Original Research

Dialysis-related transcriptomic profiling: The pivotal role of heparanase

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Abstract

Peritoneal (PD) and hemodialysis (HD) represent the leading renal replacement therapies in advanced chronic kidney disease (CKD). Although absolutely necessary to ensure patient survival, these treatments are responsible for considerable biological alterations primarily due to the un-physiological contact of blood and tissues with bioincompatible devices or plastificants. Although extensively described, this complex dialysis-related deregulated bio-molecular machinery is still not completely known. Therefore, to select a set of genes deregulated in patients on dialysis treatment and to assess the possible differences between dialysis modalities, we measured the expression level of 132 genes involved in proteoglycans (PGs) biosynthesis/metabolism by microarray in peripheral blood mononuclear cells (PBMCs), biological elements involved in the inflammatory/immune response, from 5 healthy subjects (HS), 9 CKD, 10 PD, and 17 HD patients. We focused on PGs biosynthesis/metabolism pathways because of their involvement in the onset and development of several CKD-related clinical complications. Statistical analysis/bioinformatics identified 70 genes discriminating HD/PD patients from HS/CKD subjects ($P < 0.009$, FDR $< 5\%$). Twenty-five genes were up-regulated (e.g. *HPSE, VCAN,* and *VEGFA*) and 45 down-regulated (e.g. *IDS* and *HEXA*) in PD/HD compared to HS/CKD. Gene expression and plasma activity of Heparanase (HPSE), one of the top selected up-regulated genes in PD/HD, validated microarray results. In addition, for the second part of the study, HPSE plasmatic activities were first assessed in an independent testing-group (7 HS, 10 CKD, 17 PD, and 11 HD), and then correlated with high-sensitive C reactive protein (HS-CRP) measurements. HPSE activity was higher in PD and HD versus CKD/HS and it correlated with HS-CRP levels ($R^2 = 0.37$, $P = 0.007$). Lipopolysaccharide (LPS)-stimulated PBMCs showed a significant up-regulation of HPSE mRNA level ($P = 0.04$). Our results revealed that dialysis treatments induce change in the transcriptomic pattern of biosynthetic proteoglycans in PBMCs with an up-regulation of HPSE. Our selected genes could be useful in the future as potential biomarkers and new therapeutic targets.

Keywords: Peritoneal dialysis, hemodialysis, microinflammation, microarray, proteoglycans, heparanase

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Introduction

Chronic kidney disease (CKD) is a major and growing challenge for health care systems. The prevalence rates of CKD appear to be increasing globally,[1–3] primarily as a consequence of the increased incidence of diabetes, hypertension, and aging population.[4–6]

Progression of the renal failure may be influenced by several factors, and it is well-known that the early identification of elements responsible of the onset and/or development of CKD is a great challenge in nephrology.

Additionally, it has been well described that, along with the disease progression, CKD patients develop complex biological and biochemical dysfunctions that require a rapid start of renal replacement therapies (peritoneal- and hemo-dialysis) in the last stages of renal damage (end stage renal disease).[7,8]

Although absolutely necessary to ensure patients survival, dialysis treatments are responsible for considerable molecular and cellular alterations leading to metabolic changes, enhancement of inflammation/oxidative stress, and the development of several significant

immunological deregulations.[9–11] This is primarily due to the un-physiological contact of blood (in particular peripheral blood mononuclear cells (PBMCs)) or tissues (peritoneal membrane) with dialysis devices/plastificants (e.g. the membrane, peritoneal catheter and dialysis solution in peritoneal dialysis (PD)) used to remove waste products generated from normal metabolic processes such as uremic toxins, and to normalize body fluid and electrolytes.[12,13]

Therefore, in the last few years, researchers and clinicians worldwide have worked together to identify key points for new pharmacological interventions, and to ameliorate dialysis techniques and the basic structure of materials in order to increase their biocompatibility profile.

In this context, the development of high-throughput research strategies (e.g. transcriptomic, proteomic, and metabolomic) have facilitated the identification of key biological elements involved in the onset of clinical complications (e.g. inflammation, atherosclerosis, and cardiovascular disease) which researchers and pharmaceutical or biotechnological industries may utilize to define and produce new therapeutic interventions.[14,15] However, additional studies need to be performed to better understand the complete systemic machinery associated to the renal dysfunction.

Interestingly, whilst searching for new diagnostic/prognostic targets, it has recently emerged that proteoglycans (PGs) could have a pivotal role in the biological apparatus involved in the systemic complications associated with renal failure.[16,17]

PGs are ubiquitous substances produced by a multitude of cell types including PBMCs. The enormous molecular diversity of PGs resulting from the various combinations of protein cores substituted with one or more glycosaminoglycan (GAG) chains provides the structural basis for the multitude of their biological functions, which are still partly unknown. In particular, some PGs are components of the extra cellular matrix (ECM), others are membrane PGs as cell surface receptors, and yet others are concentrated in secretory granules as a scaffold for other granule constituents.[18] Due to their capability to interact with cytokines and growth factors, these substances have been involved in several important immunological functions and PGs are now recognized for the important role they play in controlling the inflammatory response, as demonstrated in several tissues.[19]

In particular, Cohen-Mazor *et al.* have described that Heparanase (HPSE), an endoglycosidase that cleaves the heparan sulfate (HS) side chains of proteoglycans, was involved in extracellular matrix degradation and atherosclerotic lesion progression in patients on chronic hemodialysis (HD) after being released from primed circulating PBMCs.[20]

In this study, we hypothesized that the continuous pro-inflammatory stimulation and the uremia-related biochemical alterations could induce an alteration of the proteoglycan expression in circulating immune cells of CKD patients undergoing both dialysis modalities.

Therefore, to confirm this hypothesis and to select a set of biological elements deregulated in patients on chronic dialysis treatment and to assess the possible differences between modalities of dialysis, we decided to employ, for the first time, an innovative transcriptomic approach (microarray) combined with several classical biomolecular methodologies (e.g., RT-PCR, ELISA).

Moreover, we aimed to discover new key regulators of the complex proteglycan biosynthetic/metabolic intracellular system that, in future, after adequate validation and testing in independent cohorts of CKD/dialyzed patients, could be novel valuable diagnostic biomarkers and potential targets for new pharmacological/technical interventions.

Material and methods

Patients

A total of 86 subjects, after signing a written consent form, were included in the study and divided in a training group ($n = 41$) and an independent testing group ($n = 45$). The study was carried out according to the Declaration of Helsinki and approved by the institutional ethical board of the University Hospital 'Policlinico di Bari', Bari, Italy.

Training group. This population was used for the microarray analysis and to generate the initial transcriptomic model. It included 5 healthy subjects (HS), 9 CKD patients on stages III–IV (mean ±SD of estimated GFR by CKD-EPI formula: 32.27 ± 14.7 mL/min), 10 PD patients, and 17 HD patients.

Testing group. This group of patients was used for biological validation analysis and to confirm the hypothesis generated in the training group. It included 7 HS, 10 CKD patients on stages III–IV (mean ± SD of estimated GFR by CKD-EPI formula: 30.26 ± 14.89 mL/min), 17 PD patients, and 11 HD patients.

All HD patients were stably treated, three times a week (4–5 h per session) for at least 1 year using synthetic membrane dialyzers. During the study period, no CKD patients received dialysis treatment.

Twenty-seven PD patients were included in our study. Sixteen patients had been treated with continuous ambulatory peritoneal dialysis (CAPD) using a bicarbonate buffer (Physioneal, Baxter, Chicago, IL, USA) and 11 with APD using a bicarbonate buffer (Physioneal, Baxter) for a mean of 3.58 years (range 0.54–9.03).

In addition, all patients suffering from systemic autoimmune disorders, infectious diseases, diabetes, chronic lung diseases, neoplasms, or inflammatory diseases and patients receiving antibiotics, corticosteroids, or non-steroidal anti-inflammatory agents were excluded. Patients with glomerulonephritis who were included in this study did not have immune-mediated forms. No patients had symptomatic coronary artery disease or a family history of premature cardiovascular disease. The main clinical and demographic characteristics of the subjects included in the training and testing group are summarized in Tables 1 and 2, respectively.

Table 1 Demographics and clinical characteristics of the training-group patients

	HS	CKD	PD	HD	*P* value
Number	5	9	10	17	–
Gender (male/female)	2/3	6/3	4/6	10/7	NS
Age (years)	50.78 ± 10.45	51.64 ± 10.26	51.55 ± 7.12	52.86 ± 8.63	NS
Cause of CKD: GN/ADPKD/renal vascular disease/unknown	–	4,2,1,2	4,2,1,3	7,4,1,5	NS
Time on dialysis (years)	–	–	4.12 ± 0.74	3.98 ± 0.56	NS
BMI (kg/m^2)	23.1 ± 0.82	21.9 ± 0.74	21.1 ± 1.45	22.1 ± 1.01	*P* < 0.01
Systolic blood pressure (mmHg)	120 ± 4.01	136 ± 9.71	135 ± 6.26	138 ± 8.11	*P* < 0.01
Diastolic blood pressure (mmHg)	74.36 ± 5.31	82.1 ± 10.03	80.3 ± 56.12	88 ± 10.86	*P* < 0.01
Total protein (g/dL)	6.98 ± 0.41	5.98 ± 0.76	5.98 ± 0.47	6.02 ± 0.31	*P* < 0.01
Albumin (g/dL)	4.3 ± 0.31	3.71 ± 0.42	3.12 ± 0.33	3.48 ± 0.51	*P* < 0.01
Ferritin (ng/mL)	79 ± 62.3	210 ± 183.6	367 ± 221.3	451 ± 283.1	*P* < 0.001
Hemoglobin (g/dL)	13.46 ± 1.62	11.88 ± 1.87	11.68 ± 1.26	11.48 ± 1.98	*P* < 0.01

Note: *P* values calculated by ANOVA or Fisher's exact test.
GN: Glomerulonephritis; ADPKD: Autosomal dominant polycystic kidney disease; BMI: Body mass index. Values are expressed as mean ± SD.

Table 2 Demographics and clinical characteristics of the testing-group patients

	HS	CKD	PD	HD	*P* value
Number	7	10	17	11	–
Gender (male/female)	4/3	6/4	9/8	6/5	NS
Age (years)	48 ± 11.27	49.46 ± 10.78	51.25 ± 6.22	50.61 ± 7.24	NS
Cause of CKD: GN/ADPKD/renal vascular disease/unknown	–	4,2,2,2	6,4,4,3	6,2,1,2	NS
Time on dialysis (years)	–	–	3.91 ± 0.92	4,01 ± 0.42	NS
BMI (kg/m^2)	22.8 ± 0.64	20.9 ± 1.83	20.8 ± 1.16	21.7 ± 0.96	*P* < 0.01
Systolic blood pressure (mmHg)	121 ± 3.71	136 ± 9.68	140 ± 6.51	138 ± 8.11	*P* < 0.01
Diastolic blood pressure (mmHg)	75.28 ± 4,26	84.6 ± 9.25	90.04 ± 61.32	89 ± 10.93	*P* < 0.01
Total protein (g/dL)	6.87 ± 0.49	5.85 ± 0.82	5.21 ± 0.28	5.98 ± 0.34	*P* < 0.01
Albumin (g/dL)	4.1 ± 1.56	3.69 ± 0.51	3.21 ± 0.44	3.74 ± 0.48	*P* < 0.01
Ferritin (ng/mL)	89 ± 56.5	178 ± 143.2	341 ± 198.6	444 ± 212.6	*P* < 0.01
Hemoglobin (g/dL)	13.12 ± 1.49	11.75 ± 1.95	11.71 ± 1.11	11.12 ± 2.28	*P* < 0.01

Note: Values are expressed as mean ± SD. *P* values calculated by ANOVA or Fisher's exact test.
GN: Glomerulonephritis; ADPKD: Autosomal dominant polycystic kidney disease; BMI: Body mass index.

High sensitive serum C-reactive protein measurement

High sensitive serum C-reactive protein (HS-CRP) levels were measured in all patients included in the testing group using high-sensitivity immunonephelometry (Dade Behring, Marbung, Germany) according to the manufacturer's protocol.

PBMCs isolation

Twenty milliliters of whole blood were collected from all subjects included in the study. For HD patients, the biological material was obtained at the beginning of the second HD session of the week. PBMCs were isolated by density separation over a Ficoll–Paque™ (GE healthcare, Sweden) gradient (460 g for 30 min) and washed three times with PBS pH 7.4/1 mM EDTA (Sigma, Milan, Italy). Cells were counted, and viability was assessed by the trypan blue exclusion method (>90% PBMCs were viable).

RNA extraction and microarray experiments

For all subjects included in the training group, total RNA was isolated from PBMCs by a RNeasy mini kit, Qiagen (QIAGEN AG, Basel, Switzerland) from a minimum of 5×10^6 cryopreserved PBMCs. RNA was quantified by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and its integrity was assessed by electrophoresis, using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). RNA was then processed and hybridized to the GeneChip Human Genome U133A oligonucleotide microarray (Affymetrix, Santa Clara, CA, USA). The default settings of Affymetrix Microarray Suite software version 5 were used to calculate scaled gene expression values. The results of the microarray experiments are available in the Gene Expression Omnibus (Accession number GSE15072). Although the employed microarray experiment has already been extensively validated in our previous reports,[11,13] we performed an

additional experiment of validation measuring HPSE mRNA level by real-time PCR (RT-PCR) in all patients included in the training group. Microarray results were also confirmed at the protein level by measuring HPSE plasma activity by ELISA.

Plasma collection

To evaluate HPSE activity, one blood sample was collected into tubes containing EDTA from all patients included in the study at time of enrolment. The blood was then centrifuged for 15 min at 1500 *g* and the obtained plasma was aliquoted and stored at −80°C until use.

HPSE plasma assay

To measure the plasma activity of HPSE, we used a well standardized assay.[21] Briefly, HPSE was quantified by an assay based on the ability of HPSE to degrade heparan-sulphate proteoglycans present in Matrigel (Matrigel™ Basement Membrane Matrix). Twenty-five microliters of Matrigel was dissolved in ice-cold PBS at a final concentration of 200 µg/mL and used to coat an ELISA plate at room temperature for 1.5 h. Samples were diluted by a ratio 1:4 in HPSE buffer (0.1 M Sodium Acetate (pH 5.0), 0.1 mg/mL BSA, 0.01% Triton X-100, Protease Inhibitor Cocktail). The plates were washed once with PBT (PBS + 0.05% v/v Tween-20) and samples were then added and incubated at 37°C overnight. The plates were then washed with PBT and blocked with blocking buffer (PBT, 0.5% BSA, 1 mM EDTA) at room temperature for 2 h. After PBT washing, primary antibody anti-HS-specific MoAb (clone HepSS-1, Seikagaku, Tokyo, Japan) diluted 1:500 in blocking buffer was added and incubated at room temperature for 1 h. After washing, secondary antibody (goat anti-mouse IgM-HRP sc-2973, Santa Cruz Biotechnology) diluted 1:1000 in blocking buffer was added and incubated at room temperature for 1 h followed by the addition of 50 µL of the ABTS [2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid), Sigma]. OD405 absorbance was read in an ELISA plate reader. HPSE activity in each plasma sample was calculated as the difference between the OD405 value with or without heparin (Sigma) at the final concentration of 50 µg/mL. This inhibitor was used to ensure the detection of specific HPSE activity in the plasma samples.

RT-PCR for HPSE

One microgram of RNA extracted by PBMCs of all patients included in the training group was reverse transcribed into cDNA using a high-capacity cDNA Reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. RT-PCR was performed on an ABI-Prism 7700 (Applied Biosystems) using the KAPA SYBR® FAST qPCR kit (KAPA Biosystems). A quantitative analysis was performed to assess the expression of HPSE, and results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (HPSE: f—ATTTGAATGGACGGA CTGC, r—GTTTCTCCTAACCAGACCTTC; GAPDH: f—ACACCCACTCCTCCACCTTT, r—TCCACCACCCTGTT GCTGTA). Gene expression was quantified by means of the comparative CT method (ΔΔCt) and the relative quantification was calculated as $2^{-\Delta\Delta Ct}$. Melting curve analysis was performed to check for the presence of any non-specific amplification products.

In vitro study

PBMCs, isolated from three randomly selected HS from the testing-group, were resuspended in RPMI-1640 (Sigma) supplemented with 2 mM L-glutammine (1%), penicillin (100 U/mL), and streptomycin (100 µg/mL), cultured overnight at 37°C with 5% CO_2 and then stimulated with lipopolysaccharide (LPS) (10 µg/mL, Sigma) for 4 h. The dose and time-point of LPS treatment were chosen after performing time-course and dose–response experiments (data not shown). After extraction, 1 µg of RNA was reverse transcribed into cDNA and RT-PCR was performed to assess the expression of HPSE as described earlier.

Statistical analysis

Results were expressed as mean ± SD. T-test, ANOVA, and Fisher's exact test were used to assess differences in clinical and demographic features. A value of $P < 0.05$ was considered to be statistically significant. Pearson's rank test was used to measure the degree of correlation of HPSE plasma activity with HS-CRP levels.

For the microarray analysis, we selected a total of 201 gene probe sets (corresponding to 132 genes) involved in PGs biosynthesis/metabolism according to the Gene Ontology (GO),[22] a major bioinformatics initiative that aims to provide a controlled vocabulary of terms for describing gene product characteristics and gene product annotation data from GO Consortium members (set of biological databases and research groups actively involved in the GO project), as well as tools to access and process this data (more details in http://www.geneontology.org). Gene expression values for the 201 gene probe sets, scaled to the target intensity of 2500, were log transformed. Several statistical algorithms[11] and distinction calculations (DC)[23] were used to select probe sets discriminating between the study groups. The false discovery rate (FDR) was estimated using the Storey's q-value.[24] R 2.0.1 statistical software was used to perform the above analyses. Principal component analysis (PCA) and hierarchical clustering were performed using Spotfire DecisionSite 9.0 (www.spotfire.com).

RESULTS

Demographic and clinical features

As shown in Tables 1 and 2, we did not find any difference among the study groups in several demographic and clinical parameters (gender, age, cause of CKD, time on dialysis) for all patients included in the training and testing group. However, BMI, systolic and diastolic blood pressure, albumin, ferritin, and hemoglobin were significantly different among groups ($P < 0.01$).

Table 3 Top discriminating down-regulated gene probe sets in PD/HD compared to CKD/HS (cluster A)

Gene name	PD (mean ± SD)	HD (mean ± SD)	CKD (mean ± SD)	HS (mean ± SD)	*P* value*	*P* value†
Collagen, type IV, alpha 3	2.48 ± 0.74	1.47 ± 0.88	3.16 ± 0.57	2.87 ± 0.59	<0.001	<0.001
nidogen 2	2.11 ± 1.24	0.89 ± 1.20	2.33 ± 0.69	2.56 ± 1.11	0.009	0.003
Collagen, type VII, alpha 1	2.91 ± 0.59	2.60 ± 0.72	3.79 ± 0.35	3.80 ± 0.13	<0.001	<0.001
ST3 beta-galactoside alpha-2,3-sialyltransferase 2	3.25 ± 0.68	3.49 ± 0.33	4.17 ± 0.54	4.21 ± 0.36	<0.001	<0.001
Xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7	3.38 ± 0.39	3.42 ± 0.24	4.04 ± 0.34	3.64 ± 0.17	<0.001	<0.001
Syntrophin, beta 2	3.90 ± 0.33	3.81 ± 0.40	4.41 ± 0.28	4.33 ± 0.33	<0.001	<0.001
Tumor necrosis factor, alpha-induced protein 6	2.93 ± 0.57	2.35 ± 1.23	5.52 ± 0.40	4.91 ± 0.52	<0.001	<0.001
Tumor necrosis factor, alpha-induced protein 6	2.21 ± 1.19	2.19 ± 1.35	4.79 ± 0.38	4.03 ± 0.91	<0.001	<0.001
Carbohydrate sulfotransferase 15	4.99 ± 0.62	5.08 ± 0.48	6.25 ± 0.34	6.06 ± 0.37	<0.001	<0.001
Galactosamine (*N*-acetyl)-6-sulfate sulfatase	4.24 ± 0.32	4.24 ± 0.23	4.80 ± 0.34	4.82 ± 0.32	<0.001	<0.001
Exostoses (multiple)-like 3	2.52 ± 0.48	2.29 ± 0.71	3.39 ± 0.47	3.06 ± 0.87	<0.001	<0.001
Collagen, type XVIII, alpha 1	2.76 ± 0.84	2.84 ± 0.39	3.89 ± 0.40	3.95 ± 0.41	<0.001	<0.001
Hepatoma-derived growth factor	4.54 ± 0.33	4.60 ± 0.24	5.17 ± 0.33	5.19 ± 0.47	<0.001	<0.001
Hepatoma-derived growth factor	5.62 ± 0.22	5.72 ± 0.32	6.09 ± 0.35	6.35 ± 0.44	<0.001	<0.001
UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 3	4.16 ± 0.41	4.22 ± 0.24	4.99 ± 0.24	5.38 ± 0.21	<0.001	<0.001
Collagen, type XVIII, alpha 1	2.69 ± 0.57	3.00 ± 0.56	4.29 ± 0.50	3.72 ± 0.40	<0.001	<0.001
Iduronate 2-sulfatase	3.12 ± 0.89	3.56 ± 0.54	5.24 ± 0.39	5.17 ± 0.32	<0.001	<0.001
Iduronate 2-sulfatase	4.74 ± 0.23	4.81 ± 0.25	5.23 ± 0.17	5.03 ± 0.28	<0.001	<0.001
Hexosaminidase A (alpha polypeptide)	2.49 ± 0.64	2.38 ± 0.99	3.55 ± 0.63	3.53 ± 0.67	<0.001	0.002
Hyaluronan binding protein 2	1.49 ± 0.99	1.82 ± 0.64	2.90 ± 0.90	2.81 ± 0.53	<0.001	<0.001
UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	4.09 ± 0.69	4.38 ± 0.37	5.00 ± 0.40	5.10 ± 0.40	<0.001	<0.001
Dystrophin	1.54 ± 0.78	2.12 ± 0.63	3.07 ± 0.72	3.00 ± 0.51	<0.001	<0.001
UDP-GlcNAc:betaGal beta-1,3-*N*-acetylglucosaminyltransferase 1	2.56 ± 0.89	2.57 ± 0.79	3.59 ± 0.69	3.52 ± 0.51	<0.001	0.004
Sarcoglycan, delta (35 kDa dystrophin-associated glycoprotein)	1.69 ± 1.07	1.50 ± 0.97	2.65 ± 0.74	3.14 ± 0.91	<0.001	0.002
Dystrophin	1.64 ± 1.04	1.38 ± 0.99	2.58 ± 0.95	3.29 ± 0.31	<0.001	<0.001
Collagen, type V, alpha 3	1.64 ± 1.14	1.10 ± 0.81	2.52 ± 0.84	2.89 ± 0.79	<0.001	<0.001
Chondroitin polymerizing factor	2.96 ± 0.61	2.44 ± 1.01	3.69 ± 0.53	3.80 ± 0.57	<0.001	<0.001
Collagen, type V, alpha 3	2.50 ± 0.93	1.67 ± 1.15	2.88 ± 0.61	3.30 ± 0.86	<0.001	0.003
Dystrophin	1.77 ± 1.20	-0.01 ± 1.10	1.72 ± 1.40	1.21 ± 0.76	<0.001	<0.001
Laminin, alpha 2	2.75 ± 0.72	1.55 ± 1.04	2.86 ± 0.93	3.39 ± 0.46	0.002	<0.001
Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	2.67 ± 1.16	2.03 ± 0.76	2.94 ± 0.67	3.53 ± 0.25	0.003	0.003
Protease, serine, 1 (trypsin 1)	1.93 ± 1.32	1.15 ± 0.93	2.58 ± 1.04	2.61 ± 0.83	0.002	0.005
Carbohydrate (chondroitin 6) sulfotransferase 3	2.09 ± 1.21	1.62 ± 0.87	3.17 ± 0.68	3.52 ± 0.39	<0.001	<0.001
Collagen, type XIII, alpha 1	2.88 ± 1.08	2.22 ± 0.85	3.56 ± 0.51	3.69 ± 0.58	<0.001	<0.001
Transforming growth factor, beta 1	2.07 ± 0.72	1.72 ± 0.74	3.67 ± 0.60	3.61 ± 0.62	<0.001	<0.001
Peptidase inhibitor 3, skin-derived	2.29 ± 1.06	1.65 ± 0.90	6.02 ± 0.79	5.24 ± 0.54	<0.001	<0.001
Chitinase 3-like 1	1.86 ± 1.67	0.69 ± 0.88	5.75 ± 0.39	5.58 ± 0.56	<0.001	<0.001
Chitinase 3-like 1	1.43 ± 2.10	0.86 ± 0.65	6.10 ± 0.14	6.04 ± 0.45	<0.001	<0.001

(continued)

Table 3 Continued

Gene name	PD (mean ± SD)	HD (mean ± SD)	CKD (mean ± SD)	HS (mean ± SD)	*P* value*	*P* value†
Peptidase inhibitor 3, skin-derived	1.93 ± 1.62	1.70 ± 1.08	6.11 ± 0.53	5.29 ± 0.71	<0.001	<0.001
Collagen, type IX, alpha 2	2.86 ± 0.59	2.99 ± 0.20	3.82 ± 0.38	3.76 ± 0.26	<0.001	<0.001
Thrombospondin 4	1.98 ± 1.21	1.87 ± 0.77	3.66 ± 0.79	3.35 ± 0.57	<0.001	<0.001
Latent transforming growth factor beta binding protein 4	2.96 ± 0.62	2.68 ± 0.61	3.68 ± 0.53	3.85 ± 0.22	<0.001	<0.001
Collagen, type IV, alpha 3	0.92 ± 1.10	1.03 ± 0.83	2.75 ± 0.78	2.48 ± 0.46	<0.001	<0.001
N-deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 1	3.35 ± 1.00	3.18 ± 0.35	4.31 ± 0.23	4.47 ± 0.26	<0.001	<0.001
Agrin	1.80 ± 1.45	2.01 ± 0.65	3.45 ± 0.93	3.28 ± 0.38	<0.001	<0.001
Collagen, type IV, alpha 3	1.96 ± 1.17	1.00 ± 0.90	2.74 ± 1.00	3.17 ± 0.54	<0.001	<0.001
Collagen, type I, alpha 2	2.42 ± 1.18	1.93 ± 0.60	3.20 ± 0.56	3.23 ± 0.40	<0.001	<0.001
Fibromodulin	2.81 ± 1.06	2.86 ± 0.43	3.70 ± 0.95	4.14 ± 0.58	<0.001	0.002
Collagen, type XIII, alpha 1	1.84 ± 1.01	1.92 ± 0.83	3.87 ± 0.69	3.72 ± 0.96	<0.001	<0.001
Matrilin 1, cartilage matrix protein	2.05 ± 1.10	1.77 ± 0.79	3.50 ± 0.39	3.03 ± 0.45	<0.001	<0.001
Fibulin 1	2.20 ± 1.13	1.93 ± 0.80	3.12 ± 0.82	3.12 ± 0.50	<0.001	0.005
Carbohydrate (*N*-acetylgalactosamine 4-0) sulfotransferase 8	2.70 ± 0.93	1.86 ± 0.79	3.46 ± 0.69	2.91 ± 1.05	<0.001	<0.001
N-acetylglucosaminidase, alpha	1.79 ± 1.50	1.17 ± 0.89	2.48 ± 1.07	3.16 ± 0.92	<0.001	0.003
Dystonin	0.85 ± 1.49	0.60 ± 1.02	2.29 ± 1.13	1.64 ± 0.78	<0.001	0.006
Agrin	2.71 ± 1.01	2.43 ± 0.83	3.60 ± 0.43	3.33 ± 0.47	<0.001	0.004
Aggrecan	2.47 ± 1.10	2.25 ± 0.64	4.09 ± 0.54	3.30 ± 1.36	<0.001	<0.001
Dystonin	2.10 ± 1.27	2.15 ± 0.63	3.03 ± 0.78	3.56 ± 0.28	<0.001	0.002
Latent transforming growth factor beta binding protein 2	2.70 ± 0.87	2.49 ± 0.74	3.51 ± 0.88	3.52 ± 0.41	<0.001	0.007

PD: peritoneal dialysis; HD: hemodialysis; CKD: chronic kidney disease; HS: healthy subject.
**P* value by *t*-test comparison PD/HD vs. CKD/HS.
†*P* value by ANOVA comparison HD vs. PD vs. CKD vs. HS.

Table 4 Top discriminating up-regulated gene probe sets in PD/HD compared to CKD/HS (cluster B)

Gene name	PD (mean±SD)	HD (mean±SD)	CKD (mean±SD)	HS (mean±SD)	*P* value*	*P* value†
UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	1.82 ± 0.88	2.09 ± 0.59	1.58 ± 0.83	2.38 ± 0.44	0.001	0.003
Hexosaminidase A	5.06 ± 0.25	5.02 ± 0.18	4.65 ± 0.22	5.01 ± 0.23	0.002	<0.001
Structural maintenance of chromosome 3	4.05 ± 0.50	3.90 ± 0.30	2.76 ± 0.89	3.02 ± 0.63	<0.001	<0.001
N-sulfoglucosamine sulfohydrolase	5.09 ± 0.30	4.98 ± 0.28	4.51 ± 0.27	4.41 ± 0.23	<0.001	<0.001
Glucuronidase, beta	5.75 ± 0.24	5.83 ± 0.30	4.75 ± 0.26	4.60 ± 0.28	<0.001	<0.001
Hexosaminidase B (beta polypeptide)	6.25 ± 0.21	6.32 ± 0.23	5.56 ± 0.30	5.69 ± 0.20	<0.001	<0.001
Ribosomal protein L29	7.77 ± 0.16	7.72 ± 0.14	7.04 ± 0.29	6.95 ± 0.22	<0.001	<0.001
Ribosomal protein L29	8.06 ± 0.12	8.06 ± 0.08	7.66 ± 0.20	7.62 ± 0.21	<0.001	<0.001
Superoxide dismutase 1, soluble	6.42 ± 0.16	6.52 ± 0.15	5.62 ± 0.20	5.45 ± 0.38	<0.001	<0.001
Vascular endothelial growth factor A	3.84 ± 0.53	4.04 ± 0.38	2.79 ± 0.69	2.54 ± 0.76	<0.001	<0.001
60 S ribosomal protein L29-like	5.91 ± 0.18	5.99 ± 0.24	5.46 ± 0.38	5.54 ± 0.30	<0.001	<0.001
Transforming growth factor, beta 1	5.72 ± 0.36	6.03 ± 0.32	5.40 ± 0.32	5.38 ± 0.35	<0.001	<0.001
Chondroitin polymerizing factor 2	3.68 ± 0.60	3.99 ± 0.33	3.11 ± 0.84	3.27 ± 0.82	0.001	<0.001
Erbb2 interacting protein	5.30 ± 0.55	5.44 ± 0.39	4.49 ± 0.49	4.21 ± 0.32	<0.001	<0.001
Chondroitin sulfate synthase 1	4.77 ± 0.50	4.96 ± 0.39	4.15 ± 0.34	3.98 ± 0.51	<0.001	<0.001
Chondroitin sulfate *N*-acetylgalactosaminyltransferase 2	4.90 ± 0.73	5.13 ± 0.45	3.41 ± 0.90	3.38 ± 0.52	<0.001	<0.001
Chondroitin sulfate *N*-acetylgalactosaminyltransferase 2	4.48 ± 0.59	4.62 ± 0.53	3.61 ± 0.38	3.35 ± 0.45	<0.001	<0.001
Vascular endothelial growth factor A	3.99 ± 0.73	4.53 ± 0.59	2.89 ± 1.07	3.09 ± 0.36	<0.001	<0.001
Versican	6.41 ± 0.62	6.82 ± 0.41	5.04 ± 0.31	4.88 ± 0.27	<0.001	<0.001
Versican	6.70 ± 0.46	6.97 ± 0.47	5.35 ± 0.25	5.48 ± 0.09	<0.001	<0.001
Versican	7.37 ± 0.31	7.56 ± 0.21	6.57 ± 0.29	6.57 ± 0.26	<0.001	<0.001
Versican	7.59 ± 0.22	7.80 ± 0.26	6.90 ± 0.34	6.94 ± 0.13	<0.001	<0.001
Versican	5.09 ± 0.65	5.24 ± 0.46	3.80 ± 0.78	3.51 ± 0.40	<0.001	<0.001
Glucosamine (*N*-acetyl)-6-sulfatase	5.69 ± 0.58	5.87 ± 0.22	5.17 ± 0.32	5.05 ± 0.26	<0.001	<0.001
Dermatan sulfate epimerase	4.44 ± 0.57	4.54 ± 0.35	3.71 ± 0.48	3.42 ± 0.70	<0.001	<0.001
Carbohydrate (*N*-acetylglucosamine-6-O) sulfotransferase 2	4.81 ± 0.49	5.03 ± 0.36	4.03 ± 0.56	4.63 ± 0.37	<0.001	<0.001
Xylosyltransferase I	3.75 ± 0.37	3.73 ± 0.34	3.45 ± 0.33	3.04 ± 0.46	0.001	0.003
Heparin-binding EGF-like growth factor	4.00 ± 1.10	4.85 ± 0.80	1.97 ± 0.59	2.38 ± 0.85	<0.001	<0.001
Heparin-binding EGF-like growth factor	3.42 ± 1.34	4.59 ± 0.72	2.27 ± 0.59	2.41 ± 0.76	<0.001	<0.001
Carbohydrate (chondroitin 4) sulfotransferase 11	4.79 ± 0.24	4.99 ± 0.25	4.52 ± 0.28	4.36 ± 0.48	<0.001	<0.001
Structural maintenance of chromosome 3	4.45 ± 0.22	4.57 ± 0.19	3.64 ± 0.55	3.83 ± 0.80	<0.001	<0.001
UDP-GlcNAc:betaGal beta-1,3-*N*-acetylglucosaminyltransferase 2	3.72 ± 0.67	3.77 ± 0.49	2.75 ± 0.75	3.18 ± 0.69	<0.001	0.001
Glucosamine (*N*-acetyl)-6-sulfatase	4.21 ± 0.79	4.14 ± 0.46	3.14 ± 0.79	3.62 ± 0.61	<0.001	0.002
Heparan sulfate 2-O-sulfotransferase 1	3.30 ± 0.63	3.15 ± 0.48	2.79 ± 0.69	2.18 ± 0.53	0.002	0.006
Angiogenin, ribonuclease, RNase A family, 5	3.16 ± 0.45	3.45 ± 0.51	2.67 ± 0.66	2.15 ± 0.99	<0.001	<0.001
Heparanase	3.38 ± 0.44	3.21 ± 0.43	2.82 ± 0.66	2.24 ± 0.64	<0.001	<0.001

PD: peritoneal dialysis; HD: hemodialysis; CKD: chronic kidney disease; HS: healthy subject.
*P value by t-test comparison PD/HD vs. CKD/HS.
†P value by ANOVA comparison HD vs. PD vs. CKD vs. HS.

Microarray analysis

To identify specific differences in the PGs transcriptome profile between HS, CKD, PD, and HD, we analyzed the expression level of 201 gene probe sets (corresponding to 132 genes) involved in proteoglycan biosynthesis and metabolism selected by GO.

According to independent statistical algorithms and the estimated false discovery rate (FDR), we identified 94 gene probe sets (corresponding to 70 genes) able to discriminate PD/HD patients from HS/CKD subjects ($P < 0.001$, FDR $< 5\%$). We found only a slight and not significant difference in the transcriptomic profile between HS and CKD ($P = 0.14$) and PD and HD ($P = 0.16$).

Among the top selected gene probe sets, 58 (corresponding to 45 genes, e.g. *IDS, HEXA, DMD,* and *SNTB2*) were down-regulated (cluster A, listed in Table 3) and 36 (corresponding to 25 genes; e.g. *HPSE, VCAN, VEGFA,* and *FBLN1*) were up-regulated (cluster B, listed in Table 4) in PD/HD patients compared to HS/CKD.

Two-dimensional hierarchical clustering using the 94 gene probe sets clearly separated patients into two distinct groups (PD/HD versus HS/CKD) (Figure 1), and PCA illustrates the degree of separation among the study groups (Figure 2).

HPSE gene expression analysis

For the second part of the study, we focused on HPSE, one of the top selected up-regulated genes in PD/HD identified by transcriptomic analysis. As shown in Figure 3, HPSE gene expression levels evaluated by RT-PCR in all patients included in the training-group were higher in PD/HD compared to CKD/HS validating microarray results. On the contrary, HPSE levels were not significantly different in PD versus HD, and CKD versus HS groups.

HPSE plasma activity

Concordantly with results obtained by microarray and RT-PCR, HPSE plasma activity was higher in PD/HD in both training and testing groups. Additionally, its activity was similar in PD and HD patients, and CKD patients and HS (Figure 4(a) and (b)).

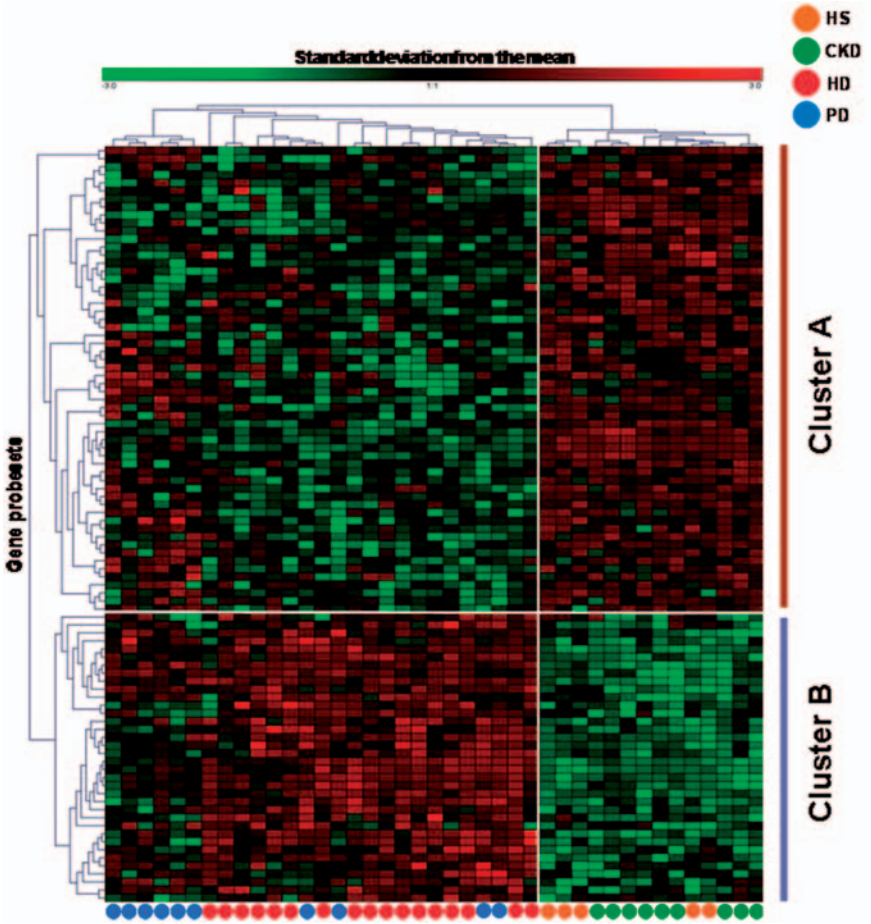

Figure 1 'Supervised' hierarchical clustering using the top selected genes by microarray analysis. Patients are depicted as vertical columns, with red symbols indicating hemodialysis (HD, $n = 17$), blue indicating PD ($n = 10$), green stage III–IV chronic kidney disease (CKD, $n = 9$) patients and yellow symbols indicating HS ($n = 5$). A total of 94 gene probe sets were used for hierarchical clustering. Clusters A and B include the top statistically significant down-regulated and up-regulated genes in PD/HD compared to CKD/HD, respectively. The relative level of gene expression is depicted from lowest (green) to highest (red) according to the scale shown at the top. As reported, there was only a slight and not significant difference in the genomic profile between HS and CKD and between PD and HD. (A color version of this figure is available in the online journal.)

Correlation between HPSE plasma activity and HS-CRP levels

There is a close link between proteoglycan biosynthesis and inflammation, as reported by several sources in the literature[25,26]; therefore, we decided to correlate HPSE plasma activity with HS-CRP levels. Statistical analysis demonstrated that the two variables were significantly correlated, as measured in the testing group (Figure 5) ($P = 0.007$, $R^2 = 0.37$).

In vitro LPS-induced HPSE gene expression

As shown in Figure 6, the *HPSE* mRNA levels measured in the PBMCs of three randomly selected HS were augmented after an acute inflammatory stimulus with LPS ($P = 0.04$).

DISCUSSION

Dialysis patients have an increased morbidity and mortality rate compared to the general population.[27,28] Biological mechanisms associated to these events are multiple and not completely understood. In particular, chronic systemic inflammation is a common feature in patients with CKD undergoing dialysis treatment[29] and it plays a central role in the development of atherosclerosis[30,31] and cardiovascular disease.[32,33]

Renal failure *per se* may possibly contribute to inflammation as a result of the accumulation of pro-inflammatory compounds.[34] In addition, the interaction of PBMCs with dialysis membranes (filters) during HD causes their

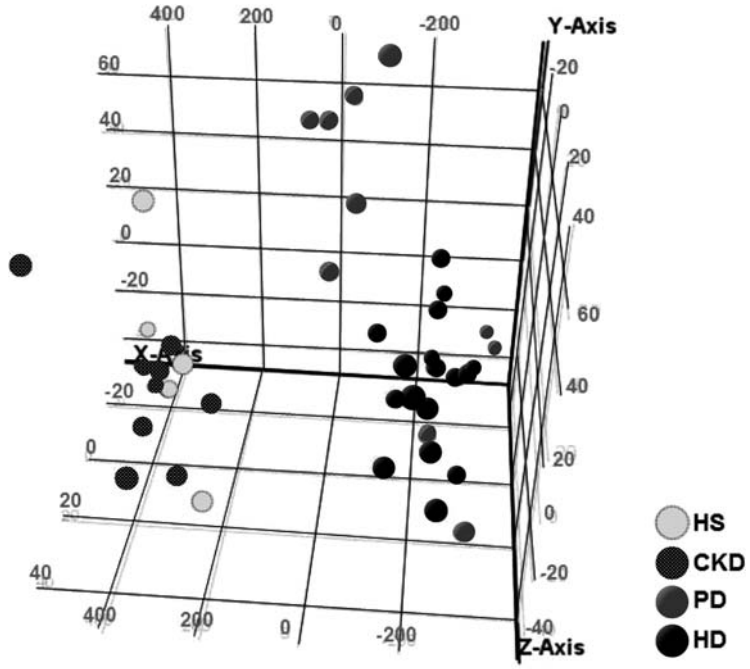

Figure 2 Principal component analysis (PCA) discriminating HD, PD, CKD and HS. PCA plot using the 94 selected gene probe sets discriminated in three-dimensional space the four groups of patients. Black dots indicate HD ($n = 17$), dark gray PD ($n = 10$), black polka CKD ($n = 9$) patients, and light gray HS ($n = 5$)

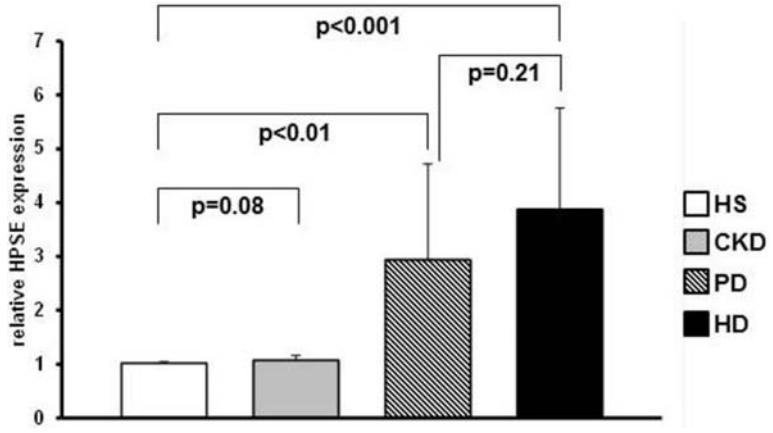

Figure 3 HPSE gene expression by RT-PCR in peripheral blood mononuclear cells (PBMC) from HS, CKD at early stages, PD, and HD patients. The histograms represent the mean ± SD of HPSE level of expression determined by RT-PCR in PBMC from 5 HS, 9 CKD, 10 PD, and 17 HD. The expression level was significantly higher in HD, PD compared to CKD and HS. No differences were found in PD versus HD and CKD versus HS

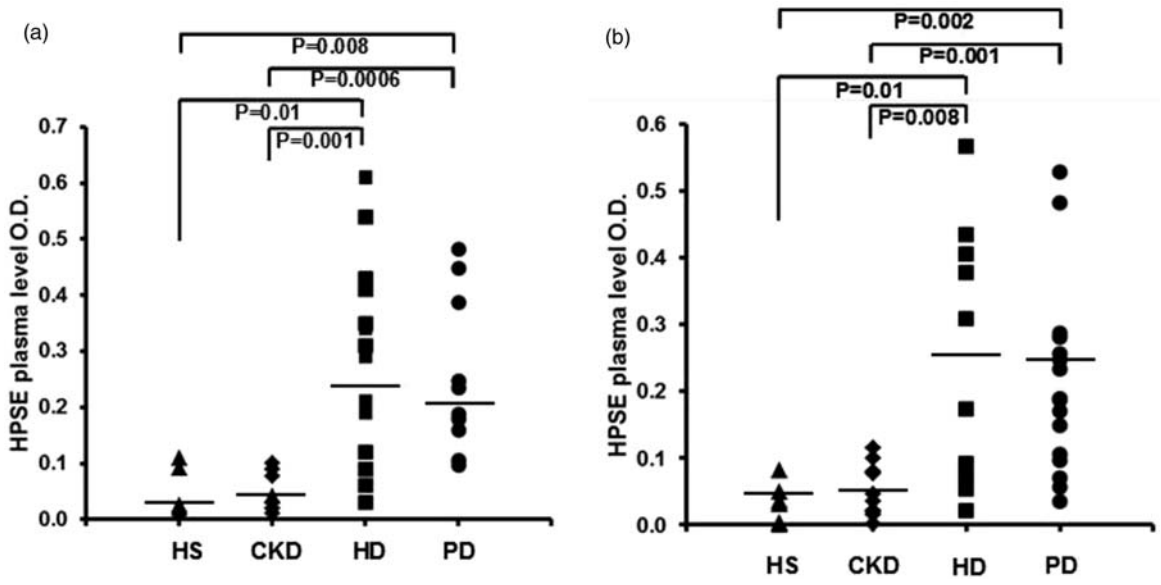

Figure 4 HPSE plasma activity in HS, CKD at early stages, PD, and HD patients. Dot-plot represents the HPSE plasma activity in (a) training group (5 HS, 9 CKD, 10 PD, and 17 HD) and (b) testing-group (7 HS, 10 CKD, 17 PD, and 11 HD) assessed by ELISA. As reported, the activity of HPSE was significantly higher in both study groups in PD and HD compared to CKD and HS. No differences were found in PD versus HD and CKD versus HS. The line represents the mean value

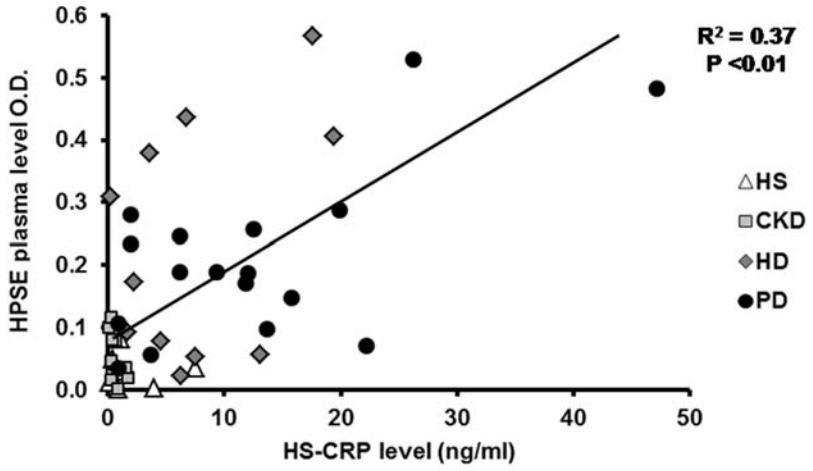

Figure 5 Correlation between high-sensitive C-reactive protein (HS-CRP) and HPSE plasma activity levels in PD ($n = 17$), HD ($n = 11$), CKD ($n = 10$), and HS ($n = 7$). As indicated in the figure, HS-CRP levels correlated with HPSE plasma activity measurements in testing-group patients ($R^2 = 0.37$, $P = 0.007$)

activation with the increased synthesis and release of pro-inflammatory cytokines as consequences.[34–37] It is well reported that the avoidance of chronic microinflammatory stimuli by the use of biocompatible PD fluids and dialysis devices is the most important criterion to enable long-term dialysis without introducing clinically significant changes in the functional characteristics of the peritoneum and systemic inflammatory effects in PD patients.[38] The effects of bioincompatibility on clinical outcome include changes in the physiology of cell populations constituting the peritoneal cavity (leukocyte, mesothelial and endothelial cells, and fibroblasts) and the gene expression of PBMC triggering alterations in cytokine, chemokine and growth factor networks, the upregulation of proinflammatory and profibrotic pathways, and the induction of oxidative stress.[39–41]

Other mechanisms involved in the release of pro-inflammatory cytokines include the generation of complement fragments as a result of plasma protein-membrane contact and the backfiltration of contaminated dialysate to the blood compartment.[12,42]

This study, using an innovative genomic approach able to simultaneously evaluate the expression of more than 15,000 genes was undertaken to select a set of genes deregulated in patients on chronic dialysis treatment and to assess the possible differences between dialysis modalities. However, to take full advantage of the opportunities offered by this high throughput method, to reduce the false positive rate and the confounding factors not directly associated with the aims of our research, we decided to use a pathway analysis to focus our research on candidate genes known to

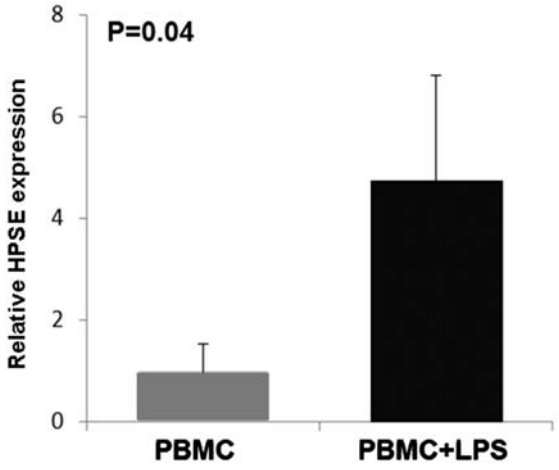

Figure 6 HPSE gene expression by RT-PCR in peripheral blood mononuclear cells (PBMC) stimulated with LPS. The histograms represent the mean ± SD of HPSE expression level of three randomly selected patients assessed by RT-PCR and the comparative CT method. LPS stimulation caused a statistically significant up-regulation of HPSE

be associated to proteoglycan biosynthesis and metabolism. This choice was also based on recent literature evidences[16,17,20] underling a key role of the above pathways in the onset and development of several CKD-related systemic clinical complications.

In our study, then, the main experimental procedures were conducted on isolated patients' PBMCs. These easily accessible cellular elements able to produce PGs[43] and primarily involved in the systemic immune/micro-inflammatory response are frequently utilized in transcriptomic studies because a small number of them may generate an adequate amount of RNA for microarray hybridization.[44–46] However, it is unquestionable that further studies are needed to evaluate whether the PBMCs subpopulations have distinctive PGs biosynthetic transcriptomic profiles.

After statistical analysis and bioinformatics, we assessed that a large number of genes ($n = 25$) involved in PGs synthesis and metabolism were up-regulated in PBMCs isolated from PD and HD patients. Among them, *HPSE, VCAN, VEGFA*, and *FBLN1* were the most significantly up-regulated genes.

Versican (VCAN) is a chondroitin sulfate proteoglycan that influences the central events involved in an inflammatory response, thanks to its capacity to interact with other ECM components, cell surface proteins (e.g. receptors), proteases, chemokines, and growth factors.[47–49] Numerous studies have reported that specific cytokines or growth factors, such as TGF-β1, -β2, -β3, IL-1β, and TNF-α regulate the synthesis of VCAN.[50,51]

Vascular endothelial growth factor (VEGF) is a well-known promoter of angiogenesis,[52] a stimulator of endothelium and vascular smooth muscle cell mitogenesis,[53,54] a regulator of vascular permeability,[55] and a modulator of the expression of adhesion molecules on the surface of endothelial cells.[56]

Fibulin-1 (FBLN1) belongs to a family of ECM proteins with functional associations with elastic fibers and basement membranes. Fibulin-1 interacts with a number of ECM molecules and a positive correlation between fibulin-1 plasma levels, and cardiovascular risk markers in patients with CKD has recently been shown.[57]

Although all genes investigated were extremely interesting, we focused on HPSE because of its close link with inflammation and the specific interests of our research group on understanding the role and the clinical utility of this molecule.[58–60]

HPSE is an endo-β-glucuronidase capable of cleaving the HS side chains of extracellular matrix proteoglycans at a limited number of sites, yielding HS fragments of ~5–7 kDa.[61] HPSE activity also results in the release of many HS-bound molecules including growth factors (e.g. FGF and VEGF), extracellular matrix molecules, enzymes, chemokines, and cytokines which are involved in processes such as inflammation, tissue repair, and tumor invasion.[62,63]

Numerous published results have shown that HPSE plasma levels are increased in disorders such as atherosclerosis,[64] cardiovascular diseases,[65] diabetic nephropathy,[66] and cancer,[67,60] and it is involved in the pathogenesis of several nephrotic syndromes and renal fibrosis,[59] suggesting its potential usefulness as a prognostic marker.

Here, we provide evidence that HPSE gene expression and plasma activity levels are considerably higher in dialysis patients (both PD and HD) compared to CKD/HS and that, interestingly, the protein level significantly correlates with HS-CRP measurements. These results are in line with a previous report performed in a relatively small study group, showing increased PBMC HPSE gene expression and plasma levels in micro-inflamed HD patients.[20] Additionally, to our knowledge, our paper has assessed the role of HPSE in PD patients for the first time.

We also stimulated PBMCs with LPS to confirm the possible influence of the inflammatory state on HPSE cellular expression. The use of this molecule was justified by a previously published *in vitro* experimental model of inflammation.[68]

As largely reported, PBMC of HD patients could be activated by the contact with bacterial-derived LPS due to the backtransport from the dialysate into the blood.[69–72]

Likewise, endotoxemia seems to be common in PD patients and the degree of circulating LPS is significantly related to the severity of systemic inflammation and features of atherosclerosis.[73] Therefore, although other inflammatory stimuli have been reported in literature (e.g., phytohemagglutinine, IL6, IL1B),[74,75] we decided to use LPS for our *in vitro* experiments.

Our *in vitro* results, although limited, clearly demonstrated an inflammatory-related transcriptional up-regulation of HPSE. However, the evaluation of HPSE alone and the use of CRP levels as a unique marker of inflammation may represent a limitation of the study. Therefore, in future, it could be useful to extend the analysis to other identified molecules and to perform a whole genome analysis including genes involved in other pathways (e.g., metabolic, apoptosis).

Taken together, our results showed that PD patients, similarly to those treated with HD, have a different biosynthesis and metabolism of proteoglycans compared to CKD and HS; and they point out on the pivotal role of HPSE in

this condition. Additionally, our data add new insights toward understanding the systemic biological machinery deregulated in dialyzed patients. Finally, the biological elements identified could help clinicians to identify new biomarkers potentially useful as new diagnostic tools and as targets for new therapeutic interventions.

Author Contributions: GZ, FS, and GG performed microarray experiment and data analysis. VM, PP, and SG carried out the biomolecular experiments. GG and LG helped in the manuscript writing and data analysis. AL supervised microarray part of the study and helped to write the paper. All authors read and approved the final manuscript.

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