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# Biomarkers of myocardial fibrosis and arterial stiffness in patients with end-stage renal disease

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par **Madonna SALIB**

## **Biomarqueurs de la fibrose myocardique et de la rigidité artérielle chez les patients en insuffisance rénale terminale**

**06 avril 2023**

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## Synthèse en Français

### 1. Préambule

L'insuffisance rénale chronique se caractérise par une détérioration progressive de la fonction rénale jusqu'à atteindre le stade terminal, c'est-à-dire un débit de filtration glomérulaire estimé (DFG)  $< 15 \text{ mL/mn}/1.73\text{m}^2$ . L'insuffisance rénale terminale (IRT) nécessite un traitement de suppléance rénale, qui peut être la dialyse (hémodialyse ou dialyse péritonéale) ou la transplantation rénale. Malgré l'amélioration des techniques de dialyse durant les dernières décennies et l'augmentation de la survie des patients, que ce soit en dialyse ou en greffe, le taux de mortalité reste significativement plus élevé pour les patients en IRT par rapport à la population générale. La mortalité cardiovasculaire (CV) est 10 à 20 fois plus élevée chez les patients traités par hémodialyse que dans la population générale, et représente la principale cause de décès chez ces patients. En outre, les pathologies CV sont la première cause de mortalité des patients transplantés rénaux.

L'association entre l'IRT et l'altération de la fonction CV est très complexe et implique plusieurs voies et mécanismes physiopathologiques tels que l'activation du système rénine-angiotensine-aldostérone, l'inflammation chronique et le stress oxydatif, qui peuvent conduire au développement d'une fibrose cardio-vasculo-rénale. En effet, ceux-ci sont directement impliqués dans le développement et la progression de la cardiomyopathie urémique et les anomalies vasculaires observées chez les patients en IRT. Les patients atteints d'IRT ne sont pas représentés de manière adéquate dans les essais cliniques randomisés dans le domaine CV.

La fibrose étant un médiateur majeur de la physiopathologie des syndromes cardio-rénaux, l'identification de sous-groupes de patients présentant une fibrogénèse active et pouvant bénéficier de molécules antifibrotiques pourrait être une stratégie efficace pour permettre une meilleure prise en charge clinique et améliorer le pronostic CV chez les patients atteints d'IRT. Ainsi, une approche potentiellement prometteuse est l'identification et la validation de biomarqueurs circulants liés à la fibrose cardiovasculaire, contribuant ainsi à un meilleur phénotypage des patients en vue d'essais cliniques dédiés et à terme d'une thérapie personnalisée.

Après transplantation rénale, les anomalies cardiaques et vasculaires observées en IRT sont partiellement améliorées, du fait de la restauration d'une fonction rénale correcte et du sevrage de la dialyse. Cependant, les patients sont exposés aux immunosuppresseurs, en particulier les anticalcineurines, pouvant avoir une toxicité vasculaire, soit directe, soit en favorisant l'apparition d'une hypertension artérielle, d'un diabète, ou d'une dyslipidémie. Les patients greffés rénaux présentent ainsi une rigidité vasculaire accrue. Des études ont par ailleurs montré une association entre l'augmentation de la rigidité artérielle et un risque CV plus élevé chez les patients transplantés. Par conséquent, des études sont nécessaires pour mieux comprendre la physiopathologie de la rigidité artérielle des patients greffés et envisager là-aussi des thérapeutiques ciblant préférentiellement les voies impliquées.

## **2. Biomarqueurs de fibrose myocardique et vasculaire**

Il n'y a pas de preuve d'une amélioration du pronostic CV par un traitement anti-fibrotique chez les patients en IRT. Une approche novatrice pourrait consister à identifier les patients présentant un risque élevé de développer une maladie CV, associé à une teneur élevée en collagène cardiaque et vasculaire, car ces patients seraient plus susceptibles de mieux répondre à une thérapie antifibrosante pour améliorer leur pronostic CV. Le phénotypage de la fibrose myocardique et vasculaire et la détermination de la réponse antifibrotique basée sur des biomarqueurs ont été raisonnablement étudiés dans le domaine de l'insuffisance cardiaque. Cependant, l'application des biomarqueurs circulants de la fibrose myocardique et vasculaire pour caractériser les patients hémodialysés et greffés n'a pas encore été testée. Par conséquent, ce travail de thèse vise à investiguer des biomarqueurs, c'est-à-dire le procollagène type I C-terminal propeptide (PICP) et galectine-3 (Gal-3), et les mécanismes profibrotiques sous-jacents potentiels qui peuvent être associés à la fibrose myocardique ou vasculaire dans l'IRT.

## **2.1. Procollagène type I C-terminal propeptide**

### **2.1.1. Physiopathologie**

Les molécules de propeptide C jouent un rôle central lors de l'assemblage intracellulaire des procollagènes à partir de leurs chaînes polypeptidiques, puis un rôle important dans l'assemblage du collagène en fibrilles dans la matrice extracellulaire (MEC). Jusqu'à 300 mutations dans des maladies graves ont été montrées comme étant associées au C-propeptide des procollagènes I, II, III et V. Par exemple, la mutation dans l'assemblage des molécules matures de collagène de type I est corrélée à la sévérité des maladies telles que la fibrose, ce qui suggère que le C-propeptide a des rôles pathogènes dans le remodelage fibrotique. De plus, les modifications du collagène de la matrice extracellulaire, c'est-à-dire les changements dans la synthèse ou la dégradation du collagène, sont des déterminants majeurs de la dysfonction cardiaque dans plusieurs troubles tels que l'infarctus du myocarde (IDM) et l'insuffisance cardiaque (IC). En outre, il a été prouvé que le degré de calcification vasculaire est lié à l'expression du collagène de type I dans les vaisseaux sanguins.

Le PICP est un procollagène de 100 kDa qui est libéré durant la synthèse du collagène de type I et qui est éliminé du sang par le foie en raison de son poids moléculaire élevé et il est donc considéré comme un marqueur de la synthèse du collagène de type I en l'absence de cirrhose du foie.

### **2.1.2. Procollagène type I C-terminal propeptide dans la maladie rénale chronique, la dialyse et la transplantation rénale**

Les collagènes I et III sont associés à la rigidité artérielle dans l'insuffisance rénale (IR) chronique, soulignant le rôle du collagène de type I dans la calcification vasculaire urémique. De plus, l'activation chronique du Système Rénine-Angiotensine-Aldostérone (SRAA) et de son effecteur l'angiotensine II semble qu'il est impliqué dans la fibrose, en induisant un dépôt de collagène interstitiel conduisant à une fibrose CV chez les patients urémiques. Les concentrations plasmatiques de PICP étaient significativement plus élevées chez les patients présentant une hypertrophie du ventricule gauche (VG), ayant une IR chronique ou une hypertension essentielle. Ces résultats suggèrent que l'insuffisance rénale n'est pas le seul facteur influençant les concentrations de PICP et que des concentrations plasmatiques élevées de PICP peuvent refléter un remodelage du VG déclenché par la fibrose.



Dans une étude portant sur le rôle du collagène de type I dans l'athérosclérose chez les patients atteints d'IRT, les valeurs élevées du collagène de type I étaient un facteur de risque indépendant de la rigidité artérielle. Chez les patients hémodialysés, des concentrations accrues de PICP ont été corrélées avec la dysfonction diastolique, indiquant une augmentation de la pression de remplissage du VG, de la rigidité et de la fibrose myocardique.

Les concentrations de PICP dans différentes catégories de patients à haut risque CV étaient les suivantes :  $119,09 \pm 3,13$  ug/L chez les patients atteints d'IC avec un DFG normal contre  $70,92 \pm 4,48$  ug/L chez les patients sans maladie cardiaque et  $66 \pm 29$  chez les patients atteints d'IR chronique sans hypertrophie du VG contre  $89 \pm 40$  ng/mL chez les patients atteints d'IR chronique avec hypertrophie du VG. Ces concentrations augmentent pour atteindre  $999,8 \pm 67,6$  ng/mL chez les patients hémodialysés présentant une dysfonction diastolique. Chez les receveurs de transplantation rénale, les concentrations de PICP étaient de  $245 \pm 100$  ug/L six mois après la transplantation. Il est important de noter que la méthodologie (kit de réactifs) pour mesurer le PICP était différente dans les articles susmentionnés, ce qui rend difficile la comparaison de sa concentration dans les différents sous-ensembles de patients. En outre, l'évolution et l'association de la concentration de PICP avec les événements CV chez les patients transplantés rénaux restent indéterminées, car la pertinence de PICP après la transplantation n'a pas encore été étudiée.

**À cet égard, le PICP, qui est un biomarqueur circulant de la synthèse du collagène, pourrait constituer un outil pertinent pour l'évaluation du remodelage et de la fibrose du myocarde. Cela pourrait ensuite être utile pour mieux comprendre la réponse aux futurs traitements (antifibrotiques) chez les patients atteints d'IRT et transplantés.**

## **2.2. Galectine-3**

### **2.2.1. Physiopathologie**

Gal-3 est une lectine se liant aux  $\beta$ -galactosides qui se lient aux glucides complexes. Les données disponibles suggèrent que Gal-3 est une petite protéine (~ 30-35 kDA) qui est exprimée dans plusieurs tissus tels que le cœur, les vaisseaux sanguins et le rein. La Gal-3 est sécrétée par les macrophages activés et se lie à plusieurs protéines de la MEC. En réponse à une maladie aiguë ou chronique, Gal-3 se lie aux myofibroblastes et les active, ce qui a pour effet de réguler à la hausse la formation de collagène dans la MEC, entraînant une fibrose myocardique.

Des études récentes démontrent que Gal-3 est impliquée dans divers processus biologiques tels que le remodelage de la MEC, l'inflammation, la croissance et la différenciation cellulaire, l'apoptose et la fibrogène. De plus, la surexpression de Gal-3 au niveau extracellulaire a été observée dans plusieurs maladies telles que la fibrose, l'athérosclérose, le diabète sucré et l'IC. Cela suggère que Gal-3 est sécrétée par des macrophages activés avec des sites de liaison dans les fibroblastes cardiaques et la MEC, qui est probablement régulée à la hausse par le SRAA (l'aldostérone induit la sécrétion de galectine-3), induisant une fibrose myocardique et vasculaire. De plus, dans la population générale, les concentrations de Gal-3 augmentent avec l'âge et sont plus élevées chez les femmes que chez les hommes, et des concentrations plus élevées de Gal-3 sont significativement associées à des taux plus élevés de mortalité toutes causes confondues, indépendamment des facteurs de risque CV.

### **2.2.2. Galectine-3 dans l'insuffisance rénale chronique et la dialyse**

L'IR est significativement corrélée à une augmentation de la concentration de Gal-3 dans la population générale et chez les patients atteints d'IC. Gal-3 est l'un des médiateurs de la fibrose induite par l'aldostérone, car c'est une molécule impliquée dans l'inflammation chronique ainsi que dans le stress oxydatif. La Gal-3 agit également comme un agent profibrotique dans le rein et sa concentration sanguine élevée est associée à un risque accru d'IR chronique.

L'association de Gal-3 avec les événements chez 1168 patients atteints de diabète de type 2 et hémodialysés a été évaluée à partir de l'étude 4D et 2579 patients ayant subi une coronarographie de l'étude Ludwigshafen Risk and Cardiovascular Health (LURIC), les patients étant stratifiés en trois groupes en fonction de leur DFG. Les résultats ont montré que chez les

patients ayant un DFG  $\geq 90$  mL/mn/1.73m<sup>2</sup>, le Gal-3 n'était pas significativement associé aux événements à long terme, alors que Gal-3 était significativement associée à la mortalité toutes causes confondues, à la mort CV et à la mort cardiaque subite chez les patients ayant un DFG compris entre 60 et 89 mL/mn/1.73m<sup>2</sup>. La concentration de Gal-3 était associée à l'infarctus du myocarde et au décès lié à l'IC chronique chez les patients ayant un DFG de 60-89 mL/mn/1.73m<sup>2</sup>. Il est important de noter qu'une concentration élevée de Gal-3 pourrait prédire la mortalité toutes causes confondues, les événements CV, les accidents vasculaires cérébraux et les décès liés à une infection.

Chez 88 patients hémodialysés suivis pendant 3 ans, une concentration plasmatique élevée de Gal-3 (> 23,73 ng/mL) était un facteur prédictif indépendant de la mortalité toutes causes confondues en utilisant le modèle de cox multivariable pas à pas rétrograde.

### **2.2.3. Galectine-3 dans la transplantation rénale**

Les concentrations sériques de Gal-3 chez 41 sujets témoins, 32 patients hémodialysés et 41 patients transplantés rénaux ont été étudiées. Cette étude a montré qu'il n'y avait pas de différence significative dans la concentration de Gal-3 au départ entre les patients hémodialysés et les patients transplantés rénaux. Cependant, les concentrations de Gal-3 étaient significativement plus faibles chez les sujets témoins par rapport aux groupes hémodialysés et les patients transplantés. Notamment, les concentrations sériques de Gal-3 ont diminué de manière significative 3 mois après la transplantation rénale, alors que les patients hémodialysés n'ont montré aucun changement significatif des concentrations de Gal-3.

Malgré l'amélioration de l'accès au traitement de suppléance rénale, de la technologie de dialyse et de la survie des patients en dialyse et après la transplantation rénale au cours des dernières décennies, les décès CV et non CV restent plus élevés chez les patients dialysés et les patients transplantés que dans la population générale. Étant donné que les profils d'expression de Gal-3 et leur association avec les événements CV dans l'hémodialyse sont peu étudiés et qu'aucune étude n'a évalué l'association de Gal-3 avec les événements CV dans la transplantation rénale, il nous a semblé important d'évaluer l'impact de concentrations élevées de Gal-3 car sa concentration élevée et soutenue peut non seulement être un marqueur, mais aussi contribuer à l'apparition et à la progression des maladies rénales et cardiaques.

**La nature exacte de l'association entre l'augmentation des concentrations de Gal-3 et la progression de l'IR chronique n'est toujours pas claire. Deux mécanismes potentiels peuvent permettre d'expliquer l'augmentation de la concentration de Gal-3 dans le cadre de l'IR chronique. Premièrement, il est possible que la concentration élevée de Gal-3 soit une conséquence du déclin de la fonction rénale, ce qui peut s'expliquer par le fait que la Gal-3 est éliminée par le rein en raison de son petit poids moléculaire, et donc que son taux sanguin est inversement corrélé à la fonction rénale et au DFG. Deuxièmement, la Gal-3 pourrait être produite par le rein et sa concentration élevée pourrait entraîner une perte supplémentaire de la fonction rénale et le dysfonctionnement d'autres tissus tels que le cœur, le foie et les poumons.**

**Cette relation peut représenter des processus parallèles affectant à la fois le cœur et les reins ou le syndrome cardio-rénal. La Gal-3 semble refléter l'inflammation, qui est un mécanisme physiopathologique important dans le contexte du syndrome cardio-rénal.**

### **3. Objectifs**

Au cours de ce travail de thèse, notre objectif principal était d'étudier certains biomarqueurs de fibrose myocardique et vasculaire (PICP et Gal-3) afin d'évaluer si ces marqueurs sont pertinents pour améliorer la stratification du risque CV parmi divers groupes de patients atteints d'IRT, à savoir les patients hémodialysés et les patients greffés. Il s'agissait également de déterminer les phénotypes biologiques des patients hémodialysés sur la base de biomarqueurs multiples grâce à la technologie Olink®, en utilisant des outils méthodologiques de type intelligence artificielle. En outre, nous avons étudié l'association du PICP et de la Gal-3 avec la rigidité artérielle mesurée par la vitesse de l'onde de pouls (VOP) et la morbi-mortalité CV chez les patients greffés à 2 ans de greffe. La VOP est un facteur important et indépendant de morbidité et de mortalité CV en insuffisance rénale terminale.

### **4. Hypothèse**

Notre hypothèse est que :

1. L'utilisation du dosage de PICP et de Gal-3 peuvent améliorer la prédiction du risque CV chez les patients hémodialysés et les patients transplantés.
2. L'identification des phénotypes pourrait refléter les voies mécanistiques sous-jacentes en hémodialyse en utilisant une approche intégrée considérant un panel de biomarqueurs et des outils de type intelligence artificielle.

### **5. Publications**

Dans le cadre de ce doctorat, j'ai écrit 4 articles en qualité de premier auteur, dont 2 sont publiés, 1 est accepté, et 1 dernier en cours de relecture finale par les multiples coauteurs.

#### **5.1. Les patients hémodialysés de l'essai clinique AURORA**

La première partie de notre travail a porté sur les patients hémodialysés de l'essai clinique AURORA (A study to evaluate the Use of Rosuvastatin in subjects On Regular hemodialysis : an Assessment of survival and cardiovascular events), dont la base de données est hébergée au CIC-P, et pour lequel il existe une collection biologique. Brièvement, il s'agit d'un essai international, en double aveugle, randomisé, incluant 2773 hommes et femmes âgés de 50 à 80 ans. AURORA est un essai à grande échelle visant à évaluer l'effet de la rosuvastatine sur la morbidité et la mortalité CV chez des patients atteints d'IRT et traités par hémodialyse chronique depuis au moins

3 mois. Après avoir fourni un consentement éclairé écrit, les patients éligibles ont été randomisés dans un rapport 1:1 pour recevoir soit 10 mg de rosuvastatine par jour, soit un placebo, indépendamment des niveaux de lipides de départ. Les événements cliniques ont été adjudiqués en aveugle. Le critère de jugement principal était le temps écoulé avant un événement CV majeur (c'est-à-dire un décès cardiovasculaire, un infarctus du myocarde non fatal ou un accident vasculaire cérébral non fatal) et les critères de jugement secondaires comprenaient le décès CV et la mortalité toutes causes confondues. La période de suivi médiane était de 3,8 ans. Le résultat de l'essai s'est avéré neutre, nous autorisant à considérer l'ensemble de la population de l'étude comme une unique cohorte.

Nous avons étudié la valeur pronostique des biomarqueurs circulants reflétant la fibrose myocardique, à savoir le PICP et la Gal-3, pour prédire la mortalité CV et la mortalité toutes causes confondues (*1<sup>er</sup> article – publié dans Journal of Clinical Research in Cardiology*). Nos analyses ont montré que l'ajout de la PICP, de la Gal-3 et de leur interaction au modèle clinique et biologique améliore la prédiction de la mortalité CV et de la mortalité toutes causes. De plus, l'interaction entre le collagène et Gal-3 en ce qui concerne la mortalité CV et la mortalité toutes causes confondues soutient l'hypothèse du rôle de Gal-3 dans l'induction d'une fibrose médiée par l'inflammation chez les patients hémodialysés.

Dans un second travail, nous avons cherché à identifier des phénotypes à risque CV chez les patients hémodialysés en utilisant des outils de type intelligence artificielle intégrant un panel de biomarqueurs (*2<sup>ème</sup> article – en cours de relecture finale par les multiples coauteurs - dont steering committee AURORA*). Nous avons identifié trois phénotypes distincts liés à la signalisation des orages cytokiniques, à la signalisation des TLRs (toll-like receptors), ou à la fibrose médiée par l'inflammation, se distinguant d'un quatrième phénotype de référence présentant peu de perturbations biologiques. Nous avons également montré que les phénotypes biologiques identifiés ont une pertinence clinique car ils étaient associés aux événements cliniques et à la réponse au traitement.

## 5.2. Les patients greffés de l'étude TRANSARTE

La deuxième partie de notre recherche s'est centrée sur les patients greffés de l'étude TRANSARTE. Brièvement, il s'agit d'une étude monocentrique observationnelle qui a inclus 100 patients atteints d'IRCT inscrits sur la liste d'attente de transplantation rénale du service de transplantation du CHRU de Nancy. Cette étude avait pour objectif d'évaluer l'évolution de la rigidité artérielle à long terme après la greffe en comparaison à celle des patients restés en dialyse.

Dans un premier temps, nous avons utilisé les données de cette étude pour mieux comprendre les facteurs associés à la rigidité artérielle, supposée comme étant en partie liée à une fibrose excessive, et le pronostic CV des patients greffés. Nous avons évalué l'association entre l'évolution de la rigidité artérielle, évaluée par VOP, mesurée à différents moments et les événements CV au cours du suivi (*3ème article – publié dans Journal of Clinical Medicine*). Nos résultats ont montré que l'absence d'aggravation de la VOP au cours du suivi était associée de manière significative à un risque plus faible d'événements CV, alors que les changements précoces de la VOP (à 1 an de greffe) ne l'étaient pas. Cette étude a évalué l'évolution de la rigidité artérielle sur une longue période (i.e. 4 ans) chez des patients greffés, alors que la plupart des études ont évalué la rigidité artérielle 6 à 12 mois après la greffe. La présente étude confirme que l'amélioration de la rigidité artérielle à long terme chez les patients greffés était associée à un meilleur pronostic CV. Ceci suggère qu'il serait possible d'améliorer le pronostic des patients greffés en identifiant des stratégies thérapeutiques à même de réduire la rigidité.

Nous avons aussi étudié dans une 4<sup>ème</sup> étude l'association du PICP et du Gal-3 avec la rigidité artérielle et la morbi-mortalité CV chez patients greffés à 2 ans de greffe (*4ème article – accepté dans Journal Annals of Transplantation*). Nous avons montré que des concentrations accrues de Gal-3, et non celles de PICP, étaient associées à la morbi-mortalité CV chez les patients greffés. La fibrose myocardique, médiée par la voie Gal-3 pourrait être majoritairement impliquée dans la morbi-mortalité des patients greffés rénaux. Il serait intéressant d'étudier l'impact de la réduction des concentrations de Gal-3 en utilisant un inhibiteur compétitif de Gal-3 dans des essais cliniques dédiés. Cette stratégie pourrait être prometteuse pour améliorer le risque CV dans cette population.

## **6. Conclusion**

Les différentes études constituant ce travail de thèse soutiennent notre hypothèse de travail selon laquelle la prise en compte des marqueurs de la fibrose myocardique et vasculaire pourrait apporter une meilleure caractérisation du profil CV individuel en insuffisance rénale terminale. En outre, L'identification de phénotypes biologiques basés sur des biomarqueurs et l'implémentation d'outils de type intelligence artificielle pourraient aider les cliniciens à identifier les patients à haut risque CV. Proposer des essais cliniques stratifiés sur le phénotype biologique des patients pourrait en effet permettre à terme d'établir des stratégies de traitement individualisées.



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## Abbreviations and acronyms

CKD: chronic kidney diseases

eGFR: estimated glomerular filtration rate

ACR: albumin to creatinine ratio

CV: cardiovascular

ESRD: end-stage renal disease

RRT: renal replacement therapy

PD: Peritoneal dialysis

KT: kidney transplantation

RAAS: renin-angiotensin-aldosterone system

HF: heart failure

CNI: calcineurin inhibitors

ECM: extracellular matrix

TGF: transforming growth factor

MI: myocardial infarction

LV: left ventricular

MRI: magnetic resonance imaging

HFrEF: heart failure with reduced ejection fraction

VSMCs: vascular smooth muscle cells

PWV: pulse wave velocity

HMG-CoA: hydroxy-methylglutaryl-coenzyme A reductase inhibitors known as statins

LDL-C: low-density lipoprotein cholesterol

KDOQI: Kidney Disease Outcome Quality Initiative

RCTs: randomized controlled trials

MACE: major adverse cardiovascular event

MR: mineralocorticoid receptor

PICP: procollagen type I C-terminal propeptide

Gal-3: galectin-3

ECCM: extracellular collagen matrix

BNP: B-type natriuretic peptide

MCP: modified citrus pectin

hs-CRP: high sensitivity C-reactive protein

TLRs: toll-like receptors

IPA: Ingenuity Pathways Analysis

HFpEF: heart failure with preserved ejection fraction

MRAs: mineralocorticoid receptor antagonists

LPS: lipopolysaccharides

MR: mineralocorticoid receptor

NT-proBNP: N-terminal pro b-type natriuretic peptide

CITP: collagen type-1 C-terminal telopeptide

MMP-1: matrix metalloproteinase-1

PIIINP: amino-terminal propeptide of type III procollagen

LCM: latent class method

IL: interleukins

TNF: tumor necrosis factors

PTH: parathyroid hormone

RRF: residual renal function

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# 1. Introduction

## 1.1. Overview

Chronic kidney disease (CKD) is defined as a persistent abnormality in kidney structure or function (i.e., persistent estimated glomerular filtration rate (eGFR)  $<60$  mL/min/1.73 m<sup>2</sup> or persistent urine albumin-to-creatinine ratio (ACR)  $\geq 30$  mg/g) for more than 3 months<sup>1</sup>. Cardiovascular (CV) diseases are the leading cause of mortality in patients with CKD<sup>2-4</sup>. There is growing evidence that decline in kidney function is an independent risk for CV diseases and that patients with CKD (i.e. stage 3 to 4 CKD) and end-stage renal disease (ESRD) (i.e. eGFR persistently below 15 mL/min per 1.73 m<sup>2</sup>) mostly die from CV causes<sup>1,4,5</sup>. Renal replacement therapy (RRT) can be achieved by several methods including hemodialysis, peritoneal dialysis (PD), or kidney transplantation (KT)<sup>5</sup>. Hemodialysis is the most commonly used method of RRT while the CV mortality in patients undergoing dialysis is 20 times higher compared to the general population<sup>4,5</sup>. Furthermore, compared to dialysis patients, the CV prognosis of KT patients is improved, but there is still high CV mortality compared to the general population<sup>5,6</sup>.

Considering that hypertension and diabetes are the most frequent causes of CKD worldwide, it is obvious that traditional CV risk factors (age, atherosclerosis, diabetes mellitus, hypertension, smoking, and obesity) contribute to the high incidence of CV mortality among CKD patients<sup>5,7</sup>. It is also widely recognized that the relationship between CKD and the impaired CV function is highly complex and involves several pathophysiological pathways and mechanisms<sup>8</sup>. Activation of the renin-angiotensin-aldosterone system (RAAS), chronic inflammation, and oxidative stress and fibrosis were demonstrated to be directly involved in the development or progression of uremic cardiomyopathy (Figure 1) and cardiorenal syndromes<sup>9,10</sup> (Figure 2). Cardiorenal syndromes are classified into 5 clinical types that are characterized by a series of interrelated disorders between the kidney and the heart<sup>11,12</sup>. Type 4 cardiorenal syndrome is known as CKD cardiomyopathy where CKD triggers cardiac disease that includes many well-characterized changes in the myocardium resulting in heart failure (HF)<sup>11,13</sup>. Importantly, fibrosis was suggested as a marker and mediator of several pathophysiological mechanisms and interactions contributing to cardiorenal syndrome<sup>12</sup>.

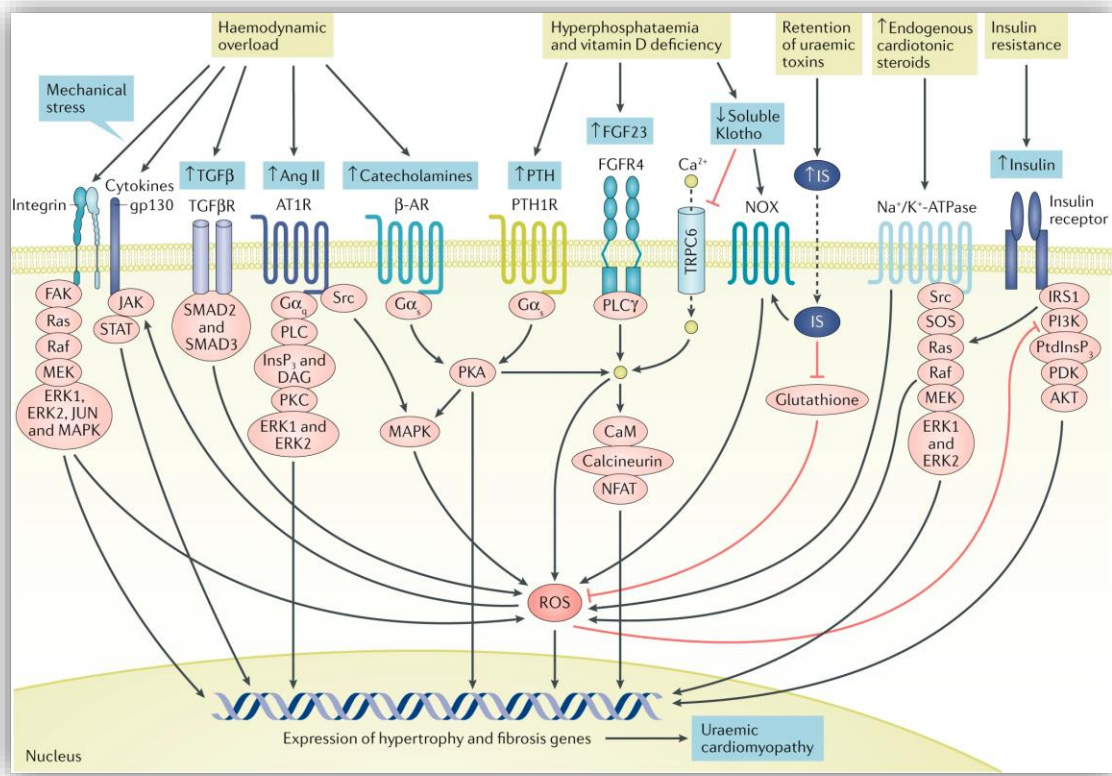


Figure 1: Crosstalk between signaling pathways involved in uremic cardiomyopathy. (From Wang et al.<sup>10</sup>)

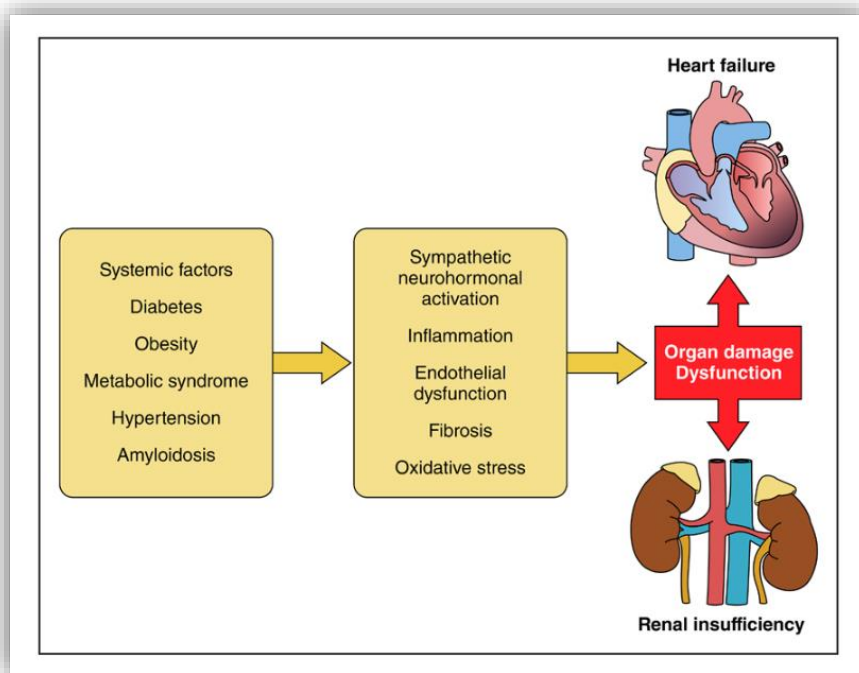
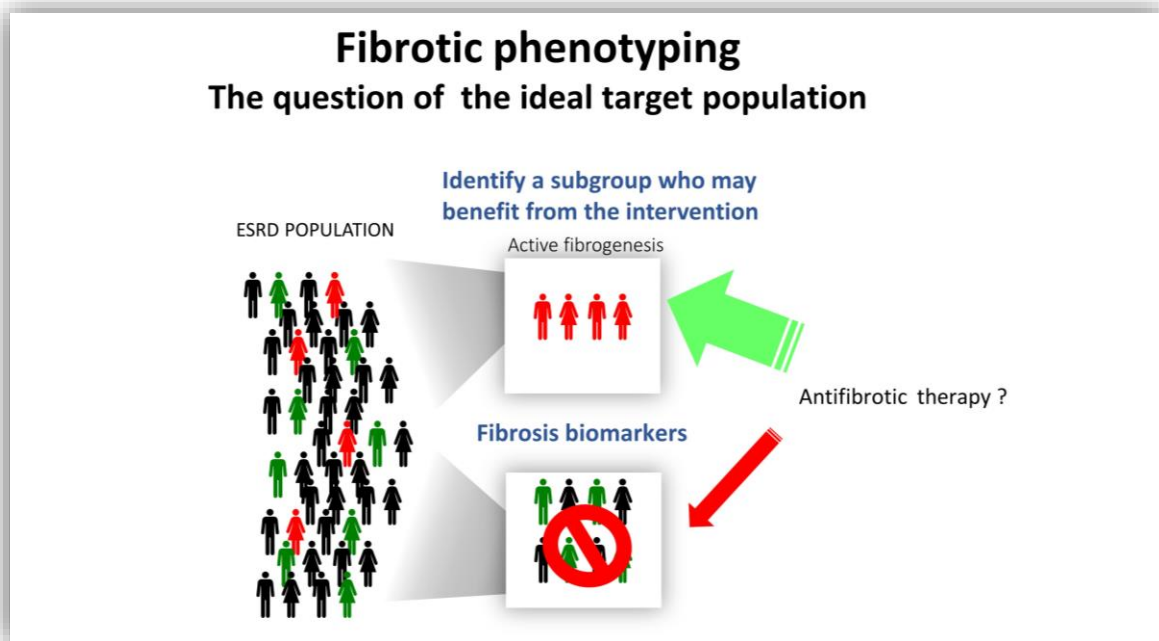


Figure 2: Conceptual framework for an unified cardiorenal syndrome. (From Zannad and Rossignol.<sup>12</sup>)



Underlining the fact that fibrosis is the major mediator of pathophysiology in cardiorenal syndromes, the identification of subgroups of patients with active fibrogenesis who may benefit from antifibrotic treatments could be a promising strategy for better clinical management and improved prognosis in patients on dialysis<sup>12</sup>. A potential approach is the identification as well as the validation of circulating myocardial fibrosis-related biomarkers, thus contributing to a better phenotyping of patients possibly leading to a personalized therapy in the future<sup>12,14,15</sup>. Therefore, targeted strategies should be considered in ESRD patients for better clinical management of this population (Figure 3).

After KT, the cardiac and vascular abnormalities observed in ESRD are partially ameliorated due to the restoration of proper renal function and withdrawal from dialysis<sup>16-18</sup>. However, patients are exposed to immunosuppressive therapy, in particular calcineurin inhibitors (CNI), which are associated with vascular toxicity, either directly or by promoting the development of hypertension, diabetes mellitus, or dyslipidemia<sup>19</sup>. Several studies have reported an association between increased arterial stiffness and a higher risk of CV outcomes in KT<sup>20-22</sup>. Therefore, studies are needed to investigate the mechanisms leading to arterial stiffness KT recipients, especially fibrosis.



**Figure 3: The identification and validation of circulating myocardial fibrosis-related biomarkers for a better phenotyping of ESRD patients possibly leading to a personalized therapy.**

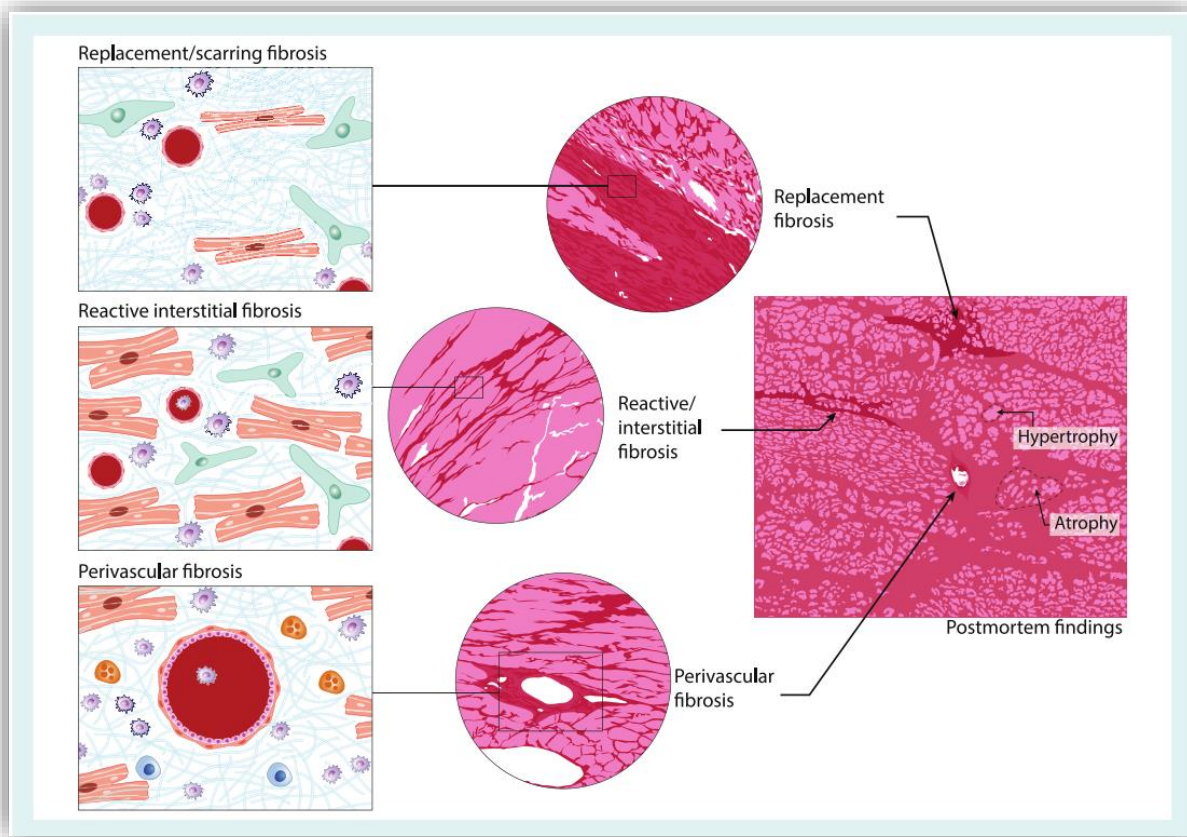
In this thesis, we investigated biomarkers of myocardial fibrosis and possibly arterial stiffness, namely procollagen type I C-terminal propeptide (PICP) and galectin-3 (Gal-3). We evaluated whether these biomarkers could be valuable to improve CV risk stratification among various groups of patients with ESRD, including patients on hemodialysis and KT recipients. We also determined biological phenotypes of hemodialysis patients based on biomarkers using machine learning approaches to better understand patients' profiles and ultimately enable personalized treatment strategies to improve CV outcomes.

## **1.2. Myocardial fibrosis**

### **1.2.1. Mechanism and pathophysiology of myocardial fibrosis**

In the healthy heart, a structural protein network mainly consisting of fibrillar collagen, connects cardiomyocytes, fibroblast (the most common cell type in the heart for normal cardiac function i.e. regulation of extracellular matrix (ECM) and wound-healing response “scar formation”), and vascular cells (Figure 4). The myocardial interstitial represents a complex ECM matrix which is made of 80% of collagen type I and 11% of collagen type III. This complex matrix is essential in maintaining the structural integrity and elasticity of the myocardium<sup>23-25</sup>.

In response to a cardiac ischemic insult or any other systemic disease, several quantitative and qualitative alterations occur in the myocardial collagen network and progressively affect the myocardium. During the wound healing, myofibroblasts (phenotypically transformed fibroblasts) initiate the formation of a scar tissue to replace the lost cardiac tissue. Inflammatory responses and persistent activated myofibroblasts stimulate the formation and the activation of angiotensin II and fibrogenic transforming growth factor (TGF)- $\beta$ , and subsequently myocardial fibrosis<sup>23,26</sup>. Further myocardial impairment in response to fibrosis may lead to morbidity and mortality<sup>25</sup>.



**Figure 4: Different forms of fibrosis are not mutually exclusive. The left panels show replacement (upper panel), reactive interstitial (middle), and perivascular (lower) fibrosis, with different cells playing the major role: fibroblasts (green), inflammatory cells (blue), and myocytes (red), with fibrillar debris interpositioned. In reality, in a typical failing heart, all forms may occur (middle panel and right histology panels). (From De Boer et al.<sup>27</sup>)**

Cardiac fibrosis is a common pathophysiologic process in most heart diseases such as myocardial infarction (MI) and hypertensive heart disease. In addition, left ventricular (LV) dysfunction is a major clinical consequence of myocardial fibrosis<sup>28</sup>. For instance, endomyocardial biopsies from a small cohort of 38 hypertensive patients with HF showed that increased collagen type I deposition was associated with an elevated risk of hospitalization for HF and mortality<sup>28</sup>. Myocardial focal fibrosis assessed by magnetic resonance imaging (MRI) was significantly associated with high rates of mortality and hospitalization in patients with heart failure with reduced ejection fraction (HFrEF)<sup>29</sup>. Indeed, cardiac fibrosis is characterized by alterations of the ECM which has a key role in cardiac remodeling and the development as well as the progression of cardiac changes leading to HF<sup>23,26,30</sup>.

### 1.2.2. Myocardial fibrosis in end-stage renal disease and kidney transplantation

Traditional CV risk factors such as diabetes mellitus, hypertension, dyslipidemia, obesity, and smoking are associated with CKD<sup>5</sup>. CV dysfunction in ESRD patients is also associated with several non-traditional CV risk factors including activation of the sympathetic nervous system, abnormal calcium-phosphate metabolism, accumulation of uremic toxins, and oxidative stress. This subsequently induces uremic cardiomyopathy (i.e. LV hypertrophy and myocardial fibrosis), accelerated atherosclerosis, and arteriosclerosis (i.e. collagen deposition)<sup>9</sup>.

The interplay between the renal and CV systems plays a pivotal role in maintaining the blood volume, hemodynamic stability, and vascular tone. However, the relationship between the various mechanisms, signaling pathways and outcomes in the cardiorenal syndrome is multifaceted and remains unclear to be translated into therapeutic interventions<sup>9,10,12</sup>. For example, it is estimated that up to 63% of HF patients suffer from some sort of cardiorenal syndrome. The interaction between HF and kidney disease leads to complex clinical outcomes in a way that it may be difficult to unravel whether volume overload results from the poor prognosis of HF or the progressive renal impairment<sup>12</sup>.

**ESRD patients undergoing hemodialysis are prone to present with myocardial fibrosis due to chronic fluid overload, hypertension, and hyperparathyroidism<sup>31</sup>.** Hemodialysis may negatively affect the CV system as a result of fluid removal which cause hemodynamic instability and systemic inflammation<sup>9</sup>. Moreover, increased LV thickness and diastolic dysfunction are common structural and functional cardiac abnormalities associated with myocardial fibrosis in patients undergoing chronic hemodialysis<sup>32</sup> (Figure 5). Furthermore, longer dialysis duration in patients with ESRD receiving hemodialysis is associated with more severe interstitial myocardial fibrosis when compared to patients with mild CKD and with levels of interstitial fibrosis similar to those found in patients with dilated cardiomyopathy<sup>32,33</sup>. This extensive myocardial fibrosis eventually leads to HF and death<sup>31</sup>.

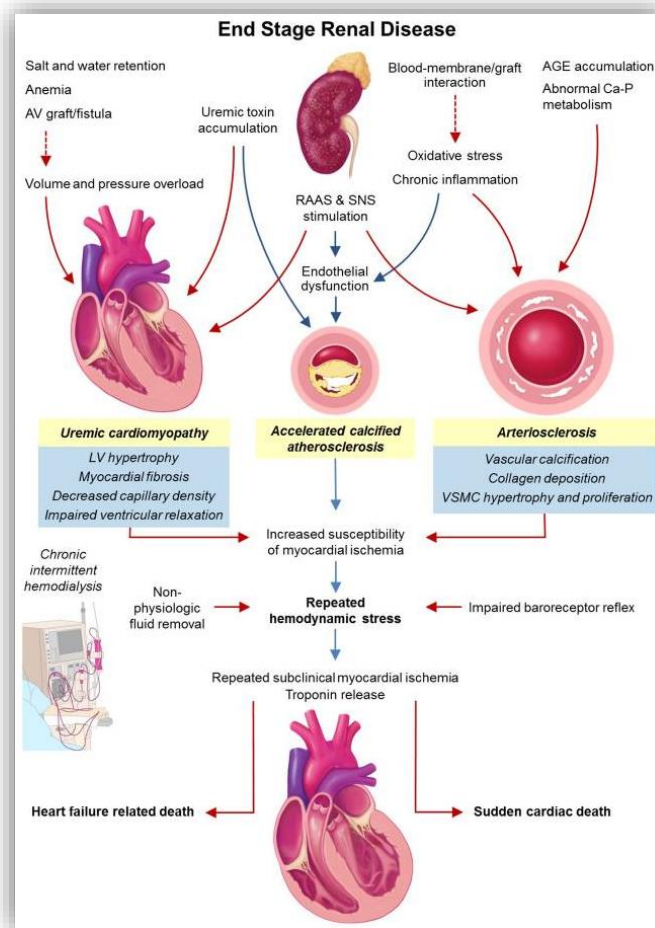


Figure 5: Pathophysiology of hemodialysis-induced myocardial injury. (From Chirakarnjanakorn et al.<sup>9)</sup>

### 1.3. Arterial stiffness

#### 1.3.1. Pathophysiology

Both vascular smooth muscle and ECM of the vascular wall play an important role in arterial stiffening. Arterial walls are made of 90% of elastic fibers known as elastin which is synthesized only at young age by the vascular smooth muscle cells (VSMCs). In normal aging, elastin may become fragmented, a process associated with the accumulation of collagen, and hence a structural remodeling of the arterial wall<sup>34,35</sup>. Increased arterial stiffness exposes the myocardium to higher systolic pressure and greater pressure fluctuations, resulting in myocardial hypertrophy stiffness and fibrosis, associated with a higher risk of stroke<sup>36</sup>.

**Arterial stiffness is a remarkable CV complication in CKD and ESRD patients, where high blood pressure gets the arterial wall stiffer and resistant to distention. In ESRD,**

atherosclerosis as well as arteriosclerosis are accelerated, as patients develop mechanical and physiological abnormalities of arteries due to fluid overload, chronic inflammation, hypertension, bone disorder, and endothelial dysfunction<sup>9,37</sup>. This subsequently may result in vascular fibrosis<sup>9,10</sup>. Increased arterial stiffness is known to be a major determinant leading to LV hypertrophy and HF<sup>38</sup> (Figure 6).

Patients on hemodialysis have a high prevalence of arterial calcifications, in the setting of secondary hyperparathyroidism and vitamin D deficiency which leads to inadequate endothelial function<sup>20,37</sup>. Also, the immune system can be stimulated by the dialysis itself which leads to chronic inflammation and oxidative stress<sup>4,5</sup>. These processes together with uremic toxins may lead to elastin fragmentation, collagen degradation, vascular calcification and arterial stiffness. Importantly, chronic inflammation is widely known to be associated with arterial stiffness<sup>8</sup>. This subsequently may result in LV hypertrophy and HF<sup>35,37</sup>.

### 1.3.2. Epidemiology

Pulse wave velocity (PWV), the gold standard method for assessing arterial stiffness, is a strong and an independent factor of CV morbidity and mortality in ESRD and KT<sup>20-22,38</sup>. In hemodialysis, arterial stiffness is considered an intermediate vascular phenotype, which reflects the cumulative influence of pre-existing hypertension, comorbidities, and dialysis vintage<sup>39</sup>. Following a successful KT, improvement of renal function probably has beneficial effects on cardiac and vascular structure and function, but KT patients receive immunosuppressive therapy with deleterious effects on metabolic and vascular parameters<sup>16-19</sup>. Several studies reported that KT is associated with an improvement of arterial stiffness in ESRD patients<sup>8,38</sup>. However, it is not clear whether the improvement in CV complications and disease persists in the long-term<sup>39</sup>. It should be mentioned that chronic inflammation in KT patients may lead to a prothrombotic state as well as chronic hypoxia associated with excessive accumulation of ECM in the vascular tree. The concomitant presence of this persistent inflammatory state and other risk factors such as diabetes mellitus, dyslipidemia, and hypertension may contribute to the increased CV morbidity and mortality in KT recipients<sup>40</sup>.

Given the strong prognostic association of PWV with CV morbidity and mortality, the longitudinal changes in PWV have greater prognostic significance than PWV at a single time-point<sup>39</sup>. However, KT studies include small number of patients and the relationship of PWV and

CV morbi-mortality at late period following KT remains controversial, as it is not fully recognized whether KT can fully counteract the deleterious effects of classical and non-classical CV risk factors. In addition, there are several factors that may have an influence on the evolution of PWV such as the type of kidney donor (living versus deceased) and the deleterious effect of the immunosuppressive drugs<sup>8</sup>. It is therefore of great interest to identify more precise mechanisms of the pathophysiology of arterial stiffness to improve CV risk prediction beyond the classical risk model. It would be worth investigating the association and interplay of biomarkers of CV fibrosis with arterial stiffness measured by PWV in KT population to stratify patients at high risk of developing CV complications. This could provide prognostic and therapeutic tools that could help normalizing arterial stiffness and preventing CV events in KT patients.

### 1.3.3. Method of evaluation

PWV is a non-invasive and reproducible tool which is widely used method to quantify arterial stiffness, as it determines the velocity at which the pressure wave propagates<sup>20</sup>. The gold standard method to measure PWV is carotid-femoral PWV, as it is used and validated in the majority of epidemiological studies<sup>20</sup>.

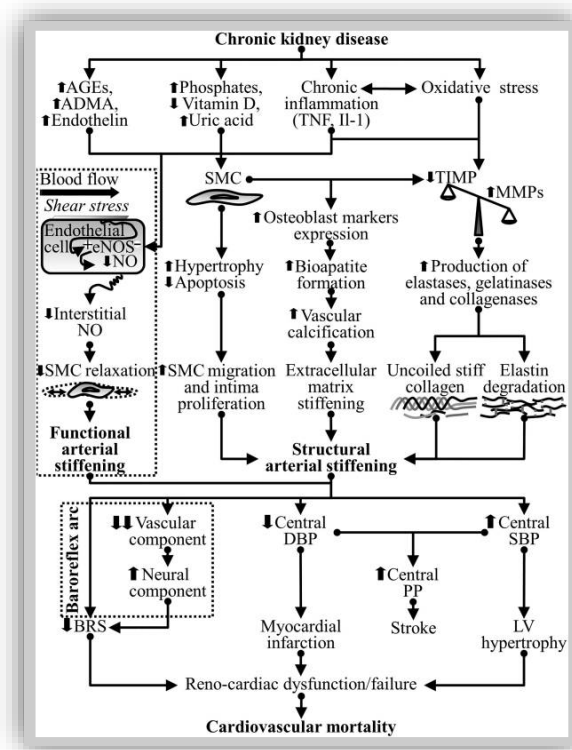


Figure 6: The vascular pathway in CKD. (From Zanoli et al.<sup>8</sup>)

#### 1.4. Clinical management of cardiovascular complications in patients with end-stage renal disease

Hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors known as statins were proposed to be potentially beneficial in the treatment or regression of CV diseases in ESRD and KT patients.

Dyslipidemia is a major CV risk factor in CKD and KT patients; however, the relationship and the pathophysiology of CV outcomes to the lipid profile in these patients may not be similar and are not yet fully understood<sup>41</sup>. Serum lipid profile abnormalities were proven to be one of the main risk factors associated with atherosclerotic disease in CKD and the relationship of atherosclerotic CV disease and dyslipidemia in KT recipients is similar to that in the general population<sup>41,42</sup>. Several studies have demonstrated that reducing low-density lipoprotein cholesterol (LDL-C) plays a pivotal role in decreasing the risk of CV morbidity and mortality as a primary prevention or in patients at high risk of CV events, such as patients with severe hypercholesterolemia, diabetes mellitus, smoking, hypertension<sup>43</sup>. In addition, lipid-lowering therapy has a beneficial effect in reducing markers of inflammation and improving endothelial dysfunction in patients with coronary atherosclerosis<sup>44-46</sup>.

Statin use showed significant benefits in HF patients without CKD, including reduction of inflammation and improvement of endothelial function and cardiac function<sup>45</sup>. For instance, in a *post-hoc* analysis of the EPHESUS trial that included 6632 acute post-MI acute HF patients randomized to a mineralocorticoid receptor antagonist vs. placebo, statin prescription was significantly associated with a lower risk of all-cause mortality due to the lower risk of CV death associated with statin use<sup>47</sup>.

The relationship of lipid profile and CV outcomes in CKD (i.e. pre-dialysis), hemodialysis, and KT patients is highly complex<sup>48</sup>. Statins have an efficient impact on lowering LDL-C and they are also highly beneficial across the lipid profile. Statin treatment has also been reported to act as anti-oxidant. This could be a key factor in the prognosis of ESRD patients, as oxidative stress is a major pathophysiological mechanism in CV diseases<sup>49</sup>. In addition, statin treatment is recommended by KDOQI practice guidelines to prevent CV diseases in pre-dialysis CKD patients with high LDL-C<sup>50</sup>. Importantly, the American college of Cardiology and the 2014 National Lipid



Association Guidelines do not provide any guidance related to the treatment of dyslipidemia in dialysis patients<sup>51</sup>.

Indeed, several large randomized controlled trials (RCTs) showed that statins have little or no effect on CV events in ESRD patients on hemodialysis<sup>42,49,52,53</sup>. In the AURORA (A study to evaluate the Use of Rosuvastatin in subjects On Regular hemodialysis: An Assessment of survival and cardiovascular events) trial in patients receiving hemodialysis, rosuvastatin showed no significant effect on all-cause mortality and the primary composite outcome of major adverse cardiovascular event (MACE) in the statin group compared to the placebo group during a median follow-up of 3.8 years<sup>54</sup>. These results suggest that the mechanistic pathways underlying CV diseases in dialysis patients differ from that in other patient population<sup>54</sup>.

The 4D (Deutsche Diabetes Dialyse Studie) Study with median follow-up of 4 years, included patients with type 2 diabetes mellitus with a mean age of 65.7 years and who were on hemodialysis, reported no significant reduction in the incidence of the composite primary outcome (i.e. death from cardiac causes, nonfatal MI, and stroke) in the statin group despite that LDL-C concentrations were lowered throughout the study period. However, this unanticipated result may be due the relatively complex pathogenetic pathways involved in CV diseases in such patients. In this 4D study, there was an increase in the relative risk for stroke in statin group and there was no significant reduction in the rate of all-cause mortality. This study supports the limited beneficial effect of prescribing statin in patients who have already reached ESRD and undergoing hemodialysis<sup>42,48,55</sup>.

In the Assessment of Lescol in Renal Transplantation (ALERT) study, about 2100 KT patients were randomized to receive 40-80 mg of Fluvastatin daily versus placebo to assess the relationship between KT and CV outcomes<sup>53</sup>. During a median follow-up of 5.4 years, there was no significant risk reduction with regard the composite primary CV endpoint of cardiac death, non-fatal MI, or coronary revascularization procedure in the statin group, despite showing a reduction of > 30% in the concentration of LDL-C. However, statin showed a significant reduction in the incidence of MI and cardiac death<sup>53,56</sup>. Of note, a 2-year extension of ALERT trial showed that 80 mg of statin lowered LDL-C concentrations below the recommended target of 100mg/dL, which, in turn, contributed to a significant reduction in the risk of MACE in KT recipients<sup>57</sup>. In a *post-hoc* analysis of the same study, it was found that the incidence of cardiac death and non-fatal MI

was reduced in patients who were treated by fluvastatin at early years (< 4.5 years) post-transplantation compared to patients who started the treatment later (> 6 years)<sup>58</sup>.

**Taking into consideration the previous RCTs, it was found that the majority of cardiac events in dialysis patients are non-atherosclerotic diseases such as HF and arrhythmia because of non-traditional risk such as oxidative stress<sup>51,55,59</sup>. The ongoing debate about the benefits of statins in ESRD patients including hemodialysis and KT emerges the need to identify new mechanistic pathways and to translate these mechanisms into personalized diagnostic and therapeutic tools<sup>26</sup>.**

## **1.5. Circulating protein molecules proposed as biomarkers of myocardial fibrosis**

There is experimental and clinical proof that myocardial fibrosis can be reversed or reduced with treatment at early stage of prognosis<sup>60</sup>. It may therefore be useful to identify patients at high risk of developing CV disease, probably with high cardiac and vascular collagen content, as those patients are more likely to respond better to a targeted therapy to improve their CV prognosis. Phenotyping of myocardial fibrosis and determination of the antifibrotic response based on biomarkers have been reasonably studied in the field of HF<sup>15</sup>. However, the application of the circulating biomarkers of myocardial fibrosis to characterize patients undergoing hemodialysis has not yet been tested, as dialysis patients have been excluded from CV trials due to their related comorbidities<sup>49</sup>. **Therefore, it appeared to be promising to investigate biomarkers (i.e. PICP and Gal-3 in the present PhD) and the potential underlying profibrotic mechanisms that may be associated with myocardial and vascular fibrosis in ESRD. This may enable the development of potential targeted diagnosis (i.e. patients at higher risk of developing CV diseases) and individualized therapies<sup>24,61</sup>.**

### **1.5.1. Procollagen type I C-terminal pro-peptide**

#### **1.5.1.1. Pathophysiology**

C-propeptide molecules play a pivotal role during the intracellular assembly of the procollagens from their polypeptides chains, and then an important role in the assembly of collagen into fibrils in the ECM<sup>62</sup>. Up to 300 mutations in serious diseases have been shown to be associated with C-propeptide of procollagens I, II, III, and V. For example, the mutation in the assembly of mature collagen type I molecules is correlated with the severity of the diseases such as fibrosis,

suggesting pathogenic roles of C-propeptide in the fibrogenic process<sup>62</sup>. Moreover, modifications in extracellular collagen matrix (ECCM), described as changes in collagen synthesis or degradation, are major determinants of cardiac dysfunction in several disorders such as MI and HF<sup>30,63</sup>. Furthermore, the degree of vascular calcification was proven to be related to the expression of type I collagen in the vascular matrix<sup>64,65</sup>.

PICP, a 100 kDa procollagen, is released into the blood as it cleaved from procollagen type I during the synthesis of fibril-forming collagen type I by the procollagen type I carboxy-terminal proteinase<sup>66</sup>. PICP is cleared from blood via the liver due to its high molecular weight and therefore, it is considered as a marker of collagen type I synthesis in the absence of liver cirrhosis<sup>66,67</sup>.

#### 1.5.1.2. Procollagen type I C-terminal pro-peptide outside the scope of chronic kidney disease

Experimentally<sup>68</sup> and clinically<sup>69</sup>, it was proven that high concentrations of collagen is related to myocardial stiffness and abnormal cardiac function, whereas their reduction improves cardiac and vascular stiffness and function.

Diez and his group found that serum PICP concentrations are correlated with total myocardial collagen volume fraction in patients with hypertensive heart disease. Moreover, they have shown that serum PICP is secreted through the coronary sinus of the heart in hypertensive heart disease patients<sup>70,71</sup>. In a study evaluating myocardial fibrosis by measuring PICP concentrations, in relation to blood pressure, LV mass and diastolic function, compared with the normotensive patients, PICP concentrations were elevated and this increase in PICP concentrations was independent of the presence of LV hypertrophy. They found that PICP was related to blood pressure, but not to LV mass, suggesting that PICP concentrations, as a direct index of myocardial collagen deposition, are raised in hypertension before the development of LV hypertrophy and are associated with diastolic dysfunction<sup>72</sup>. Moreover, in hypertensive patients with HF, increased PICP concentrations were significantly found associated with cardiotrophin-1 overexpression which is a profibrotic cytokine inducing fibrosis in the heart<sup>73</sup>.

In the Optimizing Congestive Heart Failure Outpatient Clinic (OPTIMAL) study of 208 elderly patients (60 years or older) with HF, higher PICP concentrations were significantly associated with higher B-type natriuretic peptide (BNP) concentrations, larger LV size, and

relative wall thickness<sup>74</sup>. Moreover, the same group has recently shown that high concentrations of serum PICP were significantly associated with CV death and all-cause mortality, supporting the association of elevated PICP concentration with cardiac remodeling and poor prognosis in elderly patients with HF<sup>75</sup>. Indeed, PICP concentrations were increased in patients with HF compared to patients with normal eGFR >60 mL/min per 1.73 m<sup>2</sup> and no cardiac disease<sup>76</sup>.

#### 1.5.1.3. Procollagen type I C-terminal pro-peptide in chronic kidney disease, dialysis, and kidney transplantation

Collagens I and III are associated with arterial stiffness in CKD, emphasizing the role of collagen type I in uremic vascular calcification<sup>77,78</sup>. Moreover, chronic activation of RAAS and its effector Angiotensin II appears to be involved in fibrosis, by inducing interstitial collagen deposition leading to CV fibrosis in uremic patients<sup>9,10,28</sup>. Plasma concentrations of PICP were significantly increased in patients with LV hypertrophy, having CKD or essential hypertensive<sup>79</sup>. These results suggest that renal insufficiency is not the only factor influencing PICP concentrations and that elevated plasma PICP concentrations may reflect LV remodeling triggered by fibrosis.

In a study examining the role of type I collagen in atherosclerosis in ESRD patients, elevated type I collagen expression was found to be an independent risk factor of arterial stiffness<sup>77</sup>. In patients with ESRD on maintenance hemodialysis, increased PICP concentrations were correlated with diastolic dysfunction, indicating increased LV filling pressure, stiffness, and myocardial fibrosis<sup>80</sup>.

PICP concentrations in different categories of high CV risk patients were as follows:  $119.09 \pm 3.13$  ug/L in HF patients with normal eGFR versus  $70.92 \pm 4.48$  ug/L in patients with no identifiable cardiac disease<sup>76</sup> and  $66 \pm 29$  in CKD patients without LV hypertrophy versus  $89 \pm 40$  ng/mL in CKD patients with LV hypertrophy<sup>79</sup>. These concentrations increased to reach  $999.8 \pm 67.6$  ng/mL in hemodialysis patients with diastolic dysfunction<sup>80</sup>. In KT recipients, the concentrations of PICP were  $245 \pm 100$  ug/L at 6 months post-transplantation<sup>81</sup>. It is important to note that the methodology (Reagent Kit) for measuring PICP was different in the above-mentioned papers, which makes it difficult to compare its concentration in the different subsets of patients. Furthermore, the evolution and the association of PICP concentration with CV events in KT recipients remain unclear, as the relevance of PICP post transplantation has been not studied yet.

In this regard, PICP which is a circulating biomarker of collagen synthesis may provide a relevant tool for the assessment of myocardial remodeling and fibrosis. This subsequently may be useful to better understand future (antifibrotic) treatment response in ESRD and KT patients.

## **1.5.2. Galectin-3**

### **1.5.2.1. Pathophysiology**

Gal-3 is a  $\beta$ -galactoside-binding lectin that can bind to complex carbohydrates. The available evidence suggests that Gal-3 is a small protein (~ 30-35 kDA) that is expressed in several tissues such as the heart, blood vessels, and the kidney. The upregulation of Gal-3 is secreted by activated macrophages and it binds with several ECM proteins<sup>82-85</sup>. In response to acute or chronic disease, Gal-3 binds to and activates myofibroblasts which, in turn, upregulate the formation of collagen into ECM leading myocardial fibrosis<sup>84</sup>.

Recent findings demonstrated that Gal-3 is involved in various biological processes such as ECM remodeling, inflammation, cell growth and differentiation, apoptosis and fibrogenesis<sup>84,86</sup>. Moreover, the overexpression of Gal-3 extracellularly has been observed in several diseases such as fibrosis, atherosclerosis, diabetes mellitus, and HF<sup>85</sup>. This suggests that Gal-3 is secreted by activated macrophage with binding sites in cardiac fibroblasts and ECM which is likely upregulated by RAAS (aldosterone induces galectin-3 secretion), inducing myocardial and vascular fibrosis<sup>87,88</sup>. Additionally, in the general population, Gal-3 concentrations increased with age and were higher in women compared to men and higher Gal-3 concentrations were significantly associated with higher rates of all-cause mortality, independent of CV risk factors<sup>89</sup>.

### **1.5.2.2. Pre-clinical studies**

In animal models, the overexpression of Gal-3 was found to be associated with heart diseases such as hypertensive rats with a high risk of developing HF indicating that Gal-3 plays an important role in the progression to HF<sup>85</sup>. Moreover, Calvier et al.<sup>90</sup> showed that the upregulation of Gal-3 expression in in rats VSMCs activated collagen type I synthesis leading to aldosterone-induced vascular inflammation, remodeling, and fibrosis. Furthermore, Vergaro et al.<sup>91</sup> observed that the combined used of a Gal-3 inhibitor and a mineralocorticoid receptor antagonist in mice could reserve drug-induced left ventricular dysfunction by preventing the development of myocardial inflammation and fibrosis. Importantly, Martinez-Martinez and his group

demonstrated that the inhibition of Gal-3 using modified citrus pectin (MCP) reduced the inflammatory markers as well as cardiac fibrosis induced by high fat diet in rats without provoking changes in body weight or blood pressure. Inhibition of Gal-3 activity was found to reduce concentrations of Gal-3, total collagen, and collagen type I, which in turn decreased CV inflammation and fibrosis<sup>92-95</sup>. These experimental studies demonstrated that Gal-3 plays a major role in the pathophysiology of HF and is involved in the mechanisms of cardiac remodeling and myocardial fibrosis.

#### 1.5.2.3. Galectin-3 outside the scope of chronic kidney disease

Gal-3 is an important mediator in the pathophysiology of HF, mainly by promoting fibrosis<sup>96</sup>. In the field of HF, natriuretic peptides and echocardiography showed a high accuracy in the diagnosis of patients with HF. Interestingly, several studies have shown that serum Gal-3 concentrations were significantly increased in patients with HF<sup>97-99</sup>.

High serum Gal-3 was found to be correlated with poor progression of ventricular remodeling in chronic HF<sup>100</sup>. Moreover, it was found that the persistent increase of Gal-3 concentrations was significantly associated with a higher risk of CV outcomes, suggesting that Gal-3 could serve as a biomarker in the prediction and progression of cardiac fibrosis and HF<sup>85,96,101</sup>. Furthermore, Ueland et al.<sup>99</sup> found that HF patients with increased Gal-3 concentrations also had increased C-reactive protein (CRP) concentrations, suggesting the link between Gal-3 and inflammation.

#### 1.5.2.4. Galectin-3 in chronic kidney disease and dialysis

Renal insufficiency was found to be significantly correlated with increased Gal-3 concentration in the general population and in HF patients<sup>102</sup>. Gal-3 is one mediator of aldosterone-induced fibrosis, as it is a molecule involved in chronic inflammation as well as oxidative stress. Gal-3 also acts as a profibrotic agent within the kidney and its high blood concentration is associated with a higher risk of CKD<sup>103</sup>.

Drescher et al.<sup>104</sup> studied the association of Gal-3 with outcomes in 1168 patients with type 2 diabetes mellitus and undergoing hemodialysis from the 4D study and 2579 patients who had coronary angiography from Ludwigshafen Risk and Cardiovascular Health (LURIC) study, as the patients were stratified into three groups according to their eGFR. The results showed that in

patients with  $\geq 90$  mL/min per  $1.73 \text{ m}^2$ , Gal-3 was not significantly associated with long-term events, whereas Gal-3 was significantly associated with all-cause mortality, CV death, and sudden cardiac death in patients with eGFR of 60–89 mL/min per  $1.73 \text{ m}^2$ . Gal-3 concentration was associated with MI and death related to chronic HF in patients with an eGFR of 60–89 mL/min per  $1.73 \text{ m}^2$ . Importantly, an elevated concentration of Gal-3 could predict all-cause mortality, CV outcomes, stroke, and death related to an infection<sup>104</sup>.

In 88 patients on hemodialysis followed for 3 years, high plasma Gal-3 concentrations ( $> 23.73$  ng/mL) were an independent predictor of all-cause mortality using backward stepwise multivariable cox model<sup>105</sup>.

#### 1.5.2.5. Galectin-3 in kidney transplantation

Tan et al.<sup>106</sup> studied serum concentrations of Gal-3 in 41 control subjects, 32 hemodialysis patients, and 41 KT recipients. Their study showed that there was no significant difference in Gal-3 concentration at baseline between hemodialysis and KT patients. However, Gal-3 concentrations were significantly lower in the control subjects compared to hemodialysis and KT groups. However, the kinetics of Gal-3 concentrations in KT recipients is poorly determined. Notably, serum Gal-3 concentrations significantly decreased 3 months post KT, whereas hemodialysis patients showed no significant changes in Gal-3 concentrations<sup>106</sup>.

Despite the improvement in access to RRT, dialysis technology, and patient survival in dialysis and after KT over the last decades, CV and non-CV death remain higher among dialysis patients and KT recipients than in the general population<sup>5,107</sup>. Since the expression profiles of Gal-3 and their association with CV events in hemodialysis are poorly studied and no studies have evaluated the association of Gal-3 with CV outcomes in KT<sup>107</sup>, it appeared to us the importance to understand how Gal-3 is regulated and handled, as its sustained high concentration may not only be a marker, but it may also contribute to the onset and progression of kidney and heart diseases<sup>102</sup>.

#### 1.5.2.6. Prognostic impact of galectin-3

**Prognosis impact of GAL-3 in heart failure.** There is substantial evidence that high Gal-3 concentrations may predict poor prognosis in patients with HF<sup>100</sup>. It was reported that higher serum Gal-3 concentrations were associated with higher rates of all-cause mortality, rehospitalization, and CV death in HF patients, independently of age, sex, and adjustment for natriuretic peptides.

This indicates that increased serum Gal-3 concentrations may be associated with poor prognosis in HF patients, pointing to Gal-3 as a biomarker for risk stratification and of important prognostic role in the setting of HF<sup>100,102</sup>. Importantly, in a study with 260 HF patients, Gal-3 concentrations were significantly associated with death and HF hospitalization, independent of the concentrations of natriuretic peptides, however, this association was no longer significant in eGFR adjusted model. This highlights the foremost importance of cardiorenal interactions in the setting of HF, probably involving a profibrotic process as a common mediator (i.e. Gal-3 as a profibrotic molecule)<sup>108</sup>. The circulating concentrations of Gal-3 in HF can go from 15 ng/mL up to 30 ng/mL in the presence of comorbidities such as renal diseases, whereas the concentrations of Gal-3 is 10 to 13 ng/mL in the general population<sup>102</sup>.

**Prognostic impact of GAL-3 in CKD progression (i.e. eGFR decline).** In the general population, plasma Gal-3 was highly correlated with traditional risks of CV diseases such as diabetes mellitus, hypertension, smoking, dyslipidemia, and kidney function.

In the Framingham Heart Study (FHS), the association of plasma Gal-3 concentration with renal function decline was assessed in 2450 participants during a mean follow-up of 10.1 years and it was found that higher Gal-3 concentrations were significantly associated with rapid eGFR decline as well as higher risk of incident CKD in multivariable adjusted model including age, sex, diabetes mellitus, hypertension, proteinuria, and baseline eGFR<sup>109,110</sup>.

Higher concentrations of Gal-3 were significantly associated with risk of progression to ESRD or eGFR < 15 mL/min per 1.73 m<sup>2</sup> in a pooled study of two longitudinal cohorts of 841 patients with CKD (i.e. 51 ± 27 mL/min per 1.73 m<sup>2</sup>)<sup>111</sup>.

**The exact nature of the association between increased Gal-3 concentrations and CKD progression remains unclear. There are two potential mechanisms that may unravel the increased Gal-3 concentration in the setting of CKD. First, it is possible that the elevated concentration of Gal-3 is a consequence of the decline in renal function, which can be explained by the fact that Gal-3 is cleared via the kidney due to its small molecular weight, and therefore its blood level is inversely correlated with the renal function and eGFR<sup>82,102,112</sup>. Second, Gal-3 could be produced by the kidney and its high concentration may lead to further kidney function loss and dysfunction of other tissues such as the heart, liver, and lungs<sup>102,112</sup>.**



**This relationship may represent parallel processes affecting both the heart and the kidneys or cardiorenal syndrome. Gal-3 appeared to reflect inflammation, which is an important pathophysiological mechanism in the context of cardiorenal syndrome<sup>89,90,96,97</sup>.**

#### 1.5.2.7. Interpretation of galectin-3 in the context of renal insufficiency

**The association of galectin-3 with outcomes in heart failure after adjustment for eGFR.** The accuracy of Gal-3 as an independent prognostic factor in HF when glomerular filtration estimation is included in the analysis is highly controversial. In a large cohort of 3314 CKD patients with a mean eGFR of 45 mL/min per 1.73 m<sup>2</sup>, Gal-3 was significantly associated with HF within 4 years of follow-up but this association was no longer significant after 4 years of follow-up time. This suggests the relevance of Gal-3 in HF risk stratifying and that high Gal-3 concentrations may reflect short-term risk of HF<sup>113</sup>.

The association between Gal-3 and renal function was studied in a cohort of 876 patients with HF<sup>114</sup>. It was found that serum Gal-3 concentrations were inversely correlated with eGFR in multivariable analysis. Moreover, Gal-3 was significantly associated with all-cause mortality and CV death but Gal-3 lost its prognostic significance when the model was adjusted for eGFR. Importantly, the authors stratified patients according to eGFR and they found that Gal-3 was not associated with all-cause death or CV mortality in the multivariable analysis in patients without renal insufficiency (i.e. eGFR = 60 ml/min/1.73 m<sup>2</sup>). On the contrary, Gal-3 had high predictive capacity for both outcomes in patients with moderate renal insufficiency (i.e. eGFR 30 to < 60 ml/min/1.73 m<sup>2</sup>). However, Gal-3 lost its predictive capacity when both eGFR and the interaction Gal-3\*eGFR were included in the analysis<sup>114</sup>. These findings have shown that the prognostic value of Gal-3 for CV disease was attenuated after adjusting for eGFR.

**Handling of galectin-3 in dialysis. It is unclear whether Gal-3 is modulated by dialysis.** Meijers et al.<sup>102</sup> measured Gal-3 concentration and creatinine before and after the dialysis membrane and it was shown that Gal-3 was filtered, however, at a lesser extent than creatinine.

## 1.6. Objectives and hypothesis

ESRD is a global health burden and ESRD patients on chronic hemodialysis have much higher morbidity and mortality rates than the general population<sup>5</sup>. KT is considered the best treatment for ESRD and it is widely delivered in developed countries. However, despite the improvement in patient survival following KT in comparison with dialysis, mortality rate remains significantly higher compared to the general population<sup>115</sup>. With the exception of a very small number of trials, patients with ESRD and undergoing regular dialysis are not adequately represented in RCTs in the CV field because the high rate of comorbidities in this population and the challenges in defining and developing standardized CV endpoints<sup>49,116</sup>. KT recipients are commonly excluded from RCTs due to the risk that may be induced by the possible interactions between the study treatment and immunosuppressive therapy<sup>115</sup>.

**Fibrosis is a fundamental component of CV structural modeling. A fibrosis mechanistic phenotyping using biomarkers of CV fibrosis, namely PICP and Gal-3, may further improve CV outcomes in patients with HF<sup>66,70-75,97-100,102</sup>. Therefore, the assessment of PICP and Gal-3 in hemodialysis and KT patients may be an asset step for CV risk stratification, raising the hypothesis that anti-fibrogenesis could be proposed as personalized therapy according to patients' fibrosis profile.**

### 1.6.1. Objectives

The quantification of myocardial fibrosis using biological approaches may be useful for the clinical management and prevention of poor cardiac prognosis of ESRD and KT patients, as risk stratification is a key factor in identifying of patients with a higher risk of poor outcomes. Therefore, it may appear to be critical to track the development and progression of CV fibrosis noninvasively in the setting of ESRD and KT. This can be done on several steps: first, the association between fibrosis biomarkers reflecting cardiac remodeling and CV outcomes. Then, phenotyping patients based on the validated CV biomarkers to optimize personalized therapy<sup>12,15</sup>.

In this PhD, we aimed to investigate the relevance of biomarkers of myocardial fibrosis and possibly arterial stiffness, namely PICP and Gal-3, to improve CV risk stratification among various groups of patients with ESRD, including patients on hemodialysis patients and KT recipients. We studied the association of these biomarkers with CV outcomes in patients on

hemodialysis. We also determined biological phenotypes of hemodialysis patients based on biomarkers available from Olink® using machine learning approaches. In addition, we studied the association of PICP and Gal-3 with arterial stiffness, measured by PWV, and CV morbidity and mortality at post-transplantation. PWV is an important and independent factor of CV morbidity and mortality in ESRD and KT. These approaches could be valuable in improving risk-stratification among various setting of patients with ESRD.

### 1.6.2. Hypotheses

Our hypotheses were as follows:

- ✦ The use of PICP and Gal-3 as biomarkers can improve CV risk prediction in patients on hemodialysis and KT recipients.
- ✦ The identification of biological phenotypes that may reflect underlying mechanistic pathways in hemodialysis using an integrated approach considering panels of biomarkers (dosing a few biomarkers at a time) and machine learning approaches may improve the patient prognosis evaluation.

## 2. Articles in thesis

Over the time spent working on the present PhD, I first-authored 4 manuscripts related to my thesis, 2 of which are already published, one is accepted, and a last one will be soon submitted.

The focus of the first part of our research was on hemodialysis patients from the AURORA trial. First, we investigated the prognostic value of the circulating biomarkers reflecting myocardial fibrosis, namely PICP and Gal-3, in predicting CV death and all-cause mortality (**1<sup>st</sup> published paper in the journal “Clinical Research in Cardiology”**). Our results showed that adding PICP, Gal-3, and their interaction on top of the clinical and biological model improved prediction of CV death and all-cause mortality. This prediction improvement was of similar magnitude to the one derived from high sensitivity CRP (hs-CRP). Second, we sought to identify phenotypes in hemodialysis patients using an integrated biomarkers and machine learning approaches (**2<sup>nd</sup> paper to be submitted soon - under final review by multiple co-authors, including AURORA steering committee**). Our findings showed that clustering based on biomarkers could identify different biological patterns in patients on hemodialysis. We identified four distinct phenotypes related to ‘cytokine storm signaling’, ‘toll-like receptors (TLRs) signaling’, ‘multiple pathways related to inflammation and fibrosis’ phenotypes, as well as a ‘reference phenotype’ which exhibited the least biological abnormalities. We also showed that the biological phenotypes identified have clinical relevance as they were associated with clinical outcomes.

The second part of our research was focused on KT recipients from the TRANSARTE study. Third, we evaluated the associations of PWV measured at different time-points with CV events, as well as the evolution between these measurements with CV events (**3<sup>rd</sup> published paper in the “Journal of Clinical Medicine”**). Our findings showed that the absence of PWV worsening in the late post-transplantation period was significantly associated with a lower risk of CV events, whereas early changes in PWV were not. Finally, we investigated the association of PICP and Gal-3 with arterial stiffness measured by PWV and CV morbi-mortality in KT recipients (**4<sup>th</sup> paper accepted in the “Annals of Transplantation”**). Our results demonstrated that increased Gal-3 concentrations were associated with CV morbi-mortality in KT patients independently of PWV but not elevated PICP concentrations.

## 2.1. Hemodialysis patients from the AURORA trial

AURORA (A study to evaluate the Use of Rosuvastatin in subjects On Regular hemodialysis: an Assessment of survival and cardiovascular events) was an international, double-blind, randomized, study including 2773 men and women aged 50–80 years. This large-scale trial evaluated the effect of rosuvastatin on CV morbidity and mortality in patients with ESRD and who had been treated with chronic hemodialysis for at least 3 months. After providing written informed consent, eligible patients have been randomly assigned in a 1:1 ratio to receive either 10 mg daily rosuvastatin or a matching placebo, irrespective of baseline lipids levels. During a median follow-up period of 3.8 years, a clinical end-point committee reviewed and adjudicated the outcomes of the study. The primary outcome was the time to a major CV event (i.e. cardiovascular death, non-fatal MI or non-fatal stroke) and the secondary endpoints included CV death and all-cause mortality<sup>49,52,54</sup>.

### 2.1.1. Serum markers of fibrosis, cardiovascular and all-cause mortality in hemodialysis patients: the AURORA trial (1<sup>st</sup> article)

In this article, we studied the association of serum PICP and Gal-3 concentrations with MACE, CV death, all-cause mortality using continuous and tertiles variables in cox models. Moreover, we evaluated the interaction between PICP and Gal-3 with regard to the pre-specified outcomes using the net reclassification improvement.

Our results demonstrated that the combined use of PICP and Gal-3, on top of usual clinical and biological risk scores (i.e. age, history of CV disease, diabetes mellitus, albumin, hs-CRP, sex, dialysis vintage, body mass index, and systolic blood pressure), could be valuable in optimizing CV risk prediction in hemodialysis patients. Indeed, the association of PICP and Gal-3 with CV death was stronger in comparison to their association to MACE, suggesting the significance of these biomarkers in predicting mortality rather than morbidity. Moreover, the interaction between collagen and Gal-3 with regard to CV death and all-cause mortality supports the role of Gal-3 in inducing inflammation by binding to the ECM<sup>84-88,90</sup>.

Since there was no association between PICP and mortality and no interaction between PICP and hs-CRP, the overexpression of PICP and Gal-3 and their interplay with regard to higher rates of mortality may reflect ongoing CV fibrosis rather than chronic inflammation in

hemodialysis patients. Fibrosis was suggested to be the primary driver of cardiorenal syndrome<sup>12</sup>. Importantly, Gal-3 is widely presented as a pro-fibrotic molecule and its elevated concentrations are associated with poor CV prognosis<sup>12,14,108</sup>.



# Serum markers of fibrosis, cardiovascular and all-cause mortality in hemodialysis patients: the AURORA trial

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## Abstract

**Background** Biomarkers of fibrosis are associated with outcome in several cardiovascular diseases. However, their relevance to chronic kidney disease and dialysis is uncertain, as it remains unclear how the kidneys and the dialysis procedure itself affect their elimination and degradation. We aimed to investigate the relationship of the blood levels of two markers associated with fibrosis: procollagen type I C-terminal pro-peptide (PICP) and galectin-3 (Gal-3) with mortality in dialysis patients.

**Methods** Procollagen type I C-terminal pro-peptide and galectin-3 were measured at baseline in 2773 patients enrolled in the AURORA trial, investigating the effect of rosuvastatin on cardiovascular outcomes, in patients on hemodialysis, and their interaction with CV death or all-cause mortality using survival models. The added prognostic value of these biomarkers was assessed by the net reclassification improvement (NRI).

**Results** The median follow-up period was 3.8 years. Blood concentrations of PICP and Gal-3 were significantly associated with CV death [adjusted HR per 1 SD = 1.11 (1.02–1.20) and SD = 1.20 (1.10–1.31), respectively] and all-cause mortality (all adjusted  $p < 0.001$ ). PICP and Gal-3 had a synergistic effect with regard to CV death and all-cause mortality (interaction  $p = 0.04$  and  $0.01$ , respectively). Adding PICP, Gal-3 and their interaction on top of clinical and biological covariates, resulted in significantly improved prognostic accuracy NRI = 0.080 (0.019–0.143) for CV death.

**Conclusion** In dialysis patients, concomitant increase in PICP and Gal-3 concentrations are associated with higher rates of CV death. These results suggest that concomitantly raised PICP and Gal-3 may reflect an activated fibrogenesis relevant to risk stratification in dialysis, raising the hypothesis that anti-fibrotic therapy may be beneficial for cardiovascular protection in such patients.

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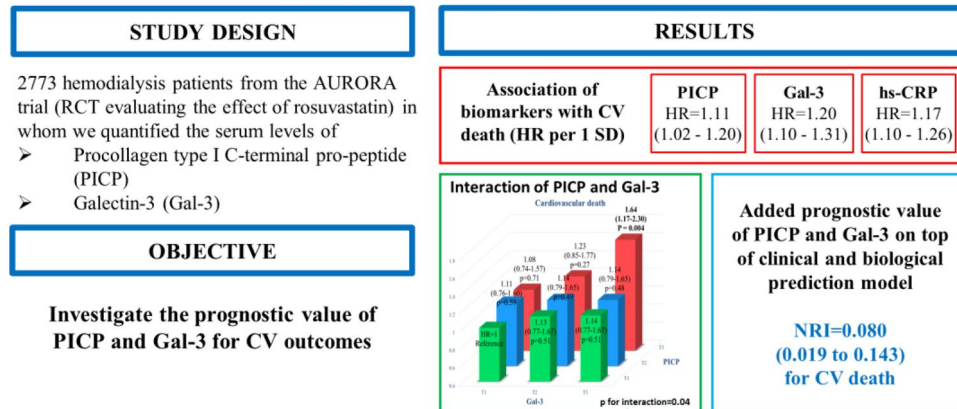
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## Graphic abstract

## Serum markers of fibrosis, cardiovascular and all-cause mortality in hemodialysis patients: the AURORA trial



**Keywords** Hemodialysis · Collagen · Fibrosis · Biomarker · Galectin-3 · Cardiovascular diseases

## Introduction

Cardiovascular (CV) diseases are the leading cause of mortality in patients on dialysis. But for now, no therapeutic intervention was proven to improve CV outcomes [1, 2].

Several studies reported that patients with end-stage renal disease (ESRD) and undergoing hemodialysis (HD) have increased level of inflammation and oxidative stress, which are associated with a higher risk of CV death [3–6]. These mechanisms can lead to high collagen turnover resulting in fibrosis [7], which could be a therapeutic target.

In heart failure (HF), circulating biomarkers of myocardial fibrosis, in particular collagen turnover biomarkers, may be useful for predicting clinical risk or response to treatment [8, 9]. Collagen I and III are also identified as relevant biomarkers of vascular disease in chronic kidney disease (CKD), as they were associated with arterial stiffness [10, 11]. Nevertheless, in non-dialysis CKD patients, the interpretation of collagen biomarkers may be difficult as they may reflect the process of renal fibrosis, the decrease of the glomerular filtration rate (for biomarkers of low molecular weight), increased bone collagen turnover, as well as fibrosis in non-renal and non-cardiovascular tissue (i.e., liver or lungs) [12].

Serum procollagen type I C-terminal pro-peptide (PICP) was found to be correlated with total myocardial collagen volume fraction in patients with hypertensive heart disease. This would suggest that PICP is a key marker of the development of myocardial fibrosis [13, 14].

Galectin-3 (Gal-3) is a marker of inflammation and is involved in aldosterone-mediated fibrosis [15, 16]. According to guidelines [17], Gal-3 can be used for additional risk

stratification in HF. Gal-3 acts as a profibrotic agent within the kidneys and therefore high plasma concentrations precede the development of CKD [15, 16]. Considering the negative association between Gal-3 and renal functions, its prognostic utility for CV disease in non-dialyzed CKD is controversial [18], but in dialysis, several studies reported an association between the concentration of Gal-3 and CV mortality [19–22].

In this framework, the investigation of PICP, a circulating collagen synthesis biomarker, in combination with Gal-3 could be a future strategy to identify patients at particularly high CV risk in hemodialysis, because these biomarkers may reflect active cardiovascular fibrogenesis.

We primarily aimed to evaluate the association of PICP and Gal-3 with adjudicated CV outcomes in the large multicenter, randomized control AURORA trial (a study to evaluate the use of rosuvastatin in subjects on regular hemodialysis: an assessment of survival and cardiovascular events). In addition, as PICP and Gal-3 could complementarily investigate ongoing active fibrosis, we investigated their interplay with regard to clinical outcomes, and further attempted to identify a fibrotic biomarker phenotype [23].

## Methods

### Study population

The description, baseline data and main results of the AURORA study have been published previously [24–26]. In short, AURORA is a double-blind, randomized, multicenter study involving 2773 men and women aged 50–80 years,



who had been treated with maintenance hemodialysis or hemofiltration for at least 3 months. After providing written informed consent, eligible patients were randomly assigned in a 1:1 ratio to receive either rosuvastatin, 10 mg daily or matching placebo.

There was no significant effect of rosuvastatin on the composite primary endpoint of major adverse cardiovascular event (MACE) (i.e., nonfatal myocardial infarction, nonfatal stroke, or death from CV causes) [HR 0.96 (95% CI 0.84–1.11),  $p=0.59$ ]. Rosuvastatin also exhibited no significant effects on the secondary endpoint of all-cause mortality [HR 0.96 (95% CI 0.86–1.07),  $p=0.51$ ].

## Outcomes

The pre-specified considered outcomes are CV death, all-cause mortality, and the composite primary endpoint of the AURORA trial (MACE). All the recorded events were reviewed and adjudicated by a clinical endpoint committee to ensure consistency of the event diagnosis. The committee members were unaware of the randomized treatment assignments.

## Biomarkers

PICP [reference range, 69–163 ng/mL] was measured in serum samples by an ELISA (Quidel Corporation, Santa Clara, USA). The dynamic range for this assay is 0.2–80 ng/mL and interassay coefficients of variation were 7%.

Gal-3 was measured in serum by a chemiluminescent microparticle immunoassay (CMIA, Abbott GmbH, Wiesbaden, Germany) on an Abbott ARCHITECT i2000 analyzer. Interassay coefficients of variation were 4.4, 5.2, and 1.6% at low, middle and high concentrations.

PICP was available for all the 2349 patients of the AURORA trial and Gal-3 as available for 2343 patients of the study.

## Statistical analysis

Categorical variables are expressed as frequencies (%) and continuous variables are expressed as means  $\pm$  standard deviation or median (25th and 75th percentiles), depending on the variable distribution.

Associations of PICP and Gal-3 with CV death, all-cause mortality or MACE were assessed using both continuous and categorized variables (tertiles) using Cox models. Models were adjusted on clinical and biological covariates based on previously published data in AURORA [23] (age, history of CV disease, diabetes mellitus, albumin, and high sensitivity C-reactive protein), and variables

correlated to PICP and Gal-3 concentrations (sex, dialysis vintage, body mass index, systolic blood pressure). History of CV disease is defined by history of coronary heart disease (i.e., prior myocardial infarction, prior coronary angioplasty or stent, and coronary artery bypass graft), history of vascular disease (i.e., peripheral artery disease, abdominal aortic aneurysm, carotid artery disease, carotid stenosis  $\geq 50\%$ , and carotid endarterectomy), and history of neurovascular disease (i.e., prior ischemic vascular accident and transient ischemic attack). High sensitivity C-reactive protein (hs-CRP) was best modeled by using its natural logarithm. Interaction between PICP and Gal-3 was assessed using a multiplicative interaction term in the Cox models.

The added prognostic value of PICP and Gal-3 in predicting CV death or all-cause mortality was assessed by the net reclassification improvement (NRI) on top of routine prognostic variables used as adjustment variables (i.e., age, history of CV disease, diabetes mellitus, sex, dialysis vintage, body mass index, systolic blood pressure, albumin and log hs-CRP at baseline).

All analyses were performed using R version 3.6.1 (R Development Core Team, Vienna, Austria). The two-sided significance level was set at  $p$  value  $< 0.05$ .

## Results

### Baseline characteristics of patients across the tertiles of each biomarker

The mean PICP was  $176 \pm 91$  ng/mL and mean Gal-3 concentration was  $69 \pm 25$  ng/mL. The correlation between PICP and Gal-3 was weak (Pearson correlation 0.068,  $p=0.002$ ). The correlation between hs-CRP and Gal-3 was also weak (Pearson correlation 0.124,  $p < 0.0001$ ), whereas there was no correlation between hs-CRP and PICP (Pearson correlation  $-0.009$ ,  $p=0.64$ ).

Patients with higher PICP concentrations were younger (median age 63 in tertile (T) 3 vs. 66 in T1,  $p < 0.001$ ) and had longer dialysis vintage (median 3.88 years vs. 2.04,  $p < 0.001$ ). Concentrations of hs-CRP were similar across the PICP tertiles. Patients with higher PICP concentrations were more likely to be women ( $p < 0.001$ ) and had lower BMI ( $p < 0.001$ ), and lower hemoglobin levels ( $p < 0.001$ ) (Table 1).

Patients with higher Gal-3 concentrations were younger (median age 63 in T3 vs. 65 in T1,  $p=0.008$ ), had longer dialysis vintage (median 3.82 years vs. 1.89,  $p < 0.001$ ) and higher levels of hs-CRP ( $p < 0.001$ ) (Table 2).

The hs-CRP (mg/L) tertiles are presented in Supplementary Table 1.

**Table 1** Baseline characteristics according to PICP tertiles

PICP (ng/mL)	1st tertile (11.3–130)	2nd tertile (> 130–186)	3rd tertile (> 186–800)	<i>p</i> value
<i>n</i>	<b>783</b>	<b>784</b>	<b>782</b>	
Female gender (%)	271 (34.6)	284 (36.2)	343 (43.9)	<b>&lt; 0.001</b>
Age (years)	66 [58–73]	66 [57–73]	63 [56–71]	<b>&lt; 0.001</b>
Dialysis vintage (years)	2.04 [0.91–4.35]	2.53 [1.05–4.86]	3.88 [1.77–7.69]	<b>&lt; 0.001</b>
Measured ( $K_t/V$ )	1.36 [1.19–1.58]	1.36 [1.20–1.56]	1.36 [1.19–1.59]	0.915
Albumin (g/L)	39.73 (3.35)	39.71 (3.47)	39.37 (3.53)	0.065
Hemoglobin (g/dL)	11.89 (1.51)	11.78 (1.53)	11.45 (1.65)	<b>&lt; 0.001</b>
hs-CRP (mg/L)	1.01 (1.14)	1.02 (1.17)	1.01 (1.18)	0.984
BMI (kg/m <sup>2</sup> )	25.53 (4.90)	25.73 (5.05)	24.71 (4.73)	<b>&lt; 0.001</b>
Systolic blood pressure (mmHg)	134 (23.15)	135 (24.01)	138 (25.46)	<b>0.003</b>
Diastolic blood pressure (mmHg)	74 (12.35)	75 (12.11)	77 (13.01)	<b>&lt; 0.001</b>
Pulse pressure (mmHg)	60 [49–70]	60 [46–71]	60 [50–73]	0.368
Current smoker (%)	139 (17.8)	123 (15.7)	116 (14.8)	0.271
Diabetes (%)	199 (25.4)	200 (25.5)	190 (24.3)	0.827
Peripheral artery disease (%)	136 (17.4)	111 (14.2)	116 (14.8)	0.179
History of coronary heart disease (%)	106 (13.5)	106 (13.5)	100 (12.8)	0.883
History of cardiovascular disease (%)	284 (36.3)	270 (34.4)	237 (30.3)	<b>0.038</b>
Cause of ESRD, <i>n</i> (%)				0.088
Diabetes	136 (17.4)	146 (18.6)	133 (17.0)	
Genetic conditions	100 (12.8)	106 (13.5)	104 (13.3)	
Glomerulonephritis or vasculitis	126 (16.1)	160 (20.4)	173 (22.1)	
Nephropathy or nephrosclerosis	180 (23.0)	145 (18.5)	134 (17.1)	
Pyelonephritis or interstitial	119 (15.2)	109 (13.9)	128 (16.4)	
Unknown/unspecified	80 (10.2)	75 (9.6)	76 (9.7)	
Other	42 (5.4)	43 (5.5)	34 (4.3)	

hs-CRP high sensitivity C-reactive protein, BMI body mass index, ESRD end-stage renal disease

Results with *p* value less than 5% were emphasized using bold letters

### Association of PICP, Gal-3, and hs-CRP with CV mortality, all-cause mortality, and MACE

In multivariable analysis (Table 3), after adjusting for key prognostic factors (including age which was inversely correlated to PICP concentrations), PICP was associated with CV death [HR per 1 SD = 1.11 (1.02–1.20)]. PICP was also significantly associated with all-cause mortality [HR per 1 SD = 1.12 (1.05–1.19) and HR for T3 vs. T1 = 1.26 (1.08–1.46)] (Table 3).

The association of Gal-3 either considered as a continuous variable or categorized variable with CV death and all-cause mortality were significant in multivariable models [adjusted HR per 1 SD = 1.20 (1.10–1.31) and 1.18 (1.10–1.25), respectively; adjusted HR for T3 vs. T1 = 1.36 (1.11–1.67) and 1.26 (1.08–1.46), respectively] (Table 3).

Gal-3 as a continuous variable was significantly associated with MACE [crude HR per 1 SD = 1.11 (1.03–1.19) and adjusted HR per 1 SD = 1.15 (1.07–1.24)]. However, PICP either considered as a continuous variable or categorized variable was not significantly associated with MACE

(Supplementary Table 2). Age, diabetes, history of CV disease, as well as a low serum albumin or an elevated hs-CRP were also significantly associated with MACE in the models (Supplementary Table 3).

When further including an interaction term between biomarkers tertiles (either PICP or Gal-3) and dialysis vintage in the multivariable models, we did not identify a significant modification of the associations of biomarkers with CV death, all-cause mortality and MACE related to dialysis vintage (*p* for interaction > 0.05, data not shown). Also, further including the underlying cause of ESRD in the multivariable models did not significantly change the association of biomarkers (either PICP or Gal-3) as a continuous variable or categorized variable with CV death, all-cause mortality, and MACE (data not shown).

The effect of PICP (per 1-SD) and Gal-3 (per 1-SD) on CV death was homogenous across pre-specified subgroups. In contrast, the association of PICP with all-cause mortality was stronger in patients without history of CV disease [*p* for interaction = 0.012 (Fig. 1); *p* for interaction in multivariable adjusted analysis = 0.010 (Supplementary

**Table 2** Baseline characteristics according to Gal-3 tertiles

Gal-3 (ng/mL)	1st tertile (11.2–56.3)	2nd tertile (> 56.3–78)	3rd tertile (> 78–228)	<i>p</i> value
<i>N</i>	<b>782</b>	<b>780</b>	<b>781</b>	
Female gender (%)	284 (36.3)	291 (37.3)	313 (40.1)	0.283
Age (years)	65 [57–73]	65 [57–72]	63 [56–71]	<b>0.008</b>
Dialysis vintage (years)	1.89 [0.83–4.02]	2.87 [1.24–5.34]	3.82 [1.78–7.01]	<b>&lt;0.001</b>
Measured ( $K_t/V$ )	1.34 [1.20–1.56]	1.38 [1.19–1.60]	1.36 [1.20–1.56]	0.508
Albumin (g/L)	39.73 (3.44)	39.95 (3.46)	39.26 (3.53)	<b>&lt;0.001</b>
Hemoglobin (g/dL)	11.76 (1.49)	11.68 (1.63)	11.57 (1.66)	0.067
hs-CRP (mg/L)	0.88 (1.07)	0.95 (1.12)	1.22 (1.27)	<b>&lt;0.001</b>
BMI (kg/m <sup>2</sup> )	25.16 (4.82)	25.40 (4.64)	25.31 (5.30)	0.618
Systolic blood pressure (mmHg)	138 (21.69)	138 (25.01)	136 (26.32)	0.342
Diastolic blood pressure (mmHg)	76 (12.12)	76 (12.36)	76 (13.51)	0.867
Pulse pressure (mmHg)	60 [50–72]	60 [49–74]	60 [48–70]	0.100
Current smoker (%)	128 (16.4)	117 (15.0)	117 (15.0)	0.685
Diabetes (%)	220 (28.1)	197 (25.3)	187 (23.9)	0.153
Peripheral artery disease (%)	113 (14.5)	124 (15.9)	108 (13.8)	0.496
History of coronary heart disease (%)	102 (13.0)	103 (13.2)	93 (11.9)	0.704
History of cardiovascular disease (%)	262 (33.5)	256 (32.8)	241 (30.9)	0.522
Cause of ESRD, <i>n</i> (%)				0.119
Diabetes	155 (19.8)	141 (18.1)	136 (17.4)	
Genetic conditions	101 (12.9)	111 (14.2)	89 (11.4)	
Glomerulonephritis or vasculitis	126 (16.1)	133 (17.1)	163 (20.9)	
Nephropathy or nephrosclerosis	161 (20.6)	156 (20.0)	151 (19.3)	
Pyelonephritis or interstitial	131 (16.8)	115 (14.7)	115 (14.7)	
Unknown/unspecified	67 (8.6)	94 (12.1)	91 (11.7)	
Other	41 (5.2)	30 (3.8)	36 (4.6)	

BMI body mass index, ESRD end-stage renal disease

Results with *p* value less than 5% were emphasized using bold letters

Fig. 1)] and without history of coronary diseases [*p* for interaction = 0.084 (Fig. 1); *p* for interaction in multivariable adjusted analysis = 0.048 (Supplementary Fig. 1)]. The association of Gal-3 with all-cause mortality was also stronger in patients without history of coronary diseases [*p* for interaction = 0.077 (Fig. 2); *p* for interaction in multivariable adjusted analysis = 0.085 (Supplementary Fig. 2).

### Interaction of PICP, Gal-3 and hs-CRP to predict CV mortality, all-cause mortality, and MACE

We identified a significant positive interaction between PICP and Gal-3 with regard to CV death and all-cause mortality (*p* for interaction with continuous variables = 0.04 and 0.01, respectively).

The magnitude of the association of an elevated concentration of PICP with either CV death or all-cause mortality was more pronounced among patients having also an elevated concentration of Gal-3 (Fig. 3). Patients with both raised PICP and Gal-3 had sizeable increase in rates for CV death (HR in patients in T3 of PICP and T3 of Gal-3 = 1.64,

*p* = 0.004), whereas patients with isolated Gal-3 or PICP increase, respectively, had no significant increase in rates of CV death (all *p* > 0.20, Fig. 3). In contrast, there was no significant interaction between hs-CRP and PICP regarding the association with either CV death or all-cause mortality (Supplementary Fig. 3).

Interaction between PICP and Gal-3 with regard to MACE tended to be significant (*p* = 0.055), and only patients with elevated Gal-3 and PICP had significantly increased from the risk of MACE [HR = 1.36 (1.00–1.84), Supplementary Fig. 4].

There was no significant interaction between rosuvastatin therapy and PICP and Gal-3 concentration (data not shown).

### Added value of PICP, Gal-3 and hs-CRP to predict CV mortality and all-cause mortality

PICP per 1 SD increase significantly improved the prediction [NRI = 0.072 (0.012–0.115)] of all-cause mortality on top of the routine clinical and biological model based on the variables used for adjustment (i.e., age, history of CV

**Table 3** Association of PICP, Gal-3 and hs-CRP with cardiovascular death and all-cause mortality

Variables	Univariable HR (95% CI)	p value	Multivariable			
			Model 1 HR (95% CI)	p value	Model 2 HR (95% CI)	p value
Cardiovascular death (n = 648)						
PICP						
Per 1SD	1.06 (0.98–1.15)	0.13	1.12 (1.03–1.21)	<b>0.01</b>	1.11 (1.02–1.20)	<b>0.017</b>
Tertiles						
1st tertile	1	–	1	–	1	–
2nd tertile	1.00 (0.82–1.23)	0.98	1.01 (0.82–1.25)	0.89	1.01 (0.82–1.24)	0.96
3rd tertile	1.14 (0.93–1.39)	0.20	1.22 (0.99–1.50)	0.061	1.22 (0.99–1.50)	0.068
Gal-3						
Per 1SD	1.15 (1.06–1.24)	<b>0.0006</b>	1.23 (1.15–1.31)	<b>&lt;0.0001</b>	1.20 (1.10–1.31)	<b>&lt;0.0001</b>
Tertiles						
1st tertile	1	–	1	–	1	–
2nd tertile	1.11 (0.91–1.36)	0.31	1.16 (0.94–1.42)	0.16	1.14 (0.93–1.40)	0.21
3rd tertile	1.29 (1.06–1.57)	<b>0.012</b>	1.46 (1.19–1.79)	<b>0.0003</b>	1.36 (1.11–1.67)	<b>0.003</b>
hs-CRP <sup>a</sup>						
Log hs-CRP	1.24 (1.16–1.32)	<b>&lt;0.0001</b>	1.22 (1.15–1.30)	<b>&lt;0.0001</b>	1.17 (1.10–1.26)	<b>&lt;0.0001</b>
Tertiles						
1st tertile	1	–	1	–	1	–
2nd tertile	1.14 (0.94–1.40)	0.19	1.14 (0.93–1.40)	0.20	1.09 (0.89–1.34)	0.39
3rd tertile	1.66 (1.38–2.01)	<b>&lt;0.0001</b>	1.62 (1.33–1.97)	<b>&lt;0.0001</b>	1.42 (1.16–1.73)	<b>0.0008</b>
All-cause mortality (n = 1296)						
PICP						
Per 1SD	1.07 (1.01–1.13)	<b>0.021</b>	1.12 (1.06–1.19)	<b>0.0002</b>	1.12 (1.05–1.19)	<b>0.0002</b>
Tertiles						
1st tertile	1	–	1	–	1	–
2nd tertile	1.09 (0.94–1.26)	0.24	1.09 (0.94–1.26)	0.25	1.09 (0.94–1.26)	0.28
3rd tertile	1.18 (1.02–1.36)	<b>0.027</b>	1.26 (1.08–1.46)	<b>0.003</b>	1.26 (1.08–1.46)	<b>0.003</b>
Gal-3						
Per 1SD	1.14 (1.08–1.21)	<b>&lt;0.0001</b>	1.21 (1.14–1.29)	<b>&lt;0.0001</b>	1.18 (1.10–1.25)	<b>&lt;0.0001</b>
Tertiles						
1st tertile	1	–	1	–	1	–
2nd tertile	1.05 (0.91–1.22)	0.50	1.10 (0.95–1.28)	0.22	1.08 (0.93–1.26)	0.30
3rd tertile	1.23 (1.06–1.42)	<b>0.006</b>	1.38 (1.19–1.61)	<b>&lt;0.0001</b>	1.26 (1.08–1.46)	<b>0.003</b>
hs-CRP <sup>a</sup>						
Log hs-CRP	1.28 (1.22–1.33)	<b>&lt;0.0001</b>	1.28 (1.22–1.34)	<b>&lt;0.0001</b>	1.22 (1.17–1.28)	<b>&lt;0.0001</b>
Tertiles						
1st tertile	1	–	1	–	1	–
2nd tertile	1.31 (1.14–1.51)	<b>0.0002</b>	1.34 (1.16–1.56)	<b>&lt;0.0001</b>	1.29 (1.11–1.49)	<b>0.0007</b>
3rd tertile	1.90 (1.66–2.18)	<b>&lt;0.0001</b>	1.91 (1.66–2.20)	<b>&lt;0.0001</b>	1.68 (1.45–1.94)	<b>&lt;0.0001</b>

Model 1: adjusted for age, diabetes, history of cardiovascular disease, sex, dialysis vintage, body mass index, and systolic blood pressure (at baseline)

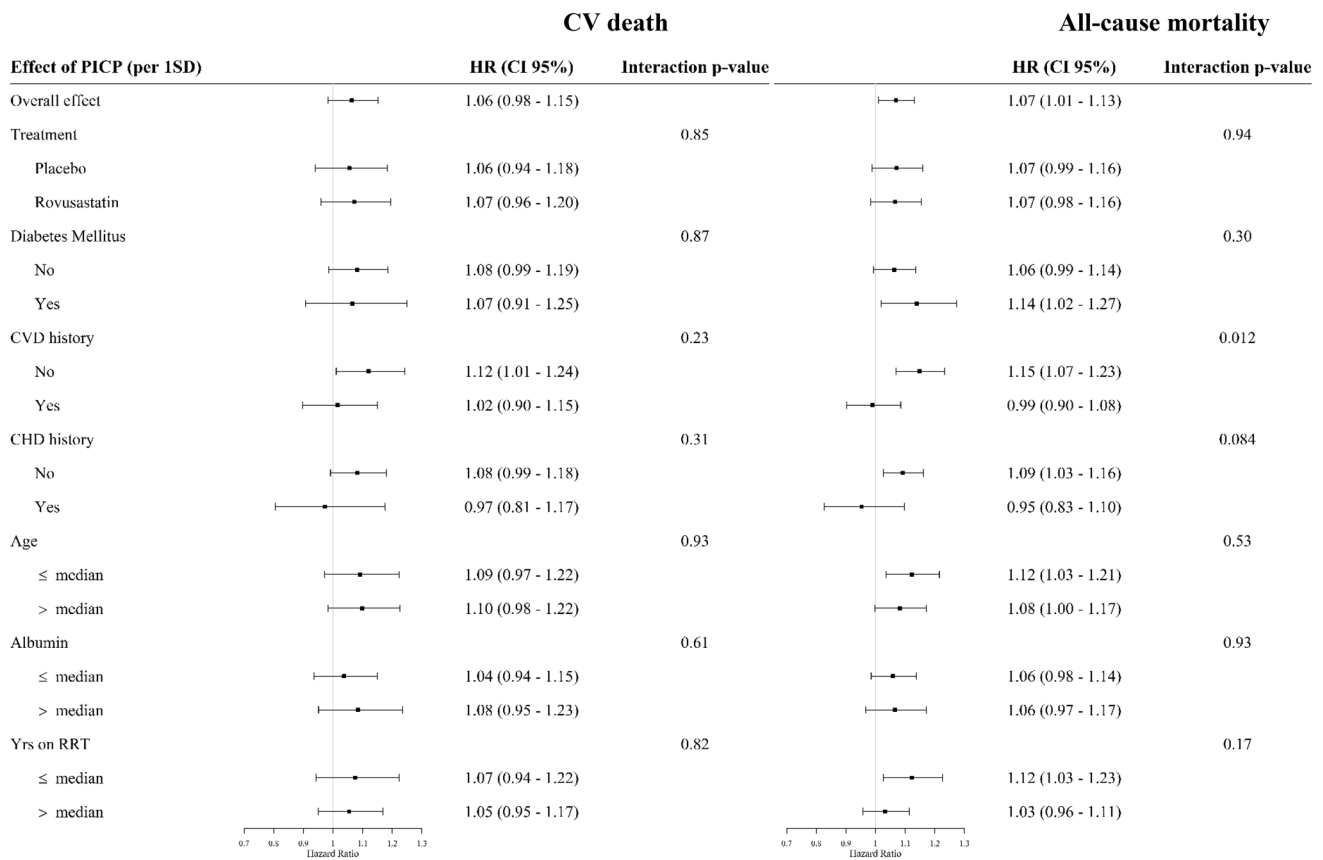
Model 2: model 1 + albumin and log hs-CRP (at baseline)

<sup>a</sup>Model 2: model 1 + albumin (at baseline)

Results with p value less than 5% were emphasized using bold letters

disease, diabetes mellitus, sex, dialysis vintage, body mass index, systolic blood pressure, albumin and log hs-CRP at baseline). Gal-3 significantly improved the prediction of

both CV death and all-cause mortality on top of the clinical and biological model [NRI = 0.101 (0.024–0.146) and NRI = 0.080 (0.015–0.113), respectively].



**Fig. 1** Association between PICP and cardiovascular death or all-cause mortality in subgroups of patients (non-adjusted analysis). *CVD* cardiovascular diseases, *CHD* coronary heart diseases, *Yrs RRT* years on renal replacement therapy (dialysis vintage)

Adding PICP, Gal-3 and their interaction on top of the clinical and biological model (i.e., age, history of CV disease, diabetes mellitus, sex, dialysis vintage, body mass index, systolic blood pressure, albumin and log hs-CRP at baseline) resulted in significant NRI for CV death and all-cause mortality [NRI=0.080 (0.019–0.143) and 0.085 (0.029–0.129), respectively]. Of note, this prediction improvement was of similar magnitude to the one derived from hs-CRP on top of usual clinical/biological variables (i.e., age, history of CV disease, diabetes mellitus, sex, dialysis vintage, body mass index, systolic blood pressure, and albumin but obviously excluding log hs-CRP at baseline) [NRI for CV death=0.108 (0.050–0.167) and NRI for all-cause mortality=0.132 (0.088–0.171), respectively].

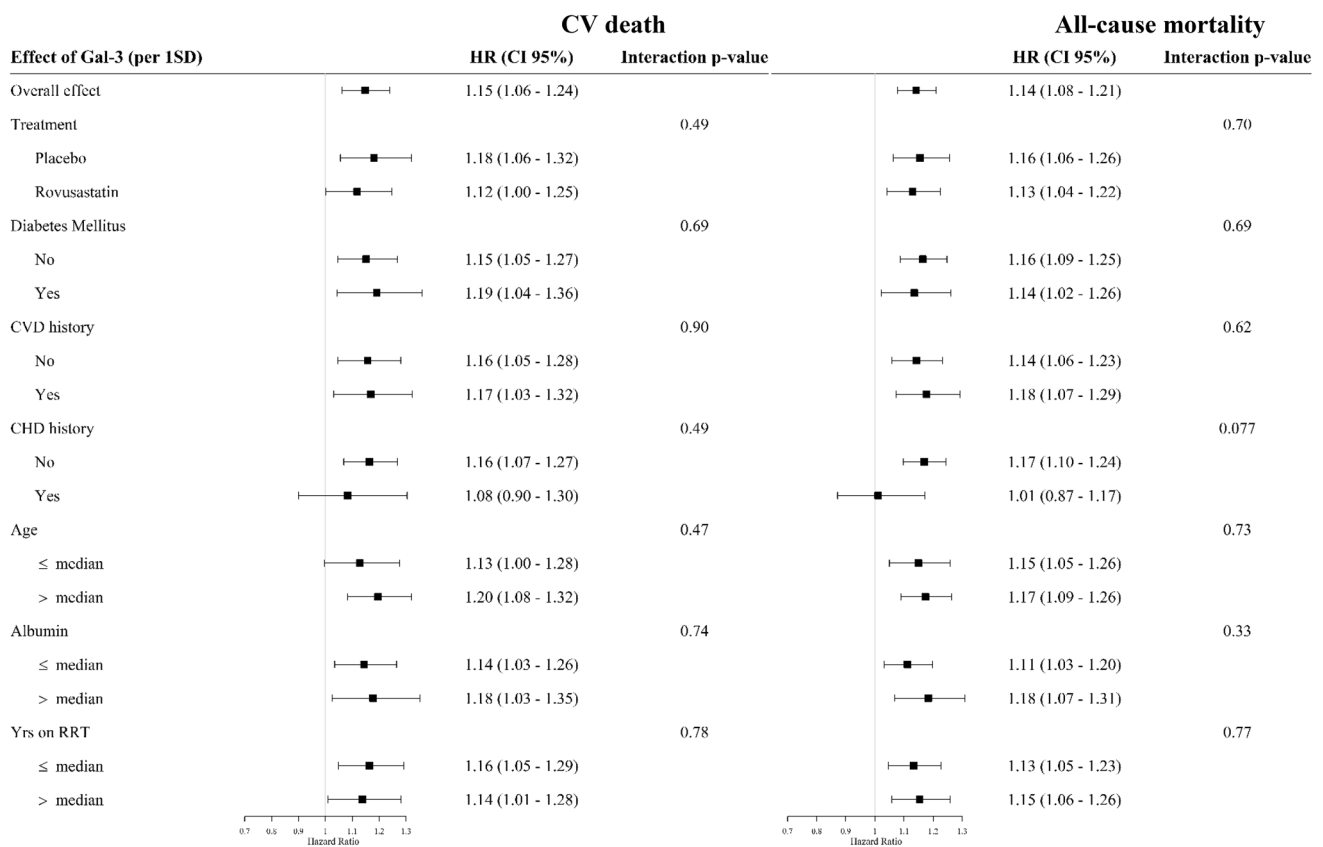
**Discussion**

To the best of our knowledge, the current study is the first to assess the combined and synergistic association of PICP and Gal-3 with CV death and all-cause mortality in hemodialysis patients. Our main findings are as follows: (1) increased concentrations of PICP or Gal-3 are significantly associated

with CV death and all-cause mortality even after adjusting on a previously validated clinical and biological score in the AURORA trial [23], and the correlated variables; (2) the increment of association with CV death provided by PICP and Gal-3 was of similar magnitude of the one provided by hs-CRP [HR per 1 SD 1.11 (1.02–1.20)=1.20 (1.10–1.31) and 1.17 (1.10–1.26) for PICP, Gal-3, and hs-CRP; respectively]; (3) there is a positive interaction between Gal-3 and PICP regarding the association with CV death and all-cause mortality whereas no significant interaction is found between PICP and hs-CRP.

The pathophysiology of CV complications is very complex in patients on dialysis, but chronic inflammation certainly plays a major role [3, 27, 28]. Moreover, cardiac and vascular fibrosis are likely to be the main histological pathways involved in the generation of CV complications related to uremic cardiomyopathy and vascular stiffness [7].

Uremic cardiomyopathy refers to histological modifications of the heart, generated by chronic fluid overload, chronic mineral bone disorder, as well of chronic inflammation, all of them ultimately leading to cardiac fibrosis [4]. Arterial stiffness is associated with an increased mortality in ESRD [29], and it is now well-established that fibrosis



**Fig. 2** Association between Gal-3 and cardiovascular death or all-cause mortality in subgroups of patients (non-adjusted analysis). *CVD* cardiovascular diseases, *CHD* coronary heart diseases, *Yrs RRT* years on renal replacement therapy (dialysis vintage)

also is the main histological finding of this vascular pathology [6].

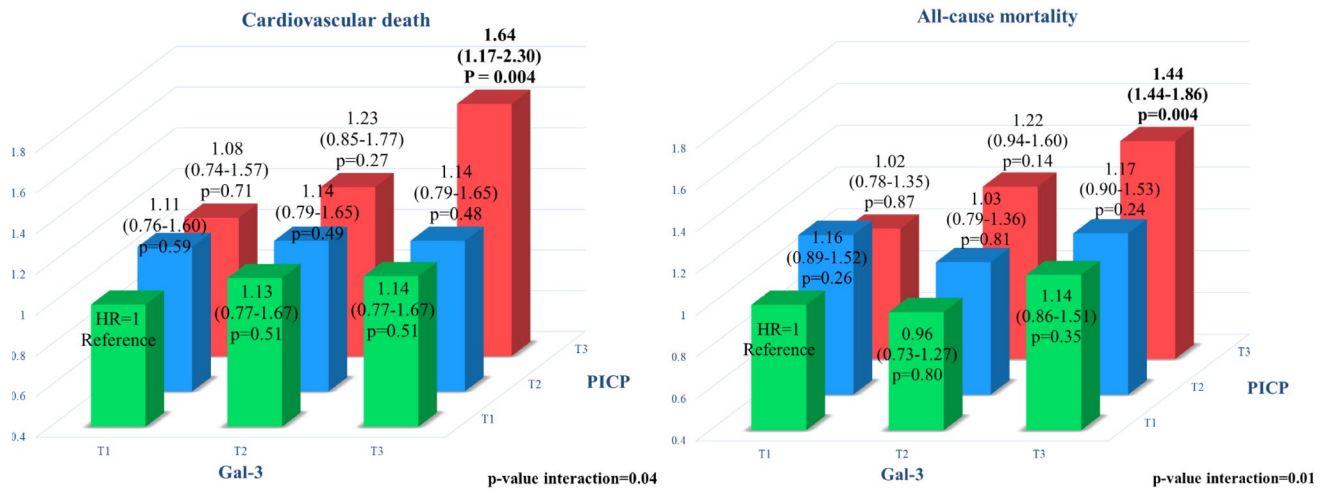
Outside the field of CKD, PICP has been shown to be raised in HF, hypertensive or ischemic heart disease with or without HF, and arterial stiffness [9, 14]. Moreover, in HF, serum concentrations of PICP are associated with worse prognosis [30–32], but little is known concerning its clinical risk prediction in dialysis.

The catabolism of high molecular weight PICP is mainly hepatic [33]. Thus, PICP does not accumulate as a consequence of impaired kidney function. Moreover, among prevalent dialysis patients, the concentration of PICP unlikely reflects active renal fibrosis, as patients have established ESRD for long periods. Finally, patients with liver cirrhosis were excluded from the AURORA trial [24]. Consequently, we hypothesized in this study that the concentration of PICP may partly reflect cardiac and vascular fibrosis. Indeed, increased PICP concentrations indicate diastolic dysfunction in ESRD patients undergoing chronic dialysis [34].

Gal-3, a 29–35 kDa protein, is a member of the β-galactin binding lectin family, which is mainly secreted by macrophages, fibroblasts, mast cells and neutrophils [35]. Gal-3 plays a major role in the pathophysiology of HF, as a marker

and a mechanism of inflammation potentially leading to fibrosis [36], but little is known about the implication of Gal-3 in the CV complications among patients on dialysis. Gal-3 appears to be related to chronic systemic inflammation [22, 37].

Of note, the interplay between Gal-3 and collagen biomarkers has not been evaluated in the setting of dialysis. The association of Gal-3 and CV outcome in patients with chronic kidney disease (CKD) and ESRD, was previously reported in a pooled analysis of the LURIC (Ludwigshafen risk and cardiovascular health) and the 4D (die deutsche diabetes dialyse studie) trial. The authors reported that elevated Gal-3 was significantly associated with CV death and all-cause mortality among patients with CKD and ESRD [19]. Gal-3 plays a pivotal role in the inflammatory response by binding to the extracellular matrix (ECM) proteins and modulating adhesions of the immune cells, including T cells, neutrophils, monocytes, and mast cells [38, 39]. In preclinical models, the overexpression of Gal-3 in rats enhanced collagen type I synthesis leading to aldosterone-induced vascular inflammation, remodeling, and fibrosis [40]. Vergaro et al. supported the role of Gal-3 in the development of myocardial inflammation and fibrogenesis. In their study,



CV death		PICP		
		T1	T2	T3
		Nevent/N (%)	Nevent/N (%)	Nevent/N (%)
Gal-3	T1	57/245 (23)	55/221 (25)	51/206 (25)
	T2	59/234 (25)	56/215 (26)	57/215 (27)
	T3	48/196 (25)	56/226 (25)	83/247 (34)

All-cause mortality		PICP		
		T1	T2	T3
		Nevent/N (%)	Nevent/N (%)	Nevent/N (%)
Gal-3	T1	108/245 (44)	109/221 (49)	92/206 (45)
	T2	95/234 (41)	96/215 (45)	107/215 (50)
	T3	91/196 (46)	108/226 (48)	137/247 (56)

Fig. 3 Interaction between PICP and Gal-3 (in tertiles) for the association with CV death and all-cause mortality

they showed that inhibition of Gal-3 in mice could reverse drug-induced left ventricular dysfunction by reducing myocardial inflammation and fibrosis [41]. Martinez-Martinez et al. reported in a preclinical study that Gal-3 expression is upregulated by cardiotrophin-1 (CT-1) which mediates the proinflammatory and profibrotic myocardial effects [35, 40, 42–45].

In the general population, the mean Gal-3 concentrations fluctuate between 11 and 14 ng/mL [16, 46–48], whereas

they have been reported to be as high as 54 ng/mL in the 4D patients undergoing hemodialysis [19]. Similar to the latter study, the mean Gal-3 concentration in our study was 69 ng/mL. Moreover, Gal-3 concentrations reach up to 26 ng/mL in congestive heart failure (CHF) [49–51]. This association between Gal-3 and kidney function may explain the increased oxidative stress partially explaining chronic inflammation. PICP, Gal-3, and hs-CRP concentrations in the current dialysis population were compared to

Table 4 CRP, PICP, and Gal-3 concentrations in the AURORA population in comparison to other populations of high CV risk patients

Reference	Studied conditions	CRP (mg/L)	PICP (ng/mL)	Gal-3 (ng/mL)
Eschalier et al. [52] Tarjus et al. [48]	Abdominal obesity	4.0 ± 6.2	87.0 ± 52.0	12.2 (10.9–15.0)
Barasch et al. [53] Gopal et al. [54]	HFrEF	3.3 (1.5–7.7)	406 (353–477)	23.0 ± 12.0
Barasch et al. [53] Gopal et al. [54]	HFpEF	4.1 (1.4–9.1)	395 (329–503)	22.0 ± 10.0
Drechsler et al. [19]	CKD	6.5 (2.5–14.9)	N/A	23.1 ± 9.9
Drechsler et al. [19] Sawamura et al. [55]	Hemodialysis	10.6 ± 17.2	162.0 ± 66.0	54.1 ± 19.6
Current study (AURORA)	Hemodialysis	1.0048 ± 1.16 (hs-CRP)	175.9 ± 91.2	69.3 ± 25.1

HFrEF heart failure with reduced ejection fraction, HFpEF heart failure with preserved ejection fraction, CKD chronic kidney disease, N/A not applicable

other categories of high CV risk patients [19, 48, 52–55], as presented in Table 4.

Excessive collagen production, fibroblasts and accumulation of ECM are stimulated by the high concentrations of Gal-3 within the myocardium [14, 21, 56]. This explains the significant interaction between PICP and Gal-3 with regard to CV death and all-cause mortality. The synergistic association of PICP and Gal-3 on mortality, in AURORA study, suggests that in dialysis patients, the process of active fibrosis was driven by Gal-3 overexpression and oxidative stress, more specifically than by chronic inflammation [57]. Indeed, the association between PICP and mortality was found only in dialysis patients without history of CV disease and coronary heart disease, and, importantly, no interaction was found between PICP and hs-CRP.

Importantly, PICP was more associated with CV death than morbid events, as there was no significant association between PICP and MACE. In addition, Gal-3 was less associated with MACE than with CV death, further strengthening the relevance of these biomarkers to predict mortality rather than morbidity.

Patients with ESRD suffer from uncontrolled secondary hyperparathyroidism where PICP concentrations may reflect bone degradation [58]. Although this mechanism may account for some of the rise in PICP in our dialysis patients, it is unlikely to be prominent since it would not explain the significant interaction found between Gal-3 and PICP. This interaction may reflect an active process of fibrosis, which is probably more often observed among younger patients in dialysis, without history of coronary heart disease.

## Clinical implications

The combined use of PICP and Gal-3, as a non-invasive assessment of fibrosis, could improve CV risk prediction on top of validated clinical and biological risk scores in patients undergoing chronic hemodialysis. Hence, the identification of patients with active fibrosis may be a promising approach as they probably have the highest probability to benefit from anti-fibrotic interventions, similarly to what is performed among non-dialyzed patient at risk of heart failure [59]. Yet, the application of this strategy will depend on the prospective validation of a panel of circulating markers in a large-scale population, and the stratification of the latter according to their fibrosis' profiles and response to a personalized therapy.

In addition, we tested the hypothesis of possible interaction between rosuvastatin therapy on PICP and Gal-3 concentrations [60, 61], and we did not identify any significant interaction in this post hoc setting. However, this does not necessarily mean that other therapeutic intervention directly targeting fibrosis could not be proposed, based on these biomarkers in the future.

Of note, the anti-fibrotic drug class prototype of the mineralocorticoid receptors antagonists is currently tested in two multicenter randomized study CV prevention studies in hemodialysis achieve (aldosterone blockade for health improvement evaluation in end-stage renal disease trial; NCT03020303), and alchemist (aldosterone antagonist chronic hemodialysis interventional survival trial; NCT01848639) [62].

Our work suggests that Gal-3 may be a key mechanistic pathway underlying fibrosis in dialysis patients, anti-Gal3 therapy for CV prevention, such as with modified citrus pectin (MCP) may be worth investigating in this setting. As a competitive inhibitor of Gal-3, MCP binds to intracellular and extracellular Gal-3, reduces level of Gal-3 and prevents cardiac fibrosis, inflammation and functional alterations associated in a number of experimental animal models [35, 40, 43].

## Limitations

As in most blood biomarkers studies, it is not possible to firmly validate the fact that the amount of circulating PICP only reflects cardiac and vascular fibrosis. The measurement of parathyroid hormone (PTH) was not carried out among the patients of AURORA study. It would have been insightful in excluding the possibility that high concentrations of PICP were not reflecting bone turnover. We did not adjust our analysis on NT-proBNP, which is an important prognostic factor [63]. In this multicenter prospective randomized trial with more than 2000 patients and adjudicated outcomes, it was not possible to assess other potentially relevant fibrosis biomarkers [8, 64]. Finally, dedicated trials are still needed to test as well as to validate more powerful treatment strategies based on PICP and Gal-3 concentrations.

## Conclusion

Increased concentrations PICP and Gal-3 were synergistically associated with both CV death and all-cause mortality in patients undergoing chronic hemodialysis. Their significant synergistic interaction with regard to CV death and all-cause mortality may reflect Gal-3 driven active CV fibrosis. The measurement of these biomarkers could be useful in stratifying patients in dialysis according to their CV risk. The use of these biomarkers to target patients with high probability to benefit from anti-fibrotic, anti-Gal-3 therapy should be further studied.

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## Declarations

**Conflict of interest** There is no conflict of interests to declare.

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## Supplementary Material

**Supplementary Table 1 Tertiles for hs-CRP (mg/L)**

<b>1<sup>st</sup> tertile (T1)</b>	<b>2<sup>nd</sup> tertile (T2)</b>	<b>3<sup>rd</sup> tertile (T3)</b>
0.0252-0.29	>0.29-0.843	>0.843-4
916	913	915

**Supplementary Table 2 Association between PICP, Gal-3, and hs-CRP with the composite primary endpoint of major adverse CV event (MACE)**

Variables	Univariable		Multivariable			
	HR (95% CI)	p-value	Model 1 HR (95% CI)	p-value	Model 2 HR (95% CI)	p-value
<b>1° endpoint of CV event (n=804)</b>						
<b>PICP</b>						
Per 1SD	1.02 (0.95 - 1.10)	0.52	1.07 (0.99 - 1.15)	0.10	1.06 (0.98 - 1.15)	0.15
Tertiles						
1 <sup>st</sup> tertile	1	-	1	-	1	-
2 <sup>nd</sup> tertile	1.08 (0.90 - 1.29)	0.42	1.09 (0.91 - 1.31)	0.36	1.08 (0.90 - 1.29)	0.42
3 <sup>rd</sup> tertile	1.07 (0.89 - 1.28)	0.48	1.12 (0.93 - 1.36)	0.23	1.11 (0.92 - 1.35)	0.27
<b>Gal-3</b>						
Per 1SD	1.11 (1.03 - 1.19)	<b>0.004</b>	1.17 (1.09 - 1.26)	<b>&lt;0.0001</b>	1.15 (1.07 - 1.24)	<b>0.0003</b>
Tertiles						
1 <sup>st</sup> tertile	1	-	1	-	1	-
2 <sup>nd</sup> tertile	1.05 (0.88 - 1.26)	0.57	1.09 (0.91 - 1.31)	0.37	1.06 (0.88 - 1.28)	0.52
3 <sup>rd</sup> tertile	1.18 (0.99 - 1.41)	0.069	1.31 (1.09 - 1.57)	<b>0.009</b>	1.22 (1.02 - 1.47)	<b>0.034</b>
<b>hs-CRP*</b>						
Log hs-CRP	1.21 (1.14 - 1.28)	<b>&lt;0.001</b>	1.20 (1.13 - 1.27)	<b>&lt;0.0001</b>	1.15 (1.08 - 1.22)	<b>&lt;0.0001</b>
Tertiles						
1 <sup>st</sup> tertile	1	-	1	-	1	-
2 <sup>nd</sup> tertile	1.18 (0.98 - 1.40)	0.074	1.16 (0.96 - 1.37)	0.11	1.12 (0.93 - 1.34)	0.23
3 <sup>rd</sup> tertile	1.58 (1.33 - 1.87)	<b>&lt;0.001</b>	1.51 (1.27 - 1.81)	<b>&lt;0.0001</b>	1.35 (1.12 - 1.61)	<b>0.001</b>

**Model 1:** Adjusted for age, diabetes, history of cardiovascular disease, sex, dialysis vintage, body mass index, and systolic blood pressure (at baseline)

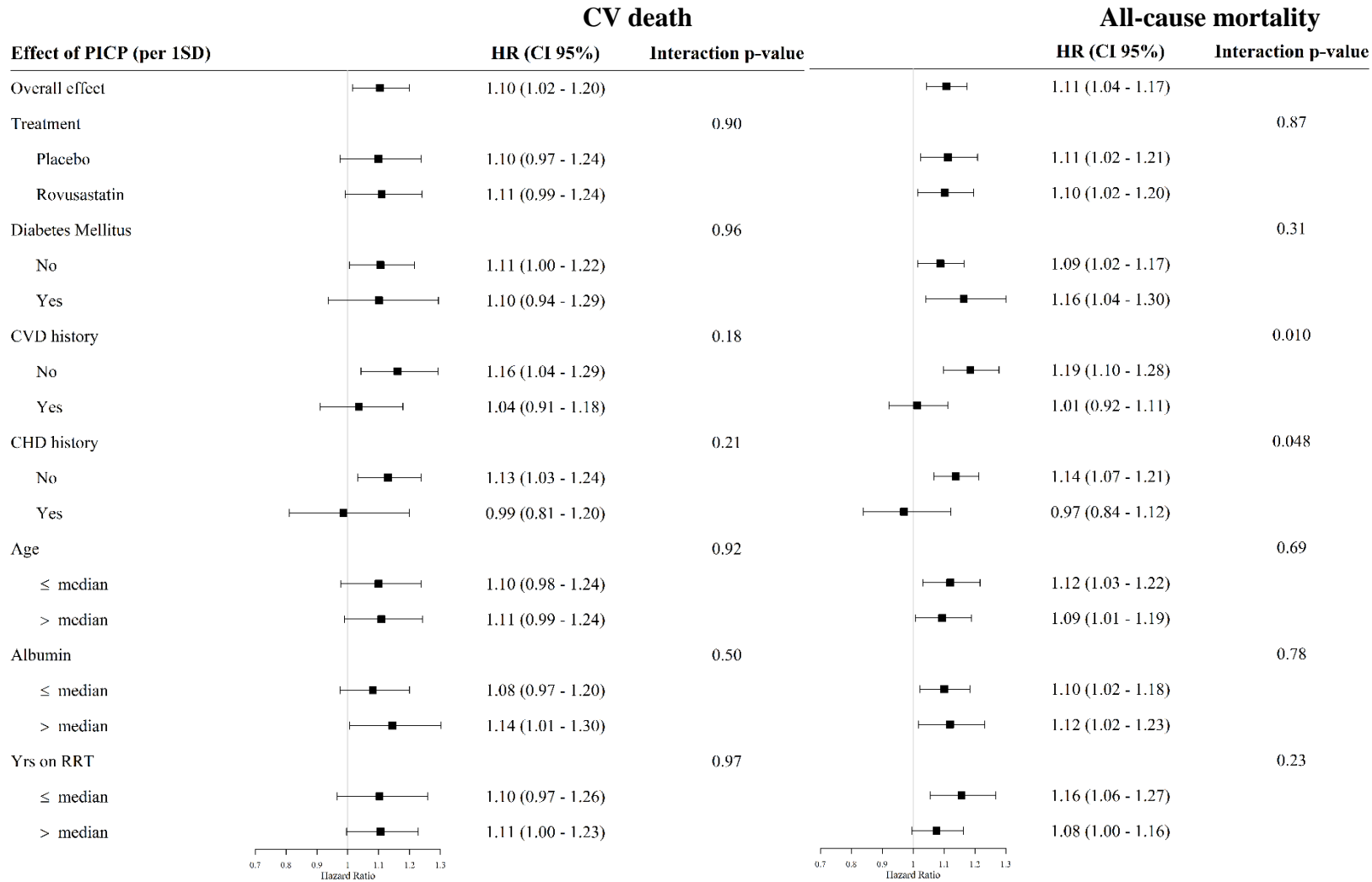
**Model 2:** model 1 + albumin and log hs-CRP (at baseline)

\***Model 2:** model 1 + albumin (at baseline)

**Supplementary Table 3 Complete PICP and Gal-3 models with the composite primary endpoint of major adverse CV event (MACE) as outcome**

	Multivariable			
	<b>PICP Model</b> HR (95% CI)	p-value	<b>Gal-3 Model</b> HR (95% CI)	p-value
<b>1° endpoint of CV event (n=804)</b>				
Per 1SD	1.06 (0.98 - 1.15)	0.15	1.15 (1.07 - 1.24)	<b>0.0003</b>
<b>Adjustment Variables</b>				
Age (years)	1.03 (1.02 - 1.04)	<b>&lt;0.0001</b>	1.03 (1.02 - 1.04)	<b>&lt;0.0001</b>
Diabetes Mellitus (1=Yes, 0=No)	1.49 (1.26 - 1.77)	<b>&lt;0.0001</b>	1.52 (1.29 - 1.80)	<b>&lt;0.0001</b>
History of cardiovascular disease (1=Yes, 0=No)	1.41 (1.20 - 1.65)	<b>&lt;0.0001</b>	1.48 (1.27 - 1.74)	<b>&lt;0.0001</b>
Sex (1=Male, 2=Female)	0.87 (0.74 - 1.03)	0.099	0.91 (0.78 - 1.07)	0.25
Dialysis Vintage (years)	1.01 (0.99 - 1.02)	0.31	1.00 (0.99 - 1.02)	0.79
Body mass index (kg/m <sup>2</sup> )	1.00 (0.98 - 1.02)	0.97	0.99 (0.98 - 1.01)	0.34
Systolic blood pressure (mmHg)	1.00 (1.00 - 1.01)	0.20	1.00 (1.00 - 1.01)	0.19
Albumin (g/L)	0.95 (0.92 - 0.97)	<b>&lt;0.0001</b>	0.95 (0.93 - 0.97)	<b>&lt;0.0001</b>
log hs-CRP (mg/L)	1.16 (1.09 - 1.23)	<b>&lt;0.0001</b>	1.15 (1.08 - 1.23)	<b>&lt;0.0001</b>

**Supplementary Fig. 1 Association between PICP and cardiovascular death or all-cause mortality in subgroups of patients (adjusted analysis)**

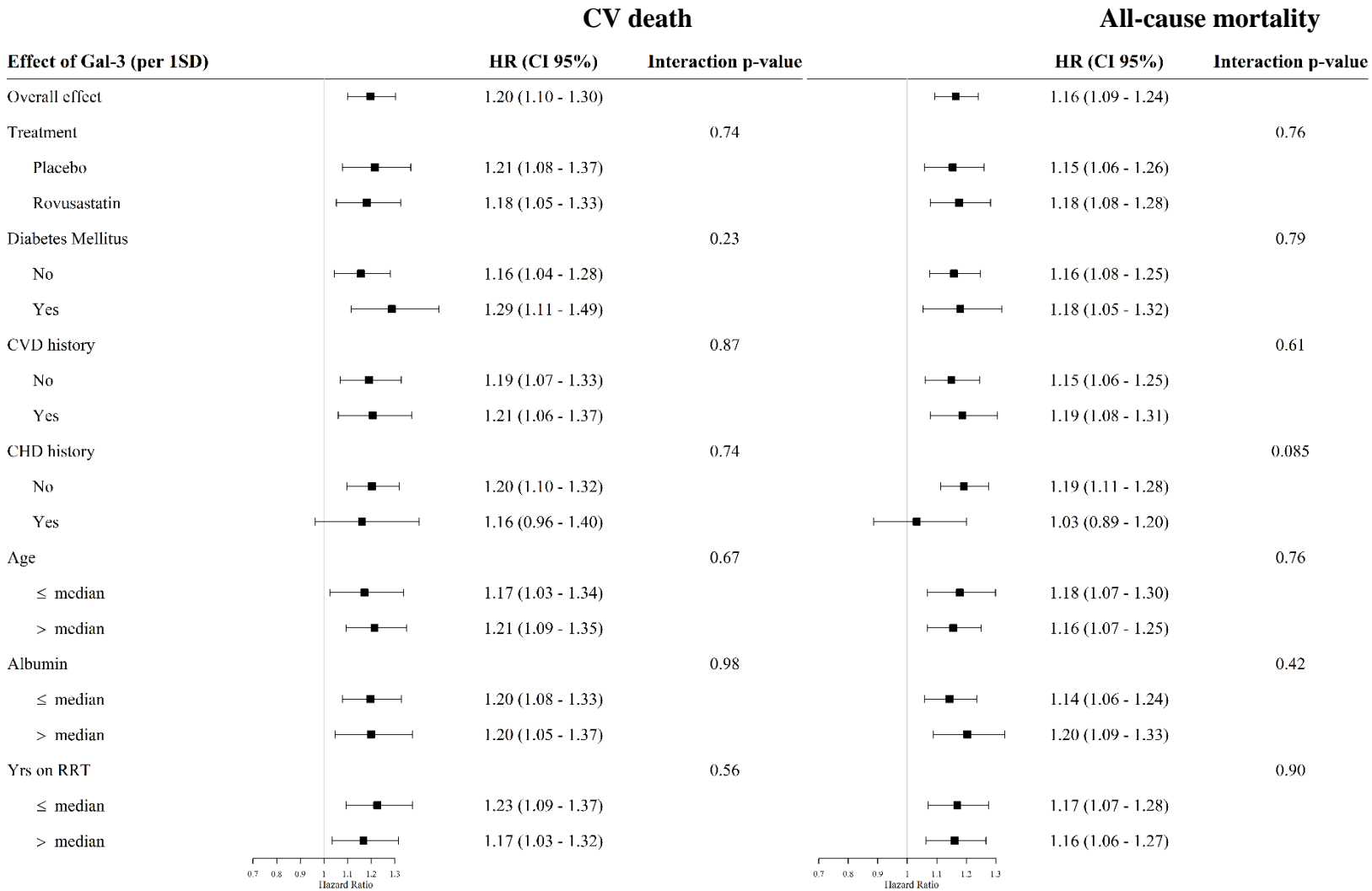


*CVD, Cardiovascular diseases; CHD, Coronary heart diseases; Yrs on RRT, years on renal replacement therapy (dialysis vintage)*

Adjusted for age, diabetes, history of cardiovascular disease, sex, dialysis vintage, body mass index, systolic blood pressure, albumin, and log hs-CRP (at baseline).



**Supplementary Fig. 2 Association between Gal-3 and cardiovascular death or all-cause mortality in subgroups of patients (adjusted analysis)**

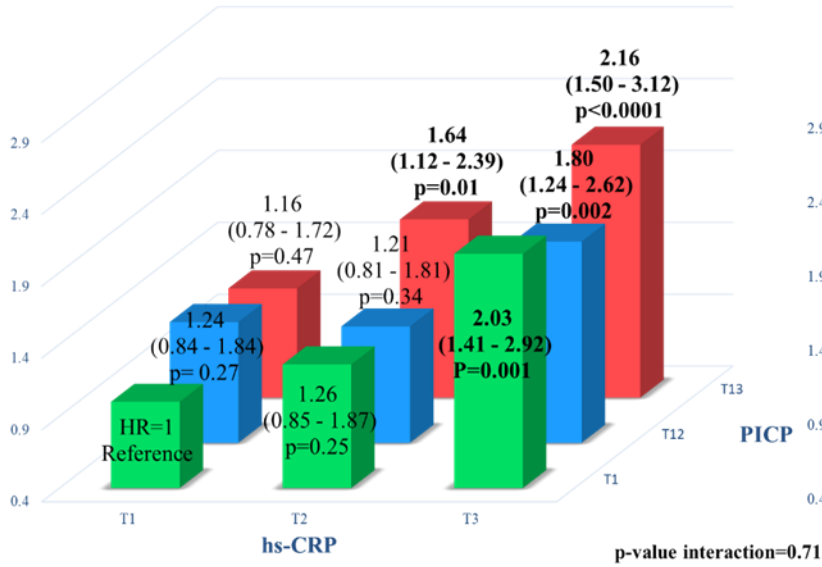


*CVD, Cardiovascular diseases; CHD, Coronary heart diseases; Yrs on RRT, years on renal replacement therapy (dialysis vintage)*

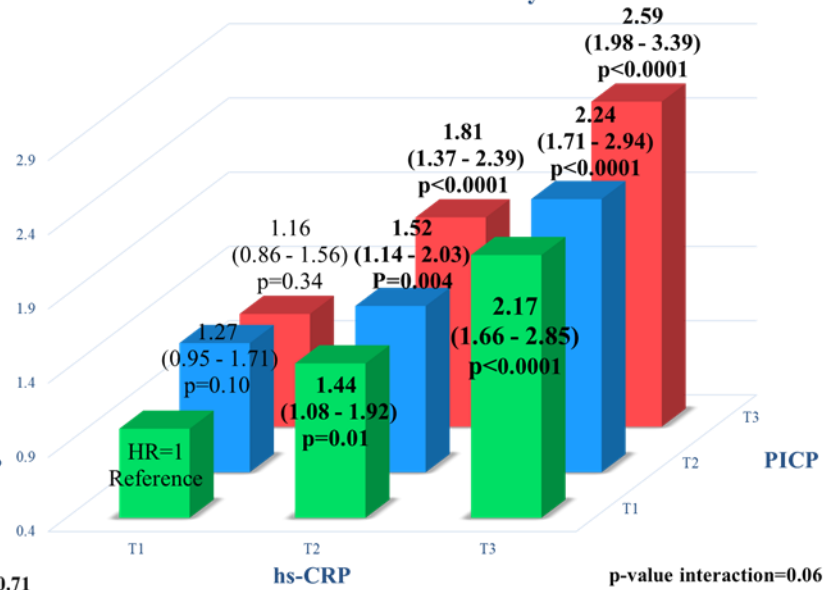
Adjusted for age, diabetes mellitus, history of cardiovascular disease, sex, dialysis vintage, body mass index, systolic blood pressure, albumin, and log hs-CRP (at baseline).

**Supplementary Fig. 3 Interaction between PICP and hs-CRP (in tertiles) for the association with CV death and all-cause mortality**

**Cardiovascular death**



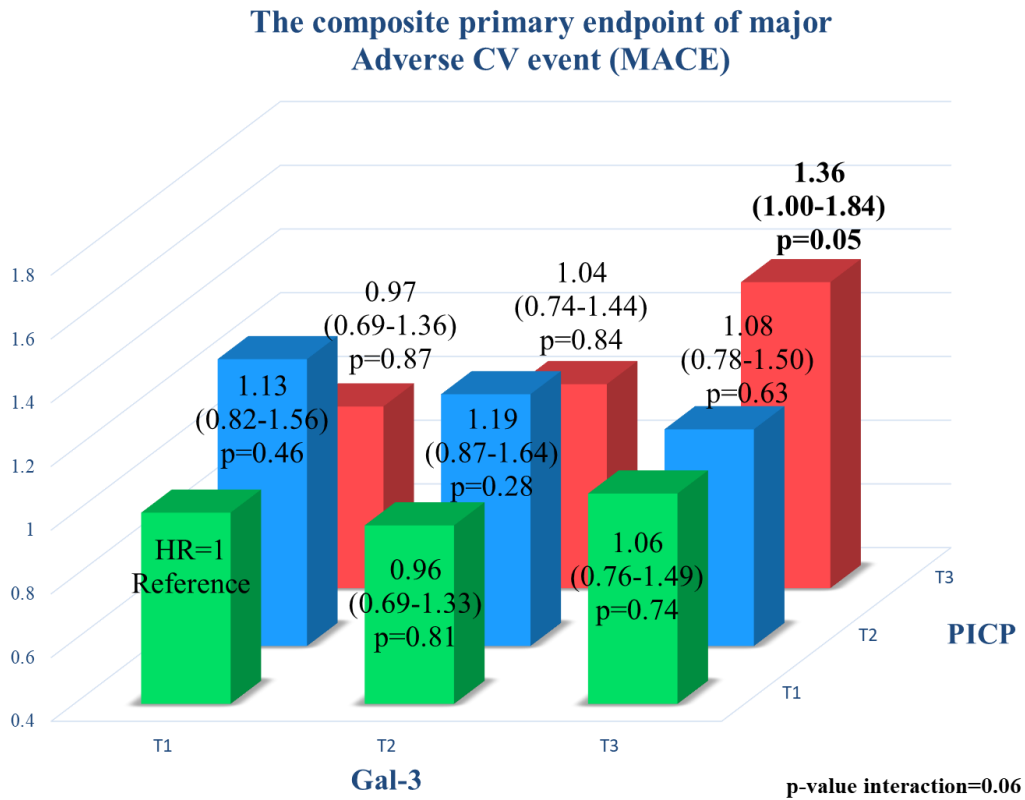
**All-cause mortality**



CV death		PICP		
		T1	T2	T3
		Nevent/N (%)	Nevent/N (%)	Nevent/N (%)
hs-CRP	T1	45/246 (18.3)	58/268 (21.6)	54/262 (20.6)
	T2	55/258 (21.3)	52/250 (20.8)	67/259 (25.9)
	T3	83/276 (30.1)	71/264 (26.9)	79/255 (30.9)

All-cause mortality		PICP		
		T1	T2	T3
		Nevent/N (%)	Nevent/N (%)	Nevent/N (%)
hs-CRP	T1	79/246 (32.1)	104/268 (38.8)	95/262 (36.3)
	T2	110/258 (42.6)	114/250 (45.6)	129/259 (49.8)
	T3	154/276 (55.8)	153/264 (58.0)	164/255 (64.3)

**Supplementary Fig. 4 Interaction between PICP and Gal-3 (in tertiles) for the association with the composite primary endpoint of major adverse CV event (MACE) (non-adjusted analysis)**



Composite primary endpoint of CV event (MACE)		PICP		
		T1	T2	T3
Gal-3	T1	N <sub>event</sub> /N (%)	N <sub>event</sub> /N (%)	N <sub>event</sub> /N (%)
	T2	75/245 (30.6)	74/221 (33.5)	62/206 (30.1)
	T3	68/234 (29.1)	77/215 (35.8)	66/215 (30.7)
	T3	60/196 (30.6)	71/226 (31.4)	93/247 (37.7)

### 2.1.2. Phenotypes using machine learning based on proteomic data in hemodialysis patients: Insights from the AURORA trial (2<sup>nd</sup> article)

The pathophysiology of CV diseases is distinct and complex in patients on hemodialysis and most CV trials have provided neutral results in hemodialysis<sup>5,9</sup>. Moreover, there is no adequate and standard pharmacological strategies to reduce CV outcomes in patients on dialysis because of the different pathophysiology and altered drug metabolism<sup>117</sup>. Therefore, investigating possible mechanistic pathways in hemodialysis for a better understanding of the patient profile may pave the way for personalized therapy which, in turn, will improve CV outcomes in this patient population.

In this study, we aimed to identify biological phenotypes associated with biomarkers from the Olink<sup>®</sup> Proseek Multiplex Cardiovascular II, Cardiovascular III, and Inflammation panels. We also investigated the association of the identified phenotypes with CV outcomes. To do so, we performed clustering analysis using 253 plasma protein biomarkers in 382 patients (dataset 1 - unsupervised machine learning "clustering" derivation analysis). Then we used a simple decision tree to predict cluster membership in another subset of the trial (n= 389 patients - dataset 2 - validation analysis). The predicted clusters were used to assess the association with CV outcomes.

We identified 4 biological phenotypes, namely 'cytokine storm signaling', 'TLRs signaling', 'multiple pathways related to inflammation and fibrosis' phenotypes, and a 'reference phenotype' which showed the least biological abnormalities (i.e. it had the lowest concentration of biomarkers and was mainly linked to the downregulation of inflammation and fibrosis). In multivariable analysis of the validation study, after adjusting for key prognostic factors (i.e. age, history of CV disease, diabetes mellitus, albumin, and hs-CRP), the 'TLRs signaling' phenotype was significantly associated with CV death, all-cause mortality, and MACE [HR=1.65 (1.13 - 2.41), 1.43 (1.03 - 1.98), and 1.48 (1.04 - 2.10), respectively]. The 'multiple pathways related to inflammation and fibrosis' phenotype experienced worse outcome when treated by statin (HR=4.55 (1.25 - 16.55) and 2.66 (1.00 - 7.09) for CV death and all-cause mortality, respectively)

We concluded that machine learning approach (i.e. clustering) based on proteomic data could identify relevant biological phenotypes in patients on hemodialysis. We also showed that the application of complex network analysis (i.e. Ingenuity Pathways Analysis (IPA)) can help understand the mechanistic pathways related to the identified phenotypes. Recently, there were

several initiatives to determine subgroups of patients in the field of KT and HF<sup>118-120</sup>. For instance, Shah et al.<sup>121</sup> used biomarkers and machine learning approaches including hierarchical cluster analysis in the field of HF. They could identify three distinct groups of patients with heart failure with preserved ejection fraction (HFpEF) with remarkable differences in clinical features, cardiac function and clinical outcomes. Similarly, Tromp. et al. performed unsupervised clustering analysis based on CV biomarkers to identify phenotypes with distinct features in clinical outcome and response to treatment in patients with HF<sup>119</sup>. In the field of KT, an unsupervised learning approach of comprehensive pathology findings and clinical, immunologic, and outcome data was used to identify five different profiles in patients with transplant glomerulopathy associated with graft survival<sup>118</sup>. However, clustering approaches based on biomarkers have not been widely used in the field of hemodialysis.

In our study, we were able to identify different biological phenotypes using clustering based on a small number of biomarkers in patients on hemodialysis. We also demonstrated that the identification of TLRs signaling pathway could be of practical therapeutic importance, as TLRs upregulation has been shown to play an important role in the development and progression of CV diseases<sup>122-124</sup>. Importantly, preclinical studies have shown that blocking TLRs with a TLR4 antagonist<sup>125,126</sup> or a soluble TLR2 inhibitor<sup>126,127</sup> could be a promising tool for improving myocardial infarction size and fibrosis.

In the ‘multiple pathways related to fibrosis and inflammation’ phenotype, statin use was surprisingly significantly associated with a higher risk of CV outcomes. Although any *post hoc* analysis may lead to spurious findings, we may postulate that the observed detrimental effect of statins observed in this phenotype may be associated with the complex pathophysiology of CV disease in the uremic milieu making it difficult for patients on dialysis to benefit from the treatment<sup>48,51</sup>. Moreover, a reverse causation cannot be ruled out. Further research may be warranted to determine whether statin can improve the development and prognosis of CV disease in hemodialysis. In addition, the potential benefits of antifibrotic therapies in patients with inflammation and fibrosis needs to be investigated in the field of hemodialysis in light of our results.

In summary, the identification of subgroups of patients on hemodialysis could pave the way for personalized treatment and improved CV prognosis in this population. Our results showed

that machine learning approaches based on proteomic data could achieve this phenotyping, as we identified four biological phenotypes with differences in clinical outcomes and response to treatment.

## **Phenotypes using machine learning based on proteomic data in hemodialysis patients: Insights from the AURORA trial**

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**Word count (excluding tables and references):** 3950



## **ABSTRACT**

### **Objectives**

This study sought to identify biological phenotypes based on proteomic data using machine learning approaches in patients undergoing hemodialysis.

### **Background**

Most cardiovascular (CV) trials have provided neutral results in hemodialysis. A better understanding of patients' profile is needed to personalize treatment strategies in order to improve CV outcomes in this setting.

### **Methods**

We performed clustering analysis using 253 plasma protein biomarkers in 382 patients (machine learning derivation analysis) from the AURORA trial which tested the effect of rosuvastatin on CV outcomes in patients on hemodialysis. We then built a decision tree to predict cluster membership and assess its association with CV outcomes in another subset of the trial (n= 389 patients - validation analysis).

### **Results**

Four phenotypes were identified, namely 'cytokine storm signaling', 'toll-like receptors (TLRs) signaling', 'multiple pathways related to inflammation and fibrosis' phenotypes, as well as a 'reference phenotype' which exhibited the least biological abnormalities. In multivariable analysis of the validation study, after adjusting for key prognostic factors, the TLRs phenotype was significantly associated with CV death, all-cause mortality, and MACE (HR=1.65 [1.13 - 2.41], 1.43 [1.03 - 1.98], and 1.48 [1.04 - 2.10], respectively).

### **Conclusion**

Using unsupervised machine learning on proteomic data, we identified 4 mechanistic biological phenotypes involving cytokine storm and TLRs signaling, inflammation and fibrosis. These biological phenotypes may contribute to CV prognosis and pave the way for personalized therapy in hemodialysis.

**Keywords:** Machine learning, Clustering, hemodialysis, cardiovascular diseases

## INTRODUCTION

Cardiovascular (CV) diseases are the leading cause of death in end-stage renal disease (ESRD), as hemodialysis patients exhibit several structural and functional CV alterations leading to left ventricular hypertrophy (LVH) and heart failure (HF)<sup>1</sup>. CV death in patients undergoing chronic hemodialysis is about 20 folds higher than in the general population<sup>2</sup>. However, there is no evidence to guide the use of pharmacological therapies in hemodialyzed population<sup>3</sup>. Several meta-analyses reported that statins had no significant effect on all-cause mortality, CV events, or CV death in dialysis<sup>4,5</sup>. Therefore, there is urgent need for a novel approach to expand our understanding of the mechanisms underlying the development and the progression of CV diseases in patients on hemodialysis. This approach could improve the effectiveness of the medications in the targeted subsets of patients.

In recent years, several clustering attempts were crucial for identifying disease phenotyping in the field of HF<sup>6-8</sup>. Ahmad et al.<sup>6</sup> reported novel HF phenotypes using clustering based on clinical and proteomic data in a population of chronic HF. Moreover, Shah et al.<sup>7</sup> used machine learning approaches to identify distinct phenotypes in HFpEF patients based on biomarker profiles. In the field of nephrology, machine learning approaches are scarce. In kidney transplantation, Aubert et al.<sup>9</sup> reported distinct groups of transplant glomerulopathy based on clinical, immunologic and histologic variables associated with graft outcomes by using an unsupervised learning approach. Clustering in dialysis has been previously reported in small observational cohorts but only for the analysis of symptoms<sup>10</sup>. Of note, the application of the circulating biomarkers to characterize patients undergoing hemodialysis has not yet been tested in large multicenter randomized clinical trials (RCTs).

Importantly, the validation of biomarkers relevant to the biological mechanisms underlying hemodialysis phenotypes may allow effective detection of patients at higher risk of developing CV diseases. This will improve the accuracy of diagnosis and consequently lead to a better treatment response<sup>11</sup>. The AURORA trial<sup>12</sup> gives us the opportunity to perform a clustering analysis, confirm its potential association with all-cause and CV mortality in a setting of an RCT with adjudicated outcomes, and evaluate whether it predict treatment response. This new approach based on biological clustering may shed a new light on treatment effect heterogeneity in end-stage renal disease.

In this study, we sought to identify mechanistic pathways associated with circulating biomarkers in patients on hemodialysis from the AURORA trial<sup>12</sup>. Then we attempted to investigate the association of the identified clustering patterns with CV outcomes and its interaction with statin effect.

## **METHODS**

### **Patient population**

The description, baseline data and main results of the AURORA study have been published previously<sup>12-14</sup>. In short, AURORA is a double-blind, randomized, multi-center study involving 2773 men and women aged 50 to 80 years, who had been treated with maintenance hemodialysis or hemofiltration for at least 3 months. Eligible patients were randomly assigned in a 1:1 ratio to receive either rosuvastatin, 10 mg daily or matching placebo and had been followed-up for 3.8 years. There was no significant effect of rosuvastatin on the composite primary endpoint of major adverse cardiovascular event (MACE) (i.e. nonfatal myocardial infarction, nonfatal stroke, or death from CV causes) [HR 0.96 (95% CI 0.84 to 1.11, p=0.6)]. Rosuvastatin also exhibited no significant effects on the secondary endpoint of all-cause mortality [HR 0.96 (95% CI 0.86 to 1.07), p=0.5].

In the present study, there were two datasets: unsupervised machine learning (clustering) derivation analysis (dataset 1) and validation analysis (dataset 2). Of the 402 patients available in dataset 1, we excluded 20 samples because they did not pass the quality control for biomarker analysis (n=18) or because they were identified as outliers by the clustering method (n=2). Of the 400 patients in dataset 2, we excluded 11 samples because they did not pass the quality control for biomarker analysis. Analyses were conducted on 382 patients in dataset 1 and 389 patients in dataset 2. The overview of included patients is presented in Supplementary Figure S1.

For the unsupervised machine learning (clustering) analysis (dataset 1), 19 mixed biological and clinical data were considered, including continuous and categorical candidate variables. Continuous variables included dialysis vintage (years), body mass index (BMI) (kg/m<sup>2</sup>), albumin (g/L), hemoglobin (g/dL), log high sensitivity C-reactive protein (hs-CRP) (mg/L), phosphate (mmol/L), fasting glucose level (mmol/L), creatine kinase (IU/L), aspartate aminotransferase (IU/L), calculated LDL-Cholesterol (mmol/L), neutrophil-lymphocyte ratio (NLR), systolic blood (mmHg), and pulse pressure (mmHg). Categorical variables included history of coronary heart

disease (i.e. prior myocardial infarction, prior coronary angioplasty or stent, and coronary artery bypass graft), history of vascular disease (i.e. peripheral artery disease, abdominal aortic aneurysm, carotid artery disease, carotid stenosis  $\geq$  50%, and carotid endarterectomy), history of neurovascular disease (i.e. prior ischemic vascular accident and transient ischemic attack), diabetes mellitus, cause of ESRD, and smoking status.

For the prediction of cluster membership (using decision tree) as well as for the validation analysis (dataset 2), 37 variables were considered as follows: the 19 biological and clinical variables mentioned above with an additional set of 18 variables including age (years), sex, calculated  $K_t/V$ , years on hemodialysis treatment, diastolic blood pressure (mmHg), heart rate (beats/min), current dialysis treatment (hemodialysis, hemofiltration / hemodiafiltration), type of access (arterial venous fistula, central dialysis catheter, or graft), and symptoms related to dialysis (i.e. hypotension, hypertension, restless legs, nausea, vomiting, headache, muscle cramps, itching, fatigue, and catheter infection).

## **Outcomes**

The pre-specified considered outcomes are CV death, all-cause mortality, and the composite primary endpoint of the AURORA trial (MACE). All the recorded events were reviewed and adjudicated by a clinical end-point committee to ensure consistency of the event diagnosis. The committee members were unaware of the randomized treatment assignments.

## **Proteomic biomarkers**

Plasma samples were analyzed for 274 protein biomarkers using the Olink<sup>®</sup> Proseek Multiplex Cardiovascular II, Cardiovascular III, and Inflammation panel. The proteins were measured by 96 × 96 kits. Each kit uses Proximity Extension Assay (PEA) technology with a dual recognition DNA-coupled readout, where 92 oligonucleotide-labeled antibody probe pairs bind to their respective targets. When binding to their correct targets, they give rise to new DNA amplicons each ID-barcoding their respective antigens. The amplicons are subsequently quantified using a Fluidigm BioMark HD realtime Polymerase Chain Reaction (PCR) platform. The platform provides log<sub>2</sub> Normalized Protein Expression (NPX) values with relative quantification. A detailed description of the Olink technology is provided on the company's website (<https://www.olink.com/>). Protein biomarkers with more than 25% non-detectable values (n=21)

were excluded, hence 253 biomarkers were ultimately studied, as we published<sup>15</sup>. The complete list of biomarkers is presented in Supplementary Table S1.

### **Statistical analysis**

Before using biological variables in the clustering analysis, we computed residuals from fitted linear regression models, where each protein biomarker was used as dependent variable and sex, age, and calculated  $K_t/V$  were used as independent variables. Residual values of 100 protein biomarkers with the highest standard deviation (Supplementary Table S1) were retained and used as input for the clustering analysis.

Latent Class Model (LCM) clustering was conducted for the residuals for the 100 protein biomarkers and the 19 mixed clinical and biological variables using VarSelLCM R package<sup>16</sup>. The estimation of the number of clusters is achieved by running the Integrated Complete-data Likelihood (ICL) criterion and is plotted to facilitate the selection of the number of clusters. In general, the optimal number of clusters is the one preceding the first plateau in the plot. Clusters differences were assessed using the Kruskal-Wallis test for continuous variables (i.e. biomarkers and biological data) and Chi-square test for categorical data (i.e. clinical data) (Supplementary Table S2). A Radar chart was generated for 50 biomarkers with the smallest resulting p-value to visualize patients' profiles by clusters.

We used the values of the 253 biomarkers from dataset 1 and the 37 candidate variables previously listed to build a decision tree to predict cluster membership using the rpart R program<sup>17</sup>, where the program decides on the levels of the expressed biomarkers to set the cutoff. The threshold of the observations in any terminal leaf of the decision tree was set to be not less than 5%.

The resulting decision tree was used to identify patients classified in each cluster in dataset 2. The association of the predicted clusters, which we have called phenotypes, with outcome was then evaluated. Furthermore, the association of phenotypes with the use of rosuvastatin was assessed.

Baseline clinical, and demographic participants characteristics were compared across the phenotypes using analysis of variance (ANOVA), Kruskal-Wallis, and chi-squared tests, as appropriate. Categorical variables are expressed as frequencies (percentages) and continuous variables are expressed as means  $\pm$  standard deviation for normally distributed data and median interquartile ranges (25<sup>th</sup> and 75<sup>th</sup> percentiles) for non-normally distributed data.

The associations of phenotypes, with CV death, all-cause mortality, and MACE were assessed using univariate and multivariate Cox proportional hazard regression models. An additional Cox proportional hazards regression analysis was constructed to assess the association between baseline prescription rosuvastatin and the pre-specified outcomes. For multivariable analyses, models were corrected for relevant clinical and biological confounders, which were previously published<sup>18</sup> (i.e. age, history of CV disease, diabetes mellitus, albumin, and high sensitivity C-reactive protein (hs-CRP)), dialysis vintage, and B-type natriuretic peptide (BNP). History of CV disease is defined as history of coronary heart disease, history of vascular disease, and history of neurovascular disease. BNP and hs-CRP were best modeled by using their natural logarithm.

Statistical approaches for each dataset are resumed in Figure 1. All analyses were performed using R version 4.0.2 (R Development Core Team, Vienna, Austria). The two-sided significance level was set at p-value < 0.05.

### **Ingenuity Pathways Analysis**

Residuals for each biomarker (n=253) were averaged across patient samples of each phenotype and compared across phenotypes and only significant biomarkers (p-value  $\leq 0.05$ ) were kept (n=215, Supplementary Table S3). The resulting four lists of biomarkers (i.e. UniProt ID is provided from the Olink website) were forwarded to Ingenuity Pathways Analysis (IPA) (version 70750971, release date 2021-10-23, Ingenuity Systems) to extract canonical pathways associated with the candidate biomarkers. The canonical pathways enriched by differential proteins were generated based on the Ingenuity Pathway Knowledge Data Base. IPA uses z-score to indicate a predicted activation or inhibition of a certain pathway, where values of  $|z| > 2$  were defined as the threshold of significance. A negative z value ( $\leq -2$ ) connotes an overall inhibition of the pathway and a positive z value ( $\geq 2$ ) connotes an overall activation of the pathway.

## **RESULTS**

### **Identification of Clusters**

We identified 4 phenotypes that included from 48 to 154 patients. The 50 biomarkers most significantly associated with patient phenotype were plotted in a radar chart (Figure 2). Phenotype 4 had the lowest concentration of the tested biomarkers together with the largest number of patients

(n=154). Phenotype 3 had usually the highest level of biomarkers, whereas the 2 other phenotypes were overall intermediate.

### **Labeling of clusters using enriched canonical pathways**

After excluding cancer pathways, a total of 88 enriched canonical pathways were identified using IPA (Supplementary Figure S2). To prevent the inclusion of redundant pathways, we ranked pathways on IPA and restricted the analysis to the highest-scoring 20 pathways (using absolute z-score > 2) that are significantly activated or inhibited pathways in each phenotype. Biomarkers that were strongly expressed in phenotype 1 were associated with cytokine storm signaling (mainly the adaptive immune system) pathway referred to thereafter as “cytokine storm signaling phenotype” (Supplementary Figure S3). Phenotype 2 was particularly linked to toll-like receptors (TLRs) (the innate immune system) signaling (“toll-like receptors signaling phenotype”, Supplementary Figure S4). Phenotype 3 was linked to the activation of multiple pathways related to inflammation and fibrosis (“multiple pathways related to inflammation and fibrosis phenotype”, Supplementary Figure S5), whereas phenotype 4, “reference phenotype”, was mainly linked to the inhibition of the same pathways as it included patients with the least biological abnormalities (Supplementary Figure S6).

### **Baseline characteristics of patients across the phenotypes in dataset 1**

Patients in cytokine storm signaling phenotype (n=91) had the longest dialysis vintage (median 4.3 years in cytokine storm signaling phenotype versus 2.0 years in toll-like receptors signaling phenotype,  $p<0.01$ ) and the highest concentration of hs-CRP (mean concentration 1.6 mg/L in cytokine storm signaling phenotype versus 0.8 in reference phenotype,  $p<0.01$ ). Patients with the lowest BMI ( $p<0.01$ ) and lowest creatine kinase concentrations (IU/L) ( $p=0.02$ ) were in cytokine storm signaling phenotype (Table 1).

### **Decision tree**

The decision tree retained caspase-3 (CASP-3), axin-1 (AXIN1), hepatocyte growth factor (HGF), and tumor necrosis factor ligand superfamily member 14 (TNFSF14) as the most relevant biomarkers to correctly classify patients in the identified phenotypes. The global accuracy of the decision tree was very good i.e. 84% (Figure 3).

## **VALIDATION ANALYSIS**

### **Baseline characteristics of patients across the phenotypes in dataset 2**

Using the aforementioned decision tree, patients in the validation dataset were classified into the four aforementioned phenotypes, which ranged from a minimum of 40 (phenotype “multiple pathways related to inflammation and fibrosis phenotype”) to a maximum 196 (phenotype “reference”) patients. Patients in phenotype “cytokine storm signaling” (n=49) were older (p=0.03), had lower albumin levels (p<0.01), the highest hs-CRP concentration (p=0.04), and the highest mortality rate (p=0.05) (Supplementary Table S4).

### **Association of predicted phenotypes with CV death, all-cause mortality, and MACE**

Phenotype “cytokine storm signaling” was significantly associated with all-cause mortality in crude model. However, this association had a trend to be significant in multivariable adjusted analysis [crude HR=1.59 (1.09 - 2.34) and adjusted HR=1.44 (0.97 - 2.15)] (Table 2).

Phenotype “toll-like receptors signaling” was significantly associated with CV death, all-cause mortality, and MACE in crude models [HR=1.51 (1.05 - 2.16), 1.38 (1.02 - 1.88), and 1.44 (1.03 - 2.01), respectively] (Table 2). In multivariable analysis (Table 2), after adjusting for key prognostic factors, Phenotype “toll-like receptors signaling” remained significantly associated with the pre-specified outcomes [HR=1.65 (1.13 - 2.41), 1.43 (1.03 - 1.98), and 1.48 (1.04 - 2.10), respectively].

### **Association between rosuvastatin and clinical outcomes according to phenotypes**

In univariate analysis, statin use was associated with an increase in the risk of CV death and all-cause mortality in comparison to placebo in patients with phenotype “multiple pathways related to inflammation and fibrosis” (crude HR=5.03 (1.43 - 17.67) and 2.95 (1.14 - 7.61), respectively) (Table 3) and that persisted after adjustment (HR=4.55 (1.25 - 16.55) and 2.66 (1.00 - 7.09), respectively) (Table 3). The p-value for interaction between the use of statin and phenotypes with regards to CV death was significant (p=0.09) (Table 3).

## **DISCUSSION**

Our main findings are that clustering analysis can identify biological phenotypes in patients treated with hemodialysis that can be predicted with a limited number of biomarkers. The underlying pathways we identified were mainly related to “cytokine storm signaling”, “TLRs signaling”,



“multiple pathways related to inflammation and fibrosis” (Central Illustration). These phenotypes were associated with CV outcome, and identified a subgroup of patients (labelled as multiple pathways related to inflammation and fibrosis) that actually had worse outcome when being treated with statin.

### **Machine Learning and complex network use in medicine**

Clustering analysis was previously used to identify phenotypes in a number of diseases/syndromes such as HF<sup>6-8</sup>. Proteomic biomarkers can further fuel clustering approaches to identify distinct biologic profiles. A study by Tromp et al.<sup>8</sup> used biomarker profiles constructed using clustering approaches to determine distinct clinical profile/evolution and treatment response in patients with HF. Similarly, in chronic kidney disease (CKD), Iatropoulos et al.<sup>19</sup> identified distinct pathogenetic patterns of patients with C3 glomerulopathy and immune complex-mediated membranoproliferative glomerulonephritis to improve risk assessment for ESRD using unsupervised hierarchical clustering. In addition, in kidney transplant patients, a study by Aubert et al.<sup>9</sup> distinguished the diagnostic and prognostic characteristics associated with 552 graft biopsies using an unsupervised analysis approach. Yet, clustering has not been widely applied in the field of hemodialysis. Our results show that it is indeed possible to identify biological profiles, which have clinical relevance, as they are associated with clinical outcomes. In addition, the fact that these phenotypes could be identified using a simple decision tree may ease their identification in the future, since only a few biomarkers are needed.

Following the identification of clusters, complex network analysis can help better understanding the profiles identified by machine learning. This approach has been recently used by our group<sup>20</sup> to identify proteomic profiles which could reflect the pathophysiological mechanisms in HF. In the analysis presented herein, the complex network approach helps us label phenotypes, and emphasized the importance of cytokine storm signaling and TLRs signaling, along with fibrosis and inflammation that are usual suspects of the cardiorenal physiopathology<sup>21</sup>.

### **Cytokine storm signaling and toll-like receptors signaling phenotypes**

We identified two phenotypes mainly characterized by an involvement of ‘cytokine storm signaling’ or ‘TLRs signaling’, and a third associated with ‘multiple pathways related to inflammation and fibrosis’.

CKD and ESRD are associated with disturbances of the immune system characterized by chronic inflammation, as well as immunosuppression<sup>22</sup>. Both the innate and the adaptive immunity are modified by CKD. The inability to maintain an effective immune response in ESRD patients is dependent on a number of features including increased oxidative stress and inflammation, uremia, intestinal dysbiosis, renin–angiotensin–aldosterone stimulation, disrupted salt homeostasis, T helper 17 cell polarization, and dialysis itself<sup>22,23</sup>.

Cytokine have been repeatedly shown to be associated with outcome in hemodialysis including interleukins (IL)-1<sup>24</sup>, IL-6<sup>24-26</sup>, IL-8<sup>26</sup>, tumor necrosis factors (TNF)- $\alpha$ <sup>24,25</sup>, and IL-13<sup>24</sup>. Cytokine storm involves uncontrolled excessive release of proinflammatory cytokines including IL-1 $\beta$  and IL-6 and TNF<sup>22,27,28</sup>. During dialysis, the interaction between blood constituents (i.e. the leukocytes) and dialyzer and soluble membrane components trigger the production of the proinflammatory cytokines<sup>27,29</sup>.

TLRs belong to the family of signaling pattern-recognition receptors of the innate immunity system and play an important role in triggering inflammatory cytokines and immune response to pathogens. Therefore, altered TLRs expression may contribute to the increased susceptibility to inflammation and infections in patients with ESRD<sup>30-32</sup>. TLR2 and TLR4, in particular, are referred as damage-associated molecular patterns (DAMPs) that trigger pro-inflammatory and profibrotic responses<sup>30</sup>. Indeed, Gollapudi et al.<sup>33</sup> found that TLR2 and TLR4 expressions in monocytes were upregulated in 21 patients undergoing hemodialysis when compared to normal controls. TLR4 is known to be expressed on several tissues, including cardiomyocytes, macrophages (whose concentrations increase preferentially in lipid-rich plaques), and endothelial and smooth muscle cells<sup>34</sup>. Overall, TLR4 is stimulated by various agents that bridge inflammation, infection, and hyperlipidemia. It should be noted that the uraemic environment stimulates the upregulation of TLR4, which in turn contributes to the development of dialysis-associated endothelial dysfunction<sup>35</sup>. Of note patients from the group “TLRs signaling phenotype” experienced the worst outcomes.

As there is increasing evidence that TLRs play an important role in several CV diseases such as atherosclerosis<sup>36,37</sup> and HF<sup>37,38</sup>, TLRs might actually represent a therapeutic target. Shimamoto et al.<sup>39</sup> showed that eritoran, a pharmacological TLR4 antagonist that has been shown to be safe in human, significantly reduced myocardial infarct size and inflammatory mediators during

myocardial ischemia-reperfusion injury in mice. In peritoneal dialysis (PD), a soluble form of TLR2 (sTLR2), a therapeutic TLR inhibitor, was found to prevent the development of PD-associated peritoneal inflammation and fibrosis in mice<sup>40,41</sup>. This therapeutic strategy may represent in the future a useful approach to improve CV fibrosis in patients undergoing dialysis. Another strategy is to investigate the potential use of the systemic levels of DAMPs as biomarkers to phenotype patients at higher risk to develop CV diseases<sup>42</sup>. However, further experimental studies and RCTs are needed for a deeper understanding of the role of TLRs in CV diseases, as TLRs are necessary for a normal immune response and bacterial infiltration. Importantly, preclinical studies have shown the potential of administering anti-TLR2/TLR4 antibodies in addition to antibiotics to reduce inflammation while controlling infection<sup>43,44</sup>.

### **Multiple pathways related to inflammation and fibrosis phenotype**

Fibrosis is known to be a major pathway involved in CV complications in the setting of CKD<sup>21</sup>. We previously reported among the AURORA trial patients that an increased concentration of procollagen type I C-terminal pro-peptide (PICP) and galectin-3 (Gal-3) - that are fibrosis markers - was synergistically associated with CV death and all-cause mortality in patients treated with hemodialysis<sup>45</sup>. Of note, there is a continuum between inflammation and fibrosis, and the mineralocorticoid receptor (MR) activation plays a major role in this cascade<sup>46</sup>. Patients in phenotype 3 had the highest inflammation profile together with fibrosis.

In the current study, the statin use was associated with a higher risk of CV death and all-cause mortality in the phenotype ‘multiple pathways related to inflammation and fibrosis’. Two trials<sup>47,48</sup> investigating the effect of statin use in HF, showed no clinical benefit on several outcomes, including the number of CV hospitalizations and mortality. However, the benefit of statin for CV risk reduction is well established<sup>49</sup>. The suspected underlying explanation of the increased CV risk under statins among patients of the group ‘multiple pathways related to inflammation and fibrosis’ could be that the pathophysiology of CV disease in patients on hemodialysis may be different (i.e. more complex), making it difficult for patients on dialysis to benefit from statins<sup>50,51</sup>. Studies have shown that statins can potentially accelerate vascular calcifications, thus contributing to LVH accompanied by myocardial fibrosis and HF<sup>52</sup>. These points may explain the detrimental effect of statins observed in this phenotype.

## **Association of phenotypes with outcomes – Basis for personalized approach**

The goal of this study was to identify patterns in hemodialysis patients, using machine learning approaches based on proteomic biomarkers. This approach allowed us to reduce patient heterogeneity and identified meaningful biological profiles regarding signaling pathways associated with inflammation as well as innate and adaptive immunity.

The use of biomarkers is a promising way in order to improve the selection of patients who are likely to benefit from a drug intervention<sup>53,54</sup>. However, the identification of a set of biomarkers to characterize patients undergoing hemodialysis has not yet been tested in large trials. In addition, there is no clear evidence that robustly determines a standard treatment protocol for patients on hemodialysis to reduce the risk of CV morbidity and mortality<sup>2,3,49</sup>. Our study revealed that the use of machine learning approaches with proteomic data could be a promising tool to characterize different pathways in patients on hemodialysis who could benefit from an individualized treatment. The identification of a TLRs signaling phenotype may be of practical importance in the future as some treatment do specifically target TLRs. Considering the tolerance issue of mineralocorticoid receptor antagonists (MRAs) in dialysis (especially the risk of hyperkalemia), targeting patients with an upregulated fibrosis pathway could also help selecting patients who may be likely to benefit from these drugs (or help restrict the potential indications for patients with an upregulated fibrosis pathway), even if a narrow biological survey is mandatory in these selected patients. This therapeutic avenue might be worth exploring in the field of hemodialysis in light of our results.

## **CONCLUSION**

To the best of our knowledge, this is the first study performing clustering analysis in hemodialyzed patients based on proteomic data. The identified phenotypes reflected underlying mechanistic pathways and association with all-cause and CV mortality in a subset of a large multicenter RCT in dialysis. They are related to “cytokine storm signaling”, “TLRs signaling,” and “multiple pathways related to inflammation and fibrosis”.

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**Phenotypes using machine learning based on proteomic data in  
hemodialysis patients: Insights from the AURORA trial**

**Tables & Figures**

**Table 1. Baseline characteristics according to phenotypes in dataset 1**

	Global (n=382)	Phenotype 1 (n=91)	Phenotype 2 (n=89)	Phenotype 3 (n=48)	Phenotype 4 (n=154)	p-value
		Cytokine storm signaling	Toll-like receptors signaling	Multiple pathways related to inflammation and fibrosis	Reference	
<b>Baseline characteristics</b>						
Female gender (%)	79 (20.7)	13 (14.3)	17 (19.1)	14 (29.2)	35 (22.7)	0.18
Age (years)	67 [60, 74]	67 [60, 74]	67 [60, 74]	67 [60, 74]	67 [59, 73]	0.86
BMI (kg/m <sup>2</sup> )	25.0 (3.5)	23.8 (3.2)	24.8 (3.4)	25.0 (3.1)	25.8 (3.6)	<b>&lt;0.01</b>
Current smoker	64 (16.8)	20 (22.0)	13 (14.6)	7 (14.6)	24 (15.6)	0.5
<b>Medical history, n (%)</b>						
Years on RRT	2.8 [1.0, 6.0]	4.3 [2.2, 10.5]	2.0 [0.7, 5.4]	3.4 [1.1, 6.0]	2.1 [0.9, 4.2]	<b>&lt;0.01</b>
Diabetes	80 (20.9)	12 (13.2)	24 (27.0)	12 (25.0)	32 (20.8)	0.13
History of cardiovascular disease	166 (43.5)	38 (41.8)	35 (39.3)	25 (52.1)	68 (44.2)	0.53
Peripheral artery disease	66 (17.3)	16 (17.6)	19 (21.3)	7 (14.6)	24 (15.6)	0.66
History of coronary heart disease	72 (18.8)	20 (22.0)	15 (16.9)	7 (14.6)	30 (19.5)	0.7
History of vascular disease	95 (24.9)	19 (20.9)	25 (28.1)	14 (29.2)	37 (24.0)	0.61
History of neurovascular disease	65 (17.0)	14 (15.4)	13 (14.6)	10 (20.8)	28 (18.2)	0.76
<b>Cause of ESRD, n (%)</b>						
Diabetes	51 (13.4)	7 (7.7)	17 (19.1)	7 (14.6)	20 (13.0)	0.16
Genetic conditions	49 (12.8)	9 (9.9)	8 (9.0)	7 (14.6)	25 (16.2)	
Glomerulonephritis or Vasculitis	68 (17.8)	19 (20.9)	21 (23.6)	6 (12.5)	22 (14.3)	
Nephrosclerosis	89 (23.3)	17 (18.7)	17 (19.1)	14 (29.2)	41 (26.6)	
Pyelonephritis or Interstitial	60 (15.7)	16 (17.6)	13 (14.6)	8 (16.7)	23 (14.9)	
Unknown/unspecified	43 (11.3)	13 (14.3)	7 (7.9)	5 (10.4)	18 (11.7)	
Other	22 (5.8)	10 (11.0)	6 (6.7)	1 (2.1)	5 (3.2)	
<b>Characteristics</b>						
Systolic Blood Pressure (mmHg)	139 (24.2)	141 (23.1)	139 (22.1)	137 (20.8)	138 (26.9)	0.8
Diastolic Blood Pressure (mmHg)	77 (12.8)	78 (12.3)	75 (13.5)	76 (10.9)	77 (13.3)	0.56
Pulse pressure (mmHg)	60 [50, 73]	67 [49, 75]	63 [51, 72]	60 [50, 72]	60 [45, 71]	0.44
Calculated K <sub>t</sub> /V	1.1 [1.0, 1.3]	1.1 [1.0, 1.3]	1.1 [0.9, 1.3]	1.1 [1.0, 1.3]	1.1 [1.0, 1.2]	0.72
Albumin (g/L)	39.4 (3.0)	39.2 (3.0)	39.3 (3.4)	39.8 (2.6)	39.4 (3.0)	0.77
Hemoglobin (g/dL)	11.8 (1.5)	11.9 (1.6)	11.9 (1.4)	12.1 (1.5)	11.6 (1.5)	0.32
hs-CRP (mg/L)	1.2 (1.2)	1.6 (1.3)	1.3 (1.3)	1.3 (1.2)	0.8 (0.9)	<b>&lt;0.01</b>
Fasting Glucose level (mmol/L)	4.9 [4.6, 5.7]	4.9 [4.4, 5.4]	5.0 [4.7, 5.5]	5.1 [4.7, 6.0]	4.9 [4.5, 5.7]	0.19
Creatine Kinase (IU/L)	56 [36, 84]	44 [33, 72]	59 [33, 85]	50 [36, 78]	61 [39, 88]	<b>0.02</b>
Aspartate Aminotransferase (IU/L)	16.0 (8.7)	16.1 (8.4)	16.9 (10.3)	16.3 (6.5)	15.4 (8.6)	0.62
Calculated LDL-Cholesterol (mmol/L)	2.6 (0.9)	2.5 (0.8)	2.6 (1.0)	2.6 (1.0)	2.6 (0.8)	0.83
<b>At follow-up</b>						
MACE	190 (49.7)	54 (59.3)	44 (49.4)	20 (41.7)	72 (46.8)	0.16
CV death	147 (38.5)	42 (46.2)	31 (34.8)	16 (33.3)	58 (37.7)	0.34

Death	217 (56.8)	59 (64.8)	45 (50.6)	30 (62.5)	83 (53.9)	0.17
Non-CV death	69 (18.1)	17 (18.7)	14 (15.7)	13 (27.1)	25 (16.2)	0.34

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*BMI, body mass index; Years on RRT, years on renal replacement therapy; ESRD, end-stage renal disease; hs-CRP, high sensitivity C-reactive.*

*Results with p value less than 5% were emphasized using bold letters*

**Table 2. Association between phenotypes and clinical outcomes**

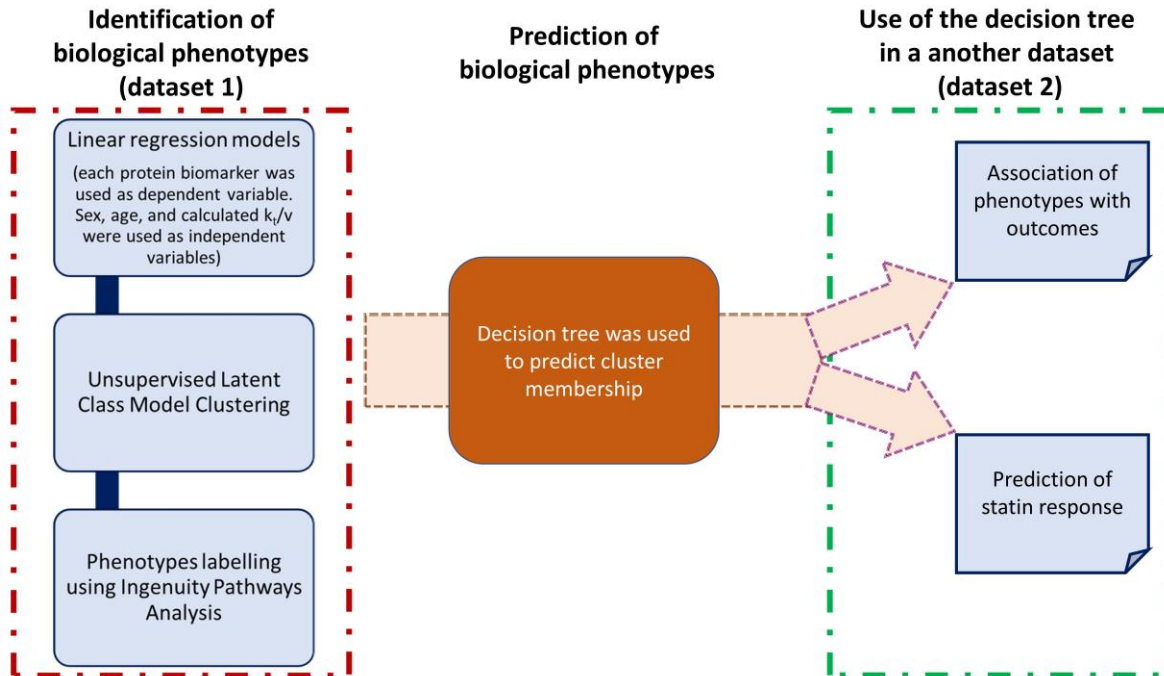
	Univariable		Multivariable*	
	HR (95% CI)	p-value	HR (95% CI)	p-value
<b>CV death (n=162)</b>				
<b>Per phenotype</b>				
Cytokine storm signaling	1.49 (0.93 - 2.39)	0.094	1.31 (0.81 - 2.14)	0.27
Toll-like receptors signaling	1.51 (1.05 - 2.16)	<b>0.025</b>	1.65 (1.13 - 2.41)	<b>0.010</b>
Multiple pathways related to inflammation and fibrosis	1.08 (0.63 - 1.86)	0.77	1.02 (0.57 - 1.82)	0.95
Reference	-	-	-	-
<b>All-cause mortality (n=227)</b>				
<b>Per phenotype</b>				
Cytokine storm signaling	1.59 (1.09 - 2.34)	<b>0.017</b>	1.44 (0.97 - 2.15)	0.07
Toll-like receptors signaling	1.38 (1.02 - 1.88)	<b>0.038</b>	1.43 (1.03 - 1.98)	<b>0.030</b>
Multiple pathways related to inflammation and fibrosis	0.98 (0.62 - 1.57)	0.95	0.96 (0.59 - 1.58)	0.88
Reference	-	-	-	-
<b>MACE (n=193)</b>				
<b>Per phenotype</b>				
Cytokine storm signaling	1.51 (0.99 - 2.32)	0.057	1.36 (0.88 - 2.11)	0.17
Toll-like receptors signaling	1.44 (1.03 - 2.01)	<b>0.034</b>	1.48 (1.04 - 2.10)	<b>0.028</b>
Multiple pathways related to inflammation and fibrosis	1.26 (0.79 - 2.01)	0.33	1.29 (0.79 - 2.12)	0.31
Reference	-	-	-	-

\*Adjusted for age, albumin, log hs-CRP, history of CV disease, diabetes mellitus, dialysis vintage and log BNP

**Table 3. Association between rosuvastatin and clinical outcomes**

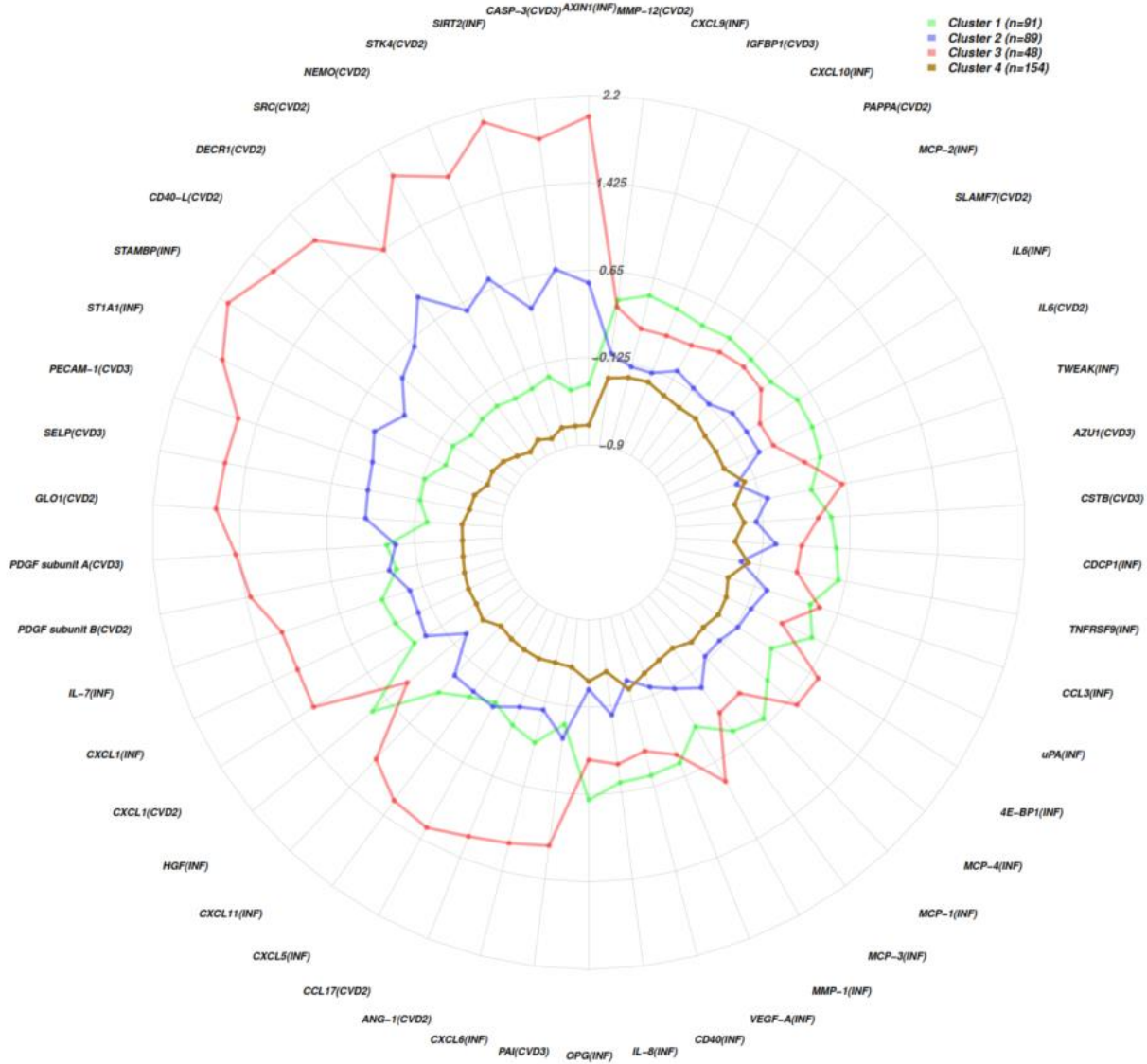
	Univariable	p-value	Multivariable*	
	HR (95% CI)		HR (95% CI)	p-value
<b>CV death (n=162)</b>				
<b>Per phenotype</b>				
Cytokine storm signaling	1.75 (0.77 - 3.98)	0.18	1.61 (0.70 - 3.69)	0.26
Toll-like receptors signaling	1.03 (0.59 - 1.79)	0.91	0.83 (0.47 - 1.47)	0.53
Multiple pathways related to inflammation and fibrosis	5.03 (1.43 - 17.67)	<b>0.012</b>	4.55 (1.25 - 16.55)	<b>0.021</b>
Reference	0.99 (0.62 - 1.57)	0.96	1.02 (0.63 - 1.65)	0.95
	-	0.079	-	0.090
<b>All-cause mortality (n=227)</b>				
<b>Per phenotype</b>				
Cytokine storm signaling	1.20 (0.61 - 2.34)	0.60	1.18 (0.60 - 2.33)	0.63
Toll-like receptors signaling	0.87 (0.54 - 1.41)	0.57	0.72 (0.44 - 1.18)	0.19
Multiple pathways related to inflammation and fibrosis	2.95 (1.14 - 7.61)	<b>0.025</b>	2.66 (1.00 - 7.09)	<b>0.049</b>
Reference	1.20 (0.82 - 1.76)	0.36	1.19 (0.80 - 1.78)	0.40
	-	0.16	-	0.10
<b>MACE (n=193)</b>				
<b>Per phenotype</b>				
Cytokine storm signaling	1.58 (0.75 - 3.33)	0.22	1.50 (0.71 - 3.17)	0.28
Toll-like receptors signaling	1.20 (0.71 - 2.03)	0.50	1.01 (0.59 - 1.73)	0.97
Multiple pathways related to inflammation and fibrosis	1.96 (0.82 - 4.68)	0.13	1.75 (0.71 - 4.33)	0.23
Reference	1.08 (0.71 - 1.65)	0.72	1.13 (0.73 - 1.76)	0.57
	-	0.59	-	0.69

\*Adjusted for age, albumin, log hs-CRP, history of CV disease, diabetes mellitus, dialysis vintage and log BNP

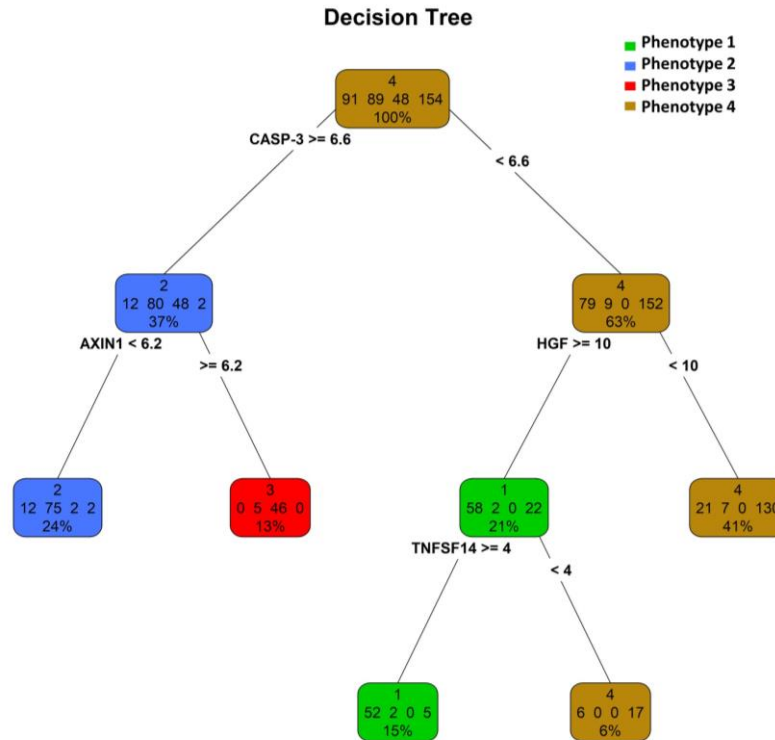


**Figure 1. Summary of the analyses performed in dataset 1 and dataset 2**





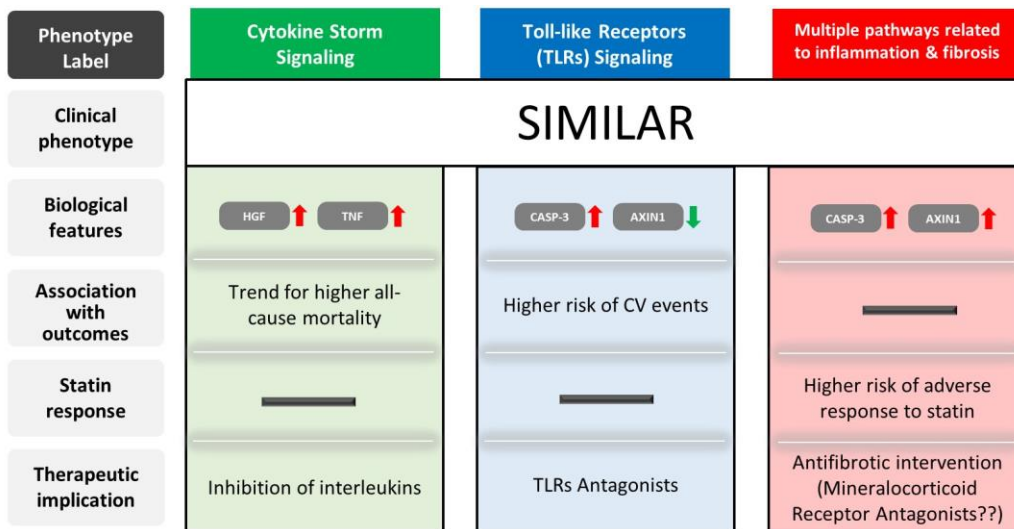
**Figure 2: Radar chart of the 50 biomarkers with the lowest resulting p-value from assessing phenotypes differences using the Kruskal-Wallis test or Chi-square test, as appropriate**



**Figure 3. Decision tree of the expressed protein biomarkers.**

CASP-3, caspase-3; AXIN1, axin-1; HGF, hepatocyte growth factor; TNFRSF14, tumor necrosis factor ligand superfamily member 14.

**Central Illustration. Mechanistic biological phenotypes in patients on hemodialysis.**



HGF, hepatocyte growth factor; TNF, tumor necrosis factor; CASP-3, caspase-3; AXIN1, axin-1

## Supplementary Data

**Supplementary Table S1: Protein names and respective Olink® panel sorted in alphabetical order**

Protein full name	Entry name	Olink® Panel*	Uniprot ID**	>25% undetectable values
Angiotensin-converting enzyme 2	ACE2	CVD II	Q9BYF1	
Adenosine Deaminase	ADA	INF	P00813	
A disintegrin and metalloproteinase with thrombospondin motifs 13	ADAM-TS13	CVD II	Q76LX8	
ADM	ADM	CVD II	P35318	
Agouti-related protein	AGRP	CVD II	O00253	
CD166 antigen	ALCAM	CVD III	Q13740	
Protein AMBP	AMBP	CVD II	P02760	
Angiopoietin-1	ANGPT-1	CVD II	Q15389	
Aminopeptidase N	AP-N	CVD III	P15144	
Artemin	ARTN	INF	Q5T4W7	X
Axin-1	AXIN1	INF	O15169	
Tyrosine-protein kinase receptor UFO	AXL	CVD III	P30530	
Azurocidin	AZU1	CVD III	P20160	
Beta-nerve growth factor	Beta-NGF	INF	P01138	
Bleomycin hydrolase	BLM hydrolase	CVD III	Q13867	
Bone morphogenetic protein 6	BMP-6	CVD II	P22004	
Natriuretic peptides B	BNP	CVD II	P16860	
Brother of CDO	BOC	CVD II	Q9BWW1	
Carbonic anhydrase 5A, mitochondrial	CA5A	CVD II	P35218	
Caspase-3	CASP-3	CVD III	P42574	
Caspase-8	CASP-8	INF	Q14790	
Eotaxin-1	CCL11	INF	P51671	
C-C motif chemokine 15	CCL15	CVD III	Q16663	
C-C motif chemokine 16	CCL16	CVD III	O15467	
C-C motif chemokine 17	CCL17	CVD II	Q92583	
C-C motif chemokine 19	CCL19	INF	Q99731	
C-C motif chemokine 20	CCL20	INF	P78556	
C-C motif chemokine 23	CCL23	INF	P55773	
C-C motif chemokine 24	CCL24	CVD III	O00175	
C-C motif chemokine 25	CCL25	INF	O15444	
C-C motif chemokine 28	CCL28	INF	Q9NRJ3	
C-C motif chemokine 3 (CVD2)	CCL3	CVD II	P10147	
C-C motif chemokine 3 (Inflammation)	CCL3	INF	P10147	
C-C motif chemokine 4	CCL4	INF	P13236	
Scavenger receptor cysteine-rich type 1 protein M130	CD163	CVD III	Q86VB7	
Natural killer cell receptor 2B4	CD244	INF	Q9BZW8	
T-cell surface glycoprotein CD4	CD4	CVD II	P01730	
CD40 receptor	CD40	INF	P25942	
CD40 ligand	CD40-L	CVD II	P29965	
T-cell surface glycoprotein CD5	CD5	INF	P06127	
T cell surface glycoprotein CD69	CD69	INF	Q07108	
SLAM family member 5	CD84	CVD II	Q9UIB8	
Complement component C1q receptor	CD93	CVD III	Q9NPY3	
CUB domain-containing protein 1	CDCP1	INF	Q9H5V8	
Cadherin-5	CDH5	CVD III	P33151	

Carcinoembryonic antigen-related cell adhesion molecule 8	CEACAM8	CVD II	P31997	
Chitinase-3-like protein 1	CHI3L1	CVD III	P36222	
Chitotriosidase-1	CHIT1	CVD III	Q13231	
Contactin-1	CNTN1	CVD III	Q12860	
Collagen alpha-1(I) chain	COL1A1	CVD III	P02452	
Carboxypeptidase A1	CPA1	CVD III	P15085	
Carboxypeptidase B	CPB1	CVD III	P15086	
Macrophage colony-stimulating factor 1	CSF-1	INF	P09603	
Cystatin D	CST5	INF	P28325	
Cystatin-B	CSTB	CVD III	P04080	
Chymotrypsin C	CTRC	CVD II	Q99895	
Cathepsin D	CTSD	CVD III	P07339	
Cathepsin L1	CTSL1	CVD II	P07711	
Cathepsin Z	CTSZ	CVD III	Q9UBR2	
Fractalkine	CX3CL1	INF	P78423	
C-X-C motif chemokine 1 (CVD2)/ growth-regulated alpha protein	CXCL1	CVD II	P09341	
C-X-C motif chemokine 1 (Inflammation)/ growth-regulated alpha protein	CXCL1	INF	P09341	
C-X-C motif chemokine 10	CXCL10	INF	P02778	
C-X-C motif chemokine 11	CXCL11	INF	O14625	
C-X-C motif chemokine 16	CXCL16	CVD III	Q9H2A7	
C-X-C motif chemokine 5	CXCL5	INF	P42830	
C-X-C motif chemokine 6	CXCL6	INF	P80162	
C-X-C motif chemokine 9	CXCL9	INF	Q07325	
Decorin	DCN	CVD II	P07585	
2,4-dienoyl-CoA reductase, mitochondrial	DECR1	CVD II	Q16698	
Dickkopf-related protein 1	DKK-1	CVD II	O94907	
Protein delta homolog 1	DLK-1	CVD III	P80370	
Delta and Notch-like epidermal growth factor-related receptor	DNER	INF	Q8NFT8	
Eukaryotic translation initiation factor 4E-binding protein 1	4E-BP1	INF	Q13541	
Epidermal growth factor receptor	EGFR	CVD III	P00533	
Protein S100-A12	EN-RAGE	INF	P80511	
Epithelial cell adhesion molecule	EP-CAM	CVD III	P16422	
Ephrin type-B receptor 4	EPHB4	CVD III	P54760	
Fatty acid-binding protein, intestinal	FABP2	CVD II	P12104	
Fatty acid-binding protein, adipocyte	FABP4	CVD III	P15090	
Tumor necrosis factor receptor superfamily member 6	FAS	CVD III	P25445	
Fibroblast growth factor 19	FGF-19	INF	O95750	
Fibroblast growth factor 21 (CVD2)	FGF21	CVD II	Q9NSA1	
Fibroblast growth factor 21 (Inflammation)	FGF21	INF	Q9NSA1	
Fibroblast growth factor 23 (CVD2)	FGF-23	CVD II	Q9GZV9	
Fibroblast growth factor 23 (Inflammation)	FGF-23	INF	Q9GZV9	
Fibroblast growth factor 5	FGF-5	INF	P12034	

Fms-related tyrosine kinase 3 ligand	FIT3L	INF	P49771	
Follistatin	FST	CVD II	P19883	
Galectin-3	Gal-3	CVD III	P17931	
Galectin-4	Gal -4	CVD III	P56470	
Galectin-9	Gal -9	CVD II	O00182	
Growth/differentiation factor 15	GDF-15	CVD III	Q99988	
Growth/differentiation factor 2	GDF-2	CVD II	Q9UK05	
Glial cell line-derived neurotrophic factor	GDNF	INF	P39905	
Growth hormone	GH	CVD II	P01241	
Gastric intrinsic factor	GIF	CVD II	P27352	
Lactoylglutathione lyase	GLO1	CVD II	Q04760	
Granulins	GRN	CVD III	P28799	
Gastrotropin	GT	CVD II	P51161	
Hydroxyacid oxidase 1	HAOX1	CVD II	Q9UJM8	
Proheparin-binding EGF-like growth factor	HB-EGF	CVD II	Q99075	
Hepatocyte growth factor	HGF	INF	P14210	
Heme oxygenase 1	HO-1	CVD II	P09601	
Osteoclast-associated immunoglobulin-like receptor	hOSCAR	CVD II	Q8IYS5	
Heat shock 27 kDa protein	HSP 27	CVD II	P04792	
Intercellular adhesion molecule 2	ICAM-2	CVD III	P13598	
Alpha-L-iduronidase	IDUA	CVD II	P35475	
Interferon gamma	IFN-gamma	INF	P01579	X
Insulin-like growth factor-binding protein 1	IGFBP-1	CVD III	P08833	
Insulin-like growth factor-binding protein 2	IGFBP-2	CVD III	P18065	
Insulin-like growth factor-binding protein 7	IGFBP-7	CVD III	Q16270	
Low affinity immunoglobulin gamma Fc region receptor II-b	IgG Fc receptor II-b	CVD II	P31994	
Interleukin-1 alpha	IL-1 alpha	INF	P01583	X
Interleukin-10 receptor subunit alpha	IL-10RA	INF	Q13651	X
Interleukin-10 receptor subunit beta	IL-10RB	INF	Q08334	
Interleukin-12 subunit beta	IL-12B	INF	P29460	
Interleukin-15 receptor subunit alpha	IL-15RA	INF	Q13261	X
Interleukin-17A	IL-17A	INF	Q16552	X
Interleukin-17C	IL-17C	INF	Q9P0M4	
Interleukin-17D	IL-17D	CVD II	Q8TAD2	
Interleukin-17 receptor A	IL-17RA	CVD III	Q96F46	
Interleukin-18-binding protein	IL-18BP	CVD III	O95998	
Interleukin-18 receptor 1	IL-18R1	INF	Q13478	
Interleukin-1 receptor antagonist protein	IL-1rA	CVD II	P18510	
Interleukin-1 receptor type 1	IL-1RT1	CVD III	P14778	
Interleukin-1 receptor type 2	IL-1RT2	CVD III	P27930	
Interleukin-20	IL-20	INF	Q9NYY1	X
Interleukin-20 receptor subunit alpha	IL-20RA	INF	Q9UHF4	X
Interleukin-22 receptor subunit alpha-1	IL-22 RA1	INF	Q8N6P7	X
Interleukin-24	IL-24	INF	Q13007	X

Interleukin-27	IL-27	CVD II	Q8NEV9 Q14213	
Interleukin-2 receptor subunit beta	IL-2RB	INF	P14784	X
Interleukin-4 receptor subunit alpha	IL-4RA	CVD II	P24394	
Interleukin-6 receptor subunit alpha	IL-6RA	CVD III	P08887	
Interleukin-10	IL10	INF	P22301	
Interleukin-13	IL-13	INF	P35225	X
Pro-interleukin-16	IL-16	CVD II	Q14005	
Interleukin-18 (CVD2)	IL-18	CVD II	Q14116	
Interleukin-18 (Inflammation)	IL-18	INF	Q14116	
Interleukin-1 receptor-like 2	IL1RL2	CVD II	Q9HB29	
Interleukin-2	IL-2	INF	P60568	X
Interleukin-2 receptor subunit alpha	IL2-RA	CVD III	P01589	
Interleukin-33	IL-33	INF	O95760	X
Interleukin-4	IL-4	INF	P05112	X
Interleukin-5	IL-5	INF	P05113	X
Interleukin-6 (CVD2)	IL6	CVD II	P05231	
Interleukin-6 (Inflammation)	IL6	INF	P05231	
Interleukin-7	IL-7	INF	P13232	
Interleukin-8	IL-8	INF	P10145	
Melusin	ITGB1BP2	CVD II	Q9UKP3	X
Integrin beta-2	ITGB2	CVD III	P05107	
Junctional adhesion molecule A	JAM-A	CVD III	Q9Y624	
Kidney injury molecule 1	KIM1	CVD II	Q96D42	
Kallikrein-6	KLK6	CVD III	Q92876	
Latency-associated peptide transforming growth factor beta 1	LAP TGF-beta-1	INF	P01137	
Low-density lipoprotein receptor	LDL receptor	CVD III	P01130	
Leptin	LEP	CVD II	P41159	
Leukemia inhibitory factor	LIF	INF	P15018	X
Leukemia inhibitory factor receptor	LIF-R	INF	P42702	
Lectin-like oxidized LDL receptor 1	LOX-1	CVD II	P78380	
Lipoprotein lipase	LPL	CVD II	P06858	
Lymphotoxin-beta receptor	LTBR	CVD III	P36941	
Macrophage receptor MARCO	MARCO	CVD II	Q9UEW3	
Myoglobin	MB	CVD III	P02144	
Monocyte chemotactic protein 1 (CVD3)/ C-C motif chemokine 2	MCP-1 (CCL2)	CVD III	P13500	
Monocyte chemotactic protein 1 (Inflammation) / C-C motif chemokine 2	MCP-1 (CCL2)	INF	P13500	
Monocyte chemotactic protein 2	MCP-2	INF	P80075	
Monocyte chemotactic protein 3	MCP-3	INF	P80098	
Monocyte chemotactic protein 4 / C-C motif chemokine 13	MCP-4	INF	Q99616	
Matrix extracellular phosphoglycoprotein	MEPE	CVD III	Q9NQ76	
Tyrosine-protein kinase Mer	MERTK	CVD II	Q12866	
Matrix metalloproteinase-1/ Interstitial collagenase	MMP-1	INF	P03956	
Matrix metalloproteinase-10	MMP-10	INF	P09238	
Matrix metalloproteinase-2	MMP-2	CVD III	P08253	



Matrix metalloproteinase-3	MMP-3	CVD III	P08254	
Matrix metalloproteinase-9	MMP-9	CVD III	P14780	
Matrix metalloproteinase-12	MMP-12	CVD II	P39900	
Matrix metalloproteinase-7	MMP-7	CVD II	P09237	
Myeloperoxidase	MPO	CVD III	P05164	
NF-kappa-B essential modulator	NEMO	CVD II	Q9Y6K9	
Neurogenic locus notch homolog protein 3	Notch3	CVD III	Q9UM47	
Neurturin	NRTN	INF	Q99748	X
Neurotrophin-3	NT-3	INF	P20783	
N-terminal prohormone brain natriuretic peptide	NT-proBNP	CVD III	NA	
Osteoprotegerin (CVD3)	OPG	CVD III	O00300	
Osteoprotegerin (Inflammation)	OPG	INF	O00300	
Osteopontin	OPN	CVD III	P10451	
Oncostatin-M	OSM	INF	P13725	
Plasminogen activator inhibitor 1	PAI	CVD III	P05121	
Pappalysin-1	PAPPA	CVD II	Q13219	
Proteinase-activated receptor 1	PAR-1	CVD II	P25116	
Poly [ADP-ribose] polymerase 1	PARP-1	CVD II	P09874	
Proprotein convertase subtilisin/kexin type 9	PCSK9	CVD III	Q8NBP7	
Programmed cell death 1 ligand 1	PD-L1	INF	Q9NZQ7	
Programmed cell death 1 ligand 2	PD-L2	CVD II	Q9BQ51	
Platelet-derived growth factor subunit A	PDGF subunit A	CVDIII	P04085	
Platelet-derived growth factor subunit B	PDGF subunit B	CVD II	P01127	
Platelet endothelial cell adhesion molecule	PECAM-1	CVD III	P16284	
Placenta growth factor	PGF	CVD II	P49763	
Peptidoglycan recognition protein 1	PGLYRP1	CVD III	O75594	
Elafin	PI3	CVD III	P19957	
Polymeric immunoglobulin receptor	PIgR	CVD II	P01833	
Perlecan	PLC	CVD III	P98160	
Paraoxonase	PON3	CVD III	Q15166	
Prolargin	PRELP	CVD II	P51888	
Serine protease 27	PRSS27	CVD II	Q9BQR3	
Prostasin	PRSS8	CVD II	Q16651	
Myeloblastin	PRTN3	CVD III	P24158	
P-selectin glycoprotein ligand 1	PSGL-1	CVD II	Q14242	
Pulmonary surfactant-associated protein D	PSP-D	CVD III	P35247	
Pentraxin-related protein PTX3	PTX3	CVD II	P26022	
Receptor for advanced glycosylation end products	RAGE	CVD II	Q15109	
Retinoic acid receptor responder protein 2	RARRES2	CVD III	Q99969	
Renin	REN	CVD II	P00797	
Resistin	RETN	CVD III	Q9HD89	
Stem cell factor (CVD2)	SCF	CVD II	P21583	
Stem cell factor (Inflammation)	SCF	INF	P21583	
Secretoglobin family 3A member 2	SCGB3A2	CVD III	Q96PL1	
E-selectin	SELE	CVD III	P16581	

P-selectin	SELP	CVD III	P16109	
Serpin A12	SERPINA12	CVD II	Q8IW75	
Tyrosine-protein phosphatase non-receptor type substrate 1	SHPS-1	CVD III	P78324	
SIR2-like protein 2	SIRT2	INF	Q8IXJ6	
Signaling lymphocytic activation molecule	SLAMF1	INF	Q13291	
SLAM family member 7	SLAMF7	CVD II	Q9NQ25	
Superoxide dismutase [Mn], mitochondrial	SOD2	CVD II	P04179	
Sortilin	SORT1	CVD II	Q99523	
Spondin-1	SPON1	CVD III	Q9HCB6	
Spondin-2	SPON2	CVD II	Q9BUD6	
Proto-oncogene tyrosine-protein kinase Src	SRC	CVD II	P12931	
Sulfotransferase 1A1	ST1A1	INF	P50225	
ST2 protein	ST2	CVD III	Q01638	
STAM-binding protein	STAMPB	INF	O95630	
Serine/threonine-protein kinase 4	STK4	CVD II	Q13043	
Tissue-type plasminogen activator	t-PA	CVD III	P00750	
Tissue factor	TF	CVD II	P13726	
Trefoil factor 3	TFF3	CVD III	Q07654	
Tissue factor pathway inhibitor	TFPI	CVD III	P10646	
Transforming growth factor alpha	TGF-ALPHA	INF	P01135	
Protein-glutamine gamma-glutamyltransferase 2	TGM2	CVD II	P21980	
Thrombospondin-2	THBS2	CVD II	P35442	
Thrombopoietin	THPO	CVD II	P40225	
Angiopoietin-1 receptor	TIE2	CVD II	Q02763	
Metalloproteinase inhibitor 4	TIMP4	CVD III	Q99727	
Trem-like transcript 2 protein	TLT-2	CVD III	Q5T2D2	
Thrombomodulin	TM	CVD II	P07204	
Tumor necrosis factor	TNF	INF	P01375	X
Tumor necrosis factor receptor 1	TNF-R1	CVD III	P19438	
Tumor necrosis factor receptor 2	TNF-R2	CVD III	P20333	
TNF-beta	TNFB	INF	P01374	
Tumor necrosis factor receptor superfamily member 10A	TNFRSF10A	CVD II	O00220	
Tumor necrosis factor receptor superfamily member 10C	TNFRSF10C	CVD III	O14798	
Tumor necrosis factor receptor superfamily member 11A	TNFRSF11A	CVD II	Q9Y6Q6	
Tumor necrosis factor receptor superfamily member 13B	TNFRSF13B	CVD II	O14836	
Tumor necrosis factor receptor superfamily member 14	TNFRSF14	CVD III	Q92956	
Tumor necrosis factor receptor superfamily member 9		INF	Q07011	
Tumor necrosis factor ligand superfamily member 13B	TNFSF13B	CVD III	Q9Y275	
Tumor necrosis factor ligand superfamily member 14	TNFSF14	INF	O43557	
Tartrate-resistant acid phosphatase type 5	TR-AP	CVD III	P13686	
Transferrin receptor protein 1	TR	CVD III	P02786	
TNF-related apoptosis-inducing ligand	TRAIL	INF	P50591	
TNF-related apoptosis-inducing ligand receptor 2	TRAIL-R2	CVD II	O14763	
TNF-related activation-induced cytokine	TRANCE	INF	O14788	
Thymic stromal lymphopoietin	TSLP	INF	Q969D9	X

Tumor necrosis factor (Ligand) superfamily, member 12	TWEAK	INF	O43508	
Urokinase plasminogen activator surface receptor	U-PAR	CVD III	Q03405	
Urokinase-type plasminogen activator (CVD3)	uPA	CVD III	P00749	
Urokinase-type plasminogen activator (Inflammation)	uPA	INF	P00749	
Vascular endothelial growth factor A	VEGF-A	INF	P15692	
Vascular endothelial growth factor D	VEGF-D	CVD II	O43915	
V-set and immunoglobulin domain-containing protein 2	VSIG2	CVD II	Q96IQ7	
von Willebrand factor	vWF	CVD III	P04275	
Lymphotactin	XCL1	CVD II	P47992	

*Legend: \*CVII: cardiovascular II; CVIII: cardiovascular III; INF: inflammation panels; \*\*UniProt ID from UniProt Knowledge base*

*The 100 biomarkers with the highest standard deviation*

**Supplementary Table S2: Comparison for the residuals of biomarkers and mixed biological and clinical data across phenotypes using the Kruskal-Wallis test**

	<b>Biomarkers and mixed biological and clinical data</b>	<b>p-value</b>
1	AXIN1	4.89E-60
2	CASP-3	1.10E-58
3	SIRT2	2.58E-58
4	STK4	4.24E-58
5	NEMO	5.74E-58
6	SRC	4.91E-57
7	DECR1	1.24E-56
8	CD40-L	2.33E-54
9	STAMBP	2.22E-53
10	ST1A1	4.78E-49
11	PECAM-1	5.73E-41
12	SELP	2.13E-37
13	GLO1	9.48E-35
14	PDGF subunit A	1.59E-29
15	PDGF subunit B	3.39E-29
16	IL-7	2.71E-25
17	CXCL1 (INF)	7.10E-25
18	CXCL1 (CVD2)	1.28E-24
19	HGF	1.29E-24
20	CXCL11	1.45E-24
21	CXCL5	1.48E-24
22	CCL17	2.00E-22
23	ANG-1	2.74E-22
24	CXCL6	6.22E-22
25	PAI	2.04E-21
26	OPG	4.85E-16
27	IL-8	6.26E-15
28	CD40	7.04E-14
29	VEGF-A	9.05E-14
30	MMP-1	1.83E-13
31	MCP-3	2.95E-13
32	MCP-1	2.32E-12
33	MCP-4	2.39E-12
34	4E-BP1	5.29E-12
35	uPA	4.02E-11
36	CCL3	4.11E-11
37	TNFRSF9	8.88E-11
38	CDCP1	2.08E-10
39	CSTB	3.63E-10
40	AZU1	4.88E-10
41	TWEAK	1.27E-09
42	IL6 (CVD2)	3.16E-09

43	IL6 (INF)	4.48E-09
44	SLAMF7	2.52E-08
45	MCP-2	6.65E-08
46	PAPPA	8.38E-08
47	CXCL10	1.02E-07
48	IGFBP-1	3.71E-07
49	CXCL9	5.86E-07
50	MMP-12	9.01E-07
51	PARP-1	9.05E-07
52	Dialysis Vintage (years)	2.33E-06
53	PRTN3	2.48E-06
54	BMP-6	6.64E-06
55	Log hs-CRP (mg/L)	9.06E-06
56	ST2	1.04E-05
57	t-PA	5.69E-05
58	EN-RAGE	7.14E-05
59	MMP-9	1.31E-04
60	Body Mass Index (kg/m <sup>2</sup> )	1.39E-04
61	IL10	1.42E-04
62	IL-18	3.28E-04
63	CCL23	7.13E-04
64	CHI3L1	9.84E-04
65	IL-12B	1.55E-03
66	CCL19	1.57E-03
67	OSM	2.26E-03
68	FGF21	3.72E-03
69	KIM1	6.84E-03
70	MMP-7	8.77E-03
71	NT-proBNP	1.10E-02
72	LEP	1.19E-02
73	VWF	1.22E-02
74	FGF19	1.50E-02
75	FGF21	1.54E-02
76	Creatine Kinase (IU/L)	1.74E-02
77	MMP-3	1.86E-02
78	DLK1	2.69E-02
79	IGGFCRECEPTORIIIB	2.69E-02
80	FABP4	3.53E-02
81	GT	3.61E-02
82	CCL11	3.78E-02
83	Cause of ESRD	7.21E-02
84	CST5	1.00E-01
85	GH	1.04E-01
86	CCL20	1.16E-01
87	Diabetes mellitus	1.26E-01
88	BNP	1.53E-01
89	CPA1	1.60E-01
90	HAOX1	1.76E-01
91	CHIT1	1.79E-01

92	Fasting glucose level (mmol/L)	1.86E-01
93	Phosphate (mmol/L)	2.78E-01
94	IL-17C	3.01E-01
95	CPB1	3.02E-01
96	Hemoglobin (g/dL)	3.02E-01
97	CA5A	3.05E-01
98	GIF	3.54E-01
99	FGF23 (INF)	3.57E-01
100	Aspartate aminotranferase (IU/L)	3.76E-01
101	FGF23 (CVD2)	4.25E-01
102	Pulse pressure (mmHg)	4.39E-01
103	CCL24	4.78E-01
104	SCGB3A2	4.85E-01
105	Smoking status	4.97E-01
106	Neutrophil-lymphocyte ratio	5.95E-01
107	EPCAM	5.97E-01
108	History of vascular disease	6.15E-01
109	GDF2	6.41E-01
110	Systolic blood pressure (mmHg)	6.93E-01
111	History of coronary heart disease	7.00E-01
112	REN	7.28E-01
113	History of neurovascular disease	7.58E-01
114	Albumin (g/L)	8.16E-01
115	SERPINA12	9.13E-01
116	Calculated LDL-Cholesterol (mmol/L)	9.19E-01
117	CTRC	9.33E-01
118	TRANCE	9.71E-01
119	PSP-D	1.00E+00

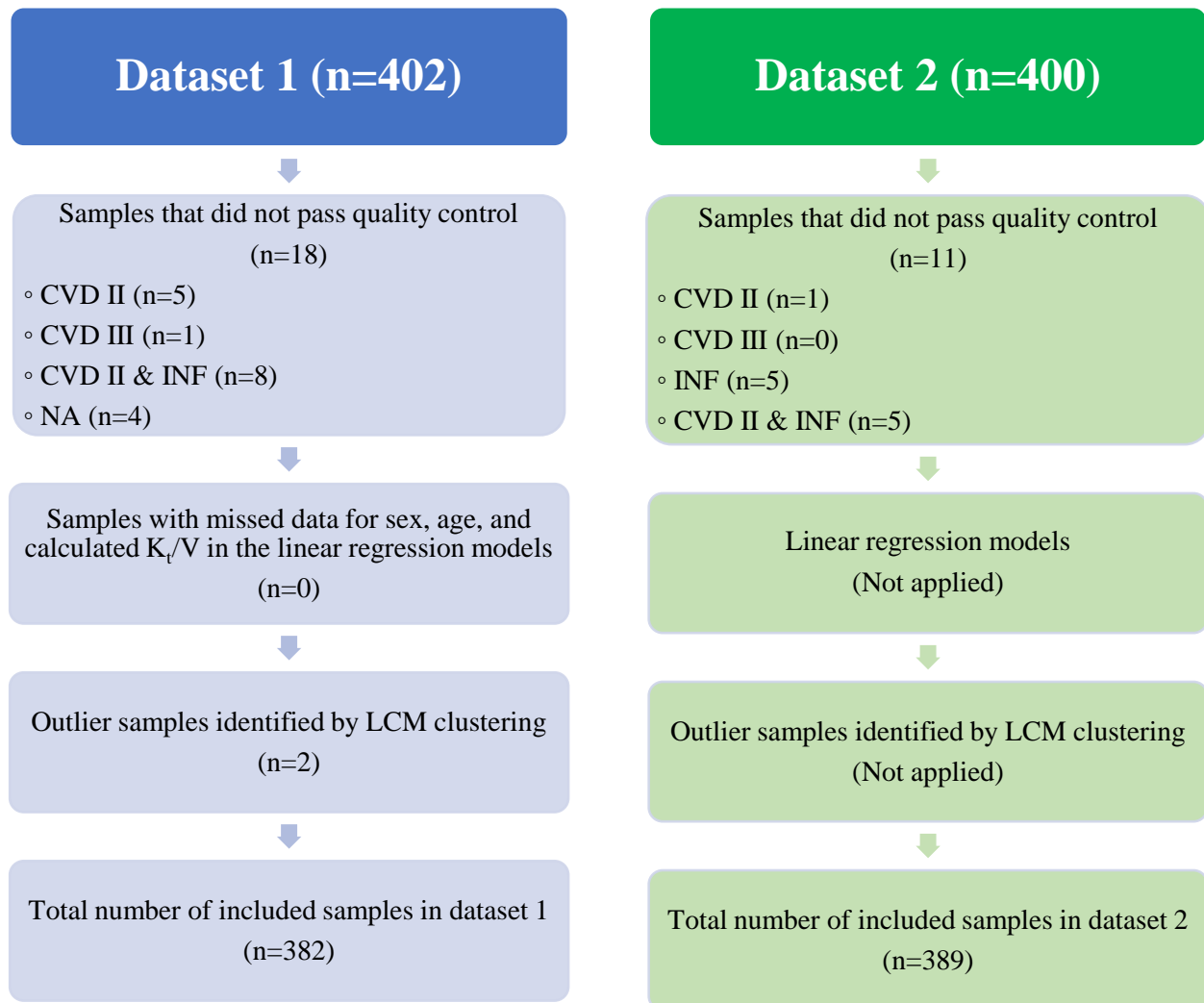


**Supplementary Table S4: Baseline characteristics according to phenotypes in dataset 2**

	<b>Global (n=389)</b>	<b>Phenotype 1 (n=49)</b>	<b>Phenotype 2 (n=104)</b>	<b>Phenotype 3 (n=40)</b>	<b>Phenotype 4 (n=196)</b>	<b>p-value</b>
<b>Baseline characteristics</b>						
Female gender (%)	164 (42.2)	24 (49.0)	48 (46.2)	12 (30.0)	80 (40.8)	0.24
Age (years)	65 [57, 72]	68 [59, 77]	63 [56, 71]	61 [54, 70]	66 [57, 73]	<b>0.03</b>
BMI (kg/m <sup>2</sup> )	25.4 (5.2)	24.9 (5.2)	26.3 (5.7)	26.1 (5.3)	24.9 (4.7)	0.09
Current smoker	62 (15.9)	8 (16.3)	17 (16.3)	9 (22.5)	28 (14.3)	0.64
<b>Medical history, n (%)</b>						
Years on RRT	2.4 [1.2, 5.3]	2.9 [1.5, 7.3]	3.1 [1.2, 5.9]	3.0 [0.9, 5.7]	2.2 [1.2, 4.5]	0.16
Diabetes	117 (30.1)	13 (26.5)	38 (36.5)	13 (32.5)	53 (27.0)	0.34
History of cardiovascular disease	129 (33.2)	14 (28.6)	39 (37.5)	13 (32.5)	63 (32.1)	0.69
Peripheral artery disease	55 (14.1)	8 (16.3)	15 (14.4)	4 (10.0)	28 (14.3)	0.86
History of coronary heart disease	54 (13.9)	4 (8.2)	15 (14.4)	6 (15.0)	29 (14.8)	0.67
History of vascular disease	68 (17.5)	9 (18.4)	18 (17.3)	5 (12.5)	36 (18.4)	0.84
History of neurovascular disease	45 (11.6)	7 (14.3)	14 (13.5)	4 (10.0)	20 (10.2)	0.76
<b>Cause of ESRD, n (%)</b>						<b>0.04</b>
Diabetes	88 (22.6)	11 (22.4)	30 (28.8)	10 (25.0)	37 (18.9)	
Genetic conditions	51 (13.1)	9 (18.4)	15 (14.4)	0 (0.0)	27 (13.8)	
Glomerulonephritis or Vasculitis	71 (18.3)	10 (20.4)	16 (15.4)	12 (30.0)	33 (16.8)	
Nephropathy or Nephrosclerosis	67 (17.2)	1 (2.0)	17 (16.3)	10 (25.0)	39 (19.9)	
Pyelonephritis or Interstitial	63 (16.2)	8 (16.3)	18 (17.3)	5 (12.5)	32 (16.3)	
Unknown/unspecified	31 (8.0)	5 (10.2)	5 (4.8)	2 (5.0)	19 (9.7)	
Other	18 (4.6)	5 (10.2)	3 (2.9)	1 (2.5)	9 (4.6)	
<b>Characteristics</b>						
Systolic Blood Pressure (mmHg)	137.5 (23.6)	135.9 (26.4)	136.8 (25.8)	138.2 (20.7)	138.2 (22.3)	0.92
Diastolic Blood Pressure (mmHg)	75.5 (12.3)	73.0 (12.0)	74.6 (12.3)	77.9 (11.1)	76.1 (12.6)	0.2
Pulse pressure (mmHg)	60 [49, 72]	60 [49, 75]	60 [45, 75]	60 [50, 70]	60 [49, 71]	0.94
Calculated Kt/V	1.2 [1.0, 1.4]	1.2 [1.0, 1.4]	1.2 [1.1, 1.4]	1.2 [1.1, 1.5]	1.2 [1.0, 1.4]	0.78
Albumin (g/L)	39.3 (3.6)	37.8 (4.6)	39.6 (3.5)	40.5 (3.7)	39.4 (3.3)	<b>&lt;0.01</b>
Hemoglobin (g/dL)	11.5 (1.7)	11.7 (1.8)	11.4 (1.6)	11.7 (1.7)	11.5 (1.6)	0.62
hs-CRP (mg/L)	1.0 (1.2)	1.4 (1.3)	1.1 (1.3)	1.2 (1.5)	0.9 (1.1)	<b>0.04</b>
Fasting Glucose level (mmol/L)	5.1 [4.5, 5.9]	4.8 [4.6, 6.0]	5.2 [4.6, 6.2]	5.1 [4.7, 5.7]	5.0 [4.5, 5.8]	0.34
Creatine Kinase (IU/L)	57 [40, 93]	45 [31, 73]	55 [42, 82]	69 [40, 105]	62 [41, 97]	<b>0.02</b>
Aspartate Aminotransferase (IU/L)	17.1 (7.8)	19.0 (8.1)	16.6 (8.4)	17.4 (10.8)	16.9 (6.7)	0.31
Calculated LDL-Cholesterol (mmol/L)	2.5 (1.0)	2.4 (0.9)	2.6 (0.9)	2.3 (0.9)	2.6 (1.0)	0.18
<b>At follow-up</b>						
CV death	162 (41.6)	23 (46.9)	51 (49.0)	16 (40.0)	72 (36.7)	0.18
Death	227 (58.4)	35 (71.4)	67 (64.4)	21 (52.5)	104 (53.1)	<b>0.05</b>
Non-CV death	64 (16.5)	11 (22.4)	16 (15.4)	5 (12.5)	32 (16.3)	0.61
MACE	193 (49.6)	28 (57.1)	57 (54.8)	22 (55.0)	86 (43.9)	0.15



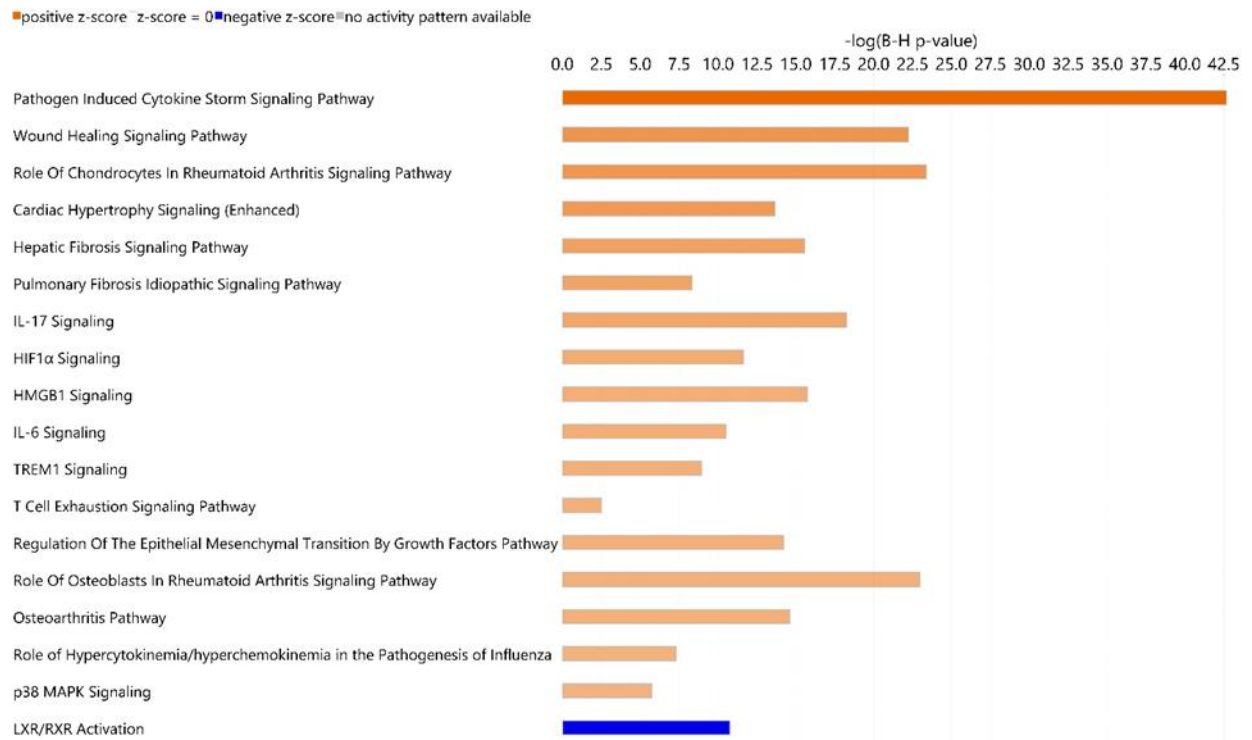
### Supplementary Figure S1: Overview of included patients in each dataset



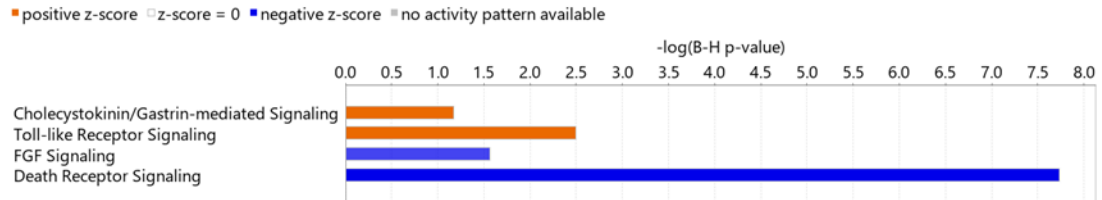
## Supplementary Figure S2: The enriched canonical pathways for all phenotypes



### Supplementary Figure S3: phenotype 1 was associated with cytokine storm signaling



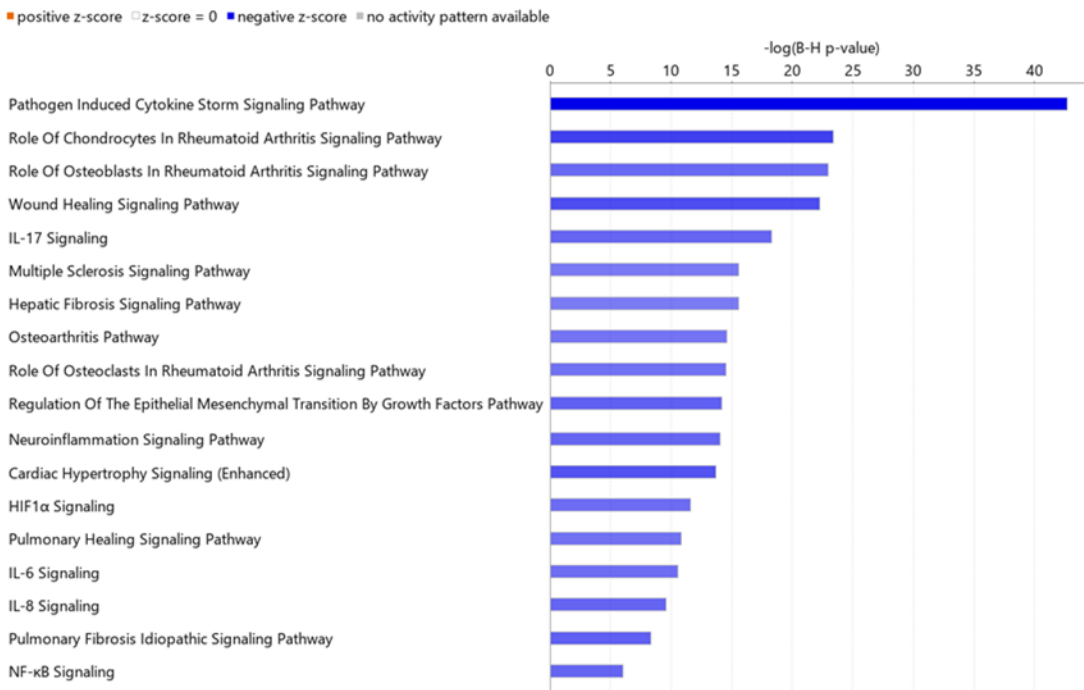
**Supplementary Figure S4:** phenotype 2 was associated with toll-like receptors



**Supplementary Figure S5:** phenotype 3 was associated with multiple pathways related to inflammation and fibrosis



**Supplementary Figure S6:** phenotype 4 includes patients with the least biological abnormalities (reference phenotype)



## Supplementary addendum 1. AURORA OLINK Dataset I and II – Bridging normalization

- Dataset I (NOV 2016): 276 biomarkers, 3 panels (CVD II, CVD III, INF)
- Dataset II (JUN 2017): 276 biomarkers, 3 panels (CVD II, CVD III, INF)
- Bridging samples (DEC 2020): 460 biomarkers, 5 panels (CVD II, CVD III, INF, CM, OD), 8 samples from dataset I and another 8 from dataset II
- In dataset I and dataset II, all values less than limit of detection (LOD) have been imputed by LOD value and we do not have access to the raw data. Since we have access to the raw data in the bridging file (DEC 2020), all data <LOD has been imputed by LOD value in order to keep consistency between files.
- The panels OLINK, between JUN 2017 and DEC 2020, were modified. 2 biomarkers were replaced.
  - ✚ CCL22 measured in datasets I-II, was replaced by GP6 in the bridging samples.
  - ✚ BDNF measured in datasets I-II, was replaced par CD8A in the bridging samples.
  - ✚ There are 274 biomarkers in common between datasets I-II and the bridging samples
- The statistical approach for normalization of dataset 1 and dataset 2:
  1. The bridging samples were used to normalize data in dataset 1 and dataset 2 using OLINK strategy i.e. bridging normalization using olink\_normalization function in the package OlinkAnalyze).
    - P1: values NPX in the file to be normalized (dataset I or II file)
    - P2: values NPX in the file to be used for the normalization (bridging file)
- ✚ The adjustment for each biomarker was carried out using the median absolute differences (MAD) between the bridging samples across the 2 files.
$$\text{Adj}_{\text{factor}} = \text{median} (P2 - P1)$$
$$P1_{\text{normalized}} = P1 + \text{Adj}_{\text{factor}}$$
$$\text{LOD}_{\text{normalized}} = \text{LOD} + \text{Adj}_{\text{factor}}$$
  2. The normalized data from Dataset I and those from Dataset II were then merged by replacing the LOD with the LOD max between the two normalized files, and the NPX <= LOD max values were replaced by the LOD max.

*NPX, Normalized Protein eXpression; Adjfactor, Adjustment factor*

## 2.2. Kidney transplantation recipients from the TRANSARTE study

The TRANSARTE study (Transplantation and Arteries) was a prospective monocentric study that included one hundred ESRD patients from the kidney transplant waiting list of the Transplant Department of Nancy University Hospital. To our knowledge, TRANSARTE was the first study to characterize the evolution of arterial stiffness using PWV after KT in comparison with patients remaining on dialysis<sup>128</sup>.

PWV was measured using the Complior® technique (ALAM Medical), which simultaneously records arterial pulse waves at carotid and femoral sites. The propagation time (t) of the incident wave was measured using mechanotransducer probes and the carotid–femoral distance (D) was determined with a tape measure. The following formula  $PWV = D \times 0.8/(t)$  was used to calculate PWV (m/s).

### 2.2.1. Association between Long-Term Change in Arterial Stiffness and Cardiovascular Outcomes in Kidney Transplant Recipients: Insights from the TRANSARTE Study (3<sup>rd</sup> article)

In this study, we assessed the association of PWV measured at pre-transplantation, early post-transplantation at 1-year and the latest post-transplantation at 4-years with CV events in 40 KT recipients. We also assessed the association the evolution between these measurements and CV events. Furthermore, we performed backward linear regression to identify the factors associated with log transformed PWV at 1-year post-transplantation and late PWV change (delta between PWV at 1-year and the latest PWV)

This study showed that a limited arterial stiffening during follow-up was significantly associated with lower rates of CV events (HR for the delta PWV = 0.76 (0.58–0.98), p = 0.036). This suggests that arterial structural changes are very likely due to the impact of non-classical factors in the late post-KT period including the deleterious effect of immunosuppressive drugs<sup>19,38,129,130</sup>. This result emphasizes beneficial effects of KT on vascular structure and function on the long-term period<sup>19</sup>. Moreover, this finding explains the effect of the chronic hypoxia and inflammation associated with excessive accumulation of ECM (i.e. as result of dialysis) in the vascular tree leading to inadequate endothelial function which in turn contributes to the increased CV morbidity and mortality<sup>20,37,40</sup>.



Age at KT was significantly associated with increased PWV at 1-year and less improvement in the latest post-transplantation period (adjusted for PWV at 1-year). This small study population had relatively young patients at the time of KT. Arterial stiffness is age-related with a linear increase after the fifth decade<sup>35,131</sup>.

We demonstrated that increased PWV at 1-year is associated with increased potential improvement in the latest post-transplantation period. Generally, KT has been demonstrated to significantly improve CV survival in consequence to the restoration of kidney function which improve blood pressure, endothelial dysfunction and arterial stiffness<sup>132</sup>. Moreover, it is reasonable to assume that arterial stiffness related to dialysis could be improved following KT. Importantly, the history of diabetes mellitus was retained as determinant of PWV at 1-year post-transplantation but not the late PWV change, suggesting the stability of PWV in diabetic patients after 1-year of the KT. It is certain that traditional risk factors such as age and diabetes mellitus are associated with higher PWV at 1-year post KT<sup>38</sup>.

It should be mentioned that mean blood pressure was not retained either in the 1-year PWV model or the late PWV change model. This could be because the reduction in blood pressure is marked shortly after KT (i.e. 3 months post KT), whereas blood pressure does not significantly change after 1-year of KT<sup>35,133</sup>.

In summary, the present study suggests that KT improves overall CV outcomes in renal transplant patients and argues that it is possible to improve the prognosis of KT recipients by reducing PWV in the long-term period. Therefore, the measurement and assessment of PWV could be help stratify and identify KT recipients at higher CV risk, which is a step forward toward the development of personalized therapies<sup>24,26</sup>.

Brief Report

# Association between Long-Term Change in Arterial Stiffness and Cardiovascular Outcomes in Kidney Transplant Recipients: Insights from the TRANSARTE Study

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**Abstract:** (1) Background: Increased arterial stiffness is associated with cardiovascular (CV) diseases in end-stage renal disease (ESRD) patients, and CV mortality remains higher in kidney transplantation (KT) recipients compared to in the general population. KT is associated with an improvement in arterial stiffness in the early post-transplant period, followed by a potential re-worsening in the late period. In a cohort of KT patients, we evaluated the associations of pulse-wave velocity (PWV) measured at different time-points (pre-transplant, and early and late post-transplant periods) with CV morbi-mortality, as well as the evolution between these measurements with CV morbi-mortality. (2) Methods: Forty KT recipients with a 10-year follow-up were included. The association of PWV with CV events was assessed with multivariable cox analysis. Backward linear regressions were conducted to identify the determinants of PWV at 1 year and those of the long-term evolution of PWV after KT (delta PWV at 1 year—latest PWV). (3) Results: The absence of arterial stiffening during the long-term follow-up after KT is associated with a lower CV outcome rate (HR for the delta PWV = 0.76 (0.58–0.98),  $p = 0.036$ ). Age at KT is associated with the worsening of arterial stiffness in the late post-transplantation period ( $\beta$  for the delta PWV =  $-0.104$ ,  $p = 0.031$ ). A high PWV at 1 year was associated with a potential for recovery during follow-up ( $\beta = 0.744$ ,  $p < 0.0001$ ). (4) Conclusions: The absence of PWV worsening in the late post-transplantation period was significantly associated with a lower risk of CV events, whereas early changes in PWV were not. Finding an intervention capable of reducing long-term PWV could improve the prognosis of KT recipients.

**Keywords:** arterial stiffness; cardiovascular diseases; kidney transplantation; pulse-wave velocity

## 1. Introduction

Cardiovascular (CV) diseases are the leading cause of death in patients with end-stage renal disease (ESRD). After kidney transplantation (KT) and the restoration of renal function, the risk of CV mortality remains high compared with the general population [1]. Increased PWV, a marker of arterial stiffness, has been reported to be a strong risk factor of CV morbi-mortality in ESRD [2]. In KT, some studies have also reported this association [3,4].

The mechanisms underlying arterial stiffness in KT are numerous, involving the pre-transplant history of patients (the duration of ESRD or dialysis, and co-morbidities such as

hypertension or diabetes). Age also remains a key determinant of PWV in KT [5]. Nevertheless, data on the evolution of PWV suggest a decrease in PWV during the first year after transplantation [6,7] followed by an increase in PWV after that point [1]. This long-term deterioration of PWV may indicate that factors arising with transplantation, e.g., immunosuppressive drugs, could have a detrimental impact on PWV [8]. It was also reported that donor age was an independent factor for PWV in KT with a deceased donor [9].

Therefore, considering the potential improvement of arterial stiffness in the early post-transplantation period [6,7]—and potential re-worsening in the late-transplantation period [1]—we aimed to evaluate the associations between CV morbi-mortality and PWV measured at different time-points (pre-transplantation, early post-transplantation, and late post-transplantation periods), as well as the evolution between these measurements with CV morbi-mortality, in a cohort of kidney transplant patients. In this framework, we investigated the association of arterial stiffness with CV events in KT recipients from the monocentric TRANSARTE study (Transplantation and Arteries).

## 2. Materials and Methods

### 2.1. Study Population

A detailed description of the TRANSARTE study has been published previously [10]. Briefly, 100 ESRD patients were recruited from the waiting list of the University Hospital of Nancy to characterize the evolution of arterial stiffness after KT in a population of ESRD patients. Among the kidney-transplanted patients, the present study was carried out on 40 renal transplant recipients with available predefined PWV measurements, as previously described [10]. The composite primary outcome of the study is a CV event (defined by acute coronary syndrome, myocardial infarction, angioplasty, vascular angioplasty, coronary bypass, ischemic or hemorrhagic stroke, angioplasty of lower extremity arteries, amputation, and atrial fibrillation).

### 2.2. Measurement of PWV

PWV measurements were performed using the Complior<sup>®</sup> technique (ALAM Medical, Saint Quentin Fallavier, France), which simultaneously records arterial pulse waves at carotid and femoral sites. The propagation time (t) of the incident wave was measured through mechanotransducer probes, the carotid–femoral distance (D) was evaluated with a tape measure, and PWV (m/s) was obtained by the formula  $PWV = D \times 0.8/t$ .

### 2.3. Statistical Analysis

Analyses were performed using R version 4.0.2 (R Development Core Team, Vienna, Austria). The means are presented with SD and median, with 25th and 75th percentiles. The *p*-values are considered significant if they are <0.05. The association of PWV with CV events was assessed using Cox models. Backward linear regression was performed to identify the determinants of PWV at 1 year, and absolute changes in PWV post-transplantation (delta between PWV before transplantation and at 1-year, and between 1- and 4-years post-transplantation). Gender, age at transplantation; mean arterial blood pressure (MBP); hypercholesterolemia; diabetes mellitus; smoking; history of angina or myocardial infarction; previous strokes; CNI (Tacrolimus/Cyclosporine); and cause of ESRD were considered as independent variables. In addition, PWV at one-year post-transplantation was considered in the model focusing on late change in PWV. All variables with a *p* < 0.15 in univariable analysis were used as candidate variables in the multivariable model prior to backward selection procedures.

## 3. Results

### 3.1. Baseline Characteristics of Patients

In the 40 patients receiving either a living (*n* = 6) or a deceased (*n* = 34) donor allograft, the median age at transplantation (years) was 53 [47–60]. 19 (47.5%) were female. Of the subjects, 5 (12.5%) were diabetic, 2 (5.0%) had history of angina or myocardial infarction,

and 2 (5.0%) had previously experienced strokes. The mean pre-transplantation, 1-year post-transplantation and latest post-transplantation PWVs (m/s) (respectively, measured at a median delay of 7 [2–11] months before KT, 13 [11–25] months after KT, and 52 [37–62] months after KT) were  $8.9 \pm 2.45$ ,  $9.0 \pm 2.8$ , and  $8.9 \pm 2.4$  (m/s), respectively ( $p = 0.96$ ). The majority of patients received calcineurin inhibitors (CNI) (95%), most of them cyclosporine (55.3%). During the median follow-up period of 10.9 years, 12 patients died, 11 patients had a CV event and 6 patients returned to dialysis (Table 1).

**Table 1.** Baseline characteristics and outcomes of patients.

	<b>Overall Population N = 40</b>
Baseline characteristics	
Female (%)	19 (47.5)
Age at transplantation (years)	53 [47–60]
Medical history	
Hypercholesterolemia (%)	20 (50.0)
Smoking (%)	16 (40.0)
Diabetes (%)	5 (12.5)
History of angina or myocardial infarction (%)	2 (5.0)
Previous Strokes (%)	2 (5.0)
Cause of ESRD (%)	
Diabetes	2 (5.0)
Glomerulopathy	7 (17.5)
Malformation	8 (20.0)
Polycystic kidney disease	10 (25.0)
Vascular	6 (15.0)
Other	7 (17.5)
Pre-transplantation dialysis (%)	
Hemodialysis (%)	23 (76.7)
Peritoneal dialysis (%)	7 (23.3)
Dialysis duration (years)	2.7 [2.1–3.5]
Transplantation characteristics	
Calcineurin inhibitors (%)	38 (95.0)
Cyclosporine (%)	21 (55.3)
Tacrolimus (%)	17 (44.7)
Donor type	
Living donor (%)	6 (15.0)
Deceased donor (%)	34 (85.0)
Standard-criteria donors (SCD)	17 (51.5)
Expanded-criteria donors (ECD)	16 (48.5)
Arterial stiffness characteristics	
Duration between pre-transplantation PWV and transplantation (months)	7 [2–11]
Pre-transplantation PWV (m/s)	$8.9 \pm 2.5$

**Table 1.** *Cont.*

	<b>Overall Population N = 40</b>
Pre-transplantation mean arterial blood pressure (mmHg)	101 ± 14
Duration between transplantation and 1-year post-transplantation PWV (months)	13 [11–25]
1-year post-transplantation PWV (m/s)	9.0 ± 2.8
1-year post-transplantation mean arterial blood pressure (mmHg)	94 ± 12
Duration between transplantation and the latest post-transplantation PWV (months)	52 [37–62]
The latest post-transplantation PWV (m/s)	8.9 ± 2.4
The latest post-transplantation mean arterial blood pressure (mmHg)	91 ± 12
Post-transplantation characteristics	
eGFR at 1 year	48.5 [40.5–54.3]
eGFR at the last contact	
All patients	48.0 [25.8–53.8]
Not back to dialysis patients	48.5 [42.3–56.8]
Follow-up time (years)	10.9 [8.2–12.0]
Back to dialysis or preemptive re-transplantation (%)	6 (15.0)
Death (%)	12 (30.0)
CV events post-transplantation (%)	11 (27.5)

Data presented as mean ± standard deviation; number (percent); and median [interquartile range]. Abbreviations: ESRD: end-stage renal disease; PWV: pulse-wave velocity; eGFR: estimated glomerular filtration rate (mL/min/1.73 m<sup>2</sup>); CV: cardiovascular. CV events post-transplantation, the composite primary outcome of the study, is defined by acute coronary syndrome, myocardial infarction, angioplasty, vascular angioplasty, coronary bypass, ischemic or hemorrhagic stroke, angioplasty of lower extremity arteries, amputation, and atrial fibrillation.

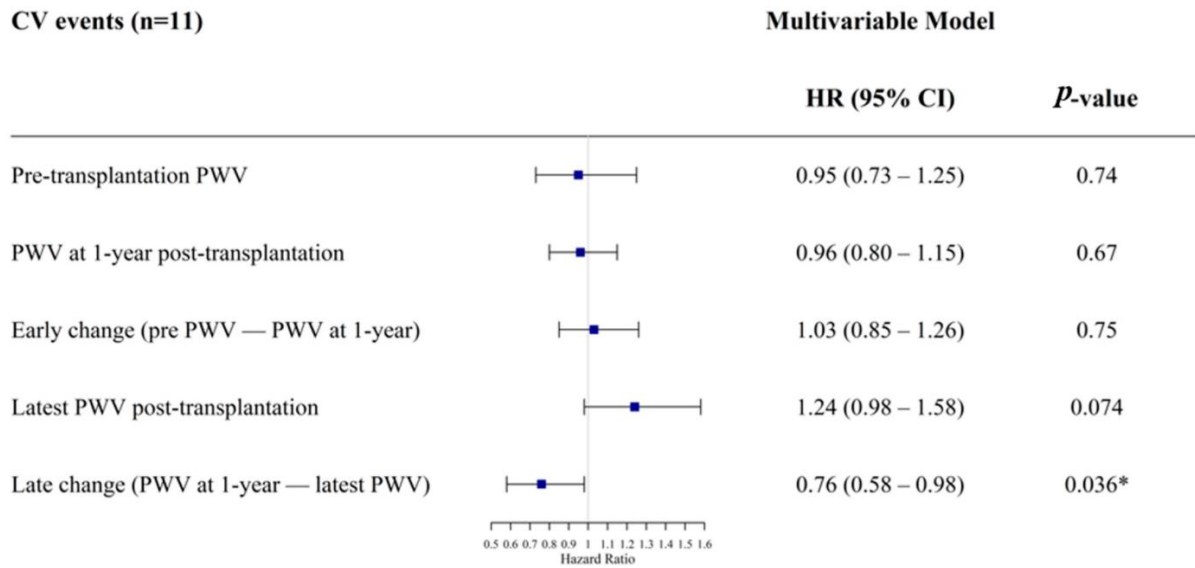
### 3.2. Association of PWV with CV Events

In multivariable analysis adjusted for PWV post-transplantation at 1 year, and age at transplantation, the absence of arterial worsening in the late post-transplantation period was significantly associated with a lower risk of CV events (HR for the delta PWV at 1 year—latest PWV = 0.76 (0.58–0.98)). On the contrary, the evolution of PWV between the pre-transplant period and the early post-transplantation period, as well as the PWV at 1 year, were not significantly associated with CV morbi-mortality (Figure 1). The complete output of the Cox models is provided in Supplementary Table S1.

### 3.3. Determinants of PWV Using Backward Linear Regression

The backward multivariate regression analyses identified that age at KT and diabetes were significantly associated with an increased arterial stiffness at 1 year post-transplantation ( $\beta = 0.013$ ,  $p = 0.006$  and  $\beta = 0.509$ ,  $p < 0.0001$ , respectively). Age at KT was also associated with the worsening of arterial stiffness in the late post-transplantation period, even after adjusting for PWV at 1 year ( $\beta$  for the delta (PWV at 1 year—PWV at 4 years =  $-0.104$ ,  $p = 0.031$ )). A higher PWV at 1-year was associated with a PWV decrease over the late post-transplantation period ( $\beta = 0.744$ ,  $p < 0.0001$ ) (Figure 1). Importantly, MBP was not retained as a significant factor in the selection process. A detailed description of the backward regression model is provided in Supplementary Table S2.

A

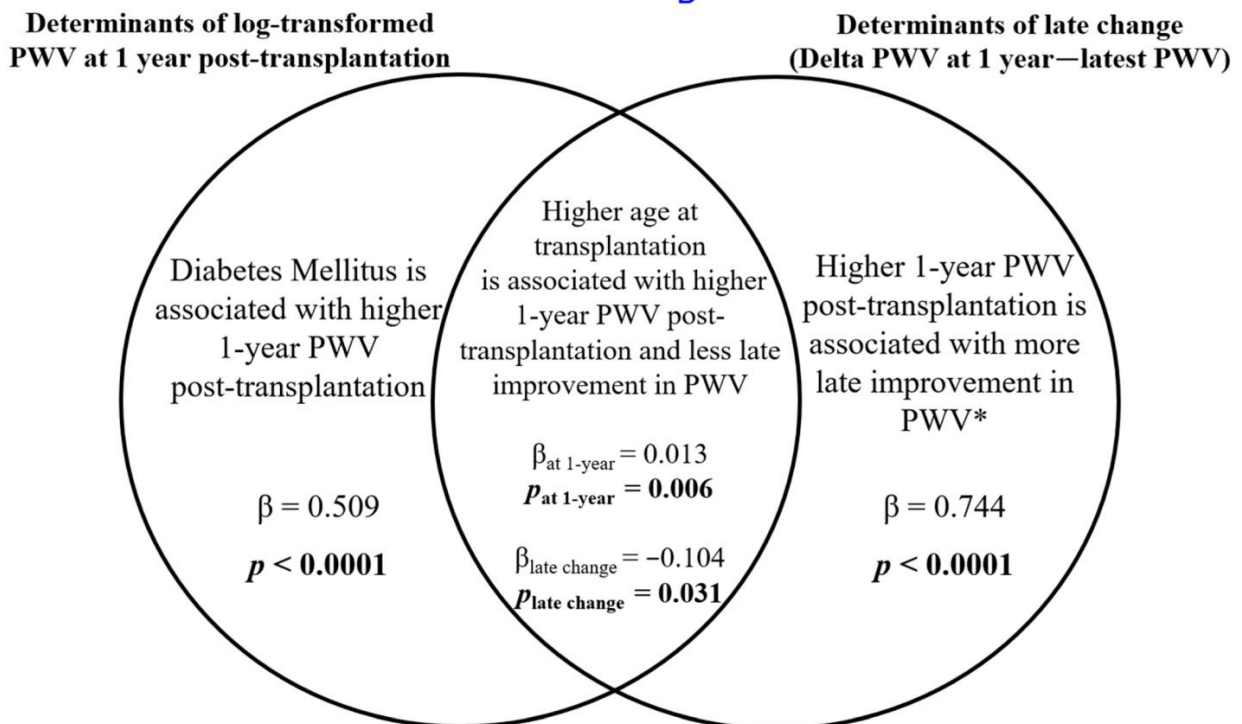


\*p-value <0.05;

All models were adjusted for age at transplantation;

PWV change was adjusted for the baseline PWV on top of age at transplantation

B



Age at transplantation: PWV at 1-year was put into the model of late change in PWV as independent variable.

\*PWV at 1-year post-transplantation was used alone in the model.

Figure 1. (A) Forest plot of the association between pulse-wave velocity (PWV) and cardiovascular (CV) events; (B) the determinants of PWV using backward linear regression.

#### 4. Discussion

Despite the improvement of renal function conferred by KT, CV mortality remains higher in KT recipients compared with the general population. In KT, some studies demonstrated that an increased arterial stiffness was associated with CV mortality [3,4]. Nevertheless, the evolution of arterial stiffness is complex after KT; some studies reported an early improvement [6,7], and others reported a long-term re-worsening [1]. Vascular remodeling is complex and involves alterations of the extracellular matrix by uremic toxins, which are not fully reversed by KT [7]. Moreover, immunosuppressive therapy is associated with hypertension, diabetes and dyslipidemia, and may indirectly or directly impact the vascular system.

The present study focuses on the association between CV morbi-mortality and the PWV measured at different time-points, as well as the evolution of the PWV through time. Notably, despite the low number of patients, we had, for the first time, the opportunity to measure the arterial stiffness in the pre-KT, the early post-KT, and the late post-KT periods. Moreover, we analyzed CV outcomes after a ten-year follow-up period.

In a study on 220 kidney transplant recipients, carotid–femoral PWV evaluated at 3 and 12 months after KT was significantly associated with morbi-mortality 5.5 years after KT, whereas the delta of PWV between month 12 and month 3 was not [11]. However, the present study added that an increased PWV between month 12 and month 48 is associated with CV events (adjusted for PWV at 1-year after KT and age at KT) in kidney recipients 10 years after KT. This suggests an impact of non-traditional factors in the late post-KT period, probably related to immunosuppressive therapy.

Unsurprisingly, age is associated with increased PWV at 1 year, but also with less improvement in the long-term post-transplantation period. Ignace et al. previously reported that older patients had a higher improvement in PWV in the early post-transplantation period at 3 months post-KT [5]. We can hypothesize that despite this early improvement, arteries from older patients are more prone to deterioration related to immunosuppressive regimens after a long-term exposition.

On the contrary, increased PWV at 1 year is associated with increased potential improvement in the long-term post-transplantation period. One can hypothesize that arterial stiffness mainly related to CKD, but not age, has a greater potential for recovery after KT, and that the susceptibility of the vascular system to immunosuppressive therapy is less pronounced among the youngest patients with ESRD.

It is worth mentioning that, similarly to the results of Kolonko et al. [12], a history of diabetes mellitus was a significant determinant of PWV 1 year after transplantation in the present study. Notably, diabetes mellitus was not related to the worsening of the PWV change post-transplantation, suggesting the stability of PWV in diabetic patients after 1 year of KT. Indeed, it was evident that patients with greater age, diabetes mellitus and higher PWV at baseline had an increased PWV 1 year after transplantation [13,14].

#### 5. Limitations

This study is a single-center study with a small sample size. This prevented the use of more advanced statistical analyses such as splines to determine the most adequate prognostic PWV thresholds, or the addition of some possibly important covariates in the multivariable analysis. However, we assessed ESRD-related arterial stiffness over a long period of time in a peculiar population of kidney-transplanted patients, something that has not been reported previously. Further long-term studies should be conducted to better ascertain the long-term changes in PWV following KT.

There was no preemptive KT in this cohort. Therefore, it was not possible to include the residual renal function before KT to assess its potential impact on the evolution of arterial stiffness after KT.

## 6. Conclusions

In this analysis, the absence of PWV- worsening in the late post-transplantation period was significantly associated with a lower risk of CV events. In contrast, the change in PWV observed early during the post-transplantation period was not associated with CV events.

In summary, the present study suggests that the worsening of aortic stiffness in the late post-KT period has a negative impact on long-term CV morbi-mortality. Ultimately, finding an intervention able to reduce PWV could improve the prognosis of KT recipients.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jcm11051410/s1>, Table S1: Association of pulse-wave velocity (PWV) with cardiovascular (CV) events; Table S2: Determinants of absolute changes in pulse-wave velocity (PWV) post-transplantation (PWV at 1 year—latest PWV) using backward linear regression.

**Author Contributions:** Conceptualization, S.G. and N.G.; Methodology, S.G., N.G. and M.S.; Formal Analysis, M.S.; Writing—Original Draft Preparation, M.S. and A.S.; Writing—Review & Editing, M.S., A.S., N.G., A.K.-S., P.R., A.B., L.F., S.G.; Supervision, S.G., N.G. and P.R. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** Approval for the study was obtained from the local research ethics committee of CPP-EST 3 (No. 06.10.03), France.

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study can be made available upon reasonable request to the corresponding author.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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## Supplementary Materials

**Supplementary Table S1: Association of pulse wave velocity (PWV) with cardiovascular (CV) events**

CV events (n=11)	Univariable	p-value	Multivariable **	p-value
	HR (95% CI)		HR (95% CI)	
Pre-transplantation PWV	1.00 (0.77 - 1.31)	0.98	0.95 (0.73 - 1.25)	0.74
PWV at 1-year post-transplantation	1.01 (0.85 - 1.18)	0.95	0.96 (0.80 - 1.15)	0.67
Early change (pre PWV – PWV at 1-year) *	0.99 (0.82 - 1.20)	0.96	1.03 (0.85 - 1.26)	0.75
Latest PWV post-transplantation	1.34 (1.08 - 1.65)	<b>0.008</b>	1.24 (0.98 - 1.58)	<b>0.074</b>
Late change (PWV at 1- year – latest PWV) *	0.71 (0.55 - 0.90)	<b>0.006</b>	0.76 (0.58 - 0.98)	<b>0.036</b>

*\*models of PWV change were adjusted for the baseline PWV*

*\*\* All models were adjusted for age at transplantation*

*Results with p value less than 5% were emphasized using bold letters*

**Supplementary Table S2: Determinants of absolute changes in pulse wave velocity (PWV) post-transplantation (PWV at 1-year – latest PWV) using backward linear regression**

Late PWV change (m/s)	Univariable*				Multivariable (backward selection) *			
	β	(95% CI)		p-value	β	(95% CI)		p-value
		Lower	Upper			Lower	Upper	
First PWV post-transplantation (m/s) **	0.662	0.405	0.918	<0.0001	0.744	0.489	1.000	<0.0001
Sex (1=Male, 2=Female)	1.125	-0.313	2.563	<b>0.12</b>	-	-	-	-
Age at transplantation (years)	-0.104	-0.198	-0.010	<b>0.031</b>	-0.104	-0.198	-0.010	<b>0.031</b>
Mean arterial blood pressure (mmHg)	-0.028	-0.095	0.033	0.35				
Hypercholesterolemia (1=Yes, 0=No)	0.532	-0.952	2.015	0.47				
Diabetes (1=Yes, 0=No)	1.565	-1.405	4.536	0.29				
Smoking (1=Yes, 0=No)	-0.828	-2.291	0.634	0.26				
History of angina or myocardial infarction (1=Yes, 0=No)	1.207	-2.331	4.746	0.49				
Previous strokes (1=Yes, 0=No)	-1.924	-5.202	1.355	0.24				
CNI (1=Tacrolimus, 2= Ciclosporin)	0.085	-1.549	0.719	0.92				
<b>Cause of ESRD</b>				<b>0.11</b>	-	-	-	-
Glomerulopathy	0.478	-3.895	4.852	0.83				
Malformation	1.023	-3.415	5.462	0.64				
Polycystic kidney disease	-0.146	-4.402	4.11	0.94				
Vascular	0.674	-3.826	5.101	0.77				
Other	0.800	-3.251	4.852	0.69				

\*Univariable and multivariable models are adjusted on first PWV post-transplantation

\*\* In this model PWV was used alone in the model

Mean arterial blood pressure is at the moment of the latest PWV; CNI, Calcineurin inhibitors; ESRD, end-stage renal disease (Reference = diabetes)

All variables with a  $p < 0.15$  in univariable analysis were used as candidate variables in the multivariable model prior to backward selection procedures.

### 2.2.2. Galectin-3 and collagen markers in kidney transplant recipients: Insights from the TRANSARTE study (4<sup>th</sup> article)

In this study, we assessed the correlation between PICP and Gal-3 measured in EDTA plasma and PWV at 2-year post KT. Moreover, we evaluated the association of biomarkers with the composite outcome of death and CV events using Cox proportional hazards models. This association was adjusted for age at transplantation, eGFR and PWV at the time of biomarkers measurement (i.e. at 2-year post KT).

Among the 44 patients included in this study, 43.2% were female patients, median age at KT was 53 [46–59] years. At mean follow-up period of 1.9 years, the median concentrations (ng/mL) of PICP and Gal-3 were 102.0 [68.5–125.1] and 15.4 [13.1–20.3], respectively. At the time of biomarkers measurement, the mean eGFR was  $42.2 \pm 14.2$  mL/min/1.73 m<sup>2</sup> and the mean of PWV was  $8.6 \pm 2.2$  m/s.

There was no correlation between PICP (Spearman's rank-order correlation ( $r = -0.16$ ,  $p = 0.3$ ) and between PWV and Gal-3 ( $r = 0.03$ ,  $p = 0.85$ ). In addition, there was no correlation between PICP and Gal-3 ( $r = -0.015$ ,  $p = 0.9$ ). The fully adjusted cox model, Gal-3 was significantly associated with a greater risk of CV morbi-mortality (HR per 1 log increase = 4.30 (1.01-18.22),  $p = 0.048$ ), whereas PICP was not significantly associated with outcome (HR per 1 log increase = 2.28(0.65-8.05),  $p = 0.20$ ).

It is well established that PWV is a strong and an independent factor of CV morbidity and mortality in ESRD and KT<sup>20</sup>. However, our study showed no significant correlation between the biomarkers (i.e. PICP and Gal-3) and PWV. This suggests that high biomarkers concentrations do not reflect vascular damage, but instead other pathophysiological features. With regard to the collagen biomarker (i.e. PICP), a number of studies showed that type I and III collagen were co-distributed in the media of blood vessels wall and aortic wall of human tissues<sup>134,135</sup>. However, in vitro studies have demonstrated different data in diseased tissue, there was a change in favor of collagen type III rather than type I. It is likely that type III collagen is strongly present as it arises from the endothelium. Chamiot Clerc et al.<sup>136</sup> showed that stiffer and thicker aortas of rats have a significantly higher density of type III collagen but not type I collagen. With regard to Gal-3, its expression is known in many tissues including fibroblasts, endothelial cells, and inflammatory cells such as macrophages, and its overexpression appears to play an essential role in fibrosis and

tissue remodeling as a consequence of inflammation and oxidative stress<sup>89</sup>. Therefore, Gal-3 is known to be a biomarker of fibrosis in the myocardium, and its high concentrations are associated with the progression of CV diseases rather than vascular damage<sup>85,103</sup>.

In the present study, Gal-3 was significantly associated with CV morbi-mortality after adjusting for prognostic factors (including eGFR at the time of biomarkers measurement, which was inversely correlated to Gal-3 concentrations and was not significantly associated with the outcome). Indeed, Gal-3 appeared to reflect chronic inflammation inducing fibrosis, which is a primary driver in pathophysiological mechanism in the context of cardiorenal syndrome<sup>89,90,96,97</sup>. Gal-3 has been suggested as a marker of myocardial fibrosis and inflammation, two associated processes involved in myocardial modelling<sup>15,90,109,137-139</sup>. In addition, high serum Gal-3 concentrations were found to be correlated with poor progression of ventricular remodeling in chronic HF. This suggests that Gal-3 could serve as a biomarker in the prediction and progression of cardiac fibrosis and HF<sup>85,96,101</sup>. Indeed, our results suggest that high concentrations of Gal-3 may reflect CV histological modifications related to uremic cardiomyopathy, the main feature of cardiac diseases in patients with ESRD, which are not fully reversed in KT<sup>9,32</sup>.

In summary, the present study suggests that Gal-3 may be a key mechanistic pathway representing another dimension of inflammation associated with ESRD. It would be interesting to investigate the impact of reducing Gal-3 concentrations using a competitive inhibitor of Gal-3 in dedicated trials<sup>93</sup>. This strategy could be promising to improve CV risk prediction in this population and subsequently benefit from anti-fibrotic therapies.

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**Galectin-3 and collagen markers in kidney transplant recipients: Insights from the TRANSARTE study**

*Madonna Salib, Nicolas Girerd, Arnaud SIMON et al*

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## **Galectin-3 and collagen markers in kidney transplant recipients: Insights from the TRANSARTE study**

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**Abstract:**

**Background:** Cardiovascular (CV) mortality remains high despite the improvement of kidney function after kidney transplantation. In heart failure (HF), high concentrations of biomarkers of fibrosis, related to cardiac and/or vascular impairment, are associated with CV outcomes but their significance in kidney transplantation is still unclear. Our aim was to investigate the association of procollagen type I C-terminal pro-peptide (PICP) and galectin-3 (Gal-3), markers of fibrosis, with arterial stiffness measured by pulse wave velocity (PWV) and CV morbidity in kidney transplantation recipients from the prospective monocenter TRANSARTE study (Transplantation and Arteries), which compared the evolution of arterial stiffness in transplanted patients and patients remained on dialysis.

**Methods:** PICP and Gal-3 were measured at 2-year post-transplantation in 44 kidney transplantation patients. Spearman's rank-order correlation was conducted to assess the relationship between biomarkers and PWV. Association of biomarkers with CV morbidity was evaluated using cox regression analysis adjusted on age, renal function, and PWV.

**Results:** There was no significant correlation between PWV and PICP ( $r = -0.16$ ,  $p = 0.3$ ) or Gal-3 ( $r = 0.03$ ,  $p = 0.85$ ). Gal-3, after adjusting for key prognostic factors including PWV, was significantly associated with CV morbidity [HR (95% CI) = 4.30 (1.01-18.22),  $p = 0.048$ ], whereas PICP was not significantly associated with outcome.

**Conclusions:** In multivariable adjusted analysis, elevated Gal-3 concentrations were associated with CV morbidity in kidney transplantation patients whereas PICP was not. As Gal-3 were not related to PWV, other sources of fibrosis (i.e. cardiac fibrosis) may be underlying the prognostic value of Gal-3 in kidney transplantation.

**Keywords:** kidney transplantation; collagen; galectin-3; pulse wave velocity; cardiovascular diseases, fibrosis, arterial stiffness.

## **Background**

The urgent need for a novel approach to better stratify cardiovascular (CV) risk in kidney transplantation recipients is derived from the high prevalence/incidence of CV diseases (CVD) in this population. Despite the restoration of renal function after kidney transplantation, CV death remains higher in kidney transplantation recipients compared with the general population [1].

Atherosclerosis, arteriosclerosis, and arterial stiffening are accelerated in end-stage renal disease (ESRD) [2,3]. Patients develop mechanical and physiological abnormalities of arteries due to fluid overload, bone disorder, chronic inflammation, hypertension, and endothelial dysfunction [3]. This subsequently may lead to cardiovascular fibrosis [2,4].

Improvement of renal function probably has beneficial effects on vascular structure and function but kidney transplantation patients receive immunosuppressive therapy with deleterious effects on metabolic and vascular parameters [5]. The net output of these opposing factors is yet insufficiently documented. Notably, the evolution of arterial stiffness in kidney transplantation is complex and has been reported to be associated with poor CV prognosis [6,7].

A number of cardiovascular markers could predict CV outcomes. Pulse wave velocity (PWV) has been shown to be a strong predictor of CV outcomes, independent of conventional prognostic factors such as age, left ventricular (LV) hypertrophy, and hypertension [8,9]. The biological factors underlying this association of PWV with outcome is currently not well understood. Arterial stiffness has been shown in other settings to be associated with fibrosis. Harvey et al.[10] emphasized that molecular mechanisms underlying vascular stiffening follows vascular fibrosis and are related to increased expression and activation of matrix metalloproteinases, upregulation of galectin-3 (Gal-3), and activation of profibrotic pathways. Collagen peptides, that actually measure circulating levels of collagen fibers degradation and/or production, and Gal-3 are commonly used biomarkers to explore biological fibrosis.

C-propeptide molecules control the assembly of collagen fibrils in the extracellular matrix (ECM) [11]. Serum procollagen type I C-terminal pro-peptide (PICP), a collagen type I synthesis biomarker, was suggested as a key marker in the development of myocardial fibrosis, as it was proven to be correlated with myocardial collagen in patients with hypertensive heart disease [12,13]. Moreover, PICP has been shown to be associated with higher rates of CV death in hemodialysis patients [14]. Notably, arterial stiffness, assessed by PWV, showed to be significantly correlated with PICP in hypertensive patients with LV hypertrophy [15].

Gal-3 is a profibrotic molecule that has been shown to be involved in cardiac fibrosis, and is associated with CV outcomes in heart failure (HF) [16]. In dialysis patients, Gal-3 was found to be associated with a higher risk of all-cause mortality and CV death [14,17]. Importantly, concomitant increase in Gal-3 concentration and PWV was demonstrated to be significantly associated with higher rates of all-cause mortality and major CV and cerebrovascular events in hemodialysis patients [18].

The relevance of PICP and Gal-3 and their relationship with PWV in kidney transplantation has not been studied yet. In this regard, we aimed to study the association of PICP and Gal-3, on top of PWV, with the composite outcome of death and CV events among kidney transplantation recipients in the TRANSARTE Study (Transplantation and Arteries) [19] which characterized the evolution of arterial stiffness after kidney transplantation in ESRD patients.

## **Material and methods**

### **Study Population**

The description of this single-center study of the prospective TRANSARTE (Transplantation and Arteries) has been previously published [19]. In short, 100 ESRD patients were recruited from the waiting list of the University Hospital of Nancy to characterize the evolution of arterial stiffness after kidney transplantation. Forty-four renal transplant recipients who had available post-transplantation PWV measurement at 2-year follow-up were considered in this subsequent analysis. Informed consent was obtained from all subjects involved in the TRANSARTE study. The study protocol was reviewed and approved by the local research ethics committee of CPP-EST 3 (No. 06.10.03), France. This analysis was conducted under the umbrella of the sponsor of the study (Nancy University Hospital) under the supervision of the principal investigator of the study (LF).

### **Follow-up**

All patients included in the TRANSARTE study were routinely followed up at least once a year at the Nancy University Hospital. Clinical events occurring during follow-up were collected from the clinical charts until December 31, 2020. The following CV events were recorded from clinical chart reviews by a trained senior physician: acute coronary syndrome, myocardial infarction, angioplasty, vascular angioplasty, coronary bypass, ischemic or hemorrhagic stroke, angioplasty of lower extremity arteries, amputation, and atrial fibrillation.

## **Measurement of PWV**

PWV was measured using the Complior® technique (ALAM Medical, Saint Quentin Fallavier, France), which simultaneously records arterial pulse waves at carotid and femoral sites. The propagation time (t) of the incident wave was calculated through mechanotransducer probes and the carotid–femoral distance (D) was determined with a tape measure. The time delay that represents the difference between the R-wave and the foot of the pressure pulse waveform at each site and the distance traveled by the pulse wave between the 2 recording sites, divided by the time, were used in the calculation of PWV. PWV (m/s) was calculated using the following formula  $PWV = D \times 0.8/(t)$ .

## **Biomarkers**

PICP and Gal-3 were measured in EDTA plasma. PICP was measured by ELISA (Quidel Corporation, Santa Clara, USA) and Gal-3 was measured by ELISA (BG MEDICINE, Foxboro, USA) at PWV assessment post kidney transplantation (~ 2-year).

## **Statistical analysis**

Continuous data are presented as mean  $\pm$  standard deviation or median [25<sup>th</sup> and 75<sup>th</sup> percentiles], whereas categorical data are expressed as numbers (%). Spearman's rank-order correlation (r) was performed to assess the relationship between biomarkers and between PWV and biomarkers. In univariable and multivariable Cox proportional hazards models, we assessed associations of biomarkers (as log-transformed continuous variables) with the composite outcome death and CV events (CV events are defined by acute coronary syndrome, myocardial infarction, angioplasty, vascular angioplasty, coronary bypass, ischemic or hemorrhagic stroke, angioplasty of lower extremity arteries, amputation, and atrial fibrillation). In multivariable analysis, covariates included age at transplantation, estimated glomerular filtration rate (eGFR) and PWV. Moreover, associations between biomarkers and the composite outcome were assessed after adjusting for graft rejection as a time-dependent variable using Cox proportional hazards models. Analyses were performed using R version 4.0.2 (R Development Core Team, Vienna, Austria).

## **Results**

### **Baseline characteristics of patients**

Of the 44 studied participants (median age 53 years [46–59], 43.2% female), 6 (13.6%) had diabetes mellitus, 1 (2.3%) had previous myocardial infarction or angina and 2 (4.5%) had

previous strokes. The majority of patients received a deceased donor allograft (90.9%) and calcineurin inhibitors (CNI) (97.7%), most of them cyclosporine (53.5%). PWV was  $8.7 \pm 2.5$  m/s and  $8.6 \pm 2.2$  at pre-transplantation and post-transplantation, respectively.

At mean follow-up time of 1.9 years, median levels of PICP and Gal-3 were 102.0 ng/mL [68.5–125.1] and 15.4 [13.1–20.3], respectively. Estimated glomerular filtration rate (eGFR) at time of biomarkers measurement was  $42.2 \pm 14.2$  mL/min/1.73 m<sup>2</sup>. During the median follow-up period of 10.8 years, 14 patients experienced a CV event, 5 patients returned in dialysis, and 13 patients died (Table 1).

### **Correlation and association of PICP and Gal-3 with PWV and outcome**

No correlation was found between PWV and PICP ( $r = -0.16$ ,  $p = 0.3$ ) and between PWV and Gal-3 ( $r = 0.03$ ,  $p = 0.85$ ). There was no correlation between PICP and Gal-3 ( $r = -0.015$ ,  $p = 0.9$ ). In continuous unadjusted analysis, PICP was not significantly associated with CV morbidity, whereas Gal-3 was significantly associated with the outcome (HR per 1 log increase in Gal-3=5.82 (1.97 - 17.16),  $p = 0.001$ ). In the fully adjusted model for age at transplantation, eGFR, and PWV, one log unit increase in Gal-3 was associated with a 4.36% greater risk of CV morbidity (HR per 1 log increase in Gal-3=4.36 (1.03 - 18.50),  $p = 0.046$ ), whereas PICP was not significantly associated with outcome (Figure 1). Of note, further including diabetes mellitus in the multivariable models did not significantly change the association of biomarkers with the outcome (data not shown).

### **Sensitivity analysis**

When further adjusting for type of CNI (i.e. cyclosporine or tacrolimus) or smoking in the multivariable models, we did not identify a significant modification of the associations of biomarkers with CV morbidity (Supplementary Table). We also performed a forward variable selection for the Cox proportional hazards models with p-to-enter set at 15% for the following candidate variables: age at transplantation, eGFR at post-transplantation PWV measurement, diabetes mellitus, post-transplantation PWV, smoking, sex, previous CV events (i.e. previous myocardial infarction or angina and previous stroke), and type of CNI. Only previous CV events was retained in the model. The association of biomarkers remained unchanged in this additional model adjusted for previous CV events (HR per 1 log increase in Gal-3=5.67 (1.42 - 22.65),  $p=0.014$ ) (Supplementary Table S1).

We did not identify a significant modification of the associations between biomarkers, PICP and Gal-3, and CV morbi-mortality when adjusting for graft rejection as time dependent variable (Supplementary Table S2).

## **Discussion**

It is well established that ESRD patients develop structural and functional pathophysiological CV changes including myocardial fibrosis, arterial stiffness, LVH, and HF [2]. Moreover, despite the improvement in renal function after kidney transplantation, CV mortality remains high in kidney transplantation recipients compared to the general population [1]. Furthermore, the impact of kidney transplantation on arterial stiffness is complex, considering the beneficial impact of the renal function improvement but the deleterious impact of immunosuppressive drugs [5,6]. The present study is the first study that aimed to investigate the association of CV biomarkers, PICP and Gal-3, on top of PWV, with CV morbi-mortality in kidney transplantation patients.

PICP is released into the blood as it cleaves from procollagen type I during the synthesis of fibril-forming collagen type I by the procollagen type I carboxy-terminal proteinase [20]. PICP is cleared from blood via the liver due to its high molecular weight (a 100 kDa procollagen) and therefore, it is considered as a marker of collagen type I synthesis in absence of liver cirrhosis [20,21]. Experimentally [22] and clinically [23], it was proven that high concentrations of collagen is related to myocardial stiffness and cardiac dysfunction/fibrosis.

Gal-3 is a  $\beta$ -galactoside-binding lectin that can bind to complex carbohydrates. The available evidence suggest that Gal-3 is a small protein (~ 30-35 kDA) that is expressed in several tissues such as the heart, blood vessels, and the kidney. The upregulation of Gal-3 is secreted by activated macrophages and it binds with several ECM proteins [23-26]. In response to acute or chronic disease, Gal-3 binds to and activates myofibroblasts which, in turn, upregulate the formation of collagen into ECM leading myocardial fibrosis [25].

Patients in the TRANSARTE study had a mean PICP of  $105 \pm 42$  ng/mL and mean Gal-3 of  $18 \pm 10$  ng/mL. These values are lower than those found in ESRD patients on chronic hemodialysis from the AURORA trial (mean PICP was  $176 \pm 91$  ng/mL and mean Gal-3 was  $69 \pm 25$  ng/mL) [14].

Arterial stiffness, which is an independent predictor of morbidity and mortality in ESRD [24]. It should be noted that there was no significant difference in PWV in transplanted patients

(median PWV 9.2 (7.9–11.9) m/s) in comparison to those remaining on dialysis (median PWV 9.8 (7.7–12.1) m/s) in TRANSARTE study [19]. In a meta-analysis, Sidibe et al. [25] showed that there is a significant reduction in overall PWV after successful kidney transplantation: a decrease of 1.20 m/s in central PWV (measured by carotid–femoral or aorta-femoral PWV), a decrease of 1.17 m/s in peripheral PWV (measured by carotid-radial PWV or femoral-distal PWV) and a decrease of 1.21 m/s in brachial-ankle PWV.

In the present study, there was no sizeable correlation between the biomarkers and PWV. Collagen type III was found to be the most abundant form in vascular walls and big arteries such as the aorta [26]. However, in the specific context of kidney transplantation, type I collagen expression may be related to other pathophysiological (possibly related to graft fibrosis) features [27,28]. PICP concentrations have been shown to be associated with myocardial fibrosis [12] but no direct evidence exists for vascular systemic damage. Actually, consistent with our study, plasma PICP concentrations were reported to be uncorrelated with arterial stiffness, measured by PWV, in hypertensive patients in two distinct studies [29,30]. Even if some interplay exists between the heart and vessels, in the setting of kidney transplantation, PICP may be more closely related to cardiac features than vascular ones, explaining the low correlation in our study between PICP and PWV.

Increased Gal-3 concentrations have been documented to be involved in cardiac modeling, such as myocardial fibrogenesis and the development of HF [31]. Based on these findings, we assessed the correlation between Gal-3 and PWV. Our study showed no correlation between Gal-3 concentrations and arterial stiffness in kidney transplantation patients, suggesting that cardiac disease more probably drives the association between Gal-3 and CV mortality than vascular damage.

Gal-3 acts as a marker of cardiac fibrosis in consequence of chronic inflammation and oxidative stress related to chronic kidney disease (CKD) [32]. Indeed, the current analysis showed that patients who were on dialysis before the transplantation had higher Gal-3 concentrations. This is likely related to the immune system which be stimulated by the dialysis itself leading to chronic inflammation and oxidative stress [33] but could be partly related to Gal-3 renal clearance [34]. Of note, chronic inflammation in kidney transplantation patients may lead to a prothrombotic state as well as chronic hypoxia associated with excessive accumulation of extracellular matrix in the vascular tree [33]. In the LURIC study, Gal-3 was reported to be associated with CV death and all-cause mortality in patients with impaired kidney function [17].

Moreover, previous studies reported an incremental prognostic value provided by Gal-3 in hemodialysis patients for all-cause mortality [35] or for CV morbi-mortality or all-cause mortality in hemodialysis patients [14]. Furthermore, Zhang et al. demonstrated that hemodialysis patients with both high PWV and Gal-3 had a significant risk increase of a composite of all-cause mortality and major adverse cerebrovascular and CV events [18].

In the present study, Gal-3 was significantly associated with the composite outcome CV morbi-mortality while adjusting for age at transplantation, eGFR, and PWV. This may suggest that despite the restoration of renal function following kidney transplantation, the histological CV modifications generated by uremic cardiomyopathy are not fully reversed [4] and continue to have an impact on patients' clinical outcome. Higher concentrations of Gal-3 may also be a proxy for ongoing inflammation in the presence of arterial stiffness [36,37].

Our findings are promising in that they provide the basis for further development strategies to validate a panel of circulating markers in a large-scale of kidney transplantation population and to stratify patients according to their biomarker profiles, which could provide novel pathways for personalized treatment.

It should be noted that this study is a single-center study with a small sample size. This moderate statistical power limited the number of covariates for which the model could be adjusted for. However, it is the first study, to our knowledge, that assesses the association of PICP and Gal-3 with PWV and CV morbi-mortality in kidney transplantation patients.

## **Conclusions**

In summary, higher Gal-3 concentrations conferred greater risk of CV morbi-mortality in kidney transplantation patients, while it was not associated with vascular modifications (as measured by PWV), thus suggesting that non-vascular (probably cardiac) fibrosis is an important predictor of outcome following kidney transplantation. These preliminary findings need further investigation to determine whether anti-Gal-3 treatment may be a path to consider in the setting of kidney transplantation.

## **Declaration of Figures Authenticity**

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.



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**Conflicts of Interest:** None.

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**Galectin-3 and collagen markers in kidney transplant recipients: Insights from the  
TRANSARTE study**

**Tables and Figures**

**Table 1** Baseline characteristics of patients.

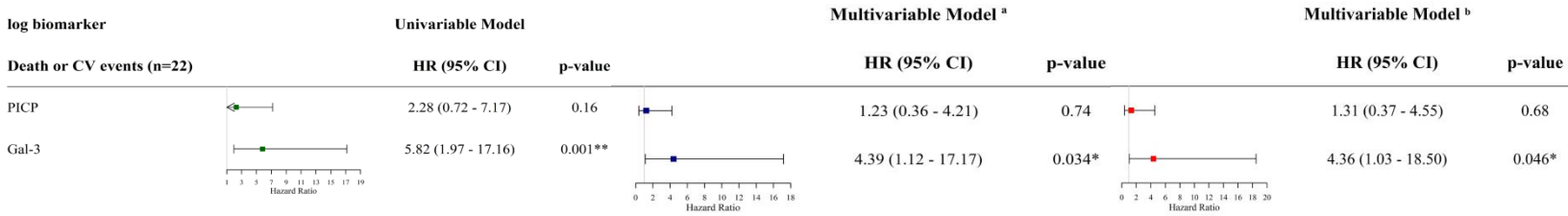
	<b>Total N = 44</b>
<b>Demographic characteristics</b>	
Female (%)	19 (43.2)
Age at transplantation (years)	53 [46–59]
<b>Medical history (%)</b>	
Hypercholesterolemia	21 (47.7)
Smoking	21 (47.7)
Diabetes	6 (13.6)
Previous myocardial infarction or angina	1 (2.3)
Previous strokes	2 (4.5)
<b>Cause of ESRD (%)</b>	
Diabetes	2 (4.5)
Glomerulopathy	11 (25.0)
Malformation	8 (18.2)
Polycystic kidney disease	10 (22.7)
Vascular	4 (9.1)
Other	9 (20.5)
<b>Pre-transplantation dialysis</b>	
Hemodialysis (%)	34 (77.3)
Peritoneal dialysis (%)	27 (79.4)
Dialysis duration (years)	7 (20.6)
	3.1 [2.1–3.8]
<b>Transplantation characteristics</b>	
Calcineurin inhibitors (%)	43 (97.7)
Cyclosporine (%)	23 (53.5)
Tacrolimus (%)	20 (46.5)
<b>Donor type</b>	
Living donor (%)	4 (9.1)
Deceased donor (%)	40 (90.9)
Standard criteria donors (SCD)	26 (65.0)
Expanded-criteria donors (ECD)	14 (35.0)
<b>Arterial stiffness characteristics</b>	
Duration between the last pre-transplantation PWV and the transplantation (months)	6.4 [2.6–10.4]
Pre-transplantation PWV (m/s), n=43	8.7 ± 2.5
Pre-transplantation mean arterial blood pressure (mmHg)	103 ± 14

Duration between transplantation and post-transplantation PWV (months)	21.6 [12.8–33.7]
Post-transplantation PWV (m/s)	8.6 ± 2.2
Mean blood pressure at post-transplantation PWV (mmHg)	96 ± 11
<b>Post-transplantation characteristics</b>	
eGFR at one year	48.0 [38.8–55.2]
Follow-up time (years)	10.8 [8.1–11.9]
eGFR at the last contact	48.0 [24.5–57.5]
Back to dialysis or preemptive re-transplantation (%)	5 (11.4)
Graft rejection (%)	17 (38.6)
Death (%)	13 (29.5)
CV event post-transplantation (%)	14 (31.8)
<b>Biomarkers at post-transplantation PWV</b>	
Duration between transplantation and biomarkers measurement (months)	21.6 [12.8–34.0]
eGFR at biomarkers measurement (mL/min/1.73m <sup>2</sup> )	42.2 ± 14.2
PICP (ng/mL)	102.0 [68.5–125.1]
Gal-3 (ng/mL)	15.4 [13.1–20.3]

ESRD, end stage renal disease; PWV, pulse wave velocity; eGFR, estimated glomerular filtration rate; CV, cardiovascular; PICP, procollagen type I C-terminal propeptide; Gal-3, galectin-3.



**Figure 1** Forest plot of the association of procollagen type I C-terminal pro-peptide (PICP) and galectin-3 (Gal-3) with CV morbi-mortality



\*p-value <0.05, \*\*p-value <0.01

<sup>a</sup>Adjusted for age at transplantation and eGFR at post-transplantation PWV measurement

<sup>b</sup>Adjusted for age at transplantation, eGFR at post-transplantation PWV measurement, and post-transplantation PWV measurement

## Supplementary data

**Supplementary Table S1: Association of biomarkers with cardiovascular morbi-mortality in multivariate cox models**

Log-transformed biomarkers	Multivariable Model 1 <sup>a</sup>		Multivariable Model 1 <sup>b</sup>		
	HR (95% CI)	p-value	HR (95% CI)	p-value	
<b>Death or CV events (n=22)</b>					
<b>PICP</b>	0.96 (0.24 - 3.81)	0.95	1.03 (0.25 - 4.19)	0.97	*
	1.24 (0.36 - 4.29)	0.74	1.31 (0.37 - 4.58)	0.67	**
	1.39 (0.38 - 5.12)	0.62	1.47 (0.39 - 5.52)	0.57	***
<b>Gal-3</b>	4.39 (1.16 - 16.69)	<b>0.030</b>	4.40 (1.06 - 18.27)	<b>0.041</b>	*
	4.45 (1.14 - 17.31)	<b>0.031</b>	4.43 (1.06 - 18.57)	<b>0.042</b>	**
	5.22 (1.47 - 18.58)	<b>0.011</b>	5.67 (1.42 - 22.65)	<b>0.014</b>	***

Model<sup>a</sup>: Adjusted for age at transplantation, eGFR at post-transplantation PWV measurement, and \*smoking OR \*\*type of CNI (cyclosporine or tacrolimus) OR \*\*\*previous CV events (previous myocardial infarction or angina and previous stroke)

Model<sup>b</sup>: Adjusted for age at transplantation, eGFR at post-transplantation PWV measurement, post-transplantation PWV, and \*smoking OR \*\*type of CNI (cyclosporine or tacrolimus) OR \*\*\*previous CV events (previous myocardial infarction or angina and previous stroke)

**Table S2: Association of biomarkers with CV morbi-mortality after adjusting for graft rejection as a time-dependent variable**

Log biomarker	Model adjusted on graft rejection as a time-dependent variable	
	HR (95% CI)	p-value
<b>Death or CV events</b>		
<b>PICP</b>	2.19 (0.66 - 7.31)	0.20
<b>Gal-3</b>	10.22 (2.94 - 35.48)	<b>0.0003</b>

### 3. Discussion

In this thesis, in patients on hemodialysis, we showed that the quantification of Gal-3 and PICP concentrations in blood could be a relevant strategy to stratify hemodialysis patients at higher CV risk. We also demonstrated that the combination of machine learning approaches, complex network analysis, and proteomic data could phenotype patients on hemodialysis into distinct biological patterns with a remarkable difference in clinical outcomes and response to treatment. In KT recipients, we observed that improvement of PWV during follow-up is associated with better CV prognosis, and that an intervention to reduce arterial stiffness would be worth exploring. Furthermore, we showed that high concentration of Gal-3, measured at 2-year post-transplantation, was associated with a greater risk of CV morbi-mortality, whereas PICP was not. This suggests that cardiac remodeling is involved in the poor CV prognosis of KT patients, as it is a frequent cause of raised Gal-3 concentrations. These results reinforced the high potential of improving CV risk stratification among various groups of patients with ESRD using biomarkers of cardiac and vascular fibrosis.

#### 3.1. Do we need to optimize the management of patients with end-stage renal disease?

In 45 European countries, 690173 patients (985 per million population) received RRT by the end of 2016. France had a prevalence of ESRD of 1278 per million population, with a prevalence of patients of dialysis patients of 648 per million population. Hemodialysis remains the most common RRT modality in Europe, despite several studies showing the high CV mortality rate and poor quality of life associated with hemodialysis<sup>4,5,140</sup>. Organ shortage is the major limitation of the development of KT across the world, as well as the poor development of living donor KT in some countries. Life expectancy is 5 years in hemodialysis patients aged from 65 to 74 years (common age group for dialysis in Europe), which is 50% lower than in the same age group in patients without ESRD<sup>5</sup>. The incidence of CV death in patients on dialysis and older than 45 years old is 10-20 times higher compared to the general population and seems to increase to be more than 100 folds higher in younger patients (less than 45 years old)<sup>5</sup>.

KT recipients have higher survival rate but they continue to remain at high risk for CV death<sup>130</sup>. However, the number of patients who received KT is low; this can be explained by several factors such as the lack of kidney donors, the number of patients with life expectancy of less than

2 years in dialysis, medical contraindications (e.g. malignancy), and socioeconomic factors<sup>5,140</sup>. 26008 KT procedures were performed in 44 European countries in 2016 and Spain had the highest rate with 64 per million population. Although France was among the countries with the highest KT rates (54 per million population), the number of KT remains low compared to the number of patients on hemodialysis, because of the policy of dialysis centers to enroll older patients, the opposition to organ donation by family donors and the poor development of living donor KT<sup>140</sup>.

CV and non-CV death remain several folds higher in patients on dialysis and post KT recipients than in the general population<sup>5,116,130</sup>. This may be due to the lack of evidence-based strategies to prevent or improve CV outcomes in these patients because of the inadequate representation of patients with advanced CKD in CV outcome studies<sup>116</sup>. A relevant strategy is to stratify patients with higher CV risk using fibrosis biomarkers. One major challenge in the application of fibrosis biomarkers in the clinical setting is that fibrosis is associated with a large number of coexisting comorbidities and diseases<sup>24,141</sup>. However, recent preclinical and clinical studies have demonstrated that PICP and Gal-3 play a role in the development of myocardial fibrosis and could serve novel biomarkers for cardiac fibrosis<sup>14,15,26,141,142</sup>. Yet, these novel mechanistic markers of myocardial fibrosis should be sufficiently tested and validated in large trials that would help significantly to identify subgroups of patients who may benefit from novel or proposed therapies such as MRAs<sup>24,26,143,144</sup>. Moreover, a deeper understanding of the mechanistic pathways involved in the development of CV outcomes in the setting of ESRD is crucial in the application of personalized medicine. One possible step forward is a biomarker-based phenotyping (i.e. using a mono-biomarker or a panel of circulating biomarkers approach) which enables the stratification of patients based on distinct fibrotic profiles<sup>15,24,145</sup>.

The success of clinical application of biomarkers and personalized medicine lies in defining adequate CV endpoints for ESRD population<sup>116</sup>. Therefore, the universal and consistent use of CV endpoints is a key issue in conducting new clinical trials in ESRD. Standardization of terminology describing CV (e.g. stroke, myocardial infarction, and HF) and non-CV outcomes would be a key component in the evaluation of clinical outcomes and comparisons of clinical findings within and across research and clinical entities (i.e. reproducibility and accuracy), thereby improving the quality of patient management. This will then be a key element of biomarker assessment and the establishment of thresholds for biomarker concentrations<sup>146</sup>.

Patients with advanced CKD are commonly excluded or underrepresented in RCTs of CV diseases interventions due to the high comorbidities and altered pathophysiology of CV diseases in ESRD patients. In addition, clinicians and trialists are concerned about the potential risks and complications of CV interventions used in trials<sup>147</sup>. Therefore, dedicated CV trials are needed in to test as well as to validate more powerful treatment strategies the setting of ESRD.

One may consider to design trials including the patients at highest CV risk and/or those more likely to respond to antifibrotic therapeutics; therefore, there is a need to study cardiac and vascular markers (i.e. the assessment of PICP, Gal-3, in this PhD) in patients on hemodialysis and KT. Furthermore, the use of new approaches (i.e. the use of machine learning approaches based on proteomic data in this PhD) to understand the mechanistic pathways underlying the development and the progression of CV diseases in patients on hemodialysis should be considered further.

### **3.2. Are PICP and Gal-3 relevant biomarkers in patients with end-stage renal disease and kidney transplantation?**

In this thesis, our findings strengthen the relevance of PICP and Gal-3 in reflecting an ongoing CV fibrogenesis. Indeed, Gal-3 is a direct profibrotic agent and its high concentrations in the myocardium stimulate excessive collagen production, via the stimulation of fibroblast activity and accumulation of ECM. Current data suggest that Gal-3 is a marker of myocardial fibrosis and remodeling, as it represents a link between inflammation and fibrosis<sup>82-85,98</sup>. Furthermore, Gal-3 is involved in macrophages chemotaxis, proliferation, inflammation, and oxidative stress. These mechanisms are involved in CV diseases such as atherosclerosis and HF<sup>148</sup>.

Our analysis from the AURORA trial also showed that the association of PICP and Gal-3 with all-cause mortality was stronger in patients without history of CV or coronary diseases. Align with this aspect, Gal-3 was found to be elevated prior to CV disease<sup>84</sup>. Furthermore, it was demonstrated that inhibition of Gal-3 activity decreased the concentrations of Gal-3, total collagen, and collagen I on the CV levels. The elevation of both PICP and Gal-3 identifies patients with a higher risk of CV diseases, as both molecules are associated with cardiac inflammation and fibrosis<sup>92-95</sup>.

We showed that Gal-3 was significantly associated with the composite outcome of death or CV events whereas PICP was not in KT recipients at 2-years post-transplantation in multivariable cox models adjusted for key prognostic factors including age at transplantation, eGFR, and PWV. This suggests that high concentrations of Gal-3 may reflect ongoing inflammation associated with cardiac features and that phenotyping of KT patients according to Gal-3 concentrations may be an approach to consider in managing this high CV risk population. Indeed, PWV for the assessment of arterial stiffness (i.e. arterial functional testing) and CV fibrosis biomarkers, possibly combined, could be used to evaluate patients who might benefit from the administration of a specific treatment such as mineralocorticoid receptor antagonists (MRAs), yet to be tested in this clinical setting.

It should be mentioned that plasma PICP concentration were increased in hypertensive patients compared to patients with normal blood pressure; however, there was no correlation between PICP and PWV. This may suggest that PICP reflects cardiac features rather than arterial stiffness<sup>149</sup>. Additionally, a study in 100 hypertensive female, serum PICP concentrations were not correlated with PWV indicating that marker of collagen synthesis is likely to be linked to CV fibrosis rather than vascular rigidity<sup>150</sup>. Indeed, PWV was significantly correlated with PICP concentrations in hypertensive patients with LV hypertrophy<sup>151</sup>.

Our work shows the clinical relevance of biomarkers of myocardial fibrosis in hemodialysis and their combination with PWV in KT recipients. The integration of these biomarkers could provide a personalized profiling in patients with ESRD in the future. This approach could ultimately contribute to change in medical practices and improve CV outcomes.

### **3.3. Association of statin with morbi-mortality in biomarker-based phenotypes of hemodialysis patients. Statins shouldn't be prescribed in some patients on hemodialysis? Any other beneficial therapeutic drugs?**

#### **3.3.1. Statins**

Biomarkers have been proposed as important molecules in every phase of drug development: from drug discovery, preclinical studies and clinical trials. They are used to determine the optimal effective dose of a drug, predict clinical outcomes or serve as surrogate endpoints. Additionally, biomarkers have been shown effective in linking between diagnosis and



prognosis of a disease to outcome, as well as in predicting patient response to drug treatment for clinical decision making and personalized medicine<sup>145</sup>. Gal-3 has been proposed as a potential marker of atherothrombosis because it regulates chronic inflammation by promoting macrocalcifications in atherosclerotic plaques<sup>105,148</sup>. It has been reported that cardiac dysfunction can be induced by intrapericardial injection of exogenous Gal-3 in rats with healthy hearts. It was also been shown that LV myocardium of Gal-3 injected rats had a three-fold increase in type I/III collagen ratio. The distinct abundance of collagen type I (i.e. which is embedded by promoter sites) underlines the fact that Gal-3 induces cardiac fibroblast proliferation and collagen I production, which are relevant in myocardial fibrosis<sup>152</sup>.

Atherosclerosis is the most common underlying cause of CV outcomes. The pathogenic inflammation in the vessel wall and the accumulation of immune cells such T-cells lead to the initiation and progression of atherosclerosis<sup>153</sup>. The benefits of reducing the concentration of LDL-cholesterol and immune-mediated inflammatory effects, and thus improving CV prognosis in patients on chronic hemodialysis by the use of statins could not be demonstrated by outcome trials so far<sup>154</sup>.

Acknowledging the former, we assessed the association between rosuvastatin prescription at baseline and outcomes across the biological phenotypes identified in patients on hemodialysis (i.e. from our 2<sup>nd</sup> paper).

Importantly, there was no significant effect of rosuvastatin on the composite primary endpoint of MACE in the AURORA trial. Rosuvastatin also exhibited no significant effects on the secondary endpoint of all-cause mortality<sup>54</sup>. In this thesis, we found that the statin use was associated with a higher risk of CV death and all-cause mortality in the subgroup of patients with the phenotype ‘multiple pathways related to inflammation and fibrosis’ (findings from our 2<sup>nd</sup> paper). Although the benefit of statin in reducing CV risk is well established outside the field of dialysis, a possible explanation of the lack of association between statin use and better CV outcome/prognosis in patients on hemodialysis is that the pathophysiology of CV disease in these patients is more complex and that the pattern of CV disease in this population reflects severe LV hypertrophy associated with myocardial fibrosis, therefore hemodialysis patients could not benefit from statins<sup>48</sup>. Indeed, arteriosclerosis is the first CV changes observed in CKD patients and represents a hallmark of arterial remodeling in ESRD patients, whereas atherosclerosis partially

contributes to the increased CV burden and its prevalence increases with age and is aggravated, but not specifically induced, by CKD<sup>9,155</sup>. This demonstrates that atherosclerosis is only of secondary importance for CV morbidity and mortality in ESRD and that cholesterol lowering therapy needs to be started before patients with CKD develop ESRD<sup>155</sup>. Consistent with this, the 4D study, which included patients with type 2 diabetes mellitus on hemodialysis with a mean age of 65.7 years, reported a higher risk for fatal stroke in the statin than in the placebo group<sup>42</sup>. This may explain our finding of the ‘multiple pathways related to inflammation and fibrosis’ phenotype, as patients from this phenotype had poorer outcome when treated with statin.

The lack of efficacy of statins in patients on hemodialysis does not mean that these drugs cannot serve as powerful treatment for CV outcomes in selected populations, different from that included so far. In contrast, this opens the door to individualized treatment which, in turn, characterizes the optimal treatment for the right patient at the right time of the disease progression. In this context, phenotyping of patients with fibrosis biomarkers or a panel of biomarkers that can stratify patients in subgroups for better treatment and clinical outcomes<sup>116,145</sup>.

### 3.3.2. Mineralocorticoid receptor antagonists

The excess activation of mineralocorticoid receptor (MR) by its mediator, aldosterone, in non-classical tissues such as the endothelium, inflammatory cells, and fibroblasts accelerate the poor prognosis of CV events in CKD patients<sup>137</sup>. Indeed, several preclinical and clinical studies have shown that MR activation is involved in several CV diseases such as hypertension, HF, and MI<sup>156</sup>. MR activation induces oxidative stress in both classic and non-classic tissues such as endothelial, VSMCs, cardiomyocytes and macrophages. Moreover, inappropriate MR activation is associated with poor cardiac and vascular outcomes and infiltration of the arterial wall, as MR activation may interfere with inflammatory mechanisms. This may suggest that MRAs can prevent the inflammation preceding the development of fibrosis. In fact, MR blockage has proven to reduce fibrosis in HF<sup>157</sup>.

MRAs administration is a promising treatment worth exploring in the management of CV morbidity in the ESRD population, due to its beneficial impact on CV remodeling, including collagen turnover and beneficial effects on oxidative stress, endothelial function, and immune response<sup>30,158</sup>. A reduction in the concentrations of serum PICP with spironolactone was associated with improved diastolic dysfunction in patients at high risk of developing HF<sup>159</sup>. HF patients

randomized to MRA have been shown to have persisted reduction in PICP concentrations, systolic blood pressure, left atrial volume, and N-terminal pro b-type natriuretic peptide (NT-proBNP) levels<sup>143,144</sup>. These results suggest the benefits of MRAs in reducing or reversing the accumulation of type I collagen synthesis, which may be useful in limiting or reversing myocardial fibrosis<sup>143,160</sup>. Similarly, in patients with HF, MRA reduced serum concentrations of PICP as a direct index of myocardial collagen deposition, but did not modify the serum concentrations of C1P:MMP-1 (collagen type-1 C-terminal telopeptide : matrix metalloproteinase-1), an inverse of myocardial collagen cross-linking<sup>161</sup>. Furthermore, a direct association was found between PICP and NT-proBNP and atrial size at baseline<sup>161</sup>. These results are relevant, as Diez and his group have shown a correlation between PICP concentrations and total myocardial collagen volume fraction using myocardial samples with collagen-specific staining in patients with hypertensive and HF<sup>70,71</sup>. Altogether, it can be speculated that MRAs could affect myocardial collagen content but are not able to modify the characteristics of the collagen fiber that affect its stability (i.e. its insolubility, stiffness and resistance to degradation)<sup>15,161</sup>. Importantly, a previous study showed that myocardial collagen cross-linking is associated with increased LV filling pressures in HF patients<sup>162</sup>.

In patients with MI, MRA treatment (i.e. eplerenone) predicted a decrease in the concentration of amino-terminal propeptide of type III procollagen (PIIINP) compared to placebo, suggesting biomarkers could help identify patients who may benefit from antifibrotic treatment (e.g. MRAs). Indeed, PICP and PIIINP reflect collagen synthesis and their increased accumulation of collagen in the cardiac interstitium is the hallmark of myocardial fibrosis<sup>163</sup>.

PICP may also have a direct pathogenic role in the fibrogenic process, as it plays a key role in the assembly of collagen type I molecules into collagen type I fibrils<sup>62</sup>. Therefore, the reduction of PICP by MRAs may have a double anti-fibrotic beneficial interpretation: as they reduce the availability of mature collagen type I molecules and reduce the assembly of these molecules to form collagen type I fibrils.

In all, the encouraging findings regarding the beneficial effects of MRAs in HF and their safety with respect to hyperkalemia in patients on hemodialysis<sup>164-166</sup> have encouraged the conduct of RCTs to provide definitive answers to the role of MRAs in patients on hemodialysis as an effective therapy in this high CV risk population. The anti-fibrotic drug class prototype of the MRAs is currently being tested in two multicenter randomized study CV prevention studies in

hemodialysis ACHIEVE (aldosterone blockade for health improvement evaluation in end-stage renal disease trial; NCT03020303, ongoing) and the recently completed ALCHEMIST (aldosterone antagonist chronic hemodialysis interventional survival trial; NCT01848639) led by our team.

We have previously discussed that CNI are likely to play an important role in the persistence increased arterial stiffness in KT recipients with restored renal function<sup>19</sup>. We may hypothesize that finding an intervention able to reduce PWV could improve the prognosis of KT recipients (findings from our 3<sup>rd</sup> paper). We also reported that high Gal-3 concentrations were associated with a greater risk of CV morbi-mortality in KT recipients (findings from our 4<sup>th</sup> paper). Gal-3 was unrelated to PWV, suggesting that cardiac remodeling is involved (as it is a frequent cause of raised Gal-3 concentrations).

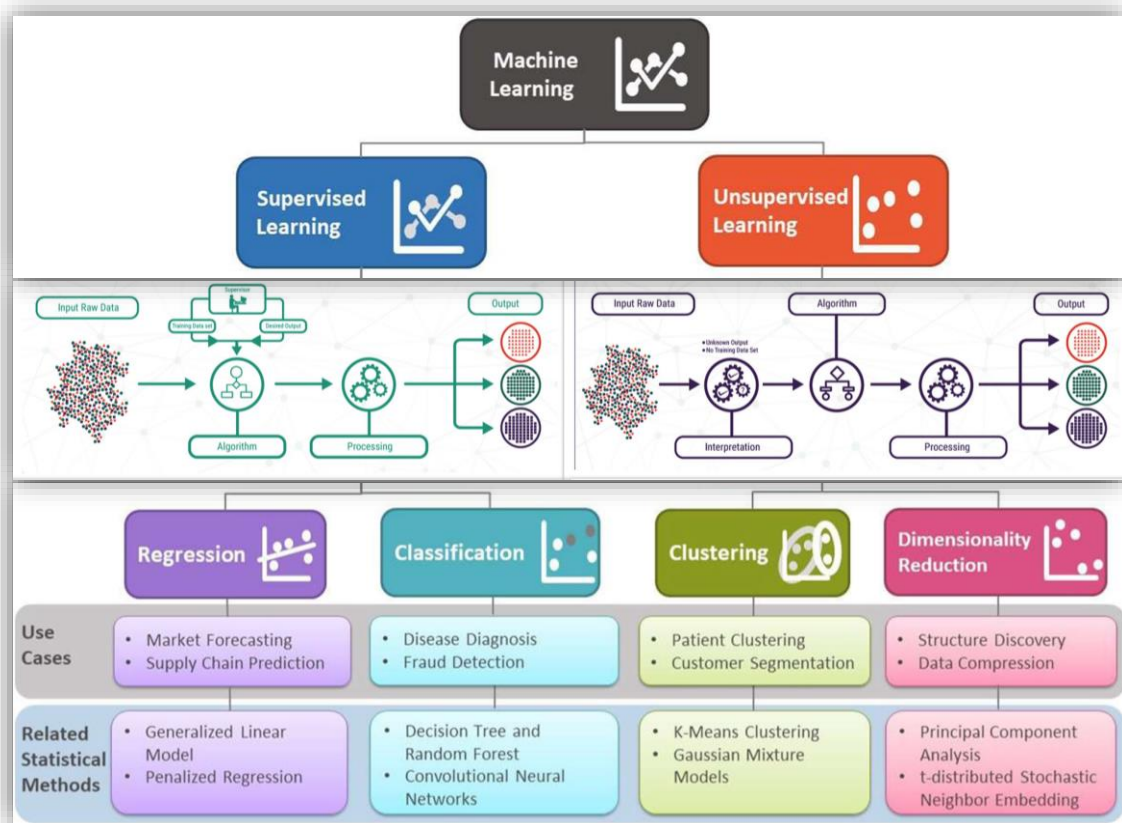
The role of MR activation in arterial stiffness is also well documented in non-transplanted patients<sup>167</sup>. Spironolactone administration was shown to reduce arterial stiffness in CKD patients with controlled blood pressure<sup>168</sup>. Importantly, MRAs administration have shown to have a good safety profile in KT recipients<sup>137</sup>. MRAs are currently tested by our team in EVATRAN (effet de l'Eplérénone sur l'évolution de la VAsculopathie du patient TRANsplanté rénal; NCT04450953) study at Nancy Hospital. This pilot study is the first randomized clinical trial that aims to investigate the impact of 6 months of MRA administration, eplerenone, on the evolution of arterial stiffness one year after transplantation in KT patients on CNI treatment. Echocardiographic examinations will be performed for the evaluation of cardiac function as a secondary objective of the study. Biobank samples for the measurement of biomarkers, including P1NP and Gal-3, will be also available in the EVATRAN study.

### **3.4. Statistical and machine learning approaches**

Statistical and machine learning methods have shown a pivotal role in the management of real-world data in clinical research. The application of machine learning techniques has a number of advantages over conventional statistical methods. Unlike classical statistical methods, machine learning can accommodate multicollinearity data in multivariable modeling. Additionally, statistical methods have limited statistical power in interaction tests, whereas machine learning techniques are powerful in this setting. Furthermore, machine learning allows the identification of

phenotypes or patterns, unlike statistical methods that can only evaluate associations with predefined outcome<sup>169,170</sup>.

There are two main strategies for a machine to learn (Figure 6) : (1) supervised learning, in which variables are selected and processed to find the best combination to fit a particular outcome of interest (i.e. successfully applied in prediction, diagnosis, and treatment); (2) unsupervised learning, in which machine tries to discover the relationship between variables to identify novel disease mechanisms or phenotypes by finding consistent patterns in the data<sup>170,171</sup>.



**Figure 7: Supervised learning versus unsupervised learning. (Adapted from Kolluri et al.<sup>172</sup> and Singh Pathania et al.<sup>173</sup>)**

There is a need for the integration of data from in silico (computer biomedical) and findings from clinical research to identify new profibrotic pathways and mechanisms which could be translated into personalized diagnostic, prognostic and therapeutic tools<sup>26</sup>. There has been tremendous interest in characterizing omics and genes related to the underlying mechanistic pathways in the development and the progression of CV diseases. However, the use of machine learning approaches to characterize ESRD patients undergoing dialysis has not yet been explored.

However, machine learning approaches were applied to phenotype HF patients and KT recipients<sup>118,119</sup>.

A promising use of unsupervised methods is precision phenotyping of CV diseases<sup>169</sup>. Applying clustering methods to clinical data such as the unsupervised Latent Class Model (LCM) using VarSelLCM package has shown encouraging results in identifying distinct clinical profiles across clusters, providing the best prognostic performance, and characterization of patient subgroups with substantial therapeutic benefit<sup>174,175</sup>. This algorithm is a probabilistic data-driven model<sup>171</sup>. Moreover, this clustering algorithm is considered one of the most appropriate machine learning methods for mixed data (i.e. continuous and categorical variables), as it has important features such as handling missing data, covariates selection (i.e. determine whether a variable is relevant for clustering) as well as the optimal number of clusters<sup>174</sup>. However, LCM can take a lengthy time to converge because it is computationally intense<sup>171,174</sup>.

Hemodialysis patients need more effective clinical management and personalized therapies. Physicians have long needed to improve the interpretation of the sophisticated data to improve patient care and reduce CV outcomes and mortality in this setting. Given the advances in the use of machine learning in the field of cardiology<sup>119-121</sup>, we propose the use of machine learning rather than classical statistical methods to overcome the high dimensional and possible highly correlated variables in real-world data of patients with ESRD. Recently, Tromp et al.<sup>119</sup> performed cluster analysis using a large panel of biomarkers to identify distinct clinical profiles and treatment response in patients with HF. In KT, Aubert et al.<sup>118</sup> identified distinct groups of patients associated with different allograft outcomes on the basis of post-transplantation biopsies by using a learning approach.

In this thesis, we applied unsupervised LCM technique on the basis of a large panel of biomarkers to identify groups of hemodialysis patients from the AURORA trial. Then, we built a decision tree to identify a threshold of the most relevant biomarkers. Finally, the decision tree was used to predict clusters in a second set of patients from the same trial. We identified four biological phenotypes with different outcomes and response to treatment, related to ‘cytokine storm signaling’, ‘TLRs signaling’, ‘multiple pathways related to inflammation and fibrosis’, and a ‘reference phenotype’ with the least biological abnormalities.

Patients with the phenotype labelled ‘multiple pathways related to inflammation and fibrosis’ responded differently to statin treatment; they had worse outcomes (i.e. we have previously discussed this in detail in the subsection 3.3.1). Overall, further research is needed to investigate the potential antifibrotic role of statin in CV diseases<sup>176</sup>. In addition, there is a need to study the different mechanisms underlying CV diseases in hemodialysis, as the characteristics of CV diseases in this population may differ from that found in the general population. This will clarify the potential effect of specific therapies such as statins<sup>48</sup>.

In support of our findings in this project, the use of machine learning techniques based on biomarkers profiles may allow the identification of new subgroups of patients on hemodialysis based on differences of underlying mechanistic pathways. Phenotyping patients on hemodialysis can be used to optimize CV risk stratification and provide targeted therapy based on the identified phenotypes.

### **3.5. Limitations**

In the AURORA trial, concentrations of the circulating biomarkers were only available at baseline. Therefore, we were not able to assess the association of PICP and Gal-3 patterns/changes with outcomes and response to statin longitudinally over extended periods of time. We also investigated a limited number of biomarkers of CV fibrosis and did not find an interaction between the use of statin and the outcomes in this neutral trial. Echocardiographic data were not collected during the AURORA trial. These data would have been useful to evaluate the correlation of biomarkers with cardiac physiology and CV outcomes. In addition, the sample size was relatively small for a proteomic approach in the second paper.

The TRANSARTE study, despite being the first study, to our knowledge, to assess the association of biomarkers of CV fibrosis with vascular modifications and outcomes in KT patients, is of single-center nature. This study is of a small size and consequently has limited statistical power. Furthermore, there was a time difference between blood sampling for biomarkers analysis and measurement of PWV, which limited the number of patients included in the analysis.

### 3.6. Strengths

It is now recognized that ECM remodeling leading to myocardial interstitial fibrosis is a final common mediator following cardiac insult or extracardiac comorbidities such as hypertension, diabetes mellitus, and CKD<sup>12,26,28</sup>. Fibrosis affects cardiac functionality in different ways: by deteriorating the filling and pumping capacity of the heart, or by inducing cardiac remodeling, thus contributing to the worsening of HF and sudden death<sup>108</sup>. The fact that fibrosis, probably driven by cardiac remodeling, is of interest following hemodialysis and KT was, in our opinion, worthy of investigation in the course of this PhD.

The AURORA trial, which was extensively used during in this PhD, has a large patient size, blindly randomized, and all the recorded events were reviewed and adjudicated by a clinical endpoint committee to ensure consistency of the event diagnosis. In addition, we were able to assess the combined and synergistic association of P1CP and Gal-3, as a non-invasive assessment of fibrosis, with CV death and all-cause mortality in patients on hemodialysis. However, to date, no large study has evaluated the interplay of collagen biomarkers and Gal-3 in the setting of hemodialysis. The identification of patients with active fibrogenesis could be a promising approach in precision-medicine for personalized care and we believe this paper is a first step in a better understanding of fibrosis in hemodialysis.

Furthermore, we had 274 protein biomarkers available from the Olink<sup>®</sup> Cardiovascular II, Cardiovascular III, and Inflammation panels in the AURORA trial. This allowed us to determine biological phenotypes based on proteomic data using machine learning approaches; this means that this strategy characterizes different pathways in patients on hemodialysis who could benefit from an individualized treatment. Our work strengthens the importance of biomarkers as a promising tool in the identification of patients at higher CV risk as they probably have the highest probability to benefit from a personalized therapy.

We used TRANSARTE study to better understand the links between fibrosis, vascular modifications and outcome following KT. As we had PWV measurements available, a non-invasive method for the evaluation of vascular stiffness, we could evaluate the association of biomarkers of CV fibrosis with arterial stiffness which is considered a validated surrogate marker of CV morbi-mortality, as well as CV outcomes. We showed that the identification of patients with



active fibrogenesis could be a promising approach in precision-medicine for personalized care and we believe this paper is a first step in a better understanding of fibrosis in KT.

## 4. Perspectives

The CV outcomes for patients with ESRD are poor and the clinical management of CV complications in the setting of ESRD is an ongoing challenge<sup>9</sup>. In particular, the distinct CV pathophysiology and uremic cardiomyopathy and arteriosclerosis as well as the altered pharmacokinetics of drugs in ESRD add another element of complexity with regard to the management of patients<sup>9,139</sup>. Therefore, the assessment of the circulating biomarkers of myocardial fibrosis to identify ESRD patients at higher CV risk might be clinically relevant. Also, phenotyping ESRD patients could be useful in tracking the evolution of myocardial fibrosis which may allow individualized management (e.g. anti-fibrotic therapy) of the patients and the improvement CV outcomes<sup>28</sup>.

PD, a home-based RRT, has become an alternative to hemodialysis in the management of patients with ESRD<sup>177</sup>. However, the access to PD is limited, especially in some central and eastern European countries. This may be due to the high cumulative cost of PD solutions and the large availability and widespread of hemodialysis centers<sup>140</sup>. At the end of 2016, Cyprus (114 per million population), followed by Denmark (97 per million population), and Sweden (90 per million population) had the highest prevalence of PD. It was 45 per million population in France<sup>140</sup>.

It is well established that residual GFR, known as residual renal function (RRF), is better preserved in patients treated with PD than in those treated with hemodialysis. RRF is associated with a reduction in blood pressure, LV hypertrophy, inflammatory markers, and an improved lipid profile<sup>177</sup>. Importantly, several studies have found that RRF is associated with improved patient survival. Indeed, two landmark studies in PD have shown that RRF is an independent marker of survival<sup>178,179</sup>. Bargman et al.<sup>178</sup> demonstrated that RRF and fluid removal were significantly associated with better survival in 601 PD patients from the multicenter prospective CANUSA study. The authors showed that there was a 12% reduction in the risk of death for each 5 L per week per 1.73 m<sup>2</sup> (i.e. a residual GFR of 1 mL/min is equivalent to a weekly peritoneal clearance of about 10 L) increment in eGFR (estimated from mean of renal urea and creatinine clearance)<sup>178</sup>. In the prospective, randomized, controlled ADEMEX (ADEquacy of PD in MEXico) trial, RRF

was an independent predictor of survival in ESRD patients on PD, even after adjustment for factors known to be associated with survival in PD patients, such as age, diabetes mellitus and serum albumin concentrations<sup>179</sup>. Despite the benefits of PD, patients on PD still have poor outcomes and CV events remain the leading cause of mortality.

The cumulative and progressive inflammation of the peritoneal membrane, peritonitis, has been well documented. The acidic pH (5–5.8), high osmolality, and glucose degradation products of conventional PD solutions contribute to local and systemic inflammatory responses leading to peritoneal injury, resulting in peritoneal fibrosis, endothelial damage, atherosclerosis, and eventually CV dysfunction<sup>180</sup>.

**Progressive peritonitis and RRF may represent challenges for the assessment of biomarkers of myocardial fibrosis in PD patients, as the concentrations of biomarkers might be altered.** Furthermore, to our knowledge, no studies have been conducted to assess the clinical relevance of these biomarkers and their association with outcomes in PD patients. It would therefore be insightful to investigate the association between Gal-3 and PICP and clinical outcomes in PD patients. The prospective multinational GLOBAL Fluid Study, available from the European consortium of IMPROVE-PD, which funded this PhD, involving 959 PD patients from 10 centers in Korea, Canada, and the United Kingdom, could be an asset in understanding the implication of biomarkers of myocardial fibrosis in PD<sup>181</sup>. We were recently granted access to this database and biobank.

## 5. Conclusion

We concluded that biomarkers of myocardial fibrosis, PICP and Gal-3 have a significant clinical relevance in ESRD, and as such they may represent a key strategy for patient risk stratification that, in turn, could reduce CV mortality in patients on hemodialysis and KT recipients. We also showed that the identification of biomarker-based biological phenotypes using machine learning approaches, which exhibited significant differences in circulating biomarkers, risk of CV outcomes, and response to treatment, provides new insights into our understanding of mechanistic pathways in patients on hemodialysis. Our findings may have significant clinical implications in the design of trials of CV disease prevention in ESRD patients. Furthermore, we suggested that the worsening of arterial stiffness in the late post-KT period has a negative impact

on long-term CV prognosis and that finding an intervention that can reduce PWV may improve the prognosis of KT patients. Sequential assessments of the applicability of these markers (i.e. biomarkers and PWV) could help clinicians manage individuals at high risk of CV diseases by providing individualized treatment strategies.

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## Résumé (Version anglaise)

The management of end stage renal disease (ESRD) requires renal replacement therapy (RRT), including dialysis (hemodialysis or peritoneal dialysis) or kidney transplantation (KT). However, despite improvements in the last decades in access to RRT, dialysis technology, and patient survival in dialysis and after KT, the mortality rate in patients with ESRD remains significantly higher than the general population. Cardiovascular (CV) mortality is 10 to 20 times higher in patients on hemodialysis than in the general population and is the most common cause of death. In addition, CV diseases are the leading cause of mortality in KT recipients. Underlining the fact that fibrosis is the major mediator of pathophysiology in cardiorenal syndromes, the identification of subgroups of patients with active fibrogenesis who may benefit from antifibrotic treatments could be a promising strategy for better clinical management and improved prognosis in patients with ESRD.

In this PhD, we aimed to investigate the relevance of biomarkers of myocardial fibrosis and possibly arterial stiffness, namely procollagen type I C-terminal propeptide (PICP) and galectin-3 (Gal-3). We evaluated whether these markers could be valuable to improve CV risk stratification among various groups of patients with ESRD, including patients on hemodialysis and KT recipients. We also determined biological phenotypes of hemodialysis patients based on biomarkers available from Olink® using machine learning approaches. In addition, we studied the association of PICP and Gal-3 with arterial stiffness, measured by pulse wave velocity (PWV), and CV morbidity and mortality in patients at 2-year post-transplantation. PWV is an important and independent factor of CV morbidity and mortality in ESRD.

We showed that the quantification of Gal-3 and PICP concentrations in blood could be a relevant strategy to stratify hemodialysis patients at higher CV risk. We also demonstrated that the combination of machine learning approaches and proteomic data could phenotype patients on hemodialysis into distinct biological patterns with a remarkable difference in clinical outcomes and response to treatment. In KT recipients, we observed that the improvement of PWV during follow-up is associated with a better CV prognosis, and that an intervention to reduce arterial stiffness would be worth exploring. Furthermore, we showed that high concentration of Gal-3, measured at 2-year post-transplantation, was associated with a greater risk of CV morbi-mortality, whereas PICP was not. This suggests that cardiac remodeling is involved in the poor CV prognosis of KT patients, as it is a frequent cause of raised Gal-3 concentrations.

These results reinforced the high potential of improving CV risk stratification among various groups of patients with ESRD, using biomarkers of cardiac and vascular fibrosis.

**Keywords:** end stage renal disease, cardiovascular diseases, fibrosis, collagen, galectin-3, biomarkers.

## Résumé (Version française)

L'insuffisance rénale terminale (IRT) nécessite un traitement de suppléance rénale, qui peut être la dialyse (hémodialyse ou dialyse péritonéale) ou la transplantation rénale. Malgré l'amélioration des techniques de dialyse durant les dernières décennies et l'augmentation de la survie des patients, que ce soit en dialyse ou en greffe, le taux de mortalité reste significativement plus élevé pour les patients en IRT par rapport à la population générale. La mortalité cardiovasculaire (CV) est 10 à 20 fois plus élevée chez les patients traités par hémodialyse que dans la population générale, et représente la principale cause de décès chez ces patients. En outre, les pathologies CV sont la première cause de mortalité des patients transplantés rénaux. La fibrose étant le médiateur majeur de la physiopathologie des syndromes cardio-rénaux, l'identification de sous-groupes de patients présentant une fibrogénèse active et pouvant bénéficier de molécules antifibrotiques pourrait être une stratégie efficace pour permettre une meilleure prise en charge clinique et améliorer le pronostic CV des patients atteints d'IRT.

Au cours de ce travail de thèse, notre objectif principal était d'étudier certains marqueurs de fibrose myocardique et vasculaire, c'est-à-dire le procollagène type I C-terminal propeptide (PICP) et galectine-3 (Gal-3), afin d'évaluer si ces marqueurs sont pertinents pour améliorer la stratification du risque CV parmi divers groupes de patients atteints d'IRT, à savoir les patients hémodialisés et les patients greffés. Il s'agissait également de déterminer les phénotypes biologiques des patients hémodialisés sur la base de biomarqueurs multiples grâce à la technologie Olink®, en utilisant des outils méthodologiques de type intelligence artificielle. En outre, nous avons étudié l'association du PICP et de la Gal-3 avec la rigidité artérielle mesurée par la vitesse de l'onde de pouls (VOP) et la morbi-mortalité CV chez les patients greffés à 2 ans de greffe. La VOP est un facteur important et indépendant de morbidité et de mortalité CV en insuffisance rénale terminale.

Nous avons montré que les marqueurs de la fibrose myocardique et vasculaire, PICP et Gal-3, pourraient apporter une meilleure caractérisation du profil CV des patients hémodialisés. Nous avons également montré que l'identification de phénotypes biologiques basés sur des biomarqueurs et l'implémentation d'outils de type intelligence artificielle pourraient aider les cliniciens à identifier les patients à haut risque CV. Les phénotypes biologiques identifiés ont une pertinence clinique car ils étaient associés aux événements cliniques et à la réponse au traitement. Nous avons par ailleurs montré que l'amélioration de la rigidité artérielle à long terme chez les patients greffés était associée à un meilleur pronostic CV. Ceci suggère qu'il serait possible d'améliorer le pronostic des patients greffés en identifiant des stratégies thérapeutiques à même de réduire la rigidité. Enfin, nous avons montré que des concentrations accrues de Gal-3, et non celles de PICP, étaient associées à la morbi-mortalité CV chez les patients greffés à 2 ans de greffe. La fibrose myocardique, médiée par la voie Gal-3 pourrait être majoritairement impliquée dans la morbi-mortalité des patients greffés rénaux.

Les différentes études constituant ce travail de thèse soutiennent notre hypothèse de travail selon laquelle la prise en compte des marqueurs de la fibrose myocardique et vasculaire pourrait apporter une meilleure caractérisation du profil CV individuel des patients en insuffisance rénale terminale.

**Keywords:** insuffisance rénale terminale, maladies cardiovasculaires, fibrose, collagène, galectine-3, biomarqueurs.