Mass spectrometry as a novel method for detection of podocyturia in pre-eclampsia

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ABSTRACT

Background. Podocyturia, i.e. urinary loss of viable podocytes, may serve as a diagnostic tool for pre-eclampsia and as a marker of active renal disease. The current method to detect podocyturia is technically complex, lengthy and requires a high level of expertise for interpretation. The aim of this study was to develop a new technique for the identification of urinary podocytes, based on the detection of podocyte-specific tryptic peptides by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), which will provide an operator-independent and highly reproducible method.

Methods and Results. The diagnosis of pre-eclampsia was confirmed in the presence of hypertension (>140/90 mmHg) and proteinuria (>0.3 g/24 h urine). The diagnosis of HELLP was confirmed based on the accepted clinical criteria of hemolysis, elevated liver enzymes and low platelet count.

Random urine samples within 24 h prior to delivery were collected and centrifuged. One half of the sediment was cultured for 24 h to select for viable cells and then stained with a podocin antibody, followed by a secondary fluorescein isothiocyanate-labeled antibody to identify podocytes. The second half of the pellet was solubilized, digested and analyzed by LC-MS/MS using an internal standard. We have recruited 13 patients with pre-eclampsia and 6 patients with pre-eclampsia/HELLP syndrome. The presence of podocytes was confirmed in all patients by the podocyte culture method. In the respective samples, the presence of a podocin-specific tryptic peptide was confirmed with LC-MS/MS technology.

Conclusion. The LC-MS/MS method is a reliable technology for the identification of urinary podocytes, based on the presence of podocyte-specific proteins in the urine.

INTRODUCTION

Pre-eclampsia is a pregnancy-specific disorder, clinically characterized by hypertension (≥140/90 mmHg) and proteinuria (≥300 mg in a 24-h urine sample) [1]. Accumulating evidence suggests that endothelial dysfunction, caused by dysregulation of angiogenic factors, may play a central role in the pathogenesis of pre-eclampsia [2], but the underlying signaling mechanisms that lead to proteinuria are not well understood. Specifically, it is unclear how endothelial dysfunction translates into dysregulation of the glomerular epithelial cells, podocytes, which play a major role in maintaining the selective permeability of the glomerular capillary wall. The foot processes of the neighboring podocytes interdigitate and connect by specialized cell-to-cell junctions, also known as glomerular slit diaphragms, which provide the main size-selective filtration barrier in the kidney [3]. Several proteins that localize either to the slit diaphragm or foot process cytoskeleton have been identified, including nephrin [4], podocin [5], synaptopodin [6] and podocalyxin [7]. These proteins, through
complex interactions, maintain the structural and functional integrity of the slit diaphragm.

Both experimental and clinical studies support the role of podocyte damage and ultimate loss in the development of proteinuria. Podocyte loss due to apoptosis has been demonstrated in experimental [8] and human glomerular diseases [9]. Another plausible mechanism is detachment of podocytes from the glomerular basement membrane resulting in urinary loss of viable podocytes, i.e. podocyturia. With the acquisition of a mature phenotype, podocytes develop a highly differentiated cytoarchitecture and lose their mitotic activity [3]. Therefore, the loss of a critical number of podocytes may well impair the filtration barrier and subsequently proteinuria may ensue [10]. Urinary podocyte loss, which has been suggested to be a more specific marker of ongoing glomerular damage than proteinuria, has been demonstrated in experimental membranous nephropathy [11] and in the urinary sediments of patients with a variety of active glomerular diseases [10], including pre-eclampsia [12]. Despite increasing evidence suggesting that podocyturia may serve both as a reliable diagnostic tool for pre-eclampsia [13–15] and as a marker of the active phases of other proteinuric diseases, reservations regarding the clinical utility of this test exist, mainly due to its technical complexity, length of time to obtain results and the level of expertise and training required for interpretation of the results [16].

The aim of this study was to develop a new technique to identify podocyturia, which would be an operator-independent and highly reproducible method that would facilitate future studies of renal injury in proteinuric renal diseases, including pre-eclampsia. We report a new technique for the identification of urinary podocytes, based on the detection of a podocin trypic peptide by high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (LC/MS/MS), in urine samples from women with pre-eclampsia. This methodology is based on the principle that tryptic peptides may serve as surrogate markers for the quantification of their respective proteins [17]. The previously described urinary podocyte culture method [12] served as the gold standard by which the presence of podocytes in the respective urinary samples was confirmed.

**MATERIALS AND METHODS**

**Methods**

**Diagnostic criteria for pre-eclampsia.** This study was approved by the Mayo Clinic Institutional Review Board, and all participating women were consented before inclusion in the study. The diagnosis of pre-eclampsia was ascertained based on the presence of the following criteria [1]: (i) hypertension after 20 weeks’ gestation, defined as a blood pressure ≥140/90 mmHg, (ii) proteinuria, defined as ≥300 mg of protein in a 24-h urine specimen and/or 1+ (30 mg/L) dipstick urinalysis, in the absence of urinary tract infection and (iii) resolution of hypertension and proteinuria by 12 weeks post-partum. The diagnosis of HELLP (an acronym that stands for hemolysis, elevated liver enzymes and low platelet count), a severe form of pre-eclampsia, was confirmed by the presence of microangiopathic hemolytic anemia, elevated liver enzymes and thrombocytopenia, according to previously accepted diagnostic criteria [18]. Random urine samples (100–150 mL each) were obtained from 13 pre-eclamptic and 6 pre-eclamptic/HELLP syndrome consecutive patients who agreed to participate in the study (Table 1). Urine samples were used both for the podocyturia assay and LC-MS/MS technology (n = 15) and for LC-MS/MS technology only (n = 4). For the controls, urine was obtained from four normotensive consecutive pregnant women at the time of delivery.

**Podocyturia assay.** Random urine samples (∼50 mL each) were centrifuged for 8 min at 700 g at room temperature and processed as previously described [12]. Briefly, 1-mL aliquots were plated on collagen-coated tissue culture slides, followed by overnight incubation at 37°C in 5% CO₂. The next day, slides were fixed with 1 mL of ice-cold methanol. Each slide was incubated with a podocin antibody (1:200, Sigma). After washing with phosphate-buffered saline, a secondary fluoroscein isothiocyanate-labeled antibody was added at a dilution of 1:40 for 30 min. The sediment was counterstained with Hoechst nuclear stain to facilitate the differentiation of whole cells from cell fragments. Nucleated, positive staining cells were considered to be podocytes. Podocyturia was expressed as a ratio of the number of podocytes to the creatinine content of the respective urine sample and was confirmed in the presence of ≥0.85 podocin-positive cells per milligram creatinine (Table 1); this threshold value has been previously determined to provide 100% sensitivity and specificity of the method in the diagnosis of pre-eclampsia [12].

**LC-MS/MS technology: materials and methods.** Recombinant human podocin was obtained from Novus Biologicals. The synthetic stable isotope-labeled peptide, with the same sequence as the podocin trypic peptide, was synthesized by the peptide synthesis facility at the Mayo Clinic, Rochester, MN. Random urine samples (∼50 mL each) were centrifuged for 8 min at 700 g at room temperature. The supernatant was discarded and the pellet was re-suspended in methanol fixative and stored at 4°C. Prior to digestion, the methanol-fixed pellets were centrifuged at 600 g for 10 min, the supernatant was removed and the pellet was re-suspended in 50 µL of RapiGest™ SF at a concentration of 0.1% in 50 mM ammonium bicarbonate, pH 8.0. The sample was sonicated for 5 min, then 100 µg of trypsin was added and the sample was sonicated again for 5 min. The sample was then digested in a shaking incubator set at 37°C for 4 h. After digestion, the sample was acidified with 2 µL of formic acid and centrifuged for 10 min at 14 000 g. A volume of 18 µL of patient digest was put into a well of a 96-well sample tray. A stable isotope-labeled internal standard peptide was added to each sample and then analyzed by LC-MS/MS.

**LC-MS.** All samples were analyzed using a Thermo LTQ-MS system coupled to an ABSciex API 5000 triple quadrupole mass spectrometer. A 20 µL injection was made from each sample and separations were carried out on a
Recently grown in popularity as there is a need for absolute quantification of proteins using the sensitivity and specificity of LC-MS/MS [19-21].

The choice of podocin in this study was based on the fact that it is highly podocyte-specific [16], and that, in the previous study, podocin exhibited 100% sensitivity and specificity in the diagnosis of pre-eclampsia [12]. Also, its respective recombinant protein is commercially available. The tryptic peptide, QEAGPEPSGSR, was chosen as the best peptide for quantifying podocin from urine for the following reasons: this sequence is unique to human podocin and gave the best response of all of the podocin tryptic peptides in a digest of recombinant podocin, as measured by LC-MS/MS. The identity of the peptide was confirmed by full scan MS/MS and by standard addition of recombinant podocin digests to the frozen cell digests. The reproducibility of the digestion protocol was examined using frozen mouse podocytes as a surrogate matrix. Conditionally immortalized mouse podocytes were a generous gift from Dr Peter Mundel. In this experiment, differentiated podocytes were used, by changing from permissive to non-permissive conditions, as previously described [22]. All digests contained the same volume of frozen mouse podocytes that were thawed and solubilized in RapiGest™ buffer. Figure 1 shows LC-MS/MS chromatograms specific to the human podocin tryptic peptide, QEAGPEPSGSR, digested in the mouse podocyte matrix. The chromatogram in Figure 1A shows the control mouse podocyte digest without recombinant human podocin added prior to digestion, and Figure 1B shows the same mouse podocytes spiked with recombinant human podocin prior to digestion. Figure 1B clearly shows the highlighted peak representing the tryptic peptide, QEAGPEPSGSR, from the recombinant human podocin spiked into the mouse podocytes. The response for this peptide is at the same retention time as the internal standard added to the sample after digestion and before injection (chromatogram

### RESULTS

**Tryptic peptide from podocin**

Applied to our study, LC-MS/MS methodology assumes a 1:1 stoichiometric ratio between the tryptic fragment isolated from podocin and the concentration of intact podocin in the sample. Adding a known amount of stable isotope-labeled internal standard peptide with the same amino acid sequence as the tryptic peptide, but with a different mass, allows the mass spectrometer to distinguish between the two forms and thus provide relative quantification. The technique has recently grown in popularity as there is a need for absolute quantification of proteins using the sensitivity and specificity of LC-MS/MS [19-21].

### Statistical methods

Statistical analyses were performed using JMP version 7.0.0 (SAS Institute Inc., Cary, NC). All data are expressed as mean values ± SD and compared using the Wilcoxon rank-sum test. For correlation studies, a two-tailed probability value of a P-value of <0.05 was pre-specified as being statistically significant.

### Table 1. Demographic and clinical data of pre-eclamptic (n = 19) and normotensive pregnant patients (n = 4) who underwent podocyturia studies

<table>
<thead>
<tr>
<th></th>
<th>Pre-eclamptic pregnancies, mean ± SD</th>
<th>Normotensive pregnancies, mean ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>30.5 ± 7.1</td>
<td>29.7 ± 0.6</td>
<td>1</td>
</tr>
<tr>
<td>Days of gestation</td>
<td>235.6 ± 24.3</td>
<td>280.3 ± 8.7</td>
<td>0.0086</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2.12 ± 1.5</td>
<td>1.33 ± 0.57</td>
<td>0.3996</td>
</tr>
<tr>
<td>Parity</td>
<td>0.81 ± 1.12</td>
<td>0.33 ± 0.58</td>
<td>0.5379</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>154.4 ± 11.35</td>
<td>118.3 ± 8.02</td>
<td>0.0085</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>93.3 ± 13.4</td>
<td>68 ± 3.46</td>
<td>0.0139</td>
</tr>
<tr>
<td>Protein/creatinine ratio (grams/24 h urine)</td>
<td>3.896 ± 3.22</td>
<td>0.109 ± 0.034</td>
<td>0.0086</td>
</tr>
<tr>
<td>Podocyturia (number of podocytes/mg creatinine)</td>
<td>3.1 ± 2.2</td>
<td>Absent</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*In addition to the diagnostic criteria for pre-eclampsia, six patients had laboratory abnormalities characteristic of HELLP syndrome: hemolysis, with a mean lactate dehydrogenase of 350 ± 32 (normal, 122–222 U/L); elevated liver enzymes, with a mean aspartate aminotransferase of 188 ± 102 (normal 8–43 U/L) and a low platelet count, with a mean platelet count of 67 ± 34 (normal 150–450 × 10⁹/L).*

100 × 3.0 mm Atlantis T3 column, with a 3 μm particle size and 120 Å pore size, run at a flow rate of 250 μL/min. A gradient consisting of mobile phase A (100% water and 0.1% formic acid) and mobile phase B (100% acetonitrile and 0.1% formic acid) was used to resolve the peptides with a 15 min gradient. All MS/MS conditions were optimized by infusing the synthetic stable isotope-labeled podocin tryptic peptide, 39QEAGPEPSGSR50. The transition that gave the best signal-to-noise was the doubly charged precursor ion to the singly charged y6 ion i.e., [M + 2H+]+2 → y61+.

**PRELIMINARY COMMUNICATION**

Response of HIV-associated kidney disease to ART

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Table 2. Peak areas for the tryptic peptide and the internal standard for each of the three replicates of the control mouse podocyte digest spiked with the recombinant human podocin

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Analyte peak area (counts)</th>
<th>Internal standard peak area (counts)</th>
<th>Area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse podocyte digest Spike 1</td>
<td>3290</td>
<td>159 000</td>
<td>0.021</td>
</tr>
<tr>
<td>Mouse podocyte digest Spike 2</td>
<td>2890</td>
<td>153 000</td>
<td>0.019</td>
</tr>
<tr>
<td>Mouse podocyte digest Spike 3</td>
<td>3210</td>
<td>155 000</td>
<td>0.021</td>
</tr>
<tr>
<td>Average (mean)</td>
<td>3130</td>
<td>155 667</td>
<td>0.020</td>
</tr>
<tr>
<td>SD</td>
<td>212</td>
<td>3055</td>
<td>0.013</td>
</tr>
<tr>
<td>% Coefficient of variation</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

(FIGURE 1: LC-MS/MS chromatograms: Multiple reaction monitoring transition for the tryptic peptide from podocin for a digest of immortalized mouse podocyte cell line without [Control: (A)] and with recombinant human podocin added [Spike: (B)].

(FIGURE 2: LC-MS/MS chromatograms: Multiple reaction monitoring transition for the tryptic peptide from podocin for a digest of fixed cells from the urine of a patient with pre-eclampsia on the top (with a calculated tryptic peptide concentration of 3.4 fmol/mg creatinine) and the peak from the internal standard at the bottom.)

not shown). Table 2 shows the peak areas for the tryptic peptide and the internal standard for each of the three replicates, along with their respective coefficient of variation values. The results suggest that the method is reproducible using the frozen mouse podocyte matrix.

All 15 urine samples obtained from pre-eclamptic patients demonstrated the podocin tryptic peptide of interest. Figure 2 shows an LC-MS/MS chromatogram representative of the results found using cells from a patient classified as having pre-eclampsia: the highlighted podocin tryptic peptide peak from the pre-eclampsia patient on the top and the peak from the internal standard at the bottom. The figure demonstrates that the tryptic peptide derived from podocin in the patient sample has the same retention time as the synthetic stable isotope-labeled internal standard peptide. In pre-eclamptic women, a positive correlation was present between the quantity of the podocin peptide in their urines and both the amount of proteinuria in the respective urine samples ($P = 0.03$) and their systolic blood pressures at the time of urine collection ($P = 0.001$). The presence of podocytes in the respective urine samples at the time of delivery was further confirmed by the overnight podocyte culture method.
(Figure 3), indicating the presence of ≥0.85 podocin-positive cells per milligram creatinine in all tested samples [12]. At the 6-week post-partum visit, podocyturia was absent in all women in whom it was present at the time of delivery.

In the final step, we collected urinary samples from four patients with normotensive pregnancies and from four patients with pre-eclampsia, and each whole urine sample was used for the detection and quantification of the podocyte tryptic peptide of interest. The quantification of the podocin peptide was performed by a single-point calibration (using a known amount of the internal standard, expressed in femt mole units, that was added to each sample) and expressed in femt mole of podocin per milligram of creatinine in the respective samples. Podocin tryptic peptide from fixed cells taken from normotensive pregnant patients’ urines, as compared to pre-eclampsia patients’ urines, showed a significantly lower response: 0.4 ± 0.04 versus 4.6 ± 2.3 fmol/mg creatinine, respectively, P = 0.01 (Figure 4). The reproducibility of fixed cell digests was evaluated by using solubilized fixed cells from a single patient, splitting the sample three ways, then performing the digestion and LC-MS/MS analysis. The results, presented in Table 3, demonstrate that the method is reproducible for fixed cells derived from patient urine.

**DISCUSSION**

This study validates a new method for the detection of podocytes in the urine from women with pre-eclampsia, based on the detection of a podocin tryptic peptide by the LC/MS/MS technology. The presence of podocytes was inferred by the detection of a tryptic peptide of the podocyte-specific protein, podocin, by LC-MS/MS in a reproducible and operator-independent manner. The presence of this peptide correlated with the urinary excretion of viable podocytes, as confirmed by culturing urinary sediment and staining for podocyte-specific proteins. We suggest that the use of LC-MS/MS technology to confirm podocyturia is a new, observer-independent and highly reproducible technique, which may facilitate the application of podocyturia both as a diagnostic test and as a research tool.

Using a tryptic peptide as a surrogate marker for quantifying proteins has been in place since Barr et al. [17] described the process in 1996. The basic principle of this technology is the use of a recombinant internal standard, which, except for mass, is identical to the native protein. The concentration of the protein of interest is determined by comparing the ratio of a tryptic digest peak area in a patient sample versus that of the internal standard of a known concentration. This approach has been used successfully for the quantification of several proteins. One notable example is urinary albumin [23], where the application of LC-MS/MS technology has resulted in the

![Figure 3: Podocyturia assay: Hoechst nuclear stain (blue); podocin antibody followed with a secondary, fluorescein isothiocyanate-labeled antibody (green).](image)

![Figure 4: LC-MS/MS chromatograms from fixed cells: normotensive pregnant patient (top), pre-eclamptic patient (bottom), with a calculated tryptic peptide concentration of 0.42 versus 5.4 fmol/mg creatinine, respectively.](image)

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Analyte peak area (counts)</th>
<th>Internal standard peak area (counts)</th>
<th>Area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient fixed cell Digest 1</td>
<td>921</td>
<td>220 000</td>
<td>4.2E-03</td>
</tr>
<tr>
<td>Patient fixed cell Digest 2</td>
<td>777</td>
<td>173 000</td>
<td>4.5E-03</td>
</tr>
<tr>
<td>Patient fixed cell Digest 3</td>
<td>947</td>
<td>201 000</td>
<td>4.7E-03</td>
</tr>
<tr>
<td>Average (mean)</td>
<td>882</td>
<td>198 000</td>
<td>4.46E-03</td>
</tr>
<tr>
<td>SD</td>
<td>92</td>
<td>23 643</td>
<td>0.25E-03</td>
</tr>
<tr>
<td>% Coefficient of variation</td>
<td>10</td>
<td>12</td>
<td>6</td>
</tr>
</tbody>
</table>
of developing a highly sensitive assay, which allows for urinary albumin quantification in the normo-albuminuric (i.e. <30 mg in 24 h urine) range. These studies have suggested that LC-MS/MS technology may be useful in identifying the urinary markers of renal disease and set the stage for our studies of the detection of podocyte-specific tryptic peptides as indicators of ongoing podocyturia.

We opted to study podocyturia by LC-MS/MS in patients with pre-eclampsia and not in those with main glomerular diseases, such as focal glomerulosclerosis, for the following reasons. It has been previously postulated that proteinuria is present in both active and chronic phases of glomerular damage, while podocyturia seems to be confined to active disease only. In contrast to the natural progressions of most glomerular diseases, which are characterized by remission/relapse cycles, proteinuria in pre-eclampsia happens suddenly, typically after 20 weeks of gestation, and resolves after delivery, usually by 12 weeks post-partum. We postulated that the acuteness of the development of proteinuria in pre-eclampsia, and its known clinical course, offers a unique clinical scenario and the opportunity to study the role of podocyturia in acute proteinuric renal injury. In addition, we have reported previously that the urinary excretion of viable podocytes, i.e., podocyturia, was present in all patients with pre-eclampsia and was absent in all normotensive controls [12]. Our findings of urinary loss of viable podocytes in pre-eclamptic pregnancies have been confirmed independently by three groups: by the presence of messenger RNA transcripts of nephrin and podocin [14] and by the presence of cells that stain positive for podocalyxin [13] or nephrin [15]. Of note, urinary excretion of viable podocytes has been demonstrated in the urinary sediments of patients with a variety of glomerular diseases, including diabetic nephropathy [24], lupus nephritis [25] and focal segmental glomerulosclerosis [10], but mainly during the active phases of the disease processes. Therefore, we considered the high-yield urine samples, with respect to podocyturia from patients with pre-eclampsia, as the optimal means by which to develop new methodologies for detection of urinary podocytes, which will be applied to studies of other proteinuric renal disease in the future.

The research and clinical applications of podocyturia have been limited by the performance characteristics of the available methodology, which requires the overnight culture of urinary sediment, followed by staining for podocyte-specific proteins and final interpretation of the results by an individual who requires special training [16]. Moreover, there are additional limitations of the current podocyturia assay that significantly restrict its use in animal experiments. First, the relatively small urine samples obtained from animals do not allow for the creation of more than one slide, thus staining of only one podocyte-specific protein. Second, as the current technology identifies viable podocytes, i.e. those that are able to attach to collagen-plate slides, samples (i.e. urinary sediment) need to be processed within 3 h and cannot be stored. LC-MS/MS technology will allow for the simultaneous processing of several different podocyte proteins in relatively small urine samples. Also, the samples can be stored and re-analyzed, thus decreasing the need to repeat animal experiments. This technology may be a useful novel tool to:

- Study the mechanisms that regulate podocyte attachment in the animal models of proteinuric renal diseases, including pre-eclampsia. These studies may not only provide information regarding the signaling pathways that underlie podocyte detachment and urinary loss but may also provide novel therapeutic targets.
- Assess disease activity, disease type and response to treatment in other proteinuric renal diseases.

The major limitation of our study is the small sample size and the evaluation of only a single podocin-specific protein, podocin. Despite these limitations, our data demonstrate that using tryptic digestion of cells coupled with LC-MS/MS highly correlates with the presence of cultured podocytes, and provides an operator-independent, reliable and reproducible method, and is a viable approach to identifying podocytes in the urine. At this time, a larger sample set of patients with pre-eclampsia and those with normal pregnancies is needed to establish a normal range for podocin in urine and to determine whether the presence of either this tryptic peptide, other podocin-specific tryptic peptides or other podocyte-specific proteins (such as nephrin, synaptopodin and podocalyxin) may differentiate between normotensive and pre-eclamptic pregnancies. It would be of particular interest to study the podocyte protein, podoplanin, as its altered distribution has been associated in pre-eclampsia with the disturbed expressions of two polarity proteins [15], which may contribute to podocyte detachment and consequent podocyturia. These studies are the focus of our current research efforts. We also plan to expand our experiments to clinical studies and animal models of proteinuric renal diseases, including pre-eclampsia. In addition, the presence of podocin may indicate the presence of not only podocytes but parietal epithelial cells as well as a readout for parietal cell activation and ongoing regeneration [26]. In future experiments, we plan to address this possibility by co-staining cultured cells with PGP9.5, a marker of parietal epithelial cells, and CK8-18, a cytokeratin, which is not regularly expressed in mature podocytes.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

Dr V.D.G. is the inventor of technology used in this research; the technology has been licensed to a commercial entity; Dr V.
D.G. and Mayo Clinic have contractual rights to receive royalties from the licensing of this technology.

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