TRANSCRIPTOMIC ANALYSIS REVEALS A MICROINFLAMMATION-RELATED UPREGULATION OF HEPARANASE IN PERITONEAL AND HEMODIALYSIS PATIENTS.

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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] Additionally, along to the disease progression, CKD patients develop complex biological and biochemical dysfunctions that, in the last stage of renal damage (end stage renal disease), require a rapid start of renal replacement therapies (peritoneal- and hemo-dialysis) which are responsible of considerable molecular and cellular alterations leading to metabolic changes, enhancement of inflammation/oxidative stress and significant development of several immunological deregulations [2-3]. Interestingly, 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 to renal failure [4]. PGs are ubiquitous substances produced by a multitude of cell types including peripheral blood mononuclear cells (PBMCs). Thanks to their capability to interact with cytokines and growth factor, these substances have been involved in important immunological functions and, as demonstrated in several tissues, PGs are now recognized for the important role they play in controlling the inflammatory response [5]. In particular, Cohen-Mazor M et al have described that Heparanase, an endoglycosidase that cleaves heparan sulfate (HS) side chains of proteoglycans, after being released from circulating PBMCs, were involved in extracellular matrix degradation and atherosclerotic lesion progression of patients on chronic

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 Declaration of Helsinki and approved by the institutional ethical board of the University Hospital "Policlinico di Bari", Bari, Italy. A) Training-group. This population was used for the microarray analysis and to generate the initial transcriptomic model. It included 5 healthy subject (HS), 9 CKD patients on stage stage IIIIV (mean SD of estimated GFR by CKD-EPI formula: 32.27 14.7 ml/min), 10 peritoneal dialysis (PD) patients and 17 hemodialysis (HD) patients. B) 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 stage stage III-IV, 17 PD patients and 11 HD patients.

High sensitive serum C-reactive protein (HS-CRP) measurement. HS-CRP levels were measured in all patients included in the testing-group using highsensitivity immunonephelometry according to the manufacturer's protocol.

Peripheral Blood Mononuclear Cells (PBMCs) isolation and plasma collection. Twenty ml 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™ gradient (460 g for 30 min). Cells were counted, and viability was assessed by trypan blue exclusion method (>90% PBMCs were viable). A total of 3 ml of peripheral blood were collected into tubes containing EDTA from all patients included in the study. The blood was, then, centrifuged 15 minutes at 1500 g and the obtained plasma was aliquoted and stored at -80 C until use.

RNA extraction and microarray experiments. Total RNA was isolated from PBMCs by RNeasy mini kit Qiagen. RNA was quantified by a NanoDrop ND-1000 Spectrophotometer and its integrity was assessed by electrophoresis, using the Agilent 2100 Bioanalyzer. RNA was, then, processed and hybridized to the GeneChip Human Genome U133A oligonucleotide microarray (Affymetrix). The default settings of Affymetrix Microarray Suite software version 5 was utilized to calculate scaled gene expression values. Results of the microarray experiments are available in Gene Expression Omnibus (Accession number GSE15072).

Heparanase plasma assay. To measure the heparanase (HPSE) plasma activity, we used a well standardized assay. Briefly, HPSE was quantified by an assay based on the ability of HPSE to degrade heparan-sulphate proteoglycans present in the Matrigel (Matrigel TM Basement Membrane Matrix).

In vitro study and real time (RT)-PCR for HPSE. PBMCs, isolated from 3 randomly selected healthy subjects 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% CO2 and, then, stimulated with Lipopolysaccharide (LPS) (10 μg/ml) for 4 hr. Subsequently, after extraction, 1 μg of RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed on an ABI-Prism 7700 (Applied Biosystems) using KAPA SYBR® FAST qPCR kit (KAPA Biosystems). A relative quantitative analysis was performed to assess HPSE expression normalized to GAPDH.

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 $P \leq 0.05$ 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. Several statistical algorithms and distinction calculation were used to select probe sets discriminating the study groups. False discovery rate was estimated using the Storey's q-value. 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

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

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 Gene Ontology. 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 selected gene probe sets, 36 (corresponding to 25 genes; e.g., HPSE, VCAN, VEGFA, FBLN1) were up-regulated and 58 (corresponding to 45 genes; e.g., IDS, HEXA, DMD, SNTB2) were down-regulated in PD/HD patients compared to HS/CKD (for details see supplemental table A and B). The 2D hierarchical clustering using the 94 gene probe sets clearly separated patients into 2 distinct groups (PD/HD versus HS/CKD) [**Figure 1**] and PCA illustrates the degree of separation among the study groups [**Figure 2**].

Heparanase (HPSE) plasma activity

For the second part of the study, we focused on HPSE, one of the top selected upregulated gene in PD/HD identified by transcriptomic analysis. As shown in Figure 3, HPSE plasma activity was higher in PD/HD in both training- and testing-group confirming microarray results. Additionally, we did not find any significant difference in HPSE activity among PD and HD patients, and CKD and HS [**Figure 3**].

Correlation between HPSE plasma activity and HS-CRP levels

Because, as reported by several literature evidences, there is a close link between

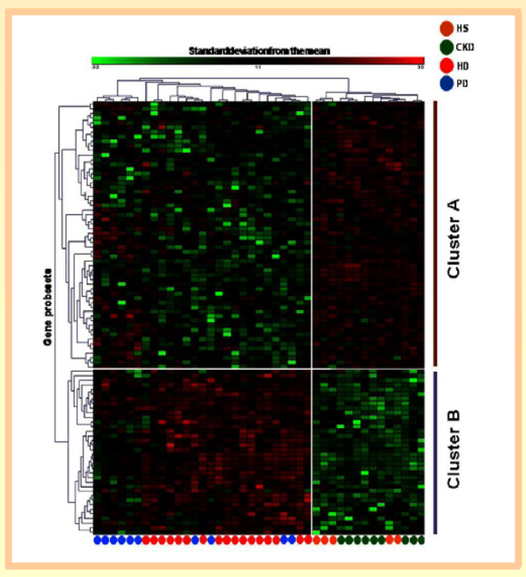

Figure 1: "Supervised" hierarchical clustering using the top selected genes by microarray analysis.

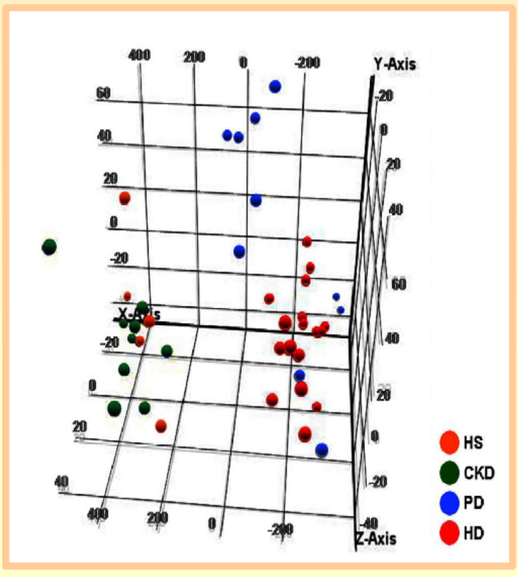

Figure 2: Principal component analysis (PCA) discriminating hemodialysis (HD), peritoneal dialysis (PD), chronic kidney disease (CKD) and healthy

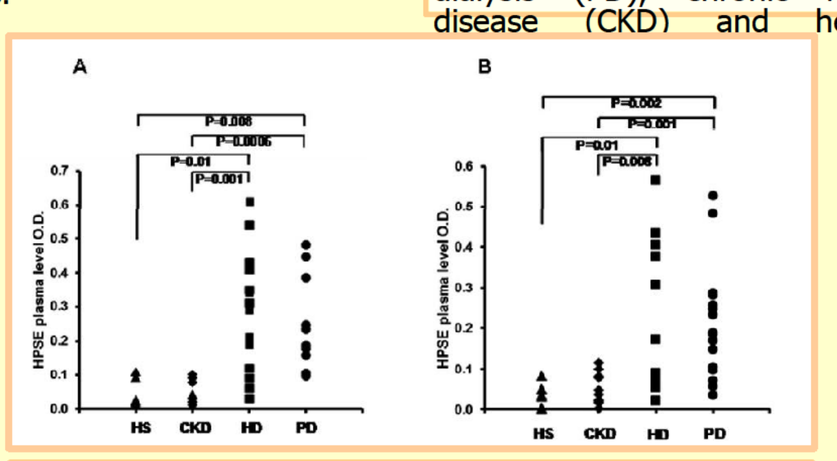

Figure 3: Heparanase (HPSE) plasma activity in healthy subjects (HS), chronic kidney disease at early stages (CKD), peritoneal dialysis (PD) and hemodialysis (HD) patients assessed by ELISA.

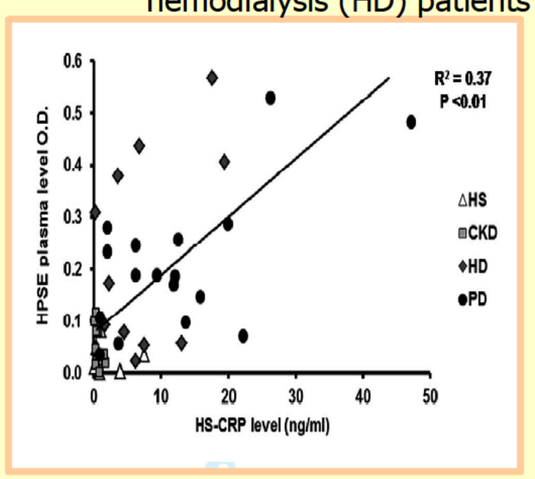

Figure 4: Correlation between high-sensitive C-reactive protein (HS-CRP) and HPSE plasma activity levels in PD (n=17), HD (n=11), CKD (n=10) and HS (n=7).

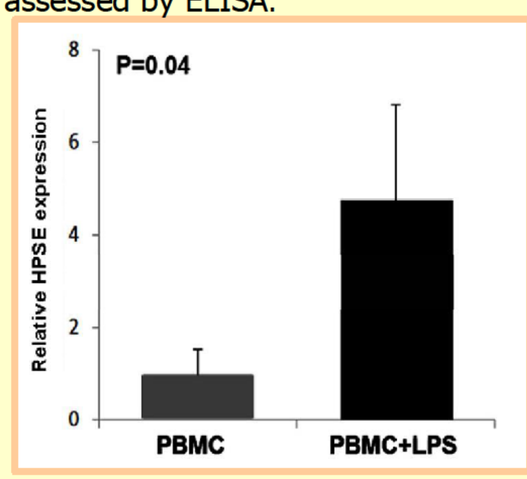

Figure 5: HPSE gene expression by Real-Time PCR in peripheral blood mononuclear cells (PBMC) stimulated with Lipopolysaccharide (LPS).

CONCLUSION

Taken together our results showed that peritoneal dialysis patients, similarly to those treated with hemodialysis, have a different biosynthesis and metabolism of proteoglycans compared to CKD and HS, and they point out on the pivotal role of Heparanase in this condition. Additionally our data, all together, add new insights towards understanding the systemic biological machinery deregulated in dialyzed patients. Finally, the identified biological elements could help clinicians to identify new biomarkers potentially useful as new diagnostic tools and as targets for new therapeutic interventions.

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