Alteration of the platelet transcriptome in chronic kidney disease

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Summary
Bleeding and thrombotic disorders are major complications affecting patients with chronic kidney disease (CKD). Exposure of circulating platelets to uraemic toxins and contact with artificial surfaces during dialysis induce platelet abnormalities and alter the platelet proteome. We hypothesised that these changes may be subsequent to changes in the composition and/or regulation of the platelet transcriptome. In this study, we investigated the circulating platelets of 10 CKD patients (i.e. five chronic haemodialysis patients and five stage 4 CKD uraemic patients) and five age- and sex-matched healthy subjects. We observed an alteration of the platelet messenger RNA (mRNA) and microRNA transcriptome in CKD patients. Impaired in uraemic platelets, the levels of some mRNAs and of most microRNAs appeared to be corrected by dialysis, which is consistent with a beneficial effect of dialysis and a mRNA regulatory role of platelet microRNAs. Reduced in platelets of uraemic patients, phosphatidylcholine transfer protein (PCTP) and WD repeat-containing protein 1 (WDR1) were found to be regulated by microRNAs, the latter of which involving hsa-miR-19b, a microRNA increased in platelets of uraemic patients and involved in platelet reactivity. These results suggest that an alteration of microRNA-based mRNA regulatory mechanisms may underlie the platelet response to uraemia and entail the development of platelet-related complications in CKD.

Keywords
Chronic kidney disease, platelets, gene expression, mRNA, microRNA

Introduction
Cardiovascular disease (CVD) is a major cause of morbidity and mortality among patients suffering from chronic kidney disease (CKD) and is the leading cause of death in patients on chronic haemodialysis (1–3). The high prevalence of established traditional risk factors in CKD patients, such as lipid disorders, diabetes and hypertension, as well as non-traditional risk factors (e.g. oxidative stress, inflammation) and platelet abnormalities (4–7), may contribute to accentuate the risks of CVD and thrombotic complications. Patients with CKD suffer from complex haemostatic disorders. On one hand, uraemic patients present abnormalities of primary haemostasis (i.e. platelet dysfunction and impaired platelet-vessel wall interactions) and increased risk of bleeding (8–10). On the other hand, several studies have shown that platelet reactivity is increased in CKD patients undergoing haemodialysis (11), which is associated with increased risk of thrombo-embolic events (12–14). CKD is thus characterised by a delicate balance in which deficient haemostasis paradoxically coexists with enhanced risk of thrombosis, and in which platelet abnormalities may play an important role.

Playing a critical role in the maintenance of haemostasis, platelets may directly contribute to cardiovascular complications, such as myocardial infarction and stroke, in CKD patients (8–10). Although devoid of a nucleus and lacking genomic DNA, circulating human platelets retain as much as 45% of the Refseq genes in the form of mRNAs (15), inherited mainly from their megakaryocyte progenitor cells (16). Several reports confirmed that platelet mRNAs can support de novo protein synthesis (17), including a recent study reporting the translation and secretion of the metalloproteinase inhibitor Temp 2 by activated platelets (18). Weyrich et al. (19) had previously demonstrated that synthesis of Bcl-3 by activated platelets participates in blood clot retraction, while Evangelista et al. (20) reported that de novo synthesis of cyclooxygenase-1 counteracts the suppression of platelet thromboxane biosynthesis by aspirin.

Proteomic analyses of platelets revealed that their protein expression profile is altered in CKD patients (21, 22). Although reticulated platelets sustain changes in terms of platelet volume, morphology and RNA content in subjects treated with haemodialysis (23, 24), whether the mRNA profile of platelets is altered in CKD and haemodialysis patients remains unknown. These observations prompted us to formulate and verify the hypothesis that the biochemical conditions (e.g. increased uraemic toxins) prevailing in patients with CKD, leading or not to end-stage kidney failure and dialysis, might negatively affect the transcriptome of circulating platelets, which is constituted mainly of mRNAs and of non-coding microRNAs.
Materials and methods

Recruitment of the subjects

After obtaining informed consent, 10 CKD patients (n=5 chronic haemodialysis patients and n=5 stage 4 CKD patients, later referred as dialysis and uraemic patients, respectively) were recruited to participate in this study, which was approved by our Institutional Human Ethics Committees. 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 1. 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. Haematological and biochemical data were recorded for each subject and are summarised in Table 2.

Purification of blood platelets

Venous blood was centrifugated at 170 g for 15 minutes (min) to obtain platelet-rich plasma (PRP), which was cleared by another centrifugation at 600 g for 10 min and filtered through leukocyte depletion filters (Pall corporation, Port Washington, NY, USA). 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, Reston, VA, USA). Approximately two thirds of purified platelets were lysed in TRIzol solution (Invitrogen, Carlsbad, CA, USA) 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 (25).

RNA extraction and analysis

Platelet total RNA was extracted using TRizol (Invitrogen) according to the manufacturer’s protocol. Purity of the platelet preparations was assessed by reverse-transcription (RT) polymerase chain reaction (PCR) amplification of the leukocyte marker CD45 and platelet marker GPIIIa, as previously described (25). 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 RNAeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The integrity of the total RNA samples was assessed by Bioanalyzer 2100 (Agilent Technology, Santa Clara, CA, USA) prior to mRNA and microRNA profiling.

Microarray profiling and analysis of platelet mRNAs

mRNA profiling was performed through the CRCHUQ Genomics platform (CHUQ research center/CHUL, Quebec, QC, Canada) using Human Gene 1.0 ST DNA BioChip (Affymetrix, Santa Clara, USA), according to the manufacturer’s protocol. Analysis of mRNA profiles from Human Gene 1.0 ST microarray data was performed on protein-coding genes. Differentially expressed mRNAs were defined as mRNAs two-fold more (or less) expressed among the uraemic or dialysis patients as compared to healthy subjects.

Quantitative real-time PCR (qPCR) experiments

Expression of selected genes of interest was assessed by qPCR using Quantitect SYBR green PCR kit (Qiagen) on a StepOnePlus Real-Time PCR system (Applied Biosystem, Foster City, CA, USA). The ΔΔCt method (26) was used to perform the relative quantification of the target genes using GAPDH mRNA as the reference gene. Expression of microRNAs of interest was assessed by qPCR using the miScript PCR system (Qiagen). MiR-191 was used as the reference for relative quantification, since this microRNA displayed no variation in expression.

Table 1: Characteristics, causes of renal disease and pharmacological treatments of the subjects.

<table>
<thead>
<tr>
<th>Subjects (n = 5 in each group)</th>
<th>Healthy</th>
<th>Kidney failure</th>
<th>Uraemic</th>
<th>Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>60 ± 11</td>
<td>58 ± 17</td>
<td>56 ± 12</td>
<td></td>
</tr>
<tr>
<td>Gender (Male:Female)</td>
<td>4:1</td>
<td>4:1</td>
<td>3:2</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td></td>
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<tr>
<td>Cause of renal disease</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Polycystic kidney</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Obstructive urethropy</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Polynephritis</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Hypertensive nephropathy</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Amyloidosis</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lithium nephropathy</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Treatments</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dialysis†</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Anti hypertensives</td>
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<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Hypolipemiant</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Anti-platelet agents</td>
<td>-</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Hypoglycaemic</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (SD). †Dialysis treatment was complemented with the same pharmacological treatment for all the patients, which comprised Darbepoetin and Iron Sucrose (anaemia treatment), 1-OH-Vitamin D and furosemide. Other agents: phosphate binders (calcium or non calcium), sodium polystyrene and allopurinol.
levels in our microarray data; similar observations were reported by Nagalla et al. upon analysis of platelet miR-191 from 17 healthy donors (27). All results were normalised 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). The three samples (healthy, uraemic and dialysis platelets total RNA pool) were labelled using the miRCURY™ Hy3/Hy5 Power labelling kit and hybridised on the miRCURY™ Locked Nucleic Acid (LNA) Array (5th generation arrays covering microRNA registered in miRBase 15.0 – Sanger Institute, Cambridge, UK [28]). Differentially expressed microRNAs were defined as microRNA whose expression level was two-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) (29).

**Statistical analyses**

Results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using InStat 3 software (GraphPad). For all analyses, a p < 0.05 was considered as statistically significant.

**Results**

**Recruitment and characteristics of the subjects**

In this study, we characterised the mRNA and microRNA transcriptomes of circulating platelets from a total of 10 CKD patients: (i) five patients suffered from end-stage renal disease (stage 5) and...
were treated by chronic haemodialysis (dialysis patients) (Table 1), whereas (ii) the other five patients suffered from stage 4 CKD (uraemic patients), the last stage before renal replacement therapy (e.g. haemodialysis or renal transplantation), which corresponds to an eGFR ranging from 15 to 29 ml/min/1.73m² (31).

Table 1 shows chronic medication use in study participants. Although the dialysis patients were treated for anaemia, their red blood cell count, haemoglobin content and haematocrit remained relatively lower than the other subjects (Table 2). None of the subjects presented obvious platelet disorders, as platelet count and platelet mean volume were within a normal range.

Figure 1: Differential platelet mRNA profile of uraemic and dialysis patients. Platelet RNA samples were obtained from each subject, and equivalent amounts were pooled and analysed by DNA-based microarray (Affymetrix, human Gene 1.0 chip). Platelet mRNA profile of the uraemic and dialysis patients were compared to the healthy subjects. A) Platelet mRNA expression level correlation between healthy and uraemic (left panel) or dialysis (right panel) subjects. Data are expressed as log2-transformed relative fluorescence unit (RFU). The mRNAs that varied by more than two-fold were numbered, as indicated in B. B) Platelet mRNAs displaying more than two-fold changes among the uraemic and dialysis patients, as compared to the healthy subjects. A total of 23 and 17 mRNAs are differentially expressed in platelets from dialysis and uraemic patients, respectively, as compared to healthy subjects. Seven mRNAs are differentially expressed in both patient groups.

MicroRNAs in vascular biology: Circulating miRNAs

Differential platelet mRNA profile of uraemic and dialysis patients

The previously reported alteration of the protein expression profile in circulating platelets of CKD patients (21, 22) prompted us to examine the impact of CKD on the platelet mRNA transcriptome. Microarray profiling of platelet mRNAs on pooled RNA samples revealed that the majority of platelet mRNAs is not markedly affected in uraemic and dialysis patients, as compared to control healthy subjects. However, we did observe an altered expression (>2-fold vs. healthy subjects) of a total of 33 platelet mRNAs in CKD patients: (i) 17 platelet mRNAs were differentially expressed in uraemic patients (Fig. 1A, left panel), whereas (ii) in dialysis patients, 23 platelet mRNAs showed a differential expression (Fig.
Analysing those differentially expressed platelet mRNAs among our uraemic and dialysis patient cohorts (Fig. 1B), seven genes showed a similar deregulation in both cohorts of CKD patients, whereas 15 genes were altered specifically in dialysis patients.

Validation of selected platelet mRNAs by qPCR analyses

Next, we wished to confirm the differential expression observed for selected mRNAs, on an individual basis, by qPCR. Four genes were selected based on their different patterns of expression and potential relevance to platelet function. Assessment of their mRNA expression levels by qPCR accurately reflected those obtained by microarray profiling (Table 3), thereby confirming the validity of our microarray approach using pooled RNA samples. As expected, the level of two of them, serine incorporator 1 (SERINC1) (Fig. 2A) and lysophosphatidic acid receptor 4 (LPAR4) (Fig. 2B), was reduced by ~50% in CKD patients, as compared to healthy subjects. In contrast, the mRNAs encoding for oxysterol binding protein 2 (OSBP2) and metallothionein 1X (MT1X) were upregulated in CKD patients (Fig. 2C and D, respectively). Although in some cases statistical significance could not be reached, the differential mRNA expression assessed by microarray and qPCR displayed similar tendencies.

### Table 3: Comparison of gene expression data obtained by microarray and qPCR.

<table>
<thead>
<tr>
<th>Gene symbol/ Gene name</th>
<th>Subjects</th>
<th>mRNA expression level (fold changes)</th>
<th>Microarray</th>
<th>qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERINC1</td>
<td>Uremic</td>
<td>0.48</td>
<td>0.49 ± 0.10 **</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td>0.73</td>
<td>0.61 ± 0.19 *</td>
<td></td>
</tr>
<tr>
<td>LPAR4</td>
<td>Uremic</td>
<td>0.49</td>
<td>0.50 ± 0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td>0.83</td>
<td>0.61 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>OSBP2</td>
<td>Uremic</td>
<td>2.17</td>
<td>3.81 ± 2.06 **</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td>2.93</td>
<td>3.40 ± 2.39</td>
<td></td>
</tr>
<tr>
<td>MT1X</td>
<td>Uremic</td>
<td>2.48</td>
<td>4.69 ± 4.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td>4.11</td>
<td>4.59 ± 3.80</td>
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</tbody>
</table>

qPCR data are provided as mean ± standard deviation. * p<0.05; ** p<0.01 versus healthy subjects (Mann-Whitney Test).

Alteration of the platelet microRNA profile in uraemic patients

The conditions associated to CKD may affect the ability of platelets to synthesise or mediate the function of microRNAs, a family of small non-coding RNAs known as key regulators of mRNAs. To verify that possibility, we performed Dicer activity assays in vitro using protein extracts prepared from purified circulating platelets.
We observed no significant changes in the ability of platelet Dicer to produce microRNAs among the uraemic or dialysis patients, as compared to healthy subjects (see Suppl. Fig. 1A, available online at www.thrombosis-online.com), although we noted a certain degree of inter-individual variations. A similar observation was made when monitoring the expression level of the ribonuclease Dicer and of its cofactor TRBP (see Suppl. Fig. 1B, available online at www.thrombosis-online.com), which are known to mediate platelet microRNA biogenesis.

Using a similar approach, we also assessed the functionality of microRNA effector complexes, composed of the ribonuclease Argonaute 2 (Ago2), by RISC activity assay in vitro. Our assays unveiled a platelet Ago2 activity that is relatively similar among all the subjects, which is in accordance with the relatively similar Ago2 expression level among healthy uraemic and dialysis donors (see Suppl. Fig. 2, available online at www.thrombosis-online.com). Altogether, our results suggest that the ability of platelets to synthesise or mediate the function of microRNAs is not affected by CKD.

Uraemic toxins that accumulate in the blood of CKD patients may nevertheless exert systemic effects, including on platelet precursor megakaryocytic cells, and influence the microRNA content of circulating platelets. Examination of the microRNA profile of platelets isolated from CKD patients identified 21 microRNAs that were differentially expressed in uraemic platelets, as compared to the healthy subjects (Fig. 3A, left panel), thereby supporting an important alteration of the platelet microRNA profile in CKD.

When analysing the differentially expressed platelet microRNAs among our uraemic and dialysis patient cohorts (Fig. 3B), we observed that microRNAs of the same family, as exemplified by
hsa-miR-33a and hsa-miR-33b, are regulated similarly. Notably, we noted a similar trend in the level of some microRNAs deriving from both strands of a precursor in uraemic platelets. Originating from pre-miR-142, both hsa-miR-142–5p and 3p were upregulated. These tandem variations in mature microRNA expression levels are indicative of a microRNA pathway that is altered upstream of the pre-miRNA processing step in uraemic patients. In contrast, hsa-miR-340 and –340* were altered in the opposite way, which is indicative of a differential strand selection process. Together, these observations suggest that microRNA biogenesis may be altered at multiple levels by uraemia.

Figure 4: The level of some platelet mRNAs is corrected by dialysis. A) Differential expression of mRNAs corrected by dialysis in platelets of CKD patients. Data are expressed as log2-transformed fold changes compared to the healthy subjects. B) List of platelet mRNAs corrected by dialysis, together with their corresponding predicted regulatory microRNAs and differential expression in uraemic and dialysis patients. Data are expressed as fold changes compared to the healthy subjects. PCTP (C), RAB1A (D) and ATP6V1C1 (E) mRNAs were quantified by qPCR using the ΔΔCt method and GAPDH mRNA as a reference. Results were normalised to the average expression level of the healthy subjects and expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using the non-parametric Mann-Whitney Test. * p<0.05; ** p<0.01 between depicted groups.

Restoration of the platelet microRNA profile upon dialysis

Importantly, only one microRNA (hsa-miR-551b) was found to be differentially expressed in the dialysis patients cohort, as compared to the healthy cohort (Fig. 3C). Contrasting with the 21 microRNAs that are differentially expressed in platelets of uraemic patients, these results suggest that haemodialysis may restore the microRNA expression profile of platelets in patients suffering from severe CKD.
To verify that possibility, we analysed our microarray data with bioinformatic microRNA target predictive tools in order to identify such platelet microRNA:mRNA pairs. Among a total of 81 platelet mRNAs, whose levels were corrected by dialysis, eight mRNAs were differentially expressed in uraemic patients (Fig. 4A). The level of all 8 mRNAs was decreased in platelets of uraemic patients. Changes in expression of the three mRNAs that were the most affected in the platelets of uraemic patients, i.e. PCTP, RAB1A and ATP6V1C1 mRNAs, were validated by qPCR (Fig. 4C). For three of these eight mRNAs, we observed a concomitant decrease in the expression of the corresponding regulatory miRNAs, a direct correlation consistent with a mRNA stabilising role of miRNAs in platelets. This is the case of the following pairs: HPGD mRNA:hsa-miR-26b, COP52 mRNA:hsa-miR-26b/hsa-miR-1297 and USP15 mRNA:hsa-miR-26b/hsa-miR-1297 (Fig. 4B).

On the opposite, the remaining 5 mRNAs showed an inverted correlation with the corresponding microRNAs, which is expected for microRNAs that regulate platelet mRNAs through destabilisation/degradation, as observed in the case of the PCTP mRNA:hsa-miR-599 pair (Fig. 4B). The level of PCTP mRNA was decreased in platelets of uraemic patients, whereas that of hsa-miR-599 was increased, which is consistent with a role for hsa-miR-599 in destabilising PCTP mRNA. These changes in PCTP mRNA and hsa-miR-599 levels were corrected upon dialysis. Suggesting that the procedure helped restore their expression levels closer to those observed in healthy subjects, these data support a functional link between PCTP mRNA and hsa-miR-599.

### MicroRNA regulation of platelet mRNAs in CKD

Computational analyses predicted the presence of several binding sites for microRNAs, including hsa-miR-599, in the 3’UTR of PCTP mRNA (Fig. 5A). As shown in Fig. 5B, we were able to document the regulatory control of hsa-miR-599 on the PCTP 3’UTR element in cultured HEK293 cells. These results support a role for hsa-miR-599 in regulating PCTP gene expression, which may explain, at least in part, their opposite variations in expression levels in platelets of uraemic and dialysis patients.

Since microRNAs may also mediate their function by interfering with mRNA translation without altering mRNA levels, and may have escaped our correlative analyses, we examined proteins whose expression was reported to be altered either in uraemic or dialysis patients. Among these proteins, WD repeat domain 1 (WDR1 or AIP1) is reduced in uraemic patients that display platelet defects (22). We observed no significant changes in WDR1 mRNA levels, either upon micro-array or qPCR analysis, in platelets (Fig. 6C). Bioinformatic analyses revealed that WDR1 mRNA 3’UTR harbors several putative binding sites for microRNAs, including hsa-miR-19b (Fig. 6A), whose levels are increased in platelets of uraemic patients. MiR-19b expression, assessed by qPCR, confirmed that the expression of this microRNA is markedly increased in platelets of some uraemic subjects (Fig. 6D).

### Restoration in the level of some platelet mRNAs upon dialysis

Some of the microRNAs deregulated in platelets of uraemic patients belong to the 20 most abundant platelet microRNAs in healthy subjects, such as hsa-miR-26a, hsa-miR-26b, hsa-miR-142–5p and 3p, and may likely influence platelet mRNAs. Incidentally, analyses of differentially expressed platelet microRNAs revealed that microRNAs sharing the same seed region, like hsa-miR-26b and hsa-miR-1297, are regulated similarly in uraemic patients. Knowing that the microRNA seed region (i.e. nucleotides 2 to 8 from the 5’ end) plays a critical role in mRNA recognition (32), these observations are consistent with a possible link between microRNAs and mRNAs in platelets.
Using a Rluc reporter gene system in cultured HEK293 cells, we confirmed the ability of hsa-miR-19b to regulate WDR1 mRNA through its 3'UTR element (Fig. 6B). Together, these findings suggest that (i) both PCTP and WDR1 mRNAs may be under microRNA control in human platelets, (ii) the deregulation of the platelet mRNA transcriptome may be linked to that of microRNAs, and (iii) alteration of this microRNA-based mRNA regulatory mechanism may underlie the platelet response to uraemia.

**Discussion**

Uraemic toxins that accumulate in the blood of patients suffering from CKD may affect platelet function, induce haemostatic imbalances and mediate thrombotic disorders (1, 2, 4, 8–10). These effects have been related to an alteration of the platelet proteome that is observed in these patients (21, 22). In the present study, we observed that the platelet mRNA and microRNA transcriptome was altered in CKD patients and could be restored partially upon dialysis. Known to contribute to the platelet proteome, it may be the deregulation of the platelet transcriptome that underlies, and forms the molecular basis of, platelet-related disorders in CKD patients.

The level of metallothionein-encoding genes, such as MT1X, was increased in platelets of both uraemic and dialysis patients, in which plasma zinc deficiency is common (33). Particularly abundant in platelets and involved in platelet reactivity and haemostasis (34), zinc imbalances are involved in oxidative stress (35), a state that induces expression of metallothionein genes (36). Elevated levels of platelet metallothionein genes may thus result from the altered zinc homeostasis and oxidative stress conditions that prevail in uraemic and dialysis patients, and that may contribute to the relatively high prevalence of CVD in these patients (6, 37).

Additional genes deregulated in platelets of CKD patients could be linked to platelet-related disorders observed in uraemia. For instance, the mRNA encoding for LPAR4, which is suspected to in-

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**Figure 6:** WDR1 protein expression, which is involved in uraemic platelet defects, is subjected to microRNA regulation. A) Upper panel: Schematic representation of human WDR1 mRNA with putative microRNA binding sites predicted by at least two bioinformatic programs among miranda, TargetScan and PicTar. MicroRNAs highlighted in black are expressed in platelets. Lower panel: Topology of the interaction between hsa-miR-19b and its putative binding site, as predicted using RNA hybrid, with its calculated minimum free energy (MFE) value. B) Reporter gene activity assay. HEK293 cells were co-transfected with a pre-miR-19b expression vector and a reporter gene construct in which the sequence of WDR1 3'UTR was inserted downstream of the Rluc coding sequence. Rluc and Fluc activities were measured, and the values were normalised to those obtained with a non-relevant short hairpin expression vector (n = 4 experiments, in duplicate). **p<0.01 (Student's paired t-test). WDR1 mRNA (C) and miR-19b (D) were quantitated by qPCR using GAPDH mRNA or miR-191 as a reference, respectively. Results were normalised to the average expression level of the healthy subjects and expressed as mean ± standard error of the mean (SEM).
hibit platelet reactivity to lysophosphatidic acid (38), is reduced by
~50% in CKD patients. Lysophosphatidic acid accumulation in
atherosclerotic lesions represents an important risk factor for the
development of atherosclerosis and thrombosis (39), and the ob-
served decrease in LPAR4 expression in CKD patients may explain
the increased risk of cardiovascular events observed in these clini-
cases. We also identified genes involved in lipid metabolism,
such as glycine N-acyltransferase-like 2 (GLYATL2), phospholi-
pase C eta 1 (PLCH1), phosphatidylincholine transfer protein
(PCTP), OSBP2 and SERINC1, whose levels are deregulated in
ulairemic conditions. This may partially explain the altered syn-
thesis of bioactive lipids (40, 41) and membrane lipid composition
(42, 43) observed in platelets of uraemic patients.

The conditions associated with CKD did not affect the ability
of platelets to synthesise or mediate the function of microRNAs.
However, we observed an important alteration of the platelet
microRNA profile in uraemic patients, as a total of 21, out of 247
microRNAs expressed in platelets, displayed more than two-fold
changes, as compared to healthy subjects. Considering that
microRNA expression in mammalian cells can be regulated at
multiple steps (gene transcription, processing, transport, strand
selection/separation, microRNP assembly, stability) (44) and that
uraemia is usually part of a complex clinical portrait, the aim of
identifying which component(s) and/or step(s) of the platelet
microRNA pathway is(are) altered in CKD patients, and how, ap-
ppears to be challenging.

Correction of the level of most microRNAs and of some
mRNAs, impaired in uraemic platelets, upon dialysis is consistent
with a mRNA regulatory role of platelet microRNAs. We were able
to confirm the microRNA regulation of two mRNAs (PCTP and
WDR1) that are reduced in platelets of uraemic patients, the latter
of which involving hsa-miR-19b. Using a proteomic approach,
Marques et al. (22) demonstrated that WDR1 protein expression
was reduced in platelets of uraemic patients with low platelet reac-
tivity and bleeding tendency. The fact that WDR1 mRNA levels
were not altered in platelets of uraemic or dialysis patients sup-
ports a role for hsa-miR-19b in repressing WDR1 mRNA trans-
lation, along a process independent of WDR1 mRNA levels (45).
Interestingly, hsa-miR-19b is among a list of seven microRNAs that
were identified as good predictors of platelet hyperreactivity to
epinephrine (27). Upregulated by >2-fold in platelets of uraemic
patients, hsa-miR-19b may thus be involved in the altered reac-
tivity of uraemic platelets and the bleeding disorders observed in
these patients.

When attempting to correlate platelet microRNA and mRNA
levels, the caveat has to be taken into account that microRNAs may
silence platelet mRNAs through translational repression (46),
without inducing their cleavage and degradation. Another major
limitation is the plurality of microRNA targets which, together
with the functional interplay of a myriad of microRNAs that may
act in concert to regulate specific platelet mRNAs, makes reliable
genome-wide assessment of functionally relevant platelet microR-
NA:mRNA pairs almost unattainable.

Our results suggest that the biochemical conditions prevailing
in patients with CKD, whether they lead to end-stage kidney failure
and dialysis or not, may alter the mRNA and microRNA transcrip-
tome of circulating platelets which, in turn, may affect platelet
function and entail the development of platelet-related clinical
complications, such as atherosclerosis and thrombosis. This study
complements previous studies describing the relationship between
platelet microRNAs and platelet reactivity (27, 47) or human dis-
eases, including premature coronary artery disease (48). The mo-
olecular mechanisms underlying the deregulation of platelet mRNA
and microRNA transcriptome in CKD patients, which may be
multifaceted considering the complexity of the medical condition
of these patients, warrants further investigations.

Acknowledgements
P. P. is a Senior Scholar from the Fonds de la Recherche en Santé du
Québec (FRSQ).

Conflicts of interest
None declared.

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