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LES TOXINES UREMIQUES PROVOQUENT UN PHENOTYPE PROCOAGULANT DE L'ENDOTHELIUM PAR LA VOIE DU FACTEUR DE TRANSCRIPTION AHR.

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INTRODUCTION

L'insuffisance rénale chronique (IRC) conduit à l'intoxication progressive de l'organisme par l'accumulation dans le sang et les tissus de substances appelés solutés urémiques ou encore toxines urémiques lorsqu'un effet toxique a été démontré[1]. Les patients avec une IRC présentent une mortalité cardiovasculaire accrue par rapport à la population générale, avec une prévalence pouvant être 100 fois plus importante en fonction de l'âge [2,3]. Ces patients présentent une athérosclérose accélérée, des calcifications vasculaires et un remodelage cardiaque [4]. Chaque année, 10 à 20 % des patients en dialyse décéderont, dans 45 % des cas de complications cardiovasculaires. Cette mortalité cardiovasculaire élevée n'est pas seulement due à la présence de facteurs de risque classique (diabète, hypertension, dyslipidémie…). Il existe chez l'IRC des facteurs de risques non classiques comme l'inflammation, le stress oxydant et le dysfonctionnement endothélial [5]

L'endothélium est un constituant essentiel des vaisseaux et son rôle n'est plus à démontrer dans le maintien de l'homéostasie vasculaire [6]. Il occupe une place importante à l'interface entre compartiments sanguin et tissulaire. Il exerce de nombreuses fonctions, telles que la régulation du tonus vasomoteur, la réponse inflammatoire, l'angiogenèse et l'hémostase [7]. Le dysfonctionnement endothélial apparaît précocement au cours de l'IRC. Il participe à l'installation et à la progression des maladies cardiovasculaires. Les mécanismes à l'origine de ce dysfonctionnement sont mal connus [8].

Notre équipe s'intéresse depuis plusieurs années au rôle des toxines urémiques dans le dysfonctionnement vasculaire. Les toxines urémiques sont présentes à une concentration qui peut être supérieure à cent fois la concentration retrouvée dans la population normo rénale [9]. Les toxines urémiques sont classées en trois groupes [1]: les solutés hydrosolubles de petit poids

moléculaire (< 500 Da), les moyennes molécules, et les solutés liés aux protéines. Les solutés de petit poids moléculaire tels que l'urée sont bien épurés par la dialyse. Les moyennes molécules et les solutés liés aux protéines sont plus difficiles à éliminer. Ainsi, lors d'une séance d'hémodialyse conventionnelle, l'épuration des toxines liées aux protéines est de l'ordre de 30%, tandis qu'elle est de 80% pour l'urée[1].

Ce travail s'inscrit dans le cadre d'un réseau européen de recherche au sein duquel nous sommes référents pour l'endothélium (European Uremic Toxins Work Group, http://uremic-toxins.org). Nous avons trois objectifs : le premier vise à identifier les toxines urémiques délétères pour l'endothélium in vitro; le deuxième vise à déterminer les mécanismes d'action de ces toxines ; le troisième vise à mettre en évidence chez les patients IRC les relations entre les taux de toxines urémiques, le dysfonctionnement endothélial et la mortalité cardiovasculaire. Parmi plus d'une centaine de toxines urémiques déjà caractérisées, celles dérivées des indoles : indoxyl sulfate (IS) et indole acetic acide (IAA) se sont révélées comme étant les plus délétères pour l'endothélium. Ces toxines font partie de la catégorie des «toxines liées aux protéines » et sont produites par le métabolisme intestinal du tryptophane. Ces deux toxines ont été largement étudiées dans le laboratoire. In vitro, l'indoxyl sulfate (IS) inhibe la prolifération et la réparation endothéliale [10], induit un stress oxydant par l'activation de la NAD(P)H oxydase et la diminution des taux de glutathion [11]. Enfin, elle augmente la production endothéliale de microparticules (MP), marqueurs reflétant des processus d'activation ou d'apoptose [12]. Chez les patients, les taux sériques d'IS sont corrélés à la mortalité cardiovasculaire.

L'IAA est aussi délétère pour l'endothelium puisque il a un effet néfaste sur les progéniteurs endothéliaux [9].

Les phénomènes thrombotiques sont au cœur des complications cardiovasculaires aiguës de la pathologie athéromateuse notamment pour les syndromes coronariens aigus [13]. Il

est désormais reconnu que l'apparition d'un thrombus secondairement à la rupture d'une plaque athéromateuse sera responsable de manifestations cardiovasculaires aigues. Dans la problématique de surmortalité cardiovasculaire et d'athérosclérose accélérée observée chez les patients IRC, le facteur tissulaire est une molécule très intéressante. Le facteur tissulaire (FT) est une glycoprotéine transmembranaire de 33 kD, après glycosylation son PM est de 43 kD. Il est codé par le gène *F3* d'une taille de 12,4 kb avec 6 exons, localisé sur le chromosome 1p21-p22. La protéine est constituée de trois domaines : un domaine extracellulaire (219 acides amines) qui fixe le facteur VII (FVII) de façon très affine, un domaine transmembranaire (21 acides amines) qui ancre le FT dans la membrane cellulaire et une queue cytoplasmique (23 acides amines) vraisemblablement impliquée dans la transduction de signaux intracellulaires. II est le récepteur et le cofacteur du facteur VIIa. Cette interaction augmente d'environ 2.10^7 fois l'activité enzymatique du FVlla vis à-vis de ses substrats, les facteurs IX et X de la coagulation. Selon le schéma classique, le FT est exprimé de façon constitutive par différents types cellulaires de localisation vasculaire, comme les fibroblastes de l'adventice, et extravasculaire, notamment les cellules épithéliales. La localisation du FT forme une enveloppe hémostatique prête à activer la cascade de la coagulation en cas de brèche vasculaire. Dans ce schéma, les cellules directement en contact avec le sang circulant n'expriment pas de FT sauf dans certaines situations pathologiques (inflammation, sepsis, cancer). Dans ces conditions, la formation du complexe FT-FVIIa déclenche l'activation des cascades de la coagulation, et entraine in fine la génération de thrombine et la formation de fibrine (fig 1). Agissant de concert avec l'activation plaquettaire, cette voie d'activation est la première ligne de défense de l'organisme contre le saignement en cas de blessure vasculaire. Elle est aussi responsable des thromboses en cas d'expression intravasculaire du FT par les cellules malignes et/ou les monocytes et cellules endotheliales activées par les cytokines pro inflammatoires [14].

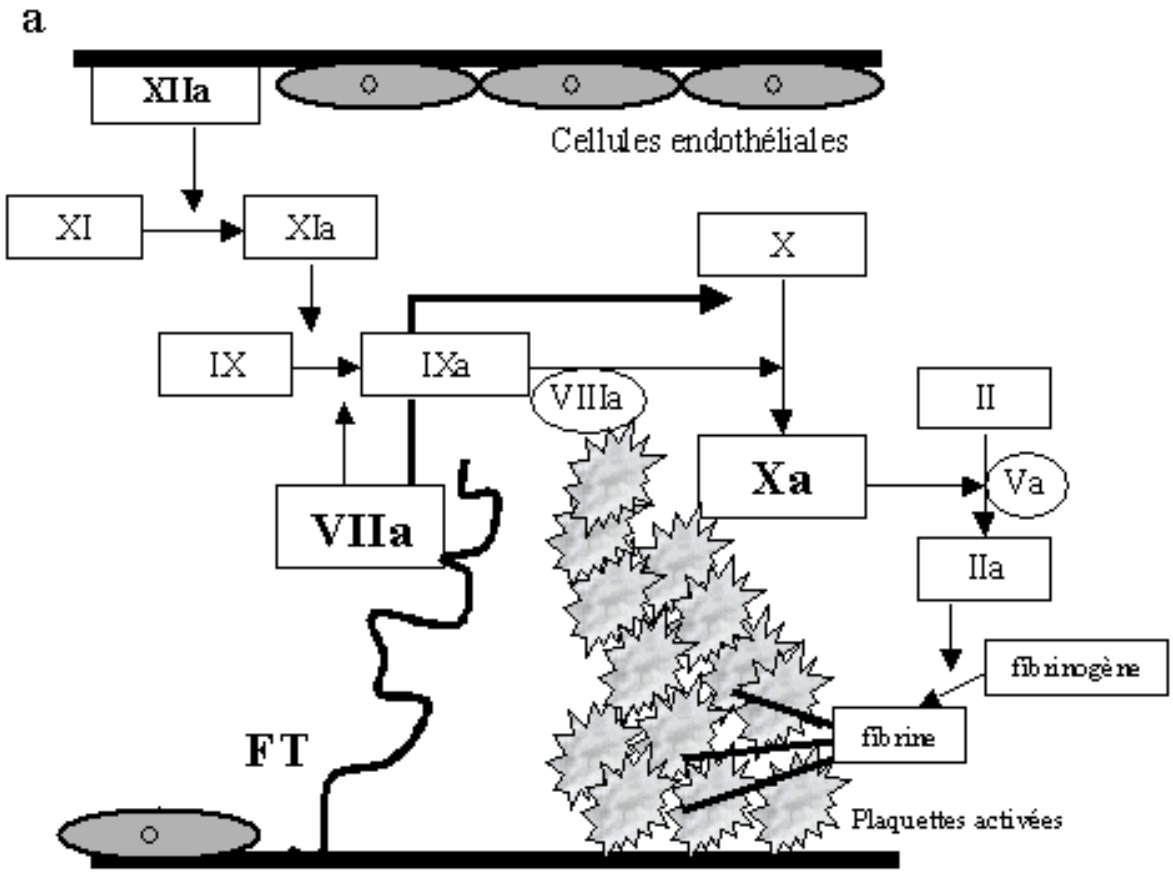

Fig 1 : Cascade de la coagulation initiée par le facteur tissulaire (FT)

Outre le fait d'être un acteur clé de la coagulation, le FT est au carrefour de deux phénomènes pathologiques majeurs retrouvés chez les patients IRC : l'inflammation et la thrombose [15]. Il est de plus un marqueur d'agression endothéliale [15]. En effet, une synthèse inappropriée de FT est corrélée à une augmentation de la morbi-mortalité par événements cardiovasculaire. Des taux élevés de FT soluble sont prédictifs d'un risque élevé de mortalité chez les patients présentant des syndromes coronariens aigus [16]. De plus, les taux de FT dans les lésions d'athérosclérose sont corrélés à la dangerosité de la lésion [17]. L'augmentation des taux de FT traduit un état d'hypercoagulabilité pouvant expliquer la survenue des complications athérothrombotiques chez des patients athéromateux ne présentant pas de rupture de plaque

[14]. Le FT est aussi porté par les microparticules dans la circulation. Chez les patients IRC, les taux plasmatiques de FT sont fortement augmentés mais peu d'études sont disponibles sur les mécanismes à l'origine de cette augmentation[18]. Des taux élevés de FT sont associés avec une maladie cardiovasculaire chez ces patients[19].

Nous proposons comme lien entre les solutés indoliques et la production de FT la voie de l'aryl hydrocarbon receptor (AHR). Le récepteur Ah ou AHR a été découvert dans les années 1990. La protéine de 96 kD (848 AA) est codée par le gène *AHR* de 47,5 kb constitué de 11 exons et localisé sur le chromosome 7p21.1. L'existence d'un locus Ah a été décrite à la fin des années 1970 et au début des années 1980 notamment par l'équipe de D.W. Nebert qui évoquait alors l'existence d'un ensemble de gènes contrôlant l'induction d'enzymes à activité « aryl hydroxylase »[20]. Le ligand canonique d'AHR est la dioxine et la première fonction décrite de ce récepteur est de participer à des mécanismes de détoxification lors d'exposition aux xénobiotiques [21]. La fonction de récepteurs aux xénobiotiques a été acquise au cours de l'évolution car les récepteurs des invertébrés étudiés jusqu'à présent (AHR-1 chez les nématodes, Spineless chez la drosophile) ne lient pas de xénobiotiques [22]. AHR est présenté généralement comme formant un complexe cytoplasmique avec des molécules chaperonnes et des co-chaperonnes (Heat Shock Proteines 70 et 90 ; XAP2 ou X associated Protein 2) [23]. Quand un organisme est exposé à un ligand d'AHR (composants de la fumée de cigarette, dioxines présentes dans notre alimentation, hydrocarbures….), la liaison de ce dernier au récepteur provoque à la fois sa translocation dans le noyau et la dissociation du complexe [24]. AHR lie alors son partenaire Aryl Hydrocarbon Nuclear Translocator (ARNT) et l'hétérodimère se lie à des éléments de réponse appelés xenobiotic responsive element (XRE ou appelés aussi DRE) localisés dans des promoteurs de gènes cibles (fig 2). Puis le couple AHR-ARNT est dégradé par le protéasome. L'IS et l'IAA ont été décrites comme ligands d'AHR [25]. Récemment, il a été démontré d'autres fonctions que celles de détoxification

initialement décrites. AHR est impliqué dans l'immunité de la barrière intestinale et de la peau [26]. Il est aussi impliqué dans la progression tumorale notamment pour le gliome cérébral. [27]. D'ailleurs, ce phénomène passe par une production tumorale de kynurenine qui est aussi une toxine urémique issue du métabolisme du tryptophane. AHR a aussi un rôle dans le développement, la maturation des cellules lymphoides B et T et probablement dans le développement de tumeurs malignes issues de ces lignée [28]. Enfin, nous pensons qu'AHR a un rôle crucial à jouer dans le développement des maladies cardiovasculaires. En effet, Wu et al ont montré récemment que l'intoxication par la dioxine, le ligand canonique d'AHR, va promouvoir un état inflammatoire chronique et le développement de lésions artérioscléreuses chez des souris Apo-E déficiente [29]. Chez les patients, on note d'ailleurs une surmortalité cardiovasculaire chez les travailleurs exposés à la dioxine [30].

Les travaux de notre équipe ont montré que ces deux toxines urémiques participaient au dysfonctionnement endothélial des patients IRC. Notre attention s'est donc naturellement focalisée sur l'endothélium.

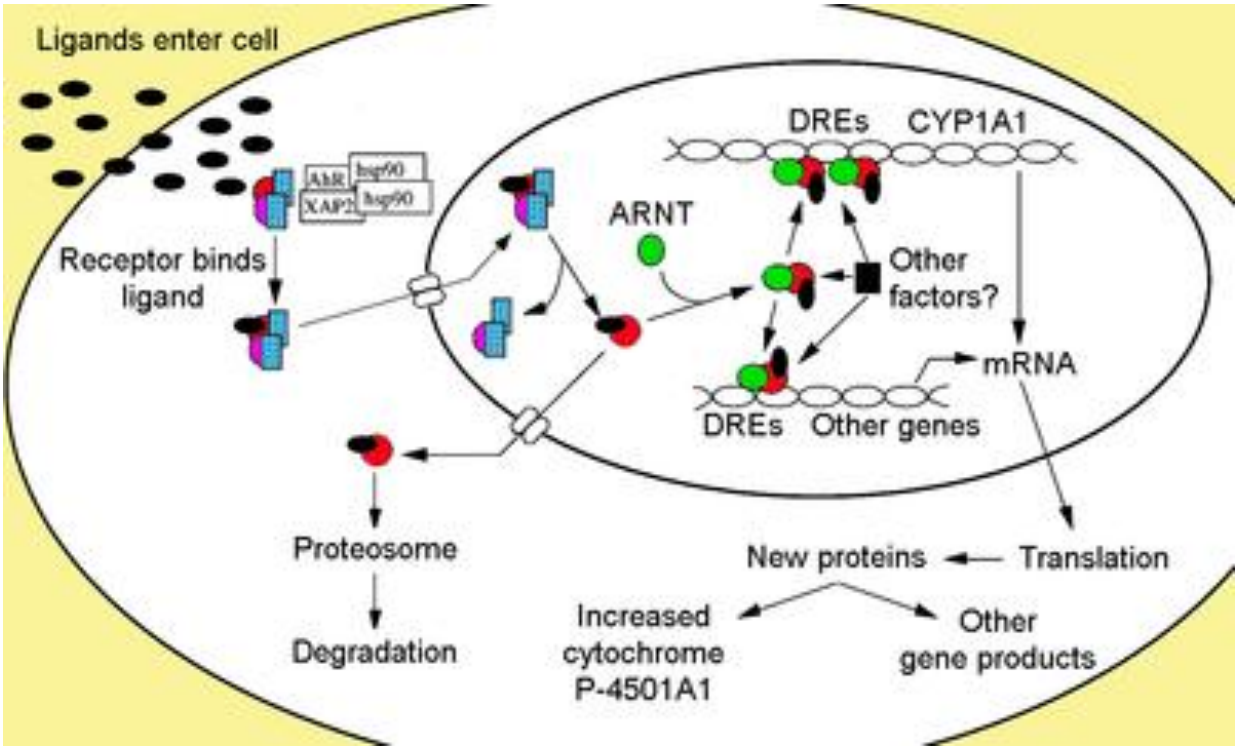

Fig 2 : Signalisation cellulaire de l'Aryl hydrocarbon receptor (AHR)

I) ETAT DES LIEUX DE LA LITTERATURE

La revue intitulée "The uremic paradox: thrombotic and bleeding events. A pivotal role for endothelium » décrit la situation paradoxale à laquelle sont confrontés les patients IRC. Ceux-ci développent plus d'évènements hémorragiques comme des AVC hémorragiques [31,32,33] ou des saignements digestifs hauts [34] lorsqu'ils sont comparés à des populations avec une fonction rénale normale. Ces patients ont de plus des médicaments antiagrégants et/ ou anticoagulants massivement prescrits du fait de leurs nombreuses comorbidités [35]. Paradoxalement, plusieurs études ont établi récemment que ces patients présentent aussi une forte prévalence d'évènements thrombotiques [36]. Outre le fait d'être susceptibles à la thrombose d'accès vasculaire pour hémodialyse [37], les patients IRC présentent une incidence plus importante de thromboses veineuses profondes et d'embolies pulmonaires [38]. De plus, le pronostic est plus grave en cas de rupture de plaques athéromateuse coronaires du fait de cet état d'hypercoagulabilité et l'IRC est un facteur de risque majeur de survenue d'AVC thrombotique en cas de trouble du rythme supra ventriculaire [39]. Nous pensons que l'endothélium joue un rôle crucial dans le développement des ces anomalies. Dans une situation physiologique, l'endothélium maintient la fluidité du sang en produisant à sa surface des inhibiteurs de la coagulation. Il participe au contrôle de l'hémostase primaire, de la coagulation et de la fibrinolyse. Ces trois mécanismes sont altérés dans la maladie rénale chronique.

Premièrement, l'hémostase primaire est déficiente avec un dysfonctionnement plaquettaire [40], une activation basale et une sécrétion plus importante de facteurs inhibant l'agréggabilité plaquettaire comme la prostacycline (PGI2) et l'oxide nitrique (NO) par l'endothélium dysfonctionnel [41]. Deuxièmement, les patients IRC ont des facteurs procoagulants augmentés comme le FT [42], le facteur Von Willebrand (vWF) et les facteurs VII et VIII [43]. La balance

penche plutôt du coté de la pro coagulation car les facteurs anticoagulants sont dans leur majorité diminués [44]. Enfin, la fibrinolyse est altérée aussi dans l'IRC ; nous retrouvons des taux élevés de PAI-1 (plasminogen activator inhibitor-1) qui inhibe la fibrinolyse naturelle. [45]. Dans ce schéma paradoxal de l'hémostase des patients IRC, l'accumulation de solutés urémiques joue un rôle. Outre les résultats montrés dans notre étude, les taux de kynurenine, une autre toxine urémique dérivée du métabolisme du tryptophane, sont corrélés aux taux de FT chez les patients IRC [46]. Il est important de noter que ces toxines (indoles et kynurénine), sont des agonistes d'aryl hydrocarbon receptor (AHR) [27]. De plus, AHR joue un rôle dans le dysfonctionnement plaquettaire [47], la production de FT [42] et la production de PAI-1[48]. Nous pensons qu'AHR est un acteur central dans l'induction des troubles de l'hémostase observés chez le patient avec IRC. Il pourrait être le facteur clé permettant d'expliquer le paradoxe hémostatique observé chez ces patients. La balance hémostatique chez le patient insuffisant rénal chronique est plus instable que chez le patient avec une fonction rénale normale du fait de la dysfonction endothéliale et de l'activation de la voie AHR. Notre hypothèse physiopathologique est celle-ci : face à une agression infra-clinique, le patient IRC va sur-répondre et basculer soit vers l'hémorragie du fait essentiellement de sa dysfonction plaquettaire soit du coté thrombotique de fait de l'augmentation des facteurs de la coagulation. Cette nouvelle approche reposant sur l'hypothèse d'un état d'équilibre plus instable permet d'envisager une nouvelle approche thérapeutique qui serait de restituer une stabilité plus importante au système. AHR pourrait être une cible thérapeutique intéressante dans cette perspective.

REVUE

The uremic paradox: thrombotic and bleeding events. A pivotal role for endothelium

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Abstract

Patients with chronic kidney disease exhibit hemostasis anomalies. These abnormalities lead to an hemostatic uremic paradox. Those patients are at increased risk for thombotic events but also at increased risk for bleeding. Thus, CKD is associated with two antagonist events, thrombosis and bleeding. We reviewed the clinical evidence, the hemostasis abnormalities associated with CKD and the mechanisms involved in this paradox. The endothelial cell plays a major role in the control of hemostasis. In CKD, endothelial dysfunction plays a major role in hemostatic processes. We propose a model where the equilibrium between procoagulant and anticoagulant factors is more unstable than in patient with normal renal function. We suggest that uremic toxins play a role via the activation of aryl hydrocarbon receptor pathway. A new therapeutic approach is to decrease the instability of the hemostatic network, AHR inhibition could be an attractive way to improve hemostasis defect in CKD.

Introduction

Chronic kidney disease (CKD) is associated with abnormal hemostasis. Paradoxically, patients with CKD have an increased risk of bleeding and thrombosis. There is no such condition similar to the one observed in CKD.

Thrombosis events mostly involve dialysis vascular access but also life threatening complications such as pulmonary embolism and stroke. New evidences suggest that uremia is a risk factor associated with thrombo embolism as other well identified risk factors like immobility, cancer, atherothrombosis or atrial fibrillation. Bleeding risk was the first hemostasis abnormality identified in CKD patients [1], it remains the main complication with a high impact on CKD associated morbidity and mortality. Yet it is unknown whether one patient would develop thrombosis and another will develop bleeding events.

In recent years, advances have been made among the understanding of this paradoxical phenomenon. Three different phenomenons are involved in haemostatic processes: primary hemostasis, coagulation cascade, fibrinolysis; all of which are impaired in CKD patients. This review is displayed in three parts. First, we will summarize clinical evidences proving increased thrombotic and haemorrhagic risk in CKD patients. Then, we will describe biological findings proving this complex pathophysiology; we will describe how the endothelium plays a major part in this impaired hemostasis. Finally, as CKD is characterised by the retention of so called “uraemic solutes”, we will describe how those molecules could have a role in this impaired hemostasis.

Thrombotic events in population with CKD

Patients with CKD are at high risk for thrombotic events. An analysis from the LITE study cohort shows a 1.71 fold increased risk for venous thromboembolism (VTE). [2]. In an observational study by Parikh et al, patients with VTE with eGFR <30 mL/min/1.73 m are at increased risk for recurrent VTE, major bleeding episodes and all-cause mortality (HR, 1.70; 95% CI, 1.12-2.57) during a 3-year follow-up [3]. This increased risk seems to be present even in early stages of CKD. [4].

Pulmonary embolism (PE) is probably the most life threatening thrombo-embolic event. A recent pooled analysis of 5 community based cohorts showed that both estimated glomerular filtration rate (eGFR) and proteinuria were independently associated with an increased risk for venous thromboembolism [5]. USRDS data analysis show an annual frequency of PE of 527 per 100,000, 204 per 100,000, and 66 per 100,000 persons with End stage renal disease (ESRD), non dialysed CKD, and normal kidney function (NKF) respectively. In-hospital mortality is also astonishingly higher in CKD patients with PE [6]. In renal transplant patients also, severe CKD is associated with an increased risk for venous thromboembolism [7].

Patients with CKD often present with vascular access (VA) thrombosis. Although, the structure of the VA itself can be pro thrombotic especially with synthetic grafts, the "uremic milieu" can provide a prothrombotic condition leading to VA thrombosis when other conditions are present. A prothrombotic reaction is initiated by damage to the vessel walls, exposing activated endothelium and subendothelial structures to blood flow, thus promoting the formation of thrombus. Fibrinogen and fibrin D Dimer, markers of activated intra vascular coagulation are independent risk factors for VA thrombosis [8]. Thus, a question arises: why some patients have frequent VA thrombosis compared to others? One could hypothesize that

increased circulating prothrombotic factors are often seen in «thrombosis susceptible» patients [8]. Another factor promoting VA thromobosis is neointimal proliferation. This causes reduction of the vessel caliber and an altered hemodynamic condition with changes in laminar flow. It induces mechanisms of vascular remodeling contributing to endothelial dysfunction with proliferation of smooth muscle cells (VSCMC) and endothelial cells [9]. The proliferation of SMC leads to an exposition of procoagulant factors to the circulating milieu. In addition, a study evaluated a variety of risk factors for thrombophilia (factor V Leiden, prothrombin gene mutation, factor XIII genotype, methylene tetrahydrofolate reductase genotype, lupus anticoagulant, anticardiolipin antibody, factor VIII, homocysteine, and lipoprotein [a] concentrations) in 419 hemodialysis patients. [10]. Overall, 59 (55%) patients with access thrombosis had at least one thrombophilic factor compared with 122 (39%) patients without access thrombosis. The association between any thrombophilia and access thrombosis remained (adjusted OR, 2.42; 95% CI, 1.47 to 3.99) after adjusting for confounding factors. Our team has already shown that antiphospholipid antibodies (APL) such as cardiolipin antibodies, lupus anticoagulant, could be found at enhanced levels in CKD patients. When histories of thrombosis were examined, vascular access thrombosis was found to be significantly more frequent in patients with LA than in patients without LA.[11] Those studies on VA could provide pathophysiologic information on other thrombo embolic events in CKD.

A high proportion of chronic hemodialysis (HD) patients die from coronary artery diseases due to atherosclerosis [12]. Compromised coronary perfusion is a classical feature of uremia with alterations in sub endocardial perfusion. Patients with versus without CKD have more extensive and severe atherosclerosis remaining in their coronary tree with plaque composed of greater necrotic core and less fibrous tissue [13,14]. A later step of atherosclerosis is the occurrence of a rupture of the atherosclerotic lesion. CKD is significantly associated with increased incidence of 1-year definite or probable stent thrombosis in patients undergoing

percutaneous coronary intervention [15]. For these reasons, CKD patients are at poor prognosis after acute coronary syndrome [16]. In a retrospective study, the discovery of the coronary heart disease (CHD) in patients with CKD was more frequently a myocardial infarction, reflecting a thrombotic event secondary to the rupture of the plaque. In patients without CKD the main entrance in CHD was angina pectoris [17]. Recently, the role of thrombosis in the manifestation of CHD was recently reviewed. Autopsy studies showed that most fatal coronary events were due to a physical disruption of coronary arterial plaques and enhanced formation of thrombus due to the exposition of endothelial and subendothelial procoagulant factors to the circulating blood [18].

Atrial Fibrillation (AF) is the most common cardiac arrhythmia in general population and is associated with an increased risk of stroke [19]. In a large observational study of outpatients with atrial fibrillation, about one third had stage 3 or 4 CKD which was found to be an independent predictor of stroke [20]. Atrial fibrillation is estimated to occur in 7 % to 20 % in CKD patients especially in ESRD patients, which is 2 to 3 fold higher than in general population. In almost all studies, the risk of mortality is increased in CKD patients with a history of AF compared with those who remain in sinus rhythm [21]. In a Danish registry analysis of patients admitted for non valvular AF, Olesen et al. found that, as compared to patients who did not have renal disease, patients with CKD had an almost 2 fold increased risk of stroke or systemic thromboembolism . The risk of bleeding was also increased in CKD patients and was further increased with warfarin, aspirin, or both [22]. Those datas suggest an independent pejorative role of uremia in the genesis of neurovascular thrombotic events. The estimated risks and benefits of anticoagulation for patients with CKD and AF still remain unclear. In the study by Chan et al, in which all patients had AF, 44.7% were on warfarin, 11.4% on Clopidogrel and 37.3 % on aspirin [23]. Paradoxically, the use of warfarin was associated with an almost 2 fold greater risk of stroke compared with warfarin non use. The

use of aspirin or clopidogrel was not associated with an increased risk of stroke. In a similar type of study, Winkelmayer et al, showed that warfarin did not decrease the risk of ischemic stroke (7.4 versus 7.8/100 patients/years) but doubled the risk of hemorrhagic stroke (2.6 versus 1.1/100 patients/years) [24]. Wizeman et al reported a 2 fold higher risk of stroke in patients with AF over the age of 75 treated with warfarin compared with elderly patients not treated with warfarin [25]. Contrary to these results, the use of warfarin was associated with a decreased thromboembolic stroke risk in another study in patients on hemodialysis [26]. But, intracerebral bleeding was not included in the stroke outcome...

Hemorragic events in CKD population

The bleeding risk in CKD population was identified before the thrombotic risk in CKD [1]. It is of major concern in clinical practice because nephrologists are often confronted to bleeding episodes. In this population the bleeding risk is outweighed by the numerous anticoagulant/anti platelets agents often prescribed.

CKD is an independent predictor of major bleeding events in patients with creatinine concentrations greater than 1.5 mg/dl [27].

A common situation is blood loss by upper gastro intestinal bleeding (UGIB) [28]. Cheung et al showed that ESRD was a predictor of worse outcomes in GI bleeding associated with peptic ulcer disease. Dialysis patients had a higher risk of new bleeding, and needed multiple transfusions and longer hospital stays when compared to normo renal patients.[29]. Patients with CKD or ESRD admitted with primary UGIB have up to three times higher risk of all cause in-hospital mortality [30].

CKD is known to be associated with intracerebral haemorrhage [31]. It is clear that CKD patients have a poorer prognosis after hemorrhagic stroke compared to general population [32].

Anticoagulant agents are also often prescribed in this population. There is a clear increased

risk of bleeding associated with the use of warfarin. The absolute risk of intracranial hemorrhage in warfarin treated patients on dialysis is 2,6% per year compared to 1,1% in those not on warfarin and 0,4% in non hemodialysed patients taking warfarin [24]

The other major class to counteract the thrombotic risk in CKD patient is antiplatelet agents (APA). The efficacy and safety of these drugs was recently reviewed [33]. The main conclusion is that there is a lack of knowledge for the efficacy of this drug compared to normo renal population studies. The benefit was clearly observed in general population, but large clinical trials excluded patients with severe CKD. The safety is the main concern for the use of these drugs. A recent meta analysis by Palmer et al evaluated safety of aspirin and clopidogrel in nine trials (all post hoc subgroup analyses for CKD) involving 9969 patients with ACS undergoing percutaneous coronary intervention (PCI) and 31 trials involving 11,701 patients with stable or no cardiovascular disease. [34]. They concluded that although the evidence was low quality there was an increase in major and minor bleeding episodes with APA use in CKD patients (RR 1.4 for Aspirin and RR 1.47 for clopidogrel). In an RCT evaluating the benefit of adding aspirin for primary prevention in CKD patients there was no increase in major bleeding but the risk of minor bleeding (epistaxis, bruising, ecchymoses) was increased by a 3 fold risk (RR: 2.8: 1.5-5.3) [35]. For the P2Y12 receptor antagonists, a retrospective study found an increased risk of mortality (HR 2.74 (1.26 to 6.00)) and hospitalization from bleeding (HR 1.39 (1.08 to 1.80)) [23]. In a randomized trial comparing clopidogrel/aspirin versus placebo, the cumulative incidence of bleeding was significantly greater in the aspirin/clopidogrel-treated group [HR 1.98; 1.19 to 3.28] [36]. In a comparative trial comparing clopidogrel vs placebo for graft patency no difference was observed for bleeding events (2,9% vs 2,8%) between both groups [37]. However, an increased risk for bleeding events in CKD stage 3 to 5 patients has been recently found in large studies evaluating novel anticoagulant agents such as the AVERROES and ARISTOTLE trials [38,39].

Endothelial wall plays a pivotal role in CKD impaired hemostasis

Endothelial wall function is critical in primary hemostasis and coagulation processes. It releases factors activating hemostasis like von Willebrand Factor (vWF) and/or plasminogen activator inhibitor 1 (PAI-1). Normal endothelium maintains blood fluidity by producing at its surface inhibitors of blood coagulation and platelet aggregation (heparan sulfate, thrombomodulin).[40] It also regulates vessel well tone by producing vasoconstrictive (endothelins) and vasodilatating products (NO, PGI2). It modulates vascular permeability and provides a protective envelope separating hemostatic blood components from reactive subendothelial structures and extracellular matrix. Thus, endothelium modulates fibrinolysis by secreting t-PA and PAI-1 and inhibits platelet aggregation by releasing prostaglandin I2 (PGI2) and nitric oxide (NO). We have shown that endothelium had a crucial role in hemostasis in CKD patients through proliferation/repair processes with endothelial progenitor cells (EPC) [41]. Patients with CKD show endothelial dysfunction resulting from endothelial injury and decreased endothelial repair [42, 43]. Endothelial dysfunction begins in the early stages of CKD [44], independently of traditional cardiovascular risk factors and is seen also in CKD children. [45,46]

In a «non pathologic» situation, there is an equilibrium between anti coagulant and procoagulant agents and this homeostasis is driven by endothelium. Normally, the balance tilts to the anticoagulant state with endothelium protecting the vessel from spontaneous activation of the coagulation cascade. After a vessel lesion, like a cut, the endothelium displays a procoagulant state to protect the organism from blood loss. During CKD, we

hypothesize that this equilibrium is more responsive to activation in one way or the other due to endothelial dysfunction. This could lead to thrombotic events or bleeding disorders after events that physiologically should not lead to it (Fig 1).

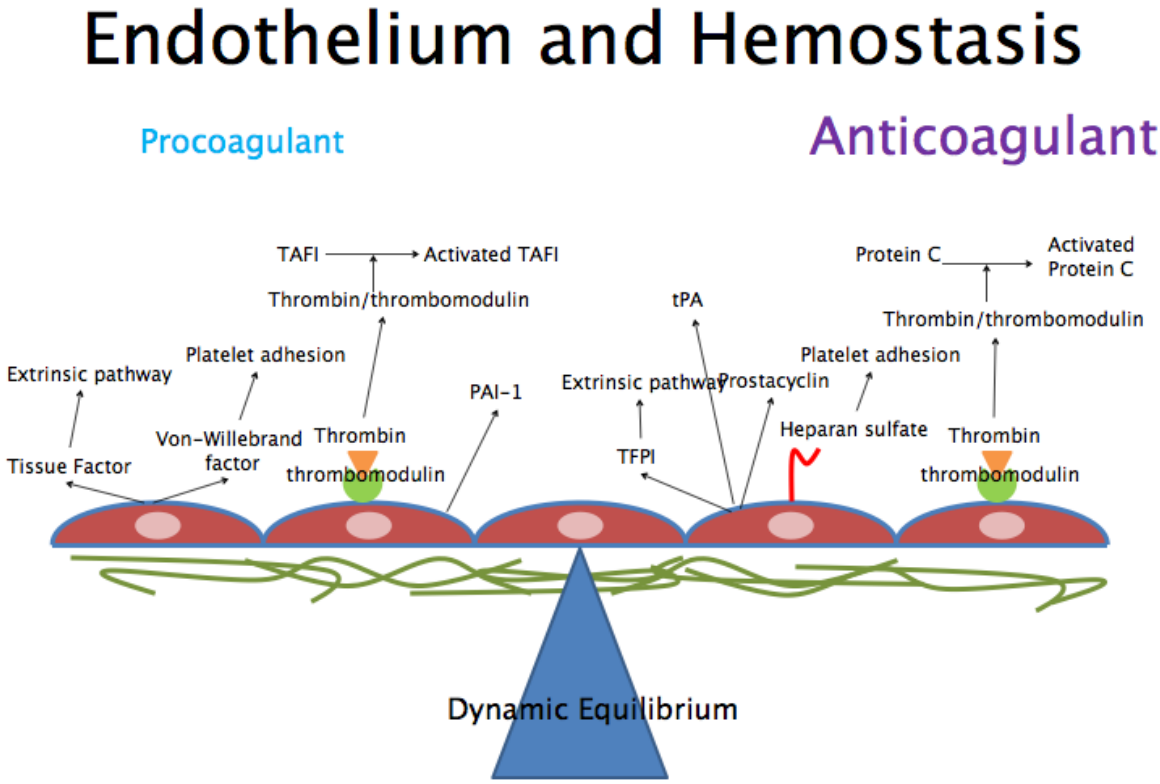

Fig 1:The balance between procoagulant and anticoagulant factors in endothelium

1. Primary hemostasis

Primary hemostasis is deficient in uremia. Abnormalities could lead to bleeding events or strengthen a pro coagulant phenotype. Numerous biological changes in platelet have been described. Dense granule content is decreased and a storage pool defect with reduction in serotonin and ADP is also present. Functional tests on platelet are also abnormal in uremic patients with a decrease in ATP release in response to thrombin stimulation. Platelets from uremic patients are unable to produce thromboxane A2 in response to platelet activating factor (PAF). [47] On the other side, platelets of uremic patients display an increased basal activation as seen by an increase in phosphatydil serine exposure and an increase in p-selectin expression at the platelet surface [48].

Molecules produced by the endothelium such as prostacyclin (PGI2) and nitric oxide (NO) inhibit platelet function and modulate the vascular tone affecting platelet - vessel wall interaction [49]. ADAMTS 13, a metalloproteinase involved in vWF shedding is reduced in uremia via a decreased production by endothelial cells. Whereas this abnormality leads to qualitative abnormality of vWF is uncertain. Overproduced circulating fibrinogen fragments in CKD patients can interfere with platelets function. They bind to the receptor GPIIb-IIIa receptor on platelets leading to a decreased adhesion to the vascular wall [50]. The endothelium is coated on the luminal side by the glycocalyx, a negatively charged mesh of proteoglycans (PGs) and glycosaminoglycans. CKD leads to the disruption of the glycocalyx by numerous factors: abnormal blood shear stress, chronic inflammatory state, oxidative stress [51]. This disruption of the glycocalyx could lead to increased platelet adhesion as described by Vink et al [52]. So far, this association has never been demonstrated in CKD animal or in vitro models. These evidences show that despite of activated basal platelet activity in CKD, the balance in primary hemostasis is in favour of a defect in primary hemostasis in CKD that could explain the increased risk of bleending and the limited efficiency of antiplatelet agents.

2. Coagulation cascade

Compared to normo renal population, patients with CKD have significantly increased levels of hemostasis markers evoking a procoagulant state. Those markers are soluble thrombomodulin (sTM), soluble tissue factor (sTF), D-Dimer, von Willebrand factor (vWF), factor VIII, plasmin-antiplasmin complex (PAP), tissue factor pathway inhibitor (TFPI), plasminogen activator inhibitor-1 (PAI-1), and fibrinogen [53]. Many of them are produced by endothelium. Furthermore, we and others have shown increased levels of circulating tissue factor (cTF) in patients with renal failure [54, 55]. Moreover, this increased cTF leads to an increased procoagulant plasma activity [55]. High plasma levels of factor VII and VIII are found in several studies [56] but whether their elevation relates to clinical situation is still debated. On that point, levels of acquired antibody against protein C and protein S were shown to be associated with thrombosis events [40]. TF is a major trigger of coagulation processes via the initiation of the intrinsic pathway. Elevated plasma TF levels are markers of poor cardiovascular outcomes [57].

Endothelial dysfunction is the common precursor and denominator of cardiovascular events. Patients with CKD have endothelial cells damages [41, 44,58]. Elevated plasma levels of endothelial dysfunction marker von Willebrand antigen (vWF Ag) in HD patients are associated with cardiovascular mortality risk factors and hyperfibrinogenaemia. Malyszko et al showed that in renal failure, there is evidence of endothelial cell injury and a higher degree of hypercoagulation shown by an increased production of thrombin antithrombin complex [59].

Renal failure is associated to an activation of tissue factor but also to a deficient tissue-factor induced response to activated protein C [60]. The activity of anti-thrombin is reduced [61]. Ghisdal et al found an antithrombin, protein C and protein S deficiency in HD patients compared to normo renal patients [62]. Anyway, the imbalance is not that clear between pro and

anti-thrombotic molecules as we can also find in the literature reports of increased of fibrinolytic molecules such as urokinase-type plasminogen activator (uPA), its soluble receptor (uPAR) and tissue plasminogen activator (tPA) [63]. Interstingly in this last paper those markers were related to VEGF levels suggesting a role of those molecules in CKD related endothelial dysfunction. Tissue factor Pathway inhibitor (TFPI) is also elevated in CKD patients compared to normo renal [53].

Available data in the literature demonstrate a clear procoagulant phenotype in CKD. Patients are in a prothrombotic state but the reported mechanisms remain unclear.

3. Fibrinolysis

Clot removal is normally conducted by the proteolytic enzyme plasmin. Its precursor, plasminogen, binds to fibrin and tissue plasminogen activator (tPA) and is converted to plasmin. Plasmin cleaves fibrin-releasing fibrin degradation products, including D-Dimer. It also cleaves fibrinogen. Its activity is regulated by the endothelium, which secretes tPA and plasminogen activator inhibitors such as PAI-1 and PAI-2. CKD patients display abnormalities of fibrinolysis. Hypofibrinolysis may be present as shown by high levels of PAI-1 [40]. PAI-1 inhibits the activation of the fibrinolytic system through inhibition of the tissue plasminogen activator (t-PA) and urokinase. Circulating PAI-1 is increased revealing an increased endothelial production [64]. Segarra et al suggested that increased levels of circulating PAI-1 could indicate a chronic endothelial activation and could be an additional tool to identify dialysis patients at risk for cardiovascular disease [65]. Paradoxically, Soluble Thrombomodulin, a fibrinolytic molecule is also increased in CKD patients [53]. Those evidences show an increased fibrinolytic process in uremia. Besides actors in fibrinolytic processes, markers of fibrinolysis are also increased such as fibrin degradation products (FDP), D-Dimers, plasmin-antiplasmin complexes [53, 66].

The activation of the fibrinolysis is probably secondary to the primary activation of coagulation. This leads to increased levels of fibrin degradation products that could impair primary hemostasis via platelet dysfunction as described earlier [50].

4. A new aspect of coagulation : Microparticules

Increased levels of MPs have been found in different diseases with an increased procoagulant risk such as cancer [67]. They are produced by membrane blebbing after activation of different cells such as platelets, monocytes/macrophages, endothelial cells and can be found in plasma at different amounts. Recently it has been described that those MPs especially from endothelial cells and monocytes have a certain role in coagulation processes [68]. MPs present on their surface an amount of activated tissue factor (TF) leading to an enhanced coagulation process [69]. Our team has already shown that plasma from CKD patients present increased levels of MPs [70]. However, it is still unknown if CKD patients have increased TF positive microparticles. One could hypothesize that the increased plasma TF levels are related to an increased levels of TF positive MP.

Uremic toxins: the missing link ?

Recent datas suggest a possible role of the uremic «milieu» itself in the genesis of altered hemostasis in CKD patients. Vaziri et al have studied various coagulation proteins before and after hemodialysis in a group of patients with end-stage renal disease [71]. They showed that despite ultrafiltration, plasma factor IX activity, von Willebrand factor, and fibrinogen concentrations decreased after hemodialysis. These data suggest a role of the correction of the uremic milieu by hemodialysis. An interesting study by Ghisdal et al compared thrombophilic factors such as antithrombin, protein-c, protein-s deficiency in patients before transplantation and 1 month after. One month after transplantation, the global prevalence of thrombophilic factors had dropped from 74.4 to 44.7% ($P < 0.001$) [62]. This study corroborates the hypothesis that the uremic milieu leads to an increased plasmatic release of pro coagulant factors such as tissue factor (TF) [72]. Recent in vitro studies tried to elucidate the mechanistics of that increase. Recently, Chitalia et al had shown that uremic serum up regulated vSMC TF levels by increasing TF stability and decreasing its ubiquitination [73]. Several uremic solutes raise TF production in different cell types: ADMA (Asymmetric dimethylarginine) increases monocyte TF antigen [74], Advanced Glycation End products (AGE) enhance endothelial TF production [75], kynurenin is associated with cTF levels and hypercoagulability in CKD patients [54]. We found in our lab that indolic uremic solutes indole acetic acid (IAA) and indoxyl sulfate (IS) derived from tryptophan enteric metabolism enhanced endothelial and monocytic TF production and TF positive MP release in the supernatant [55]. Thus in patients, circulating TF was correlated with eGFR, IAA and IS. We identified the mechanism that leads to the increase of TF production by monocyte and endothelial cells, IS and IAA activate aryl hydrocarbon receptor (AHR) and induces the expression of TF gene. AHR is the major mediator of organism response to xenobiotics and is mostly involved in detoxification processes, and it's well known exogenous ligand is TCDD (dioxin) [76]. The gene silencing of

AHR prevented the procoagulant activity of IS and IAA in our study [55]. Interestingly, another uremic toxin: kynurenin is also an agonist of AHR [77]. It is linked to TF in CKD patients [54]. Moreover, PAI-1 expression is controlled by AHR [78]. Agonists of AHR could lead to an increased activity of cyclooxygenase 2 (COX2) that could lead to an increase in prostacyclin (PGI2) production [79]. On that point, platelets dysfunction and prolonged bleeding times are associated with an increase in prostacyclin production in CKD patients [80]. So we think that AHR pathway could be a link between uremic toxins and hemostasis disorders observed in CKD. Another uremic solute, homocysteine, can play a role as a mediator between renal dysfunction and endothelial cell damage. It can inhibit the thrombomodulin-dependent activated protein c system that results in permanent activation of thrombin with subsequent formation of fibrin. It also interferes with endothelial release of tissue plasminogen activator (t-PA) predisposing to hypofibrinolysis [81].

We propose a model where the activation of cascade coagulation by the increase TF production and activity leads to a procoagulant state then to an activation of fibrinolysis. This activation of fibrinolysis leads to production of fibrin degradation products (FDP) that impaired platelets aggregation prostacyclin over production could lead to platelet dysfunction and increased bleeding risk. So our model could explain the instability of hemostatic system during CKD that could lead to thrombosis and bleeding for infraclinical triggers events. (fig 2)

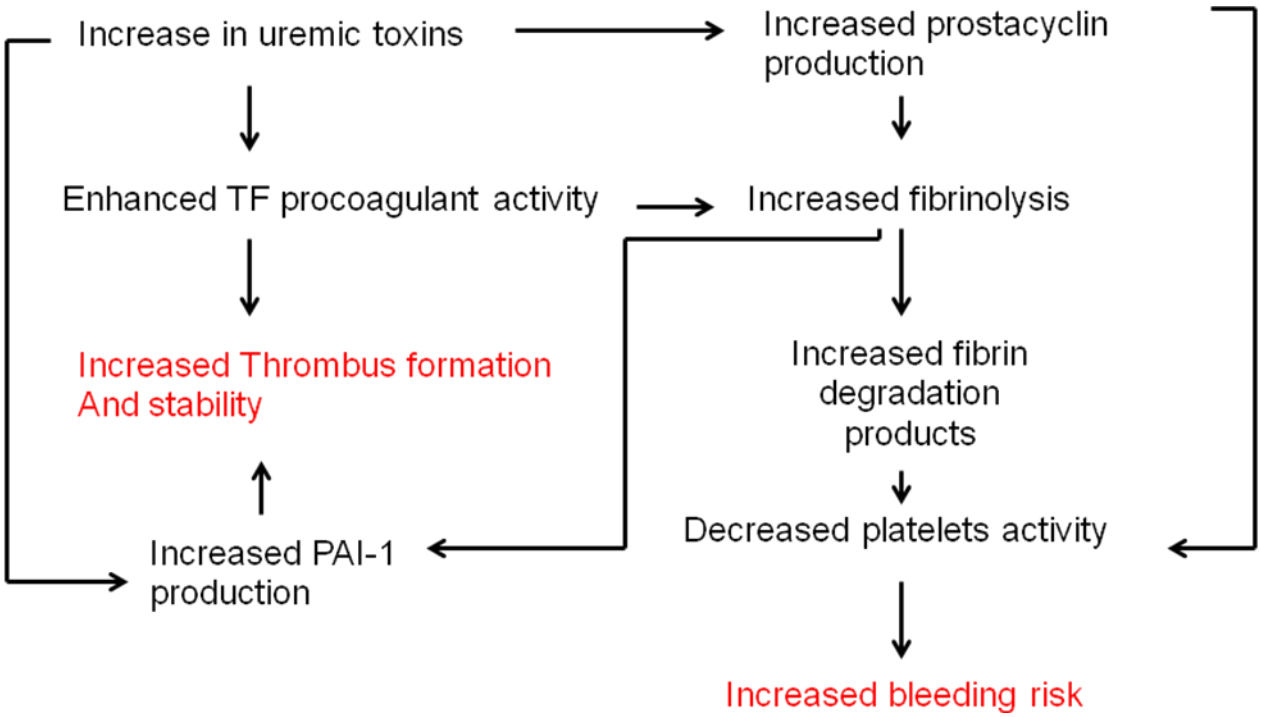

Fig 2: proposed mechanisms for CKD associated impaired hemostasis

Conclusion

Stable CKD patients are prone to bleed and to clot. At stable state, they do not present with thrombosis or bleeding event because of equilibrium between actors of hemostasis. But this equilibrium is frail and we can hypothesize that a disruption between pro thrombotic and anti thrombotic factors such as acute events can lead to more severe episodes in CKD patients.

More studies are needed to identify patients more prone to clot OR to bleed, a biochemical and molecular screening should be necessary during the early stages of CKD in order to prevent life threatening complications. Moreover, this paradox must be understood in order to adapt anti coagulation or anti aggregant therapy in those patients. AHR therapeutic inhibition could be an attractive way to reset the hemostasis disorders observed during CKD and render the endothelial wall less responsive.

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II) HYPOTHESES DE TRAVAIL ET OBJECTIFS

La pathologie cardio-vasculaire est la cause majeure de mortalité et de morbidité chez les patients IRC. Comme décrit en introduction, de nombreuses études ont montré un effet toxique de l'IS et l'IAA sur la paroi vasculaire ainsi que leur association avec des évènements cardiovasculaires chez les patients.

Notre hypothèse était que les toxines urémiques en modifiant l'expression des gènes de la cellule endothéliale pouvaient participer à la genèse d'événements cardiovasculaires des patients IRC..

Afin de mieux approcher l'implication de ces toxines dans la pathogénie cardiovasculaire par un dysfonctionnement endothélial, ce travail de thèse avait deux objectifs :

1) comprendre les mécanismes cellulaires impliqués dans le dysfonctionnement endothélial lié à la toxicité de l'IAA et l'IS
2) démontrer que l'IS et l'IAA vont promouvoir un état d'hypercoagulabilité via une production accrue de FT endothélial et de fait démontrer une implication physiopathologique de ces toxines dans la maladie cardiovasculaire de l'IRC.

III) EXPOSE DES TRAVAUX

Dans ce travail intitulé « Indolic uremic solutes increase tissue factor production in endothelial cells by the aryl hydrocarbon receptor pathway » nous avons réalisé une étude in vitro et in vivo chez des patients insuffisants rénaux chroniques.

Pour la partie in vitro, nous avons utilisé une stratégie « non a priori » en étudiant le transcriptome de cellules endothéliales humaines (culture primaire de cellules endotheliales veineuses de cordon ombilical : HUVEC) exposées à l'IS. L'analyse des micro arrays nous a permis d'identifier une up régulation significative du gène du facteur tissulaire (gène *F3*). Cette molécule nous a fortement intéressés du fait de son implication dans les maladies cardiovasculaires associées à l'IRC. De plus, l'analyse du transcriptome nous a permis d'identifier une possible implication de la voie AHR dans la toxicité endothéliale de l'IS. Nous avons ensuite confirmé l'impact de l'activation d'AHR sur l'expression du FT et son activité procoagulante par les solutés dérivés des indoles (IS et IAA) sur des HUVEC et sur des cellules mononuclées circulantes (PBMC). Le ligand canonique d'AHR étant la dioxine, nous avons évalué les effets de cette molécule sur la production endothéliale de FT. Les résultats obtenus avec IS, IAA et dioxine sont similaires ce qui nous a fait évoquer un mimétisme d'action entre ces trois molécules. L'inhibition de l'activation d'AHR aussi bien de façon pharmacologique que génétique prévient l'action procoagulante du FT lorsque l'endothelium est stimulé par l'IS et l'IAA. Enfin, comme la dioxine, les solutés indoliques sont responsables d'une translocation d'AHR du cytoplasme vers le noyau. Les toxines dérivées des indoles ont donc un effet « dioxine like » sur l'endothélium.

Pour la partie in vivo, nous avons réalisé des mesures de FT circulant dans trois groupes de malades : cinquante IRC non dialysés, soixante treize IRC dialysés et quarante trois patients contrôles sans maladie rénale. Les taux de FT sont significativement plus élevés chez les patients IRC comparés aux contrôles. Nous avons identifié une corrélation inverse entre les taux de FT et le débit de filtration glomérulaire chez les patients IRC non dialysés. De plus, les taux de FT sont corrélés aux taux plasmatiques d'IS et d'IAA. Enfin, l'activité du FT circulant est plus élevée chez les patients IRC que chez les contrôles.

Notre travail confirme notre hypothèse de départ, l'analyse du transcriptome nous a permis d'identifier une nouvelle voie de signalisation impliquée dans l'effet d'une toxine urémique sur la cellule endothéliale. Notre travail confirme que l'IS et l'IAA sont des ligands d'AHR dans l'endothelium et confèrent à celui-ci un phenotype procoagulant. Avec cette approche in vitro et in vivo nous proposons une possible explication physiopathologique à l'association entre taux de ces toxines et mortalité cardio-vasculaire chez les patients présentant une insuffisance rénale chronique.

Indolic uremic solutes increase tissue factor production in endothelial cells by the aryl hydrocarbon receptor pathway

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In chronic kidney disease (CKD), uremic solutes accumulate in blood and tissues. These compounds probably contribute to the marked increase in cardiovascular risk during the progression of CKD. The uremic solutes indoxyl sulfate and indole-3-acetic acid (IAA) are particularly deleterious for endothelial cells. Here we performed microarray and comparative PCR analyses to identify genes in endothelial cells targeted by these two uremic solutes. We found an increase in endothelial expression of tissue factor in response to indoxyl sulfate and IAA and upregulation of eight genes regulated by the transcription factor aryl hydrocarbon receptor (AHR). The suggestion by microarray analysis of an involvement of AHR in tissue factor production was confirmed by siRNA inhibition and the indirect AHR inhibitor geldanamycin. These observations were extended to peripheral blood mononuclear cells. Tissue factor expression and activity were also increased by AHR agonist dioxin. Finally, we measured circulating tissue factor concentration and activity in healthy control subjects and in patients with CKD (stages 3–5d), and found that each was elevated in patients with CKD. Circulating tissue factor levels were positively correlated with plasma indoxyl sulfate and IAA. Thus, indolic uremic solutes increase tissue factor production in endothelial and peripheral blood mononuclear cells by AHR activation, evoking a 'dioxin-like' effect. This newly described mechanism of uremic solute toxicity may help understand the high cardiovascular risk of CKD patients.

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KEYWORDS: aryl hydrocarbon receptor; tissue factor; uremic solutes

Patients with chronic kidney disease (CKD) are at a high risk for cardiovascular diseases. CKD leads to accelerated atherosclerosis and consequently to a marked increase in cardiovascular morbi-mortality.[1,2] The high rate of mortality cannot be explained merely by the traditional cardiovascular risk factors.[3] Nonclassical risk factors have emerged, including inflammation and endothelial dysfunction.[4–6] In addition, CKD patients display a prothrombotic state, revealed by elevated levels of factor VII, VIII,[7] PAI-1,[8] thrombin anti-thrombin complex,[9] and tissue factor (TF).[10] These biological abnormalities lead to increased thrombotic events (deep venous thrombosis and vascular access thrombosis) in hemodialysis patients[11] and may have a role in cardiovascular events in CKD.

CKD leads to the retention of the so-called uremic solutes, which are retained in blood and tissues instead of being excreted by kidneys.[12] These uremic solutes are classified by their behavior during dialysis in three groups: small water-soluble molecules, middle molecules, and protein-bound solutes.[12] The protein-bound uremic solutes are poorly removed by conventional dialysis.[12] Two of them, indoxyl sulfate (IS) and indole-3-acetic acid (IAA), which are tryptophan metabolites derived from indole, have deleterious effects on the endothelium. We have previously shown that IS inhibits endothelial proliferation, wound repair,[5] induces oxidative stress,[13] and enhances the production of endothelial microparticles.[14] In CKD patients, IS is associated with cardiovascular mortality and traditional risk factors.[15] We have also shown that IAA is deleterious for progenitor cells.[16]

The mechanisms leading to the deleterious effects of indolic solutes on endothelial cells are unknown. In this study, we used a non *a priori* approach, pan genomic

expression microarrays, to identify genes dysregulated by indolic uremic solutes. Microarray analysis allowed us to identify the TF as a high interest molecule in the setting of cardiovascular complications associated with CKD. In addition, microarray analysis provided the possible involvement of a new signaling factor aryl hydrocarbon receptor (AHR) in the expression of TF. We confirmed the role of AHR in increased TF expression and activity by indolic solutes in endothelial cells and peripheral blood mononuclear cells (PBMCs). We identified a relationship between indolic solutes and circulating TF (cTF) concentrations in the plasma of patients with various stages of CKD.

RESULTS

Identification of genes upregulated by indolic solutes in endothelial cells.

We performed microarray expression profiling of human umbilical vein endothelial cells (HUVECs) incubated with IS for 4 h. IS increased the expression of 50 genes (Table 1) in HUVECs. Among them, the TF gene (*F3*) was overexpressed. TF is involved in various processess, such as in coagulation cascade, endothelial dysfunction, atherosclerosis, and inflammation. TF is known to have a role in the increased cardiovascular morbid–mortality of CKD patients.[17] In addition, the expression of eight genes known to be targeted by the transcription factor AHR, *CYP1A1, CYP1B1, CYP1A2, TGFB3, PTGS2, TIPARP, CRR7,* and *AHRR*, the repressor of AHR, was increased by IS, and these data were confirmed by reverse transcriptase PCR (RT-PCR) experiments (Table 2). These results showed a strong enrichment of genes regulated by AHR and highlighted AHR as a main factor in the effects of IS. We next examined whether IAA also stimulated the expression of the genes regulated by IS, and we focused on those targeted by AHR. IAA increased the expression of the genes targeted by AHR (Table 2). Thus, we hypothesized that indolic uremic solutes could increase TF expression and activity by an AHR-dependent pathway. In addition, the expression of *MCP-1* and *ICAM-1* has been showed to be increased by IS in endothelial and tubular cells.[18,19] As our microarrays analysis did not reveal these two genes, we performed RT-PCR experiments and showed that IS induced *ICAM-1* and *MCP-1* expression, whereas IAA induced only *MCP-1* expression. The increased expression of ICAM-1 and MCP-1 was only observed after 24 h of incubation with IS and not after 4 h of incubation (Supplementary Figure S1 online).

Indolic uremic solutes increased TF gene expression

To confirm the transcriptional regulation of TF by uremic solutes, we analyzed *F3* mRNA levels in HUVECs incubated with IS or IAA (Figure 1a and b) by comparative RT-PCR using oligonucleotides specific from the full-length TF, the well-recognized form of TF involved in coagulation. IS induced a significant increase in TF gene mRNA levels after 2 h of incubation, reaching a peak after 4 h (Figure 1a). After 8 h of incubation, mRNA levels returned to baseline. The same kinetic of *F3* mRNA expression was observed with

Table 1 | Upregulated genes identified by microarray analysis after incubation of HUVECs with IS (1 mmol/l) for 4 h

P-value	Gene symbol	Description
0.002	*CYP1B1*	Cytochrome P450, family 1, subfamily B, polypeptide 1
0.002	*NPTX1*	Neuronal pentraxin I
0.003	*CYP1A1*	Cytochrome P450, family 1, subfamily A, polypeptide 1
0.002	*F2RL3*	Coagulation factor II (thrombin) receptor-like 3
0.006	*VIPR1*	Vasoactive intestinal peptide receptor 1
0.004	*AHRR*	Aryl-hydrocarbon receptor repressor
0.009	*SERPINB2*	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
0.002	*GAD1*	Glutamate decarboxylase 1 (brain, 67 kDa) (GAD1), transcript variant GAD25
0.002	*TIPARP*	TCDD-inducible poly(ADP-ribose) polymerase
0.018	*CYP26B1*	Cytochrome P450, family 26, subfamily B, polypeptide 1
0.002	*ITGA11*	Integrin, α11
0.002	*APOLD1*	Apolipoprotein L domain containing 1, transcript variant 2
0.004	*SECTM1*	Secreted and transmembrane 1
0.002	*F3*	Coagulation factor III (thromboplastin, tissue factor)
0.009	*PTGS2*	Prostaglandin G/H synthase and cyclooxygenase
0.009	*CCR7*	Chemokine (C–C motif) receptor 7
0.025	*PPM1E*	Protein phosphatase 1E (PP2C domaincontaining)
0.048	*MITF*	Microphthalmia-associated transcription factor,transcript variant 2
0.013	*RAB11FIP4*	RAB11 family interacting protein 4 (class II)
0.018	*TCHH*	Trichohyalin
0.003	*ROR1*	Tyrosine-protein kinase transmembranereceptor ROR1 precursor
0.002	*RAB38*	RAB38, member RAS oncogene family
0.025	*CYP1A2*	Cytochrome P450, family 1, subfamily A, polypeptide 2
0.025	*TNS4*	Tensin 4
0.004	*GPR68*	G protein-coupled receptor 68
0.013	*RND1*	Rho family GTPase 1 (RND1)
0.035	*ST8SIA5*	ST8 α-*N*-acetyl-neuraminide α-2,8-sialyltransferase 5
0.009	*SHISA2*	Shisahomolog 2 (*Xenopus laevis*)
0.035	*SAMD12*	Sterile α-motif domain containing 12, transcript variant 1
0.025	*POU4F3*	POU class 4 homeobox 3
0.004	*BMF*	Bcl2 modifying factor, transcript variant 1
0.009	*FOXF1*	Forkhead box F1
0.048	*LAMB4*	Laminin, β4
0.048	*SLC16A6*	Solute carrier family 16, member 6
0.009	*LIF*	Leukemia inhibitory factor (cholinergic differentiation factor)
0.002	*KCTD12*	Potassium channel tetramerization domain containing 12
0.013	*NALCN*	Sodium leakchannel, nonselective
0.004	*CDC42EP3*	Cdc42 effector protein 3 (binder of Rho GTPases 2)
0.048	*XKRX*	XK, Kell blood group complex subunit-related, X-linked
0.035	*NR0B1*	Nuclear receptor subfamily 0, group B, member 1
0.006	*TNIP3*	TNFAIP3 interacting protein 3, transcript variant 1
0.002	*OSGIN1*	Oxidative stress–induced growth inhibitor 1
0.025	*DNAH12*	Dynein, axonemal, heavy-chain 12, transcript variant 1
0.048	*TAS2R50*	Taste receptor, type 2, member 50
0.025	*ADM2*	Adrenomedullin 2
0.018	*TGFB3*	Transforming growth factor, β-3
0.018	*KCTD16*	Potassium channel tetramerization domain containing 16
0.013	*NR4A3*	Nuclear receptor subfamily 4, group A, member 3, transcript variant 2
0.018	*EDC3*	Enhancer of mRNA decapping 3 homolog (*S. cerevisiae*), transcript variant 3
0.009	*PRKG1*	Human mRNA for type I β-cGMP-dependent protein kinase

Abbreviations: ADP, adenosine-5′-diphosphate; cGMP, cyclic guanosine monophosphate; HUVEC, human umbilical vein endothelial cells; IS, indoxyl sulfate; TCDD, 2,3,7,8- tetrachlorodibenzo-p-dioxin; TNF, tumor necrosis factor.
The genes with expression controlled by AHR are in gray background. Analysis used a cutoff of 2-fold upregulated genes compared with control cells (KCl 1 mm).

Table 2 | Upregulated genes identified by RT-PCR after incubation of HUVECs with IS (1 mmol/l) or IAA (50 μmol/l) for 4 h

Gene	Fold change of expression after incubation with IS	Fold change of expression after incubation with IAA
CYP1B1	200.1 ± 91.5	101.8 ± 30.3
CYP1A1	21.4 ± 7.9	14.9 ± 5.2
CYP1A2	4.1 ± 2.0	4.0 ± 0.6
AHRR	7.3 ± 1.9	4.5 ± 1.0
CCR7	6.3 ± 4.2	3.1 ± 0.7
TIPARP	6.1 ± 5.5	2.8 ± 0.2
PTGS2	3.7 ± 1.2	2.2 ± 0.4
TGFB3	2.1 ± 0.9	1.8 ± 0.1

Abbreviations: HUVECs, human umbilical vein endothelial cells; IAA, indole-3-acetic acid; IS, indoxyl sulfate; RT-PCR, reverse transcriptase PCR.

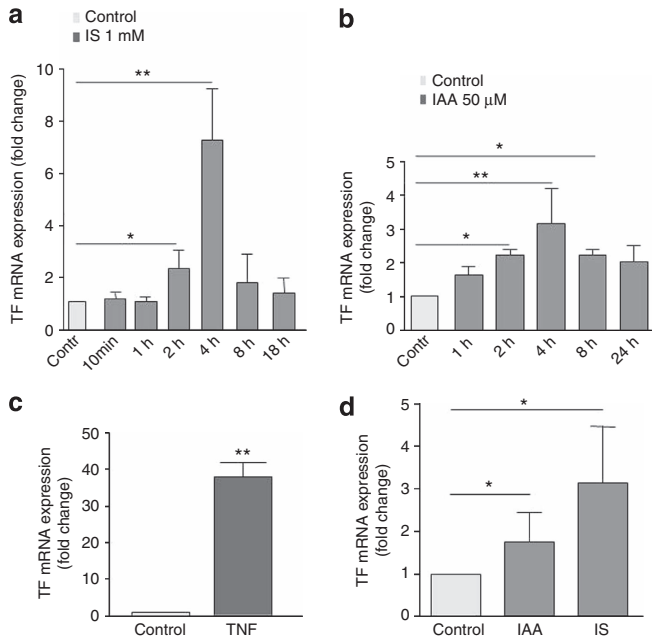

Figure 1 | Indoxyl sulfate (IS) (1 mmol/l) and indole-3-acetic acid (IAA; 50 µg/ml) increased tissue factor (TF) mRNA levels in human umbilical vein endothelial cells (HUVECs) and peripheral blood mononuclear cells (PBMCs). (**a**–**b**) HUVECs and (**d**) PBMCs obtained from healthy donors were incubated with indolic uremic solutes. mRNA levels of TF were quantified by quantitative comparative reverse transcriptase PCR. (**b**–**d**) Cells were incubated for 4 h with IS or IAA. HUVECs incubated with tumor necrosis factor (TNF) at a concentration of 25 ng/ml during 4 h were used as positive control (contr) for TF expression (**c**). Results are expressed in fold increase compared with cells exposed to control medium (KCl for IS, ethanol for IAA). Results represent the mean ± s.d. of four independent experiments. *$P<0.05$ versus control, **$P<0.01$ versus control.

IAA (Figure 1b). Indolic solutes increased TF expression in primary cultures of human endothelial cells from microvessels of heart and lung, and from coronary and pulmonary arteries (data not shown). The level of TF mRNA obtained with indolic solutes remained lower than those obtained with tumor necrosis factor (TNF), a positive control for TF induction (Figure 1c). The main circulating cells producing TF are PBMCs, and thus we studied TF gene mRNA levels in PBMCs incubated with IAA or IS. Expression of TF mRNA in PBMCs was increased after 4 h of incubation with IAA or IS (Figure 1d). In addition, an alternative splice of TF mRNA, asTF, without coding sequences for cytoplasmic and transmembrane domains was described.[20] We showed that IS and IAA could also increase asTF mRNA levels in HUVECs (Supplementary Figure S2 online).

As indolic uremic solutes upregulate TF at a transcriptional level, the next step was to determine whether TF protein was overproduced.

Indolic uremic solutes increased TF production *in vitro*

In HUVECs, we performed dose–response experiments analyzing the effect of IS and IAA (Figure 2a and b), at concentrations found in CKD patients.[12] First of all, we verified that IS at a concentration of 1 mmol/l and IAA at a concentration of 50 mmol/l did not induce endothelial lactate dehydrogenase release release (Supplementary Table S1 online). IS at concentrations of 0.1, 0.5, and 1 mmol/l increased endothelial TF production, measured with enzyme-linked immunosorbent assay, by 84% ($P<0.05$ vs. control), 106% ($P<0.05$ vs. control), and 135% ($P<0.0001$ vs. control), respectively (Figure 2a). IAA at a concentration of 50 µmol/l increased endothelial TF production by 52% ($P<0.001$ vs. control) (Figure 2b). The level of TF protein obtained with TNF is higher than those obtained with indolic solutes, and these levels were in accord with those obtained for mRNA (Figure 2c). This increase in TF protein level in HUVECs was confirmed by western blot analysis (Figure 2d). Next, we analyzed TF expression on endothelial cell surface by flow cytometry. TF membrane expression was significantly increased after 6 h of incubation with IS or IAA (Figure 2e and f). The maximum membrane expression was observed at 6 h of incubation with IS, with a return to basal levels at 12 h (Figure 2e). Moreover, the increase in TF production induced by uremic solutes IS and IAA was also found in PBMCs (Figure 2g).

Indolic uremic solutes increased TF procoagulant activity in endothelial cells and endothelial microparticles

To assess whether the increased TF production was associated with an enhanced procoagulant activity, we analyzed the TF-dependent procoagulant activity in HUVECs (Figure 3). After 6 h of incubation with IS at a concentration of 1 mmol/l (Figure 3a) or IAA at a concentration of 50 µmol/l (Figure 3b), factor Xa activity in HUVECs was significantly higher than that in control cells. A blocking TF antibody abolished TF procoagulant activity, whereas immunoglobulin G isotypic control had no effect. As IS increases the release of microparticles from endothelial cells,[14] we investigated whether these microparticles could convey procoagulant activity. We showed that microparticles from HUVECs incubated overnight with IS or IAA exhibited an increased procoagulant activity (Figure 3c and d).

Involvement of the AHR pathway in TF production by HUVECs

To confirm our hypothesis generated by microarray analysis on whether increased TF expression induced by indolic solutes was related to AHR activation, we used geldanamycin, an inhibitor of AHR.[21,22] Geldanamycin inhibits AHR by inhibiting the chaperone protein heat-shock protein 90, which is essential in the protection of the AHR from proteolysis. When geldamycin was added to HUVECs, TF expression induced by IS and IAA was markedly reduced, as shown in Figure 4a. We also confirmed the involvement of AHR in TF production by AHR silencing. AHR silencing led to a marked inhibition of AHR expression on mRNA and protein levels (Figure 4b and c). AHR silencing decreased the TF expression induced by IS and IAA at mRNA (Figure 4d and e) and protein (Figure 4f and g)

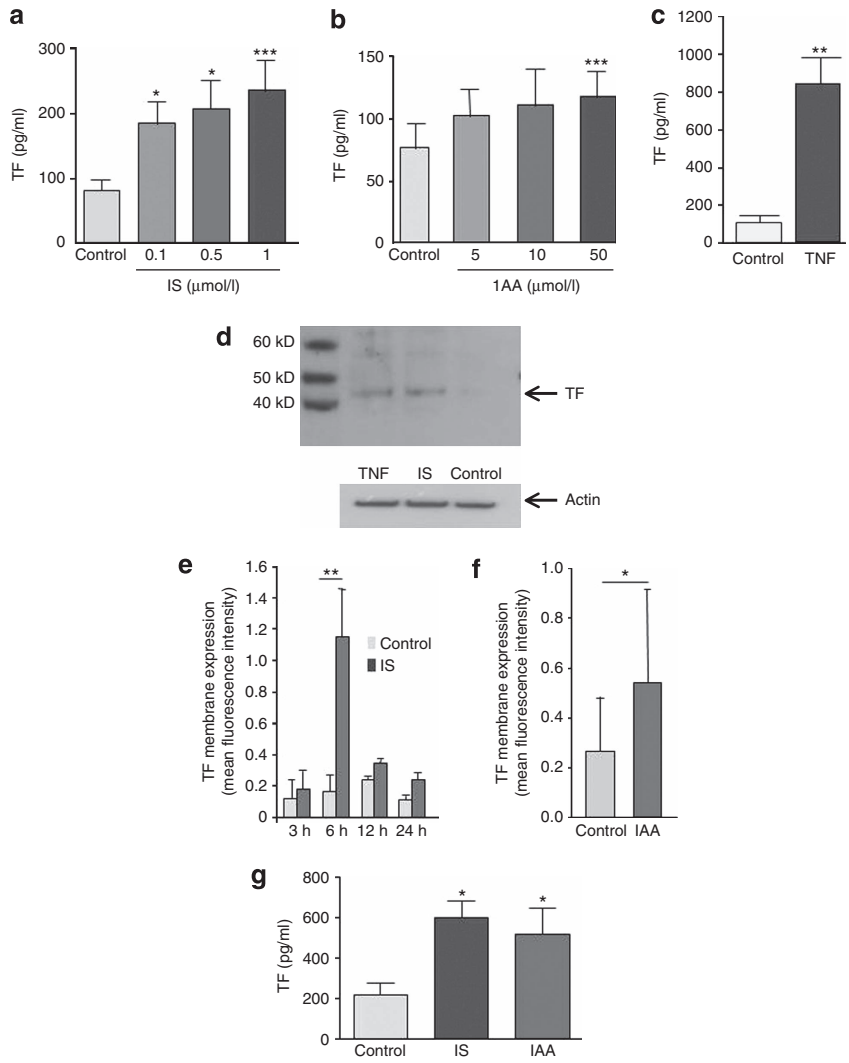

Figure 2 | Indoxyl sulfate (IS) and indole-3-acetic acid (IAA) increased tissue factor (TF) production. (**a**–**c**) TF protein levels measured by enzyme-linked immunosorbent assay (ELISA) in human umbilical vein endothelial cells (HUVECs) incubated 6 h with IS, IAA, or 25 ng/ml tumor necrosis factor (TNF). Results represent the mean ± s.d. of five, eight, and four independent experiments, respectively. (**d**) Western blot analysis of TF expression in HUVECs after 6 h of incubation with IS (1 mmol/l). (**e**) Membrane expression of TF analyzed by flow cytometry. Results represent the mean ± s.d. of five independent experiments. (**f**) Membrane expression of TF analyzed by flow cytometry in HUVECs after 6 h of incubation with IAA (50 μmol/l). Results represent the mean ± s.d. of six independent experiments. (**g**) TF protein levels measured by ELISA in peripheral blood mononuclear cells (PBMCs) obtained from healthy donors incubated with IS (1 mmol/l) and IAA (50 μmol/l) during 6 h. Results represent the mean ± s.d. of six independent experiments. *$P<0.05$ versus control, **$P<0.01$ versus control, ***$P<0.001$ versus control.

levels, whereas small interfering RNA (siRNA) control did not. TF procoagulant activity induced by IS and IAA was abolished by AHR silencing, whereas siRNA control had no effect (Figure 4h and i).

In PBMCs, the increase in TF production by IS and IAA was also AHR dependent, as geldanamycin completely abolished the effect of indolic solutes (Figure 4j–m). Furthermore, in PBMCs, IS and IAA enhanced the expression of *CYP1A1*, a well-known target of AHR, and this effect was also inhibited by geldanamycin (Supplementary Figure S3 online).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a well-known agonist of AHR.[23] We tested whether it could also lead to enhanced TF production. We found that TCDD increased the mRNA and protein levels of TF (Figure 5a and b), and consequently the procoagulant activity (Figure 5c) in HUVECs.

As nuclear factor-κB (NF-κB) was well known to induce TF expression,[24] we tested an inhibitor of NF-κB signaling pathway, wedelolactone, that inhibits NF-κB-mediated gene transcription by blocking the phosphorylation and degradation of IκBα. NF-κB inhibition by wedelolactone significantly decreased TF antigen levels induced by IS and IAA (Supplementary Figure S4 online). However, blocking the NF-κB signaling pathway did not lead to a complete inhibition of TF expression induced by indolic uremic solutes.

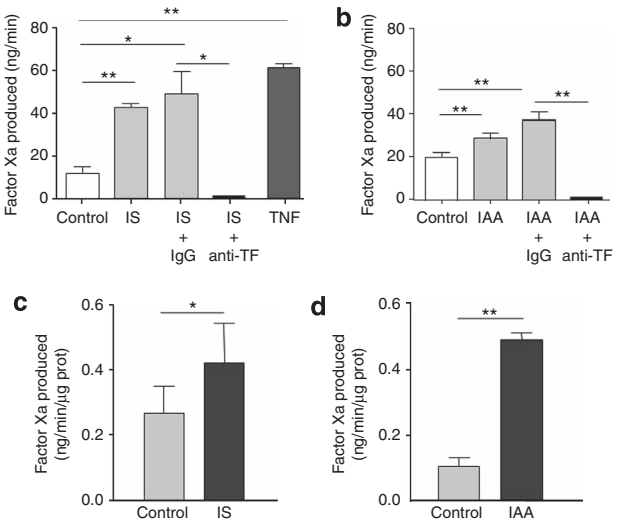

Figure 3 | Indoxyl sulfate (IS; 1 mmol/l) and indole-3-acetic acid (IAA; 50 µmol/l) increased tissue factor (TF) procoagulant activity in human umbilical vein endothelial cells (HUVECs) and in microparticles. Tissue factor activity was determined by measuring the production of factor Xa by a chromogenic assay. Tissue factor activity in cell lysates after incubation during 6 h with (**a**) IS, (**b**) IAA, and (**a**) tumor necrosis factor (TNF; 25 ng/ml, positive control). Tissue factor activity of microparticles produced by endothelial cells after overnight incubation with (**c**) IS or (**d**) IAA. Results represent the mean ± s.d. of (**a**, **b**) four or (**c**, **d**) three independent experiments. $^{*}P<0.05$ versus control,$^{**}P<0.01$ versus control.

IS and IAA induced the translocation of AHR from the cytoplasm to the nucleus

In addition, we showed by immunofluorescence microscopy that IS and IAA, as well as TCDD, could induce nuclear translocation of AHR (Figure 6a). We also showed that AHR is rapidly degraded upon IS or IAA treatment (Figure 6b and c), a process that follows AHR activation and translocation into the nucleus.[23] The effect was more pronounced in the presence of IS, as the decrease in AHR protein level persisted for a longer time than in the presence of IAA (Figure 6c).

Relationships between cTF and indolic uremic solutes in CKD patients

To strengthen our results *in vitro* that linked TF production and indolic uremic solutes, we studied 73 hemodialysis patients (hemodialyzed (HD) group), 50 undialyzed CKD patients (CKD group), and 43 control subjects. The baseline characteristics of the three groups of patients are described in Table 3. HD and CKD patients had higher blood pressure than controls. As expected, hemoglobin and albumin levels were lower in HD and CKD groups than in controls. Phosphorus, uric acid, urea, and creatinine levels were higher in CKD and HD groups. In the undialyzed CKD group, mean glomerular filtration rate (GFR), estimated by the simplified Modified Diet in Renal Disease formula (estimated GFR), was 27 ± 13 ml/min.

We measured cTF levels in plasma (Figure 7a). cTF includes asTF, TF produced by shedding of the full-length protein, and TF carried by microparticles. cTF levels were significantly higher in HD (142 ± 48 pg/ml, $P<0.0001$ vs. controls) and undialyzed CKD (86 ± 45 pg/ml, $P<0.0001$ vs. controls) patients than in controls (36 ± 14 pg/ml). Moreover, cTF levels were significantly higher in the HD group than in the CKD group ($P<0.0001$).

We analyzed the relationship between cTF levels and estimated GFR in 50 CKD patients. Estimated GFR and cTF levels were negatively correlated ($r=-0.25$; $P<0.05$) (Figure 7b). This link between the loss of renal function and the increased levels of cTF confirmed our hypothesis that some uremic solutes retained during renal impairment were implicated in this phenomenon. Therefore, we analyzed the relationships between cTF plasma levels and the serum concentrations of some uremic solutes in CKD and HD patients. cTF was positively correlated with IS ($r=0.39$; $P=0.006$) in CKD patients (Figure 7c) and with IAA ($r=0.42$, $P=0.003$) in HD patients (Figure 7d). cTF was not correlated with serum levels of the other uremic solutes measured, *p*-cresylsulfate, homocysteine, and β2-microglobulin, in both groups of patients (data not shown).

In addition, we analyzed the procoagulant activity of cTF in plasma of patients with CKD and controls by the calibrated automated thrombogram assay, which is suitable for numerous samples.[25] The lag time of thrombin generation (LT), which is a marker of TF contribution to thrombin generation,[25] was clearly shorter in undialyzed and hemodialyzed patients than in controls, indicating that their higher level of cTF level was associated with higher TF procoagulant activity (Figure 7e).

DISCUSSION

We demonstrated that indolic uremic solutes modulate TF production via the AHR pathway, a major mediator of organism response to xenobiotics.

We used a non *a priori* strategy based on expression microarray. Microarray analysis of HUVECs incubated with IS revealed an enrichment of genes controlled by the transcription factor AHR besides the TF gene (*F3*). IAA also enhanced AHR-dependent gene expression. The results of our gene-based strategy are supported by a recently published biochemical approach showing that IS and IAA are direct ligands for AHR.[26] AHR is involved mostly in detoxification processes, and its well-known exogenous ligand is TCDD.[23] AHR is constrained in a conformation receptive to ligand binding by the chaperone protein heat-shock protein 90.[21,22] We studied AHR involvement in TF induction by indolic uremic solutes. Geldanamycin, which leads to a total inhibition of AHR activity, inhibits IS- and IAA-induced TF expression in HUVECs and PBMCs. Once AHR is activated, it translocates rapidly into the nucleus to permit transcription of genes. After that, AHR joins the cytoplasm in order to be degraded. We clearly showed that IS, IAA, and TCDD were able to induce AHR translocation. In addition, we showed that the translocation of AHR induced by IS and IAA was followed by its degradation. By using endothelial cells transfected with siRNA for AHR, we showed that

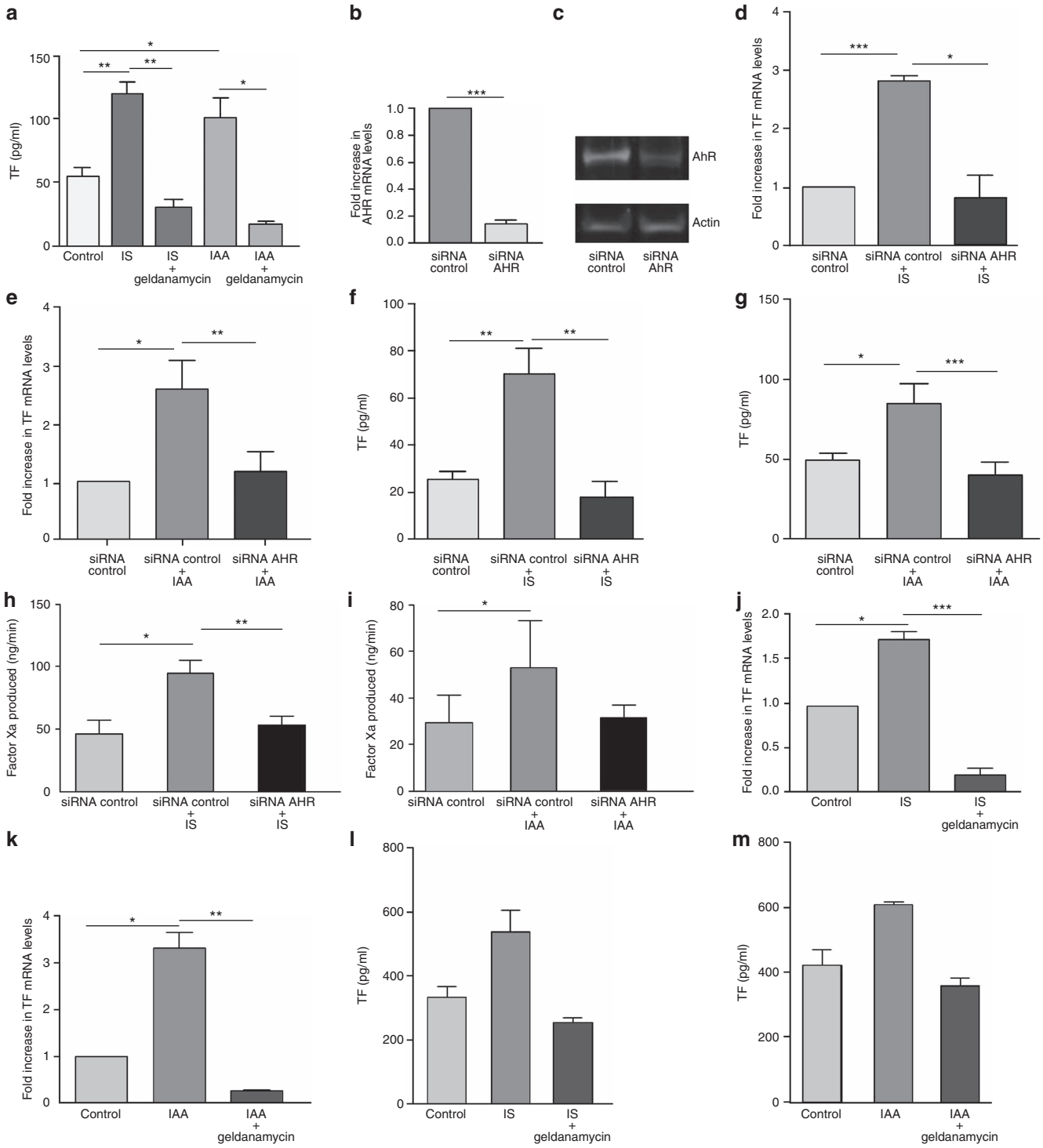

Figure 4 | Tissue factor (TF) expression and activity induced by indoxyl sulfate (IS; 1 mmol/l) and indole-3-acetic acid (IAA; 25 μmol/l) were aryl hydrocarbon receptor (AHR) dependent in human umbilical vein endothelial cells (HUVECs) and peripheral blood mononuclear cells (PBMCs). (**a**) TF protein levels measured by enzyme-linked immunosorbent assay (ELISA) in HUVECs incubated with IS or IAA for 6 h with geldanamycin, an inhibitor of the AHR pathway. Results represent the mean ± s.d. of six independent experiments. (**b**, **c**) Analysis of AHR mRNA and protein levels in HUVECs transfected with small interfering RNA (siRNA) control or siRNA AHR. Results represent the mean ± s.d. of three independent experiments. Analysis of (**d**, **e**) TF mRNA levels by reverse transcriptase PCR (RT-PCR), (**f**, **g**) TF protein levels by ELISA, and (**h**, **i**) TF activity induced by IS and IAA in HUVECs transfected with siRNA control or siRNA AHR. Results represent the mean ± s.d. of three independent experiments. Analysis of TF mRNA level by RT-PCR (**j**, **k**) and TF protein levels measured by ELISA (**l**, **m**) in PBMCs incubated with IS or IAA for 6 h with or without geldanamycin. *$P<0.05$ versus control, **$P<0.01$ versus control, ***$P<0.001$ versus control in PBMCs incubated with IS or IAA. Results represent the mean ± s.d. of (**a**–**k**) three and (**l**, **m**) two independent experiments.

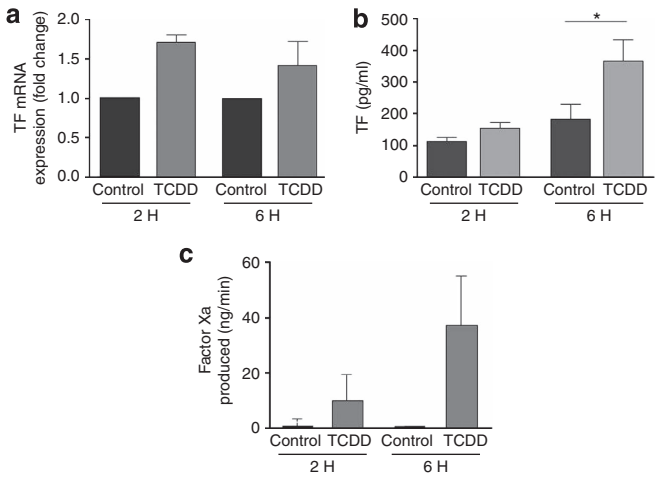

Figure 5 | 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induced tissue factor (TF) expression and activity in human umbilical vein endothelial cells (HUVECs). (**a**) TF mRNA levels quantified by reverse transcriptase PCR in HUVECs incubated with 30 nmol/l TCDD. (**b**) TF protein level in HUVECs incubated with 30 nmol/l TCDD. (**c**) TF activity in HUVECs incubated with 30 nmol/l TCDD. Results represent the mean ± s.d. of (**b**) three or (**a**, **c**) two independent experiments. *$P < 0.05$ versus control.

IS-induced TF expression was completely inhibited when AHR expression was markedly decreased. Finally, we found that the canonical AHR agonist TCDD was able to increase TF procoagulant activity. All these results demonstrate a crucial role of AHR in TF production. Thus, we provided a bunch of arguments to suggest that *F3* expression is controlled by the AHR pathway in endothelial cells and PBMCs. The peak of transcription occurs at 4 h and then decreases; this could be explained by the kinetics of AHR. We observed a complete degradation of AHR after 6 h, and it takes 24 h to replenish the cells with AHR. The molecular mechanisms leading to increased TF production upon stimulation are diverse. In endothelial cells, TF production is regulated by several signaling pathways such as MAPK/p38,[27] ERK,[24] and Akt,[28] with subsequent activation of transcription factors such as NF-κB or EGR-1; our data strongly suggest that AHR is a new transcription factor involved in the control of TF gene expression. The inhibition of NF-κB does not completely block the induction of TF, which is in contrast to AHR inhibition that completely blocks it. Therefore, the AHR has a critical role in the induction of TF dependent of uremic toxins. There is no consensus sequence for AHR binding (XRE) in the promoter of the TF gene. Three mechanisms implying AHR are possible. First, it has been shown that AHR could interact with other transcription factors such as NFκ-B to drive TF expression induced by indolic solutes, and in this case the XRE could be dispensable.[29,30] Second, AHR could participate in signaling pathways without DNA binding.[31] Third, recently, noncanonical XRE was described in the promoter of PAI-1,[32] and thus it is possible that a new noncanonical XRE sequence could be present in the *F3* promoter. More experiments are needed to conclude on the mechanism responsible for TF expression by AHR. To our knowledge, the involvement of AHR in TF production has never been described before. In the same way, another uremic toxin kynurenic acid is associated with elevated cTF levels in CKD patients,[33] and is also an AHR endogenous ligand.[34] A role of AHR activation in cardiovascular diseases has been described recently. Wu *et al.*[35] showed a time-dependent progression of atherosclerosis with an induction of inflammatory genes in ApoE-deficient mice when exposed to AHR activation by TCDD. AHR deficiency decreases lipopolysaccharide-induced expression of inflammatory genes, such as IL-8, which is involved in atherogenesis.[29] A recent study confirms the higher incidence of ischemic heart disease in former TCCD workers.[36] Therefore, the increased rate of cardiovascular diseases observed in CKD could share mechanisms with low-dose dioxin intoxication. Renal insufficiency could be seen as a 'dioxin-like' exposure. Beyond its potential role in the atherosclerotic process, AHR could be involved in the imbalance between vascular injury/regeneration in CKD. We have previously shown that CKD patients show a decreased number of CD34+ progenitor cells, and IAA is negatively correlated with CD34+ cells.[16] AHR inhibition promotes the expansion of CD34 hematopoietic stem cells ex vivo.[37] One could hypothesize that indolic uremic solutes, by activating AHR, promote a diminution of endothelial progenitor cells, leading to a deleterious effect on endothelial repair.

We showed that IS and IAA increased TF protein expression in endothelial cells and in PBMCs, which are two cellular sources of TF in blood.[38] This increased TF protein expression was associated with increased procoagulant activity. We were particularly interested in endothelial TF production, because we have already shown that IS is deleterious for the endothelium.[5,13,14,16] In addition, microparticles from endothelial cells incubated overnight with indolic solutes were able to convey a procoagulant activity. These results emphasize that TF produced by the constituents of the vessel wall after indolic solutes exposure could be a procoagulant. The PBMC product TF in response to IS or IAA. The monocytes are well-known factors in atherogenesis. The activation of PBMCs and dysfunction of endothelial cells induced by IS or IAA could have a role in accelerated thromboatherogenesis observed during CKD. In addition, even if TF expression by the endothelium is controversial, there are now *in vivo* evidences showing an endothelial-derived TF. Endothelial microparticles and circulating endothelial cells, which are positive for TF, are found in patients with sickle cell disease,[38,39] a pathology also characterized by a proinflammatory and procoagulant state. In addition, in mouse model of sickle cell disease, endothelial cells clearly express TF[40], which participates in endothelial injury.[41,42] The real toxic concentrations of IS or IAA *in vivo* are not known because of their binding to plasma proteins. Therefore, the effect observed, *in vitro*, could overestimate the real ones.

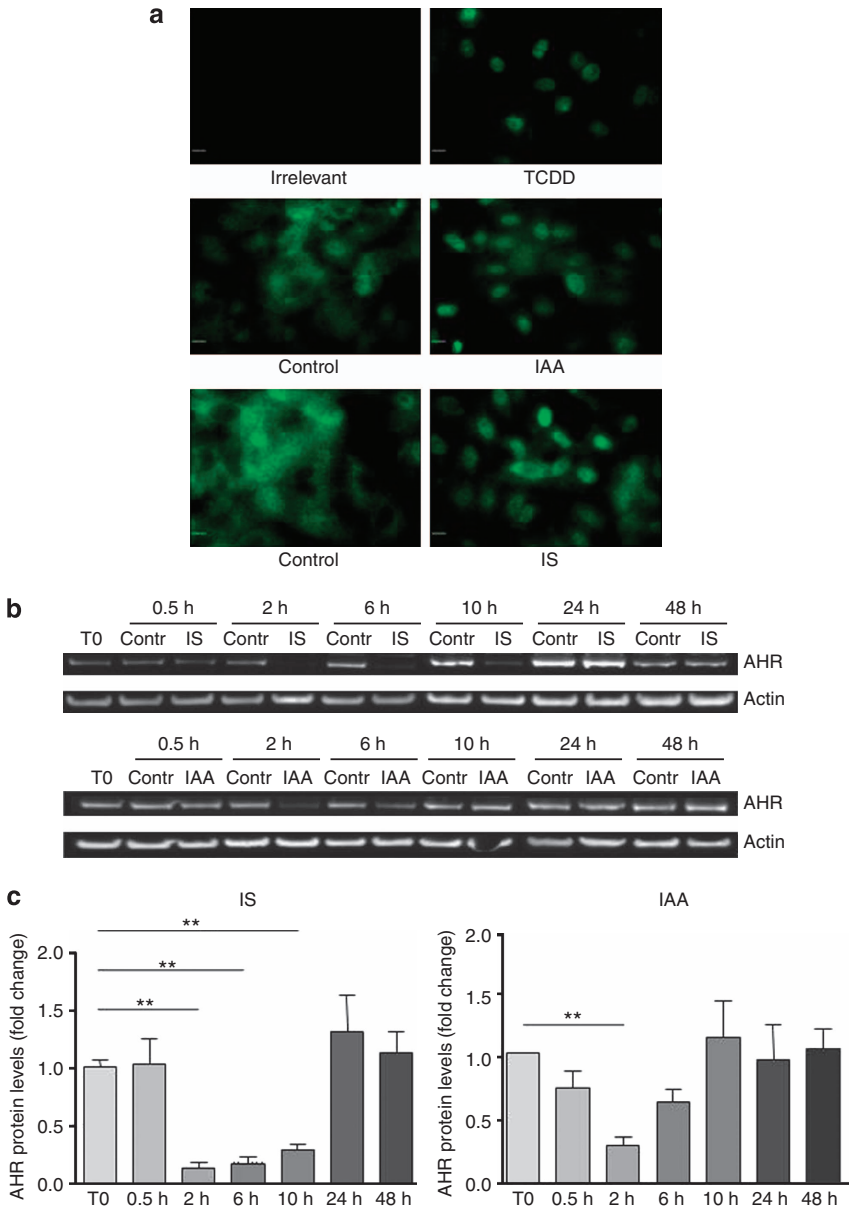

Figure 6 | Indoxyl sulfate (IS; 1 mmol/l) and indole-3-acetic acid (IAA; 50 μmol/l) induced changes in aryl hydrocarbon receptor (AHR) localization and expression in human umbilical vein endothelial cells (HUVECs). (**a**) Immunofluorescence microscopy of HUVECs incubated during 40 min in the different indicated conditions. Fixed cells were incubated with antibody against AHR or irrelevant isotype control (Contr). IS (1 mmol/l), IAA (50 μmol/l), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 30 nmol/l) induced AHR redistribution from the cytoplasm to the nucleus compared with controls. Representative images of three independent experiments. (**b**) Western blot with total cell extracts from HUVECs incubated with IS (1 mmol/l) or IAA (50 μmol/l) solutes during the indicated times and revealed with and antibody against AHR and with a secondary peroxidase-conjugated antibody. Actin staining was used as a loading control. IS and IAA induced a decrease in AHR protein levels. Representative images of three independent experiments. (**c**) Densitometry analysis of previous western blots of AHR protein performed with Genesys software. $^{**}P < 0.01$ versus control.

In addition to our *in vitro* data, we confirmed that cTF levels are highly increased in CKD patients.[10,33] Furthermore, we showed that procoagulant activity of TF was also increased in CKD patients. In addition, cTF levels were negatively correlated with estimated GFR, confirming the possible role of accumulation of uremic toxins. We therefore analyzed the relationship between some uremic solutes (β2-microgobulin, *p*-cresylsulfate, homocysteine, IS, IAA) and cTF levels in CKD patients. Among all the solutes we measured, only the indolic solutes were correlated with TF levels. It could be of great interest to study the kynurenin levels, to identify which tryptophan product of degradation is mainly involved in AHR stimulation *in vivo*. Such relationships suggested that these solutes could participate in TF production *in vivo*. cTF is a 43-kD-molecular-weight protein. It could be filtered by the glomerulus and could be

Table 3 | Baseline characteristics of controls, undialyzed CKD patients, and HD patients

	Controls ($n = 43$)	CKD ($n = 50$)	HD ($n = 73$)
Age (years)	64 (38; 78)	63 (26; 89)	68 (23; 91)
Sex ratio (F/M)	23/20	19/31	31/42
Dialysis vintage (months)	—	—	6 (0.75; 30)
Body mass index (kg/m^2)	25.12 ± 3.33	25.04 ± 4.4	24.3 ± 5.2
Kidney disease			
Glomerulonephritis	—	7 (14%)	17 (23%)
PKD	—	5 (10%)	5 (7%)
Vascular nephropathy	—	11 (22%)	22 (29%)
Interstitial nephropathy	—	15 (29%)	10 (13%)
Other hereditary	—	3 (6%)	2 (3%)
Unknown	—	9 (17%)	17 (22%)
SBP (mm Hg)	128.9 ± 19.06	141.7 ± 23.94[a]	144.4 ± 24.31[a,b]
DBP (mm Hg)	75.49 ± 10.27	81.18 ± 11.24[a]	73.26 ± 15.87[b]
Smokers	—	26 (51%)	27 (36%)
History of cardiovascular disease	0	16 (31%)	25 (34%)
Hypertension	2 (5%)	44 (86%)	54 (73%)
Statins	0	15 (29%)	25 (34%)
Antihypertensive agents	2 (5%)	44 (86%)	40 (54%)
Aspirin or antiaggregant agents	0	11 (22%)	38 (51%)
EPO therapy	0	11 (22%)	55 (74%)
Anticoagulant agents	0	8 (16%)[a]	16 (21%)[a,b]
Hemoglobin (g/dl)	14.27 ± 1.03	12.5 ± 1.8[a]	11.66 ± 1.6[a,b]
Albumin (g/l)	40 (31; 59)	37 (31; 41)[a]	35 (26; 46)[a,b]
Calcium (mmol/l)	2.31 (2.17; 2.52)	2.33 (2; 2.55)	2.36 (1.84; 2.66)
Phosphorus (mmol/l)	1.09 (0.74; 1.46)	1.14 (0.65; 2.20)	1.46 (0.54; 3.17)[a,b]
Uric acid (3 mol/l)	297 (197; 451)	438 (175; 828)[a]	342 (197; 524)[b]
Urea (mmol/l)	5.5 (3.2; 9.3)	14.8 (4.9; 44.7)[a]	19 (7.6; 39.6)[a,b]
Creatinine (mol/l)	69 (48; 107)[a,b]	209 (84; 640)[a]	783 (213; 1533)[a,b]

Abbreviations: CKD, chronic kidney disease; DBP, diastolic blood pressure; EPO, erythropoietin; HD, hemodialyzed; PKD, polycystic kidney disease; SBP, systolic blood pressure.
Results are given as mean ± s.d. if Gaussian distribution or in median (min.; max.) if not.
[a]Significantly different versus control.
[b]Significantly different versus CKD.

retained during CKD as other molecules in this molecular-weight range. The high levels observed in the plasma certainly reflect an increased production, which is in line with our *in vitro* data.

TF is implicated in the pathogenesis of several cardiovascular disorders and in the development of atherosclerotic diseases.[43] Oxidized LDL enhances TF production in endothelial cells[44] and monocytes.[45] In the early stages of atherogenesis, TF is expressed in monocytes[45] and in the later stages in foam cells, endothelial cells, and smooth muscle cells.[46] TF is also closely related to apoptosis in lipid-rich plaques[47] and to plaque vulnerability.[48] cTF is elevated in CKD patients,[10] and several uremic solutes raise TF production in different cell types: ADMA (asymmetric dimethylarginine) increases monocyte TF antigen;[49] Advanced glycation end products (AGE) enhance endothelial TF production;[50] and kynurenin is associated with cTF levels and hypercoagulability in CKD patients.[33] Here, we showed that IAA and IS are among the uremic solutes able to increase TF production. Increased production of TF by the vessel wall of CKD patients exposed to uremic solutes could lead to accelerated atherosclerosis and could explain the high rates of cardiovascular mortality observed in this population. It is noteworthy that, compared with the control population in which statin was not used, 30% of patients with CKD used statins. Statins are well known to reduce TF production by endothelial cells or monocytes. Despite the used of this drug, the TF levels is clearly increased in CKD patients, the reduced estimated GFR could lead to accumulation or more certainly to increase production not counteract by statins. It confirms the important role of AHR-dependent production of TF in CKD.

In conclusion, our study shows that two indolic uremic solutes IAA and IS induce TF production via AHR activation. We propose that at least two processes participate in accelerated atherogenesis in CKD patients related to indolic uremic solutes: procoagulant state induced by increased TF expression and a direct proatherogenic effect of AHR activation. This new concept in cardiovascular diseases linked to CKD has to be confirmed by clinical studies.

MATERIALS AND METHODS

Endothelial cell culture

HUVECs were obtained from umbilical cord vein as previously described,[51] grown in EGM-2 medium and used until the fourth passage.

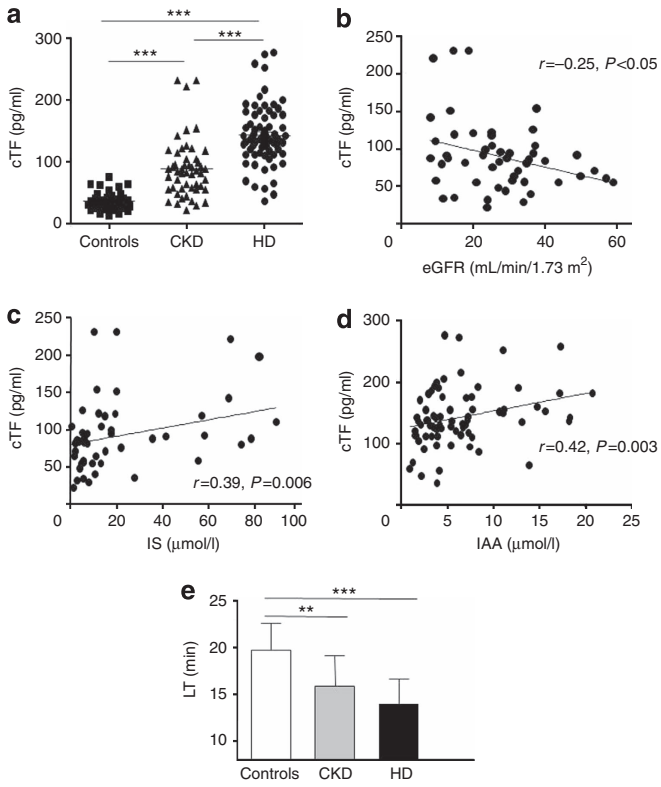

Figure 7 | Tissue factor (TF) levels and activity and relationship with Modified Diet in Renal Disease (MDRD) and indoxyl sulfate (IS) and indole-3-acetic acid (IAA) levels in chronic kidney disease (CKD) patients. (**a**) TF levels were higher in hemodialyzed (HD) and CKD patients than in controls (both $P<0.0001$). Circulating TF (cTF) levels were increased significantly in HD versus CKD ($P<0.0001$). (**b**) Levels of soluble TF in CKD patients ($n=50$) were inversely correlated with glomerular filtration rate estimated by the MDRD formula (eGFR). Data obtained using a Spearman's correlation coefficient. cTF was positively correlated with (**c**) IS in CKD patients and with (**d**) IAA in HD patients. Relationships were determined using a Spearman correlation test. (**e**) Lag time (LT) of thrombin generation of healthy, undialyzed, and HD CKD plasmas. Plasmas from undialyzed and HD CKD displayed and shorter LT than plasma controls.

Cell treatment with uremic solutes and TCDD

Details for uremic toxins and dioxin preparations are found in Supplementary Methods online.

DNA microarray analysis

HUVECs were incubated with IS at a concentration of 250 µg/ml or KCl at a concentration of 1 mmol/l for 4 h. Seven biological replicates were obtained. Processes for total RNA preparation, quantification, labeling, and hybridization are given in Supplementary Methods online.

Comparative quantification of mRNA levels

Details on RT-PCR reactions and oligonucleotide sequences are found in Supplementary Methods online.

Measurement of TF by enzyme-linked immunosorbent assay

TF was quantified in human plasma and cell lysates of HUVECs and PBMCs with the enzyme-linked immunosorbent assay kit Quantikine Human Coagulation Factor III/Tissue Factor (R&D Systems, Lille, France) according to the instructions of the manufacturer.

Immunofluorescence assay and flow cytometry analysis of TF membrane expression

Details are available in Supplementary Methods online.

Western blotting

Details are available in Supplementary Methods online.

Endothelial microparticle preparation

Details are available in Supplementary Methods online.

Procoagulant activity of TF

Total cell and microparticle-associated TF activity was measured in 96-well microplates using TF capacity to generate activated factor X, whereas factor VII (Kordia, Lille, France) and X (Kordia) were added in excess. All details of this reaction are given in Supplementary Methods online.

Gene silencing

Details are available in Supplementary Methods online.

Patients

We studied 73 HD patients and 50 nondialyzed CKD patients, selected according to the criteria listed in Supplementary Methods. The control subjects were recruited by the Centre d'Investigation Clinique of Assistance Publique-Hopitaux de Marseille. All study participants gave their written informed consent, and the study was approved by the local ethics committee and adhered to the Declaration of Helsinki Principles. The characteristics of patients and control subjects are listed in Table 3.

Uremic toxin measurement

Details are available in Supplementary Methods online.

Calibrated automated thombogram assay

Thrombin generation was measured using a calibrated automated thrombogram assay (Thrombinoscope BV, Maastricht, The Netherlands), which was performed on plasma of 10 subjects in each group (controls, undialyzed CKD and HD CKD). Details of the reaction are given in Supplementary Methods online.

Statistical analysis

Details are available in Supplementary Methods online.

DISCLOSURE

All the authors declare no competing interests.

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We thank Karim Fallague for preparing HUVECs, Tacy Santana for preparing the other primary cultures of endothelial cells, and Christine Scagliarini for technical assistance. This work was supported by a grant of 'Société Francophone de Dialyse'.

SUPPLEMENTARY MATERIAL

Figure S1. mRNA levels of ICAM-1 and MCP-1 in HUVECs incubated with IS or IAA.

Figure S2. IS (1 mmol/l) and IAA (50 µmol/l) increased mRNA levels of alternatively spliced TF.

Figure S3. Geldanamycin, an AHR pathway inhibitor, decreased *CYP1A1* expression induced by indolic solutes in PBMCs.
Figure S4. Wedelolactone decreased TF protein level induced by IS (1 mmol/l) and IAA (50 μmol/l).
Figure S5. Polymyxin B did not alter the expression of TF induced by IS and IAA.
Table S1. Effect of IS and IAA on endothelial lactate dehydrogenase release release.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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IV) DISCUSSION ET PERSPECTIVES

Dans ce travail de thèse, nous présentons une hypothèse physiopathologique originale pouvant expliquer en partie la surmortalité cardiovasculaire présentée par les patients IRC. Les toxines urémiques dérivées des indoles augmentent l'expression du facteur tissulaire, un agent procoagulant, dans les cellules constituantes de la barrière vasculaire normalement anticoagulante, les cellules endothéliales. L'hypercoagulabilité de la paroi vasculaire induite par les indoles est susceptible de jouer un rôle dans l'augmentation des événements thrombotiques et athérothrombotiques observés au cours de l'insuffisance rénale chronique. Cette activation du FT est dépendante de la voie AHR, un médiateur majeur de la réponse aux xenobiotiques. Nous avons confirmé par une approche méthodologique différente les résultats récents ayant identifiés l'IS et l'IAA comme des ligands directs d'AHR (Schroeder JC Biochemistry 2010). Le lien entre AHR et FT n'avait jamais été décrit auparavant.

Les voies de signalisation jouant sur la production du FT sont diverses. Dans les cellules endothéliales, le FT est régulé par plusieurs voies comme MAPK/p38 –Nf-kB [49] ou bien Akt [50]. Dans notre étude, nous montrons un rôle conjoint de Nf-kB dans la production du FT.

D'autres toxines urémiques comme les kynurenines sont associées aux taux de FT chez les patients IRC [46]. Elles ont récemment été décrites comme des ligands d'AHR [27]. Des données récentes nous permettent de suspecter un rôle de l'activation d'AHR dans la genèse et l'aggravation des maladies cardiovasculaires [29]. Notre étude suggère que l'intoxication endogène chronique des patients IRC par les solutés urémiques mime l'intoxication exogène par la dioxine en tout cas sur la production de FT.

De plus, le facteur tissulaire est impliqué dans la physiopathologie des atteintes cardiovasculaires et dans l'athérogénèse. Par exemple, le LDL oxydé augmente la production endothéliale et monocytaire de FT [51,52]. Les solutés indoliques semblent avoir la même

capacité. Dans les stades précoces d'athérogénèse, le FT est sur exprimé dans les plaques riches en lipides [53] et est associé à la vulnérabilité de cette même plaque [54]. Une augmentation du FT circulant est observée chez les patients IRC [55].

Le FT étant une protéine de 43 kDa, nous pourrions penser que les taux élevés rencontrés chez les IRC soient secondaires à une rétention plutôt qu'à une hyperproduction. Notre étude montre une augmentation de la production de FT à un niveau transcriptionnel.

Ce travail ouvre de nombreuses perspectives. In vitro, il sera intéressant de déterminer comment AHR active le promoteur du FT, car il n'existe pas de séquence spécifique XRE à son niveau. Une possibilité est que cette augmentation de la production passe par la voie dite non génomique ou par une voie génomique non classique. En effet, récemment, Chitalia et al ont montré que le FT est augmenté dans les cellules musculaires lisses (CML) après exposition à des toxines urémiques. La surexpression n'est pas due à une augmentation transcriptionnelle mais à des modifications posttranslationnelles: inhibition de l'ubiquitinylation et augmentation de la stabilité de la molécule. [56]. Il serait intéressant de rechercher si ce mécanisme est limité au CML ou s'il existe aussi dans les cellules endothéliales. De plus, une association AHR et Nf-kb a été décrite pour participer à la production de molécules pro inflammatoires comme l'IL-6 [57]. Ce mécanisme pourrait être envisagé pour la production de FT endothelial via l'IS et l'IAA.

Nous avons prévu dans un futur proche de confirmer les données de notre étude dans un modèle de souris rendues urémiques par une néphrectomie des 5/6 èmes et dans un modèle d'animaux perfusés avec ces toxines. Notre but est de montrer une augmentation de la production de FT au niveau de la paroi vasculaire. Enfin, chez les patients IRC, notre but sera de montrer une hyperactivation des gènes dépendants d'AHR en réalisant des études d'expression génique sur sang total et en étudiant la capacité du sérum de ces patients à activer AHR sur des lignées cellulaires dédiées.

V) CONCLUSION

Dans ce travail de thèse, nous avons montré que l'IS et l'IAA induisent une augmentation de la production et une activation du FT via une activation de la voie AHR. Cette surproduction de FT a un retentissement pathologique puisqu'elle entraine une hypercoagulabilité in vitro et un état procoagulant du plasma de patients IRC. De fait, les toxines urémiques participent au moins en partie au déséquilibre de l'hémostase rencontré chez les patients IRC. Nous décrivons ces solutés comme des acteurs importants dans la mortalité cardiovasculaire par deux phénomènes qui sont liés : un état procoagulant du à la production de FT et un effet pathogénique direct de l'activation d'AHR sur la paroi vasculaire. L'approche de ce travail est inédite puisque nous considérons des toxines urémiques endogènes, produites par métabolisme intestinal et mal éliminées chez l'IRC, au même titre que des composés toxiques exogènes comme la dioxine. Les études thérapeutiques à venir visant à diminuer la surmortalité cardiovasculaire des patients IRC pourraient avoir pour cible AHR, par l'utilisation d'inhibiteurs pharmacologiques par exemple, en essayant d'améliorer l'épuration des toxines urémiques liées aux protéines, ou en diminuer l'absorption intestinale.

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