Gene Expression Profile of HCAEC Cells Exposed to Serum From Chronic Kidney Disease Patients: Role of MAPK Signaling Pathway

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Abstract

End-stage renal disease (ESRD) patients have an elevated risk of cardiovascular (CV) complications including acute myocardial infarction (AMI); endothelial dysfunction and accumulation of uremic toxins have been associated with such CV-events. To explore which molecular pathways are involved in this CV-complication and the effects of the uremic serum on gene expression, an endothelial dysfunction model was studied through microarrays and pathway analysis. mRNA was isolated of human coronary arterial endothelial cells (HCAEC) primary cultures supplemented with 20% uremic serum from two groups of patients, USI: ESRD-patients; UCI: ESRD-AMI-patients. Affymetrix GeneChip® microarray and the LIMMA-package (Linear Models for Microarray Data) of the Bioconductor sofware17 was implemented to identify relevant DEGs between the two groups of uremic patients. Protein-protein interaction networks and pathway analysis were made to analyze the interaction and expression tendency of differentially expressed genes. 100 differentially expressed genes were identified from two data sets triggered by uremic state using bioinformatics, from 16,607. After in a new cohort, 30 genes were overexpressed in UCI group, which we identified 500 ontological genetic terms and one KEGG-pathway with $p < 0.05$. The metabolic pathway significantly represented was the *MAPK* signaling pathway. Network analysis showed six genes (*PTGS2, SELE, ICAM1, HMOX1, EGR1, and TLR2*) that represent potential markers for ESRD with AMI, as an approximation to their underlying mechanisms. The results obtained suggest that uremic toxins in patients with ESRD can alter HCAEC and modify the gene expression profile, which could have an impact on the development of cardiovascular complications in these patients.

1. Introduction

Chronic kidney disease (CKD) represents a public health problem throughout the world, it arises from many heterogeneous pathways marked by deteriorating of kidney structure and function irreversibly over time and is characterized by a high risk of cardiovascular (CV) death, that increases exponentially according to the degree of kidney damage [1]. In patients with end-stage renal disease (ESRD), who receive renal replacement therapy, it is estimated that CV mortality is greater than 50% of all deaths in this population [2, 3] of which 20% are caused by acute myocardial infarction (IAM) [4–6]. Due to the multifactorial and complex nature of CKD, non-traditional and specific risk factors for CKD have been studied, such as endothelial dysfunction and the accumulation of uremic toxins (UT) [7–9], which could help to better understand the processes involved in the cardiovascular complications in ESRD patients and it has been mentioned that analyzing the genetic profile of endothelial cell expression could help us better understand the processes involved in this disease [10].

Endothelial cells (CE) serve as the first barrier that plays a crucial role in maintaining vascular integrity, and in patients with ESRD, the uremic environment leads to endothelial dysfunction due to the toxic effects of uremia and has been associated as initial event in the development of cardiovascular diseases (CVD) [11], and also with adverse outcomes in dialysis patients [12], UTs are compounds that accumulate in the blood and tissues during the development of ESRD, and some of them exert adverse biological effects mediated by the toxins themselves to form new compounds that negatively interact with various

biological functions [13]. Studies have shown that some of these toxins behave as pollutants in the environment and induce endothelial dysfunction and leukocyte activation, promote inflammation and thrombosis, and increase vascular oxidative stress in CKD [14, 15]. Furthermore, other studies have used approaches to classify uremic metabolites that predict adverse clinical outcomes in large cohorts of end-stage renal disease, some of which have been associated with mortality in these patients [16, 17]. There are some gene expression studies in cell cultures that have revealed some possible mechanisms involved in patients with ESRD associated with EC dysfunction [18–20]. Recently, a study has explored the signaling pathways involved in cardiovascular disease processes through stimulation with specific UTs [21]. And it has been mentioned that studies focused on UTs and related pathways could have a high therapeutic value in mitigating the progression of cardiovascular disease [22]. In this work we compare the gene expression profile of HCAEC in RNA samples exposed to uremic serum obtained from ERT patients with and without MI using the microarray approach to identify unique MI-ESRD gene expression signatures using a model *in vitro* of endothelial dysfunction.

2. Methods

Experimental Design

The study was approved by the Medical Review Ethics Committee of the Instituto Mexicano del Seguro Social (R-2008-3601-113/FIS/IMSS/PROT/551), and all patients gave their consent to participate in the study. Stage 4–5 patients were selected according to the KDOQI guidelines undergoing conservative treatment at outpatient chronic kidney disease (CKD) clinics and dialysis clinics at the hospitals with which we collaborate. Six patients were included in this study, divided into two groups. Group 1, patients with end-stage renal failure (ESRD) undergoing peritoneal dialysis treatment without a history of acute myocardial infarction (AMI), called uremia without infarction (USI) and Group 2: patients with ESRD undergoing hemodialysis treatment with a history of MI infarct, identified as uremia with infarction (ICU).

Collection of the uremic serum sample

A venous blood sample was taken from the selected patients and placed in plastic tubes without anticoagulant (BD-Vacutainer Plymouth, United Kingdom), previously cooled, which were immediately sent on ice to the laboratory and cold centrifuged (1300 xg at 4 ° C for 15 min). The serum was then aliquoted and frozen at -–80°C until use.

HCAEC culture, RNA isolation and purification

Human Coronary Artery Endothelial Cells (HCAEC; No. PCS-100-020; ATCC) and Endothelial Cell Enrichment Kit-VEGF (No. PCS-100-041; ATCC) were purchased from the American Type Culture Collection (ATCC). Manassas, VA, USA). The preparation and cultivation of the HAEC was carried out according to the manufacturer's instructions. The final concentrations in the medium were: rhVEGF, rhEGF and rhFGF basic 5 ng / ml, rhIGF-1 15 ng / ml, L-glutamine 10 mM, heparin sulfate 0.75 U / ml, hydrocortisone hemisuccinate 1 µg / ml, 2% fetal bovine serum, 50 µg / ml ascorbic acid and 10 U / m-10

µg / ml penicillin-streptomycin. The endothelial cells (CE) were cultured at 37ºC in a humid atmosphere with 5% CO2, the culture medium was renewed every 2 days and they were separated in a ratio of 1: 3 in each passage; cells from 3 to 6 passages were used for this study. Serum samples from the USI and UCI groups were inactivated for 30 min at 56oC and adjusted to a concentration of 20%, then added to cell cultures and incubated for 24 h. Subsequently, the cultured CEs were trypsinized, harvested and washed with lysis buffer. Cells were lysed using a TissueLyserTM system (Qiagen, Valencia, CA, USA) for 25 seconds at 25 Hz. Total RNA was isolated using a commercially available kit (RNeasy, Qiagen, Valencia, CA, USA) and stored at —80° C until use. Total RNA quantification was done using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and the RNA integrity number (RIN) score was obtained using the Agilent 2100 bioanalyzer, the RNA 6000 Nano LabChip kit and Agilent 2100 Expert software (Agilent Technologies, Santa Clara, CA, USA). Only those samples with RIN ≥ 9.5 were included in the microarray assay.

Microarray analysis

The microarray used in the present study was Human GeneChip 1.0 (Affymetrix, Santa Clara, CA). This chip includes a total coverage of 36,079 transcripts (Ref Seq). The preparation and amplification of the samples was carried out according to the manufacturer's protocols (Affymetrix Inc.). Briefly, 100 ng of total RNA was used for cDNA synthesis. The cDNA was transcribed in vitro for labeling and cleaved by enzymatic reaction, then placed in the microarrays and hybridized in an Affymetrix hybridization oven at 60 rpms, 45 ° C for 17h. Subsequently, the microarrays were washed and stained using the GeneChip 450 fluid station and scanned using a 7G GeneArray® Scanner (Affymetrix, Santa Clara, CA). We analyzed a total of 6 microarrays, identified as Experiments: 433, 434, 435, which correspond to the USI Group and Experiments: 436, 437, 438 which correspond to the UCI Group. We use GeneChip operating software (GCOS, Affymetrix) to obtain and analyze the images.

Bioinformatic Analysis

Data preprocessing

The processing of the “raw data” of the intensity data of the cells of the probes (CEL files) (Affymetrix Inc.) was carried out by means of a customized protocol implemented in R language using the *affyparser, affyoi* and oligo libraries from Bioconductor [23]. An RMA (Robust Multichip Average) normalization was carried out and subsequently a filtering to eliminate the transcripts with very low levels of expression, for which a cut-off value of 4.2 was used for the median of the intensities. Subsequently, an annotation of the transcripts was carried out, using the annotation data of Affymetrix hugene10sttranscriptcluster.db (R package version 8.7.0.).

Genomic differential expression

Statistical analysis was performed with the limma package (*Linear Models for Microarray Data*) [24]. To do this, a contrast matrix was generated between two differential conditions, called USI and UCI, and then

an adjustment of the model was carried out. Statistical relevance was determined by the Bayes variance moderation method using a moderate Student's t-test (which is recommended for estimating variance in microarray experiments with few replications). The threshold for genes upregulated and downregulated according to logFC > 1 and logFC <-1, respectively, and a *p*-value ≤ 0.01. On the other hand, a Gene Ontology (GO) enrichment analysis was carried out, using Bioconductor's *topGO* package to investigate the functional categories of differentially expressed genes, and the number of differentially expressed genes included in each GO term was counted. The relevance of the enriched categories was determined using a classical Fisher test and the best 500 categories were selected. The enriched GO relations map was elaborated considering the first 5 significant nodes.

Construction of the network of molecular interactions from the stimulation of HCAEC with uremic serum

A molecular network was constructed to reveal the interactions of the selected genes using the Interacting Gene Retrieval Tool (STRING) [25]. Microarray data was publicly deposited with Gene Expression Omnibus - Accession Number GSE125898

Validation assay through real-time qPCR

Among the differentially expressed genes identified by microarray and molecular network analysis, four of them were selected for RT-qPCR analysis. Briefly, the total RNA from each sample was reverse transcribed into cDNA using reverse transcriptase, and the resulting cDNA was then used as a template for quantitative PCR amplification, the product was resolved by electrophoresis on a 2% agarose gel to know the integrity of the cDNA and to be able to use it for qPCR. Before starting qPCR, all cDNA samples were homogenized to a cDNA concentration of 50 ng / µl to facilitate methodology and analysis. The expression levels of these genes were measured using the appropriate design and all tests were performed on the StepOne ™ real-time PCR system (Applied Biosystems, Fosters City, CA, USA). The quantitative fluorescence of the data was analyzed using sequence detection software (SDS version 2.2, PE Applied Biosystems). The cycle number in which the amplification plot crossed the threshold was calculated and the cycle threshold (Ct) values were recorded. The relative expression of genes was calculated using the method $2^{-\Delta\Delta Ct}$ [26], ornithine decarboxylase Antizyme 1 (OAZ) was used as an internal control to normalize the data. The primer sequences are listed in **Supplementary Table 1**

Statistics analysis

Statistical analysis was performed using One Way ANOVA in the data analysis package in SPSS 23.0 statistical software (SPSS Inc., Chicago, USA). Student's t test was applied to comparisons between two groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

Study population

The characteristics of the patients from whom the uremic serum samples were obtained are shown in Table 1

Table 1
Clinical and analytical characteristics of the patients who provided their serum sample.

Characteristic	Description	USI Group, Uremia without infarction (n = 3)	UCI Group, Uremia with Infarction (n = 3)
Demographic	Age (years)	66 ± 21	66 ± 11
	Female Male	1/2	1/2
Cause of End-Stage Renal Disease (%)	Diabetic nephropathy	1 (33)	3 (100)
	Arterial hypertension	1 (33)	3 (100)
	Coronary heart disease	0 (0)	3 (100)
	Other or Unknown	1 (33)	1 (33)
Comorbidity (%)	Ischemic heart disease	1 (33)	3 (100)
	Myocardial infarction	0 (0)	3 (100)
	History of hypertension	3 (100)	3 (100)
	Mellitus diabetes	3 (100)	3 (100)
	Smoking	2 (67)	0 (0)
	Familial hyperlipidemia	1 (33)	1 (33)
	Familial hypercholesterolemia	1 (33)	1 (33)
Treatment (%)	Insulin	0 (0)	2 (67)
	Folic acid	0 (0)	1 (33)
	Complex B	0 (0)	2 (67)
	Calcitriol	0 (0)	1 (33)
	Enalapril	1 (33)	0 (0)
	Losartan	1 (33)	1 (33)
	Amlodipino	1 (33)	0 (0)
	Clopidogrel	0 (0)	1 (33)
	Acetylsalicylic acid	0 (0)	1 (33)
	Isosorbide	2 (67)	1 (33)
	Statins	0 (0)	1 (33)

Values express n (%), mean [range]

Characteristic	Description	USI Group, Uremia without infarction (n = 3)	UCI Group, Uremia with Infarction (n = 3)
Renal replacement therapy	Peritoneal dialysis (%)	3 (100)	0 (0)
	Hemodialysis (2–3 sessions / week)	0 (0)	3 (100)
Analytical	Glucose (mg/dl)	125 (89–167)	124 (86–189)
	Urea (mg/dl)	81 (32–108)	122 (104–134)
Treatmen (%)	Creatinine (mg/dl)	10.5 (1.1–18)	10.6 (5.8–16.1)
	Cholesterol (mg/dl)	170 (147–192)	166 (149–192)
	Triglycerides (mg/dl)	115 (100–138)	180 (115–223)
	HDL-Cholesterol (mg/dl)	43 (28–64)	29 (27–32)
	LDL-Cholesterol (mg/dl)	110 (99–126)	105 (100–112)
Values express n (%), mean [range]			

Quality control assurance

Before determining DEGs, we analyze the microarray to assess quality. Microarray quality analysis was done with *Bioconductor's arrayQualityMetrics package*. The quality was satisfactory as can be seen in the files that we include as supplementary material (**report.zip_index.htm**).

Principal Component Analysis (PCA) and Comparison of Gene Expression Profiles Across Hierarchical Groups

The cluster study using the principal component analysis (PCA) shows the grouping of the samples according to their groups: USI and UCI, which in the graph are located in opposite areas (Fig. 1, a). However, the USI group showed a high intragroup dispersion that differs from the UCI group. Differences in the gene expression pattern could be related to abnormalities that are likely to lead to cardiovascular disease in patients with IRT. We used 2-way unsupervised hierarchical clusters to analyze the expression profile of the different groups. The unsupervised analysis clearly separates the two experimental groups USI and UCI, demonstrating their dissimilarity. We consider significant genes in the framework of $p < 0.05$ and genes overexpressed with *log*FC > 1 and underexpressed with *log*FC <-1, to define differential gene expression profiles (Table 2).. We used these genes as target genes for gene ontology and annotation analysis based on the expression of 100 genes involved in end-stage kidney disease and infarction visualized through a hierarchical clustering dendrogram of the expression profile (Fig. 1, b).

Table 2
42 Differentially expressed genes

SYMBOL	GENENAME	Log2 Fold Change
OTUB2	OTU deubiquitinase, ubiquitin aldehyde binding 2	1.24784736
DDIT3	DNA damage inducible transcript 3	1.12444667
EXOC3L2	exocyst complex component 3 like 2	1.33359048
CXCR4	C-X-C motif chemokine receptor 4	1.46389778
EGR1	early growth response 1	1.21455052
CCL5	C-C motif chemokine ligand 5	1.19118113
MIR21	microRNA 21	1.09812127
FAM102A	family with sequence similarity 102 member A	1.66949083
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif 4	1.70255491
STC1	stanniocalcin 1	2.08837478
SLC39A10	solute carrier family 39 member 10	1.13058274
PLA2G4C	phospholipase A2 group IVC	1.0174256
UBD	ubiquitin D	1.35415701
UBD	ubiquitin D	1.33496747
SELE	selectin E	1.6895415
HSD17B14	hydroxysteroid 17-beta dehydrogenase 14	1.00789671
VGLL4	vestigial like family member 4	1.23549369
PLA2G4A	phospholipase A2 group IVA	1.009869
IL1A	interleukin 1 alpha	1.21211616
LYPD1	LY6/PLAUR domain containing 1	-1.1448949
NRG1	neuregulin 1	-1.2643943
DKK1	dickkopf WNT signaling pathway inhibitor 1	-1.1293126
SULT1B1	sulfotransferase family 1B member 1	-1.1046125
MT1E	metallothionein 1E	-1.3871371
RGS4	regulator of G protein signaling 4	-1.2042344
PRICKLE1	prickle planar cell polarity protein 1	-1.3330305
TGFB2	transforming growth factor beta 2	-1.7897115

SYMBOL	GENENAME	Log2 Fold Change
ICAM1	intercellular adhesion molecule 1	1.21240874
HMOX1	heme oxygenase 1	1.07180732
CCNA1	cyclin A1	-1.0558036
DHCR24	24-dehydrocholesterol reductase	-1.0625927
PTGS2	prostaglandin-endoperoxide synthase 2	1.67290225
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif 9	1.0539104
SNORA23	small nucleolar RNA, H/ACA box 23	1.02948249
GDF15	growth differentiation factor 15	1.0566471
TLR2	toll like receptor 2	1.25298093
CYP1A1	cytochrome P450 family 1 subfamily A member 1	1.18216497
THUMPD3-AS1	THUMPD3 antisense RNA 1	1.01512175
MIR186	microRNA 186	1.1629098
BDNF	brain derived neurotrophic factor	-1.0833956
CCDC190	coiled-coil domain containing 190	-1.2005495
CYP4Z1	cytochrome P450 family 4 subfamily Z member 1	1.01580511

Differential genomic expression

Microarray analysis was performed to investigate gene expression levels in HCAECs cells exposed to uremic serum from UCI group samples compared to USI group samples. Only those genes were selected that in our six microarrays: three control and three test arrangements, showed correspondence in the levels for each group, that is, that the expression in the three was greater than the cut-off value based on the expression of arrangement background. Next, the statistical analysis was performed with LIMMA, in this case an experiment design matrix was created contrasting cases of uremia with infarction and uremia without infarction. A linear model was developed for the data according to this contrast and the Empirical Bayes Statistics for Differential Expression statistical test was applied. From this study, the Volcán graph was obtained to visualize the differentially expressed transcripts in the samples of the USI and UCI group (Fig. 1, a) and two tables, one using the most significant data according to the adjusted p-value (with which The analysis of networks and Ontology was carried out) and another table based on the traditional filtering with the log Fold Change and the *p*.value (**Table Suppl 1 DE_limma_1 and Table Suppl 2 DE_limma_2**).

In total, we identified 590 genes differentially expressed in HCAECs cells cultured in the presence of uremic serum from the two study groups; 342 were over-expressed, while 250 were under-expressed ((2.0-

fold, $p < 0.05$). Of the 42 most significant differentially expressed genes (Fig. 1, d) it can be seen that the Staniocalcina-1 (STC1) gene was the most overexpressed, with a logFC of 2.09, while the TGFB2 gene was the most under-expressed, with a *log*FC of -1.8. The most significant upregulated coding genes were: *STC1, ADAMTS4, SELE, PTGS2, FAM102A, CXCR4, UBD, EXOC3L2, OTUB2, VGLL4, EGR1, ICAM1, IL1A, CCL5, SLC39A10, DDAMIT3, HMOX1, GD9F15, ADAMGTSC PLA2G4A and HSD17B14*; the genes significantly downregulated were: *CCNA1, DHCR24, BDNF, SULT1B1, DKK1, LYPD1, CCDC190, RGS4, PRICKLE1, MT1E and TGFB2*. The complete list of genes is provided as supplementary information (**Table Supl.1 DE_limma_1**).

Functional analysis through gene enrichment

Microarray analysis revealed a significantly different expression profile of 100 genes capable of discriminating patients with ERT (Fig. 1, c). By gene pool enrichment analysis, we obtained 50 genes differentially expressed between the two groups of patients: 30 genes were overexpressed and 12 were under-expressed in the ICU group (Fig. 1, d). To explore the functional similarities of the 50 differentially expressed genes, we used enrichment analysis to determine the probability that these gene clusters fall within any ontological genetic term (GO) and functional categories defined by KEGG. Specifically, we use the Panther GeneOntology tool for the functional analysis of differentially expressed genes. The significance of the enrichment was measured by P values according to Fisher's exact test. For 30 overexpressed genes in the ICU group, we identified 182 GO terms and a KEGG pathway $p < 0.05$. The GO terms identified include the response to hypoxia; negative regulation of signal transduction; the response to organic substances; the response to chemicals; regulation of cardiocyte differentiation; the regulation of apoptotic processes; regulation of neuronal death; the cellular response to chemical stimuli; regulation of signal transduction; the response to toxic substances; the inflammatory response; regulation of molecular function; the negative regulation of multicellular organic processes; the positive regulation of proteins of metabolic processes; the response to external stimuli; positive regulation in cell communication and signaling among others (Fig. 2) Further analysis using a different tool showed that the metabolic pathway that was significantly represented was the *MAPK* signaling pathway, which involves 4 genes (*PLA2G4A, IL1A, RASGRP3 and DDIT3*). Another 10 GO terms and 5 metabolic pathways were also identified, although not significant.

For the 12 under-expressed genes in the UCI group, we identified 32 GO terms and a KEGG pathway with $p < 0.05$. The GO terms identified were related to the regulation of cardioblast differentiation; negative regulation of cardiocyte differentiation; the myocardial morphogenesis of the ventricular trabecula; negative regulation of cardiac muscle cell differentiation; negative regulation in striated muscle cell differentiation; regulation of cell growth; regulation of protein kinases; tissue development; cellular regulation of proteins of metabolic processes and regulation of molecular function, among others.

Construction of a network of molecular interactions

Molecular networks were algorithmically generated by the STRING platform using significant GO terms and pathway analysis, as instructive tools to comprehensively explore the molecular mechanisms

involved in our study. As a result, some positively regulated genes and protein-encoded genes were obtained in the transduction network (Fig. 3). 6 genes: *PTGS2, SELE, ICAM1, HMOX1, EGR1* and *TLR2* are observed as core genes, which are related to: inflammation, cell adhesion, apoptosis, signal transduction and transcriptional regulation in HCAEC cells stimulated with uremic serum. **FileGO_classic_5_def.pdf**. It is a map of the best enriched GO hierarchies according to the criterion of the classic Fisher test, defining the first 5 most significant nodes. (**Figure. 3**). **GO_top_results.csv.** Contains the 500 best enriched biological processes. Some categories are very general and do not mention the genes they include. It can be seen that the MAPK category is the best enriched. The interpretation of this table is greatly complemented by Fig. 3.

Validation

Based on the analysis of the uremic serum pathway and molecular networks in HCAEC, we randomly selected 4 candidate genes: *DDIT3, PLA2G4C*, and *PTGS2. FAM102A* for validation of gene expression by qPCR analysis. The expression levels of these genes are shown in supplementary Fig. 2. And according to the analysis of the microarrays, the qPCR results showed that the expressions of the four genes increased in the UCI group and were under-expressed in the USI group in HCAEC cells treated with uremic serum.

4. Discussion

In this study, we used microarray approach to identify HCAEC gene expression signatures of ESRD with and without MI. Previous literature has revealed that there is an intrinsic interplay between ESRD and CVD, while the detailed mechanism remains unclear. We report the gene expression profile obtained from uremic serum stimulation of endothelial cells in an in vitro model. We also identify common molecular pathways linked to important physiological processes. According to the GO classification, we found that genes differentially express a variety of transcription factors that are involved in the immune response. Several studies have discussed the role of inflammation as a first step to promote endothelial dysfunction and progression of atherosclerotic processes [27]

There are studies that suggest that atherosclerosis could be caused by an immune reaction against autoantigens such as oxidized low-density lipoproteins (LDL) and heat shock proteins (HSP) [27]. Interestingly our microarray profile highlights some genes that could sustain common molecular alterations in ESRD and ECV. Some of these genes, but not all, were independently validated by RT-PCR analysis on the samples.

The cardiovascular system is the main target of uremic toxins and chronic inflammation in ESRD patients. Genetic studies with focus in endothelial dysfunction associated to cardiovascular develop are scarce. The main purpose of this study was to explore the effect of uremic serum on the gene expression pattern of HCAEC associated to adverse cardiovascular outcomes in CKD patients through a microarray analysis.

Although it is widely recognized that patients on dialysis have substantially higher cardiovascular and non-cardiovascular mortality rates compared with the general population, little is known about the genetic predisposition to mortality of these vulnerable patients. In the present study, we investigated serum of patients with ESRD have a very high mortality risk as compared with the general population. Cardiovascular disease is a major cause of death in these patients, accounting for 40–50% of total mortality [28, 29].

Currently, CKD is associated with an increased risk of CVD. In ESRD patients, CVD is responsible for almost 50% of deaths [30]. Several studies have focused on clearing out the mechanisms involved in the increase of the risk. Uremic toxins have been classified into three major groups as proposed by the European Uremic Toxin Work Group (EUTox) as well as their behavior during dialysis in: a. Water-soluble molecules of low molecular weight, such as urea; b. Middle molecules; and c. Protein-bound uremic toxins whose removal through conventional dialysis and hemodialysis treatments is problematic due to their high protein (mostly albumin)-binding affinity [31].

Endothelium, a disseminated organ, is a major component of most organs. Due to its disseminated nature and involvement in the normal physiology in the body it has a myriad of functions. Unsurprising, this functional heterogeneity requires not only a high variation in its phenotype expression which depends on the vascular bed but also needs the ability to react according to the environs in health and disease. We may appreciate the importance of EC by the fact that it is involved in almost all disease states either as a primary determinant or as an innocent bystander [32].

A permanent EC aggression as a result of chronic exposure to uremic toxins induces cellular phenotype abnormalities which may result in high serum levels of inflammatory biomarkers such as IL-8 and MCP-1 (CCL2), cytokines, and the adhesion moleculesVCAM-1 and ICAM-1 [33]. Serum levels of these entire biomarkers rise in patients with CKD a fact that suggests a link between vascular activation, inflammation, and uremic toxicity [34]. Uremic toxins have been associated with EC dysfunction in CKD patients. As a consequence, the uremic toxins may induce active free radicals [34]. Uremic toxins and chronic inflammation undoubtedly contribute to EC dysfunction associated to the CV but the complex mechanism associated to CKD alterations needs to be more elucidated. EC have multiple functions such as regulation of hemodynamics, permeability, nutrients exchange, leukocyte interaction, and blood coagulation, amongst others. Chronic EC dysfunction is also considered as the main event in atherosclerosis which progresses toward a pro-inflammatory cell pattern, senescence, and apoptosis [10].

Moreover, accumulation of uremic toxins induces oxidative stress (OS) related to reactive oxygen species and reactive nitrogen species production (RONS) [35], which, in the vessel wall are mainly produced from NADPH oxidase, xanthine oxidase, the mitochondrial respiratory chain, and uncoupled endothelial nitric oxide synthase (eNOS) in which oxygen is reduced from nitric oxide (NO) synthesis [35]. In a patient with ESRD uremic toxins promote vascular leakage by increasing EC permeability, impaired blood flow, and leukocyte adhesion [36]. Moreover, they may have pro-fibrotic and pro-hypertrophic effects on cardiac cells as well as a proinflammatory effects in monocytes by increasing gene expression of key

inflammatory cytokines involved in the progression of heart failure [37]. The cardiac profibrotic effect of these toxins are also observed in patients with renal failure and in animal models of MI with concomitant renal impairment which is likely mediated through the oxidative stress/NF-κB/TGF-β pathway [38]. Furthermore, the proteomic approaching in ESRD patients reveals changes in the expression of inflammation and oxidative stress related molecules; some of these changes correlated with NFkB activation [39]. Additionally, in animal models, elevated IL-18 levels are associated with pressure overload and inflammatory states and may play a role in cardiac hypertrophy and remodeling [40].

This methodology also revealed possible mechanisms involved in ESRD in patients with EC dysfunction and showed six genes involved in the regulation of cell-cycle progression (CDK-1, topoisomerase II, PDZ-binding kinase, CDCA1, protein SDP35, E2F transcription factor 8), and two genes of the cholesterol efflux system (ABCA1 and ABCG1), which were down-regulated in HCAECs exposed to uremic plasma [19].

We used a microarray technology to investigate the gene expression profiles in HCAECs, induced by serum from USI and UCI patients and we explored which pathways were potentially involved in this process. This microarray approach allowed us to reduce biases due to the relatively small number of patients selected and to minimize confounding factors. Microarray analysis was performed in an UCI group to assess differences in the gene expression pattern vs. USI patients. Unsupervised analysis clearly separated the groups demonstrating their differences. Microarray analysis revealed a 100-gene profile differentially expressed which discriminated CKD patients. By enrichment analysis we reduced the set to 50 genes: 30 genes were over-expressed and 12 were under-expressed in the UCI group. Although we identified candidate molecular markers, it is necessary to test these candidates in independent cohorts before any conclusion concerning their diagnostic impact [40].

The main finding of this work was the identification of key genes involved in the MAPK signaling pathway. The MAPKs signaling pathway is involved in a repertoire of biological events including proliferation, differentiation, metabolism, motility, survival, and apoptosis. And it pathway encompasses a large number of serine/threonine kinases and it is divided into four MAPK subfamilies including extracellular signal-regulated kinases (ERK1/2), c-Jun NH2-terminal kinases (JNK1, -2 and – 3), p38 kinase (α, β, γ, δ,), and big MAPK (BMK or ERK5) [41]. Studies have shown that MAPK subfamilies are involved in the pathogenesis of numerous renal diseases, including CKD and ESRD [42], and produce important signaling molecules involved in inflammatory process in the kidney [48]. Also, previous studies have focused on TGF-β and epithelial or EC for mesenchymal transition in myofibroblast transformation, which leads to fibrosis [43].

On the other hand, while we looked for a gene-set associated to MAPK signaling pathway, we found a group of four specific DEGs genes members of this pathway: *PLA2G4A, IL1A, RASGRP3* and *DDIT3*, which are molecules related to inflammation, apoptosis, signal transduction and atherosclerosis. *PLA2G4A* was one of the two most significantly overexpressed genes. Phospholipases A2 (PLA2s), a family of enzymes that hydrolyze the fatty acid at the sn-2 position of phospholipids, play pivotal roles in cell signaling and inflammation [44]. Recently, it has been reported that these enzymes also function as key regulators of

lipid droplet (LD) homeostasis [45]. Although various cellular PLA2s may contribute to generating free fatty acids from membrane phospholipids initially needed for LD synthesis, strong evidence supports that the PLA2 form, such as PLA2G4A, is also involved in ER phospholipids remodeling and LD expansion processes [44, 45]. IL1A was also significantly overexpressed in this analysis. This gene codifies for Interleukin-1 (IL-1), a proinflammatory cytokine, plays a crucial role in ischemic stroke (IS) [46]. Because intracranial atherosclerosis is a risk factor for IS [47], this finding strongly suggests that IL-1 is implicated in the pathophysiology of IS. In our study, RAS guanyl nucleotide-releasing protein 3 (RASGRP3), was one of the main over-expressed genes associated with MAPK pathway. Members of the RAS subfamily of GTPases function as signal transductions, like GTP/GDP-regulated switches that cycle between inactive GDP- and active GTP-bound states, serve as RAS activators by promoting acquisition of GTP to maintain the active GTP-bound state, and are the key link between cell surface receptors and RAS activation [48]. DNA damage-inducible transcript 3 (DDIT3), was also significantly overexpressed in this analysis. This gene encodes to a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors. The protein functions as a dominant-negative inhibitor by forming heterodimers with other C/EBP members, such as C/EBP and LAP (liver activator protein) and preventing their DNA binding activity. During endoplasmic reticulum stress (such as in pancreatic beta cells or in atherosclerosis associated macrophages), CHOP can induce activation of Ero1, causing calcium release from the endoplasmic reticulum into the cytoplasm, resulting in apoptosis activation [49]. CHOP also induces apoptosis during endoplasmic reticulum stress by growth arrest and DNA damage-inducible protein GADD34 activation [62]. A recent study, which showed a significantly increased DDIT3 protein (ddit3) expression, induced by the exposure of longer MWCNTs [50]. ddit3 is a transcription factor that could regulate a number of inflammatory cytokines, such as IL-6 [50]. Interestingly, the biological network generated by the String software platform showed an important functional role in all processes described previously. Finally, our results revealed a certain genetic profile with a small set of genes which, in the future, could provide additional information about the biological basis of CVD in CKD.

Limitations of the Study

The main limitation of the present pilot study was the number of samples and microarrays analyzed. However, all measurements were performed in duplicate and confirmed by Quantitative reverse transcription PCR (RT-qPCR). The in vitro studies were performed in triplicate in order to minimize variations and confirmed by different experimental approaches.

Our study has limitations because we cannot disregard that several differentially expressed genes were actually derived from contaminating like others serum toxins, particularly environmental pollutants. Therefore, purification of the serum toxins by different methodologies should be implemented to validate all potential biomarkers. Studies in clinical samples to additional validation on independent samples seem necessary.

5. Conclusions

The results obtained in this study identify novel molecular alterations possibly involved in the pathogenesis of cardiovascular disease, and suggest that uremic toxins released into the blood of ESRD patients can alter HCAECs and modify the expression profile of genes, which could have an impact on the development of cardiovascular complications in these patients. patients. To confirm these data, future studies or strategies are necessary.

Declarations

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Conflicts of Interest: The authors declare no conflicts of interest.

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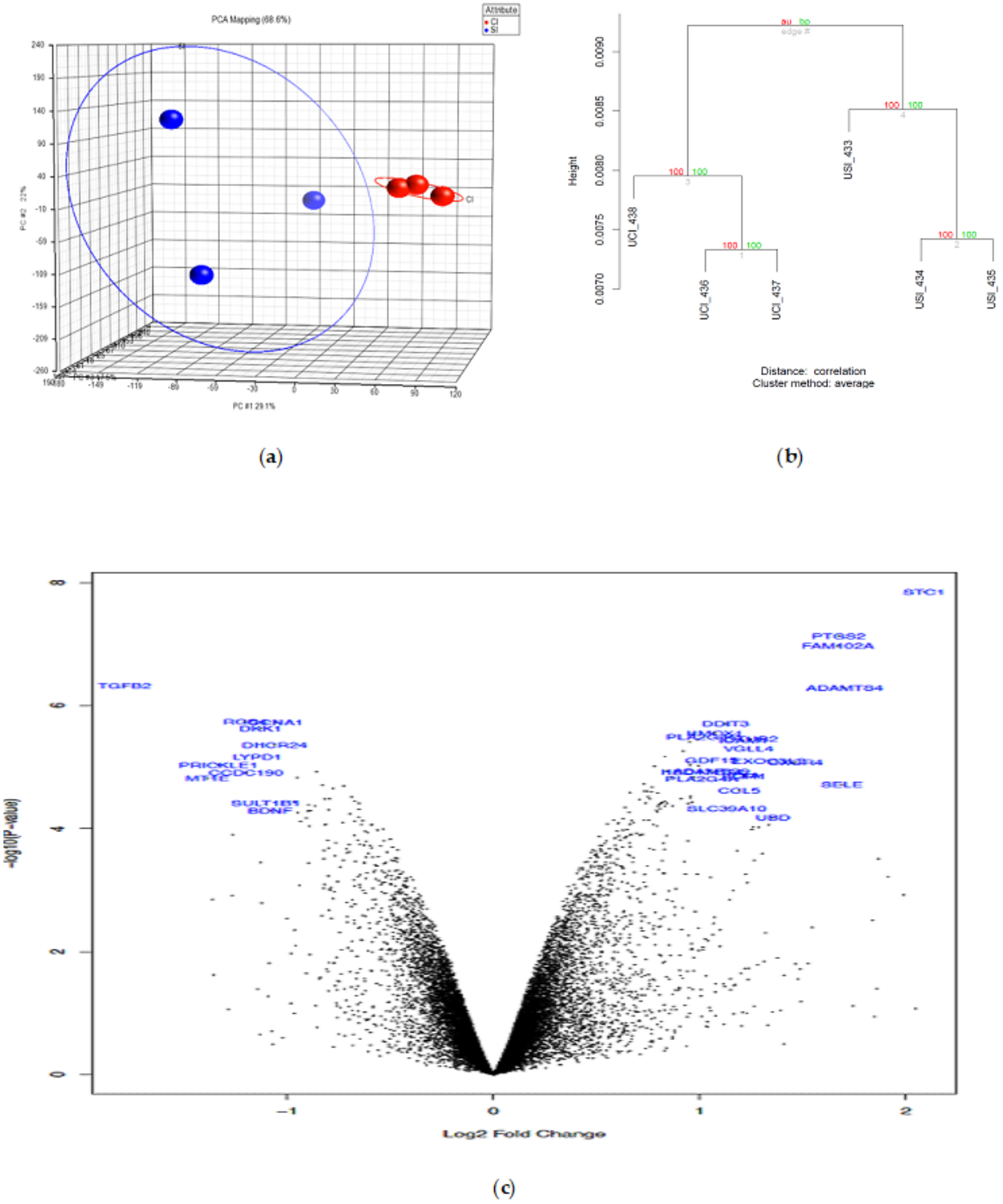
Figures



(a) (b)

(c)

Figure 1

Transcriptome analysis of primary HCAEC endothelial cell cultures exposed to uremic serum from IRT patients with and without infarction (a). Principal component analysis that describes the associated profile in the groups. In blue is the USI group and in red is the UCI group (c). Dendrogram based on the expression of 100 genes involved in IRT and infarction. (c). Representation of differentially expressed

genes through the volcano graph. The dots in the upper left and upper right quadrants represent the genes that met the requirements for them to be called differentially expressed.

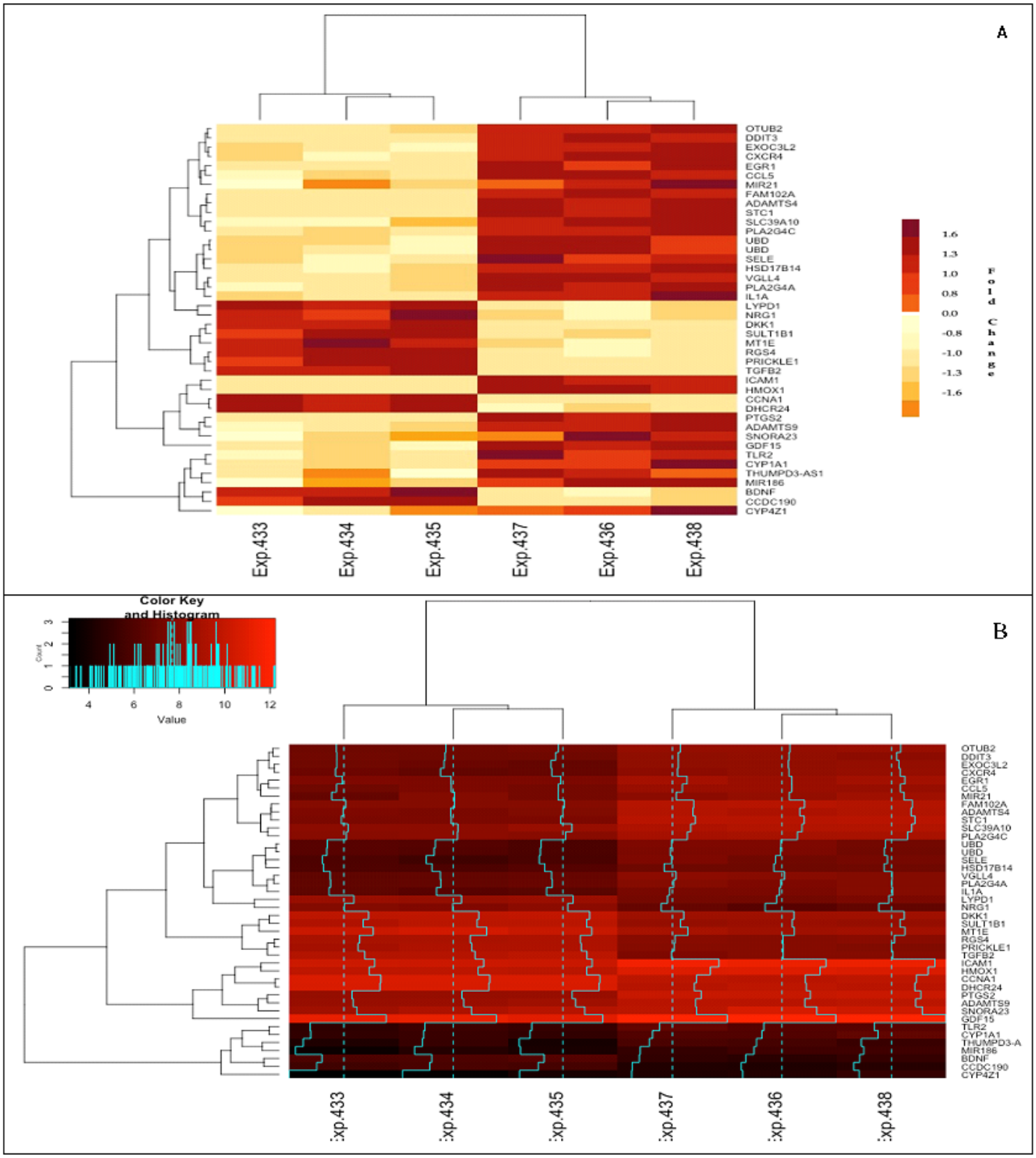


Figure 2

Transcriptome analysis of primary HCAEC endothelial cell cultures exposed to uremic serum from ESRD patients with and without infarction (A and B). Hierarchical cluster analysis of 42 differentially expressed genes. The samples are in the columns and the genes are in the rows.

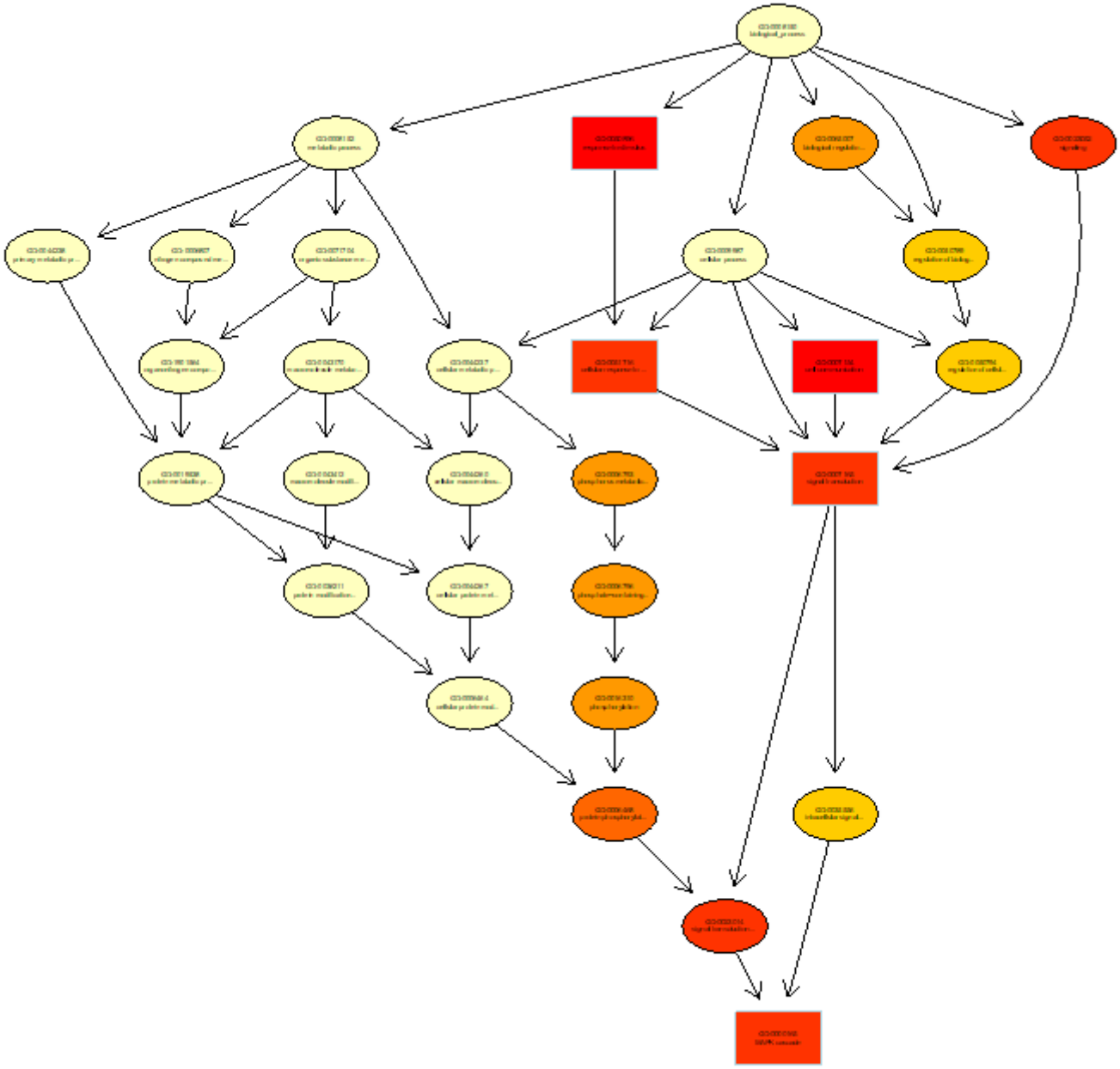


Figure 3

Interaction graph according to GenOntology data (GO: biological process). Networks of interactions were based on correlations in order to characterize cellular and molecular functions, and identify enriched canonical pathways / networks for the list of selected candidate genes, based on GenOntology data.

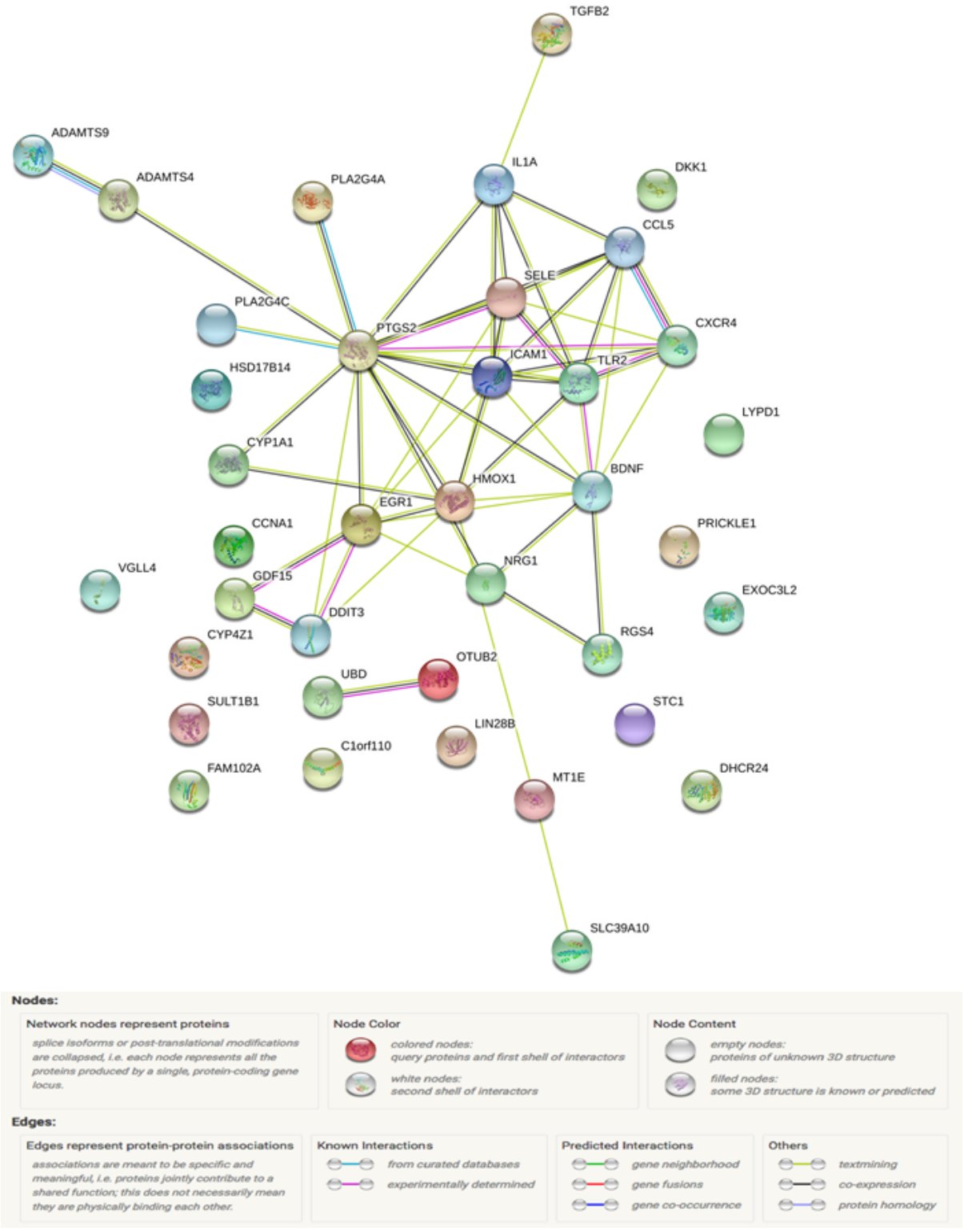


Figure 4

Molecular networks of uremic samples in HCAEC (with String). Protein-protein interaction (PPI) networks were based on PPI correlations in order to characterize cellular and molecular functions, and identify enriched canonical pathways / networks for the list of selected candidate genes, according to GenOntology data.