein is widely responsible for the antiaggregant properties of HDL³³ and several modifications were located onto key parts of the protein for cholesterol efflux, platelet aggregation and lipid binding.

To conclude, we describe herein for the first time the impaired anti-aggregant properties of HDL in CKD. To improve cardiovascular outcomes in CKD, future studies need to focus on HDL quality as well as quantity.

Author Contributions

N.F., P.M., L.J. and C.O.S. conceived and designed the study. N.F., C.C., S.L., M.M.B, C.B., J.R., N.G. and C.O.S. performed the experiments. F.D. and A.P. performed the mass spectrometry analysis. N.F. and C.O.S. analyzed the data. N.F. and C.O.S. wrote the manuscript. F.G.E edited the manuscript. N.F., C.C., S.L., M.M.B, J.M.P., M.L., P.M., F.G.E., L.J. and C.O.S. revised the manuscript. N.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1.**General characteristics of sham-operated (control) and 5/6 nephrectomized rabbits.**

	Control	CKD	P-value
N	9	8	
Biometry			
Body weight, kg	3.5 (3.2 – 3.9)	3.1 (3.0 – 3.3)	0.040
Body weight gain, kg	1.0 (0.9 – 1.2)	0.7 (0.6 – 0.8)	0.001
Kidney weight, g	19 (16 – 22)	14 (12 – 16)	0.008
Kidney weight, g/kg body weight	5 (4 – 7)	5 (4 – 5)	0.212
Biological features			
Urea, mmol/L	6.8 (5.6 – 8.4)	12.8 (12.6 – 19.2)	<0.001
Creatinine, μ mol/L	57 (52 – 75)	193 (154 – 270)	<0.001
Bicarbonate, mmol/L	20.1 (16.8 – 24.0)	21.0 (14.6 – 22.7)	0.700
Glucose, mmol/L	5.6 (4.8 – 6.9)	7.4 (7.1 – 8.1)	0.042
Total cholesterol, mg/dL	47 (37 – 68)	109 (73 – 198)	<0.001
HDL cholesterol, mg/dL	29 (18 – 37)	56 (35 – 85)	0.043
HDL-C/TC ratio	0.66 (0.33 – 0.85)	0.49 (0.35 – 0.53)	0.269
Triacylglycerols, mg/dL	270 (228 – 677)	316 (165 – 719)	0.842
Protidemia, g/L	59 (48 – 69)	39 (32 – 49)	0.015
Proteinuria, g/mmol creat	617 (60 – 1287)	19 (9 – 56)	0.198
Iohexol clearance			
GFR (mL/min/kg)	4.3 (4.1 – 4.8)	2.2 (1.8 – 2.5)	0.008
Oxidative stress markers (plasma)			
MDA, μ mol/L	0.8 (0.5 – 1.2)	2.3 (1.2 – 3.0)	0.009
AOA, mmol/L	1.0 (0.8 – 1.1)	0.8 (0.7 – 0.9)	0.021

Data are expressed as median (IQR). CKD: chronic kidney disease; HDL-C: HDL cholesterol; TC: total cholesterol; GFR: glomerular filtration rate; MDA: malondialdehyde; AOA: anti-oxidant activity (of the plasma). Mann-Whitney test, $p < 0.05$ was considered as significant. Creatinine: $\times 0.011$ for mg/dl; urea: $\times 2.8$ for mg/dl

Table 2.**General characteristics of hemodialysis, peritoneal dialysis and control patients.**

	Control	Hemodialysis (HD)	Peritoneal dialysis (PD)
N	15	25	20
General characteristics			
Age, years	46 (36 – 58)	56 (43 – 73)	67 (56 – 84)*†
Gender, % male	56	43	56
BMI, kg/m ²	26 (24 – 28)	24 (20 – 26)	25 (23 – 28)
<i>Comorbidities</i>			
HT, n(%)	3(21)	17(74)	19(95)
Stroke, n(%)	0(0)	2(9)	1(5)
CHD, n(%)	0(0)	5(22)	3(15)
Cardiopathy, n(%)	0	9(39)	8(40)
PVD, n(%)	0	4(17)	3(15)
<i>Therapies</i>			
Statins, n(%)	0	6	5
PI, n(%)	0	5	6
RASi, n(%)	1	3	11
β-blockers, n(%)	1	5	4
CCB, n(%)	1	1	6
Biological parameters			
Urea, mmol/L	5.4 (4.4 – 7.5)	17.1 (13.3 – 19.8)*	18.8 (16.1 – 22.6)*
Creatinine, μmol/L	73 (69 – 85)	702 (506 – 862)*	575 (422 – 758)*
mGFR, mL/min/1.73m ²	94 (86 – 96)	-	-
Total cholesterol, mg/dL	208 (189 – 237)	149 (113 – 180)*	209 (155 – 232)†
LDL cholesterol, mg/dL	139 (105 – 158)	71 (54 – 103)*	125 (81 – 159)†
HDL cholesterol, mg/dL	59 (53 – 66)	41 (30 – 47)*	45 (36 – 62)*
Triacylglycerols, mg/dL	91 (64 – 116)	119 (88 – 173)*	149 (118 – 207)*
CRP, mg/L	1.9 (0.5 – 3.5)	3.3 (1.9 – 8.0)	3.7 (1.7 – 5.8)

*Data are expressed as median (IQR). BMI: body mass index; HT: hypertension; CHD: coronary heart disease; PVD: peripheral vascular disease; PI: platelet inhibitor; RASi: renin-angiotensin system inhibitor; CCB: calcium-channel blocker; eGFR: estimated glomerular filtration rate by CKD-EPI formula; mGFR: measured GFR by iohexol clearance; CRP: C-reactive protein. *: p<0.05 vs controls, †: p<0.05 vs HD, Mann-Whitney U-test.*

Table 3.

List of 4-hydroxy-2-nonenal adducted amino-acid in HDL constitutive proteins from hemodialysis patients.

Protein name	Protein label	%	Site of adduction	Abundance ratio (HD 1 to 9, respectively)
Apolipoprotein B100	APOB	0.04	[K3689]	0.293 0.825 0.468 4.099 8.148 1.488 1.626 3.606 0.322
			[K4498]	- - - - 4.421 - - 2.339 -
Serum albumin	ALB	1.15	[H152]	2.646 5.05 0.373 - 0.036 0.072 1.263 0.874 6.837
			[K160 or H152]	2.956 6.049 0.229 - 0.295 - 0.718 0.405 12.994
			[K347]	- 1.452 - 0.879 - 0.582 1.767 2.442 1.966
			[H170]	1.463 - - 0.919 - 0.178 0.957 - -
			[K581]	0.861 1.148 - - - 0.668 1.944 2.204
			[K198]	1.715 - - - 2.864 - - 1.28
			[H20]	0.516 0.863 1.131 2.298 1.414 1.284 1.133 1.109 0.556
			[H179]	2.448 2.756 5.133 1.117 0.995 1.306 0.316 0.802 0.853
			[H186]	2.646 2.508 4.401 0.613 0.806 0.93 0.168 1.221 1.767
			[H223]	0.456 0.549 1.471 0.101 0.125 0.188 - 0.136 -
Apolipoprotein A1	APOA1	4.50	[H217]	1.342 0.939 3.216 0.039 0.175 0.266 - 0.14 -
			[K36]	0.89 1.362 1.181 1.64 0.996 1.36 1.873 1.863 1.128
			[K250]	1.423 3.189 0.548 4.329 31.087 1.035 3.154 30.549 2.522
			[K130]	0.332 1.236 0.856 - - 1.018 - 0.728
			[K47]	0.596 1.345 1.237 2.083 0.861 1.211 2.596 2.217 1.557
			[K83]	0.393 - 0.943 1.004 0.559 0.79 2.181 2.642 2.131
			[K64]	0.571 1.139 1.079 1.643 1.119 1.543 1.403 1.616 0.935
			[K262]	- 10.052 - 0.757 7.534 - 0.442 42.042 -
			[K215]	- 100 - - - - 100 - -
			[K26]	- 1.983 - 0.134 - - 0.963 0.29 0.689
Keratin, type I, cytoskeletal 10	KRT10	0.17	[K362]	2.598 3.076 3.419 1.44 0.596 1.462 0.778 2.66 1.595
			[H153]	1.292 0.7 1.056 1.332 0.827 5.646 0.972 0.914 0.906
UDP-glucose:glycoprotein glucosyltransferase 2	UGGT2	0.06	[H1388]	1.46 1.385 3.62 3.525 4.433 2.838 5.1 11.999 1.877

H, histidine; K, lysine. %: percentage of potential sites of adduction regarding all the amino-acids of the protein sequence. Abundance ratio was calculated against the mean of the 9 controls. -: not found

Figure legends

Figure 1. HNE-adducts were increased in HDL from CKD rabbits (A) as well as MDA concentration (B). No difference was observed concerning 8-isoprostane (C) and tocopherols (D) concentrations. HDL from CKD rabbits were more prone to oxidation after a copper-induced oxidation (E) as described in concise methods section. n=8 and 9, CKD and control respectively, p<0.05. Mann-Whitney test, p< 0.05 was considered as significant. Data are expressed as median and interquartile range.

Figure 2. CKD is responsible for impaired anti-aggregant properties of HDL in rabbits. CKD HDL exhibited blunted anti-aggregative properties compared to control HDL (A). Control HDL modified by an incubation overnight with HNE (HNE HDL) solution exhibited similar blunted properties to CKD HDL compared to Control HDL (A). Pre-incubation with anti-CD36 antibody restored to CKD and HNE HDL anti-aggregant properties like Control HDL (A). Typical aggregation curves obtained with collagen, control HDL and CKD HDL. Collagen aggregation level is considered as 100% (B). Same results were observed with a lipoprotein mix containing triglyceride-rich lipoproteins (VLDL and chylomicrons), LDL and HDL (LPP) from Control and CKD rabbits. CKD LPP exhibited blunted anti-aggregative properties compared to Control LPP. Control LPP modified by an incubation overnight with HNE solution (HNE LPP) exhibited similar properties to CKD LPP (C). Pre-incubation with anti-CD36 antibody restored to CKD and HNE LPP anti-aggregant properties like Control LPP (C). HNE HDL had increased level of HNE-adducts compared to control HDL (D). * n=8 and 9, CKD and control respectively, p<0.05. Mann-Whitney test, p<0.05 was considered as significant. Data are expressed as median and interquartile range.

Figure 3. 4-HNE adducts are increased in HDL from hemodialysis (HD) patients compared to controls. Immunoblotting (dot blot) of 4-hydroxy-nonenal (4-HNE) Michael adducts were performed as described in concise methods section (A). Identification of increased 4-HNE Michael adduct on lysine and histidine residues of HDL from HD patients by liquid chromatography—mass spectrometry/mass spectrometry assay (LC-MS/MS). HDL were reduced, alkylated and digested with trypsin. Sequence of Apolipoprotein A1 (accession number: P02647-1) indicating the position of the adducts (bold characters) and the sequences of interest (highlighted and framed, B). Typical MS/MS spectra from unmodified peptide (upper spectrum) and modified peptide (lower spectrum). The abundance of 4-HNE adduct of Lys 250 in HD HDL was 5.3-fold higher than in controls ($p < 0.01$, C). $n = 9$ and 9 , HD and control HDL respectively, Mann-Whitney test, * $p < 0.05$, data are expressed as median and interquartile range.

Figure 4. Structure of truncated human apolipoprotein A-I (pdb code 1av1). Four molecules are associated with their hydrophobic faces to form an antiparallel four-helix bundle. Lysine and histidine residues possibly modified by 4-HNE are colored in cyan with CPK representation (A). Model of HDL: Phospholipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, POPC) are colored in yellow and cholesterol in magenta. Lysine and histidine residues possibly modified by 4-HNE are colored in cyan with CPK representation (pdb code 3k2s, B).

Figure 5. HDL from peritoneal (PD) and hemodialysis (HD) patients exhibited impaired anti-aggregant properties. Regarding levels of Thromboxane B2 (TxB2), HD and PD HDL triggered over-activation of platelets compared to control HDL (n=15 for control, n=25 for HD group and n=20 for PD group, A). Same observations were found for healthy volunteers HDL modified by an incubation overnight with HNE (HNE HDL, n=5 for each group, B). HD, PD and HNE HDL exhibited blunted anti-aggregant properties compared to control HDL (n=9 for control and HD groups, n=5 for HNE group). We found a threshold effect of HNE modification on HDL as only a 100 μ M solution of HNE permitted to significantly alter the anti-aggregant properties of control HDL (n=4 for each group, D). *p<0.05, Mann-Whitney test and Kruskal-Wallis test (D), data are expressed as median and interquartile range.

Figure 6. The impaired anti-aggregant properties of HDL in peritoneal (PD), hemodialysis (HD) and from healthy volunteers HDL modified by an incubation overnight with HNE (HNE HDL) is mediated by CD36 binding and SRC kinases-mediated pathways. Regarding levels of Thromboxane B2 (TxB2), the pre-incubation of platelets with an antibody against CD36 receptor (Ab anti-CD36) significantly lowered the activation of platelets exposed with control (CTL), HD, PD and HNE compared without the antibody (A). The pre-incubation of platelets with an antibody against SRB1 receptor (Ab anti-SRB1) significantly increased the activation of platelets exposed with CTL, PD and HNE compared without the antibody (A). The pre-incubation of platelets with a pharmacological inhibitor of SRC kinases (Naphtyl PP1) significantly lowered the activation of platelets exposed with CTL, HD, PD and HNE compared without the inhibitor (A). In a fluorescent activated cell sorting assay, activation of platelets were determined with the P-selectin expression. The pre-incubation of platelets with Ab anti-CD36 and Naphtyl PP1 significantly decreased the activation of platelets exposed with CTL,

HD, PD and HNE HDL compared to the samples without the antibody/inhibitor (B). The pre-incubation of platelets with Ab anti-SRB1 significantly increased the activation of platelets exposed with CTL, HD, PD and HNE HDL compared to the samples without the antibody/inhibitor (B). n=5 for each group. Mann-Whitney test, *p<0.05 compared to Control HDL without blocker; \$p<0.05 compared to HD HDL without blocker; †p<0.05 compared to PD HDL without blocker; ‡p<0.05 compared to HNE HDL without blocker. Without blocker: without either Ab anti-CD36, anti-SRB1 or Naphtyl PP1.

Figure 7. HDL from chronic kidney disease patients and rabbit exhibited blunted anti-aggregant properties by the binding onto CD36 receptor. Carbonylation by 4-hydroxy-2-nonenal (4-HNE) of proteins from HDL was found to be part of this pathological behavior. Activation of the platelet in response of this binding was through a SRC-kinases mediated pathway. SRC kinase activation resulted with an increase of Jun-Kinase phosphorylation (pJNK). Native HDL apolipoprotein A1 binds SRB1 and inhibits platelet activation.

Figure 1

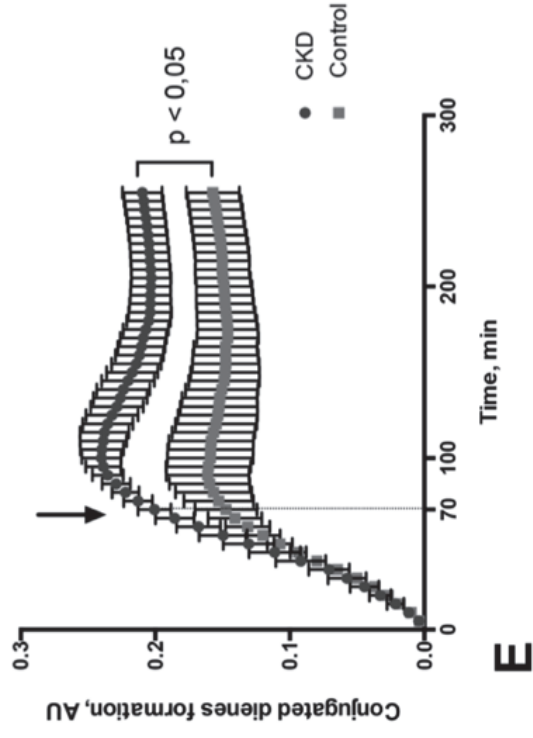
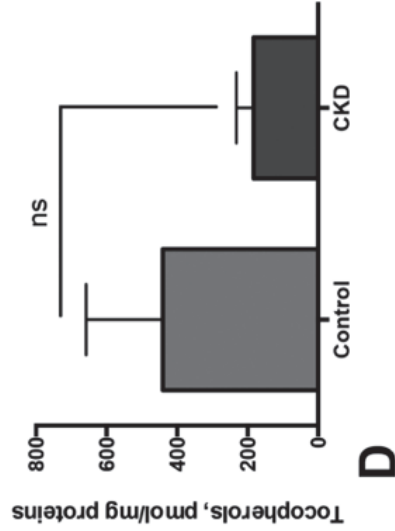
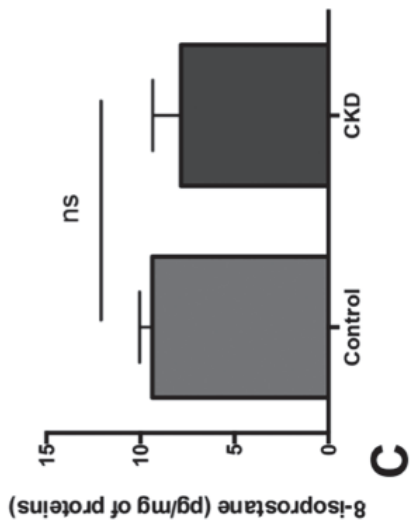
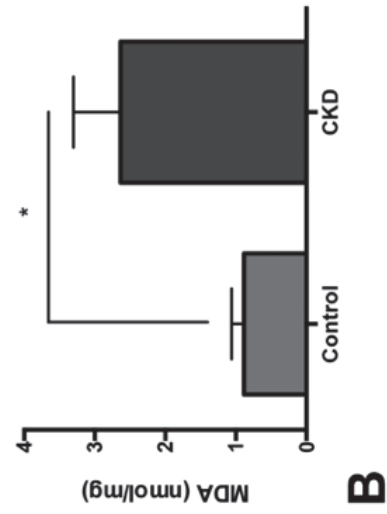
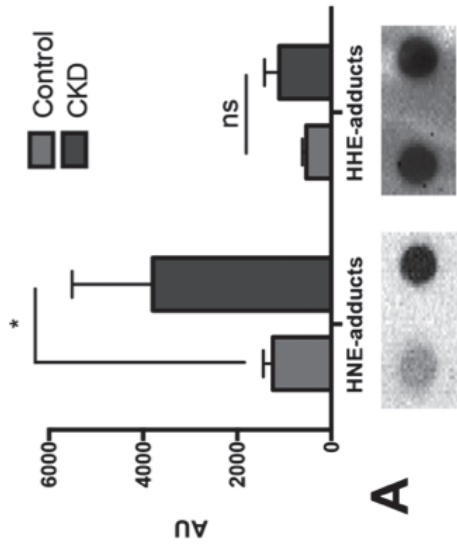


Figure 2

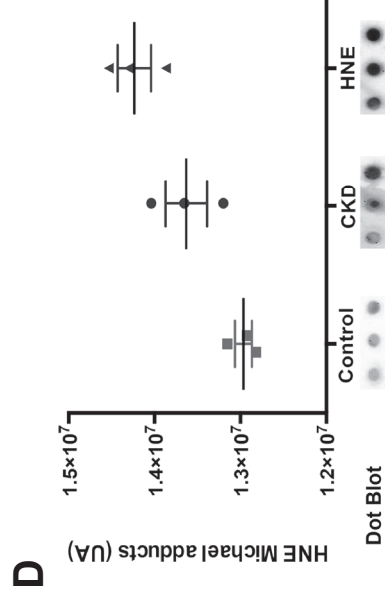
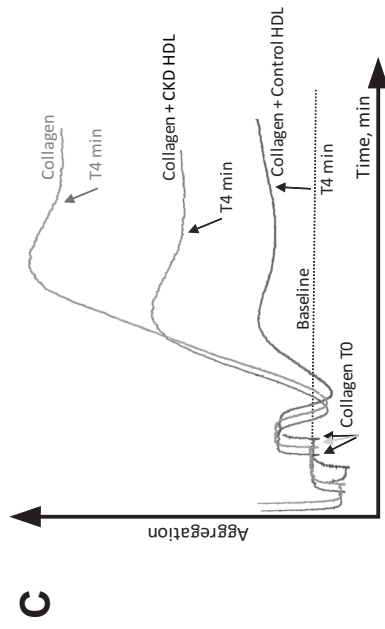
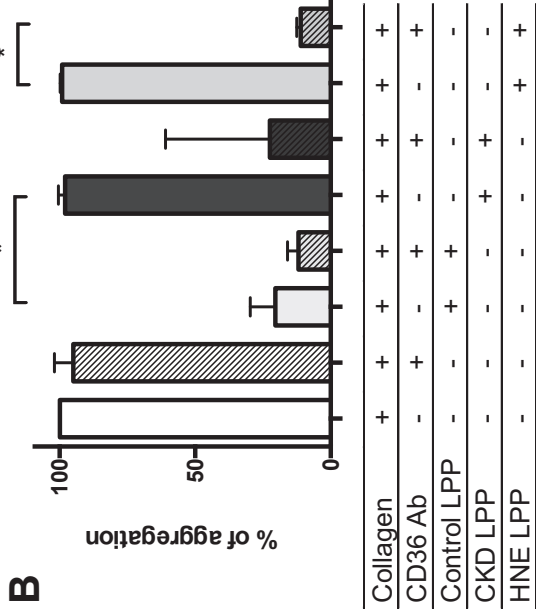
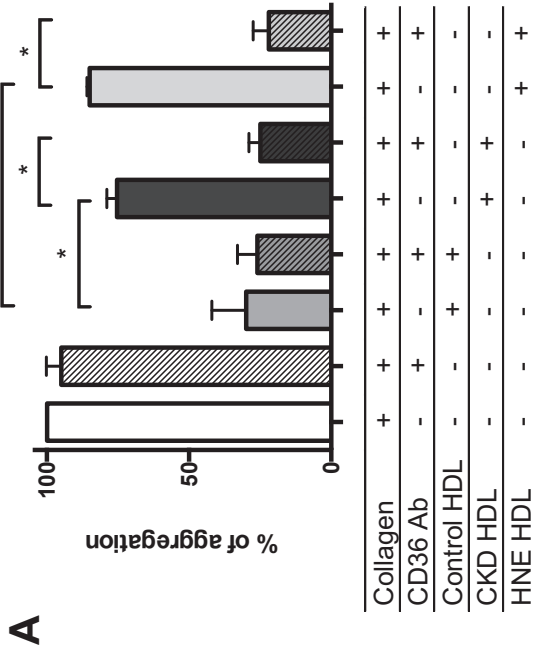
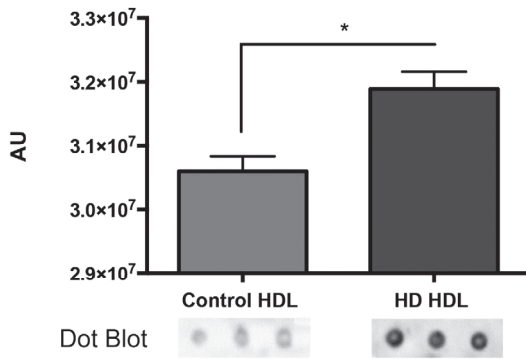
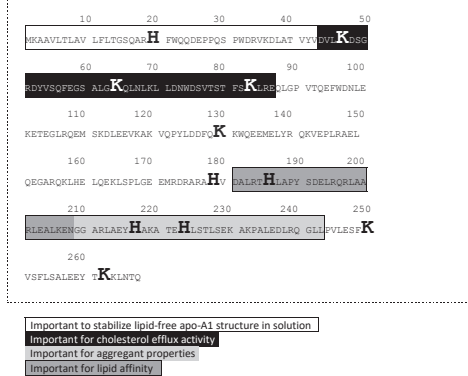


Figure 3

A



B



C

Sequence	Modifications	Theo. MH ⁺ (Da)	Abundances (Control)	Abundances (HD)	Abundance Ratio	P-Value
[R]QGLLPVLESEKVSFLSALEYTK(K)	1xHNE (K11)	2754.52156	28.2	171.8	5.3	0.009

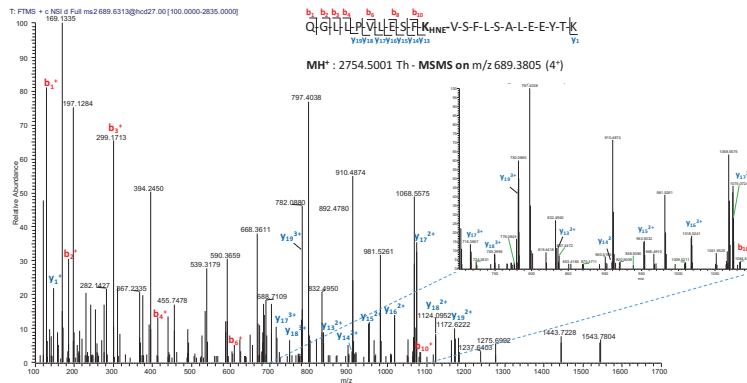
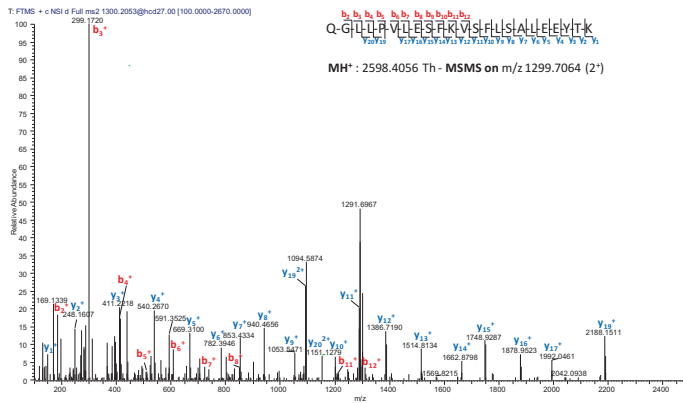


Figure 4

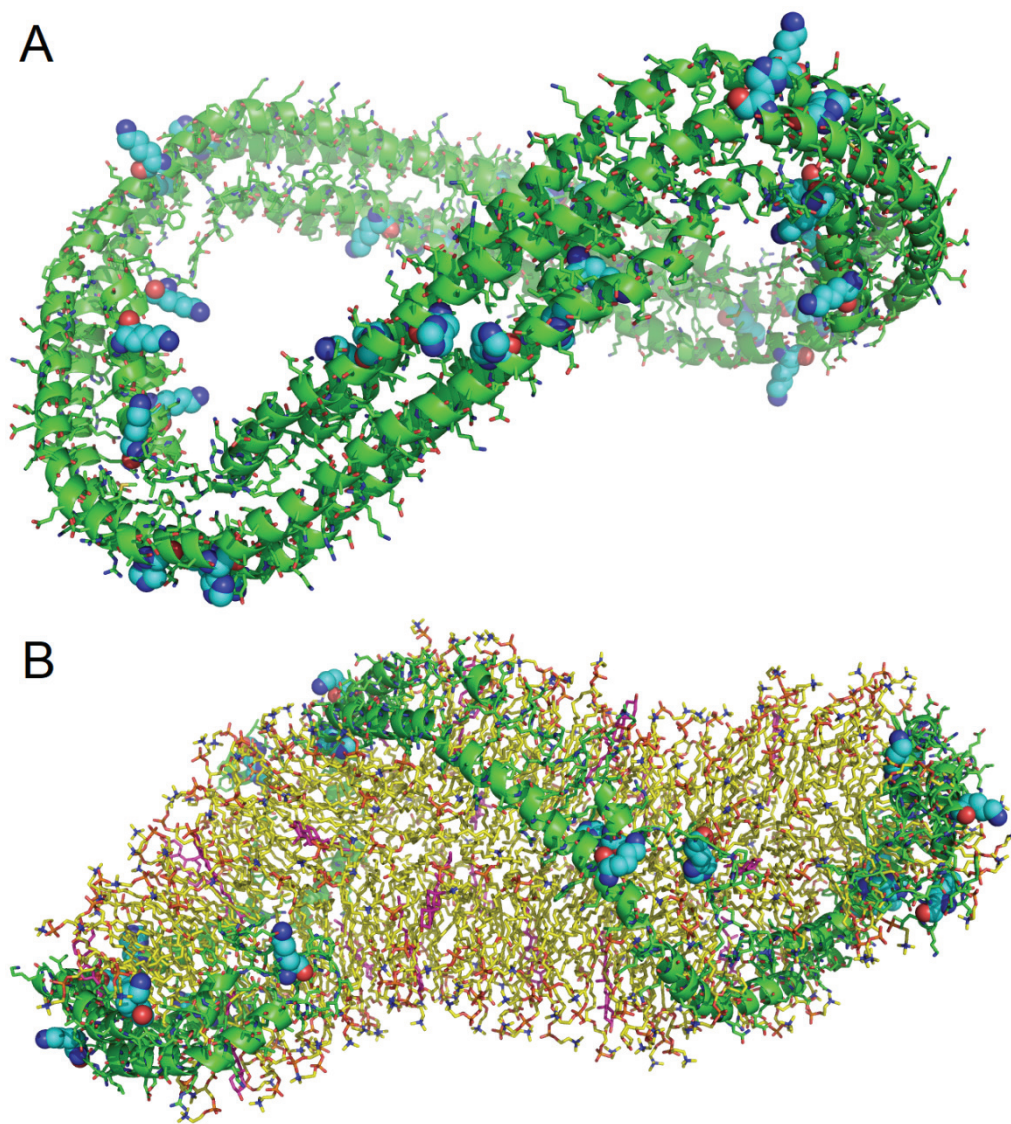


Figure 5

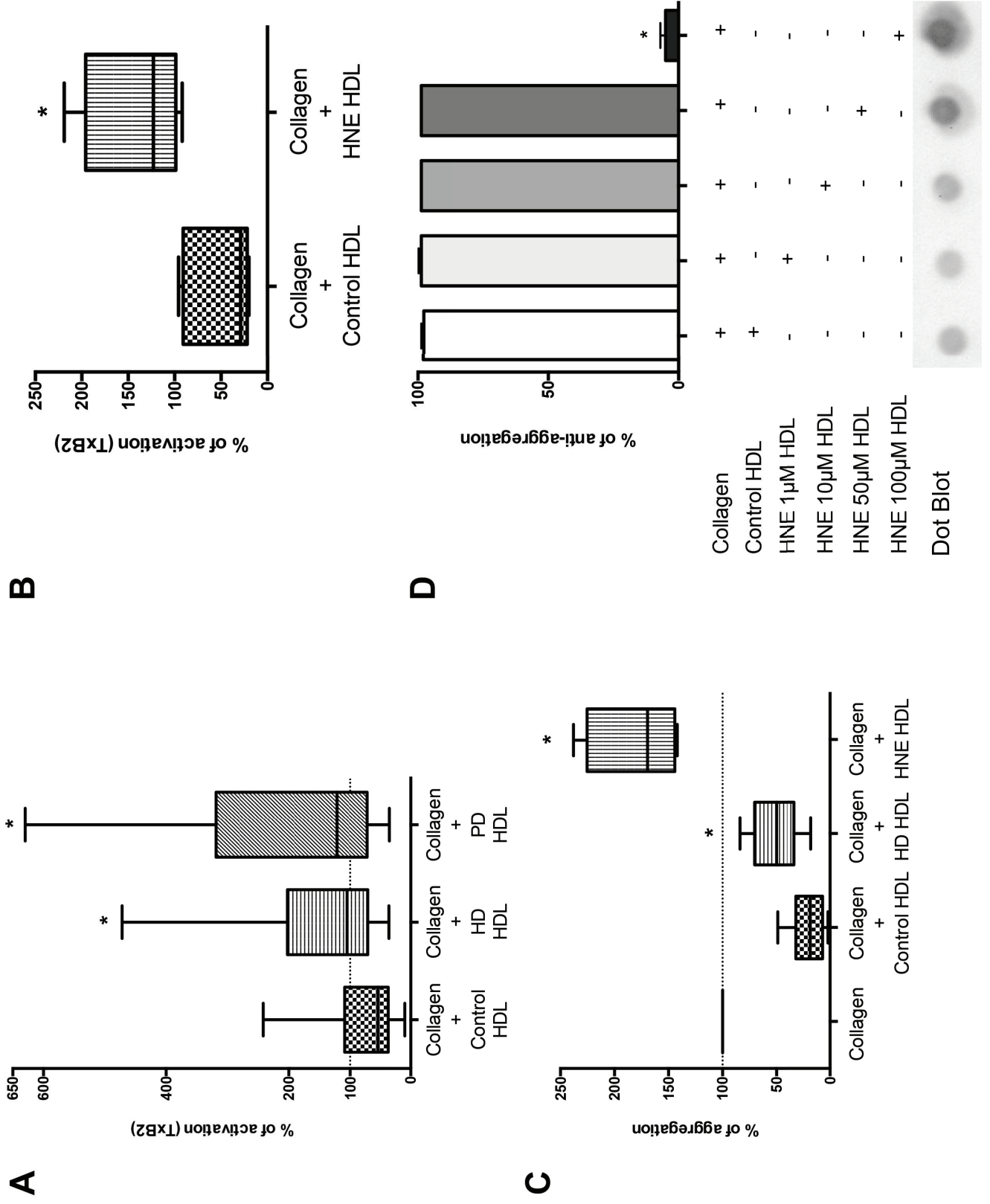


Figure 6

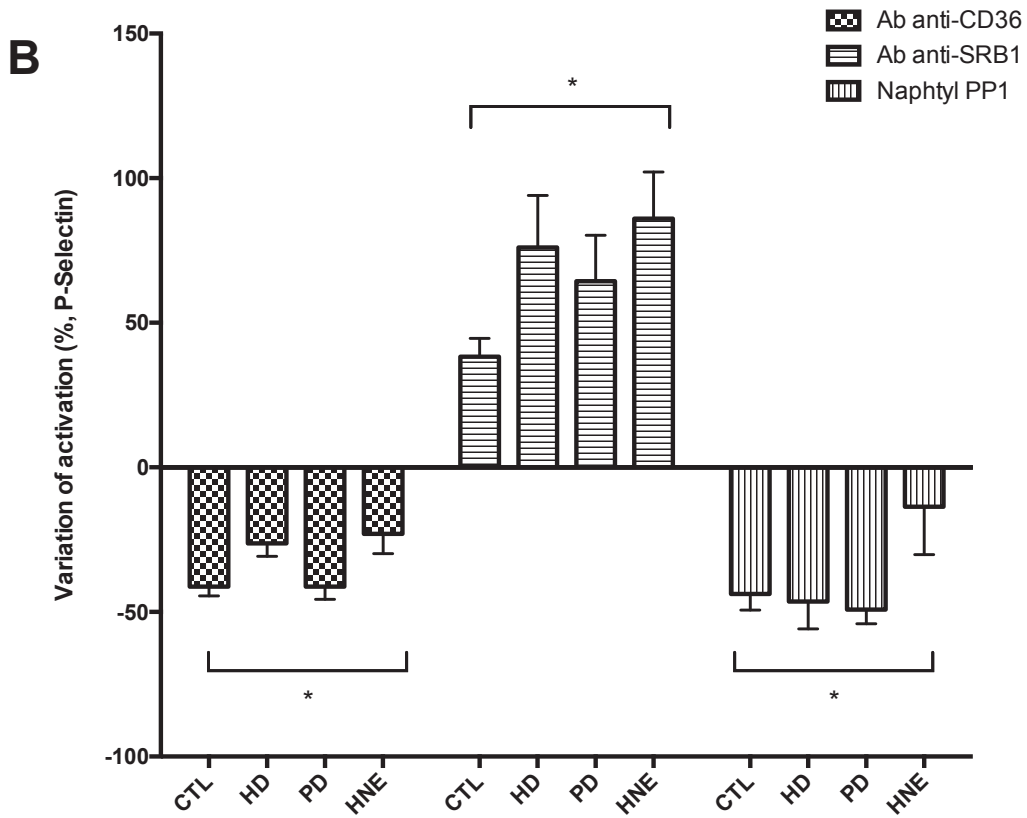
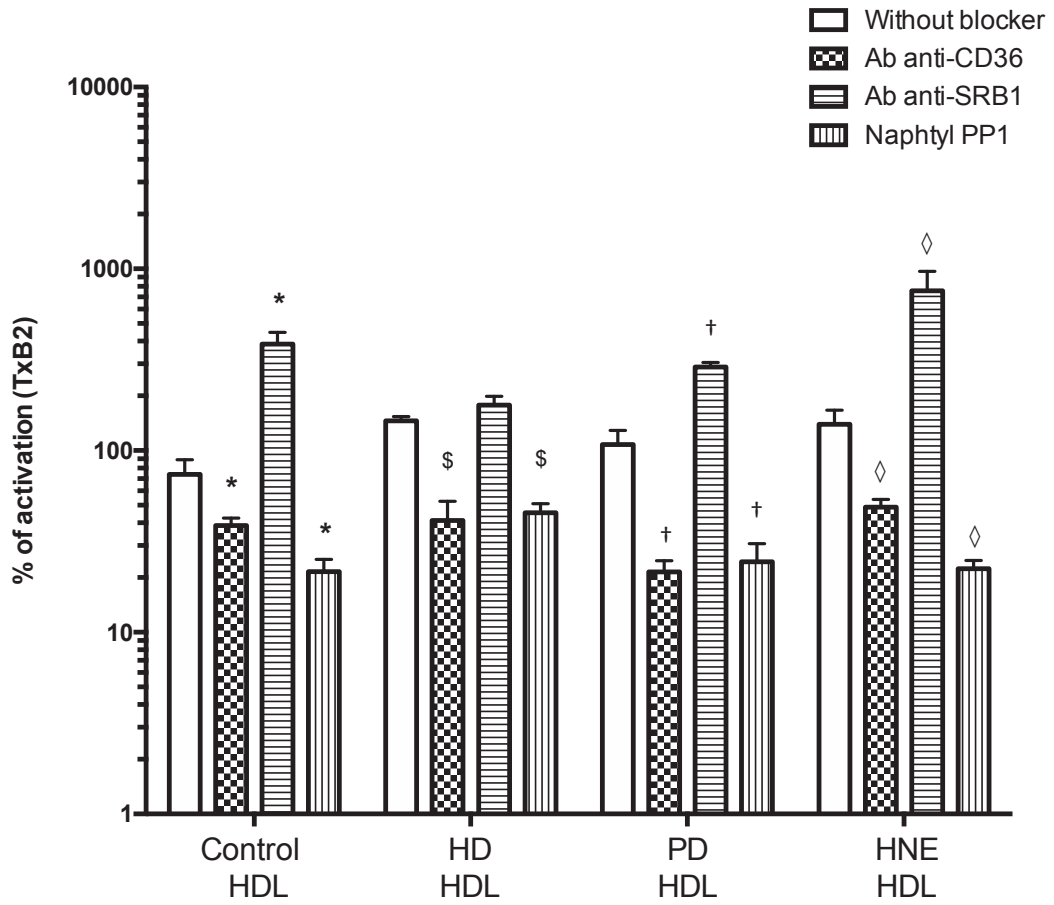
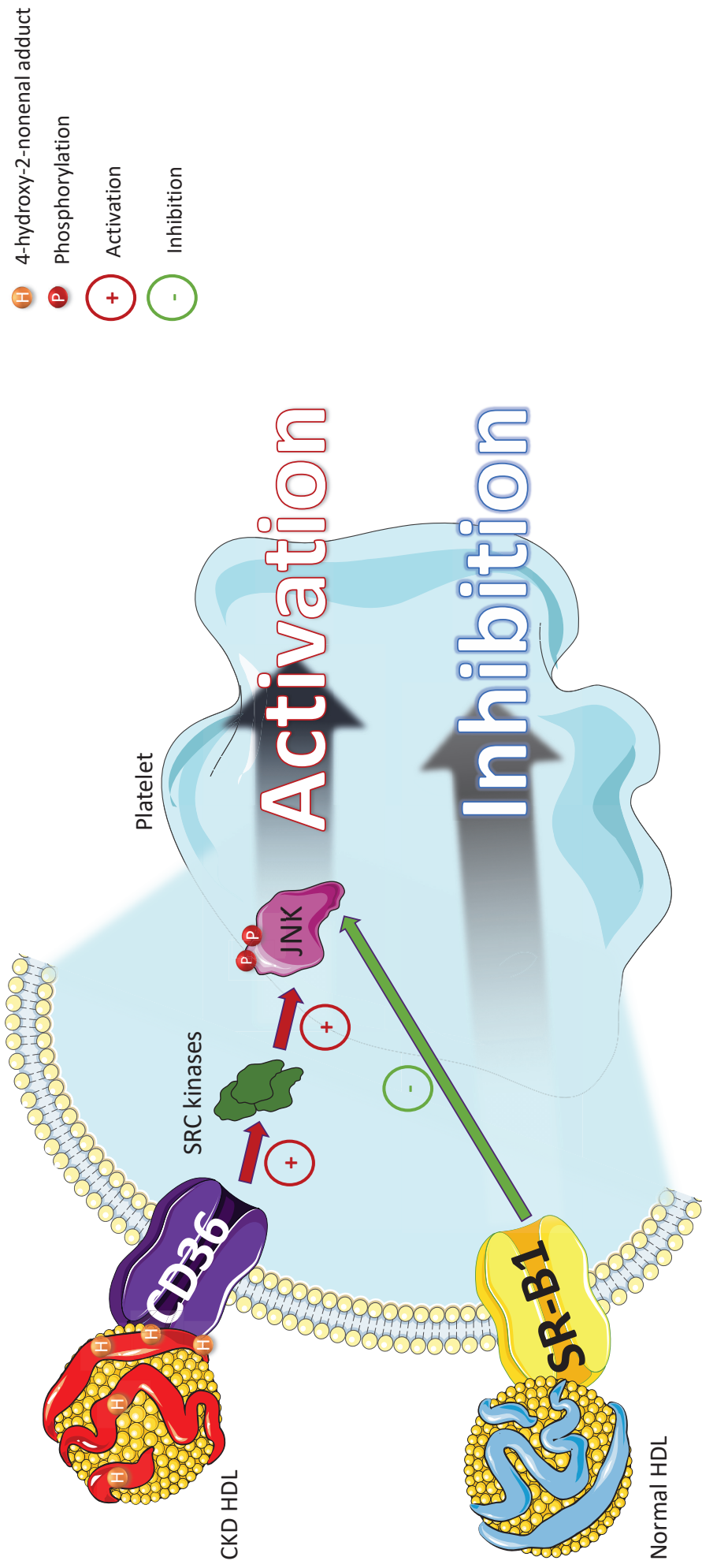


Figure 7



Supplementary materials

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Complete methods

Patients and ethics statement

Patients were sampled at the University Hospital of Lyon. Control patients were healthy volunteers for a living kidney donation, hospitalized for their pre-donation biological and clinical work-up. Hemodialysis patients were sampled in the hemodialysis unit of the Edouard Herriot hospital before the mid-week session. Inclusion criteria were age >18, undergoing hemodialysis for more than 6 months. Exclusion criteria were diabetes mellitus, ongoing inflammatory disease, liver cirrhosis, recent cardiovascular event (<3 months, cardiac myoinfarction, stroke, acute peripheral artery occlusion), uncontrolled anemia, coagulopathy and BMI higher than 35 kg.m². The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethical committee (reference L16-57, Comité de Protection des Personnes Lyon Sud Est IV). A written informed consent was obtained from all subjects.

Blood collection

Blood samples were obtained by venipuncture (on EDTA coated tubes) except for dialysis blood samples that were obtained immediately before dialysis from the arterial line of the mechanical bloodstream. Blood samples were centrifuged at 3500 x g for 10 min to isolate plasma which were collected and stored at -80°C until use.

Animal procedures

All animal experiments were performed under the authorization no. n°69-266-0501 and according to the guidelines laid down by the French Ministry of Agriculture (n° 2013-118) and the European Union Council Directive for the protection of animals used for scientific purposes of September 22, 2010 (2010/63UE). Adult male White New Zealand rabbits (CEGAVssc, Saint Mars d'Egrenne, France) were housed in individual cages at constant ambient temperature (21-23°C) and humidity (45-50%) with a 12 h light cycle. All animals had free access to tap

water. After a 7-day period of acclimation, rabbits were randomized to either the 5/6 nephrectomy group or in the control group.

Nephrectomy was performed as described by Goitloib et al.¹ Rabbits were anaesthetized using an intramuscular injection of ketamine (50mg/kg), xylazine (5mg/kg), and acepromazine (0.5mg/kg, Centravet, Lapalisse, France). The right dorsolumbar area was shaved and disinfected twice with povidone-iodine. A sterile field was applied to delimit the operative site and a sterile film was applied onto the shaved skin. The rabbit was placed on the right lateral decubitus. Local anesthesia was performed by a subcutaneous injection of xylocaine 2% (2 ml) at the incision site. Then, a skin incision of approximately 4 cm in length was performed at 2 finger-breadths from the caudal end of the rib cage and from the lumbar muscle. The left kidney was externalized and the perirenal adipose tissue was gently dissected. The two poles of the kidney were electrocoagulated using an electric needle to produce a 2/3 reduction of the left kidney mass. The muscle and wound were sutured (Prolene 4/0, Ethicon, Somerville, NJ, USA), and the wound was painted with povidone-iodine. One week after surgery, a unilateral right nephrectomy was performed. A nylon monofilament ligature was placed on the kidney pedicle. The kidney pedicle was carefully sectioned between the kidney and the ligature. Special care was taken to avoid damage to the adrenal glands. The control animals underwent the same procedures (general anesthesia, skin, and muscle incisions) followed by a simple kidney mobilization. All rabbits were given buprenorphine (0.05mg/kg sc, 2-times a day) for 2 days to prevent post-surgical pain. Food intake and body weight were measured on a daily and twice-weekly basis, respectively.

Measurement of glomerular filtration rate

The glomerular filtration rate (GFR) was measured through the kinetics of plasma iohexol concentration decrease. Rabbits were anesthetized with an intravenous injection of 27.5 mg/kg

of sodium pentobarbital (Centravet). Then, the neck was shaved and a median incision was performed after local anesthesia with a subcutaneous infiltration of xylocaine 2% (1mL). A PE-50 catheter was introduced in the left jugular vein for the injections and PE-60 catheter was disposed in the left carotid artery for the blood sampling. A 1mL bolus of iohexol (Ominpaque 300®, GE Healthcare, Chicago, IL, USA) was performed and the timer was started after flushing the residue left in the jugular catheter with a saline solution. The syringe was weighted to the nearest milligram before and after injection to precisely calculate the amount of iohexol delivered to the rabbit. Blood was sampled at 5, 15, 30, 45, 60, 120, and 180 min on lithium-heparin coated tubes. The plasma was aliquoted after centrifugation at $5,900 \times g$ for 2 min and stored at -20°C . The serum iohexol concentration was measured by HPLC as previously described² and GFR was calculated using a bi-compartmental model equation.

Sacrifice and necropsy

At the end of GFR measurement, rabbits were deeply anesthetized with an overdose of sodium pentobarbital (70 mg/kg iv). Blood was removed by cardiac puncture and placed in EDTA-coated tubes. After a centrifugation at $1,250 \times g$ for 10 min, plasma was aliquoted and stored at -80°C . Urine were obtained with a direct bladder puncture and stored at -20°C . The kidney was dissected out, weighed, and stored in buffered formalin 10% (w/v) for histological examination.

Renal histology

Kidneys were dehydrated using ethanol, embeded in paraffin and sliced. Haematoxylin Erythrosine Saffron (HES) and Sirius Red stainings were performed.

Isolation of lipoproteins from the plasma

Lipoproteins were separated from plasma by stepwise potassium bromide (KBr) density gradient ultracentrifugation.³ Plasma was fractionated in the Beckman ultracentrifuge with a rotor TAL 100.3 (Beckman, Brea, CA, USA). A first centrifugation at $100,000 \times g$ for 3h 30min at 15°C was performed to remove the top layer corresponding to VLDL and chylomicrons. Then, the plasma density was adjusted to 1.063 g.mL^{-1} with KBr ($M=119.01 \text{ g.mol}^{-1}$). After a second centrifugation at $100,000 \times g$ for 5h at 4°C the orange ring, corresponding to LDL, was collected. Finally, plasma density was adjusted to 1.21 g.mL^{-1} with KBr and after centrifugation at $100,000 \times g$ for 6 h 30 at 4°C the orange ring corresponding to HDL, was collected.

For the isolation of all the lipoproteins together, a single ultracentrifugation was performed at $100,000 \times g$ for 6h 30min at 4° after an adjustment of plasma density to 1.21 g.mL^{-1} with KBr. For platelet aggregation and copper-induced HDL oxidation, lipoproteins were extensively dialyzed against phosphate saline buffer (PBS) with 1mM EDTA for 3h, twice at room temperature and then overnight at 4°C. A last dialysis without EDTA was performed just before the platelet aggregation and copper-induced oxidation.

Biochemistry

Serum creatinine measurement was performed using the Siemens enzymatic method (on the Dimension Vista System, Siemens Healthcare, Erlangen, Germany) traceable to National Institute of Standards and Technology Creatinine Standard Reference Materials 914 (verified with National Institute of Standards and Technology SRM 967) with calibration certified by isotope dilution mass spectrometry. Urea was measured with urease test (Vista 1500). Cholesterol and triacylglycerols levels were measured using enzymatic kits (Biomerieux, Marcy l'Etoile, France). HDL concentration was measured with an enzymatic kit (Abcam, Paris, France). Malondialdehyde (MDA) was measured by HPLC coupled to UV-visible detection (Diode Array detector) as described by Grotto *et al.*⁴. Antioxidant activity (AOA) of

the plasma was measured as described by Koracevic *et al.*⁵. Proteinuria was measured using the Bradford protein assay (BioRad, Marne-la-Coquette, France) using bovine serum albumin as a standard.

Lipoproteins assays

MDA concentration in HDL was determined by HPLC according to the method described by Therasse and Lemonnier⁶. Lipoprotein samples, mixed with thiobarbituric acid (TBA, 10 mM), acetic acid and butyl-hydroxy-toluen (5mM) were heated for 60min at 95°C. The TBA-MDA adducts were then extracted with ethyl acetate and separated on a Nucleosil C18 column (5µm, 4.6 × 250 mm, Macherey-Nagel, Hoerdt, France) using methanol/water (20:80, v/v) as mobile phase. Detection was performed by measuring the absorbance at 532 nm.

Anti-HNE-Michael adduct (ref 393207) and anti- HHE-Michael adduct (ref NOF-N213730-EX) antibodies were purchased from Calbiochem (San Diego, CA, USA) and Cosmobio (Tokyo, Japan), respectively. The antibody used to detect HHE-adducts is reported to be highly specific for HHE Michael adducts on histidine residues and therefore enables the specific detection of HHE-histidine in protein samples⁷. Fifty micrograms of HDL were loaded directly onto a nitrocellulose membrane using the Bio-Dot apparatus (BioRad, Marne-la-Coquette, France). Following saturation with 5% bovine serum albumin (BSA), membranes were probed overnight with primary antibodies, anti-HHE-Michael adducts, or anti-HNE-Michael adducts (1:1000 dilution in a 5% BSA solution). After incubation with HRP-coupled secondary antibodies (1:3000; 5% BSA solution), membranes were processed for chemiluminescence (ECL plus, GE Healthcare, Chicago, IL, USA) and quantitated by densitometry using Image J software (NIH, Bethesda, MD, USA). The 8-isoprostane level was measured using an immunoassay (Bertin Pharma, Montigny-le-Bretonneux, France).

4-HNE was synthesized as described by Soulère *et al.*⁸ and was diluted in dimethylsulfoxide (DMSO). 4-HNE was added to HDL solutions at a final concentration of 1, 10, 50, or 100 μ M. After a 16h overnight incubation at 37°C in a water bath, HDL were dialyzed three times against PBS to remove the free fraction of 4-HNE.

Platelet aggregation and activation

Blood was collected at the regional blood center from healthy volunteers who had not ingested any aspirin or other non-steroidal anti-inflammatory drug in the previous 10 days. Platelets were prepared for the assays as described by Lagarde *et al.*⁹ Platelet function test was carried out according to the Born turbidimetric method.¹⁰ Platelet aggregation was measured in isolated platelets in a dual-channel aggregometer (Chrono-log; Coulter, Margency, France). Platelet suspensions were pre-incubated for 5 min at 37°C in the presence or absence of lipoproteins (0.025 mg of protein/mL for rabbit, 0.050 mg/mL for human) and stimulated with threshold concentrations of collagen (75 ± 9 ng/ml) while being continuously stirred at 1,000 rpm. The threshold concentration of collagen was defined as the concentration of collagen that induced a 50% increase in light transmission. The extent of platelet aggregation was expressed in terms of percentage of change in light transmission 4 min after the addition of collagen. Blocking of CD36 or SRB1 receptor was achieved by the pre-incubation with 10 μ L of an anti-CD36 (Ab-CD36) or anti-SRB1 (Ab-SRB1) antibodies (dilution 1:500, Abcam, Paris, France) for 10 min at 37°C before the incubation with HDL. Blocking of SRC-kinases was achieved by the pre-incubation with Naphtyl PP1 (final concentration 1 μ M, Santa Cruz Biothechnologies, Dallas, TE, USA) for 10 min at 37°C before the incubation with HDL. Aggregation values from lipoprotein assays were expressed as a percentage of the maximum aggregation induced by the collagen alone (considered as 100%).

For activation assays, platelet suspensions were pre-incubated for 15 min at 37°C in the presence or absence of lipoproteins (0.050 mg/mL for human) and stimulated with threshold concentrations of collagen (4ng/mL). Blocking of CD36 or SRB1 receptor was achieved by the pre-incubation with 10 μ L of an anti-CD36 (Ab-CD36) or anti-SRB1 (Ab-SRB1) antibodies (dilution 1:500, Abcam, Paris, France) for 10 min at 37°C before the incubation with HDL. Blocking of SRC-kinases was achieved by the pre-incubation with Naphtyl PP1 (final concentration 1 μ M, Santa Cruz Biothechnologies, Dallas, TE, USA) for 10 min at 37°C before the incubation with HDL. Levels of thromboxane B2 (TxB2) was measured using an immunoassay expressed as a percentage of the maximum level induced by the collagen alone (considered as 100%, Cayman Chemical, Ann Arbor, MI, USA). After pre-incubation with HDL and antibodies or pharmacological inhibitor, platelets were activated with collagen then fixed in paraformaldehyde 5% and stained with anti-CD62/P-Selectin (Thermo Fisher Scientific, San Jose, CA, USA) according to the manufacturer protocol and analyzed by flow cytometry.¹¹ Expression of intra-platelet phosphorylated JNK was measured by Western Blot with an anti-phospho-JNK antibody (Cell signaling technologies, Danvers, MA, USA). Quantification and expression were processed as previously described.¹²

Copper-induced HDL oxidation

Aliquots of freshly dialyzed HDL (50 μ g protein) were oxidized in the presence of 2.5 μ M of CuSO₄ within the day following the last dialysis. The oxidation was monitored by measuring the formation of conjugated dienes at 234 nm¹³ every 5 min for 3 h in a Kontron computer-linked spectrophotometer.

Mass spectrometry assay

List of reagents:

Sigma Aldrich (Saint Quentin Fallavier, France): Acetonitrile MS grade (ACN), Formic acid (FA), Trifluoroacetic acid (TFA), Iodoacetamide (IAA), Dithiotreitol (DTT), Sodium Deoxycholate (SDC), Ammonium Bicarbonate (AB)

MilliQ Water

Trypsin: Promega (Lyon, France)

Pierce C18 desalting spin column and Pierce quantitative fluorometric peptide assay: Thermo Scientific (San Jose, CA, USA)

In-solution digestion

30 μg of protein were diluted in 100 μL of 50 mM AB/SDC 1% buffer. They were reduced with 5 mM DTT for 1h at 57°C, and then alkylated with 10 mM IAA for 1h in the dark at room temperature and under agitation (850 rpm). Enzymatic digestion was performed with trypsin at a ratio 1/100 (enzyme/proteins) and overnight at 37°C. Endly, the samples were desalted on C18 spin column and dried on Speed-Vacuum before nanoLC-MS/MS analysis.

LC-MS/MS analysis

Prior to injection, the peptides concentrations in the samples were determined by the Pierce quantitative fluorometric peptide assay (Thermo scientific) according to the manufacturer's instructions. An aliquot containing 1 μg for each sample was dried and suspended in 9 μL of FA 0.1% + 1 μL of Cytochrome C digest (2 pmol/ μL) used as internal standard. 2 μL of this solution was then injected. Samples were analyzed in a Label Free quantitation strategy, in triplicate using an Ultimate 3000 nano-RSLC (Thermo Scientific) coupled on line with a Q Exactive HF mass spectrometer via a nano-electrospray ionization source (Thermo Scientific, San Jose California).

Nano-LC: Samples were injected and loaded on a C18 Acclaim PepMap100 trap-column 75 μm ID x 2 cm, 3 μm , 100Å, (Thermo Scientific) for 3.0 minutes at 5 $\mu\text{L}/\text{min}$ with 2% ACN,

0.05% TFA in H₂O and then separated on a C18 Acclaim Pepmap100 nano-column, 50 cm x 75 μm i.d., 2 μm, 100 Å (Thermo Scientific) with a 60 minutes linear gradient from 4% to 50% buffer B (A: 0.1% FA in H₂O, B: 100% ACN, 0.1% FA) and then from 50 to 95% of B in 2 min, hold for 10 min and returned to the initial conditions in 1 min for 14 min. The total duration was set to 90 minutes at a flow rate of 300 nL/min. The oven temperature was kept constant at 40°C.

MS: Samples were analysed with a TOP20 HCD method: MS data were acquired in a data dependent strategy selecting the fragmentation events based on the 20 most abundant precursor ions in the survey scan (300-1600 Th). The resolution of the survey scan was 60,000 at m/z 200 Th and for MS/MS scan the resolution was set to 15,000 at m/z 200 Th. The Ion Target Value for the survey scans in the Orbitrap and the MS/MS scan were set to 3E6 and 1E5 respectively and the maximum injection time was set to 60 ms for MS scan and for MS/MS scan. Parameters for acquiring HCD MS/MS spectra were as follows; collision energy = 27 and an isolation width of 2.0 m/z. The precursors with unknown charge state, charge state of 1 and 8 or greater than 8 were excluded. Peptides selected for MS/MS acquisition were then placed on an exclusion list for 20 s using the dynamic exclusion mode to limit duplicate spectra.

Data Analysis

Proteins were identified by database searching using SequestHT with Proteome Discoverer 2.2 software (Thermo Scientific) against the Swissprot Homo Sapiens database (2018-07 release, 24480 sequences). Precursor mass tolerance was set at 10 ppm and fragment mass tolerance was set at 0.02 Da, and up to 2 missed cleavages were allowed. Oxidation (M), acetylation (Protein N-terminus) and HNE (+ 156.115 Da on K or H) were set as variable modifications, and Carbamidomethylation (C) as fixed modification. Peptides and proteins were filtered with a false discovery rate (FDR) at 1% using percolator and proteins were identified with 1 unique peptide in rank 1. Protein quantitation was performed with Minora feature detector and

precursor ions quantifier node in Proteome Discoverer 2.2 software with protein quantitation based on pairwise ratios and ANOVA (individual proteins) hypothesis test.

Statistical analysis

Data were expressed as mean \pm standard error of the mean (SEM). All analyses were performed using GraphPad Prism version 6.0 (GraphPad software, La Jolla, CA, USA). Normality was assessed using D'Agostino & Pearson test. Dialysis vintage was classified as non-normally distributed ($P < 0.05$) and was therefore log-transformed before analysis (correlation analysis). Comparisons were performed using Student's t-test or Mann-Whitney U-test. In case of variance inhomogeneity, a Welch's correction was applied. Differences were considered as significant at the $P < 0.05$ level.

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Supplementary Table 1.**General characteristics of hemodialysis and control patients for aggregation assay**

	Control	Hemodialysis (HD)	P-value
N	9	9	
General characteristics			
Age, years	41.78 ± 4.50	57.11 ± 5.60	0.049
Gender, % male	66.67 ± 16.67	55.56 ± 17.57	0.653
BMI, kg/m ²	25.42 ± 1.06	22.89 ± 1.43	0.186
<i>Comorbidities</i>			
HT, n	2	8	
Stroke, n	0	0	
CHD, n	0	2	
Cardiopathy, n	0	4	
PVD, n	0	1	
<i>Therapies</i>			
Statins, n	0	6	
PI, n	0	5	
RASi, n	1	3	
β-blockers, n	1	5	
CCB, n	1	1	
Biological parameters			
Urea, mmol/L	6.20 ± 0.51	15.00 ± 1.62	<0.0001
Creatinine, μmol/L	78.78 ± 4.70	612.10 ± 52.95	<0.0001
mGFR, mL/min/1.73m ²	92.33 ± 4.08	-	
Total cholesterol, mg/dL	211 ± 9	153 ± 18	0.011
LDL cholesterol, mg/dL	132 ± 8	87 ± 16	0.025
HDL cholesterol, mg/dL	58 ± 2	44 ± 3	0.005
Triacylglycerols, mg/dL	105 ± 10	109 ± 9	0.749
CRP, mg/L	3.00 ± 1.13	18.83 ± 11.29	0.200

Data are expressed as mean ± SEM. BMI: body mass index; HT: hypertension; CHD: coronary heart disease; PVD: peripheral vascular disease; PI: platelet inhibitor; RASi: renin-angiotensin system inhibitor; CCB: calcium-channel blocker; eGFR: estimated glomerular filtration rate by CKD-EPI formula; mGFR: measured GFR by iohexol clearance; CRP: C-reactive protein. Creatinine: x 0.011 for mg/dl; urea: x 2.8 for mg/dl

Supplementary Figure 1

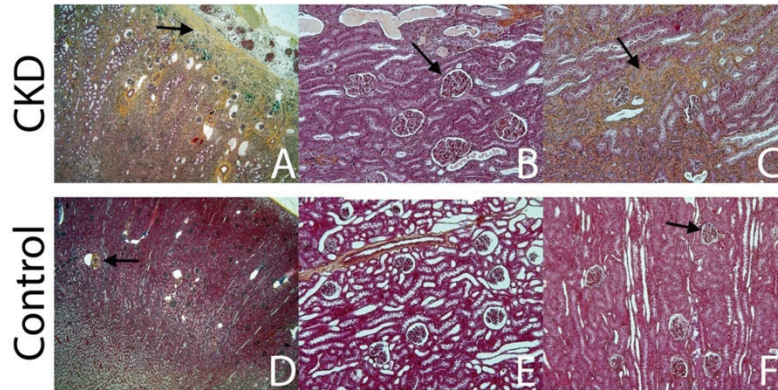
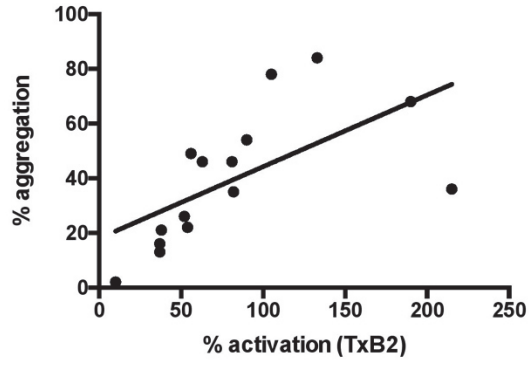


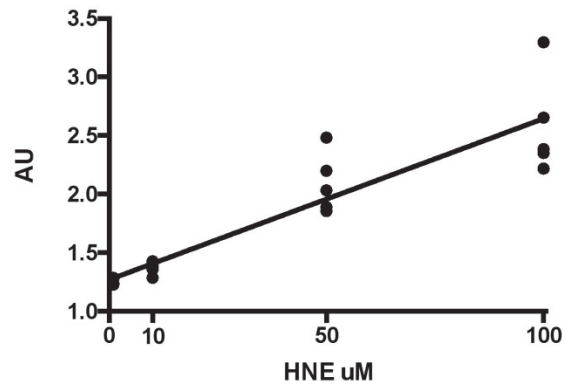
Figure 1: Histological examinations of rabbit kidneys with hematoxylin phloxine saffron (HPS) staining.
A: The electrocoagulated cortex was replaced by a fibrous layer (black arrow) and some glomeruli were surrounded by fibrosis. (x2,5)
B: Remnant glomeruli exhibiting hypertrophy (black arrow; x10). Mean glomerular radius was 23 in control group vs 29 in CKD group ($p<0.05$)
C: Peritubular fibrous involution (black arrow) (x10)
D: Note the large number of of glomeruli and the fibrosis restricted to perivascular areas (black arrow) (x2,5)
E – F: Glomeruli were smaller (black arrow) than in CKD group and fibrosis was restricted to perivascular areas (x10).

Supplementary Figure 2



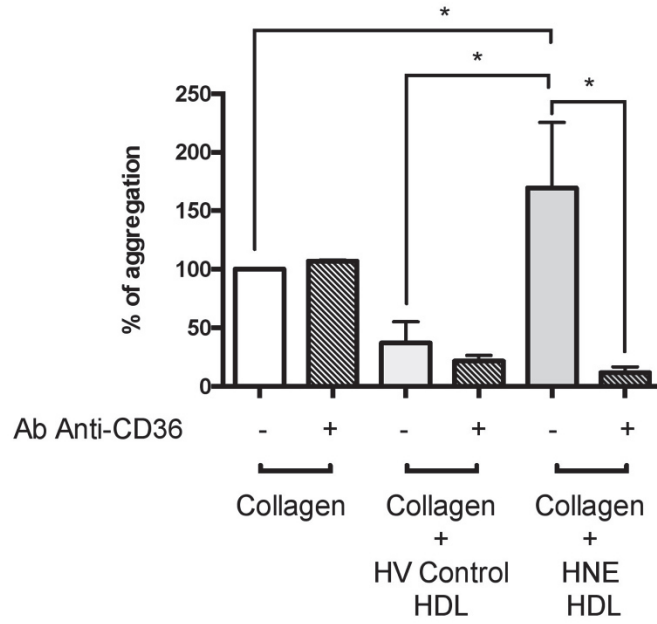
Spearman correlation of TxB2 assay and aggregation assay of 7 control patients and 8 hemodialysis patients.
 $r^2=0.3894$, $p=0.0129$

Supplementary Figure 3



Sperman correlation of the amount of 4-HNE adducts on HDL from in vitro adduction. Significant correlation was observed ($p=0.0179$)

Supplementary Figure 4



Healthy volunteers (HV) HDL modified by an incubation overnight with HNE (HNE HDL) solution exhibited pro-aggregant properties compared to non-modified HDL (HV Control HDL, C). Pre-incubation with anti-CD36 antibody restored HNE HDL anti-aggregant properties like HV Control HDL (n=4 for each group). *p<0.05

8. Protéome des patients hémodialysés non-diabétiques :

Proteomic characterization of high-density lipoprotein particles from non-diabetic hemodialysis patients

Nans Florens, Catherine Calzada, Frédéric Delolme, Adeline Page, Fitsum Guebre-Egziabher, Laurent Juillard, Christophe O. Soulage

Toxins **2019**, 11(11), 671.

Special issue on Uremia and Metabolic Complications of Chronic Kidney Disease

La quatrième partie de ce travail est la description du protéome des HDL des patients hémodialysés non-diabétiques. Outre la modification qualitative des protéines par les modifications post-traductionnelles, un certain nombre de travaux se sont intéressés à la composition des HDL *per se*. Ces travaux ont pu mettre en évidence une modification importante du protéome des HDL chez les patients hémodialysés. Les principale limite de ces travaux est l'hétérogénéité des patients étudiés en terme de co-morbidités. Dans la continuité de nos travaux, il nous semblait intéressant d'explorer le protéome des patients non-diabétiques car cette pathologie est déjà elle-même associée à un remodelage important des HDL.⁶⁵⁻⁶⁷

Premièrement, nous avons mis en évidence un grand nombre de protéines (plus de 500) composant le protéome des HDL. La spectrométrie de masse étant une technique très sensible, le risque d'une contamination plasmatique des HDL malgré leur purification par ultracentrifugation n'était pas exclue. Ainsi, nous avons donc affiné l'étude du protéome aux seules protéines présentes dans au moins 60% des patients (HD ou contrôles). L'analyse a donc été réduite à 326 protéines dont 171 étaient présentes dans tous les échantillons. Une vingtaine de protéines étaient sur- ou sous-représentées dans les HDL des patients en hémodialyse avec dans les protéines en abondance, la bêta-2-microglobuline (B2M), la protéine AMBP, le Complement Factor D (CFD) et la Pulmonary surfactant-associated protein B (SFTPB). Ces

protéines ont déjà été mises en évidence dans des études protéomiques pour certaines (B2M, SFTPb, AMPb), d'autres sont bien documentées comme ayant des rôles délétères chez le patient dialysé (B2M^{8,68}, CFD⁸).

Nous avons d'autre part mis en évidence une certaine corrélation entre la quantité de B2M et de SFTPb avec la quantité de paraoxonase-1, une enzyme importante pour les propriétés anti-oxydantes des HDL. Il s'agit potentiellement ici d'une des explications de la toxicité potentielle de la B2M dans l'IRC : dans les HDL, sa présence semble négativement impacter la quantité de cette enzyme clé. Concernant la SFTPb, cette protéine ne semble pas exercer *per se* de toxicité au niveau rénal mais sa concentration semble être relié au débit de filtration glomérulaire d'une part⁶⁹ et à l'exposition à la fumée de tabac d'autre part⁷⁰. Comme sa concentration est négativement corrélée à celle de PON-1, cela pourrait être une des nombreuses voies de toxicité de l'exposition à la fumée de cigarette.

Cette étude renforce l'idée d'un remodelage important de la composition protéique des HDL dans l'insuffisance rénale terminale. Cette modification influence très probablement les propriétés biologiques des HDL et participe à leur dysfonction chez les patients dialysés.

Article

Proteomic Characterization of High-Density Lipoprotein Particles from Non-Diabetic Hemodialysis Patients

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Abstract: Chronic kidney disease is associated with an increased cardiovascular risk, and altered biological properties of high-density lipoproteins (HDL) may play a role in these events. This study aimed to describe the HDL proteome from non-diabetic hemodialysis patients and identify potential pathways affected by the dysregulated expression of HDL proteins. HDL were sampled from nine non-diabetic hemodialysis (HD) and eight control patients. Samples were analyzed using a nano-RSLC coupled with a Q-Orbitrap. Data were processed by database searching using SequestHT against a human Swissprot database and quantified with a label-free quantification approach. Proteins that were in at least five of the eight control and six of the nine HD patients were analyzed. Analysis was based on pairwise ratios and the ANOVA hypothesis test. Among 522 potential proteins, 326 proteins were identified to be in the HDL proteome from HD and control patients, among which 10 were significantly upregulated and nine downregulated in HD patients compared to the control patients ($p < 0.05$). Up and downregulated proteins were involved in lipid metabolism, hemostasis, wound healing, oxidative stress, and apoptosis pathways. This difference in composition could partly explain HDL dysfunction in the chronic kidney disease (CKD) population and participate in the higher cardiovascular risk observed in this population.

Keywords: HDL cholesterol; lipoproteins; cardiovascular risk; proteomic; mass spectrometry; hemodialysis

Key Contribution: HDL from hemodialysis (HD) patients has a unique composition. Dysregulated protein cargo could partly explain HDL dysfunction in the HD population.

1. Introduction

Chronic kidney disease (CKD) is associated with an increased cardiovascular risk [1]. The failure of statins to reduce the cardiovascular risk of hemodialysis (HD) patients has recently led researchers to focus on the specific properties of high-density lipoproteins (HDL) among this population [2,3]. HDL are essential lipoproteins that exert several atheroprotective properties, among which are the induction of cholesterol efflux from peripheral macrophages and anti-inflammatory, anti-oxidant, vasoprotective, and anti-aggregant effects [4]. These atheroprotective properties of HDL are found to be deeply impaired in CKD and particularly in HD patients [5]. Indeed, several studies have reported

that dysfunctional HDL particles from CKD patients can lead to vasoconstrictive and pro-inflammatory properties [5,6] and a reduction in cholesterol efflux [5–8].

HDL are also essential for lipid and lipoprotein metabolism and homeostasis [9]. CKD leads to a unique phenotype of dyslipidemia with impaired lipoprotein metabolism, particularly in end-stage renal disease (ESRD) patients. In those patients, there is an accumulation of triglyceride-rich particles, mainly very-low-density lipoproteins (VLDL) and chylomicrons. ESRD patients, however, present with normal or lowered low-density lipoprotein (LDL) levels [10].

HDL protein composition directly determines the particle properties and has already been associated with poor cardiovascular outcomes in non-CKD subjects [11,12]. In ESRD patients, label-free proteomic analysis highlighted that dysregulated levels of certain proteins in HDL particles could lead to impaired biological functions [12,13]. Another study found that increased levels of serum albumin, serum amyloid A, and apolipoprotein C-III in HDL particles were correlated with a decrease in the levels of essential apolipoprotein A1 and A2 [8]. Moreover, an increased level of alpha-1-microglobulin/bikunin precursor (AMBp), β 2-microglobulin (B2M), complement factor D (CFD), cystatin-C (CST3), and retinol-binding protein 4 (RBP4) has been reported in HDL particles from ESRD patients [13]. Taken separately, these three studies have demonstrated that the HDL proteome consists of around 100 proteins.

Since HDL proteome composition can directly influence the biological functions of this lipoprotein, we chose to use a high-resolution mass spectrometry analysis and label-free quantification approach to investigate the proteomic composition of HDL isolated from non-diabetic HD patients. Such quantification of HDL protein composition should be useful in identifying new markers of HDL dysfunction and assessing cardiovascular risk among HD patients.

2. Results

Eight healthy controls (CTL) were compared to nine non-diabetic HD patients, the main characteristics of whom are presented in Table 1. HD patients exhibited significantly lower total cholesterol, LDL-cholesterol, and HDL cholesterol than the controls ($p < 0.05$). Two-thirds of HD patients were under statin therapy.

Table 1. Characteristics of hemodialysis and control patients.

N	Control (CTL) 8	Hemodialysis (HD) 9	P-value
General characteristics			
Age, years	39 (31–50)	57 (46–74)	0.045
Gender, <i>n</i> male/ <i>n</i> female	5/3	5/4	0.653
BMI, kg/m ²	22 (19–26)	26 (25–28)	0.060
<i>Comorbidities</i>			
HT, <i>n</i>	2	8	
Stroke, <i>n</i>	0	0	
CHD, <i>n</i>	0	2	
Cardiopathy, <i>n</i>	0	4	
PVD, <i>n</i>	0	1	
<i>Therapies</i>			
Statins, <i>n</i>	0	6	
PI, <i>n</i>	0	5	
RASi, <i>n</i>	1	3	
β -blockers, <i>n</i>	1	5	
CCB, <i>n</i>	1	1	

Table 1. Cont.

N	Control (CTL) 8	Hemodialysis (HD) 9	P-value
Biological parameters			
Urea, mmol/L	6.5 (5.3–7.8)	13.0 (10.9–19.8)	<0.0001
Creatinine, $\mu\text{mol/L}$	77.5 (5.3–7.8)	583 (458.0–798.0)	<0.0001
mGFR, mL/min/1.73 m ²	94 (84–96)	-	
Total cholesterol, mg/dL	217 (187–238)	153 (104–191)	0.021
LDL cholesterol, mg/dL	141 (104–157)	71 (43–124)	0.029
HDL cholesterol, mg/dL	58 (52–62)	46 (38–48)	0.016
Triacylglycerols, mg/dL	99 (86–133)	93 (87–138)	0.999
CRP, mg/L	1.7 (0.2–4.6)	2.5 (1.5–24.8)	0.145

Data are expressed as median (interquartile range). BMI: body mass index, HT: hypertension, CHD: coronary heart disease, GFR, glomerular filtration rate, PVD: peripheral vascular disease, PI: platelet inhibitor, RAASi: renin-angiotensin system inhibitor, CCB: calcium-channel blocker, mGFR: measured glomerular filtration rate by iohexol clearance, CRP: C-reactive protein. Creatinine: $\times 0.011$ for mg/dL, urea: $\times 2.8$ for mg/dL. Differences were considered significant at the $P < 0.05$ level (Mann–Whitney U tests).

2.1. Proteome Characteristics

Five hundred and twenty-two (522) proteins were identified from the mass spectrometry analysis of both control and HD patients, among which 482 were found in more than one patient (Table S1). To avoid potential plasma contamination, we decided to only analyze the proteins that were found at least in 60% of the patients (id. five out of the eight controls and six out of the nine HD patients). Three hundred and twenty-six (326) proteins were found in both conditions (Figure S1), among which 151 were found in the 17 samples. Among those 326 proteins, 10 were significantly upregulated while nine were significantly downregulated in HD patients compared to control patients ($p < 0.05$, Figure 1, Tables 2 and 3). Twenty-two (22) proteins were only found in control samples (Table S2) and 20 in HD samples (Table S3). Regarding the HDL proteome watch database (<http://homepages.uc.edu/~davidswm/HDLproteome.html>), we recovered 69 of the 95 (73%) proteins defined as appearing in three separate studies from three independent laboratories. We also found 155 of the 228 proteins cited in this database (70%).

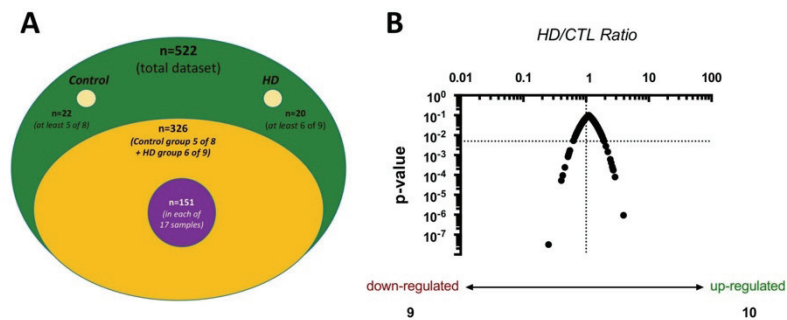


Figure 1. Among 522 proteins, 326 were at least found in 60% of the samples (id. five of the eight controls and six of the nine hemodialysis (HD) patients). Twenty-two and 20 proteins were only found in control and HD samples, respectively. Among the 522 proteins, 151 were found in every sample. (A). The hemodialysis/control patient (HD/CTL) protein ratio was calculated as the protein abundance in HD patients divided by protein abundance in control patients. Among 326 proteins, nine were significantly downregulated while 10 were upregulated (see Tables 2 and 3 for details). $P < 0.05$ was considered as significant (dot line, B).

Table 2. List of upregulated proteins in high-density lipoprotein (HDL) from hemodialysis patients.

Protein Name	Protein Label	Ratio	P-value
UDP-glucose: glycoprotein glucosyltransferase 1	UGGT1	3.948	9.34×10^{-6}
Beta-2-microglobulin	B2M	2.895	7.90×10^{-4}
Pulmonary surfactant-associated protein B	SFTPB	2.716	1.72×10^{-3}
Protein AMBP	AMBP	2.711	1.75×10^{-3}
Insulin-like growth factor II	IGF2	2.672	2.07×10^{-3}
Immunoglobulin heavy constant alpha 2	IGHA2	2.615	2.66×10^{-3}
Immunoglobulin lambda constant 2	IGLC2	2.514	4.13×10^{-3}
HLA class I histocompatibility antigen, B-58 alpha chain	HLA-B	2.427	6.05×10^{-3}
Complement factor D	CFD	2.224	1.46×10^{-2}
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	2.073	2.79×10^{-2}

Protein ratio was calculated as protein abundance in HD patients/protein abundance in control.

Table 3. List of downregulated proteins in HDL from hemodialysis patients.

Protein Name	Protein Label	Ratio	P-value
Guanylin	GUCA2A	0.553	1.71×10^{-2}
Calpain-1 catalytic subunit	CAPN1	0.538	1.32×10^{-2}
Keratin, type I cytoskeletal 16	KRT16	0.526	1.05×10^{-2}
Ras-related protein Rab-6B	RAB6B	0.519	9.23×10^{-3}
Ganglioside GM2 activator	GM2A	0.513	8.19×10^{-3}
Prostaglandin-H2 D-isomerase	PTGDS	0.458	2.40×10^{-3}
Secretoglobin family 3A member 2	SCGB3A2	0.424	9.51×10^{-4}
Thioredoxin-dependent peroxide reductase, mitochondrial	PRDX3	0.404	5.22×10^{-4}
Solute carrier family 2, facilitated glucose transporter member 2	SLC2A2	0.251	3.16×10^{-7}

Protein ratio was calculated as protein abundance in HD patients/protein abundance in control.

Gene ontology enrichment analysis of the 46 proteins with a HD/Control ratio ≥ 1.5 is shown in Figure 2. These proteins were mainly involved in the regulation of lipid metabolism and transport, activation of the immune response, coagulation and platelet activation processes, upregulation of the renewal of the extracellular matrix, generation of reactive oxygen species, and inhibition of apoptosis (Table S4). Gene ontology enrichment analysis of the 11 proteins with a HD/Control ≤ 0.66 is shown in Figure 3. Those proteins were involved in a cyclooxygenase pathway, a prostaglandin metabolic process, and a fatty acid derivate biosynthetic process (GO:0019371, GO:0006693, and GO:1901570, respectively).

Proteins only found in control samples are listed in Table S2; gene ontology enrichment analysis showed that they mainly belonged to immune response pathways. Proteins only found in HD samples are listed in Table S3; gene ontology enrichment analysis did not find relevant pathways from these proteins.

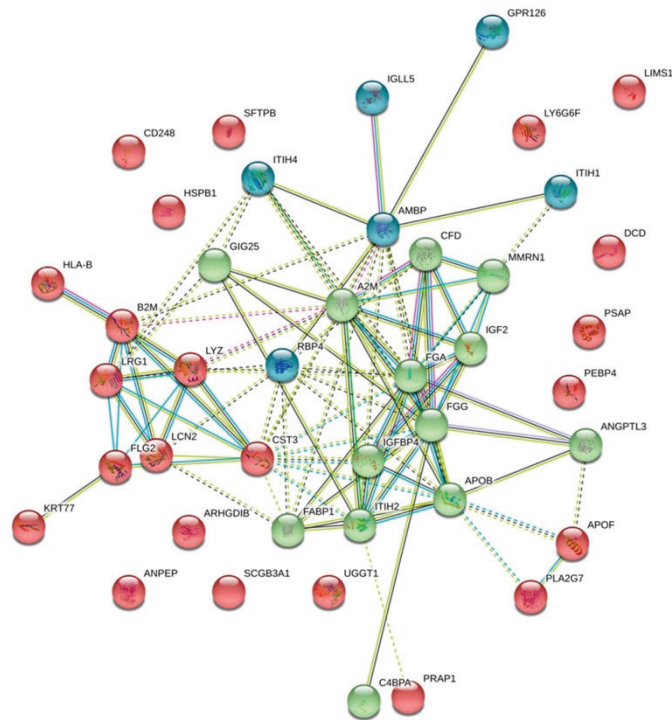


Figure 2. STRING protein–protein interaction network of upregulated proteins in HDL from HD patients. Proteins with a HD/CTL ratio ≥ 1.5 were analyzed with the STRING bioinformatic tool (<https://string-db.org>). Three clusters were identified using a k-means approach. Those clusters are highlighted in three different colors. Nodes represent proteins and lines of interactions between proteins.

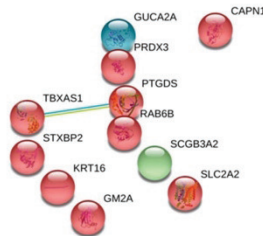


Figure 3. STRING protein–protein interaction network of downregulated proteins in HDL from HD patients. Proteins with a HD/CTL ratio ≤ 0.66 were analyzed with the STRING bioinformatic tool (<https://string-db.org>). Three clusters were identified using a k-means approach. Those clusters are highlighted in three different colors. Nodes represent proteins and lines of interactions between proteins.

2.2. HD-Associated Protein Expression Correlations in HD Patients

Ratios of up- and downregulated proteins, apolipoproteins, and key-enzymes in HDL metabolisms were expressed as each HD patient ratio versus mean control ratios. The results from the correlation analysis are represented as a correlation matrix in Figure 4.

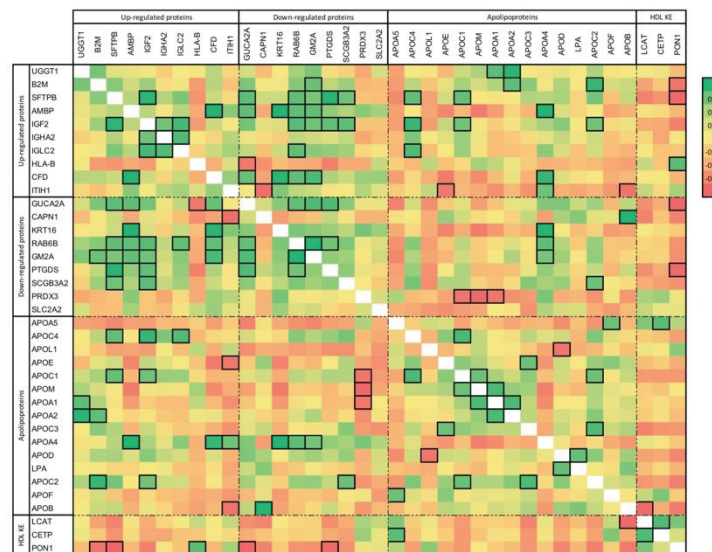


Figure 4. Correlation matrix of up- and downregulated proteins from HDL from HD patients, and apolipoproteins and key-enzymes of HDL. A positive correlation is represented in green and a negative correlation in red. A significant correlation between two proteins is framed. $P < 0.05$ was considered as significant (multiple Spearman correlation matrix).

Among these correlations, Apolipoprotein E and B (APOE and APOB) amounts were significantly negatively correlated with the inter-alpha-trypsin inhibitor heavy chain H1 (ITHI1) amount in HDL. The paraoxonase 1 (PON1) amount was also negatively correlated with beta-2-microglobulin (B2M), pulmonary surfactant-associated protein B (SFTPB), and prostaglandin-H2 D-isomerase (PTGDS) abundance in HDL.

3. Discussion

We found that the HDL proteome from HD patients contained certainly more than 300 proteins (Figure S1), among which 19 were up- or downregulated when compared to control subjects. Using a high-resolution mass spectrometer, we were able to identify 10 times more proteins associated with HDL than any previous work in the field had identified [8,14]. This finding is consistent with a study conducted in chronic heart failure patients, where the authors reported 494 proteins associated with HDL in this pathological context [15]. Simultaneously, our findings were consistent with the HDL proteome watch database (see Table S1, <http://homepages.uc.edu/~davidswm/HDLproteome.html>). This highlights not only the complexity of the HDL structure but possibly other lipoproteins with diverse roles in pathophysiological processes, such as those observed in CKD. In hemodialysis patients, many beneficial properties of HDL, such as cholesterol efflux, anti-oxidant, and vasodilatory effects, have been found to be impaired [5–8]. Gene ontology enrichment analyses of our dataset indicated that dysregulated HDL protein composition in HD patients likely underlies the altered properties of HDL observed in this population. Upregulated proteins were involved in extracellular matrix expansion and blood coagulation, platelet activation, oxidative stress, activation of immune system, and negative regulation of apoptosis. Taken together, the dysregulation of these proteins could point to poor vascular homeostasis, which would then contribute to endothelial dysfunction and premature aging of blood vessels.

In a previous study also using label-free quantification, Mangé et al. found 19 upregulated and 21 downregulated proteins in HDL from HD patients [14]. Among these, only two proteins, β 2-microglobulin and AMBP protein, were also found to be similarly dysregulated in our work. Moreover, we did not confirm the finding by Holzer et al. that ApoA1 and Apo A2 are negatively correlated with serum albumin, serum amyloid A, and ApoC3 in HDL particles from HD patients [8]. According to Shao et al., AMBP, B2M, CFD, CST3, RBP4, and PTGDS were enriched in HDL from ESRD patients [13]. Although AMBP and B2M were also found to be enriched in our study, PTGDS was shown to be downregulated herein. This latter protein is associated with renal damage, and its concentrations rise while the glomerular filtration rate (GFR) declines, especially in diabetes mellitus patients. In the present study, we only explored the protein cargo of HDL in HD patients that cannot actually be considered as an index of plasma concentration of this protein. Two factors could, however, account for the downregulation of PTGDS observed in our cohort. First, the patients included in the present study were all non-diabetic in contrast to the study by Shao et al. (in which 55% of patients had diabetes). Second, among our nine HD patients, six were anuric. This status is unfortunately not documented in the Shao et al. study [13] and could be correlated with PTGDS levels in HDL. PTGDS downregulation could be related to the residual renal function (RRF), as this protein has been reported to be an interesting marker for estimated GFR calculation in HD patients [16]. In our cohort, we noticed that all patients with an RRF exhibited a PTGDS ratio >1 , while all anuric patients exhibited a PTGDS ratio <1 (Fisher's exact test, $p = 0.012$) (see Supplementary Table S5). A major difference between the study herein and previous ones is the choice of HD patients. Most patients in the aforementioned studies were diabetic, a situation that could per se lead to significant differences in HDL proteome composition. Patients included in two of those studies were also older than patients included herein. Most importantly, quantification methods, false discovery rate (FDR) threshold, and baseline for significant change in protein expression were different in all studies.

The potential critical role of AMBP protein in lipid and metabolic dysregulation in CKD is confirmed here. The upregulation of AMBP reported in our analysis is in line with the findings from both Mangé et al. and Shao et al. Interestingly, a proteomic analysis of adipose tissue from HD patients also revealed an upregulation of AMBP [17]. AMBP is the precursor of alpha-1-microglobulin, a protein involved in the scavenging and metabolism of free radical and oxidizing residues. Its upregulation could thus be linked to the increase in oxidative stress observed in HD patients [17]. Our results show correlations between the expressions of some HDL key metabolic proteins and the observed dysregulated proteins (Figure 4). Among those correlations, levels of ITH1, PRDX3, SFTPB, and B2M were strongly associated with key known proteins of HDL expression. Those proteins are involved in different pathways and their clinical and biological relevance in HDL composition remains unknown. However, the correlation between B2M and PON1 levels strengthens the deep implication of B2M in cardiovascular complications in hemodialysis patients. The proteomic approach can indeed help to determine highly sensitive and resolute patterns of protein expression, but these should be interpreted with caution.

Limitations of the Study

Beyond the effects of CKD on proteome composition, the altered protein expressions observed herein can also be explained by the hemodialysis procedure per se. Indeed, the hemodialysis procedure is responsible for bio-incompatibility mechanisms including activation of coagulation and immune system processes (complement activation, neutrophil degranulation) that could lead to a specific enrichment of the HDL proteome. This potential contamination process could in turn also be responsible for the disturbances in HDL normal properties. Otherwise, HDL proteome composition could be parasitized by plasma proteins that stricto sensu do not belong to HDL particles. However, the two sequential ultracentrifugations with an adjusted density using potassium bromide seriously limit such contamination. Some other factors such as age or therapies could have modified the protein cargo of HDL in our patients. Nevertheless, the majority of our HD patients were under statins, mitigating the

potential effects of such medicine on the proteome in our cohort. Finally, as the number of included subjects was rather low and as we do not include a validation cohort, our study remains exploratory and extrapolations should be made with caution.

4. Conclusions

Alteration of the HDL proteome composition of HD patients could underlie HDL dysfunction among this population, leading to the increased cardiovascular risk observed in those patients [1]. Future studies need to focus on the relevance and impact of such protein dysregulation on HDL properties to provide insights on how to restore normal HDL function in CKD and HD patients.

5. Materials and Methods

5.1. Recruitment and Sample Collection

Patients were sampled at the Lyon teaching hospitals (Hospices civils de Lyon, Hôpital E. Herriot, Lyon, France). Control patients were healthy volunteers referred to the renal unit for a living kidney donation and who were hospitalized in the context of a pre-donation laboratory and clinical work-up. HD patients were recruited within the hemodialysis unit before the mid-week session. Patients aged ≥ 18 years and undergoing hemodialysis for more than six months were included. Subjects with diabetes mellitus, ongoing inflammatory disease, liver cirrhosis, a recent (less than three months' ago) cardiovascular event (i.e., myocardial infarction, stroke, acute peripheral artery occlusion), uncontrolled anemia, coagulopathy, and BMI ≥ 35 kg·m⁻² were excluded. The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional review board (CPP Lyon Sud Est IV, ref: L16-57). Written informed consent was obtained from all subjects. Blood samples were obtained by venipuncture on EDTA-coated tubes. Blood samples were centrifuged at 3500 g for 10 min to isolate plasma which was then stored at -80 °C until use. Lipoproteins were separated from plasma by stepwise potassium bromide (KBr) ultracentrifugation as described by Havel et al. [18]

5.2. Mass Spectrometry

Briefly, samples were reduced, alkylated, and digested with trypsin at 37 °C overnight. They were desalted on C18 spin columns, dried, and analyzed in triplicate using an Ultimate 3000 nano-RSLC (Thermo Fisher Scientific, Illkirch, France) coupled online to a Q-Exactive HF hybrid Quadrupole-Orbitrap Mass Spectrometer (Q-Orbitrap, Thermo Fisher Scientific). Briefly, peptides were separated on a C18 nano-column using an acetonitrile linear gradient and analyzed in a Top 20 Higher Collision Dissociation (HCD) data-dependent mass spectrometer. Data were processed by database searching using Sequest HT (Thermo Fisher Scientific) with Proteome Discoverer 2.2 software (Thermo Fisher Scientific) against a human Swissprot database and quantified with a label-free quantification approach. The quantification was expressed either as a ratio of the mean relative amount of protein in HD samples versus the mean relative amount in control samples, or the relative amount in each HD sample versus the mean relative amount in control samples. Precursor and fragment mass tolerances were set at 10 ppm and 0.02 Da, respectively. Trypsin was set as the enzyme, and up to two missed cleavages were allowed. Peptides were filtered with FDR at 1% with a Benjamini-Hochberg correction.

5.3. Gene Ontology and Protein Network

Gene ontology enrichment and protein network analysis of the data was done using the STRING tool (<https://string-db.org>). For these analyses, all proteins with a ratio of ≥ 1.5 and ≤ 0.66 were included. Enrichments with an FDR lower than 5% were considered significant.

5.4. Statistical Analysis

Data are expressed as mean \pm standard error of the mean (SEM). All analyses were performed using GraphPad Prism version 6.0 (GraphPad software, La Jolla, CA, USA) and XLSTAT software

(Addinsoft. XLSTAT 2016: Data Analysis and Statistical Solution for Microsoft Excel. Paris, France). Multiple correlations were made using Spearman's rank correlation, and the correlation matrix was presented as a heatmap. Protein quantitation was performed with a Minora feature detector and precursor ions quantifier node in Proteome Discoverer 2.2 software with protein quantitation based on pairwise ratios and the ANOVA (individual proteins) hypothesis test. Differences were considered as significant at the $p < 0.05$ level.

5.5. Associated Data

The mass spectrometry proteomics data have been deposited to the ProteomeX change Consortium via the PRIDE partner repository with the dataset identifier PXD013301 (DOI: 10.6019/PXD013301, username: reviewer26456@ebi.ac.uk, password: oMD4gyDT).

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6651/11/11/671/s1>, Figure S1: STRING protein–protein interaction network of all proteins in HDL from HD patients, Table S1: protein list and mass spectrometry analysis statistics, Table S2: Proteins only found in Control samples, Table S3: Proteins only found in HD samples, Table S4: Gene ontology enrichment of upregulated proteins (HD/CTL ratio greater than 1.5), Table S5: Reduced PTGDS abundance in anuric HD patients.

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Conflicts of Interest: The authors declare no conflicts of interest.

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9. Adductome des HDL de patients non-diabétiques hémodialysés :

Is glycation of proteins only a diabetes mellitus related problem?

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La cinquième partie de ce travail est consacré à la description d'un catalogue de modifications post-traductionnelles présentent sur les HDL de patients hémodialysés que nous appellerons « adductome ». Notre travail a pu mettre en évidence le rôle central de la carbonylation par le 4-HNE dans la perturbation des propriétés biologiques de HDL de patients hémodialysés. Cependant, il existe d'autres modifications pouvant aussi fortement impacter les propriétés biologiques de ces protéines. Nous avons donc choisi de regarder la présence et l'abondance de 7 modifications post-traductionnelles (**Figure 12**) :

- La glycation via la formation de carboxy-methyl-lysine (CML)
- La carbamoylation
- La carbonylation par le 4-HNE
- La guanidinylation des lysines (homoarginine)
- La nitration
- La nitrosylation
- La chlorination

Chacune de ces modifications correspond à un excès de masse attendu sur les acides aminés. Ceux-ci étaient donc rentrés dans le logiciel d'analyse et nous avons pu décrire précisément l'adductome des HDL concernant cette sélection de modifications post-traductionnelles.

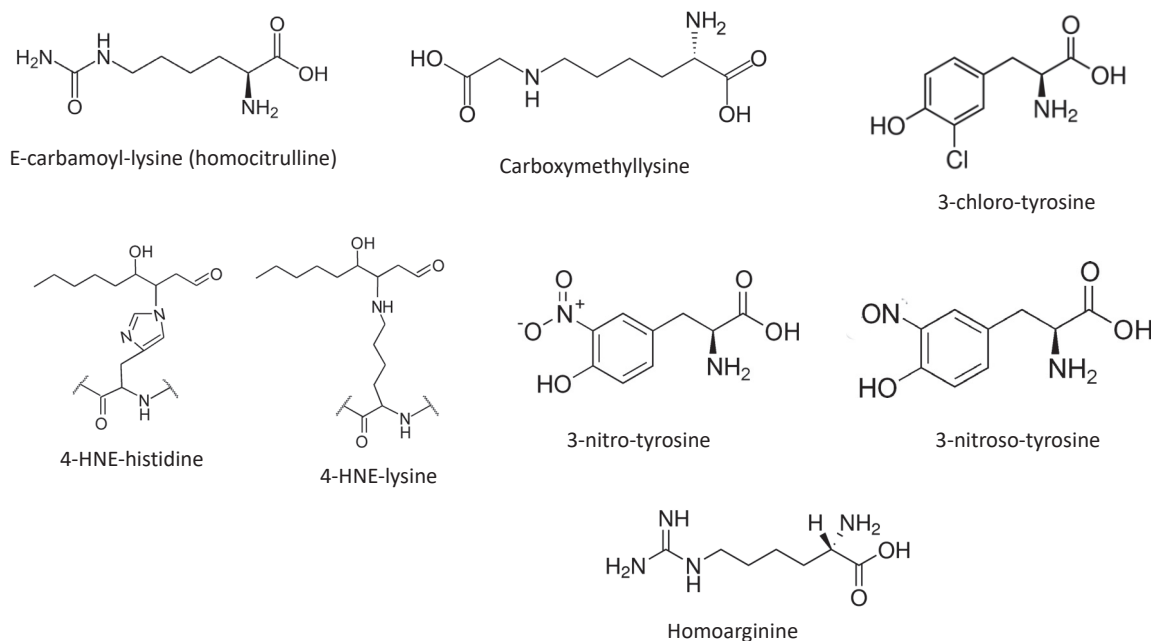


Figure 12 – Récapitulatif des différentes modifications post-traductionnelles recherchés par l’analyse en spectrométrie de masse.

Dans cette partie du travail, nous avons mis en évidence une grande diversité d’adduits présents sur une partie des protéines constituant le protéome des HDL. En effet, 14% des protéines totales retrouvées dans les échantillons présentaient au moins un adduit sus-cité. Cela représentait 74 protéines dont le détail des adduits est présenté dans la **Table 2** ci-après. La première modification post-traductionnelle en terme de fréquence (adduits rapportés au nombre d’acide-aminé constituant la protéine) était le produit de la glycation : la carboxy-méthyl-lysine (CML). La deuxième modification était non pas la carbamylation mais la guanidinylation. La carbamylation n’arrivait qu’en troisième position (voir **Figure 1** de l’article). Le pourcentage de chaque modification par protéines est présenté dans la **Figure 13**. En regardant sur un plan quantitatif sur l’Apo A1, nous avons mis en évidence 2 faits : 1) la glycation par le CML restait la plus fréquente et 2) la moyenne d’abondance des adduits excédait rarement 1%.

Cette constatation vis à vis de la carbamylation reste surprenante sur plusieurs points. Le premier est que la glycation par le CML reste la principale modification chez des patients

non diabétiques. Il est connu que les patients hémodialysés présentent un niveau élevé de glycation des protéines plasmatiques, que ce soit l'albumine ou ici les HDL, mais, cet excès de glycation est jusqu'ici décrit comme très abondant surtout chez les patients diabétiques. Le deuxième point est qu'en terme de fréquence d'adduit, la carbamoylation n'est même pas en deuxième position, devancée par la guanidinylation (**Figure 13**). Cette modification est pourtant très peu décrite dans la littérature dans l'insuffisance rénale, hormis pour son rôle sur l'albumine dans les perturbations des fonctions de transports des toxines telles que l'indoxyl-sulfate.⁴² Le dernier point est que l'abondance de la carbamoylation était la plupart du temps corrélée à la glycation par le CML. Ainsi, bien que de nombreuses preuves indiquent que la carbamoylation a des effets toxiques, il est légitime de se poser la question si les effets qui lui sont reliés dans l'IRC^{29,30} ne sont pas surtout dus à un excès de glycation.

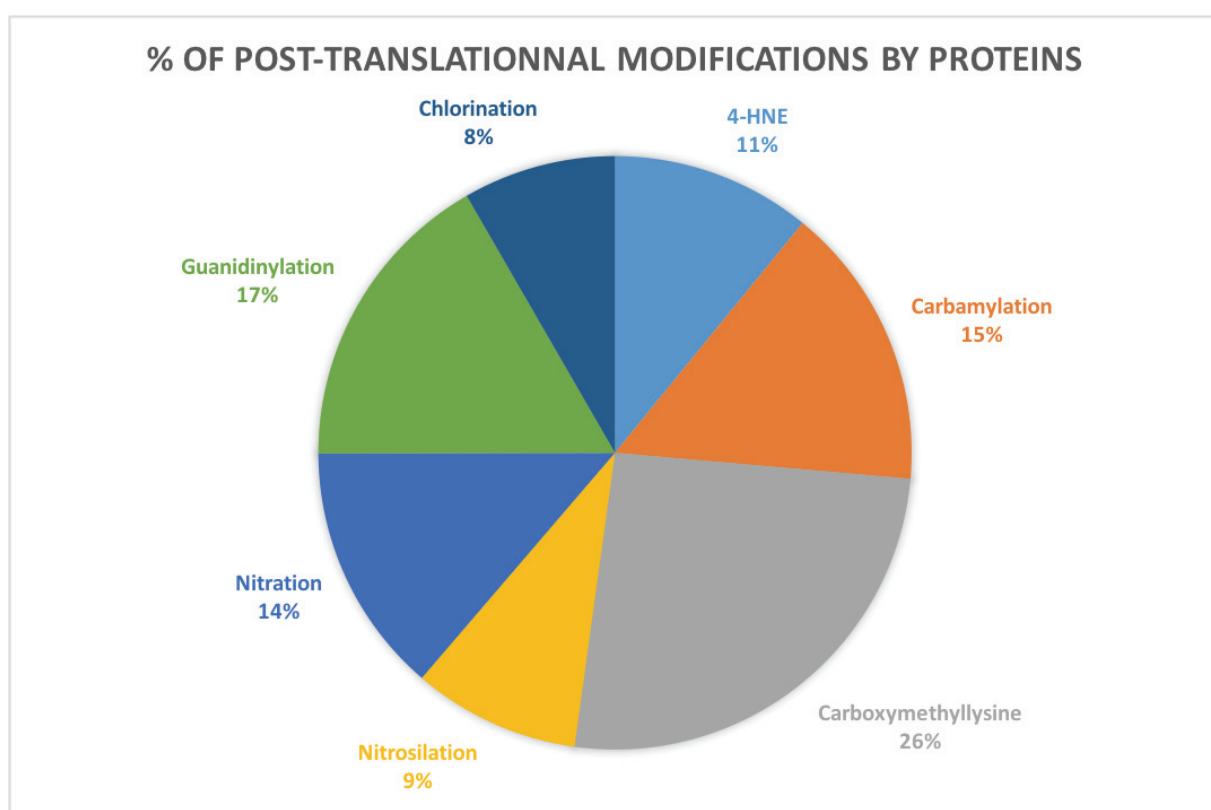


Figure 13 – Répartition des différentes modifications post traductionnelles parmi les 74 protéines adduites

Le dernier point majeur que soulève ce travail est sur l'origine de cette glycation. En effet, il existe potentiellement un rôle majeur de la dégradation de la balance anti/pro-oxydante dans l'IRC et que cette dernière entraîne une génération importante de produits de dégradation du glucose à partir des sucres endogènes. Cependant, une autre source de ces produits de dégradation du glucose potentiellement importante peut être soulevée : les concentrés acides composant le bain de dialyse. Le dialysat est formé par un subtil mélange d'un concentré acide contenant des concentrations fixes de glucose, potassium, magnésium, calcium et dont le tampon acide varie (acétate, acide chlorhydrique, citrate). Ce concentré va être dilué par l'apport d'eau ultra-pure et les concentrations de sodium et de bicarbonates vont être ajustées par l'apport effectué par le concentré « basique » généralement présenté sous forme de sel de bicarbonates de sodium. Le concentré acide contient alors avant dilution une moyenne de 250 mM soit 50g de glucose par poche (environ 5 litres). La génération spontanée de ces produits de dégradation du glucose a été bien étudiée dans les poches de dialyses péritonéales et les facteurs qui l'influencent sont le pH de la poche, la température et la durée du stockage.⁷¹ Étonnamment, aucune donnée n'est disponible concernant la quantité de ces produits de dégradation dans les poches de concentrés acides d'hémodialyse.

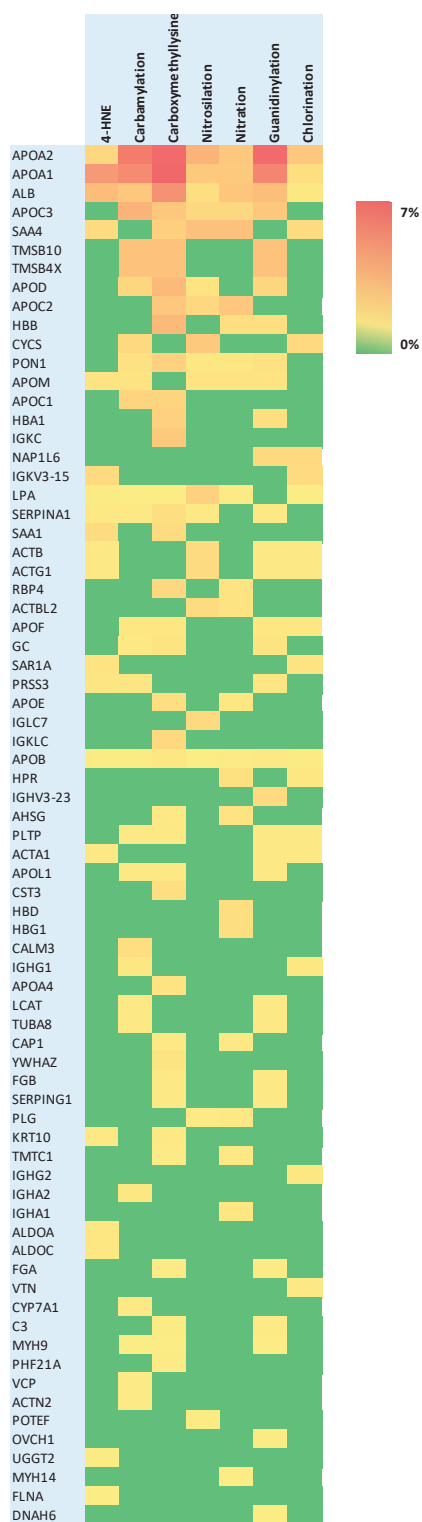


Figure 14 — Heat map des modifications post-traductionnelles des protéines présentant des adduits dans les HDL de patients hémodialysés non-diabétiques.

Les protéines sont classées par ordre décroissant de pourcentage d'adduction en rapport avec leur nombre de résidus acides aminés.

Table 2. Nature et site d'adduction sur les 74 protéines adduites dans les HDL de patients hémodialysés.

Protein name	Protein label	% PTM	Adduct (n)	Site of adduction
14-3-3 protein zeta/delta Actin, alpha skeletal muscle	YWHAZ	0.41%	Glycation (1)	Carboxymethyl [K3]
	ACTA1	0.80%	4-HNE (1) Chlorination (1) Guandrylation (1)	4-HNE [K217] Chlorination [Y220] Guandryl [K215]
Actin, cytoplasmic 1	ACTB	1.60%	Guandrylation (1)	Guandryl [K284]
			4-HNE (1) Chlorination (1) Nitrosylation (3)	4-HNE [K291] Chlorination [Y294] Nitrosyl [Y69; Y166; Y169]
Actin, cytoplasmic 2	ACTG1	1.60%	Guandrylation (1)	Guandryl [K284]
			4-HNE (1) Chlorination (1) Nitrosylation (3)	4-HNE [K291] Chlorination [Y294] Nitrosyl [Y69; Y166; Y169]
Adenylyl cyclase-associated protein 1	CAP1	0.42%	Nitration (1) Glycation (1)	Nitro [Y419] Carboxymethyl [K422]
			Glycation (3)	Carboxymethyl [K225; K367; K392]
Alpha-1-antitrypsin	SERPINA1	1.67%	Guandrylation (1) Carbamoylation (1)	Guandryl [K367] Carbamyl [K367]
			4-HNE (1) Nitrosylation (1)	4-HNE [K215] Nitrosyl [Y211]
Alpha-2-HS-glycoprotein	AHSG	0.82%	Glycation (1) Nitration (2)	Carboxymethyl [K67] Nitro [Y47; Y56]
			Carbamoylation (1)	Carbamyl [K523]
Alpha-actinin-2	ACTN2	0.11%	Glycation (19)	Carboxymethyl [K36; K47; K64; K69; K83; K101; K112; K120; K130; K131; K142; K157; K164; K206; K219; K230; K232; K250; K262]
			Nitration (5)	Nitro [Y42; Y53; Y124; Y139; Y260]
Apolipoprotein A-I	APOA1	26.97%	4-HNE (12) Carbamoylation (14) Guandrylation (15) Nitrosylation (4) Chlorination (2)	4-HNE [H20; K36; K47; K64; K83; K130; H179; H186; H217; H223; K250; K262] Carbamyl [K36; K47; K64; K69; K83; K101; K112; K131; K157; K206; K219; K230; K232; K250] Guandryl [K36; K47; K64; K69; K83; K101; K112; K120; K130; K131; K142; K164; K219; K232; K250] Nitrosyl [Y42; Y53; Y124; Y260] Chlorination [Y42; Y53]
			Glycation (2)	Nitro [Y37; Y89] Carboxymethyl [K26; K46; K51; K53; K62; K69; K78]
Apolipoprotein A-II	APOA2	28.00%	Nitrosylation (3) Chlorination (2) Carbamoylation (6) Guandrylation (7) 4-HNE (1) Nitration (7) Glycation (2)	Nitrosyl [Y37; Y44; Y89] Chlorination [Y37; Y44] Carbamyl [K26; K46; K51; K53; K62; K69] Guandryl [K26; K46; K51; K53; K62; K69; K77] 4-HNE [K26] Nitro [Y37; Y89] Carboxymethyl [K26; K46; K51; K53; K62; K69; K78]
			Glycation (2)	Carboxymethyl [K65; K233]
Apolipoprotein A-IV	APOA4	0.51%	Glycation (9)	Guandryl [K275; K742; K745; K82; K876; K1188; K1750; K3627; K4021]
			Carbamoylation (3)	Carbamyl [K876; K1750; K4021]
Apolipoprotein B-100	APOB	0.88%	Nitrosylation (1) Chlorination (4) Nitrosylation (1) 4-HNE (4) Glycation (14) Nitration (5) Glycation (1)	Chlorination [Y720; Y740; Y1746; Y4025] Nitrosyl [Y247] 4-HNE [K147; K3689; K4512; K4518] Carboxymethyl [K539; K822; K876; K1415; K2062; K2800; K2985; K3148; K3586; K3627; K3790; K3963; K4021; K4055] Nitro [Y720; Y2125; Y2568; Y3139; Y3958] Carbamyl [K56] Carboxymethyl [K56]
			Glycation (1)	Carbamyl [K56]
Apolipoprotein C-I	APOC1	2.41%	Nitrosylation (1) Nitration (2) Glycation (2)	Nitrosyl [Y85] Nitro [Y47; Y85] Carboxymethyl [K61; K98]
			Carbamoylation (3)	Carbamyl [K41; K44; K71]
Apolipoprotein C-III	APOC3	9.09%	Nitrosylation (1) Guandrylation (2) Nitration (1) Glycation (2)	Nitrosyl [Y73] Guandryl [K41; K44] Nitro [Y73] Carboxymethyl [K44; K71]
			Carbamoylation (2)	Carbamyl [K51; K176]
Apolipoprotein D	APOD	5.29%	Guandrylation (2) Nitrosylation (1) Glycation (5) Nitration (1)	Guandryl [K51; K176] Nitrosyl [Y47] Carboxymethyl [K41; K51; K164; K175; K176] Carboxymethyl [K93; K280] Nitro [Y54]
			Glycation (2)	Nitro [Y54]
Apolipoprotein E	APOE	0.95%	Nitrosylation (1) Chlorination (1) Carbamoylation (1) Glycation (1)	Chlorination [Y223] Guandryl [K246] Carbamyl [K246] Carboxymethyl [K246]
			Nitrosylation (1)	Guandryl [K50]
Apolipoprotein F	APOF	1.23%	Guandrylation (1) Carbamoylation (1) Glycation (1)	Guandryl [K50] Carbamyl [K50] Carboxymethyl [K50]
			Carbamoylation (1) Nitrosylation (1) Carbamoylation (1)	Nitrosyl [Y102] Guandryl [K99] Carbamyl [K91]
Apolipoprotein L1	APOL1	0.75%	Guandrylation (1) Glycation (1)	Guandryl [K50] Carboxymethyl [K50]
			Nitrosylation (1) Carbamoylation (1)	Nitrosyl [Y102] Guandryl [K99]
Apolipoprotein M	APOM	2.66%	Guandrylation (1) Carbamoylation (1)	Guandryl [K91] Carbamyl [K91]

Apolipoprotein(a)	LPA	1.72%	4-HNE (1) Nitration (1)	4-HNE [H103] Nitro [Y102]	Carbamyl [K4373; K4381] 4-HNE [H58; K4373; K4381] Chlorination [Y4380] Nitrosylation (64) Glycation (2) Nitration (6)	Carbamyl [K4373; K4381] 4-HNE [H58; K4373; K4381] Chlorination [Y4380] Nitrosylation (64) Glycation (2) Nitration (6)	Y1663; Y1776; Y1800; Y1890; Y1891; Y2004; Y2005; Y2118; Y2119; Y2232; Y2233; Y2346; Y2347; Y2574; Y2575; Y2688; Y2689; Y2802; Y2803; Y2916; Y2917; Y3030; Y3031; Y3144; Y3145; Y3258; Y3259; Y3372; Y3373; Y3623; Y3843; Y3957; Y4555; Y7569 Carboxymethyl [K4066; K4297] Nitro [Y3395; Y3623; Y3843; Y3957; Y7455; Y7569]
Beta-actin-like protein 2	ACTBL2	1.33%	Nitrosylation (3) Nitration (2)	Nitrosyl [Y167; Y170; Y307] Nitro [Y167; Y170]	Nitrosylation (3) Nitration (2)	Nitrosyl [Y167; Y170; Y307] Nitro [Y167; Y170]	
Calmodulin-3	CALM3	0.67%	Carbamylation (1)	Carbamyl [K141]	Carbamylation (1)	Carbamyl [K141]	
Cholesterol 7 α -methyltransferase	CYP7A1	0.20%	Carbamylation (1)	Carbamyl [K199]	Carbamylation (1)	Carbamyl [K199]	
Complement C3	C3	0.18%	Guandrylation (2) Glycation (1)	Guandinyl [K1139; K1155] Carboxymethyl [K1139]	Guandrylation (2) Glycation (1)	Guandinyl [K1139; K1155] Carboxymethyl [K1139]	
Cystatin-C	CST3	0.68%	Glycation (1)	Chlorination [Y68]	Chlorination [Y68]	Chlorination [Y68]	
Cytochrome c	CYCS	3.81%	Chlorination (1) Nitrosylation (2) Carbamylation (1)	Nitrosyl [Y47; Y49] Carbamyl [K56]	Chlorination (1) Nitrosylation (2) Carbamylation (1)	Nitrosyl [Y47; Y49] Carbamyl [K56]	
Dynamin heavy chain 6, axonemal	DNAH6	0.02%	Guandrylation (1)	Guandinyl [K4019]	Guandrylation (1)	Guandinyl [K4019]	
Fibrinogen alpha chain	FGA	0.23%	Guandrylation (1)	Guandinyl [K480]	Guandrylation (1)	Guandinyl [K480]	
Fibrinogen beta chain	FBG	0.41%	Guandrylation (1) Glycation (1)	Guandinyl [K247] Carboxymethyl [K247]	Guandrylation (1) Glycation (1)	Guandinyl [K247] Carboxymethyl [K247]	
Filamin-A	FLNA	0.04%	4-HNE (1)	4-HNE [K2346]	4-HNE (1)	4-HNE [K2346]	
Fructose-bisphosphate aldolase A	ALDOA	0.27%	4-HNE (1)	4-HNE [H197]	4-HNE (1)	4-HNE [H197]	
Fructose-bisphosphate aldolase C	ALDOC	0.27%	4-HNE (1)	4-HNE [H197]	4-HNE (1)	4-HNE [H197]	
GTP-binding protein SAR1a	SAR1A	1.01%	4-HNE (1) Chlorination (1)	4-HNE [K87] Chlorination [Y89]	4-HNE (1) Chlorination (1)	4-HNE [K87] Chlorination [Y89]	
Haptoglobin-related protein	HPR	0.86%	Chlorination (1) Nitration (1)	Chlorination [Y312] Nitro [Y184; Y222]	Chlorination (1) Nitration (1)	Chlorination [Y312] Nitro [Y184; Y222]	
Hemoglobin subunit alpha	HBA1	2.11%	Guandrylation (1) Glycation (2)	Guandinyl [K62] Carboxymethyl [K62; K128]	Guandrylation (1) Glycation (2)	Guandinyl [K62] Carboxymethyl [K62; K128]	
Hemoglobin subunit beta	HBB	4.08%	Guandrylation (1) Glycation (4) Nitration (1)	Guandinyl [K60] Carboxymethyl [K60; K83; K96; K121] Nitro [Y36]	Guandrylation (1) Glycation (4) Nitration (1)	Guandinyl [K60] Carboxymethyl [K60; K83; K96; K121] Nitro [Y36]	
Hemoglobin subunit delta	HBD	0.68%	Nitration (1)	Nitro [Y36]	Nitration (1)	Nitro [Y36]	
Hemoglobin subunit gamma-1	HBG1	0.68%	Nitration (1)	Nitro [Y183]	Nitration (1)	Nitro [Y183]	
Immunoglobulin heavy constant α 1	IGHA1	0.28%	Nitration (1)	Carbamyl [K294]	Carbamylation (1)	Carbamyl [K294]	
Immunoglobulin heavy constant α 2	IGHA2	0.29%	Carbamylation (1) Chlorination (1)	Chlorination [Y161] Carbamyl [K292]	Carbamylation (1) Chlorination (1)	Chlorination [Y161] Carbamyl [K292]	
Immunoglobulin heavy constant γ 1	IGHG1	0.61%	Chlorination (1)	Chlorination [Y157]	Chlorination (1)	Chlorination [Y157]	
Immunoglobulin heavy constant γ 2	IGHG2	0.31%	Chlorination (1)	Chlorination [K84]	Chlorination (1)	Chlorination [K84]	
Immunoglobulin heavy variable 3-23	IGHV3-23	0.85%	Chlorination (1)	Chlorination [K84]	Chlorination (1)	Chlorination [K84]	
Immunoglobulin kappa constant	IGKC	1.87%	Glycation (2)	Carboxymethyl [K62; K76]	Glycation (2)	Carboxymethyl [K62; K76]	
Immunoglobulin kappa light chain	IGKL	0.93%	Glycation (2)	Carboxymethyl [K169; K183]	Glycation (2)	Carboxymethyl [K169; K183]	
Immunoglobulin kappa variable 3-15	IGKV3-15	1.74%	4-HNE (1)	4-HNE [K59]	4-HNE (1)	4-HNE [K59]	
Immunoglobulin lambda constant 1	IGLC7	0.94%	Chlorination (1)	Chlorination [Y56]	Chlorination (1)	Chlorination [Y56]	
Keratin, type I cytoskeletal 10	KRT10	0.34%	Nitrosylation (1) Glycation (1)	Nitrosyl [Y85] 4-HNE [K362] Carboxymethyl [K207]	Nitrosylation (1) Glycation (1)	Nitrosyl [Y85] 4-HNE [K362] Carboxymethyl [K207]	
Myosin-14	MYH14	0.05%	Nitration (1)	Nitro [Y275]	Nitration (1)	Nitro [Y275]	
Myosin-9	MYH9	0.15%	Guandrylation (1) Carbamylation (1) Glycation (1)	Guandinyl [K1538] Carbamyl [K8] Carboxymethyl [K1538]	Guandrylation (1) Carbamylation (1) Glycation (1)	Guandinyl [K1538] Carbamyl [K8] Carboxymethyl [K1538]	
Oxodismutase-1	OXCHI	0.09%	Guandrylation (1)	Guandinyl [K330]	Guandrylation (1)	Guandinyl [K330]	
PHD finger protein 21A	PHF21A	0.15%	Glycation (1)	Carboxymethyl [K43]	Glycation (1)	Carboxymethyl [K43]	
Phosphatidylcholine-sterol acyltransferase	LCAT	0.45%	Guandrylation (1) Carbamylation (1)	Guandinyl [K242] Carbamyl [K242]	Guandrylation (1) Carbamylation (1)	Guandinyl [K242] Carbamyl [K242]	
Phospholipid transfer protein	PLTP	0.81%	Chlorination (1) Guandrylation (1) Carbamylation (1) Glycation (1)	Chlorination [Y217] Guandinyl [K221] Carbamyl [K221] Carboxymethyl [K221]	Chlorination (1) Guandrylation (1) Carbamylation (1) Glycation (1)	Chlorination [Y217] Guandinyl [K221] Carbamyl [K221] Carboxymethyl [K221]	
Plasma protease C1 inhibitor	SERPING1	0.40%	Guandrylation (1)	Guandinyl [K162]	Guandrylation (1)	Guandinyl [K162]	
Plasminogen	PLG	0.37%	Nitrosylation (1) Nitration (2)	Nitrosyl [Y425] Nitro [Y416; Y425]	Nitrosylation (1) Nitration (2)	Nitrosyl [Y425] Nitro [Y416; Y425]	
POT1e ankyrin domain family member F	POTEF	0.09%	Nitrosylation (1)	Nitrosyl [Y918]	Nitrosylation (1)	Nitrosyl [Y918]	

Putative nucleosome assembly protein 1-like 6	NAP1L6	1.87%	Chlorination (1) Guandrylation (1)	Chlorination [Y83] Guandryl [K78]
Retinol-binding protein 4	RBP4	1.49%	Glycation (2) Nitration (1)	Carboxymethyl [K48; K76] Nitro [Y108]
Serum albumin	ALB	15.11%	4-HNE (15) Guandrylation (15) Carbamylation (16) Nitrosylation (4) Chlorination (2) Glycation (30)	4-HNE [H152; K160; H170; K183; K198; K305; H312; K347; K426; K438; H464; K558; K581] Guandryl [K160; K214; K223; K300; K310; K337; K341; K347; K375; K402; K426; K468; K543; K565; K569] Carbanyl [K97; K100; K161; K183; K214; K223; K300; K305; K310; K337; K375; K426; K438; K549; K565; K569] Nitrosyl [Y172; Y174; Y356; Y476] Chlorination [Y476; Y521] Carboxymethyl [K36; K44; K65; K75; K88; K97; K130; K160; K161; K183; K186; K198; K214; K223; K286; K300; K305; K337; K341; K347; K402; K413; K426; K437; K438; K490; K524; K543; K549; K569] Nitro [Y108; Y164; Y172; Y174; Y343; Y356; Y358; Y365; Y377; Y394; Y425; Y476; Y521]
Serum amyloid A-1 protein	SAA1	1.64%	Nitration (13) 4-HNE (1) Glycation (1)	4-HNE [H89] Carboxymethyl [K108]
Serum amyloid A-4 protein	SAA4	7.69%	Nitrosylation (3) Chlorination (1) 4-HNE (1) Nitration (3) Glycation (2)	Nitrosyl [Y39; Y82; Y90] Chlorination [Y39] 4-HNE [K26] Nitro [Y39; Y60; Y90] Carboxymethyl [K26; K116]
Serum paraoxonase/arylesterase 1	PON1	3.10%	Nitrosylation (1) Guandrylation (2) Carbamylation (2) Glycation (5) Nitration (1)	Nitrosyl [Y128] Guandryl [K3; K290] Carbanyl [K3; K290] Carboxymethyl [K3; K70; K75; K145; K290] Nitro [Y294]
Thymosin beta-10	TMSB10	6.82%	Guandrylation (1) Carbamylation (1) Glycation (1)	Guandryl [K4] Carbanyl [K4] Carboxymethyl [K4]
Thymosin beta-4	TMSB4X	6.82%	Guandrylation (1) Carbamylation (1) Glycation (1)	Guandryl [K4] Carbanyl [K4] Carboxymethyl [K4]
Transitional endoplasmic reticulum ATPase	VCP	0.12%	Carbamylation (1)	Carbanyl [K8]
Transmembrane and TPR repeat-containing protein 1	TMTC1	0.34%	Nitration (2) Glycation (1)	Nitro [Y506; Y513] Carboxymethyl [K511]
Trypsin-3	PRESS3	0.99%	4-HNE (1) Guandrylation (1) Carbamylation (1)	4-HNE [H153] Guandryl [K155] Carbanyl [K155]
Tubulin alpha-8 chain	TUBA8	0.45%	Guandrylation (1) Carbamylation (1)	Guandryl [K124] Carbanyl [K124]
UDP-glucose:glycoprotein glucosyltransferase 2	UGGT2	0.07%	4-HNE (1)	4-HNE [H1388]
Vitamin D-binding protein	GC	1.05%	Guandrylation (2) Carbamylation (1) Glycation (2)	Guandryl [K332; K345] Carbanyl [K332] Carboxymethyl [K242; K332]
Vitreonectin	VTN	0.21%	Chlorination (1)	Chlorination [Y468]
AA, position of the modified amino-acid. H, histidine; K, lysine; Y, tyrosine. Similar sites of modifications are in bold type. % PTM, percentage of potential sites of adduction regarding all the amino-acids of the protein sequence.				

Is glycation of proteins only a diabetes mellitus-related problem?

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Cardiovascular morbidity and mortality remains a heavy burden for chronic kidney disease (CKD) and hemodialysis (HD) patients.¹ Interestingly, lowering the low-density lipoprotein cholesterol level using statins has failed to reduce the CV risk among hemodialysis patients,² and high-density lipoprotein (HDL) that are well known to be atheroprotective in the general population, are reported to have impaired biological properties in HD patients but little is known about the underlying mechanism.³ The latter could be explained, at least in part, by post-translational modifications (PTM) of proteins that is defined by the adduction onto amino-acid residues of solutes from oxidative and non-oxidative reactions.⁴ In CKD, for instance, the high level of circulating blood urea exposes patients to an increased risk of carbamoylation. This PTM results from the addition of isocyanate to lysine or histidine residues that is generated through the spontaneous degradation of urea to cyanate or the transformation of thiocyanate by myeloperoxidase,⁵ and this adduction leads to deep modifications of LDL properties (increased generation of foam cells, pro-aggregant behavior).^{6,7} In HD, the level of carbamoylated albumin is correlated with cardiovascular outcomes,⁸ and is now considered as a surrogate marker of cardiovascular (CV) risk among CKD patients, especially the HD population. Nevertheless, little is known about PTMs in general, probably owing to technical reasons which have largely been overcome with tandem mass spectrometry (MS).

In the present work, we aimed to describe the adductome (*i.e.* the catalogue of PTM) of HDL in non-diabetic hemodialysis patients. We chose non-diabetic HD patients to limit the glycation induced by diabetes mellitus *per se*. HDL was extracted from the plasma of 9 non-diabetic HD patients and 8 potential kidney donors (controls, See supplementary Table 1) using a sequential potassium bromide stepwise density gradient ultracentrifugation. The study was approved by the regional review board (CPP Lyon Sud Est IV, reference number L16-57) and written informed consent was obtained from all subjects. Samples were analyzed using a nano-

RSLC coupled to a Q-Orbitrap mass spectrometer (Thermo Fischer, San Jose, CA, USA). Proteins were identified using SequestHT with Proteome Discoverer 2.2 software against a human Swissprot database and quantified with a label-free quantitation approach. Oxidation, acetylation, carboxy-methyl-lysine (CML) resulting from glycation, carbonylation by 4-hydroxy-2-nonenal (4-HNE), carbamoylation, guanydination, chlorination, nitration, and nitrosylation were set potential modifications. Protein and peptide label-free quantitation was based on pairwise ratios and ANOVA hypothesis test. The mass spectrometry proteomics data have been deposited to the ProteomeX change Consortium via the PRIDE partner repository with the dataset identifier PXD013301 (DOI: 10.6019/PXD013301; username: reviewer26456@ebi.ac.uk; password: oMD4gyDT). See supplementary material for further details.

A total of 522 proteins were identified in HDL from HD patients and controls, among which 74 (14%) had covalent adducts. Apolipoprotein A-II and I (ApoA-II and I) were the proteins the more prone to adduction (28 and 27% of amino acids, respectively) followed by serum albumin (15%), apolipoprotein C-III (9%) and serum amyloid A4 (8%; Figure 1A). Surprisingly, the most frequent PTM was glycation, namely formation of CML (26%), followed by guanidinylation (17%), carbamoylation (15%), nitration (14%), carbonylation by 4-HNE (11%), nitrosylation (9%) and chlorination (8%). Most of the key proteins of HDL metabolism were found to be adductable and the most frequent PTM was always glycation (Figure 1B). As this analysis was mainly qualitative, we further conducted a quantitative analysis (using Peptide Mapping Software; Thermo Scientific) of ApoA-I as it was one of the most extensively modified proteins in the samples tested. On ApoA-I, 6 amino acid residues were found to be significantly more glycated in HD patients than in controls. The median proportion of glycation of such sites was less than 1%. Three amino acid residues were found to be significantly more carbamoylated in HD patients than in controls. The median percentage was also about 1%

except for lysine K117 which was 4.8%, and none of these were found in control patient samples (Figure 1C).

Taken together, these results suggest that formation of CML by glycation is more frequent than carbamoylation in HDL from non-diabetic HD patients, but also that the proportion of adducted proteins could be regarded as rather low (<1% for ApoA-I) although one should keep in mind that tiny structural modifications can strongly impair the biological activity of proteins. The results also bring into question the role of carbamoylation in HD patients as it was not the main PTM observed in the samples studied; although carbamoylation was one of the two most represented in the HD patient samples, and lysine K117 of ApoA-I was the most frequently modified by carbamoylation (Figure 1C), more generally glycation was more frequent. As these two PTM result mainly from non-enzymatic reactions and concern the same amino-acid residues, this could be explained by a competitive effect mostly in favor of CML generation in HD patients. Indeed, it is important to note that in the qualitative analysis, several PTM could occur on the same amino-acid residue (see supplementary Table 2) but in a quantitative approach, only one PTM per amino acid residue was quantified (Figure 1C). Interestingly, the CML adducts that were found to be significantly more frequent in patients than in controls were all positively correlated with carbamoylation in ApoA-I (Figure 1D); this means that for every detected and quantified carbamoylated residue in a protein there were probably significant amount of associated glycated ones. Thus, as this glycation is also associated with cardiovascular toxicity,⁹ this could explain an important part of the correlation observed between carbamoylation and hard outcomes (death, cardiovascular diseases) in HD patients.

This leads to the question of the origin of such glycation. As is the case for HD, peritoneal dialysis requires a dialysate containing glucose. The generation of advanced

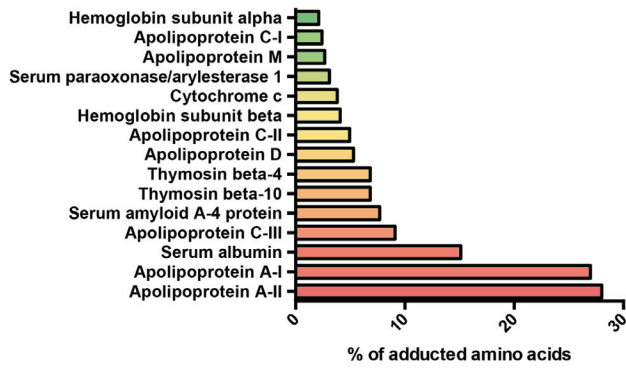
glycation end-products (AGEs) has been reported with conventional peritoneal dialysis fluids,¹⁰ and this is related to the generation of glucose degradation products (GDP) during heat sterilization and/or storage over a long period of time. To the best of our knowledge, there is however little data about the generation of GDPs in HD concentrates that contain 250mM glucose, while these could be great contributors to the glycation of proteins in non-diabetic HD patients.

To conclude, we described herein that glycation is probably the main PTM observed in HDL from non-diabetic HD patients. The origin of this excess of glycation needs to be investigated; it could be a result of a chemical favorable environment in HD patients but the role of GDPs in HD fluids needs to be addressed.

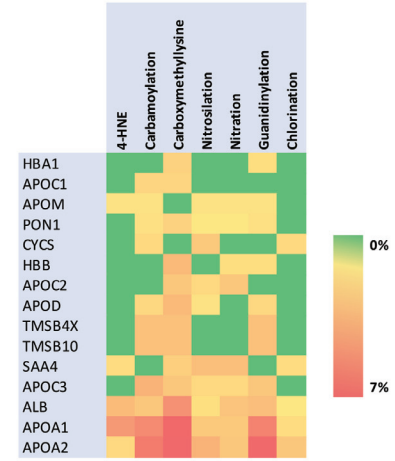
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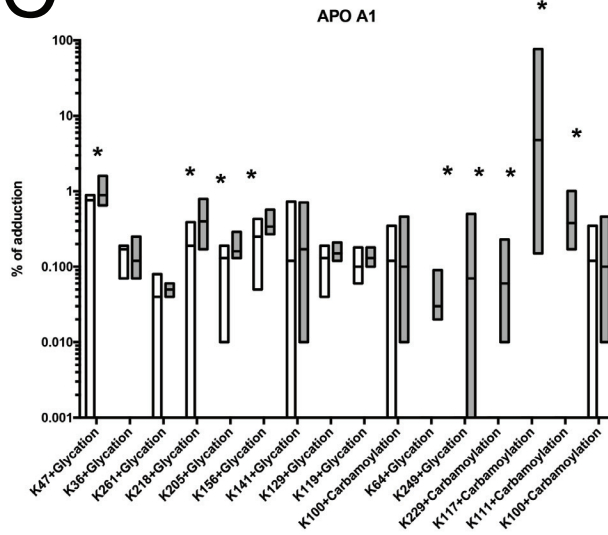
A



B



C



D

	K229+Carbamoylation	K117+Carbamoylation	K111+Carbamoylation
K47+Glycation	0.58	0.56	0.44
K36+Glycation	-0.17	-0.30	-0.38
K261+Glycation	0.56	0.47	0.44
K218+Glycation	0.65	0.48	0.55
K205+Glycation	0.57	0.58	0.39
K156+Glycation	0.49	0.43	0.39
K141+Glycation	0.13	0.19	0.04
K129+Glycation	0.51	0.39	0.39
K119+Glycation	0.55	0.40	0.49
K64+Glycation	0.96	0.88	0.85
K249+Glycation	0.72	0.46	0.55

Figure 1. A: The 15 most adducted high-density lipoprotein (HDL) proteins from hemodialysis (HD) patients. The proportion (%) of amino acids adducted with respect to the total number of amino acids of the protein is presented. B: Percentage of post-translational modifications (PTM) in the 15 most adducted proteins of HDL from HD patients. The proportion (%) of each modification with respect to the total number of amino acids of the protein is presented as a color gradation between green (0%) and red (7%). C: PTM onto Apolipoprotein A1 (ApoA-I) quantified by Peptide Mapping tool in Biopharma Finder 3.0 Software (Thermo Scientific, San Jose, CA, USA). The median proportion (%) of each post-translational modification is presented and compared between HD patients (grey) and controls (white). Boxes represent the interquartile range. HDL from HD patients showed 6 amino-acid residues significantly more glycosylated and 3 amino-acid residues carbamoylated. Mann-Whitney U test, * $p < 0.05$ D: correlation matrix of carbamoylated and glycosylated amino-acid residues of ApoA-I. Significant correlations are framed in black ($p < 0.05$; Spearman's correlation test).

Supplementary materials

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Complete methods

Patients and ethics statement

Patients were sampled at the University Hospital of Lyon. Control patients were healthy volunteers for a living kidney donation, hospitalized for their pre-donation laboratory and clinical work-up. Hemodialysis patients were sampled in the hemodialysis unit of the Edouard Herriot hospital (Lyon) before the mid-week session. Inclusion criteria were age ≥ 18 years, undergoing hemodialysis for ≥ 6 months. Exclusion criteria were diabetes mellitus, ongoing inflammatory disease, liver cirrhosis, recent cardiovascular event (≤ 3 months, myocardial infarction, stroke, acute peripheral artery occlusion), uncontrolled anemia, coagulopathy, and BMI ≥ 35 kg.m⁻². The study was conducted in accordance with the Declaration of Helsinki and was approved by the regional ethics committee (reference L16-57, *Comité de Protection des Personnes Lyon Sud Est IV*). A written informed consent was obtained from all subjects.

Blood collection

Blood samples were obtained by venipuncture (on EDTA-coated tubes) except for dialysis blood samples that were obtained immediately before dialysis from the arterial line of the mechanical bloodstream. Blood samples were centrifuged at 3500 \times g for 10 min to isolate plasma which were collected and stored at -80°C until use.

Isolation of lipoproteins from the plasma

Lipoproteins were separated from plasma by stepwise potassium bromide (KBr) density gradient ultracentrifugation.¹¹ Plasma was fractionated in the Beckman ultracentrifuge with a rotor TAL 100.3 (Beckman Coulter, Brea, CA, USA). A first centrifugation at 54,000 \times g for 3h 30min at 15°C was performed to remove the top layer corresponding to VLDL and chylomicrons. Then, the plasma density was adjusted to 1.063 g.mL⁻¹ with KBr (M=119.01

g.mol⁻¹). After a second centrifugation at 100,000 \times g for 5h at 4°C the orange ring, corresponding to LDL, was collected. Finally, plasma density was adjusted to 1.21 g.mL⁻¹ with KBr and after centrifugation at 100,000 \times g for 6 h 30 at 4°C the orange ring corresponding to HDL, was collected.

For the isolation of all the lipoproteins together, a single ultracentrifugation was performed at 100,000 \times g for 6h 30min at 4° after an adjustment of plasma density to 1.21 g.mL⁻¹ with KBr.

Biochemistry

Serum creatinine measurement was performed using the Siemens enzymatic method (on the Dimension Vista System, Siemens Healthcare, Erlangen, Germany) traceable to National Institute of Standards and Technology Creatinine Standard Reference Materials 914 (verified with National Institute of Standards and Technology SRM 967) with calibration certified by isotope dilution mass spectrometry. Urea was measured with urease test (Dimension Vista System). Cholesterol and triacylglycerols levels were measured using enzymatic kits (Biomerieux, Marcy l'Etoile, France). HDL concentration was measured using an enzymatic kit (Abcam, Paris, France).

Mass spectrometry assay

List of reagents:

Sigma Aldrich (Saint Quentin Fallavier, France): acetonitrile MS grade (ACN), Formic acid (FA), trifluoroacetic acid (TFA), iodoacetamide (IAA), dithiotreitol (DTT), sodium deoxycholate (SDC), ammonium bicarbonate (AB). MilliQ Water. Trypsin: promega (Lyon, France). Pierce C18 desalting spin column and Pierce quantitative fluorometric peptide assay: Thermo Scientific (San Jose, CA, USA)

In-solution digestion

Thirty micrograms of protein were diluted in 100 μ L of 50 mM AB/SDC 1% buffer. They were reduced with 5 mM DTT for 1h at 57°C, and then alkylated with 10 mM IAA for 1h in the dark at room temperature and under agitation (850 rpm). Enzymatic digestion was performed with trypsin at a ratio 1/100 (enzyme/proteins) and overnight at 37°C. The samples were then desalted on a C18 spin column and dried on Speed-Vacuum (Thermo Fischer) before nanoLC-MS/MS analysis.

LC-MS/MS analysis

Prior to injection, the peptide concentrations in the samples were determined by the Pierce quantitative fluorometric peptide assay (Thermo Scientific) according to the manufacturer's instructions. An aliquot containing 1 μ g for each sample was dried and suspended in 9 μ L of FA 0.1% + 1 μ L of Cytochrome C digest (2 pmol/ μ L) used as internal standard. 2 μ L of this solution was then injected. Samples were analyzed in a label-free quantitation strategy, in triplicate using an Ultimate 3000 nano-RSLC (Thermo Scientific) coupled with a Q Exactive HF mass spectrometer via a nano-electrospray ionization source (Thermo Scientific).

Nano-LC: Samples were injected and loaded on a C18 Acclaim PepMap100 trap-column 75 μ m ID x 2 cm, 3 μ m, 100Å, (Thermo Scientific) for 3.0 minutes at 5 μ L/min with 2% ACN, 0.05% TFA in H₂O and then separated on a C18 Acclaim Pepmap100 nano-column, 50 cm x 75 μ m i.d, 2 μ m, 100 Å (Thermo Scientific) with a 60 min linear gradient from 4% to 50% buffer B (A: 0.1% FA in H₂O, B: 100% ACN, 0.1% FA) and then from 50 to 95% of B in 2 min, hold for 10 min, and returned to the initial conditions in 1 min for 14 min. The total duration was set to 90 min at a flow rate of 300 nL/min. The oven temperature was kept constant at 40°C.

MS: Samples were analyzed using the TOP20 HCD method: MS data were acquired in a data-dependent strategy selecting the fragmentation events based on the 20 most abundant precursor

ions in the survey scan (300-1600 Th). The resolution of the survey scan was 60,000 at m/z 200 Th and for MS/MS scan the resolution was set to 15,000 at m/z 200 Th. The Ion Target Value for the survey scans in the Orbitrap and the MS/MS scan were set to 3E6 and 1E5 respectively and the maximum injection time was set to 60 ms for MS scan and for MS/MS scan. Parameters for acquiring HCD MS/MS spectra were as follows; collision energy = 27 and an isolation width of 2.0 m/z. The precursors with unknown charge state, charge state of 1 and 8 or greater than 8 were excluded. Peptides selected for MS/MS acquisition were then placed on an exclusion list for 20 s using the dynamic exclusion mode to limit duplicate spectra.

Data analysis

Proteins were identified using using SequestHT with Proteome Discoverer 2.2 software (Thermo Scientific) against the Swissprot Homo Sapiens database (2018-07 release, 24480 sequences). Precursor mass tolerance was set at 10 ppm and fragment mass tolerance was set at 0.02 Da, and up to 2 missed cleavages were allowed. Oxidation (methionine: M), acetylation (protein N-terminus) carboxy-methyl-lysine (CML; +204.90 Da on lysine: K or histidine: H) resulting from glycation, carbonylation by 4-hydroxy-2-nonenal (4-HNE; + 156.115 Da on K or H), carbamoylation (+43.02 Da on K or H), guanydinilation (+74.08 Da on K or H), chlorination (+ 35.45 Da on tyrosine: Y), nitration (+30 Da on Y), nitrosylation (+46 Da on Y) and carbamidomethylation (cysteine: C) were set as potential modifications. Peptides and proteins were filtered with a false discovery rate (FDR) set at 1% using percolator and proteins were identified with 1 unique peptide in rank 1. Protein quantitation was performed with Minora feature detector and precursor ions quantifier node in Proteome Discoverer 2.2 software with protein quantitation based on pairwise ratios and ANOVA (individual proteins) hypothesis test.

Peptide mapping

Raw files were submitted to peptide mapping analysis through the BioPharma Finder software (v3.1; ThermoFisher Scientific). Default parameters were used except the confidence score which was set to 80% and the recovery value which was set to a minimum of 10%. Search was performed against the Apolipoproteins A-I and A-II sequence (Uniprot P02647 and P02652). Carbamidomethyl (C), carbamylation (K,H,C), Glycation (K), HNE (K,H), Oxidation (M), Nitration (Y), Nitrosylation (Y), Chlorination (Y), Guanydilation (K), carboxymethylation (K) were set as dynamic modifications. The results of relative quantification are presented as the proportion (%) of the modification among the number of proteins in the samples.

Statistical analysis

Data were expressed as median and interquartile range [IQR]. All analyses were performed using GraphPad Prism version 6.0 (GraphPad software, La Jolla, CA, USA). Normality was assessed using D'Agostino & Pearson test. Comparisons were performed using Mann-Whitney U-test. Differences were considered significant when $P < 0.05$.

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Supplementary Table 1. Patient characteristics.

	Control	Hemodialysis (HD)	P-value
N	8	9	
General characteristics			
Age, years	39 (31 – 50)	57 (46 – 74)	0.045
Gender, n male/n female	5/3	5/4	0.653
BMI, kg/m ²	22 (19 – 26)	26 (25 – 28)	0.060
<i>Comorbidities, n</i>			
Hypertension	2	8	
Stroke	0	0	
CHD	0	2	
Cardiopathy	0	4	
PVD	0	1	
<i>Therapies, n</i>			
Statins	0	6	
PI	0	5	
RASi	1	3	
β-blockers	1	5	
CCB	1	1	
Biological parameters			
Urea, mmol/L	6.5 (5.3 – 7.8)	13.0 (10.9 – 19.8)	<0.0001
Creatinine, μmol/L	77.5 (70.5 – 91.3)	583 (458.0 – 798.0)	<0.0001
mGFR, mL/min/1.73m ²	94 (84 – 96)	Not applicable	
Total cholesterol, mg/dL	217 (187 – 238)	153 (104 – 191)	0.021
LDL cholesterol, mg/dL	141 (104 – 157)	71 (43 – 124)	0.029
HDL cholesterol, mg/dL	58 (52 – 62)	46 (38 – 48)	0.016
Triacylglycerols, mg/dL	99 (86 – 133)	93 (87 – 138)	0.999
CRP, mg/L	1.7 (0.2 – 4.6)	2.5 (1.5 – 24.8)	0.145

Data are expressed as median ± interquartile range. BMI: body mass index; CHD: coronary heart disease; GFR, glomerular filtration rate; PVD: peripheral vascular disease; PI: platelet inhibitor; RASi: renin-angiotensin system inhibitor; CCB: calcium-channel blocker; mGFR: measured GFR by iohexol clearance; CRP: C-reactive protein. Creatinine: × 0.011 for mg/dl; urea: × 2.8 for mg/dl

Supplementary Table 2. List of adducts of the 74 adducted proteins

Protein name	Protein label	% PFM	Adduct (n)	Site of adduction
14-3-3 protein zeta/delta	YWHAZ	0.41%	Chlorination (1) 4-HNE (R127) Chlorination (1) Oxidation (1)	Carbonyl (K51) 4-HNE (R127) Chlorination (Y228) Oxidation (R215)
Actin, alpha skeletal muscle	ACTA1	0.80%	Chlorination (1) Oxidation (1)	Carbonyl (K284) 4-HNE (K291)
Actin, cytoplasmic 1	ACTB	1.00%	Chlorination (1) 4-HNE (1) Chlorination (1) Nitrosation (3)	Oxidation (Y294) Nitrosyl (Y59, Y166, Y169)
Actin, cytoplasmic 2	ACTG1	1.00%	Chlorination (1) 4-HNE (1) Chlorination (1) Nitrosation (1)	Oxidation (Y294) 4-HNE (Y294) Chlorination (Y294) Nitrosyl (Y109, Y196, Y199)
Adenylyl cyclase-associated protein 1	CAP1	0.42%	Chlorination (1) Nitrosation (1)	Nitro (Y419) Carbonyl (K422)
Alpha-1-antitrypsin	SIKFN5A1	1.07%	Chlorination (3) Carbonylation (1) Carbonylation (1) 4-HNE (1) Nitrosation (1)	Carbonyl (K225, K367, K392) Oxidation (K367) Carbonyl (K367) 4-HNE (R215) Nitrosyl (R211)
Alpha-2-HS-glycoprotein	AHSG	0.82%	Chlorination (1) Nitrosation (2)	Carbonyl (K67) Nitro (Y47, Y56)
Alpha-actinin-2	ACTN2	0.11%	Carbonylation (1)	Carbonyl (K51)
Apolipoprotein A-I	APOA1	20.07%	Chlorination (9) Nitrosation (5) 4-HNE (12) Carbonylation (14) Oxidation (15) Nitrosation (4) Chlorination (2)	Carbonyl (K56, K47, K54, K69, K83, K101, K112, K120, K130, K131, K142, K157, K164, K206, K219, K230, K232, K250, K262) Nitro (Y42, Y51, Y124, Y139, Y200) 4-HNE (H20, K36, K47, K64, K69, K83, K101, K112, K131, K157, K206, K219, K230, K232, K250) Oxidation (K36, K47, K54, K69, K83, K101, K112, K120, K130, K131, K142, K164, K219, K232, K250) Nitrosyl (Y42, Y51, Y124, Y200) Chlorination (Y42, Y51)
Apolipoprotein A-II	APOA2	20.00%	Nitrosation (3) Chlorination (2) Chlorination (7) 4-HNE (1) Nitrosation (2) Chlorination (7)	Nitrosyl (Y37, Y44, Y99) Chlorination (Y37, Y44) Oxidation (K26, K46, K51, K51, K62, K69) Oxidation (K26, K46, K51, K51, K62, K69, K77) 4-HNE (K26) Nitro (Y37, Y99) Carbonyl (K26, K46, K51, K51, K62, K69, K78)
Apolipoprotein A-IV	APOA4	0.51%	Chlorination (2)	Carbonyl (K44, K218)
Apolipoprotein B-100	APOB	0.88%	Carbonylation (9) Carbonylation (4) Chlorination (4) Nitrosation (1) 4-HNE (4) Nitrosation (2)	Oxidation (K275, K742, K743, K822, K876, K1188, K1790, K3027, K4021) Carbonyl (K275, K742, K822) Chlorination (Y720, Y740, Y746, Y4025) Nitrosyl (K47) 4-HNE (K147, K369, K452, K458) Nitrosation (K369, K452, K458, K414, K420, K2800, K2888, K3148, K3886, K6267, K3790, K3900, K4021, K4055)
Apolipoprotein C-I	APOC1	2.41%	Carbonylation (1) Chlorination (1)	Carbonyl (K50) Carbonyl (K50)
Apolipoprotein C-II	APOC2	4.05%	Nitrosation (1) Nitrosation (2) Chlorination (2)	Nitrosyl (Y185) Nitro (Y187, Y189) Carbonyl (K50, K98)
Apolipoprotein C-III	APOC3	9.09%	Carbonylation (3) Nitrosation (1) Chlorination (2) Nitrosation (1) Chlorination (2)	Carbonyl (K41, K44, K71) Nitrosyl (Y71) Oxidation (K41, K44) Nitro (Y71) Carbonyl (K41, K71)
Apolipoprotein D	APOD	5.29%	Carbonylation (2) Chlorination (2) Nitrosation (1) Chlorination (5)	Carbonyl (K51, K76) Oxidation (K51, K76) Nitrosation (Y47) Carbonyl (K41, K51, K164, K175, K179)
Apolipoprotein E	APOE	0.95%	Chlorination (2) Nitrosation (1)	Carbonyl (K298, K298) Nitro (Y54)

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Apolipoprotein F	APOF	1.25%	Chlorination (1) Oxidation (1) Carbonylation (1) Chlorination (1)	Chlorination (Y221) Oxidation (K240) Carbonyl (K240) Carbonyl (K240)
Apolipoprotein L1	APOA1	0.75%	Chlorination (1) Carbonylation (1) Chlorination (1)	Oxidation (K246) Carbonyl (K246) Carbonyl (K246)
Apolipoprotein M	APOA1	2.66%	Nitrosation (1) Chlorination (1) Carbonylation (1) 4-HNE (1) Nitrosation (1)	Nitrosyl (Y102) Oxidation (K99) Carbonyl (K91) 4-HNE (H101) Nitro (Y101)
Apolipoprotein(a)	LPA	1.72%	Carbonylation (2) 4-HNE (1) Chlorination (1) Chlorination (6) Chlorination (1) Chlorination (2) Nitrosation (6)	Carbonyl (K473, K488) 4-HNE (K58, K475, K488) Chlorination (Y480) 4-HNE (Y180, Y181, Y204, Y205, Y408, Y409, Y522, Y523, Y526, Y647, Y780, Y781, Y864, Y865, Y978, Y979, Y1092, Y1207, Y1320, Y1321, Y1434, Y1435, Y1548, Y1549, Y1662, Y1663, Y1776, Y1777, Y1890, Y1891, Y2004, Y2005, Y2118, Y2119, Y2232, Y2233, Y2346, Y2347, Y2466, Y2467, Y2575, Y2576, Y2688, Y2689, Y2692, Y2800, Y2806, Y2947, Y3000, Y3001, Y3144, Y3145, Y3288, Y3289, Y3372, Y3373, Y3621, Y3643, Y3957, Y7455, Y7560) Carbonyl (K496, K4297) Nitro (Y195, Y362, Y363, Y367, Y7455, Y7560)
Beta-actin-like protein 2	ACTB2	1.35%	Nitrosation (3) Nitrosation (2) Carbonylation (1)	Nitrosyl (Y170, Y307) Nitro (Y170, Y307) Carbonyl (K141)
Calmodulin-3	CALM3	0.67%	Carbonylation (1)	Carbonyl (K141)
Cholesterol 7-alpha-monooxygenase	CYP7A1	0.20%	Carbonylation (1)	Carbonyl (K199)
Complement C3	C3	0.18%	Chlorination (2) Chlorination (1)	Oxidation (K119, K115) Carbonyl (K111, K119)
Cystatin C	CST3	0.08%	Chlorination (1)	Carbonyl (K88)
Cytochrome c	CYCS	3.81%	Chlorination (1) Nitrosation (2) Carbonylation (1)	Chlorination (Y98) Nitrosyl (Y7, Y91) Carbonyl (K50)
Dentin heavy chain 6, nonrenal	DNAH6	0.02%	Chlorination (1)	Carbonyl (K499)
Fibrinogen alpha chain	FGA	0.25%	Chlorination (1)	Oxidation (K40)
Fibrinogen beta chain	FGB	0.41%	Chlorination (1) Chlorination (1)	Oxidation (K247) Carbonyl (K247)
Fibronin A	FN1A	0.04%	4-HNE (1)	4-HNE (K236)
Fructose-bisphosphate aldolase A	ALDOA	0.27%	4-HNE (1)	4-HNE (H197)
Fructose-bisphosphate aldolase C	ALDOC	0.27%	4-HNE (1)	4-HNE (H197)
GTP-binding protein SAR1a	SAR1A	1.01%	4-HNE (1) Chlorination (1)	4-HNE (K67) Chlorination (Y89)
Hemoglobin-related protein	HBR	0.86%	Chlorination (1) Nitrosation (1)	Chlorination (Y132) Nitro (Y148, Y222)
Hemoglobin subunit alpha	HBA1	2.11%	Chlorination (1) Chlorination (2)	Oxidation (K62) Carbonyl (K62, K120)
Hemoglobin subunit beta	HBB	4.08%	Chlorination (1) Chlorination (1) Nitrosation (1)	Oxidation (K66) Carbonyl (K66, K90, K90, K121) Nitro (Y36)
Hemoglobin subunit delta	HBD	0.08%	Nitrosation (1)	Nitro (Y36)
Hemoglobin subunit gamma-1	HBG1	0.08%	Nitrosation (1)	Nitro (Y36)
Immunoglobulin heavy constant alpha 1	IHAI1	0.28%	Nitrosation (1)	Nitro (Y161)
Immunoglobulin heavy constant alpha 2	IHA2	0.29%	Carbonylation (1)	Carbonyl (K291)
Immunoglobulin heavy variable 2-23	IHV2-23	0.85%	Chlorination (1)	Chlorination (Y157)
Immunoglobulin kappa constant	IKC	1.87%	Chlorination (1)	Oxidation (K291)
Immunoglobulin kappa light chain	IKLC	0.95%	Chlorination (2)	Carbonyl (K169, K183)
Immunoglobulin lambda variable 3-15	IKV3-15	1.74%	4-HNE (1) Chlorination (1)	4-HNE (K59) Chlorination (Y50)
Immunoglobulin lambda constant 7	IKL7	0.94%	Nitrosation (1)	Nitrosyl (Y185)
Keratin, type I cytoskeletal 10	KRT10	0.14%	4-HNE (1)	4-HNE (R362)
Myosin-14	MYO14	0.09%	Nitrosation (1)	Nitro (Y275)

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Myosin-9	MYH9	0.15%	Glycosylation (1) Carbonylation (1) Oxidation (1)	Guanidiniol [K158] Carbonyl [D6] Carboxymethyl [K1308]
Oxochalase-1	OVCH1	0.09%	Glycosylation (1)	Guanidiniol [K330]
PHD finger protein 21A	PHF21A	0.15%	Oxidation (1)	Carboxymethyl [K43]
Phosphatidylcholine-sterol acyltransferase	LCAT	0.45%	Glycosylation (1)	Guanidiniol [K242]
Phospholipid transfer protein	PLTP	0.85%	Chlorination (1) Glycosylation (1) Carbonylation (1) Oxidation (1)	Chlorination [K217] Carbonyl [K242] Guanidiniol [K221] Carbonyl [K221] Carboxymethyl [K221]
Plasma protease C1 inhibitor 1	SERPINC1	0.49%	Glycosylation (1) Oxidation (1)	Guanidiniol [K432] Carboxymethyl [K432]
Plasminogen	PLG	0.37%	Nitrosylation (1) Nitrosation (2)	Nitrosyl [D 425] Nitro [Y416, Y425]
POF1 ankyrin domain family member F	POF1F	0.09%	Nitrosylation (1)	Nitrosyl [D 398]
Putative nucleosome assembly protein 1 like 6	NAP1L6	1.87%	Chlorination (1) Glycosylation (1)	Chlorination [Y 83] Guanidiniol [K74]
Retinol-binding protein 4	RBP4	1.49%	Oxidation (2) Nitration (1)	Carboxymethyl [K48, K76] Nitro [Y 89]
Serum albumin	ALB	15.11%	4-HNE (13) Glycosylation (15) Carbonylation (16) Nitrosylation (4) Chlorination (2) Oxidation (30) Nitration (1)	4-HNE [H152, K160, H170, K183, K198, K305, H112, K347, K426, K438, H164, K358, K381] Guanidiniol [K 96], K214, K223, K380, K310, K337, K341, K347, K378, K402, K426, K468, K563, K585, K609 Carbonyl [D07, K160, K361, K383, K214, K223, K300, K365, K381, K337, K375, K426, K438, K549, K565, K589] Nitrosyl [D 172, Y 174, Y196, Y476] Chlorination [D 476, Y182] Carboxymethyl [K 96, K44, K65, K75, K88, K97, K130, K160, K161, K183, K186, K198, K214, K223, K286, K300, K365, K337, K341, K347, K402, K413, K426, K437, K438, K490, K524, K541, K549, K569] Nitro [Y 188, Y 166, Y 172, Y 174, Y 243, Y 336, Y 386, Y 365, Y 377, Y 394, Y 425, Y 476, Y 821]
Serum amyloid A-1 protein	SAA1	1.04%	4-HNE (1) Oxidation (1) Nitrosylation (1)	4-HNE [H10] Carboxymethyl [K198] Nitrosyl [D 9, Y 352, Y36]
Serum amyloid A-4 protein	SAA4	7.09%	Chlorination (1) 4-HNE (1) Nitration (1) Oxidation (2)	Chlorination [Y 39] 4-HNE [K24] Nitro [Y39, Y60, Y90] Carboxymethyl [K26, K104]
Serum paraoxonase/arylesterase 1	PNP1	3.10%	Nitrosylation (1) Glycosylation (2) Carbonylation (2) Oxidation (5) Nitration (1)	Nitrosyl [D 128] Guanidiniol [K1, K290] Carbonyl [K1, K290] Carboxymethyl [K3, K30, K75, K145, K290] Nitro [Y294]
Thymosin beta-10	TMSB10	6.82%	Glycosylation (1) Carbonylation (1) Oxidation (1)	Guanidiniol [K4] Carbonyl [K4] Carboxymethyl [K4]
Thymosin beta-4	TMSB4	6.82%	Glycosylation (1) Carbonylation (1) Oxidation (1)	Guanidiniol [K4] Carbonyl [K4] Carboxymethyl [K4]
Transitional endoplasmic reticulum ATPase	VCP	0.12%	Carbonylation (1)	Carbonyl [K8]
Transmembrane and FPK repeat-containing protein 1	TMTC1	0.34%	Nitration (2)	Nitro [Y 806, Y513] Carboxymethyl [K511]
Trypsin-3	PRSS3	0.39%	4-HNE (1) Glycosylation (1) Carbonylation (1)	4-HNE [H153] Guanidiniol [K155] Carbonyl [K153]
Tubulin alpha-8 chain	TUBA8	0.48%	Glycosylation (1) Carbonylation (1)	Guanidiniol [K124] Carbonyl [K124]
UDP-glucosylglycerol glucosyltransferase 2	UGG12	0.07%	4-HNE (1)	4-HNE [H188]
Vitamin D-binding protein	GC	1.05%	Glycosylation (2) Carbonylation (1) Oxidation (2)	Guanidiniol [K332, K345] Carbonyl [K332] Carboxymethyl [K242, K332]
Vitaminin	VIN	0.21%	Chlorination (1)	Chlorination [D 66]

A1, position of the modified amino-acid; H, histidine; K, lysine; Y, tyrosine. Similar sites of modifications are in bold type.
% PTM, percentage of potential sites of inhibition regarding all the amino-acids of the protein sequence.

9. Concentration de 4-HHE et 4-HNE dans l'IRC

The toxic lipid aldehydes 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE) accumulate in adult patients with chronic kidney disease

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Manuscrit en préparation

La dernière partie de ce travail s'intéresse aux concentrations circulantes des aldéhydes issus de la peroxydation des acides gras polyinsaturés n-3 (4-hydroxy-2-hexenal, 4-HHE) et n-6 (4-HNE) dans la population IRC. Bien que de nombreux travaux aient pointé le rôle de ces aldéhydes dans des mécanismes toxiques (cf partie 4), peu de preuve de leur circulation en quantité importante chez le patient IRC sont disponibles. Une étude chez le patient en hémodialyse a montré que ces aldéhydes circulaient à plus forte concentration chez le patient indemne de toute insuffisance rénale chronique.⁵²

Cependant, aucune donnée n'est disponible chez le patient IRC non dialysé. Dans ce travail, nous avons dosé en spectrométrie de masse couplée à une chromatographie en phase gazeuse, les concentrations circulantes des deux aldéhydes 4-HHE et 4-HNE chez le patient IRC. Le stade d'IRC était validé par une mesure du débit de filtration glomérulaire.

Cette étude confirme que les aldéhydes dosés circulent en plus grande quantité chez les patients IRC et notamment dans les stades les plus avancés de la maladie (IV et V). Il est à noter que les concentrations de 4-HHE sont plus hétérogènes parmi les populations étudiés. Cet aldéhyde est fortement dépendant de l'apport alimentaire en

oméga-3 qui peut fortement varier d'un patient à l'autre. Le même constat peut être fait avec le 4-HNE et l'apport d'oméga-6 même si les différences semblent être vraiment beaucoup plus marquées entre les populations contrôle et IRC de I à III vs IV-V.

Comme ces aldéhydes sont très réactifs sur les protéines (cf partie 6 et 8), nous avons réalisés un immunoblotting sur les protéines plasmatiques totales dans ces populations. Les résultats concernant le 4-HHE montrent une quantité d'adduits plus importante dans les stades les plus sévères de l'IRC (III à V). Par contre, aucune différence n'a été vue sur les adduits de 4-HNE. Cela peut être expliqué d'une part par l'hétérogénéité des patients et du faible effectif d'une part et par la plus grande spécificité de l'anticorps anti-4-HHE (spécifique des histidines modifiées) par rapport à l'anticorps dirigé contre le 4-HNE. En effet, dans notre travail, nous avons mis en évidence un certain nombre d'adduits de 4-HNE présents en plus grande quantité chez le patient hémodialysé non-diabétique (cf partie 6) mais cela avec une méthode beaucoup plus spécifique (spectrométrie de masse).

The toxic lipid aldehydes 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE) accumulate in adult patients with chronic kidney disease

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Key words: chronic kidney disease, toxic lipid aldehydes, oxidative stress, glomerular filtration rate

Abstract

Background: Oxidative stress is a common feature of patients with chronic kidney disease (CKD). Lipid peroxidation produces many reactive by-products including lipid aldehydes, derived from the peroxidation of n-3 and n-6 polyunsaturated fatty acids. Lipid aldehydes are increased in patients undergoing maintenance dialysis, a process known to generate oxidative stress. However, there is no data for patients before initiation of renal replacement therapy (RRT).

Methods: We prospectively evaluated the level of two major lipid aldehydes, 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE) and their relationship with the decline of glomerular filtration rate (GFR) in CKD patients.

Forty patients with CKD stage 1-5 and 13 non-CKD patients were prospectively recruited. GFR was measured by iohexol or inulin clearance. 4-HNE and 4-HHE were assayed in plasma by gas chromatography–mass spectrometry and their covalent adducts on proteins were quantitated by immunoblotting.

Results: Plasma 4-HHE concentration gradually increased from CKD stage I-II to CKD stage IV-V when compared to non-CKD patients (4.5-fold higher in CKD IV-V, $P<0.005$). In contrast, 4-HNE concentration only increased for CKD stage IV-V patients (6.2 fold, $P<0.005$). The level of protein adducted with 4-HHE was 9.5-folds higher in patients with CKD ($P<0.005$) compared to controls while no difference was noticed for 4-HNE adducts.

Conclusions: Plasma levels of both 4-HNE and 4-HHE sharply increased in CKD IV-V patients unambiguously evidencing the presence of oxidative stress and lipoperoxidation before the initiation of renal replacement therapy. 4-HHE and 4-HNE accumulation in end-stage renal disease could, as documented in other pathologies, exert deleterious effects.

Introduction

Patients with chronic kidney disease (CKD) exhibit a high incidence rate of cardiovascular diseases (1, 2). Oxidative stress, i.e. an imbalance between free radical production and antioxidant defences, is a common feature of the uremic syndrome(3 – 5). Oxidative stress, which also contributes to the inflammatory process, accelerates renal injury and promotes cardiovascular dysfunctions(6–9). During this process, cell and tissue damages can result from the direct attack by free radicals, but also from several oxidation by-products resulting from the oxidative breakdown of biomolecules. Possibly more deleterious than radical species themselves, these secondary oxidation by-products can diffuse within the body fluids to propagate the noxious effects of oxidative stress. Polyunsaturated fatty acids (PUFA) are major targets of oxidative stress, and their degradation yields a myriad of toxic electrophilic compounds(10). Several oxidation by-products are the results of fatty acid peroxidation, a non-enzymatic process initiated by free radical attack on the double bonds of PUFAs. Lipid peroxidation leads to the production of many reactive carbonyl compounds (RCCs) such as malondialdehyde (MDA), acrolein or 4-hydroxy-alkenals, among which the more studied are 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxy-2-hexenal (4-HHE)(10). 4-HNE is a 9-carbon lipid aldehyde issued from the peroxidation of n-6 PUFAs (11) while 4-HHE is a 6-carbon lipid aldehyde issued from the peroxidation of n-3 PUFAs (12). Because of their high reactivity, the lipid aldehydes are thought to be involved in many pathophysiological processes associated with oxidative stress (13). 4-HNE and 4-HHE exhibit important electrophilic properties that make them prone to react with many classes of biomolecules such as phospholipids, nucleic acids or proteins (to form advanced lipoperoxidation by-products or ALEs)(13 – 15). A major determinant of the toxicity of lipid aldehydes is their ability to covalently modify

protein reactive groups. HNE has been found to promptly react with sulfhydryl and amino groups in proteins especially from cysteine and histidine residues (16,17). HNE protein adducts have been detected in many circulating proteins including serum albumin (18) and low density lipoproteins (LDL) (19). While 4-HNE received extensive attention, the toxicity of 4-HHE is however less well documented (20). We have previously demonstrated that the cytotoxicity of 4-HHE and 4-HNE was related to their ability to form covalent adducts on proteins (21) that can potentially cause functional damage to proteins. For instance, many enzymes were reported to be inactivated following structural modification with lipid aldehydes (22–25). Most RCC identified in the plasma of CKD patients are derived from carbohydrates, such as glyoxal, methylglyoxal or pentosidine (26,27). In contrast, RCCs from lipid origin only received limited attention in CKD. To the best of our knowledge, only one study reported that the concentrations of other lipid aldehydes (length 6-12 carbons) were increased in haemodialysis patients (28). In spite of this limited set of data, 4-HNE and 4-HHE were recognized in 2013 as uremic toxins, belonging to the group of water-soluble low molecular weight molecules, by the European Uremic Toxin Workgroup (EUTOx, <http://www.uremic-toxins.org/>) (29).

RRT such as hemodialysis is known to increase oxidative stress (30) and thus promote lipid peroxidation. However, there is no data in the literature focusing on the plasma levels of reactive lipid aldehydes in non-dialysed CKD patients. The present study was designed to evaluate the levels of two major lipid peroxidation products: 4-HNE and 4-HHE and their relationship with the decline in glomerular filtration rate (GFR) in CKD patients.

Material and Methods

Ethic statement

This research was approved by the local institutional review board (Comité de Protection des Personnes - Recherche Biomédicale, CPP Lyon Sud-Est IV) and conducted in accordance with the ethical standards and the principles of the Declaration of Helsinki. All subjects involved in the research signed written informed consent forms prior to enrolment.

Subjects

A total of 53 subjects (32 men), among which 13 non-CKD subjects, were recruited from Nephrology department of the Edouard Herriot University Hospital (Lyon, France). This study was proposed to CKD patients who were referred for GFR measurement excluding renal transplant recipients. Exclusion criteria were age >65 years and the main pathologies other than CKD, known to be associated with oxidative stress (diabetes, obesity defined with a body mass index >30 kg/m², systemic inflammatory, autoimmune diseases or known active malignant diseases). Non-CKD patients were subjects who were candidates for kidney donation.

Blood sampling

After an overnight fast, blood samples were obtained by venipuncture. Blood samples were centrifuged at 3500 × g for 10 min to isolate plasma supernatant which was snap frozen in liquid nitrogen and stored at -80°C until use.

Glomerular filtration rate measurements (mGFR)

GFR was measured by the gold standard method, i.e. inulin or iohexol clearance (expressed as ml/min per 1.73 m²). Urinary inulin clearance was performed for 32 subjects. Briefly, inulin (Polyfructosan[inutest], Laevosan, Linz, Austria) was infused continuously for 3 hours after a priming dose, and urine was collected every 30 minutes

by spontaneous voiding and blood samples were obtained in the middle of each period of urine collection (three to four collection periods of 30 minutes). Inulin concentration was assayed by the enzymatic method. Inulin clearance was calculated in each period (UV / P) to obtain the average (where U is urinary inulin, V is urine volume and P is plasmatic inulin). For iohexol clearance, iohexol (300 mg; Omnipaque; GE Healthcare SAS, Vélizy-Villacoublay, France) was injected. Blood collection was performed at 120, 180, and 240 minutes. The serum iohexol concentration was measured by HPLC. The GFR was calculated as $GFR = \text{slope} \times \text{dose} / \text{concentration at time 0}$ corrected with the Bröchner–Mortensen equation. CKD stages were determined according to the Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines.

4-Hydroxy-2-alkenals: derivatization, analysis, and quantification

4-Hydroxy-2-alkenals were assayed by gas chromatography coupled to mass spectrometry (GC-MS) as previously described (31). The detailed protocol of GC-MS assay and typical chromatogram are presented in the supplemental material.

Dot blot: 4-HHE and 4-HNE protein adducts

Unless mentioned, all solvents were from Carlo Erba (Peypin, France) and all chemicals from Sigma Aldrich (Saint Quentin Fallavier, France). Anti-HNE-Michael adduct (ref 393207) and anti- HHE-Michael adduct (ref NOF-N213730-EX) antibodies were from Calbiochem (San Diego, USA) and Cosmobio (Tokyo, Japan), respectively. The antibody used to detect 4-HHE-adducts (immunogen: HHE modified keyhole-lympet hemocyanine) is highly specific for 4-HHE-Michael adducts on histidine residues and therefore enables the specific detection of HHE-histidine in protein samples

4-HHE and 4-HNE Michael adducts on plasma protein were quantitated by dot blot as previously described (25,32) . Briefly, 50 μg micrograms of proteins were loaded directly on a nitrocellulose membrane using the Bio-Dot apparatus (BioRad, Marne-la-

Coquette, France). Following saturation with 5% bovine serum albumin (BSA), membranes were probed overnight with primary antibodies, anti-HHE-Michael adducts, or anti-HNE-Michael adducts. After incubation with HRP-coupled secondary antibodies, membranes were processed for chemiluminescence (ECL plus, GE Healthcare) and quantitated by densitometry using Image J software (NIH, USA).

Other biochemical measurements

Plasma urea, bicarbonate, hemoglobin, protein and albumin were measured by standard laboratory methods in a certified laboratory. Serum creatinine measurement was performed with the Siemens enzymatic method (Vista 1500; Siemens Healthcare, Erlangen, Germany) traceable to National Institute of Standards and Technology Creatinine Standard Reference Materials 914 (verified with National Institute of Standards and Technology SRM 967) with calibration certified by isotope dilution mass spectrometry.

Statistical analysis

Data were analysed using Graphpad Prism 5.0 (GraphPad softwares, La Jolla, USA) and Statview 4.5 (Abacus concept, Berkeley, USA) softwares. The data are expressed as mean \pm 1 standard deviation (SD) or as median (interquartile range - IQR) when variables were not normally distributed. Distributions were tested for normality using d'Agostino-Pearson test. Differences between groups were assessed by Kruskal & Wallis test followed when appropriated by Dunn tests. Sex ratio and medications between groups were compared using Fisher exact test. Univariate analysis was performed using the Spearman rank correlation method. A $P < 0.05$ was considered as statistically significant in all analysis.

Results

Patient data

We recruited 40 patients with CKD stage 1 to end-stage 5 (aged 47 ± 14 years) and 13 non-CKD participants (aged 44 ± 13 years, $P=0.235$). Their renal diseases were glomerulonephritis ($N=9$ but none from diabetes), renovascular disease ($N=6$, including 3 nephroangiosclerosis), 6 solitary kidney, 4 tubulointerstitial disease, 5 cystic kidney disease and of other or unknown etiologies in 10 patients. Out of the 40 CKD patients, 14 were on antihypertensive drugs (35%, $P=0.045$) while 8 were on lipid lowering drugs (20%, $P=0.322$). The main clinical, anthropometric and biochemical characteristics of the CKD patients and controls are summarized in **Table 1**. As expected, mGFR ($P<0.001$), plasma creatinine ($P<0.001$) and urea ($P<0.001$) were the main differences between the groups.

Plasma concentration of 4-hydroxy-alkenals increases in ESRD patients

We used a specific gas chromatography–mass spectrometry (GC-MS) assay to perform the measurement of 4-HHE and 4-HNE in plasma from CKD and non-CKD subjects (31) (**Figure 1**). Plasma 4-HHE concentration was 37 nM (17-63) in non CKD subjects while it was 145 nM (40-284) in CKD patients (3.9-fold, $P<0.005$) (**Figure 1A**). In contrast, no significant difference was noticed in 4-HNE concentration (non-CKD vs CKD, 13(12-19) vs 14 (11-21) nM, $P=0.679$) (**Figure 1B**). Plasma 4-HHE concentration gradually increased from CKD stage 1-2 to CKD stage 4-5 when compared to non-CKD subjects (**Figure 2A**). However, this increase was statistically significant from CKD stage 3 ($P<0.01$ vs control, $P<0.05$ vs CKD stage 1-2) and was 4.5-fold higher in CKD 4-5 patients (174.6 (interquartile range (IQR) 61.5–305.2) nmol/L) compared with non-CKD subjects (37.3 (18.6-63.0) nmol/L, Dunn's test, $P<0.005$). In contrast to 4-HHE, 4-HNE concentrations did not gradually increase from CKD stage 1-2 to CKD stage 4-5 (**Figure 2B**) since concentrations were only

significantly different for CKD stage 4-5 patients and were 6.2-fold higher (67.4 (8.6–97.8) nmol/L) compared with non-CKD subjects (12.7 (11.8 – 18.8) nmol/L, Dunn’s test, $P<0.005$).

Advanced lipoperoxidation by-products

4-HNE and 4-HHE are prone to react with proteins to form covalent adducts (10). We therefore used specific antibodies directed against 4-HNE and 4-HHE Michael adducts to detect covalent adducts on plasma proteins using immunoblotting (i.e. dot blot). Regardless of CKD stage, CKD patients exhibited an increased level of 4-HHE adducts on plasma proteins (**Figure 3**). The level of protein adducted with 4-HHE was 115 (92-147) arbitrary unit (AU) in non-CKD subjects and 1093 (587-3984) AU in patients with CKD (Mann & Whitney U test, $P<0.005$), i.e. a 9.5-fold increase. In contrast, no significant difference was noticed in 4-HNE adducts (1617 (1129-2501) and 1863 (1450-2524) AU for non-CKD and CKD patients, respectively (**Figure 4**). To get further insight, ALEs were studied according to the actual stage of CKD which was evaluated with measured GFR. 4-HHE protein adducts gradually increased with the decline of GFR (**Figure 3B**) while 4-HNE protein adducts were not correlated with the CKD stage (**Figure 4B**).

Relationships between plasma 4-hydroxy-alkenal concentration and clinical or biochemical parameters

The results of the univariate correlation analysis are summarized in **Table 2**. Plasma 4-HHE concentration positively correlated with age ($r_s=0.430$, $P<0.005$) and negatively with GFR ($r_s=-0.377$, $P<0.05$). Plasma 4-HNE concentration negatively correlated with GFR ($r_s=-0.444$, $P<0.005$) and positively correlated with age ($r_s=0.326$, $P<0.05$),

plasma creatinine level ($r_s=0.628$, $P<0.005$) and urinary albumin/creatinine ratio ($r_s=0.307$, $p<0.05$). Plasma 4-HHE concentration positively correlated with 4-HHE adducts on proteins ($r_s=0.661$, $P<0.005$) while no such trend was noticed for 4-HNE.

Discussion

Few studies reported that plasma hydroxyl-alkenals (among which 4-HNE and 4-HHE) concentrations were increased in chronic hemodialysis patients (28). However, there is no data in the literature reporting the actual concentration of 4-HNE and 4-HHE in CKD patients before initiation of renal replacement therapy, a process known to exacerbate oxidative stress(30,33). We reported in the present study that plasma levels of both 4-HNE and 4-HHE sharply increase in CKD 4-5 patients. Plasma levels of 4-HHE (4.5-fold) and 4-HNE (6-fold) were significantly higher in CKD 4-5 patients than in controls unambiguously evidencing the presence of oxidative stress and lipoperoxidation before the initiation of renal replacement therapy. These results are in good agreement with the report of Alhamdani et al (28) who observed an increase in both 4-HHE (2.5-folds) and 4-HNE (7-folds) in hemodialysis patients. These data confirmed that the oxidative stress and lipoperoxidation results from the uremic environment per se rather than from the hemodialysis process. Many works suggest that accumulation of uremic toxins could contribute to increased oxidative stress in uremia (34–41).

4-HHE and 4-HNE exhibited different patterns of accumulation. Indeed, while 4-HHE level gradually increases with the decline of GFR (reaching statistical significance at stage 3), 4-HNE only increased in CKD stage 4-5. Free 4-HHE concentration was positively correlated with the amount of 4-HHE adduct on plasma proteins while free 4-HNE concentration was not correlated with the 4-HNE adducts

on plasma proteins (see Table 2). The relative proportion of 4-HHE and 4-HNE accumulated in ESRD deserves some comments. The proportion of 4-HHE to 4-HNE depends of two main factors: First, the availability of omega 3 PUFAs and omega-6 PUFAs, which largely depends from the dietary habits (e.g. intake of low/large amount of omega-3 PUFAs). Secondly, the relative activity of the various detoxifying enzymes and their respective affinity for 4-HNE and 4-HHE. Detoxification of lipid aldehydes involves several enzymes, such as for instance glutathione S-transferase (GST), aldehyde dehydrogenase (ALD) and/or fatty aldehyde dehydrogenase (FALD). Hubatsch et al. (42) reported that 4-HHE is a poor substrate for GSTA4-4 compared to HNE. Long et al (43) demonstrated that HHE is a poorer substrate than HNE for the mitochondrial ALDH5A while both of them are detoxified by aldose reductase (44). Some differences in detoxification and disposition could therefore account for the different levels of 4-HNE and 4-HHE accumulated in ESRD patients. Some data suggest an overexpression of GST in hemodialysis patients (45) but there is further information about the other detoxifying enzymes.

In an experimental model, increased lipid peroxidation was shown to play a pathobiologic role for glomerulonephritis (46). A pathological role of lipid peroxidation was also suggested in CKD (47). Bae et al (2011) (48) demonstrated in vitro that 4-HHE induces HK-2 tubular cells apoptosis suggesting that it could contribute to pathogenesis of kidney injury. HHE treatment resulted in a dose-dependent decline of tubular cells viability associated with an over-expression of nuclear factor kappa-B (NF- κ B) and a decrease of Inhibitor of nuclear factor kappa-B kinase beta (IKK β). Nevertheless, the concentrations of 4-HHE used in this study (5-100 μ M) were unrealistic, largely exceeding the concentrations found in human plasma from CKD patients (28) (i.e. $<1\mu$ M, from the present study). However, the lipid peroxidation by-

products play an important role in inflammation. 4-HNE and many other aldehydes have been shown to display a chemotactic activity for leukocytes from a concentration of 0.1 μ M (49,50) suggesting that these compounds could contribute to micro-inflammation commonly observed in CKD.

4-HHE and 4-HNE are reactive aldehydes that are prone to covalently bind to plasma proteins. Lipid aldehydes can react with proteins through the formation of Schiff bases (with the amino group of lysine residues) and/or Michael adducts (with thiol or amino groups of cysteine, lysine or histidine residues) (51). This effect is therefore thought to be pivotal in the toxic effect of hydroxy-alkenals. Lipid aldehydes have been shown to bind to serum albumin (18) and many other plasma proteins and may even change albumin structure and, hence, also its binding capacity for other ligands. The lipid aldehydes exhibit therefore the double potency to behave as small molecules but also as protein bound uremic toxins. This type of interactions (i.e. covalent) with proteins is very different from more classical protein-bound retention solutes, such as indoxyl sulfate or p-cresyl sulfate. This covalent binding of lipid aldehydes can be regarded as irreversible in contrast to the other ligands, which display a competitive binding with 2 specific binding sites on albumin. It is worth noting that adducted albumin can actually exert deleterious biological activities (52) therefore behaving itself as a secondary toxin. In our study, in contrast to 4 HHE, the increase of plasma 4 HNE was not accompanied with a parallel increase of its protein adducts. This discrepancy may be related to the difference in the biological properties of the two aldehydes that is partially related to their carbon chain length (6 and 9 carbons for 4 HHE and 4 HNE, respectively). This structural difference may result in large difference of solubility and reactivity with amino acids. For instance, in vitro studies have demonstrated the formation of adducts incorporating multiple HNE molecules (19).

Furthermore, as we have only plasma concentrations, we can't exclude a difference in tissue accumulation of 4 HNE adducts conferring intracellular toxicity. Indeed, 1-8 % of 4 HNE that is generated in cells would react with proteins and the majority of the target enzymes for 4-HNE are mitochondrial proteins (53). Further studies are needed to investigate the difference in the metabolism of these two aldehydes in CKD.

According to the EuTox workgroup (29), a uremic toxin can be defined as a compound that first is normally excreted in urine and thus accumulates in patients with CKD as a result of the decline in renal clearance and that exerts deleterious effects on biological systems. 4-HHE and 4-HNE are partly excreted by the kidney in the form of 4-hydroxy-2-hexenoic acid (4-HHA), 4-hydroxy-2-nonenoic acid (4-HNA) or as mercapturic acid (54) but kidney is not the major site of their metabolization. Indeed, the main site of hydroxyalkenals biotransformation is the liver (54) and the subsequent metabolites are excreted by the kidneys. 4-HHE and 4-HNE do not accumulate in CKD as the sole result of a decreased renal clearance and thus according to the EuTox definition, are not *stricto sensu* uremic toxins.

Conclusion

We reported in the present study that plasma levels of both 4-HNE and 4-HHE sharply increased in CKD 4-5 patients evidencing the presence of oxidative stress and lipoperoxidation before the initiation of RRT. These data confirmed that the oxidative stress and lipoperoxidation result from the uremic environment *per se* rather than from hemodialysis process. 4-HHE and 4-HNE accumulated in ESRD as well as lipoperoxidation by-products (e.g. protein adducts) could, as documented in other

pathologies, exert toxic effect and therefore be regarded as a “uremic toxin like” compound.

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Disclosure

The authors have nothing to disclose.

Author Contributions

COS and FGE conceived and designed the study. CCP, SL and LD included the patients and collected the clinical data. COS and NF performed the experiments. COS performed statistical analysis. COS and FGE interpreted results and wrote the manuscript. CCP, NF, SL, LD and LJ edited and revised the manuscript.

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Table 1. Demographic and clinical data for the non CKD and CKD participants

	Non CKD	Stage 1-2	Stage 3a-3b	Stage 4-5	P-value
Gender, M/F	9/4	8/11	4/6	9/2	0.092
Age, y	43.9 ± 13.0	44.8 ± 13.5	52.8 ± 14.3	45.9 ± 14.9	0.424
Body weight, kg	73.9 ± 11.4	65.3 ± 16.0	67.7 ± 17.9	77.5 ± 12.5	0.129
Height, m	1.72 ± 0.10	1.72 ± 0.12	1.67 ± 0.10	1.75 ± 0.12	0.285
BMI, kg/m ²	24.9 ± 2.1	23.0 ± 2.7	23.9 ± 4.0	25.2 ± 3.0	0.158
SBP, mmHg	130 ± 14	127 ± 20	135 ± 18	136 ± 18	0.487
DBP, mmHg	82 ± 14	81 ± 14	87 ± 13	90 ± 11	0.334
mGFR, ml/min/1.73m ²	102 ± 9	75 ± 11	46 ± 8	20 ± 6	< 0.001
Creatinine, μM	66 ± 17	82 ± 17	127 ± 41	393 ± 149	< 0.001
Urea, mM	5.3 ± 1.6	6.4 ± 2.4	9.0 ± 2.4	14.7 ± 6.6	< 0.001
Bicarbonate, mM	26.3 ± 2.0	25.2 ± 2.9	24.6 ± 2.7	22.6 ± 2.9	0.143
Protein, g/L	76.7 ± 5.0	72.5 ± 15.0	75.6 ± 2.3	72.3 ± 4.9	0.808
Hypertension, %	30.0	36.8	40.0	54.5	0.468
Lipid-lowering therapy, %	0	0	20	54.5	0.006
RAA inhibitors, %	0	21.1	40	54.5	0.038

Data are expressed as mean ± SD. Abbreviations : BMI, body mass index, CKD, chronic kidney disease, DBP, diastolic blood pressure, mGFR, measured glomerular filtration rate, RAA, Renin angiotensin aldosterone, SBP Systolic blood pressure. GFR was measured using urinary inulin clearance. Differences were considered significant at the P<0.05 level CKD vs non CKD.

Table 2. Univariate correlations with 4-HNE or 4-HHE plasma concentrations

	4-HNE		4-HHE	
	r_s	P-value	r_s	P-value
Age, y	0.326	0.022	0.430	0.004
BMI, kg.m ⁻²	0.068	0.640	0.253	0.098
MAP, cm Hg	0.161	0.269	0.223	0.145
Proteins, g.L ⁻¹	0.011	0.953	-0.201	0.305
4-HHE, ng/ml	0.146	0.318	-	-
Bicarbonate, mM	-0.075	0.688	0.070	0.729
mGFR, ml/min/1.73m ²	-0.444	0.002	-0.377	0.012
Urea, mM	0.146	0.337	0.164	0.312
Creatinine, μM	0.628	<0.001	0.152	0.326
Urine albumin/Creatinine ratio	0.307	0.042	-0.067	0.685
4-HNE protein adducts, AU	0.163	0.457	0.408	0.083
4-HHE protein adducts, AU	-0.043	0.858	0.661	0.003

Abbreviations: AU, arbitrary unit, BMI, Body mass index, HDL, high density lipoprotein, 4-HHE, 4-hydroxy-2-hexenal, 4-HNE, 4-hydroxy-2-nonenal, LDL, low density lipoprotein, mGFR: measured glomerular filtration rate, MAP, mean arterial pressure, mGFR was measured using inulin or iothexol clearance. Correlations were considered significant at the P<0.05 level.

Figure legends

Figure 1. Plasma free 4-HHE concentrations is increased in CKD patients. 4-HHE (A) and 4-HNE (B) concentrations were quantified in plasma from non-CKD subjects (N = 13) and chronic kidney disease (CKD stage 1 to 5, N = 40) patients by GC-MS as described in methods. The horizontal bar indicates the median. $P < 0.05$ was considered significant.

Figure 2. Plasma free 4-HNE and 4-HHE concentrations are increased in CKD patients according to the stage of chronic kidney disease. 4-HHE (A) and 4-HNE (B) concentrations were quantified in plasma from non-CKD subjects (N = 13) and chronic kidney disease (CKD stage 1 to 5, N = 40) patients by GC-MS as described in methods. GFR was measured using inulin or iohexol clearance. Data are presented as median (interquartile range). * $P < 0.05$, ** $P < 0.01$, ns non significant.

Figure 3. Measurement of 4-HHE Michael adduct on plasma proteins from non-CKD subjects and CKD patients. Michael adducts of 4-HHE with proteins were detected after dot blot using specific antibodies to 4-HHE protein adducts. A) Typical blots. B) Quantification data, obtained by densitometry, are presented as median (interquartile range). Differences were considered significant at the $P < 0.05$ level. Note that 4-HHE adducts on proteins gradually increased with the decline of renal function.

Figure 4. Measurement of 4-HNE Michael adduct on plasma proteins from non-CKD subjects and CKD patients. Michael adducts of 4-HNE with proteins were detected after dot blot using specific antibodies to 4-HNE protein adducts. A) Typical blots. B) Quantification data, obtained by densitometry, are presented as median (interquartile range). Differences were considered significant at the $P < 0.05$ level. Note that 4-HNE adducts only increased in CKD stage 4-5 patients.

Figure 1

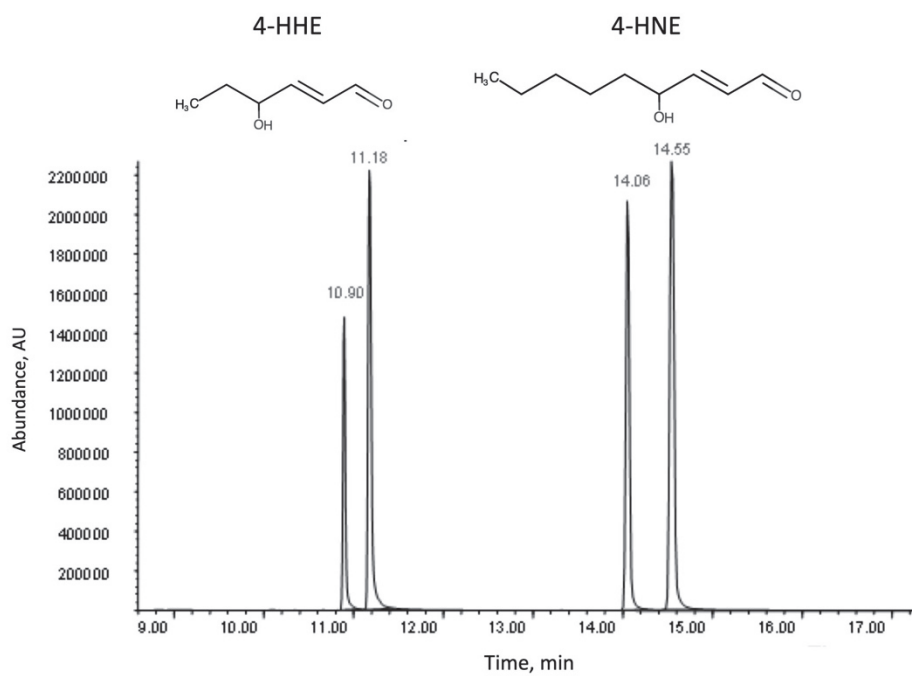


Figure 2

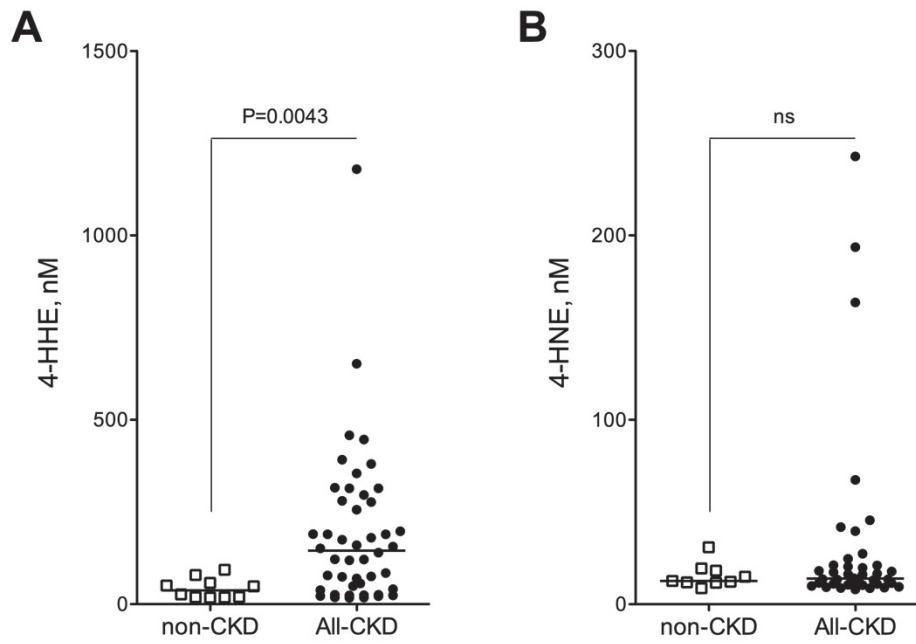


Figure 3

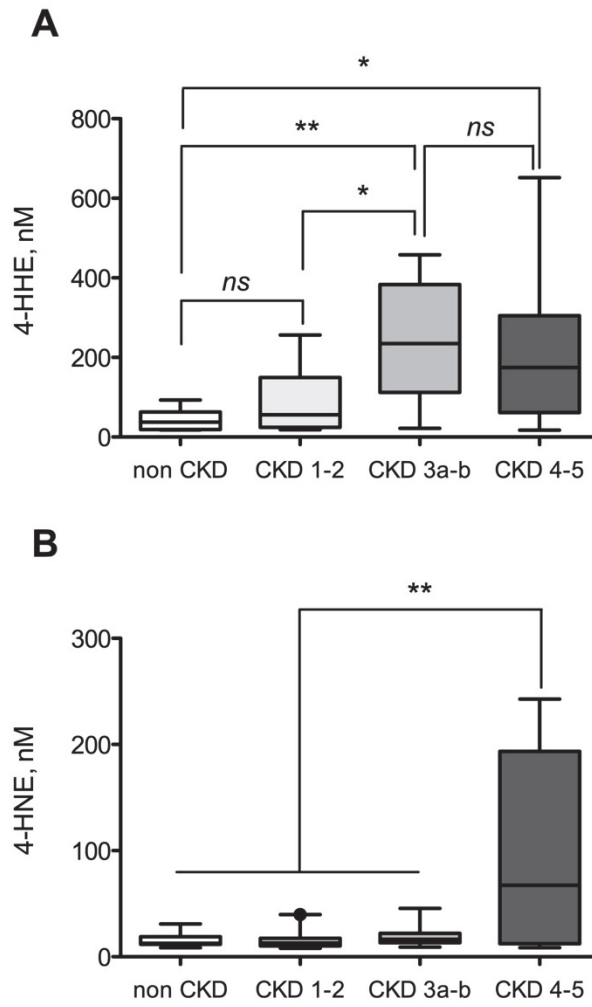
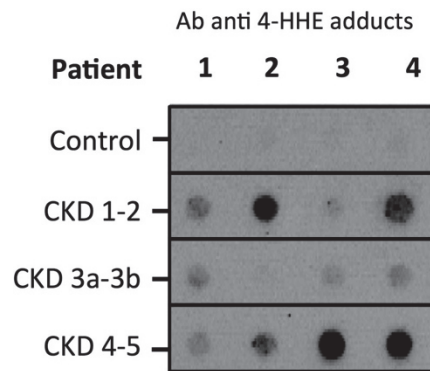


Figure 4

A



B

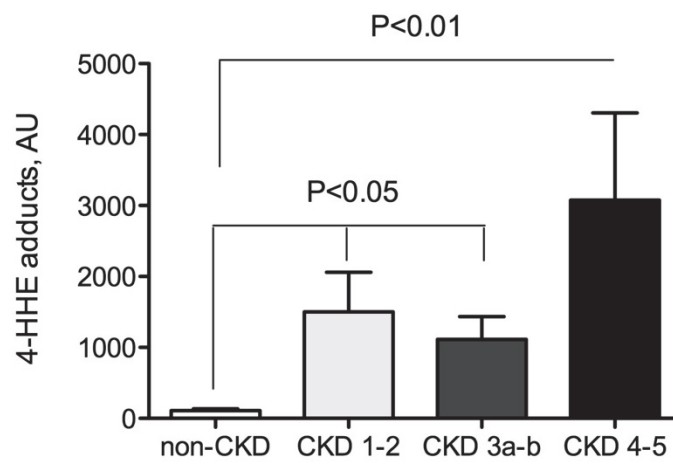
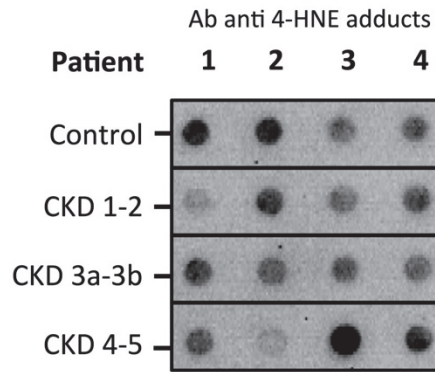
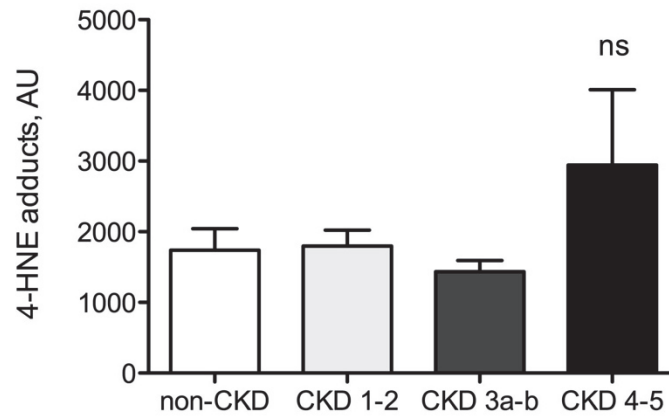


Figure 5

A



B



10. Discussion générale

L'insuffisance rénale chronique reste grevée d'une morbi-mortalité cardiovasculaire importante malgré l'amélioration continue de la prise en charge et des techniques de suppléances rénales.^{2,74} L'échec de stratégies classiques de réduction du risque cardiovasculaire comme la prescription de statines dans l'IRC dialysée^{5,6,75} nous amène à plusieurs constats. Le premier est que cette population présente une gravité sur le plan cardio-vasculaire qui rend ces thérapeutiques inefficaces. Deuxièmement, le taux de LDL circulant chez les patients en hémodialyse est déjà naturellement bas ce qui grève d'une grande partie l'effet protecteur dû à la réduction du LDL cholestérol. Troisièmement, la cible des statines n'étant vraisemblablement pas la bonne, il paraît intéressant de s'intéresser aux lipoprotéines qui ne sont pas non affectées par cette prescription, à savoir les HDL.

Les HDL sont des lipoprotéines protectrices du système cardio-vasculaire

Ces lipoprotéines sont reconnues comme anti-athérogènes car elles exercent plusieurs propriétés biologiques reconnues comme protectrices.⁷⁶ Toutes ces fonctions ont été décrites comme étant altérées dans l'IRC (**Figure 14**). Les HDL sont responsables d'un efflux de cholestérol des macrophages qui est pris en charge par ces lipoprotéines et qui va permettre la clairance du cholestérol de la périphérie vers le foie. Ce mécanisme est connu pour être important dans l'athéro-protection.⁵⁹ L'efflux de cholestérol est altéré dans l'IRC comme le souligne plusieurs publications.^{60,61,77-79} Les HDL favorisent également une vasodilatation médiée par la sécrétion de NO. Cette propriété est aussi retrouvée comme altérée dans l'IRC.^{61,80} La troisième propriété est leur vertu anti-inflammatoire qui est elle aussi altérée dans l'IRC.^{61,79} La dernière est l'effet anti-agrégant des HDL qui semble aussi être altérée dans l'IRC (cf partie 6). Ainsi, l'ensemble des propriétés protectrices des HDL ont été trouvées au moins une fois

comme altérées dans la littérature. Cela renforce l'idée d'une implication de ces lipoprotéines dans le sur-risque cardio-vasculaire observé dans l'IRC. Il persiste cependant deux questions : 1) ces dysfonctions sont-elles des bons marqueurs prédictifs du risque cardio-vasculaire en hémodialyse ? 2) quels sont les mécanismes physiopathologiques qui sous-tendent ces dysfonctions ?

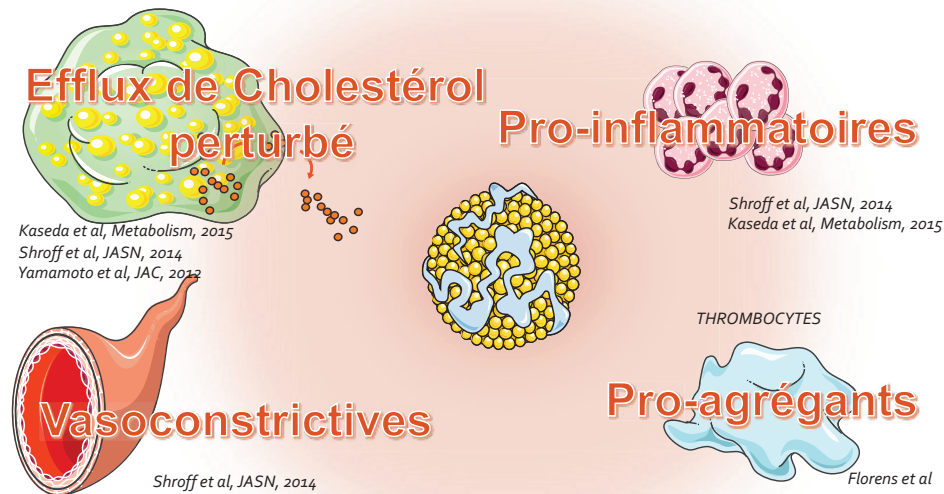


Figure 15 – Propriétés des HDL altérées dans l'insuffisance rénale chronique

Peut-on utiliser les HDL comme des marqueurs du risque cardio-vasculaire en IRC ?

Concernant le premier point, une étude chez l'hémodialysé n'a pas pu mettre en évidence de lien entre les niveaux de HDL cholestérol, d'ApoA1 ou C3 et le risque cardio-vasculaire.⁸¹ Parallèlement, une analyse de la capacité d'efflux de cholestérol induit par les HDL des patients issue de la cohorte 4D⁵ n'a pas pu mettre en évidence de relation directe entre cette dysfonction et la survenue d'évènements cardio-vasculaires en hémodialyse.⁸² Cela indique que cette propriété bien qu'essentielle n'est pas suffisante pour expliquer un sur-risque cardio-vasculaire. Cette cohorte était cependant essentiellement composée de diabétiques et n'est pas forcément extrapolable à l'ensemble des patients hémodialysés. Il n'existe cependant pas de

données concernant l'ensemble des fonctions biologiques des HDL et le risque cardio-vasculaire mais les quelques données de la littérature restent décevantes. Cependant, les HDL peuvent se révéler associées d'une autre manière au risque cardio-vasculaire en hémodialyse. Pour cela, il faut s'intéresser aux mécanismes potentiels qui sous-tendent la dysfonction des HDL dans cette population.

Les modifications du protéome ou de l'adductome des HDL dans l'IRC contribuent-elles à leur dysfonction ?

D'une part, nous avons mis en évidence dans la partie 7, une modification importante du protéome des HDL chez les patients hémodialysés chroniques. Ces modifications ont déjà été exploré auparavant dans d'autres travaux.^{60,83,84} Les HDL sont des lipoprotéines complexes dont le protéome contient plusieurs centaines de protéines. Un certain nombre de ces protéines sont impliquées dans les grandes fonctions biologiques des HDL mais pour leur vaste majorité, leurs rôles précis restent inconnus. On retrouve cependant des toxines urémiques comme la β 2-microglobuline et le Complement Factor D. Il est ainsi probable qu'elles exercent par là une certaine forme de toxicité d'autant plus que, pour certaines, leur concentrations ont été négativement corrélées à la présence de protéines fondamentales comme la paraoxonase-1 dont la concentration est associée à la survie chez le patient hémodialysé.^{85,86} D'autre comme la SFTPB ne semble pas exercer de toxicité directe mais sont associées à la fonction rénale résiduelle chez le patient dialysé et à l'exposition à la cigarette. Dans nos travaux, cette protéine était aussi négativement corrélée à la présence de paraoxonase-1, ce qui peut être considéré comme une première description de toxicité. Ainsi, les analyses protéomiques apportent un grand nombre de données qualitatives sur le protéome des HDL. A une plus large échelle, il serait probablement plus intéressant de considérer les corrélations entre la présence de ces protéines avec des protéines fondamentales plutôt que la présence *per se* d'une protéine. En

effet, le « remplacement » d'une protéine essentielle par une protéine qui au premier abord semble inutile dans les fonctions biologiques ici des HDL, pourrait également constituer une certaine forme de toxicité.

On peut d'un autre côté expliquer une partie de la dysfonction des HDL par les modifications post-traductionnelles qui interviennent sur leurs portions protéiques. En effet, les fractions oxydées des HDL (c'est à dire présentant des adduits de malondialdéhyde sur les protéines) sont associées à une surmortalité cardiovasculaire dans une étude de cohorte de patients prévalents en hémodialyse.⁸⁷ Il est cependant impossible de savoir si ces modifications sont un indicateur du risque ou en sont bel et bien responsables. De nombreuses constatations dans la littérature font état de la toxicité des protéines présentant des modifications post-traductionnelles. Dans une étude sur les LDL, Holy et al ont démontré que la carbamoylation des LDL étaient responsables de propriétés pro-agrégantes et que les LDL issues de patients IRC contenant un nombre significatif de résidus de carbamoylation et présentaient des propriétés similaires.³⁵ Dans une étude similaire, Speer et al ont démontré que ces fractions carbamoylées des LDL entraînaient aussi de la dysfonction endothéliale.⁸⁸ Les fractions carbamoylées du LDL ont aussi été associées à de la dysfonction macrophagique et endothéliale.³⁴ D'autres protéines ont aussi révélées des propriétés potentiellement toxiques lorsqu'elle présentaient des modifications post-traductionnelles. C'est le cas de l'albumine, dont un certain nombre de propriétés sont altérées par les modifications post-traductionnelles (capacité de transport de solutés, ^{89,90} agrégation des globules rouges, ⁹¹ pression oncotique^{92,93}). Ces modifications vont même influencer l'acuité des techniques de dosage, en effet la carbamoylation semble profondément modifier la précision des techniques de dosage classiques comme le bleu de bromocrésol⁹² et même de référence comme l'immunonéphélométrie.⁹⁴ Le fibrinogène peut quant à lui être carbamoylé et présenter des anomalies de structures

tridimensionnelle lors de sa transformation en réseau de fibrine et ainsi exercer des propriétés pro-inflammatoires.⁹⁵

Ainsi, les HDL peuvent elles aussi logiquement subir des modifications post-traductionnelles qui vont en altérer les fonctions. La dysfonction endothéliale était remarquée avec des HDL issues de patients en IRC terminale⁸⁰ et expliquée par des taux élevés de symétric diméthylarginine (SDMA). L'action des myéloperoxydases et la génération de 3-chloro-tyrosine sur l'ApoA1 est associée à une perturbation des capacités d'efflux de cholestérol médiées par le HDL.³⁸ Dans nos travaux, nous avons pu relier de façon directe la carbonylation par le 4-HNE et les propriétés pro-agrégantes des HDL (cf partie 6). Cependant, ces adduits n'étaient pas majoritaires lorsque nous avons exploré l'adductome de ces HDL. En effet, la glycation représentée par la présence de CML était la première modification dans une population expressément choisie comme non-diabétique. En terme de quantité d'adduits, la guanidinylation, une modification peu étudiée dans l'IRC, arrivait en deuxième position. La carbamoylation était quant à elle troisième suivie par la nitrosylation et la carbonylation par le 4-HNE (cf partie 8).

Il n'est cependant pas possible de déterminer laquelle de ces modifications est la plus délétère. Il est probablement plus pertinent de considérer le site de modification sur la protéine plutôt que sa qualité. En effet, nous avons montré qu'une certaine partie de ces adduits se situaient sur des acides aminés appartenant à des portions clés des protéines constituant les HDL (cf partie 6, Figure 3 de l'article). La modélisation in-silico pourrait à l'avenir être une aide précieuse dans la prédiction de l'effet des modifications post-traductionnelles. Il y a fort à penser qu'une modification post-traductionnelle située dans une portion clé de la protéine aura plus d'effet pathologique qu'une autre située dans une portion moins impliquée dans les fonctions biologiques de la protéine. Cependant, l'effet de l'addition d'un certain nombre de ces modifications sur une même protéine reste inconnu.

En effet, il existe de nombreuses preuves dans la littérature de la toxicité des modifications post-traductionnelles. La glycation, et notamment les taux circulants de CML, a été rapportée comme reliée à la mortalité cardiovasculaire chez le patient dialysé.⁹⁶ La carbamoylation est aussi bien décrite comme associée à des événements cardio-vasculaires et à de la mortalité en hémodialyse.^{30,36} La guanidinylation, bien que peu étudiée, a également été reliée à une altération des propriétés biologiques de l'albumine. La carbonylation par le 4-HNE est, dans ce manuscrit, aussi reliée à de la toxicité cardio-vasculaire potentielle. Il est donc indéniable que ces modifications jouent un rôle important dans le sur-risque cardio-vasculaire chez nos patients hémodialysés. Cependant, aucune étude n'a, à ce jour, étudiée l'effet conjoint de ces modifications. Il est donc impossible de prédire l'impact isolée d'une modification par rapport à une autre car ces modifications sont souvent liées entre elles comme nous l'avons montré dans la partie 8 de ce travail. En effet, en terme qualitatif, il existe une grande variété d'adduits sur les HDL de patients hémodialysés. De façon surprenante, les adduits les plus fréquents dans une population pourtant non-diabétique étaient issus de la glycation (CML). Ainsi, la carbamoylation n'était pas, en terme qualitatif, la modification la plus fréquente, derrière la glycation par le CML et la guanidinylation. D'un point de vue quantitatif, le pourcentage de modifications n'excédait pas, en médiane, 1% dans la plupart des cas ; ce qui indique que les effets toxiques des modifications post-traductionnelles, s'exercent à de très faible taux de modifications. Encore une fois, la glycation était la plus fréquente et, elle était significativement associée à la carbamoylation. Ainsi, il existe un possible effet synergique de ces deux modifications *in-vivo*. Il est aussi intéressant de noter que, bien qu'elles soient possibles sur le même acide aminé dans l'analyse qualitative, ces deux modifications n'étaient pas retrouvées sur les mêmes acides aminés dans l'analyse quantitative.

Ces résultats interrogent aussi beaucoup sur l'origine de cet excès de glycation. D'une part, il peut s'agir d'un environnement favorable à la génération de produits de dégradation du

glucose chez le patient hémodialysé, du fait d'une exacerbation du stress oxydant. Néanmoins, le rôle des solutés perfusés pendant la dialyse peut aussi être questionnée. Dans la dialyse péritonéale, il existe un certain nombre de preuves de la génération de produits de dégradation du glucose dans les première génération de dialysat.^{64,81,82} Ces produits avancés de glycation sont générés spontanément dans les poches de dialysat, influencés par la chaleur (lors de la stérilisation), le pH et les conditions de stockage (durée, température, lumière,...). Dans les procédures d'hémodialyse moderne, la génération du dialysat s'effectue avec le mélange d'eau ultra-pure, d'un concentré basique (bicarbonate de sodium) et d'un concentré acide contenant initialement près de 250 mM de glucose. De plus, les procédures actuelles d'hémodiafiltration post-dilutionnelle impose la ré-injection de plus de 20 litres de ce soluté et la présence et l'impact éventuel de la perfusion de produits avancés de la glycation sur le patient hémodialysé n'a jamais été prise en compte. S'il est confirmé, cet excès de glycation pourrait être une des explications à la difficulté pour l'hémodiafiltration d'avoir des résultats clairs en terme d'amélioration de la mortalité malgré de nombreux avantages théoriques avancés par les néphrologues et industriels convaincus par la technique.^{74,97}

Nous avons montré dans ce travail, qu'il existe potentiellement un effet seuil de la modification post-translationnelle en terme de répercussion sur les fonctions biologiques (cf Partie 6). En effet, de faibles concentrations de 4-HNE ajouté au milieu des HDL issus de contrôle ne modifient pas les propriétés anti-agrégantes de ces lipoprotéines. Seule la concentration la plus forte (100 μ M) était responsable d'une altération forte du potentiel anti-agrégant. Cette concentration peut être considérée comme non physiologique car les quantités circulantes de 4-HNE chez le patient IRC varie entre 10 et 250 nM (cf partie 9). Cependant, il s'agissait là de la concentration restante circulante mais cela implique qu'une grande partie de l'aldéhyde ait déjà réagi avec les protéines, glucides ou acides nucléiques. Il existe cependant dans certains autre fluides physiologiques, des concentrations circulantes plus importantes de

4-HNE : dans le liquide cérébro-spinal des patients Alzheimer, la concentration peut atteindre $120\mu\text{M}$ ⁹⁸ et jusqu'à $30\mu\text{M}$ dans les îlots pancréatiques.⁹⁹ Esterbauer *et al* suggèrent même que les concentrations locales peuvent monter jusqu'à l'ordre du millimolaire au contact des membranes en lieu et place de la peroxydation.¹⁰⁰ De plus, nos données suggèrent que seule une faible partie du 4-HNE ajouté en solution réagit avec les protéines du milieu car une grande partie réagit directement avec les parois des tubes et les constituants des solutions tampons (**Figure 15**).

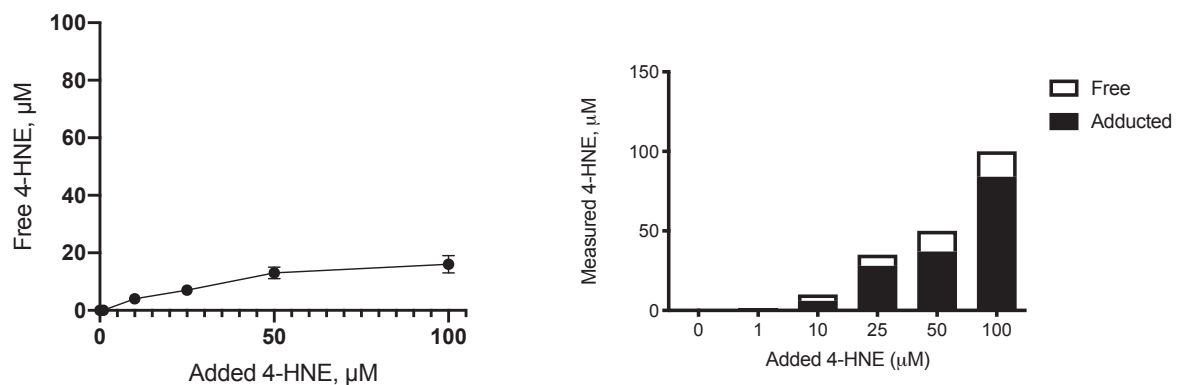


Figure 16 – Rapport entre les concentrations mesurées et ajoutées de 4-HNE dans les expérimentations de modification in-vitro.

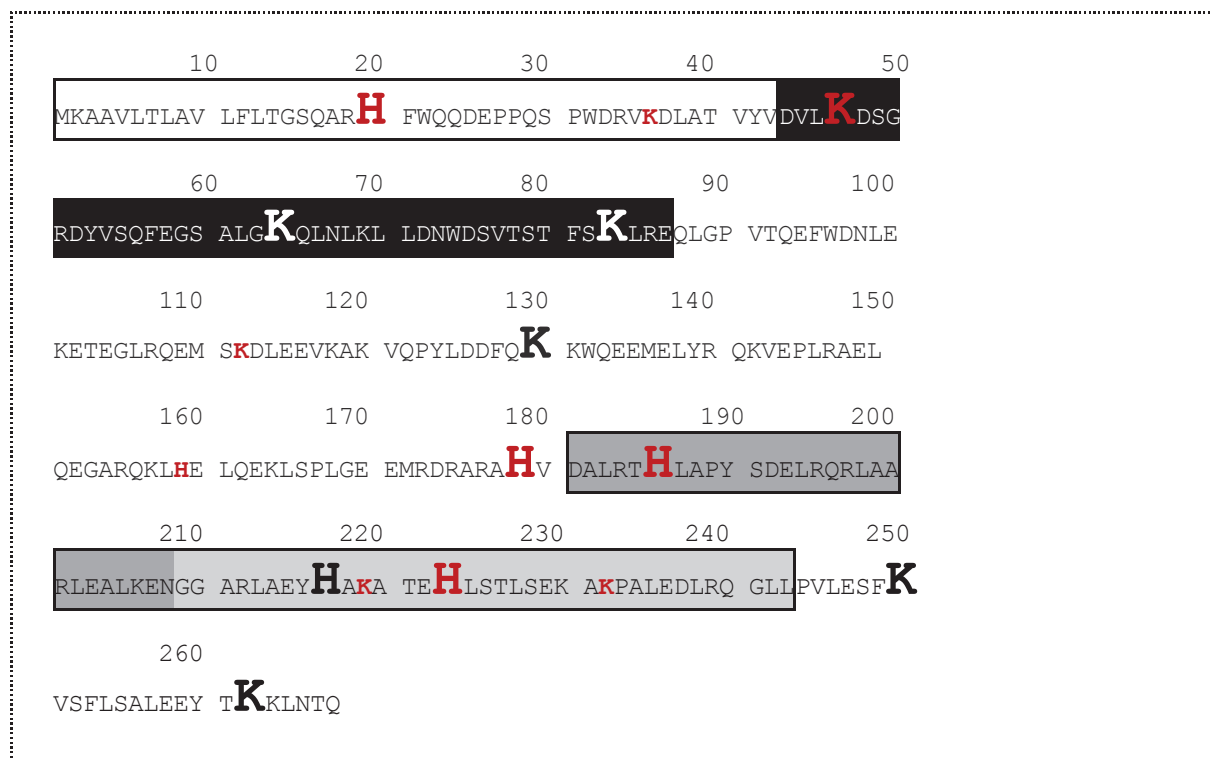
Une grande partie du 4-HNE ajouté en solution réagit avec le tampon et les parois plastiques des tubes. Pour une concentration ajoutée de $100\mu\text{M}$, seule une vingtaine de μM est effectivement disponible pour la modification.

De plus, dans nos travaux préliminaires, nous avons effectué une LC-MS/MS dans du HDL modifié par $100\mu\text{M}$ de HNE et nous avons constaté que les adduits étaient situés sur les même résidu acide aminés que sur les HDL isolés chez les patients HD pour l'ApoA1 (**Tableau 3, Figure 17**): Ainsi, cinquante pour cent des adduits (5 sur 10) étaient présents à la fois dans

les HDL de patients HD et dans les HDL natives modifiées in vitro avec de la 4-HNE ce qui valide l'étude in vitro.

AHVDALRTHLAPYSDELQR	2xHNE [H2; H9]
AKPALEDLR	1xHNE [K2]
ATEHLSTLSEK	1xHNE [H4]
DLATVYVDVLK	1xHNE [K11]
HFWQQDEPPQSPWDR	1xHNE [H1]
LAEYHAK	1xHNE [H5]
LHELQEK	1xHNE [H2]
QEMSKDLEEVK	1xHNE [K5]
VKDLATVYVDVLK	1xHNE [K2]

Table 3 : Liste des peptides de l'apolipoprotéine A1 présentant des adduits de 4-HNE après incubation d'HDL issu de patients sains avec une solution de 100µM de 4-HNE



Il existe un remodelage important des HDL dans l'IRC. Les protéines qui composent ces lipoprotéines sont en nature différente chez les patients hémodialysés et présentent un certain nombre de modifications post-traductionnelles impliquées dans la dysfonction biologique de ces protéines. Ces constatations doivent faire évoluer, pour les protéines circulantes, le postulat purement quantitatif qui est souvent appliqué en médecine vers un postulat intégrant une notion qualitative prenant en compte les modifications post-traductionnelles.

11. Conclusion

Le risque cardio-vasculaire dans l'IRC et notamment dans les stades les plus avancés reste une problématique centrale pour la prise en charge des patients par le néphrologue. En effet, malgré l'amélioration de la connaissance sur les causes de ce sur-risque cardiovasculaire, la prise en charge et la prévention de ce risque reste décevante dans les populations IRC les plus sévères.

Notre travail démontre l'implication des modifications post-traductionnelles et notamment de la carbonylation par le 4-HNE dans la dysfonction des HDL observée dans l'IRC. Chez le patient dialysé, les HDL perdent pour partie leurs propriétés anti-agrégantes, exposant potentiellement le patient à un sur-risque thrombotique. La carbonylation par le 4-HNE semble jouer un rôle dans l'avènement de ces propriétés pathologiques. Bien qu'impliquée dans ces propriétés pathologiques, notre étude montre qu'un grand nombre de modifications sont qualitativement et quantitativement présentes sur les HDL de patients hémodialysés non-diabétiques, avec au premier plan la glycation par le CML.

Les effets conjoints de l'ensemble de ces modifications reste néanmoins imprévisible et les futurs travaux devront faire la lumière sur l'impact d'une mosaïque complexe de modifications post-traductionnelles sur la morbi-mortalité cardiovasculaire. De plus, du fait de leur implication bien documentée dans des processus toxiques et de leur présence en quantité significative sur les protéines clés du métabolisme, les modifications post-traductionnelles doivent faire évoluer notre raisonnement surtout quantitatif vers un paradigme incluant une dimension qualitative lorsqu'il est question de protéines importantes (albumine, fibrinogène, lipoprotéines...).

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Annexe 1 : Liste des publications et des communications orales et écrites relatives à ce travail.

I- Publications

Revue internationale :

- Lipids and lipoproteins in CKD: a new class of uremic toxins. **Nans Florens**, Catherine Calzada, Laurent Juillard, Christophe O. Soulage. *Toxins*, 2016, 8(12), 376, doi: 10.3390/toxins8120376 (IF 3,273)
- Adenine rich diet is not a surrogate of 5/6 nephrectomy in rabbits. **Nans Florens**, Sandrine Lemoine, Caroline C. Pelletier, Maud Rabeyrin, Laurent Juillard and Christophe O. Soulage. *Nephron*, 2017, doi: 10.1159/000454990 (IF 2,203)
- Proteomic characterization of high-density lipoprotein particles from non-diabetic hemodialysis patients. **Nans Florens**, Catherine Calzada, Frédéric Delolme, Adeline Page, Fitsum Guebre Egziabher, Laurent Juillard, Christophe O. Soulage. *Toxins*, 2019 (IF 3,859)

II- Présentation des travaux de recherche

Communications affichées :

- Enhanced Oxidation of HDL in a Rabbit Model Of Chronic Kidney Disease. (Congrès ASN — Chicago 2016)
- Two toxic lipid aldehydes 2-Hydroxy-2-hexenal (4-HHE) and 4-Hydroxy-2-nonenal (4-HNE) accumulate in adult patients with chronic kidney disease (EDTA — Madrid 2017)
- Increased carbonylation of HDL in rabbits with CKD: Carbonylated-HDL exhibit blunted anti-aggregative activity on human platelets through a CD36 pathway (Congrès ERA-EDTA — Madrid 2017)
- HDL from CKD rabbits and hemodialysis patients exhibit impaired anti-aggregative activity on human platelets through CD36 pathway (Congrès ASN — Nouvelle Orléans 2017)
- Proteome of HDL from non-diabetic hemodialysis patients (Congrès ERA EDTA Budapest 2019)
- Adductome of HDL from non-diabetic hemodialysis patients (Congrès SFNDT Nancy, congrès ASN Washington 2019)

Communications orales :

- Oxidative Modification of Lipoproteins and their Effects on Platelet Aggregation in a Rabbit Model of Chronic Kidney Disease (ARN Macôn 2016)
- Carbonylated-HDL exhibit blunted anti-aggregative activity on human platelets through a CD36 pathway (NSFA Biarritz 2017)
- Altération des propriétés anti-agrégantes plaquettaires des HDL dans l'insuffisance rénale chronique : rôle de la carbonylation et du récepteur CD36 (SFNDT Nice 2017)
- Modifications post-traductionnelles et risque cardio-vasculaire dans l'IRC (ARN/ANCA Vichy 2018)
- Adductome of HDL from non-diabetic hemodialysis patients (ERA EDTA Budapest 2019)

Modifications post-traductionnelles des lipoprotéines de haute densité (HDL) et risque cardio-vasculaire dans l'insuffisance rénale chronique

Résumé : Les complications cardiovasculaires restent prépondérantes dans l'insuffisance rénale chronique bien que les causes demeurent assez méconnues. Il existe une addition chez les patients insuffisants rénaux chroniques de facteurs de risque cardiovasculaires classiques et d'autres plus spécifiques à l'insuffisance rénale. Parmi ces derniers, la formation de modifications post-traductionnelles (adduits issus de réactions oxydatives et non-oxydatives) sur les lipoprotéines HDL pourrait jouer un rôle. Dans ce travail nous avons mis en évidence d'une part, un excès de carbonylation par le 4-HNE sur les HDL dans l'IRC sur un modèle animal de lapin et chez le patient hémodialysé ou dialysé péritonéal. Cette carbonylation par le 4-HNE est responsable d'une altération des propriétés anti-agrégantes des HDL et ce par une voie médiée par le récepteur scavenger CD36. Nous avons aussi montré que le protéome des HDL était modifié chez le patient HD et qu'il existait un large éventail d'adduits covalents sur les HDL de patients hémodialysés non-diabétiques. La formation de carboxy-méthyl-lysine issue de la glycation était la principale modification post-traductionnelle observée chez les patients HD. Cela questionne sur l'origine de cet excès de glycation qui pourrait être dû à un environnement chimique favorable chez les patients mais un apport exogène par les solutés utilisés en hémodialyse est aussi possible.