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Circulating microvesicle protein is associated with renal transplant outcome

Khalid Al-Nedawi[a,b,*], Sandor Haas-Neill[a,b], Azim Gangji[a,b], Christine M. Ribic[a,b], Anil Kapoor[b,c], Peter Margetts[a,b]

[a] Division of Nephrology, Department of Medicine, McMaster University, Hamilton, Ontario, Canada
[b] St. Joseph's Healthcare Research Institute & Hamilton Center for Kidney Research, Canada
[c] Department of Surgery, McMaster University, Hamilton, Ontario, Canada

A B S T R A C T

Renal transplantation is an effective therapy with improved long-term outcomes compared with other therapies for end stage renal disease. Present methods for evaluating kidney allograft function, such as serum creatinine or allograft biopsy, are not sensitive and identify pathological changes only after any potential intervention would be effective. Thus, there is a necessity for biomarkers that would provide early prognostic information about kidney transplant outcomes. Circulating microvesicles represent an attractive source of biomarkers for different diseases including renal failure. We have studied the proteins of the circulating microvesicles from two populations of kidney transplant recipients (n = 20) with poor transplant outcomes (n = 10) or good transplant outcome (n = 10), according to their estimated glomerular filtration rate (eGFR). Microvesicles from age-matched healthy subjects (n = 10) were used as a control. Also, we performed a pilot study to assess the microvesicle protein in kidney transplant recipients before and six months after kidney transplant (n = 6), compared to healthy subjects. Proteomic analysis of microvesicles could discriminate between transplant recipients and healthy subjects, and between transplant patients based on eGFR. Our results shed light on the potential of blood microvesicles to provide a novel tool for the prediction of the outcome of kidney transplants.

1. Introduction

Kidney transplantation (KT) is the preferred therapy for end-stage renal disease. There is a pressing necessity for new markers for the prognosis of the KT. Current procedures to detect fibrosis or rejection episodes are dependent on functional parameters that lack predictive value – for example: measuring existing tissue damage. The functional parameters: serum creatinine, estimated glomerular filtration rate (eGFR), proteinuria, and histological information from renal biopsies are used to determine the prognosis of patients with renal disease [1,2]. Parameters such as eGFR are inaccurate in determining the progression of chronic kidney disease [3,4]. In addition, biopsies are an invasive procedure and provide information only on pre-existing tissue damage with modest prognostic value [2]. Herein lies the need for new molecular technologies to help in diagnosis and prognosis of renal transplant recipients and to better characterize acute and chronic complications.

Microvesicles are membrane compartments shed from cells by either direct budding from the plasma membrane or through secretion via the endocytic pathway (exosomes) [5]. Microvesicles exist in body fluids such as blood and urine, and can provide a simple non-invasive tool to look for biomarkers for their content of proteins, mRNAs, miRNAs and DNA. We previously reported that microvesicles facilitate the intercellular transfer of molecules such as receptors [6,7] or other proteins [8] conferring a new phenotype on the recipient cells. Microvesicles can also transfer mRNA, and miRNA from one cell to another - affecting the gene expression of the recipient cells [9,10].

The importance of microvesicles in the context of renal failure and kidney transplants stems from their potential role as regulators of inflammation and immunological processes [11]. It has been speculated that the cargo of circulating microvesicles may reflect possible immune rejection of a transplant, and modulation of microvesicle secretion may have therapeutic potential for transplant rejection [12]. Also, microvesicles are important in kidney transplants because they carry MHC I, and MHC II molecules [13,14], which play a major role in the immune response against the transplanted kidney. However, the role of microvesicles in organ transplantation is still poorly understood and needs further research [11]. Other studies have shown that microvesicles have a profound impact in protection against ischaemia-reperfusion-induced kidney injury. For example, microvesicles were found to account for the protective effect of mesenchymal stem cells against acute and chronic kidney injury, by the horizontal transfer of mRNAs and miRNAs in mice [10].

Thought has been given to using exosome cargo as biomarkers for the success of the outcomes of lung transplantation and other organs [15,16,17]. Urinary derived exosomes have previously been shown to be effective as biomarker vehicles for determining the outcome of kidney transplants [18]. Neutrophil gelatinase-associated lipocalin (NGAL) one of the most promising biomarker candidates for both chronic kidney disease and acute kidney injury, has previously been found to be deferentially

* Corresponding author at: Department of Medicine, McMaster University and St. Joseph's Healthcare, 50 Charlton Street East, Hamilton, Ontario L8N 4A6, Canada.
E-mail address: alnedaw@mcmaster.ca (K. Al-Nedawi).

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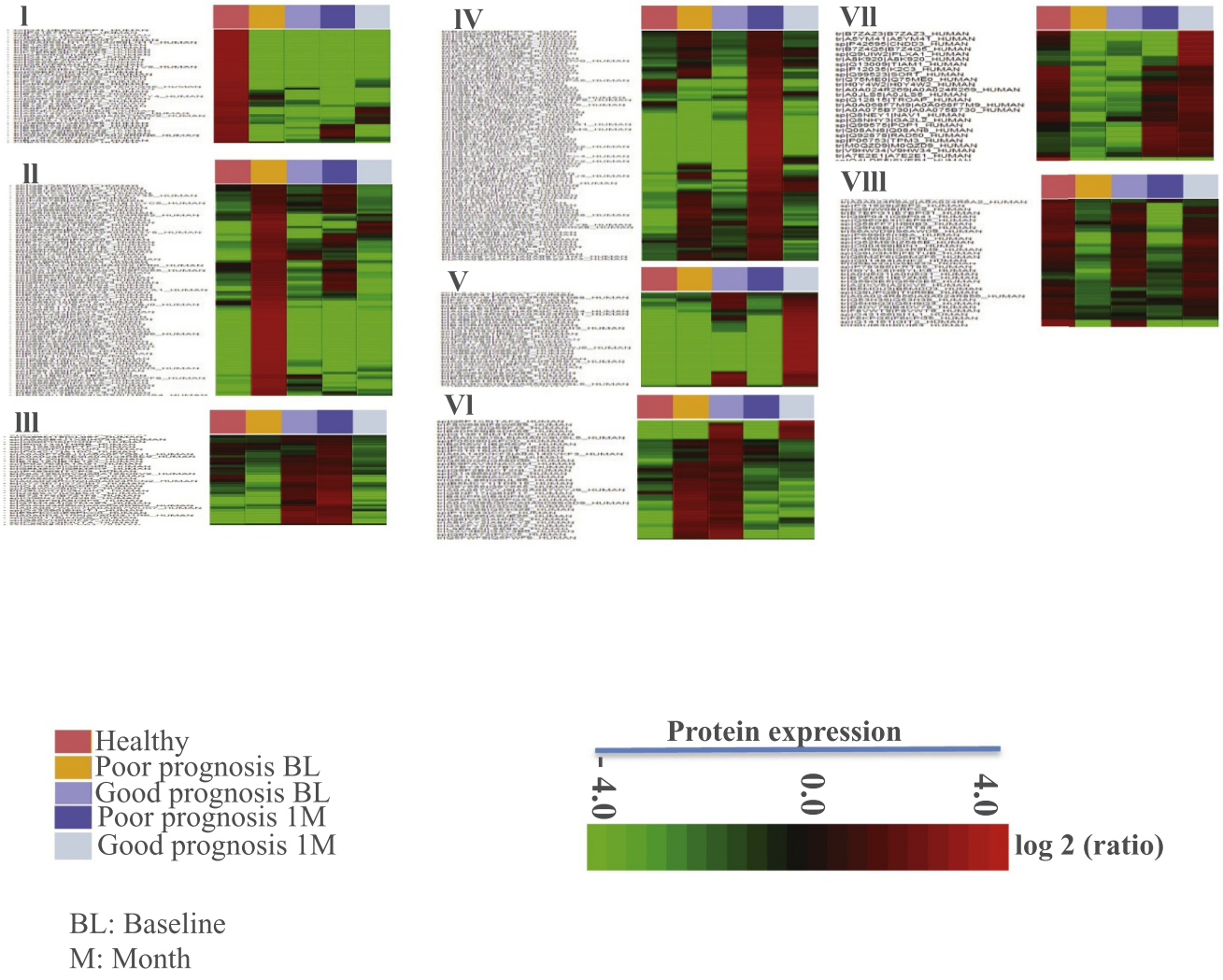

Fig. 1. Proteomic analysis for microvesicles collected from the plasma of 10 healthy subjects, 10 kidney transplant recipients with good outcome (eGFR = 81 ± 22 mL/min), and 10 kidney transplant recipients with poor outcome (eGFR = 32.3 ± 5 mL/min). For each group of kidney transplant recipients we used plasma samples before the kidney transplant as baseline (BL), and 1 month after the kidney transplant. Eight clusters of proteins that reflect different predictive value for the outcome of the kidney transplant as described in the results.

expressed in urine vesicles between patients with and without delayed graft function following kidney transplant [18]. NGAL was also found to be much more abundant in urinary exosomes than free in the urine [18]. It will be interesting and valuable to uncover what proteins in the blood vesicles could serve as good biomarkers.

2. Materials and methods

2.1. Microvesicle collection

We used plasma from 2 different transplant populations for these studies. We collected plasma from 10 healthy volunteers, 10 kidney transplant patients with poor prognosis, and 10 patients with good prognosis. The prognosis was defined by the slope of the eGFR from 3 to 12 months post transplant. For these transplant recipients, we used two time points: base line (BL), which is before the kidney transplant; and one month after the kidney transplant. Also, we performed A second proteomic analysis, where we collected plasma from six kidney transplant recipients (6 months post-transplant) and three healthy volunteers. The six transplant patients were divided into good and poor prognosis based on the 12 month eGFR. We have used a procedure that we have used before to collect the microvesicles [8]. Briefly, 2 mL of plasma was centrifuged at 300*g* for 15 min and 12,000 *g* for 20 min to remove cellular debris and large particles. The supernatant was centrifuged at 100,000 *xg* for 2 h to pellet microvesicles. The microvesicle-enriched pellet was suspended in 100 μL of sterile phosphate-buffered saline and stored at −80 °C. The following flow chart summarizes the protocol of microvesicles isolation.

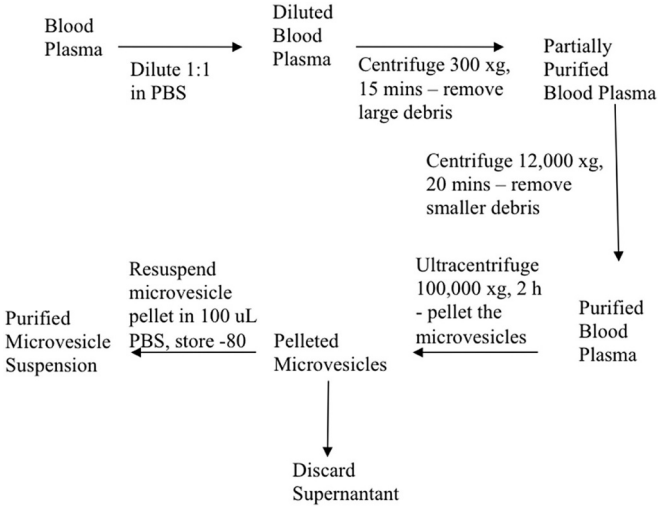

Table 1
Summarizes selected proteins from the protein groups of Fig. 1. The table shows group number as seen in Fig. 1. The table contains the protein's accession number, name, cellular location, protein function, the protein's role in kidney transplant from the literature, and the reference number.

Group 1	Name	Location	Protein function	Role in kidney transplant	Ref.
P21675 (TAF1	Transcription initiation factor TFIID subunit 1	Nucleus	TFIID basal transcription factor complex's largest subunit	Thought to be a bridging molecule for many protein-protein interactions in renal allograft rejection.	[25]
Q43318 (Q43318_ARTAN)	3-hydroxy-3-methylglutaryl coenzyme A reductase	Endoplasmic reticulum: ER membrane	Involved in the synthesis of (*R*)-mevalonate	In renal failure HMG-reductase is twice as active but in transplant survival rate is unaffected by HMG-reductase inhibition.	[26,27]
B7ZLE5 (B7ZLE5	FN1 protein	Extracellular region or secreted	Involved in blood clotting	Thought to be a bridging molecule for many protein-protein interactions in renal allograft rejection.	[25]
A0A024R0N6(A0A024R0N6)	Spectrin beta chain	Cytoskeleton	Involved in actin and phospholipid binding	When measured in serum, has been found to be significantly more associated with suboptimal outcome in kidney transplantation.	[28]
Group 2					
Sp|P02760 (AMBP	Protein AMBP	Extracellular region or secreted	Light chain of the inter-alpha trypsin inhibitor	Has been found to be upregulated in serum in association with renal allograft rejection.	[25]
Sp|P19652 (A1AG2)	Alpha-1-acid glycoprotein 2	Extracellular region or secreted	Blood stream transport protein	Precursor is present in the urine exosomes of patients with stable kidney transplants and rejected kidneys, but is far more abundant in the vesicles of the latter.	[29]
SP|Q8TD57 |DYH3_	Dynein heavy chain 3, axonemal	Cytoskeleton, cilium axoneme	Generates force toward microtubule minus end	Present in urine exosomes of patients with cell mediated kidney transplant rejection.	[29]
sp|P35568 (IRS1)	Insulin receptor substrate 1	Cytosol, Nucleus, Plasma Membrane, Caveola	Interacts with insulin receptor to mediate various cellular processes	Present in urine exosomes of patients with tubular injury kidney transplant rejection.	[29]
Group 3					
P00734 (THRB)	Prothrombin	Extracellular region or secreted	Inactive form of thrombin	Prothrombin precursor is present in urine exosomes of patients with tubular injury and antibody mediated rejection .	[29]
P02763 (A1AG1)	Alpha-1-acid glycoprotein 1	Secreted	Blood stream transport protein	Precursor is present in the urine exosomes of patients with tubular injury, cell mediated, and antibody mediated rejection.	[29]
P08582 (TRFM	Melanotransferrin	Isoform 1: Plasma membrane, GPI-anchor	Involved in cellular uptake of iron	Precursor is present in the urine exosomes of patients with tubular injury kidney rejection.	[29]
Group 4					
P04040 (CATA)	Catalase	Peroxisome	Protects from hydrogen peroxide toxicity	Present in urine exosomes of patients with transplanted kidney rejections due to cellular and antibody mediated rejection, and even more abundant in those associated with tubular injury.	[29]
Q8TF46 (DI3L1_	DIS3-like exonuclease 1	Cytoplasm	RNA exosome complex catalytic component	Present in the urine exosomes of patients with cellular mediated kidney rejection.	[29]
P50748 (KNTC1	Kinetochore-associated protein 1	Cytoskeleton: Spindle, Nucleus, Cytoplasm	Aids in the prevention of exiting mitosis prematurely	Present in urine exosomes of patients with cellular mediated kidney rejection.	[29]
Group 5					
Sp|P02647(APOA1	Apolipoprotein A-I	Extracellular region or secreted	Promotes cholesterol efflux from tissues	Has been found to be downregulated in the blood and urine in association with renal allograft rejection.	[25]
sp| O43790 (KRT86)	Keratin, type II cuticular Hb6	Cytoskeleton, keratin filament, Cytosol Extracellular region or secreted, exosome	Heterodimerizes with type I keratins	Present in the urine exosomes of patients with tubular injury, and to a much lesser extent cell mediated kidney rejection.	[29]
Tr|A0A024R914 (A0A024R914)	Neuroblast differentiation-associated protein AHNAK	Cytoskeleton, Centrosome	Anchors microtubules to centrisomes	Present in the urine exosomes of patients with cell mediated and antibody mediated kidney rejection.	[29]
Group 6					
Sp|P02649 (APOE)	Apolipoprotein E	Extracellular region or secreted	Mediates lipoprotein particle internalization catabolism and binding	Precursor present in urine exosomes of patients with tubular injury kidney rejection, and to a lesser extent cell mediated kidney rejection.	[29]
Sp|P01019 (ANGT)	Angiotensinogen	Extracellular region or secreted	Renin-angiotensin system component	Precursor found in urine exosomes of patients with stable kidney transplants, but is even more abundant in those with rejected kidneys, particularly tubular injury.	[29]
Sp|P02774 (VTDB)	Vitamin D-binding protein	Extracellular region or secreted	Aids in vitamin D storage and transport	Precursor present in the urine exosomes of patients with antibody mediated kidney rejection.	[29]

(*continued on next page*)

Table 1 (*continued*)

Group 1	Name	Location	Protein function	Role in kidney transplant	Ref.
Tr|Q580Q6 (Q580Q6)	EGF-containing fibulin-like extracellular matrix protein 1	Extracellular region or secreted	Interacts with ARAF	Precursor present in the urine exosomes of patents with cell mediated and antibody mediated kidney rejection.	[29]
Group 7					
P12035 (K2C3)	Keratin, type II cytoskeletal 3	Intermediate filament, Keratin filament Cytosol, exosome	Heterodimerizes with type I keratins	Present in the urine exosomes of patients with tubular injury, cell mediated, and to a lesser extent, antibody mediated kidney rejection.	[29]
Q92878 (RAD50)	DNA repair protein RAD50	Nucleus	MRN complex component	Isoform 2 present in the urine exosomes of patients with tubular injury kidney rejection.	[29]
B7Z4Q5 (B7Z4Q5)	Receptor-type tyrosine-protein phosphatase	Integral component of membrane	Removes phosphates from protein tyrosines	Isoform 1 precursor present in the urine exosomes of patients with tubular injury, and to a slightly lesser extent cell mediated and antibody mediated kidney rejection.	[29]
Group 8					
Tr|V9GYM3|V9GYM3	Apolipoprotein A-II	Extracellular region or secreted	Ivolved in lipid binding	Present in urine exosomes of patients with tubule injury kidney rejection.	[29]
Tr|E9KL36|E9KL36	Transthyretin	Extracellular or secreted	Binds thyroid hormones	Precursor present in urine exosomes of patients with tubular injury kidney rejection, to a lesser extent, antibody mediated rejection, and to an even lesser extent, cell mediated kidney rejection.	[29]
Hemoglobin Beta	Hemoglobin subunit beta	Cytosol, Extracellular or secreted: blood microparticle, exosome,	Involved in oxygen transport	Found in urine vesicles of all four groups examined in the study. Most abundant in tubular injury kidney rejection, second most abundant in cell mediated kidney rejection, third most abundant in healthy kidney transplants, and is least abundant in antibody-mediated kidney rejection.	[29]

We elucidated that the mean size of the obtained vesicles from both healthy and patients is 257.5 nm (using Nano-Sight analysis). Thus the mean size is within the microvesicle size range (extracellular vesicles are classified according to the size to exosomes (30–100 nm), and microvesicles (100–1000 nm)) [19].

2.2. Proteomic analysis

Plasma microvesicles were lysed in RIPA-buffer and protein concentration was assessed by Bradford assay (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). 150 μg of microvesicle protein from each subject was separated with 10% SDS-PAGE. Bands from Coomassie blue stained gels were excised, and subjected to proteomic analysis, as detailed together with database search and criteria for protein identification in our previous manuscript [20]. Extracted peptide samples were identified using MS/MS sequencing. MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.01). Scaffold (version Scaffold-4.0.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 3 identified peptides. Protein probabilities were assigned by the Prophet algorithm [21]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins were annotated with GO terms from NCBI [22].

3. Results

3.1. Patients

All transplant patients included in this study received their first renal transplant at St. Joseph's Healthcare, Hamilton, Ontario, between March 2013 and December 2014. Patients provided informed consent. eGFR was calculated using the CKD-EPI formula. Twenty patients had plasma collected pretransplant and 1 month post transplant and had microvesicles proteomic analysis. Patients were analyzed in 2 groups based on the slope of eGFR from 3 months to 12 months. Both groups were equally divided male and female. The 10 patients in the good prognosis group had an eGFR slope of 0.35 (± 0.4) ml/min/month with an average age of 54 (± 14) years. There were 6 live related transplants, 1 standard criteria donor, 2 extended criteria donors, and one donation after cardiac death in this group. The 10 patients in the poor prognosis group had an eGFR slope of −0.10 (± 0.07) ml/min/month with an average age of 56 (± 13) years. There were 3 live related transplants, 3 standard criteria donors, 1 extended criteria donors, and 3 donations after cardiac death in this group (all data shown ± standard deviation). All the samples were taken prospectively, and grouped afterwards. At the time of sampling the plasma from each patient was collected and stored in liquid nitrogen and used to collect the microvesicles for the proteomic analysis.

Another population of patients that contains six patients had plasma drawn 6 months after transplant and analyzed for microvesicle proteins. These patients were chosen either for good outcome (average 12 month eGFR 81 ± 22 mL/min) or poor outcome (average 12 month eGFR 32 ± 5 mL / min). In the good outcome group, there were 2 males and one female with an average age of 40 ± 14 years. One transplant was live related, 2 were standard criteria donors. In the poor outcome group, there was 1 male and 2 females with an average age of 58 ± 7 years. One transplant was live related, one was an extended criteria donor, and one was donated after cardiac death.

The healthy volunteers were males and females with no history of kidney complication with matching age with the kidney transplant patients (54 ± 13) years. The plasma samples were collected at the St. Joseph's healthcare, between October 2014 and June 2015. Subjects provide informed consent.

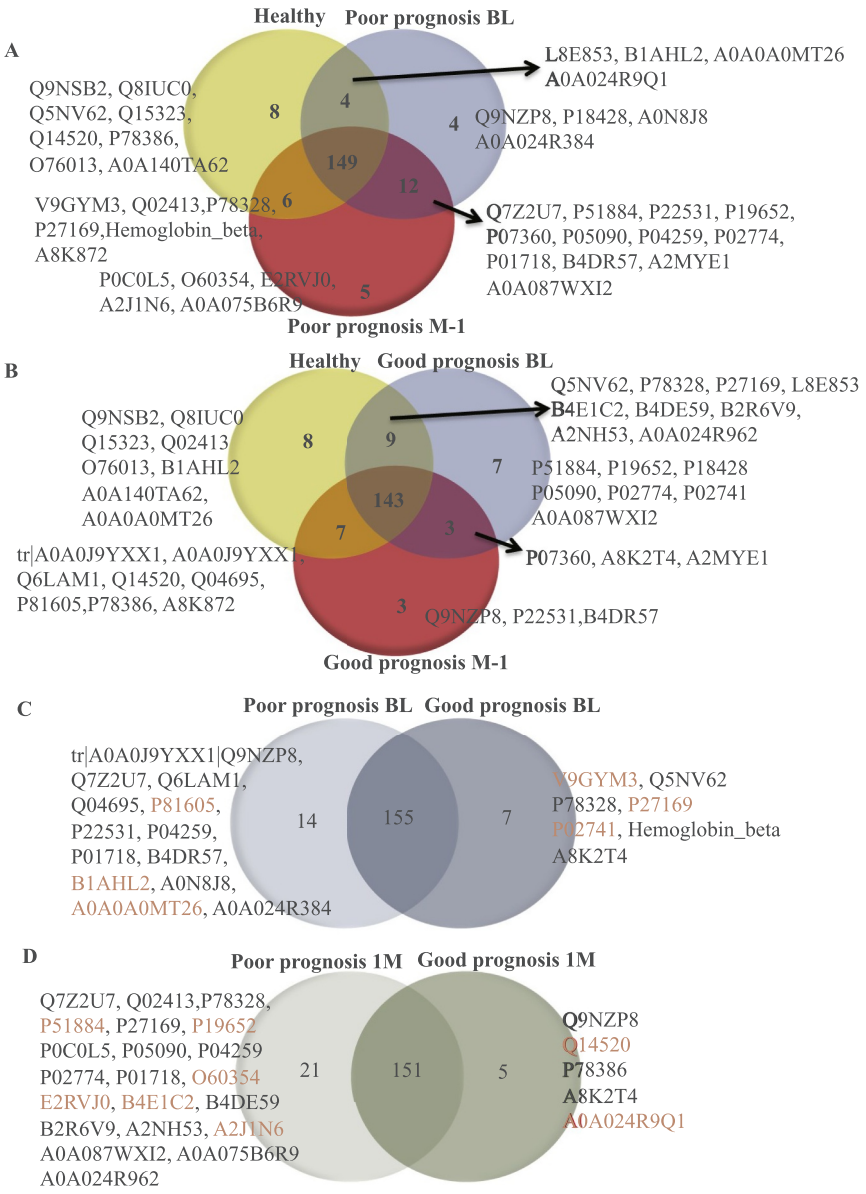

Fig. 2. Venn diagrams depicting the total number of proteins exclusively expressed in each population of the subjects used in the proteomic study. A) Shows the spectrum of proteins exclusively loaded or shared between healthy, poor prognosis BL, and poor prognosis M-1. B) The spectrum of proteins in healthy, good prognosis BL, and good prognosis M-1. C) The spectrum of proteins in poor prognosis BL, and good prognosis BL. D) shows the spectrum of proteins in poor and good prognosis after 1 M of kidney transplant. The highlighted proteins in red have direct role in renal diseases as summarized in the Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Proteomics analysis

MV lysates were subjected to SDS-PAGE, bands were excised and underwent several preparations [20]. Extracted peptide samples were identified using electrospray tandem mass spectrometry (ESI-MS/MS) sequencing. MS/MS samples were analyzed using Mascot (Matrix Science, version 2.3.01). The software Scaffold (version Scaffold_4.0.4) was used to validate MS/MS-based peptide and protein identifications. The heat map was generated using the software 'PEAKS 7.5' from Bioinformatics solutions. Scaffold (version Scaffold_4.0.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 50.0% probability by the Peptide Prophet algorithm [23]. Protein identifications were accepted if they could be established at greater than 50.0% probability and contained at least 3 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [24]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

We have analyzed the protein content of 30 subjects as outlined above (10 healthy volunteers, 20 kidney transplant patients divided between good and poor prognosis based on slope of eGFR (10 patients in each group). For the kidney transplant recipients we used two time points: base line (BL), which is before the kidney transplant; and one month after the kidney transplant. As seen in Fig. 1 we found eight (I-VIII) protein clusters that may have a diagnostic or prognostic value for the outcomes of kidney transplants because their expression is up or down- regulated in comparison to healthy subjects, as follows:

I. Proteins that are up-regulated in healthy subjects and down regulated in both kidney transplant patient populations despite the outcome of the kidney transplant and the follow up intervals.
II. Proteins that are mainly up-regulated in the BL of poor prognosis patients, some proteins in this cluster stay up-regulated in the IM follow up. This cluster may have a prognostic value for the outcome of the allograft even before the kidney transplant.
III. These proteins are up-regulated in the BL of the good prognosis group, but retain their healthy expression after IM of kidney transplant in the patients with good prognosis. This group of proteins may have a role in the good prognosis of this group of patients. Contrary to the good prognosis group, the expression of these proteins is similar in both the healthy subjects and in the BL

Table 2
The table summarizes the proteins highlighted (in red) in Fig. 2. The table contains the protein ID (accession number), protein name, protein's function, the protein's role in kidney transplant from the literature, and the reference number.

Protein ID	Protein Name	Protein Function	Role in Kidney Transplants	Ref.
P81605	Dermcidin OS = Homo sapiens GN = DCD PE = 1 SV = 2	Displays proteolytic and antimicrobial activity.	Present in the urine vesicles of patients with tubular injury kidney rejection.	[29]
B1AHL2	Fibulin-1 OS = Homo sapiens GN = FBLN1 PE = 1 SV = 1	Component of fibronectin-containing extracellular matrix.	Isoform C precursor present in patients with tubule injury kidney rejection. Isoform D is abundant in patients with tubule injury rejection and, few patients with antibody mediated rejection.	[29]
A0A0A0MT26	Sodium/potassium-transporting ATPase subunit alpha-3 OS = Homo sapiens GN = ATP1A3 PE = 1 SV = 1	ATP binding and ATPase activity.	Present in urine vesicles of patients with cell mediated kidney rejection	[29]
V9GYM3	Apolipoprotein A-II OS = Homo sapiens GN = APOA2 PE = 1 SV = 1	Lipid binding.	Present in urine vesicles of patients with tubule injury kidney rejection.	[29]
P27169	Serum paraoxonase/arylesterase 1	Hydrolyses lactones, aeromatic carboxylic acid esters, and organophosphates.	Precursor present in urine vesicles of patients with tubule injury kidney rejection.	[29]
P02741	C-reactive protein OS = Homo sapiens GN = CRP PE = 1 SV = 1	Promotes phagocytosis, and agglutination.	Precursor present in urine vesicles of patients with antibody mediated kidney rejection	[29]
P51884	Lumican OS = Homo sapiens GN = LUM PE = 1 SV = 2	Componant of the extracellular matrix, binds collagen.	Precursor present in urine vesicles of patients with tubule injury kidney rejection, and more abundant in those with cell mediated kidney rejection.	[29]
P19652	Alpha-1-acid glycoprotein 2 OS = Homo sapiens GN = ORM2 PE = 1 SV = 2	Blood stream transport protein.	Precursor is present in the urine vesicles of stable kidney transplant patients but is more abundant the kidney rejection patients	[29]
O60354	Loricrin OS = Homo sapiens PE = 2 SV = 1	Component of the epidermis	Detectable loricrin is found in few kidney transplant recipients-derived keratinocyte keratinocytes.	[30]
E2RVJ0	Anion exchange protein OS = Homo sapiens GN = SLC4A1 PE = 2 SV = 1	Exchanges inorganic ions.	Present in urine vesicles of patients with tubule injury and cell mediated kidney rejection.	[29]
B4E1C2	Kininogen 1, isoform CRA_b OS = Homo sapiens GN = KNG1 PE = 2 SV = 1	Inhibits cysteine-type endopeptidases.	Present in vesicles from patients with stable transplants as well as with cell and antibody mediated kidney rejection and it is abundant in the vesicles of patients with tubular injury kidney rejection. Also, downregulation of serum Kininogen 1 is associated with renal allograft rejection.	[29,25]
A2J1N6	Rheumatoid factor RF-ET9 (Fragment) OS = Homo sapiens PE = 2 SV = 1	Antibody specific to the Fc region of IgG.	Associated with cytomegalovirus infection in renal transplant patients, and not with transplant outcome.	[31]
Q14520	Hyaluronan-binding protein 2 OS = Homo sapiens GN = HABP2 PE = 1 SV = 1	Cleaves fibrinogen and converts plasminogen-activator to its two chain form.	Hyaluronan (substrate of HBP2) in renal tubule cells is increased following injury. This process is believed to support renal tubule regeneration and proliferation.	[32]
A0A024R9Q1	Thrombospondin 1, isoform CRA_a OS = Homo sapiens GN = THBS1 PE = 4 SV = 1	Binds heparin, the extracellular matrix, and calcium ions.	Overexpressed in biopsies of renal allografts of patients with interstitial fibrosis and tubular atrophy. Also, TSP-1 expression to be associated with kidney injury, and proceeding interstitial fibrosis.	[33,34]

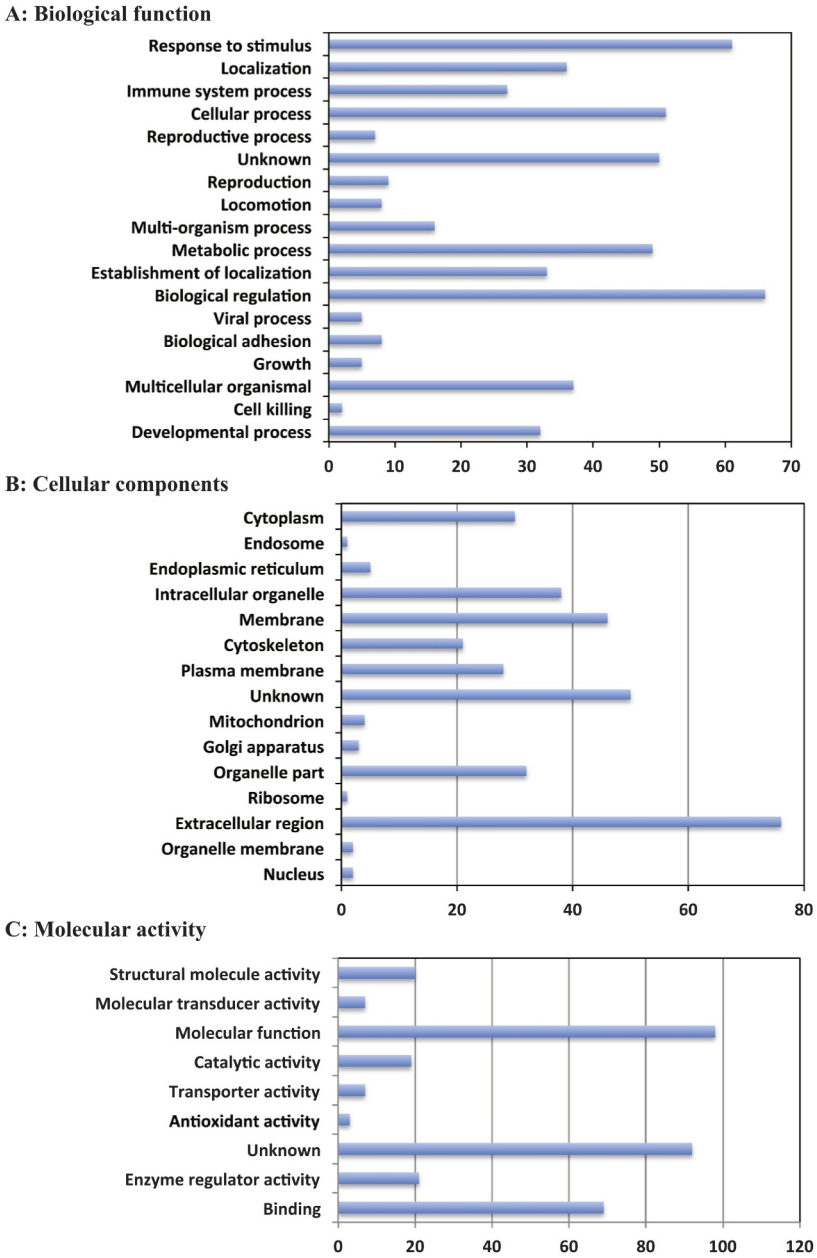

Fig. 3. Gene Ontology enrichment of the proteins incorporated into the plasma microvesicles from the kidney recipients. The figure shows A) Biological functions, B) Cellular component, and C) Molecular activity.

for the poor prognosis group. Further, they are up-regulated one month after receiving the kidney transplant, which once again indicates the correlation with the outcome of the kidney transplant.

IV. The proteins in this group are mainly up-regulated after the 1 M follow up of the poor prognosis group of patients. Proteins in this group may reflect the deleterious status of the transplanted kidney in the patients with poor prognosis.
V. Contrary to group 4 these proteins are up-regulated in the good prognosis patients after 1 M of follow-up. These proteins may reflect protective characteristics to the transplanted kidney.
VI. This cluster of proteins is up-regulated in the BL regardless of the outcome of the kidney transplant, these proteins may correlate to the pre-existing condition before the kidney transplant and are, therefore, suitable diagnostic markers.
VII. The proteins in this cluster are down-regulated in the BL of both prognosis but up-regulated after 1 M follow up in both groups to match the expression of healthy subject. These proteins may correlate with the enhancement of kidney function from the base line regardless of the global outcome.
VIII. These proteins are expressed to a similar degree in healthy subjects and the BL and 1 M follow up of the good prognosis patients. Simultaneously, these proteins are down regulated in the BL and the 1 M follow-up of the poor prognosis patients. This group of proteins may be the key to predicting if the recipient will have a successful kidney transplant.

Some of the proteins detected in this analysis are listed in Table 1. The table contains the group number (from the heatmap), protein's accession number, protein's name, location, and the role of the protein in kidney transplant from the literature with the reference number. These proteins were selected specifically for their role in kidney transplant or renal diseases.

We have used the software Scaffold-4 to generate Venn charts to

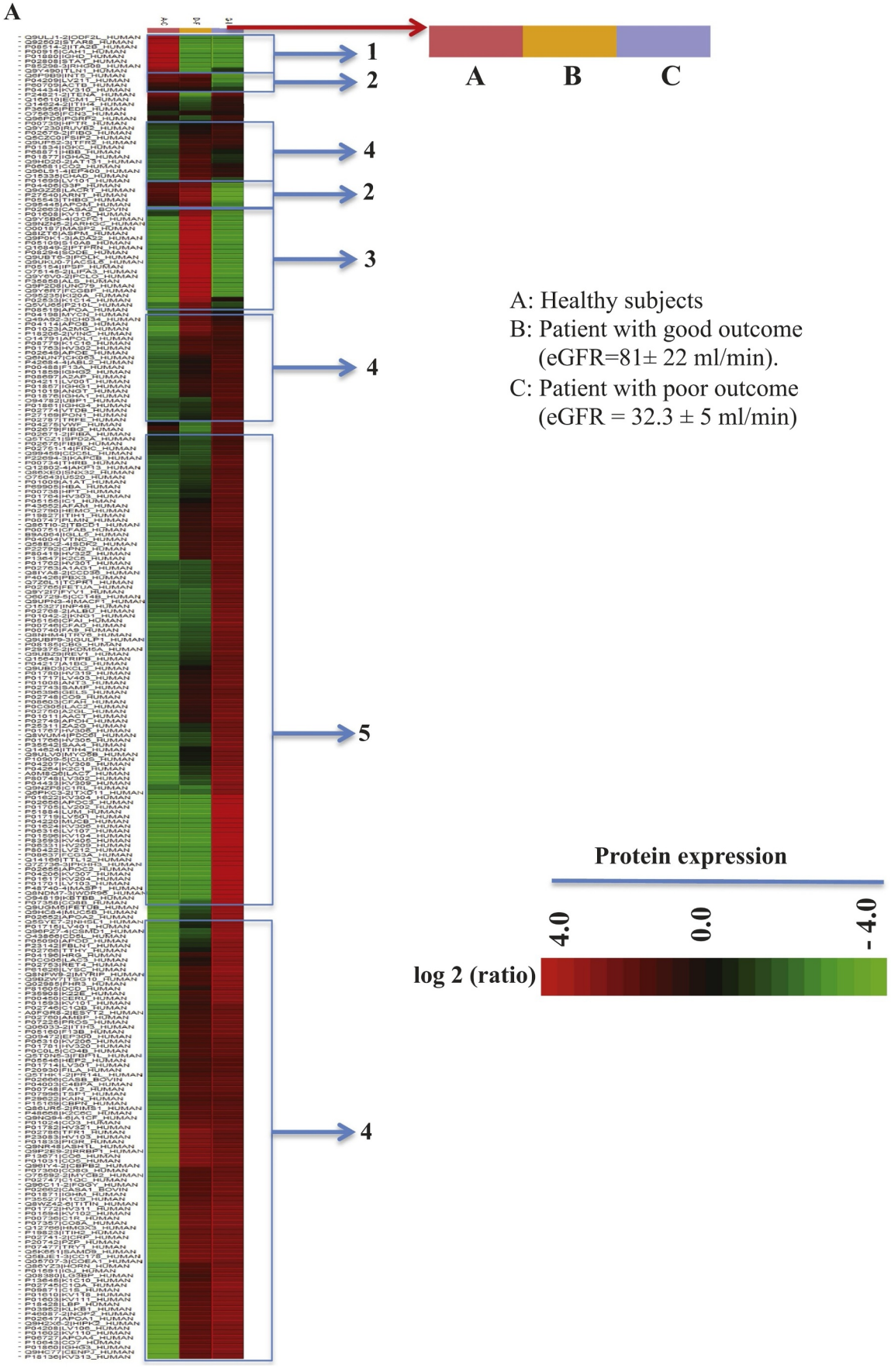

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Fig. 4. A) A heat map demonstrating the proteomic analysis for microvesicles collected from the plasma of three healthy subjects (group A), and two groups of kidney transplant patients after 6 months of the kidney transplant, three kidney transplant recipients with good outcome (eGFR = 81 ± 22 mL/min) (group B), and three kidney transplant recipients with poor outcome (eGFR = 32.3 ± 5 mL/min) (group C). Five clusters of proteins [1–5] were detected and as detailed in the results. B) Venn diagram depicting the total number of proteins exclusively expressed our shared in the healthy subjects subjects and the two groups of kidney recipients. The highlighted proteins (in red) were summarized in Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

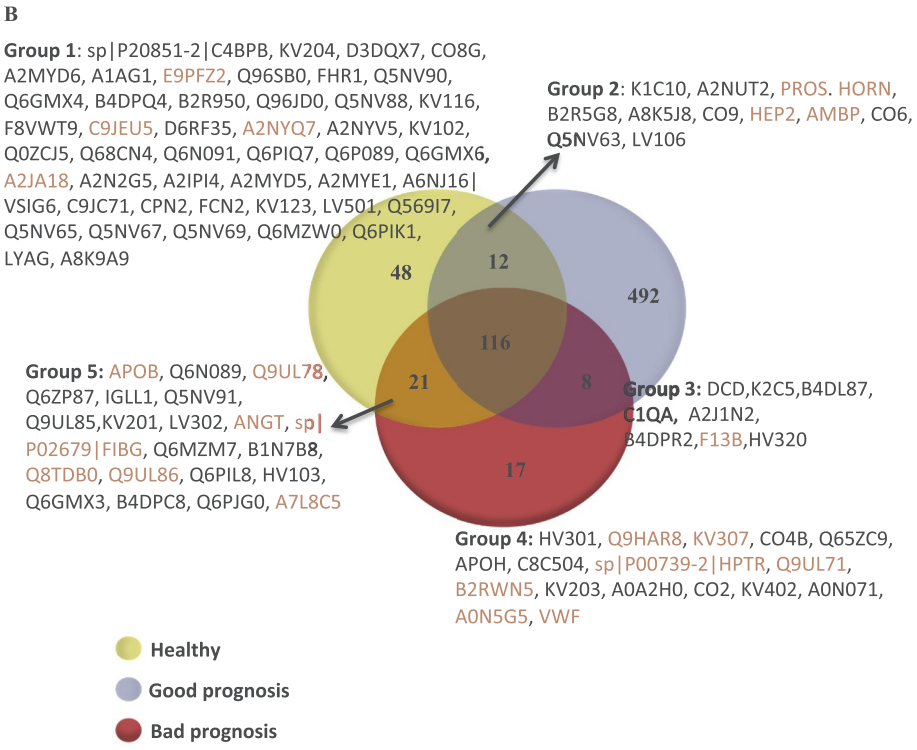

Fig. 4. (*continued*)

show the protein expression in the various blood microvesicles of the used population. Fig. 2 contains Venn charts showing the spectrum of various proteins that were expressed differently in each group, and proteins that are shared with other groups. The figure contains the accessions numbers of these proteins and comparing the following:

A) Healthy, poor prognosis base line, and poor prognosis after I month of kidney transplant. As seen from the figure most of the proteins were shared between the three groups [149 proteins]. Others proteins are shared by healthy and poor prognosis BL [4 proteins], healthy and poor prognosis 1 M [6 proteins], and poor prognosis BL and poor prognosis 1 M [12 proteins]. Some proteins are exclusively expressed in each group i.e. [8 proteins] in healthy, poor prognosis BL [4 proteins] and poor prognosis IM [5 proteins].
B) Venn diagram shows proteins that are shared or exclusively expressed in healthy, good prognosis BL, and good prognosis 1 M. As seen from the figure most of the proteins were shared between the three groups [143 proteins]. Others proteins are shared by healthy and good prognosis BL [9 proteins], healthy and good prognosis 1 M [7 proteins], and good prognosis BL and good prognosis 1 M [3 proteins]. Proteins that are exclusively expressed in each group i.e. [8 proteins] in healthy, good prognosis BL [9 proteins] and good prognosis IM [3 proteins].
C) Proteins that are shared or exclusively expressed in base line (BL) for both poor and the good prognosis patients. In this figure there are 155 proteins shared between the two groups, 14 proteins exclusively expressed in poor prognosis BL, and 7 proteins expressed in good prognosis BL.
D) Proteins that are shared or exclusively expressed after 1 month (1 M) of kidney transplant for both poor and the good prognosis patients. In this figure there are 151 proteins shared between the two groups, 21 proteins exclusively expressed in poor prognosis 1 M, and 5 proteins expressed in good prognosis 1 M.

For the Venn charts of C and D we have highlighted some proteins (in red) for their role in kidney transplant, these proteins are summarized in the Table 2. These proteins and others were highlighted for their role in renal disease as seen in the references within the table.

The gene ontology of the proteins in heatmap (Fig. 1) is obtained using the software scaffold 4 with the Go terms utilizing the NCBI database for human, and are summarized in Fig. 3. Fig. 3A shows the biological functions of these proteins. As seen from the figure these proteins contribute to a wide spectrum of biological function such as, cellular process, localization, immune system process, and others. Fig. 3B shows the cellular components, where a large number of the proteins are of extracellular region, organelle part, plasma membrane. These proteins have various molecular activities as summarized in Fig. 3C. Most of the proteins contribute to binding and molecular functions, enzyme regulator activity, catalytic activity.

Further we have performed a pilot assessment of the protein cargo of blood microvesicles after six month of kidney transplant for patients with good and bad prognosis compared to healthy subjects. We collected plasma from 6 kidney transplant patients at 6 months as outlined above. These patients were grouped according to outcome based on the 12 month eGFR for patients with good prognosis ($n = 3$), and patients with bad prognosis ($n = 3$). These groups were compared to three

Table 3
The table summarizes the proteins highlighted (in red) in Fig. 4B. The table contains the protein ID (accession number), protein name, protein's function, the protein's role in kidney transplant from the literature, and the reference number.

Accession	Protein Name	Protein Function	Kidney Transplant Literature	Ref.
Group 1				
E9PFZ2_HUMAN	Ceruloplasmin OS = Homo sapiens GN = CP PE = 4 SV = 1	Possesses a ferroxidase and copper ion binding activities.	Precursor found in equal proportion in the urine EV of patients with cell mediated and antibody mediated kidney rejection, as well patients with stable transplants. Much more upregulated in the urine EV of patients with tubule injury kidney rejection.	[29]
C9JEU5_HUMAN	Fibrinogen gamma chain OS = Homo sapiens GN = FGG PE = 4 SV = 1	Possesses signaling receptor binding activity.	Isoform gamma A precursor found in urine vesicles of all patients with kidney rejection, and not in those with stable transplants. It is most abundant in urine vesicles from those with antibody mediated kidney rejection, less abundant in those with tubule injury mediated kidney rejection, and least abundant in those with cell mediated kidney rejection. Isoform gamma B precursor is also found in urine vesicles from those with stable kidney transplants and cell mediated kidney rejection, and to a lesser extent tubule injury kidney rejection.	[29]
A2NYQ7_HUMAN	Anti-folate binding protein (Fragment) OS = Homo sapiens GN = HuC4lambda Vlambda PE = 2 SV = 1	Folate binding activity.	Mediates the reabsorption of folate into kidney tubules.	[35]
A2JA18_HUMAN	Anti-mucin1 heavy chain variable region (Fragment) OS = Homo sapiens PE = 2 SV = 1	Binds mucin-1.	T-cell immunoglobulin mucin-1, a cleaved domain KIM-1 (kidney injury molecule 1) detectable in urine, has undetectable expression during healthy kidney conditions, and is abundantly expressed and easily detectable in urine during renal injury.	[36]
Group 2				
PROS_HUMAN	Vitamin K-dependent protein S OS = Homo sapiens GN = PROS1 PE = 1 SV = 1	Circulating anti-coagulant.	Has been found to be upregulated in serum in association with renal allograft rejection.	[25]
HORN_HUMAN	Hornerin OS = Homo sapiens GN = HRNR PE = 1 SV = 2	Epidermal cornified cell envelope component.	Present in all 3 groups of urine vesicles from patients with different types of kidney rejection (tubule injury, cell mediated, and antibody mediated) and not present in the urine vesicles of stable kidney transplant recipients.	[29]
HEP2_HUMAN	Heparin cofactor 2 OS = Homo sapiens GN = SERPIND1 PE = 1 SV = 3	Inhibits thrombin following activation by glycosaminoglycans.	Precursor upregulated in the urine vesicles of patients with tubule injury kidney rejection and to a lesser extent antibody mediated kidney rejection.	[29]
AMBP_HUMAN	Protein AMBP OS = Homo sapiens GN = AMBP PE = 1 SV = 1			
Group 3				
F13B_HUMAN	Coagulation factor XIII B chain OS = Homo sapiens GN = F13B PE = 1 SV = 3	Stabilizes A subunits of coagulation factor.	Elevated levels of coagulation factors thought to be a cause of end stage renal disease in 1% of cases. Rejected kidneys produce more endothelial protein C receptor which degrades certain coagulation factors (Va, and VIIIa).	[37,38]
Group 4				
Q9HAR8_HUMAN	Mutant beta-globin (Fragment) OS = Homo sapiens GN = HBB PE = 2 SV = 1	Heme binding activity.	Wild type beta-globin mRNA identified previously as being a distinguishing factor between a stable transplant and a kidney rejection group measured in biopsies.	[39]
KV307_HUMAN	Ig kappa chain V-III region GOL OS = Homo sapiens PE = 1 SV = 1	Subunit of immunoglobin.	Has previously been found expressed at a ratio of 11:3 in acute kidney rejection patients vs stable kidney transplant patients, measured in urine.	[40]
sp|P00739–2|HPTR_HUMAN	Isoform 2 of Haptoglobin-related protein OS = Homo sapiens GN = HPR	Mediates innate human immune response against trypanosomes.	Haptoglobin-related protein thought to be a bridging molecule between interactions associated with renal allograft rejection	[25]
Q9UL71_HUMAN	Myosin-reactive immunoglobulin heavy chain variable region (Fragment)	Component of myosin-reactive immunoglobulin.	Widely expressed in the normal kidney, especially in glomerulus and peritubular vessels.	[41]
B2RWN5_HUMAN	HEAT repeat containing 1 OS = Homo sapiens GN = HEATR1 PE = 2 SV = 1	Plays a role in RNA synthesis and processing.	Slightly more blood mRNA HEAT repeat containing 1 has been found on average at a ratio of 131.9 to 101.0 in acute kidney rejection patients compared to stable transplant recipients.	[42]
A0N5G5_HUMAN	Rheumatoid factor D5 light chain (Fragment) OS = Homo sapiens	Component of the rheumatoid factor D5 antibody.	Rheumatoid factors found to be associated with cytomegalovirus infection in renal transplant patients, and not with transplant outcome.	[31]
VWF_HUMAN	von Willebrand factor OS = Homo sapiens GN = VWF PE = 1 SV = 4	Promotes platelet adhesion to vascular injury sites.	Type A found in urine vesicles of patients with tubule injury and to a lesser extent cell mediated kidney rejection.	[29]
Group 5				
APOB_HUMAN	Apolipoprotein B-100	Recognition signal for internalization and cellular binding of LDL particles	Precursor found in urine vesicles of patients with antibody mediated kidney rejection.	[29]

(*continued on next page*)

Table 3 (*continued*)

Accession	Protein Name	Protein Function	Kidney Transplant Literature	Ref.
Q9UL78_HUMAN	Myosin-reactive immunoglobulin light chain variable region	Component of myosin-reactive immunoglobilin.	Immunoglobulin light chains are directly damaging to renal epithelial cells.	[43]
ANGT_HUMAN	Angiotensinogen OS = Homo sapiens GN = AGT PE = 1 SV = 1	Regulates blood pressure and electrolyte homeostasis.		
sp|P02679|FIBG_HUMAN	Fibrinogen gamma chain OS = Homo sapiens GN = FGG PE = 1 SV = 3	Component of insoluble fibrin matrix following polymerization.	Isoform A precursor found in urine vesicles of patients with antibody mediated kidney rejection, to a lesser extent tubule injury kidney rejection, and to an even lesser extent cell mediated kidney rejection. Isoform B precursor found in urine vesicles of patients with stable transplants and cell mediated kidney rejection, and to a lesser extent in patients with tubule injury kidney rejection.	[29]
Q8TDB0_HUMAN	Apolipoprotein A-1 A175P variant (Fragment) OS = Homo sapiens PE = 2 SV = 1	Component of HDL particles in plasma.	High serum Apolipoprotein AI (normal) is associated with a lower instance of chronic kidney disease and a higher glomerular filtration rate.	[44]
A7L8C5_HUMAN	Alpha-1-antitrypsin (Fragment) OS = Homo sapiens PE = 4 SV = 1	Common circulating serine protease inhibitor.	Found to be abundant in urine vesicles of patients with tubule injury, cell mediated, and antibody meditated kidney rejection, as well as those with stable kidney transplants.	[29]

healthy subjects. MV were collected from 2 mL of plasma. The MV-enriched pellet was suspended in 100 μL sterile phosphate-buffered saline and stored at −80 °C. Protein cargo analyzed for the two groups of kidney recipients compared to a matching group of three healthy subjects, and we obtained the following results:

The analysis showed differences in protein expression between the two groups of patients and the healthy subjects. As seen in the heat map (Fig. 4A) we could determine five clusters of differentially expressed proteins. These clusters could discriminate between each group of the patients and the healthy subjects, and distinguish between the two groups of patients to reflect the outcomes of kidney transplants. The following are the clusters of the characterized proteins:

1. Proteins that are up-regulated in healthy subjects, but down regulated in both groups of patients.
2. Proteins that are up-regulated in healthy subjects, and the group of patients with good transplant outcome but down regulated in the poor outcome group.
3. Proteins that are up-regulated only in the good outcome group.
4. Proteins that are up-regulated only in the two groups of the patients, but not in healthy subjects.
5. Proteins that are up-regulated specifically in the poor outcome group.

Clusters 4 and 5 represent the two largest clusters, and proteins associated with these clusters may provide markers for poor prognosis upon validation. These results clearly demonstrate the power of blood MV to predict the outcome of kidney transplants. Among the characterized proteins, there is a distinct pattern of expression for each group compared to the healthy. The Venn diagram shows the expression of different proteins shared among the different groups, and the specific proteins expressed in each group (Fig. 4B). The Venn diagram shows spectrum of 116 proteins shared between all the groups, 21 proteins shared between healthy and bad prognosis, 12 between healthy and good prognosis, and 8 between good and bad prognosis. Also, a spectrum of proteins that are exclusively expressed in each group, those are in healthy 48 proteins, 492 proteins in good prognosis group, and 17 proteins in patients with bad prognosis. The figure contain the protein accession numbers (except for the 492 proteins), and we have highlighted (in red) some of these proteins for their role in kidney transplant and summarized them in Table 3, which contain the protein accession number, the name of the protein, protein function, the protein role in kidney transplant as recorded in the literature with the reference number.

4. Discussion

Circulating microvesicles represent a unique source of biomarkers for different diseases. Our data shows that microvesicles may have the potential to predict the outcome of the kidney transplant in a simple non-invasive procedure. Thus, microvesicles may provide a suitable substitute for the current methods used to predict the outcome of a kidney transplant. Current methods lack predictive value and identify existing pathological changes representing injury that has already occurred [1–4]. Our study shows that circulating microvesicles of kidney transplant recipients have expression levels of many proteins that differ from healthy subjects. Furthermore, microvesicles could discriminate between the transplant recipients with different transplant prognoses based on slope of eGFR. Proteomic analysis showed that microvesicles contain proteins that play vital role in signaling pathways related to kidney function and pathology. The protein clusters in Fig. 1 and the ones summarized in Table 1 represent layers of prognostic and predictive markers, which upon validation may provide a simple, non-invasive tool to follow-up the kidney transplants and predict their outcomes. Such a tool could provide a monitoring system for the progression of the pathological status of kidney transplants in a nearly

real-time manner, which in turn would enable a timely therapeutic intervention. The protein groups in Fig. 4 and Table 3 clearly demonstrate the power of blood MV to predict the outcome of kidney transplants after six months from the transplant.

Proteins that are involved in complement activation, coagulation cascades, PPAR, TGF-β, and HIF-1 signaling pathways represent key players in kidney pathology. Such proteins not only could provide predictive value as in this work but upon further validation they might provide mechanistic insights into the role of microvesicles in the pathophysiology of the kidney transplant [45–47].

Our study has certain strengths and limitations. We have selected patients from a single center, well-characterized, prospective transplant cohort. Our study is clearly preliminary and more samples and more time points, along with a validation cohort, will be required to confirm these observations. Despite these limitations, our data sheds a spotlight on the potential of circulating microvesicles to provide a simple non-invasive procedure to predict the outcomes of kidney transplants. This method could enable healthcare providers to follow up on kidney transplant outcome to deliver a suitable intervention in a timely manner.

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