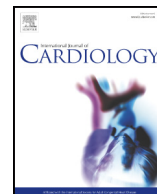




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## Review

## Cardiorenal disease connection during post-menopause: The protective role of estrogen in uremic toxins induced microvascular dysfunction

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## ABSTRACT

Female gender, post-menopause, chronic kidney disease (CKD) and (CKD linked) microvascular disease are important risk factors for developing heart failure with preserved ejection fraction (HFpEF). Enhancing our understanding of the interrelation between these risk factors could greatly benefit the identification of new drug targets for future therapy. This review discusses the evidence for the protective role of estradiol ( $E_2$ ) in CKD-associated microvascular disease and related HFpEF. Elevated circulating levels of uremic toxins (UTs) during CKD may act in synergy with hormonal changes during post-menopause and could lead to coronary microvascular endothelial dysfunction in HFpEF. To elucidate the molecular mechanism involved, published transcriptome datasets of indoxyl sulfate (IS), high inorganic phosphate (HP) or  $E_2$  treated human derived endothelial cells from the NCBI Gene Expression Omnibus database were analyzed. In total, 36 genes overlapped in both IS- and HP-activated gene sets, 188 genes were increased by UTs (HP and/or IS) and decreased by  $E_2$ , and 572 genes were decreased by UTs and increased by  $E_2$ . Based on a comprehensive *in silico* analysis and literature studies of collected gene sets, we conclude that CKD-accumulated UTs could negatively impact renal and cardiac endothelial homeostasis by triggering extensive inflammatory responses and initiating dysregulation of angiogenesis.  $E_2$  may protect (myo)endothelium by inhibiting UTs-induced inflammation and ameliorating UTs-related uremic bleeding and thrombotic diathesis via restored coagulation capacity and hemostasis in injured vessels.

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### 1. Complex interrelationship between chronic kidney disease and heart failure with preserved ejection fraction

Heart failure (HF) is a growing major public health problem that affects ~2% of the western population [1]. It has two main subtypes, HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF). Within the HF population, more than 50% suffer from HFpEF [2]. Interestingly, chronic kidney disease (CKD) occurs in 26% to 53% of the HFpEF population and the subclinical diastolic dysfunction appears to be the most common echocardiographic feature in asymptomatic CKD patients on hemodialysis, suggesting a strong link between CKD and HFpEF [3,4]. Furthermore, clinical studies showed a

linear relationship between the progression of CKD and the worsening of longitudinal function of the left ventricle in the HFpEF population [5]. The cardiac parameters in patients with CKD stage 2 and 3 already resemble early HFpEF, and the cardiac mechanics have been reported to become worse in patients with CKD stage 4 and 5. In a large cohort study on the development of heart dysfunction during 11 years of follow-up, Brouwers and colleagues demonstrated that increased urinary albumin excretion and cystatin C were more associated with the onset of HFpEF when compared to HFrEF [6]. In particular, older females with increased urinary albumin excretion or cystatin C were more vulnerable to develop HFpEF. These findings indicate a clear association between CKD and HFpEF, especially in the elderly female population.

Important findings in the field further prove that impaired renal function is a major risk for developing HFpEF [7]. Although several mechanisms underlying how CKD contribute to HF in general have been well established, including increased inflammatory responses and activated neurohormonal pathways [8], studies on the driving mechanisms on CKD-related HFpEF are limited. A recent publication by Paulus et al.

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proposed a disease mechanism in which renal dysfunction caused systemic changes in circulating factors that activated inflammation and led to microvascular disease (MVD), cardiomyocyte stiffening, and a hypertrophic response [7]. MVD often occur ubiquitously throughout the body in patients with cardiovascular disease [9]. Paulus and co-workers further proposed that identified metabolic syndrome linked cardiovascular comorbidities, such as diabetes and obesity, could act as inducers of systemic inflammation that trigger global and coronary endothelial dysfunction, leading to myocardial hypertrophy, impaired myocardial relaxation and increased myocardial stiffness [10]. In relation to cardiovascular disease in general, endothelial dysfunction is well known to be able to serve as a strong predictor of coronary artery disease onset [9]. CKD, a common cardiovascular comorbidity associated with metabolic risk factors, leads to hyperphosphatemia and accumulation of uremic toxins (UTs) that trigger inflammation and MVD, which could subsequently contribute to HFpEF onset and progression [11].

Early stage of kidney disease can already be detected by the presence of proteinuria, and a continuous retention of UTs results in a toxic circulatory environment due to the reduction of glomerular filtration rate during the progression of CKD [12]. Serum levels of some uremic toxin compounds, such as methionine sulfoxide and hydroxyproline, accumulate significantly as glomerular filtration rate declines and have been proposed as markers for detecting early stage of CKD [13]. Most UTs circulate in the bloodstream in albumin bound form, and they are not able to directly pass an intact endothelial barrier [14,15]. However, alterations in endothelial barrier do occur in response to various inflammatory mediators and atherogenic metabolic particles [16]. Many UTs, such as indoxyl sulfate and p-cresyl sulfate, have been shown to compromise the endothelial barrier function [17], which could promote protein leakage, causing direct exposure of surrounding non-vascular cells like cardiomyocytes to (protein bound) UTs. However, the exact mechanisms underlying CKD-triggered MVD and corresponding HFpEF remain to be further elucidated, especially at the molecular level.

In this review we focused on the microvasculature in female CKD patients before and after menopause, which will improve our understanding on the subsequent development of HFpEF. Firstly, female gender and aging, two major risk factors of HFpEF, will be addressed. Secondly, the evidence for the role of estrogen ( $E_2$ ) mediated protection mechanisms in the onset and progress of renal disease-related MVD and HFpEF will be summarized. Finally, we will further discuss the new information that we have gathered from the analysis of publicly available NCBI Gene Expression Omnibus (GEO) database sets for the transcriptome response of endothelial cells (ECs) to  $E_2$  and CKD associated circulatory factors. Based on this, we propose putative pathways of CKD-related MVD that are susceptible to  $E_2$  protection.

## 2. Postmenopausal women are at high risk of developing HFpEF

The prevalence of HFpEF increases with age and HFpEF patients are typically older than those with HFrEF [18]. In general, the average age of HFpEF patients are between 73 and 79 years old [19]. Aging has been proposed as an independent risk factor for abnormal diastolic function [20]. Age-dependent increase in left ventricular mass index has been observed in humans, and age-dependent increase in cardiomyocyte size has been observed in animals. In addition, increased interstitial fibrosis has also been noticed in aged myocardium. These changes due to aging contribute to myocardial stiffness, putatively leading to diastolic dysfunction in HFpEF. Unfortunately, clinical trial data of treatments for HF were mostly collected from the young and the middle-aged patients, leading to the lack of adequate evidence in treating the elderly, not to mention the older HFpEF patients specifically [21].

Besides the elderly population, women are consistently ~2 times more at risk than men to develop HFpEF and outnumber men by a 2:1 ratio in the HFpEF patient population [22]. Women also differ from the

male HFpEF population as they show less evidence of coronary artery disease but are more vulnerable for coronary MVD, indicating a sex-based difference in the underlying pathology of HFpEF [22,23]. Left ventricular diastolic dysfunction (LVDD) can be considered as a pre-stage of HFpEF. In the female population, LVDD onset and progression into HFpEF is strongly associated to the postmenopausal period [24]. High estrogen levels appear to protect the premenopausal heart from ventricular remodeling triggered by hypertension, although the specific mechanism remains to be further defined. Therapeutic interventions for HFpEF have failed to improve the mortality rate. At the moment early detection and treatment of LVDD appear to be the only effective strategy to prevent progression into HFpEF.

Since both aging and female gender appear to be important risk factors for LVDD and HFpEF, it has been postulated that gender specific hormones and changes in hormone levels may play an important role in the higher prevalence of HFpEF in women, particularly in the postmenopausal population [25]. Studies in the early 1990s already showed the beneficial effects of menopausal hormone therapy (MHT) on preventing coronary heart disease [26]. However, subsequent randomized clinical trials failed to demonstrate that MHT prevents secondary events in ischemic heart disease, cerebrovascular events, or progression of coronary atherosclerosis in postmenopausal patients with already established coronary disease [27,28]. Recently, reevaluation of those trials and the initiation of new clinical trials have shed light on how to improve MHT. In particular, the effects of early versus late MHT intervention were evaluated, as comparison between previous MHT responsive and non-responsive groups have indicated that women who received intervention at the early postmenopause stage without pre-existing coronary disease were more likely to benefit from MHT than older patients with pre-existing coronary disease [29]. A recent retrospective single-center study showed that MHT was significantly associated with improved left ventricular relaxation indices, which is in line with the reported improvement in diastolic function following MHT in postmenopausal women, pointing towards the need for further investigation of the use of MHT in treatment of HFpEF [30]. Together, these clinical studies indicate the postmenopausal women are at high risk of developing HFpEF.

## 3. Postmenopausal estrogen depletion in female CKD patients and microvascular dysfunction

A limited number of studies have started to reveal the putative disease mechanisms of LVDD and HFpEF in women with CKD. An in vitro study showed that the contraction rate in uremic toxin p-cresol treated cardiomyocytes was decreased, and p-cresol impaired cardiomyocytes gap junctions by increasing the activity of protein kinase  $C\alpha$  [31]. UTs have also been shown to induce cardiac remodeling response via estrogen receptor dependent mitogen-activated protein kinase and nuclear factor- $\kappa$ B pathways, suggesting that estrogen receptor signaling could interfere with the negative effects of UTs [32].

Brunet et al. have summarized two major mechanisms of how UTs contribute to vascular dysfunction [33]: (1) UTs promote inflammation by stimulating leukocyte activation and endothelial adhesion molecule expression. Activated inflammation and immune responses increase the migration and proliferation of vascular smooth muscle cells (VSMCs). However, UTs also inhibit the proliferation of ECs and enhance the apoptosis of endothelial progenitor cells, thus impairing vascular repair. (2) UTs stimulate the transdifferentiation of VSMCs into osteoblast-like cells and reduce digestibility of collagen and other extracellular matrix proteins by forming irreversible crosslinks, which subsequently lead to an increased vessel stiffness and vascular dysfunction. Among over 150 UTs that have been listed to date, some UTs like indole-3-acetic acid strongly accumulate in the circulation of patients who are still in an early stage of CKD as compared to normal levels observed in healthy individuals [34]. The concentrations of 11 different uremic toxins have been reported to be 2.3 to 44.7 times increased in

patients with stage 3 and stage 4 CKD (moderate to severe CKD stage but before stage 5 (dialysis stage)) versus the plasma levels found healthy controls [35]. Protein-bound UTs, such as ADMA, p-cresyl sulfate and indoxyl sulfate (IS), also accumulate in patients with early CKD stages with continuous concentration build up during CKD progression [36]. These protein-bound toxins have been shown to exhibit high endothelial and vascular toxicity [36,37]. A common pathway for these UTs is the activation of NAD(P)H oxidase, a reactive oxygen species (ROS) inducer, leading to oxidative stress in ECs and a reduction of nitric oxide (NO) bioavailability in the micro-environment [38]. NO contributes to vasorelaxation and inhibits platelet aggregation, expression of adhesion molecules and proliferation of VSMCs. Through activated oxidative stress and the subsequent activation of the p38/ mitogen-activated protein kinase pathway, UTs also affects immune cells as demonstrated by increased cell-surface expression of the immune activation marker beta2-integrin Mac-1 (CD11b/CD18) in the leukocyte and monocyte cell populations of CKD patients.

Unlike the deleterious influence of UTs on endothelium, clinical studies showed that E<sub>2</sub> administration increased flow-mediated vasodilation response, indicating that E<sub>2</sub> is an important regulator of protective endothelial function [39]. The non-genomic and genomic pathways of E<sub>2</sub> that act via its three receptors (ER $\alpha$ , ER $\beta$ , and GPER) have been shown to be able to increase endothelial NO synthase (eNOS) in various cell types [39, 40]. In E<sub>2</sub> treated human ECs, ER $\alpha$  signaling via PI3K triggers the activation of protein kinase B, extracellular-signal-regulated kinase 1/2, and phosphorylation and activation of eNOS. Increased eNOS and NO bioavailability promote vascular relaxation, ECs migration and proliferation. A rapid increase in intracellular calcium is also observed after E<sub>2</sub> stimulation [40]. A recent paper demonstrated that activated GPER increased the expression of calmodulin and prolonged cytoplasmic Ca<sup>2+</sup> signals via the transactivation of epidermal growth factor receptor and the activation of mitogen-activated protein kinase cascade in porcine aortic ECs [41]. Calmodulin is a transducer of Ca<sup>2+</sup> signals, and an activated calmodulin/Ca<sup>2+</sup> system is able to modulate eNOS function. Interestingly, E<sub>2</sub> treatment was shown to increase the number of endothelial progenitor cells in a NO-dependent way, which in return restored vascular repair activities [42]. Furthermore, Osako et al. showed that the activation of ER $\alpha$  suppressed the cascade of the receptor activator of nuclear factor- $\kappa$ B and its ligand, which subsequently promoted expression of a calcification inhibitor matrix Gla protein while inhibiting the expression of a calcification inducer bone morphogenetic protein 2 in both human aortic ECs and VSMCs, implying a beneficial effect of E<sub>2</sub> in preventing vascular calcification [43].

A community-based study showed that 18.2% of 445 women at the mean age of 45.2 years old had reduced glomerular filtration rate (<60 mL/min/1.73 m<sup>2</sup>), indicating a high prevalence of CKD in perimenopausal women [44]. Furthermore, clinical studies showed that MHT protected renal function by increasing the glomerular filtration rate in healthy postmenopausal women. This was coincided with a lower left ventricular mass index when compared to other healthy postmenopausal women with lower glomerular filtration rate who did not receive MHT [45]. Taken together, we postulate that reduced estrogen levels during and after menopause could increase the vulnerability of these CKD patients for MVD, which may lead to LVDD and HFpEF. To further elucidate this process, we analyzed published transcriptome datasets of UTs or E<sub>2</sub> treated human derived ECs from NCBI GEO database. We investigated genes and pathways that may be involved in UTs-triggered MVD in CKD. We obtained a gene set (group A) that poses deleterious effects on endothelial function and could be inhibited by E<sub>2</sub> stimulation, and a second gene set (group B) with an endothelial protective effect that could be induced by E<sub>2</sub> (Supplemental Table 1). Based on these two gene sets, we propose several target genes and their associated biological pathways, which may assist future studies in identifying valuable biomarkers and drug targets for early diagnosis and treatment of the CKD postmenopausal population at risk of developing MVD and subsequent HFpEF.

### 3.1. UTs impair microvascular homeostasis

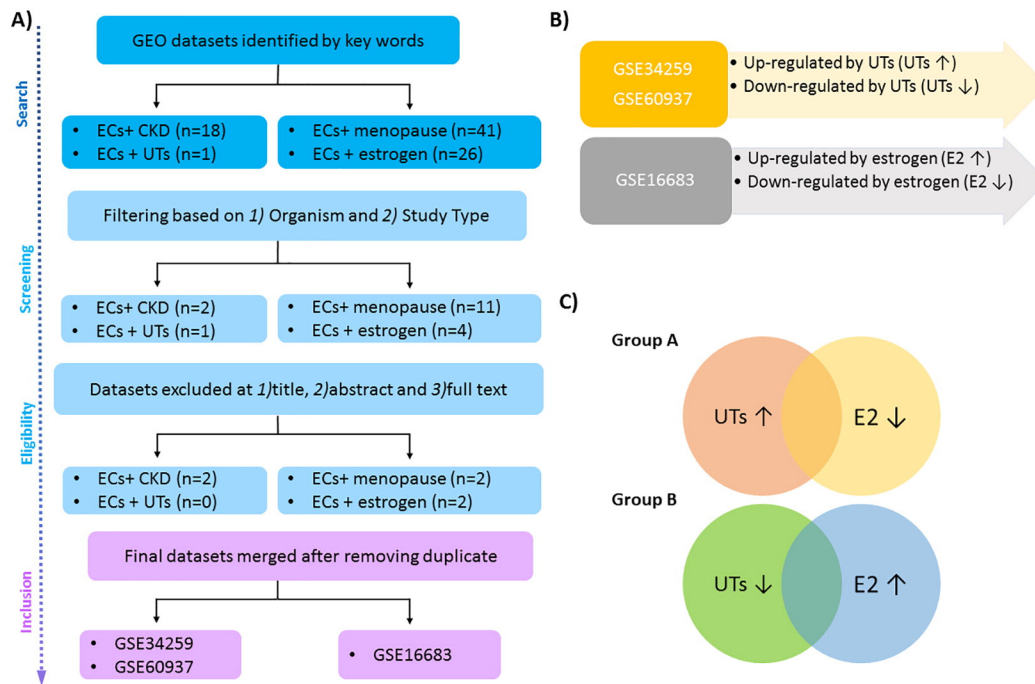
Following a systematic pipeline (Fig. 1), we obtained two GEO datasets that provide information of gene expression changes in ECs under CKD condition. Briefly, keywords “chronic kidney disease” and “uremic toxin” were coupled with “endothelial cells” separately to gather datasets from NCBI GEO database. Available datasets were further filtered for “Organism: *Homo sapiens*” and “Study type: Expression profiling by array”. Two GEO datasets were found according to these criteria (Table 1). GSE34259 contains information of the transcriptome profile of human umbilical vein endothelial cells (HUVECs) in response to IS treatment. GSE60937 contains information of the transcriptome profile of HUVECs in response to high inorganic phosphate (HP) treatment. Both raw datasets were downloaded and sorted by R script provided by NCBI's GEO2R program. These sorted gene expression datasheets were analyzed as followed: (1) Based on default setting stated in GEO2R, *p*-value below 0.05 was used to filter genes that reached significance. (2) Selected genes with logFC value above 0 represented increased expressions, and those with logFC value below 0 represented decreased expressions. (3) Genes with increased or decreased expression were mapped between IS and HP groups to obtain overlapping genes. We identified 36 genes with significantly increased expression under both IS and HP stimulation, whereas 14 gene decreased expressions under both IS and HP stimulation (Fig. 2A, Supplemental Table 2).

### 3.2. Functional annotation of UTs activated genes include positive regulation of prostaglandin synthesis and prostaglandin-related processes

Based on the 36 genes that were significantly activated by both IS and HP, we performed comprehensive functional annotations by using the ToppGene Suite tool ToppFun (Correction: FDR; *P*-Value cutoff: 0.05; Gene limits: 1 ≤ n ≤ 2000). The most enriched biological processes include positive regulation of prostaglandin biosynthetic process (GO:0031394) and prostaglandin-related processes (GO:2001280, GO:2001279, GO:0031392, GO:0046890, Supplemental Table 3). In addition, cardiac chamber morphogenesis (GO:0003206) was identified as the 7th most enriched biological process. By building a protein-protein interaction network with these 36 genes using STRING, we identified *PTGS2*, *NRG1*, *ICAM1*, *PPKCA*, *PLA2G4A* and *ADAMTS1* as key regulators within the constructed networks (Fig. 2B, confidence score ≥ 0.4). Basal levels of prostaglandin production are very low but increase significantly during inflammation, indicating UTs may promote inflammation of the endothelium. Many of the identified key mediators of this gene set, including *PTGS2*, *ICAM1* and *PLA2G4*, indeed exhibit pro-inflammatory properties. During inflammation, arachidonic acid is released from the plasma membrane by *PLA2G4*-encoded phospholipase 2, followed by *PTGS2* mediated conversion of arachidonic acid to prostaglandins [46]. *ICAM1* is a ligand for lymphocyte function-associated antigen 1 on leukocytes, and upregulation of *ICAM1* expression in ECs during endothelial activation is essential for leukocytes recruitment [47]. Interestingly, Hadad et al. showed that phospholipase 2 increased *ICAM1* expression via two transcription factors nuclear factor- $\kappa$ B and CREB in ECs [48]. These data indicate that prostaglandin regulation may represent a common pro-inflammatory pathway underlying MVD, which is induced by both IS and hyperphosphatemia.

### 3.3. Dysregulation of angiogenesis in UTs exposed vasculature

Inflammation-induced angiogenesis aids in the replacement of lost microvasculature and restores microvascular density in CKD [49]. Other identified key mediators of this gene set such as *PRKCA* and *NRG1* are known to promote angiogenesis, whereas *ADAMTS1* blocks angiogenesis: An in vitro study showed that inhibition of *PRKCA* resulted in decreased expression of *VEGF*, a crucial angiogenic factor [50]. Endothelial-derived *NRG1* has been reported to bind to ERBB receptors, and the downstream cascade involves the activation of angiogenesis-



**Fig. 1.** Overview of the workflow for identifying interesting gene groups from three included GEO datasets, representing gene expressions in HUVECs under indoxyl sulfate (IS), high level inorganic phosphates (HP) and estrogen ( $E_2$ ) stimulations. A) Workflow of dataset collection by searching for key words in GEO datasets, followed by filtering “Organism: Homo sapiens” and “Study Type: Expression profiling by array” and screening for the relevance. Three datasets were finally included after removing duplicates in filtered datasets. B) Significantly up- and down-regulated genes in HUVEC under UTs (IS and HP) and  $E_2$  were identified based on their fold change and p values. C) By mapping genes that were regulated by both UTs and  $E_2$ , we identified a group of genes (group A) that were upregulated by UTs (IS and/or HP) and downregulated by  $E_2$ , and another gene group (group B) in which genes were inhibited by UTs and increased by  $E_2$ .

related tyrosine kinase receptors, like VEGF receptors and Eph receptors [51]. *ADAMTS1* has been proposed to block angiogenesis via two mechanisms, i.e. by suppressing EC proliferation by disrupting VEGF signaling or by releasing anti-angiogenic peptides from angiogenesis-related proteins thrombospondin 1 and 2 [52]. Based on our analysis, we propose that increased circulating UTs could negatively impact renal and cardiac endothelial homeostasis by triggering inflammation and initiating vascular damage. In addition, vascular repair activity appears to be impaired by UTs-activated genes, leading to further progression of MVD.

#### 4. $E_2$ actives microvascular protective mechanisms

We also searched for gene expression changes in ECs under post-menopausal condition. Keywords “menopause” and “estrogen” were coupled with “endothelial cells” separately to gather datasets from NCBI GEO database. Available datasets were further filtered for “Organism: *Homo sapiens*” and “Study type: Expression profiling by array” (Fig. 1). One GEO dataset (GSE16683) was found according to these criteria, which showed the transcriptome response of  $E_2$  stimulated HUVECs (Table 1). Gene transcripts that were significantly increased and decreased by  $E_2$  were identified in the same way as previously described. In order to evaluate the susceptibility of UTs influenced genes to  $E_2$  regulation, we mapped genes with increased and/or decreased expressions among IS, HP and  $E_2$  groups. We identified 188 overlapping

**Table 1**  
Included GEO (Gene Expression Omnibus) datasets.

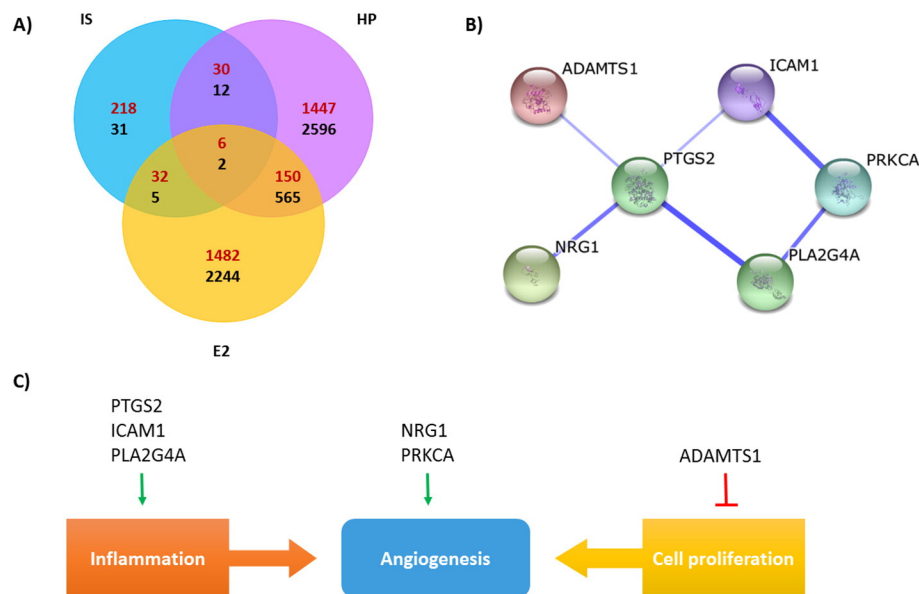
GEO series	Cell type	Stimulus	Number of up-regulated genes	Number of down-regulated genes
GSE16683	HUVECs	$E_2$	2816	1670
GSE34259	HUVECs	IS	286	50
GSE60937	HUVECs	HP	1633	3175

HUVECs: human umbilical vein endothelial cells;  $E_2$ : estradiol; IS: indoxyl sulfate; HP: high inorganic phosphate.

genes that were increased in expression by UTs (IS and/or HP) and inhibited by  $E_2$  in group A, and 572 overlapping genes that were decreased in expression by UTs and increased by  $E_2$  in group B. Group A represents genes which are possibly involved in MVD-inducing mechanistic pathways that could be suppressed by  $E_2$  protection. Group B represented genes in putative protective MVD-preventive pathways that could be induced by  $E_2$ .

#### 4.1. $E_2$ protects microvasculature by suppressing UTs-induced inflammation

Based on 188 genes in group A, the most enriched biological processes (Table 2, Supplemental Table 4) that were annotated by using ToppFun include “regulations of nitrogen compound metabolic process”, “potassium ion import” and “neutrophil migration”, which are known to influence microvascular homeostasis directly and could lead to MVD [53,54]. During inflammation, neutrophils extravasate the vasculature by migrating between ECs to inflamed sites. Increased ROS levels, produced by neutrophils, disrupt endothelial integrity by inhibiting endothelial occludin expression in tight junctions and by activating the phosphorylation of VE-cadherin,  $\beta$ -catenin and P120 catenin in adherens junctions, leading to increased diapedesis of inflammatory cells [53]. Excessive ROS also activates the JNK cascade, which is responsible for apoptosis, and could lead to tissue injury. Vasodilation regulated by “positive regulation of nitrogen compound metabolic process” and “potassium ion import”, further promotes a persistent inflammatory response [55]. In the cardiac microvasculature, it has been reported that increased ROS levels lower the activity of protein kinase G and titin hypophosphorylation, which resulted in an increased resting tension of cardiomyocytes [56]. In addition, lower activity of protein kinase G contributed to cardiomyocyte hypertrophy, and subsequently increased left ventricle wall stiffness. Based on these observations, we conclude that  $E_2$  could protect the (myo)endothelium



**Fig. 2.** Identification of genes with significantly increased and decreased expressions in response to IS, HP and/or E<sub>2</sub> stimulation in HUVECs. A) Venn diagram of genes stimulated by UTs and suppressed by E<sub>2</sub> (group A, indicated in red) and genes inhibited by UTs and enhanced by E<sub>2</sub> (group B, indicated in black). B) Among 36 genes increased by both IS and HP, ADAMTS1, PTGS2, NRG1, ICAM1, PRKCA and PLA2G4A were identified as the key mediators by STRING (confidence score  $\geq 0.4$ ). Stronger associations are represented by thicker lines. C) Biological functions of identified key genes regarding endothelium homeostasis based on literature studies. Green arrow: gene promotes the process; red indicator: gene inhibits the process.

by inhibiting UTs-induced inflammation, especially via downregulation of genes involved in ROS signalling.

#### 4.2. E<sub>2</sub> facilitates vascular repair activity by activating platelet coagulation and hemostasis at injured sites

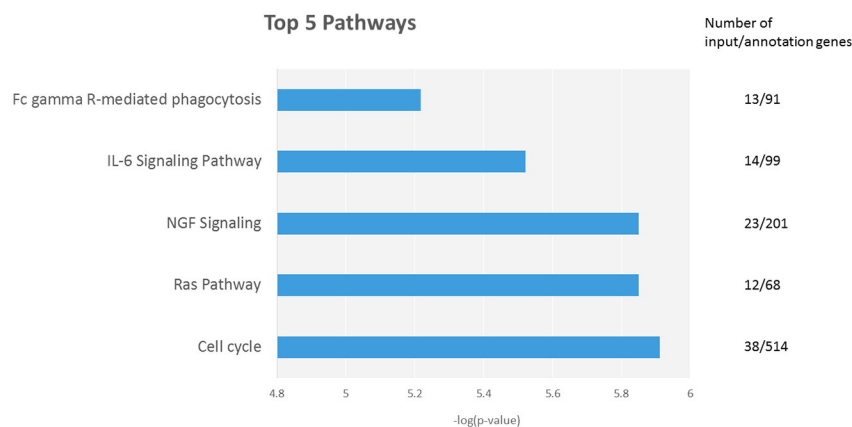
Based on the 572 genes in group B, the most enriched biological processes that are annotated by using ToppFun include “responses to stress”, “wounding and growth factors” and “hemostasis” (Table 2, Supplemental Table 4). These processes have been linked in literature to mechanisms that restore microvascular homeostasis [57–60]. In addition, nerve growth factor signaling (ID106459) was one of the top pathways annotated in this gene set (Fig. 3) and is known to play a regulatory role in promoting angiogenesis in vascular disease. For example, Park et al. showed that the binding of nerve growth factor to its receptor TrkA increased matrix metalloproteinase 2 expression via

the activation of PI3K/Akt pathway and the AP-2 transcription factor [57]. Another significantly enriched pathway is the interleukin 6 signaling pathway (ID198864, Fig. 3), which has been positively linked to coagulation [58]. Blood coagulation, a significantly enriched Biological process (GO:0007596,  $p$ -value  $< 0.01$ ), is in line with the observations of estrogen-related pro-coagulation capacity in clinical studies and represents an important step in hemostasis [61]. During hemostasis, tissue factor and the serine protease factor VIIa activate factor X and IX, which initiate the coagulation cascade and lead to the formation of thrombin [62]. Thrombin cleaves fibrinogen to generate insoluble fibrin, forming a fibrin mesh to strengthen and stabilize the blood clot and stop bleeding at the site of injury. By cleaving 2 protease activated receptors, PAR1 and PAR4, thrombin also activates platelets. Activated platelets express receptor GPIb-IX-V and receptor GPVI to bind von Willebrand factor and collagen in the sub-endothelial matrix, which further facilitates platelet adhesion. In addition, activated platelets secrete both pro- and anti-

**Table 2**

Most enriched biological processes in uremic toxins-increased and estrogen-inhibited groups (group A) and uremic toxins-decreased and estrogen-enhanced groups (group B).

GO rank	ID	Name	FDR-corrected p-value	Number of input/annotation genes
<i>Group A</i>				
1	GO:0031328	Positive regulation of cellular biosynthetic process	7.410E-6	37/1872
2	GO:0097368	Establishment of Sertoli cell barrier	7.799E-6	3/5
3	GO:0051173	Positive regulation of nitrogen compound metabolic process	8.883E-6	37/1887
4	GO:0010107	Potassium ion import	1.431E-4	4/29
5	GO:0002027	Regulation of heart rate	2.602E-4	6/95
6	GO:0010657	Muscle cell apoptotic process	3.711E-4	5/66
7	GO:1902622	Regulation of neutrophil migration	4.168E-4	4/38
8	GO:0001666	Response to hypoxia	4.293E-4	10/293
<i>Group B</i>				
1	GO:0007010	Cytoskeleton organization	1.747E-9	72/1142
2	GO:0007049	Cell cycle	4.609E-9	97/1780
3	GO:0033554	Cellular response to stress	4.610E-3	97/1983
4	GO:0033043	Regulation of organelle organization	2.126E-6	62/1116
5	GO:0007599	Hemostasis	4.903E-6	38/574
6	GO:0016032	Viral process	6.011E-6	48/810
7	GO:0009611	Response to wounding	7.165E-6	60/1109
8	GO:0022008	Neurogenesis	8.600E-6	88/1848
9	GO:0070848	Response to growth factor	9.128E-6	53/944
10	GO:0044764	Multi-organism cellular process	9.172E-6	48/823



**Fig. 3.** Top 5 statistically significant pathways based on 572 genes in group B using the ToppGene Suite tool ToppFun (Correction: FDR; P-Value cutoff: 0.05; Gene limits:  $1 \leq n \leq 2000$ ). The number of input genes and genes involved in each pathway are also listed.

angiogenetic factors to repair and replace damaged blood vessels and restore homeostasis [59].

Based on these findings, we suggest that  $E_2$  could counter the UTs-induced inhibition of the platelet coagulation and hemostasis response of the (micro)vasculature. Indeed, it has been long recognized that the coagulation system of patients with renal insufficiency and uremia is profoundly affected, demonstrating frequent symptoms of severe bleeding or thrombosis that lead to significant increase in morbidity and mortality [63]. The expression data of group B imply that UTs may contribute to the pathogenesis of uremic bleeding and thrombotic diathesis, which can be counteracted by the effects of  $E_2$  protection. In relation to uremic bleeding, treatment with conjugated  $E_2$  has been proposed and shown to be effective [64]. Interestingly, uremic bleeding has been linked to high NO bioavailability. Early studies have shown that monomethyl-L-arginine mediated inhibition of NO could normalize platelet dysfunction and bleeding time of uremic rats [65]. Likewise, systemic inhibition of NO production in healthy human volunteers could significantly shorten the bleeding time [66].  $E_2$  has been shown to normalize vascular expression of NO-producing enzymes and NO plasma levels in uremic rats [67], whereas induction of NO diminished the protective effects of estrogen in uremic bleeding [68]. As NO has been indicated to play a central role in cardiac endothelial dysfunction, linking microvascular disease to onset of HFpEF, the uncovered putative mechanism of UTs regulation of the coagulation system may be an important step in the pathogenesis that requires further study, particularly in relation to post-menopausal estrogen levels.

#### 4.3. Possible UTs and $E_2$ regulate gene targets in EC-cardiomyocyte paracrine crosstalk

Since ECs are located at a distance of maximum 5  $\mu$ M from cardiomyocytes, UTs and/or  $E_2$  induced signaling in ECs could further influence cardiac function in a paracrine fashion. In order to identify possible genes that are involved in EC-cardiomyocyte paracrine crosstalk, genes in Group A and B were re-analyzed using ToppFun regardless of their  $p$ -value ( $p$ -value cutoff: 1), and the obtained enrichments (including Molecular function, Biological process, Pathway, Mouse phenotype, Human phenotype and Disease) were further filtered for terms including “cardio”, “cardiac”, “myocard” and “heart”. Following this procedure, an enrichment list of 58 genes (31%) in group A, and 138 genes (24%) in group B were produced that matched the above described criteria (Fig. 4C and D).

Gene functions annotated from these 58 cardiac related genes in group A include “leukocyte migration”, “immune system development”, “positive regulation of cell motility”, “response to lipid”, “regulation of JNK cascade”, “vascular process in circulatory system”, and “regulation of cellular response to stress” (Fig. 4A). Immune responses are known

to be linked to endothelial dysfunction, which in turn is related to a lower activity of protein kinase G, titin hypophosphorylation, and an increased resting tension of cardiomyocytes and a hypertrophic response [56]. Endothelial dysfunction-related impaired NO bioactivity also results in endothelial-to-mesenchymal transition, during which ECs differentiate into (myo)fibroblasts and contribute to cardiac fibrosis. The identification of inflammation and MVD related mechanisms in the gene set of group A, thus imply that these initiating factors that contribute to LVDD and HFpEF could be mainly induced by circulating UTs during post-menopause.

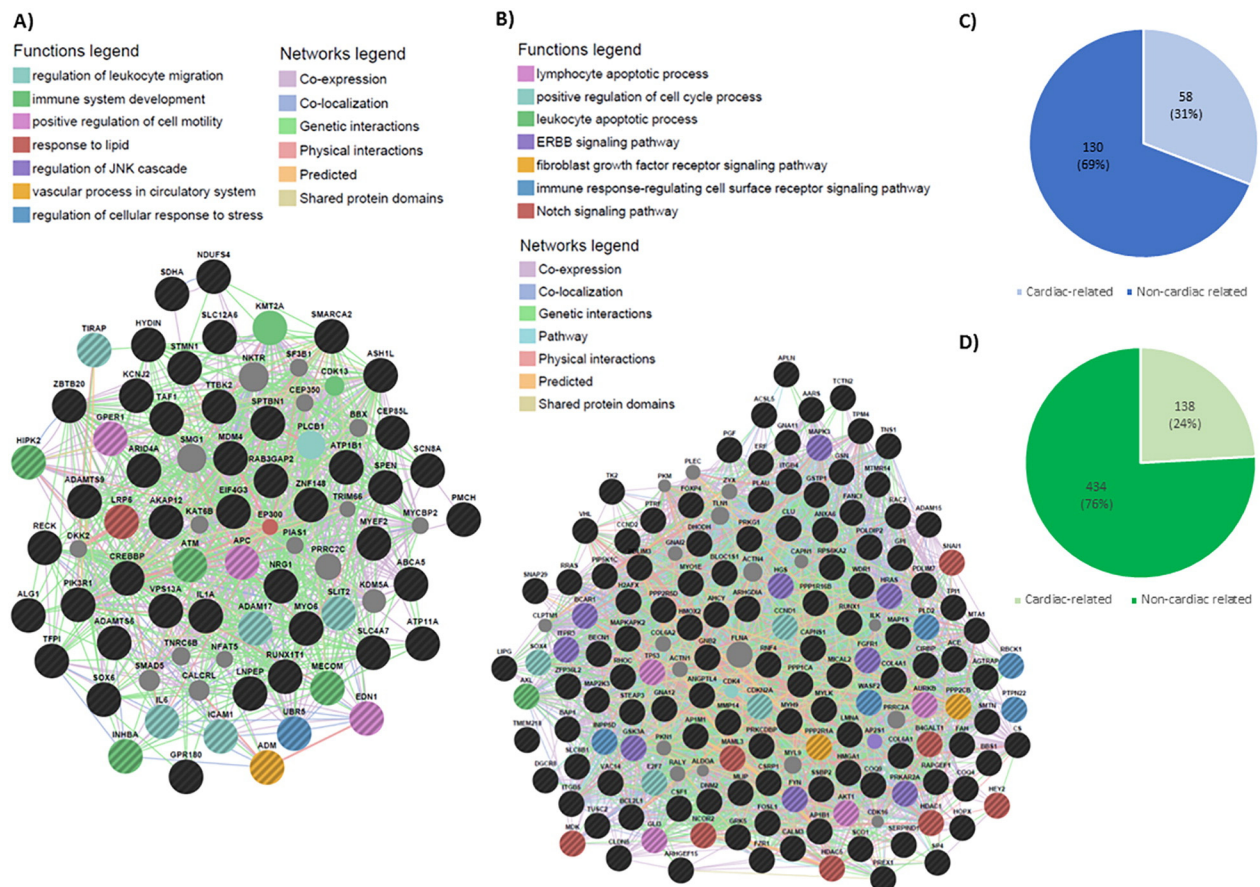
Gene functions annotated from the 138 cardiac related genes in group B include “Notch signaling pathway”, “ERBB signaling pathway”, “fibroblast growth factor receptor signaling pathway” and “downregulation of immune process” (Fig. 4B). During embryonic ventricular development, Notch activation induces the expression of *EPHRINB2*, *NRG1* and *BMP10*, resulting in trabecular differentiation and the proliferation of trabecular cardiomyocytes. In addition, Notch pathway, especially *NOTCH1*, is important in regulating endothelial function in the aortic valve, whereas endothelial dysfunction is associated with aortic valve calcification [69]. In response to pressure overload of the left ventricle due to aortic valve calcification, the myocardium becomes hypertrophic, eventually leading to diastolic dysfunction [70,71]. A recent paper showed an increased level of Notch ligand *DLL1* was associated with diastolic dysfunction, but no significant association between serum level of *DLL1* and HF patients with LVEF > 50% was found [72].

ERBB signaling is activated by endothelium-derived neuregulins. Parodi et al. indicated that during heart development, neuregulins-ERBB4 complex initiates dimerization with ERBB2, leading to a neuregulins/ERBB signaling cascade that promotes cardiomyocytes proliferation and differentiation [73]. Protein levels of ERBB2 and ERBB4 have been reported to be extremely reduced in HF patients, suggesting a key role of ERBB signaling in maintaining cardiac function [73]. In line with this notion, genetic knockdown of FRG receptor 1 and 2 in ECs of myocardial infarcted mice led to decreased vessel density, increased apoptosis and worsened cardiac function measured by echocardiography, suggesting a protective role of fibroblast growth factor signaling in vascular adaptation for cardiomyocyte function during ischemic heart injury [74].

To conclude, these findings imply that  $E_2$  might protect the heart against UTs by suppressing inflammatory responses, protecting cardiac vessel density and facilitating cardiomyocyte proliferation in an EC-cardiomyocyte paracrine crosstalk dependent manner.

## 5. Conclusion

In the current review, we aimed to gain more insight into the gene expression profiles of ECs that are predisposing and preventing MVD



**Fig. 4.** Gene annotations based on 38 genes and 138 genes related to cardiac function in group A and B. A, B) Gene functions including; immune response, apoptosis, response to lipid and stress signals in blood vessel cells were annotated by GeneMANIA (default parameters) in group A, whereas gene functions including; downregulation of immune-related process, ERBB signaling pathway, Notch signaling pathway and fibroblast growth factor receptor signaling were annotated in group B. Genes in black and/or with lines within the nodes are input gene and genes in grey and/or without lines within the nodes are annotation genes. C, D) Proportions of cardiac function-related genes within group A and B, respectively.

and MVD-related HFpEF in postmenopausal women with CKD. By analyzing three published microarray datasets based on the transcriptome responses of IS-, HP- and E<sub>2</sub>-treated HUVECs, we conclude that transcriptional changes of ECs exposed to CKD-associated circulatory risk factors IS and HP are characterized by increased expression of genes that are related to activation of inflammation and dysregulation of angiogenesis. In addition, these deleterious pathogenic expression profiles may be ameliorated by E<sub>2</sub> protection during the premenopausal period by a gene expression profile that promotes suppression of UTs-induced inflammation and facilitates angiogenesis by restoring coagulation and hemostasis in injured vessels. Reduced estrogen levels during and after menopause could accelerate the development of cardiorenal syndrome and CKD-associated HFpEF in CKD patients due to loss of E<sub>2</sub> protection of the endothelium in the deleterious environment of UTs. Our analysis provides, for the first time to our knowledge, a comprehensive in silico analysis of possible genes involved in CKD associated MVD that are regulated by E<sub>2</sub> and may be more affected during postmenopause period. Other mechanisms, which could also contribute to CKD-related HFpEF, might interact or work in parallel with our proposed mechanism. Clinical studies of the high-risk population of postmenopausal patients with cardiorenal syndrome are required to further verify our findings and to reveal the precise disease mechanisms involved. Eventually, improved insight into the link between loss of estrogen protection, MVD onset and progression in the CKD postmenopausal population could yield new and more dedicated biomarkers and drug targets for the development of new diagnostic tools and pharmacotherapies for HFpEF for this specific patient population.

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#### Author disclosure statement

The authors declare that they have no competing interests.

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#### References

- [1] C.S. Lam, E. Donal, E. Kraigher-Krainer, R.S. Vasan, Epidemiology and clinical course of heart failure with preserved ejection fraction, *Eur. J. Heart Fail.* 13 (2011) 18–28.
- [2] F. Edelmann, R. Stahrenberg, G. Gelbrich, K. Durstewitz, C.E. Angermann, H.D. Dungen, T. Scheffold, C. Zugck, B. Maisch, V. Regitz-Zagrosek, G. Hasenfuss, B.M. Pieske, R. Wachter, Contribution of comorbidities to functional impairment is higher in heart failure with preserved than with reduced ejection fraction, *Clin. Res. Cardiol.* 100 (2011) 755–764.
- [3] R. Pecoits-Filho, S. Bucharles, S.H. Barberato, Diastolic heart failure in dialysis patients: mechanisms, diagnostic approach, and treatment, *Semin. Dial.* 25 (2012) 35–41.
- [4] K. Sharma, D.A. Kass, Heart failure with preserved ejection fraction: mechanisms, clinical features, and therapies, *Circ. Res.* 115 (2014) 79–96.
- [5] E.D. Unger, R.F. Dubin, R. Deo, V. Daruwalla, J.L. Friedman, C. Medina, L. Beussink, B.H. Freed, S.J. Shah, Association of chronic kidney disease with abnormal cardiac mechanics and adverse outcomes in patients with heart failure and preserved ejection fraction, *Eur. J. Heart Fail.* 18 (2016) 103–112.

- [6] F.P. Brouwers, R.A. de Boer, P. van der Harst, A.A. Voors, R.T. Gansevoort, S.J. Bakker, H.L. Hillege, D.J. van Veldhuisen, W.H. van Gilst, Incidence and epidemiology of new onset heart failure with preserved vs. reduced ejection fraction in a community-based cohort: 11-year follow-up of PREVEND, *Eur. Heart J.* 34 (2013) 1424–1431.
- [7] J.M. Ter Maaten, K. Damman, M.C. Verhaar, W.J. Paulus, D.J. Duncker, C. Cheng, L. van Heerebeek, H.L. Hillege, C.S. Lam, G. Navis, A.A. Voors, Connecting heart failure with preserved ejection fraction and renal dysfunction: the role of endothelial dysfunction and inflammation, *Eur. J. Heart Fail.* 18 (2016) 588–598.
- [8] D.H. Smith, M.L. Thorp, J.H. Gurwitz, D.D. McManus, P. Robert, J. Goldberg, L.A. Allen, G. Hsu, S.H. Sung, D.J. Magid, A.S. Go, Chronic kidney disease and outcomes in heart failure with preserved versus reduced ejection fraction, *Circ. Cardiovasc. Qual. Outcomes* (2013).
- [9] R.J. Widmer, A. Lerman, Endothelial dysfunction and cardiovascular disease, *Glob. Cardiol. Sci. Pract.* 2014 (2014) 291–308.
- [10] W.J. Paulus, C. Tschope, A novel paradigm for heart failure with preserved ejection fraction: comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation, *J. Am. Coll. Cardiol.* 62 (2013) 263–271.
- [11] A. Clementi, G.M. Virzi, C.Y. Goh, D.N. Cruz, A. Granata, G. Vescovo, C. Ronco, Cardiorenal syndrome type 4: a review, *Cardiorenal Med.* 3 (2013) 63–70.
- [12] A. Regeniter, H. Freidank, M. Dickenmann, W.H. Boesken, W.H. Siede, Evaluation of proteinuria and GFR to diagnose and classify kidney disease: systematic review and proof of concept, *Eur. J. Intern. Med.* 20 (2009) 556–561.
- [13] T. Toyohara, Y. Akiyama, T. Suzuki, Y. Takeuchi, E. Mishima, M. Tanemoto, A. Momose, N. Toki, H. Sato, M. Nakayama, A. Hozawa, I. Tsuji, S. Ito, T. Soga, T. Abe, Metabolomic profiling of uremic solutes in CKD patients, *Hypertens. Res.* 33 (2010) 944–952.
- [14] X. Tao, S. Thijssen, P. Kotanko, C.H. Ho, M. Henrie, E. Stroup, G. Handelman, Improved dialytic removal of protein-bound uremic toxins with use of albumin binding competitors: an in vitro human whole blood study, *Sci. Rep.* 6 (2016) 23389.
- [15] R. Vanholder, U. Baurmeister, P. Brunet, G. Cohen, G. Glorieux, J. Jankowski, G. European Uremic Toxin Work, A bench to bedside view of uremic toxins, *J. Am. Soc. Nephrol.* 19 (2008) 863–870.
- [16] M. Aepfelbacher, M. Essler, Disturbance of endothelial barrier function by bacterial toxins and atherogenic mediators a role for RhoRho kinase, *Cell. Microbiol.* (2001).
- [17] N. Jourde-Chiche, L. Dou, C. Cerini, F. Dignat-George, P. Brunet, Vascular incompetence in dialysis patients—protein-bound uremic toxins and endothelial dysfunction, *Semin. Dial.* 24 (2011) 327–337.
- [18] J.E. Ho, A. Lyass, D.S. Lee, R.S. Vasan, W.B. Kannel, M.G. Larson, D. Levy, Predictors of new-onset heart failure: differences in preserved versus reduced ejection fraction, *Circ. Heart Fail.* 6 (2013) 279–286.
- [19] T. Bhuiyan, M.S. Maurer, Heart failure with preserved ejection fraction: persistent diagnosis, therapeutic enigma, *Curr. Cardiovasc. Risk Rep.* 5 (2011) 440–449.
- [20] F.S. Loffredo, A.P. Nikolova, J.R. Pancoast, R.T. Lee, Heart failure with preserved ejection fraction: molecular pathways of the aging myocardium, *Circ. Res.* 115 (2014) 97–107.
- [21] K. Alagiakrishnan, M. Banach, L.G. Jones, A. Ahmed, W.S. Aronow, Medication management of chronic heart failure in older adults, *Drugs Aging* 30 (2013) 765–782.
- [22] D.C. Scantlebury, B.A. Borlaug, Why are women more likely than men to develop heart failure with preserved ejection fraction? *Curr. Opin. Cardiol.* 26 (2011) 562–568.
- [23] A.H.E.M. Maas, Y.E.A. Appelman, Gender differences in coronary heart disease, *Neth. Hear. J.* 18 (2010).
- [24] A.A. Oktay, J.D. Rich, S.J. Shah, The emerging epidemic of heart failure with preserved ejection fraction, *Curr. Heart Fail. Rep.* 10 (2013) 401–410.
- [25] E.Z. Gorodeski, Is early menopause a risk factor for heart failure? *Menopause* 21 (2014) 558–560.
- [26] B.E. Henderson, A. Paganini-Hill, R.K. Ross, Decreased mortality in users of estrogen replacement therapy, *Arch. Intern. Med.* 151 (1991) 75–78.
- [27] H.N. Hodis, W.J. Mack, S.P. Azen, R.A. Lobo, D. Shoupe, P.R. Mahrer, D.P. Faxon, L. Cashin-Hemphill, M.E. Sanmarco, W.J. French, T.L. Shook, T.D. Gaarder, A.O. Mehra, R. Rabbani, A. Sevanian, A.B. Shil, M. Torres, K.H. Vogelbach, R.H. Selzer, Hormone therapy and the progression of coronary-artery atherosclerosis in postmenopausal women, *N. Engl. J. Med.* 349 (2003).
- [28] S. Hulley, D. Grady, T. Bush, C. Furberg, D. Herrington, B. Riggs, E. Vittinghoff, Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women, *JAMA* 280 (1998).
- [29] O.M. Reslan, R.A. Khalil, Vascular effects of estrogenic menopausal hormone therapy, *Rev. Recent Clin. Trials* 7 (2012).
- [30] M.A. Duzenli, K. Ozdemir, A. Sokmen, K. Gezginc, A. Soylu, C. Celik, B.B. Altunkeser, M. Tokac, The effects of hormone replacement therapy on myocardial performance in early postmenopausal women, *Climacteric* 13 (2010) 157–170.
- [31] Y.S. Peng, H.C. Ding, Y.T. Lin, J.P. Syu, Y. Chen, S.M. Wang, Uremic toxin p-cresol induces disassembly of gap junctions of cardiomyocytes, *Toxicology* 302 (2012) 11–17.
- [32] J.A. Tumlin, M.R. Costanzo, L.S. Chawla, C.A. Herzog, J.A. Kellum, P.A. McCullough, C. Ronco, Cardiorenal syndrome type 4: insights on clinical presentation and pathophysiology from the eleventh consensus conference of the Acute Dialysis Quality Initiative (ADQI), *Contrib. Nephrol.* 182 (2013) 158–173.
- [33] P. Brunet, B. Gondouin, A. Duval-Sabatier, L. Dou, C. Cerini, F. Dignat-George, N. Jourde-Chiche, A. Argiles, S. Burtey, Does uremia cause vascular dysfunction? *Kidney Blood Press. Res.* 34 (2011) 284–290.
- [34] H.A. Mutsaers, L.P. van den Heuvel, L.H. Ringens, A.C. Dankers, F.G. Russel, J.F. Wetzels, J.G. Hoenderop, R. Masereeuw, Uremic toxins inhibit transport by breast cancer resistance protein and multidrug resistance protein 4 at clinically relevant concentrations, *PLoS One* 6 (2011), e18438.
- [35] H.A. Mutsaers, U.F. Engelke, M.J. Wilmer, J.F. Wetzels, R.A. Wevers, L.P. van den Heuvel, J.G. Hoenderop, R. Masereeuw, Optimized metabolomic approach to identify uremic solutes in plasma of stage 3–4 chronic kidney disease patients, *PLoS One* 8 (2013), e71199.
- [36] O. Deltombe, W. Van Biesen, G. Glorieux, Z. Massy, A. Dhondt, S. Eloit, Exploring protein binding of uremic toxins in patients with different stages of chronic kidney disease and during hemodialysis, *Toxins (Basel)* 7 (2015) 3933–3946.
- [37] P.R. Gajjala, M. Sanati, J. Jankowski, Cellular and molecular mechanisms of chronic kidney disease with diabetes mellitus and cardiovascular diseases as its comorbidities, *Front. Immunol.* 6 (2015) 340.
- [38] S. Ito, M. Yoshida, Protein-bound uremic toxins: new culprits of cardiovascular events in chronic kidney disease patients, *Toxins (Basel)* 6 (2014) 665–678.
- [39] C.W. Usselman, N.S. Stachenfeld, J.R. Bender, The molecular actions of estrogen in the regulation of vascular health, *Exp. Physiol.* (2016).
- [40] S. Menazza, E. Murphy, The expanding complexity of estrogen receptor signaling in the cardiovascular system, *Circ. Res.* 118 (2016) 994–1007.
- [41] Q.K. Tran, R. Firkins, J. Giles, S. Francis, V. Matnishian, P. Tran, M. VerMeer, J. Jasadra, M.A. Burgard, B. Gebert-Oberle, Estrogen enhances linkage in the vascular endothelial calmodulin network via a feedforward mechanism at the G protein-coupled estrogen receptor 1, *J. Biol. Chem.* 291 (2016) 10805–10823.
- [42] A. Blum, Endothelial progenitor cells are affected by Meidcations and estrogen, *Isr. Med. Assoc. J.* 17 (2015).
- [43] M.K. Osako, H. Nakagami, N. Koibuchi, H. Shimizu, F. Nakagami, H. Koriyama, M. Shimamura, T. Miyake, H. Rakugi, R. Morishita, Estrogen inhibits vascular calcification via vascular RANKL system: common mechanism of osteoporosis and vascular calcification, *Circ. Res.* 107 (2010) 466–475.
- [44] H. Salve, S. Mahajan, P. Misra, Prevalence of chronic kidney diseases and its determinants among perimenopausal women in a rural area of North India: a community-based study, *Indian J. Nephrol.* (2012).
- [45] E. Vitolo, M. Comassi, M.T. Caputo, A. Solini, Hormone replacement therapy, renal function and heart ultrasonographic parameters in postmenopausal women: an observational study, *Int. J. Clin. Pract.* 69 (2015) 632–637.
- [46] E. Ricciotti, G.A. FitzGerald, Prostaglandins and inflammation, *Arterioscler. Thromb. Vasc. Biol.* 31 (2011) 986–1000.
- [47] J.M. Cook-Mills, T.L. Deem, Active participation of endothelial cells in inflammation, *J. Leukoc. Biol.* 77 (2005) 487–495.
- [48] N. Hadad, L. Tuval, V. Elgazar-Carmom, R. Levy, R. Levy, Endothelial ICAM-1 protein induction is regulated by cytosolic phospholipase A2alpha via both NF-kappaB and CREB transcription factors, *J. Immunol.* 186 (2011) 1816–1827.
- [49] D.A. Long, J.T. Norman, L.G. Fine, Restoring the renal microvasculature to treat chronic kidney disease, *Nat. Rev. Nephrol.* 8 (2012) 244–250.
- [50] H. Xu, P. Czerwinski, M. Hortmann, H.Y. Sohn, U. Forstermann, H. Li, Protein kinase C alpha promotes angiogenic activity of human endothelial cells via induction of vascular endothelial growth factor, *Cardiovasc. Res.* 78 (2008) 349–355.
- [51] K.S. RUSSELL, D.F. STERN, P.J. POLVERINI, J.R. BENDER, Neuregulin activation of ErbB receptors in vascular endothelium leads to angiogenesis, *Am. Physiol. Soc.* (1999).
- [52] S. Kumar, N. Rao, R. Ge, Emerging roles of ADAMTSs in angiogenesis and cancer, *Cancers (Basel)* 4 (2012) 1252–1299.
- [53] M. Mittal, M.R. Siddiqui, K. Tran, S.P. Reddy, A.B. Malik, Reactive oxygen species in inflammation and tissue injury, *Antioxid. Redox Signal.* 20 (2014) 1126–1167.
- [54] W.F. JACKSON, Potassium channels in the peripheral microcirculation, *Microcirculation* (2005).
- [55] A.H. Sprague, R.A. Khalil, Inflammatory cytokines in vascular dysfunction and vascular disease, *Biochem. Pharmacol.* 78 (2009) 539–552.
- [56] C. Tschope, S. Van Lintouth, New insights in (inter)cellular mechanisms by heart failure with preserved ejection fraction, *Curr. Heart Fail. Rep.* 11 (2014) 436–444.
- [57] M.J. Park, H.J. Kwak, H.C. Lee, D.H. Yoo, I.C. Park, M.S. Kim, S.H. Lee, C.H. Rhee, S.I. Hong, Nerve growth factor induces endothelial cell invasion and cord formation by promoting matrix metalloproteinase-2 expression through the phosphatidylinositol 3-kinase/Akt signaling pathway and AP-2 transcription factor, *J. Biol. Chem.* 282 (2007) 30485–30496.
- [58] E. de Jonge, P.W. Friederich, G.P. Vlasuk, W.E. Rote, M.B. Vroom, M. Levi, T. van der Poll, Activation of coagulation by administration of recombinant factor VIIa elicits interleukin 6 (IL-6) and IL-8 release in healthy human subjects, *Clin. Vaccine Immunol.* 10 (2003) 495–497.
- [59] T. Browder, J. Folkman, S. Steven Pirie-Shepherd, The hemostatic system as a regulator of angiogenesis, *Biol. Chem.* 275 (2000).
- [60] B.M. Doshi, G.A. Perdrizet, L.E. Hightower, Wound healing from a cellular stress response perspective, *Cell Stress Chaperones* 13 (2008) 393–399.
- [61] K.K. Koh, B.K. Yoon, Controversies regarding hormone therapy: insights from inflammation and hemostasis, *Cardiovasc. Res.* 70 (2006) 22–30.
- [62] A.J. Gale, Current understanding of hemostasis, *Toxicol. Pathol.* 39 (2011) 273–280.
- [63] A.S. Sohal, A.S. Gangji, M.A. Crowther, D. Treleven, Uremic bleeding: pathophysiology and clinical risk factors, *Thromb. Res.* 118 (2006) 417–422.
- [64] S.J. Hedges, S.B. Dehoney, J.S. Hooper, J. Amanzadeh, A.J. Busti, Evidence-based treatment recommendations for uremic bleeding, *Nat. Clin. Pract. Nephrol.* 3 (2007) 138–153.
- [65] S. Aiello, M. Noris, M. Todeschini, S. Zappella, C. Foglieni, A. Benigni, D. Corna, C. Zoja, D. Cavallotti, G. Remuzzi, Renal and systemic nitric oxide synthesis in rats with renal mass reduction, *Kidney Int.* 52 (1997) 171–181.
- [66] D. Simon, J. Stampler, E. Loh, J. Loscalzo, S. Francis, M. Creager, Effect of nitric oxide synthase inhibition on bleeding time in humans, *J. Cardiovasc. Pharmacol.* 26 (1995).
- [67] M. Noris, G. Remuzzi, Uremic bleeding closing the circle after 30 years of controversies, *Blood* 94 (1999).
- [68] C. Zoja, M. Noris, D. Corna, G. Viganò, N. Perico, G. de Gaetano, G. R., L-arginine, the precursor of nitric oxide, abolishes the effect of estrogens on bleeding time in experimental uremia, *Lab. Invest.* (1991).



- [69] K. Niessen, A. Karsan, Notch signaling in cardiac development, *Circ. Res.* 102 (2008) 1169–1181.
- [70] A. Ozkan, Low gradient “severe” aortic stenosis with preserved left ventricular ejection fraction, *Cardiovasc. Diagn. Ther.* 2 (2012) 19–27.
- [71] R.V. Freeman, C.M. Otto, Spectrum of calcific aortic valve disease: pathogenesis, disease progression, and treatment strategies, *Circulation* 111 (2005) 3316–3326.
- [72] H.M. Norum, L. Gullestad, A. Abraitte, K. Broch, S. Aakhus, P. Aukrust, T. Ueland, Increased serum levels of the notch ligand DLL1 are associated with diastolic dysfunction, reduced exercise capacity, and adverse outcome in chronic heart failure, *J. Card. Fail.* 22 (2016) 218–223.
- [73] E.M. Parodi, B. Kuhn, Signalling between microvascular endothelium and cardiomyocytes through neuregulin, *Cardiovasc. Res.* 102 (2014) 194–204.
- [74] S.L. House, A.M. Castro, T.S. Lupu, C. Weinheimer, C. Smith, A. Kovacs, D.M. Ornitz, Endothelial fibroblast growth factor receptor signaling is required for vascular remodeling following cardiac ischemia-reperfusion injury, *Am. J. Physiol. Heart Circ. Physiol.* 310 (2016) H559–H571.