1. COMPLETE METHOD

Recruitment of the subjects

After obtaining informed consent, 10 CKD patients (n=5 chronic hemodialysis patients and n=5 stage 4 CKD patients, later referred as dialysis and uremic patients, respectively) were recruited to participate in this study. Five age- and sex-matched healthy subjects with normal renal function were recruited as control group. For all participants, data on demographics, medical history and current pharmacological treatment regimen were collected, as detailed in Table I. Approximately 400 ml of venous blood was collected from each subject using collection bag containing sodium citrate as anticoagulant. For dialysis patients, blood collection was performed immediately prior to dialysis treatment. Hematological and biochemical data were recorded for each subject and are summarized in Table II.

Purification of blood platelets

Venous blood was centrifugated at 170 g for 15 min to obtain the platelet-rich plasma (PRP), which was cleared by another centrifugation at 600 g for 10 min and filtered through leukocyte depletion filters (Pall corporation). Platelets were collected by centrifugation at 1,500 g for 15 min and subjected to negative selection based on magnetic cell sorting using human CD45+ depletion kit (EasySep, Stemcell technologies). Approximately two thirds of purified platelets were lysed in TRIzol solution (Invitrogen) for RNA extraction, whereas the remaining platelets were flash-frozen in a dry-iced ethanol bath and stored at -80°C for subsequent enzymatic and Western blot experiments, as described previously (1).
**RNA extraction and analysis**

Platelet total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s protocol. RNA concentration and purity was determined by 260 nm and 280 nm absorbance using Nanodrop 2000 (Thermo Scientific). cDNA was synthesized from 0.5 µg total RNA with random hexameric primers using SuperScript III Reverse Transcriptase kit (Invitrogen). Purity of the platelet preparations was assessed by polymerase chain reaction (PCR) amplification of the leukocyte marker CD45 and platelet marker GPIIIa, as previously described (1). For microarray analysis, 1.5 µg of total RNA from each subject were pooled together for each cohort. Pooled total RNA was purified further using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. The integrity of the total RNA samples was assessed by Bioanalyzer 2100 (Agilent Technology) prior to mRNA and microRNA profiling.

**Microarray profiling and analysis of platelet mRNAs**

mRNA profiling was performed through the CRCHUQ Genomics platform (CHUL research center/CHUQ). Labeled DNA probes were synthesized from total RNA samples and hybridized on Human Gene 1.0 ST DNA BioChip (Affymetrix) according to the manufacturer’s protocol. Robust multi-Array data (RMA) method (2) was applied for the normalization of GeneChips array data obtained for the 3 samples.

Analysis of mRNA profiles from Human Gene 1.0 ST microarray data was performed on protein-coding genes. The mRNA for which relative fluorescence intensity values were lower than the detection threshold (arbitrarily set at 7 in log2 values) in all three samples were excluded and assumed not to be expressed in platelets. Among the remaining 2568 mRNAs, differentially expressed mRNAs
were defined as mRNAs 2 fold more (or less) expressed among the uremic or dialysis patients as compared to healthy subjects.

Gene expression analysis for enrichment in functional or structural categories was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7; http://david.abcc.ncifcrf.gov/), as described (3). Two gene lists were analyzed, i.e. the differential gene expression in platelets of dialysis and uremic patients (>1.5 fold relative to the expression in platelets of healthy subjects). Gene annotation enrichment was performed using all 2568 mRNAs expressed in platelets as background, in order to avoid artificial enrichment in platelet-specific terms. Platelet genes restored upon dialysis were defined as genes whose expression level in platelets of uremic patients displayed at least 1.5 fold change as compared to the healthy cohort, and whose expression in dialysis patients was closer to the healthy control than to the uremic, corresponding to at least a 1.5 fold correction of expression in platelets of the dialysis patients cohort.

**Quantitative real-time PCR (qPCR) experiments**

Expression of selected genes of interest was assessed by qPCR using QuantiTect SYBR green PCR kit (Qiagen). For each subject, the PCR reaction was performed in triplicate using 0.25 to 0.5 µl of cDNA reaction and 0.5 µM of each of the oligonucleotide primers per reaction. The primer sequences and annealing temperatures are provided in Supplementary Table S1. The PCR reactions were performed on a StepOnePlus Real-Time PCR system (Applied Biosystem) with the recommended cycling temperatures provided in the QuantiTect PCR kit guide. The ∆∆Ct method (4) was used to perform the relative quantification of the target genes using StepOnePlus software, using GAPDH mRNA as the reference gene. The results were normalized to the average expression level obtained for the healthy subjects group, and arbitrarily set at 1.
Expression of miR-19b was assessed by qPCR using miScript PCR system (Qiagen). For each subject, the PCR reaction was performed in triplicate using 2 µl of cDNA reaction (miScript II RT kit, Qiagen), diluted 1:20 as recommended by the manufacturer. The PCR reactions were performed on a StepOnePlus Real-Time PCR system (Applied Biosystem) with the recommended cycling temperatures provided in miScript PCR system guide. The ∆∆Ct method (4) was used to perform the relative quantification of the target genes using StepOnePlus software, using miR-191 as the reference gene. The results were normalized to the average expression level obtained for the healthy subjects group, and arbitrarily set at 1.

Microarray profiling and analysis of platelet microRNAs

MicroRNA profiling was performed by Exiqon microRNA profiling service (Vedbaek, Denmark). Samples were labeled using the miRCURY™ Hy3/Hy5 Power labeling kit and hybridized on the miRCURY™ Locked Nucleic Acid (LNA) Array (5th generation arrays covering microRNA registered in miRBase 15.0 - Sanger Institute (5)). The 3 samples (healthy, uremic and dialysis platelets total RNA pool) were labeled with Hy3 dye, whereas a pool of the 3 RNA samples was labeled with Hy5 as normalization control. The quantified signals were normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm (6), to minimize the intensity-dependent differences between the dyes.

MicroRNA detection was considered as positive when the calculated relative fluorescence intensity (RFI) for a given microRNA capture probe was above the detection threshold, which was conservatively set at 2 times the highest mean background signal observed for negative controls in the array experiments. Differentially expressed microRNAs were defined as microRNA whose expression
level was 2 fold higher or lower than that observed in healthy subject group. Predicted mRNA targets of differentially expressed microRNAs were determined using miRecords (http://mirecords.biolead.org), which allows the integration of predicted microRNA targets produced by multiple microRNA target prediction programs (7). For each differentially expressed microRNA, predicted targets had to be identified by at least 2 prediction programs among miRanda (8), TargetScan (9) and PicTar (10). For some microRNAs (e.g. hsa-miR-376a, 551b, 599), no predictions are available in PicTar. As a result, mRNA targets of these microRNAs were defined as mRNAs predicted by both TargetScan and Miranda. Hsa-miR-129*, miR-340*, miR-593* and miR-1973 were excluded from this study as neither PicTar nor miRanda could provide predictions.

**Reporter gene activity assays**

HEK293 cells (2 × 10⁵) were plated in 24-well plates 24 hours before cotransfection with a pGeneClip (Promega) construct expressing pre-miR-599 or pre-miR-19b-1 (2.5 to 250 ng DNA) and a psiCHECK (Promega) reporter construct, in which the 3'UTR of phosphatidylcholine transfer protein (PCTP) or WDR1 (100 ng DNA) was inserted downstream of the Rluc reporter gene, using Lipofectamine 2000 (Invitrogen). The vector encoding pre-miR-599 generated functional hsa-miR-599 species, as attested by its ability to induce a decrease in the activity of a Rluc reporter gene harboring a perfectly complementary binding site (H.P. and P.P., data not shown). A pGeneClip-Neg vector (11), encoding a short hairpin RNA (shRNA) directed against a deleted region in Rluc mRNA, was used as a negative control. Forty-eight hours later, cells were harvested and protein extracts were prepared for measurement of Renilla luciferase (Rluc) and Firefly luciferase (Fluc) activities, essentially as described previously (11).
**Statistical analyses**

Results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using InStat 3 software (GraphPad). A non-parametric Kruskal-Wallis ANOVA followed, when significant, by the non-parametric Mann-Whitney test with a Bonferonni correction, was used to compare qRT-PCR mRNA expression data as well as the hematological and biochemical data between the healthy subject group and each patient cohort. The reporter gene activity data were analyzed using paired Student’s T-tests. For all analyses, a p < 0.05 was considered as statistically significant.

**2. SUPPLEMENTARY METHODS**

**Protein extracts and analysis**

Platelet protein extracts were prepared in lysis buffer (7 M Urea, 2 M Thiourea, 3% v/v 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM dithiothreitol (DTT), 5 mM tris, (2-carboxyethyl)phosphine (TCEP), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1x Roche Complete antiprotease cocktail ethylenediaminetetraacetic acid (EDTA) free) and cleared by centrifugation at 10,000 g for 10 min. Platelet protein extracts (20 and 50 µg) were analyzed by 10% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting, followed by enhanced chemiluminescence detection (ECL plus; Amersham) using anti-Dicer (12), anti-transactivating response RNA-binding protein 2 (TRBP2) (1), anti-Ago2 (1C9 ; Abanova) and anti-β-actin (AC-40 ; Sigma) antibodies.

**Dicer activity assay**

Platelets were lysed in 2x Dicer lysis buffer (100 mM KOAc, 40 mM Hepes pH 7.6, 4 mM MgCl₂, 5 mM DTT, 1 mM PMSF, 1x Roche Complete Ethylenediaminetetraacetic acid (EDTA) free antiprotease cocktail), and lysates were cleared by centrifugation at 10,000 g for 10 min. Dicer processing activity was assessed using platelet protein extracts (50 µg) incubated with randomly ³²P-labeled human let-7a-
3 pre-microRNA in the presence of 1 mM adenosine 5’-triphosphate (ATP). Following proteinase K digestion and RNA extraction, pre-microRNA processing was analyzed by denaturing PAGE (15%) and autoradiography, as described previously (1, 13). For each subject, Dicer activity was determined by densitometric quantitation of the substrate and product bands. Dicer activity was expressed as mature microRNA/pre-microRNA ratio, and normalized to the average activity determined for the healthy subject group.

**RNA-induced silencing complex (RISC) activity assay**

Platelets were lysed in 2x RISC lysis buffer (100 mM KOAc, 40 mM Hepes pH 7.6, 5 mM MgCl₂, 2 mM DTT, 0.35% v/v Triton, 1 mM PMSF, 1x Roche Complete EDTA free antiprotease cocktail) and lysate was cleared by centrifugation at 100,000 g for 1 h. Ago2-mediated cleavage activity was assessed using platelet protein extracts (30 µg) incubated with a hsa-miR-223 sensor RNA 5’ end-labeled with $^{32}$P-[ATP] (Perkin Elmer) in the presence of 1 mM ATP and 0.2 mM guanosine 5’-triphosphate (GTP). Following proteinase K digestion and RNA extraction, sensor cleavage by Ago2-miR-223 complexes were analyzed by denaturing PAGE (8%) and autoradiography, as described previously (1, 13). Quantification of RISC activity was determined essentially as described for Dicer activity assays.

3. SUPPLEMENTARY RESULTS

**Platelet microRNA biogenesis remains functional in uremic and dialysis patients**

The differential expression of platelet mRNAs observed in CKD patients led us to examine a major class of mRNA regulatory RNAs, i.e. microRNAs. Representing an important component of the platelet transcriptome and generated from pre-microRNAs, microRNAs are short 21- to 24-nucleotide (nt) RNA species that act as key regulators of mRNAs in the vast majority of eukaryotes, including humans. We have previously reported that human platelets contain a functional microRNA pathway
capable of mediating the biogenesis and regulatory action of microRNAs on mRNAs (1). The abundance and diversity of platelet microRNAs has been corroborated by independent groups, and their relationship with platelet reactivity and protein expression was further demonstrated (14, 15). Therefore, we examined if the ability of platelets to produce microRNAs was altered in uremic or dialysis patients, as compared to the healthy subjects. For that purpose, we purified circulating platelets and prepared protein extracts in order to perform in vitro Dicer activity assays. As shown in Figure S1A (upper panel), addition of platelet protein extracts to a pre-microRNA species (pre-let-7a-3) led to the formation of 21-nt RNA species, which corresponds to the size expected for a mature microRNA produced by Dicer. The production of mature microRNAs in this assay indicated that the platelet microRNA biogenesis pathway remains functional in CKD patients. Although we observed a certain degree of interindividual variations in Dicer activity, no significant changes in the ability of platelet Dicer to produce microRNAs could be detected among the uremic or dialysis patients (see Figure S1A, lower panel). We noticed an enhanced platelet Dicer activity in one uremic (Subject 6) and one dialysis (Subject 13) patient, and a reduced Dicer activity in a dialysis patient (Subject 12), as compared to the healthy subjects group, which might be explained by their individual conditions independent from CKD.

The differences observed in platelet Dicer activity prompted us to ask whether this was related to a differential expression of the protein components of the platelet Dicer complex, i.e. Dicer and its cofactor TRBP (1). Analysis of platelet Dicer and TRBP expression level by immunoblotting (see Figure S1B) showed that the proteins are expressed at different levels among the subjects, with no relationship with the uremic or dialysis condition. No correlation between Dicer or TRBP expression level and the enzymatic activity of Dicer could be established, suggesting that the activity of the pre-microRNA processing complex may be affected by one or more factors or prevailing conditions.
**Platelet Ago2•microRNA complexes remain functional in uremic and dialysis patients**

Our data indicate that the microRNA processing machinery remains functional in platelets of uremic and dialysis patients, and allow the production of mature microRNAs. Next, we tested whether these platelet microRNAs remained functional in platelets of uremic and dialysis patients, as any alteration of the microRNA effector complex might impair platelet protein expression control and explain the platelet proteome changes observed in patients suffering from CKD (16, 17). For that purpose, we measured the microRNA-guided silencing activity of Ago2, which is the main component of microRNA effector complexes. Platelet protein extracts were prepared from purified platelets and incubated with an RNA sensor bearing a sequence perfectly complementary to hsa-miR-223, which is a myeloid cell-specific microRNA particularly abundant in human blood platelets (1). As shown in Figure S2A (upper panel), addition of platelet protein extracts to the hsa-miR-223 RNA sensor led to the formation of a 39-nt RNA fragment of the size expected from cleavage by the Ago2• hasa-miR-223 complex. Our data indicate that, despite a certain degree of interindividual variations, platelet Ago2 activity is very similar among all the subjects (see Figure S2A, lower panel), suggesting that microRNA function is not altered in platelets of uremic and dialysis patients.

These findings were corroborated by the similar platelet Ago2 protein expression level among our subjects (see Figure S2B), suggesting that platelet Ago2 function is not altered in CKD patients. We noticed a reduced Ago2•hsa-miR-223 activity in one healthy (Subject 3) and one dialysis patient (Subject 11), as compared to the healthy subjects, which might be explained by undetermined factors and/or conditions specific to these individuals.
## 4. SUPPLEMENTARY TABLE

Supplementary Table S1. PCR primer sequences and annealing temperatures

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<th>Gene ID</th>
<th>Primer Sequence (5' → 3')</th>
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<th>Annealing T°</th>
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F, forward; R, reverse
Suppl. Figure 1: Analysis of platelet microRNA biosynthesis in uremic and dialysis patients. (A) The microRNA biosynthetic capacity of platelets from each subject was assessed in *in vitro* Dicer activity assays using platelet protein extracts and a synthetic $^{32}$P-labeled pre-microRNA substrate. The reactions were analyzed by PAGE and autoradiography (upper panel). The data are representative of two technical replicates. Dicer activity was normalized to the average activity obtained for the healthy subjects (lower panel). (B) Expression of platelet Dicer and TRBP proteins, which are known to mediate microRNA biogenesis in platelets (1), was assessed in each subject by Western blot using anti-Dicer and anti-TRBP antibodies, and anti-β-actin as a loading control.
Suppl. Figure 2: Analysis of platelet microRNA function in uremic and dialysis patients. A) The ability of platelet microRNAs to target and regulate mRNA was assessed in each subject in in vitro RISC activity assays using platelet protein extracts and a synthetic 5' end, $^{32}$P-labeled RNA sensor bearing a sequence complementary to hsa-miR-223. The reactions were analyzed by PAGE and autoradiography (upper panel). The data are representative of two technical replicates. RISC activity was normalized to the average activity obtained for the healthy subjects (lower panel). (B) Expression of platelet Argonaute 2 (Ago2), which is the core component of the microRNA effector complex in platelets (1), was assessed in each subject by Western blot using anti-Ago2 antibody, and anti-β-actin as a loading control.
6. SUPPLEMENTARY REFERENCES