

# **Identification of $\beta$ 2-microglobulin as a urinary biomarker for chronic allograft nephropathy using proteomic methods.**

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**Running title:**  $\beta$ 2 microglobulin and chronic allograft nephropathy

**Abbreviations:** CAN (chronic allograft nephropathy), ROC (receiver operator characteristic), SELDI-TOF MS (surface enhanced laser desorption and ionisation time of flight mass spectrometry)

**Keywords:** CAN,  $\beta$ 2-MICROGLOBULIN, SELDI-TOF MS, Biomarkers, Renal Transplantation

**Clinical Relevance:**

There is a significant clinical need to identify renal transplant patients at risk of developing CAN, as it causes between 50-80% of graft losses after the first year. This study has provided a potential non-invasive protein biomarker,  $\beta$ 2-microglobulin, which could be used to detect and diagnose CAN at an early stage ensuring better healthcare provision for patients with CAN.

## **Abstract**

**Purpose:** Chronic allograft nephropathy (CAN) remains the leading cause of renal graft loss after the first year following renal transplantation. This study aimed to identify novel urinary proteomic profiles, which could distinguish and predict CAN in susceptible individuals.

**Experimental Design:** The study included 34 renal transplant patients with histologically proven CAN and 36 patients with normal renal transplant function. High-throughput proteomic profiles were generated from urine samples with three different ProteinChip arrays by surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). Following SELDI a biomarker pattern software analysis was performed which led to the identification of a novel biomarker pattern that could distinguish patients with CAN from those with normal renal function.

**Results:** An 11.7 kDa protein identified as  $\beta$ 2 microglobulin was the primary protein of this biomarker pattern, distinguishing CAN from control patients (ROC = 0.996). SELDI-TOF-MS comparison of purified  $\beta$ 2 microglobulin protein and CAN urine demonstrated identical 11.7 kDa protein peaks. Significantly higher concentrations of  $\beta$ 2 microglobulin were found in the urine of patients with CAN compared to the urine of normal renal function transplant recipients ( $p < 0.001$ ).

**Conclusions and clinical relevance:** Whilst further validation in a larger more diverse patient population is required to determine if this  $\beta$ 2 microglobulin protein biomarker will provide a potential means of diagnosing CAN by non-invasive methods in a clinical setting, this study clearly shows a capability to stratify control and disease patients.

## 1 Introduction

Chronic allograft nephropathy (CAN) is the principle cause of late graft loss after the first year of renal transplantation (1), accounting for 50-80% of graft losses after this time (2). The reasons for this are multi-factorial including immune and non-immune factors e.g. acute rejection (3,4), delayed graft function (5) and acute calcineurin-inhibitor toxicity (6,7). Also, despite the significant improvement in the rate of acute renal rejection over the last decade (8), CAN remains the leading cause of late graft lost after renal transplantation (9).

At present, a histological diagnosis with a renal transplant biopsy is the 'gold standard' for determining CAN. CAN is a descriptive term for histological lesions in a renal allograft that include atherosclerosis, glomerulosclerosis, interstitial fibrosis, and tubular atrophy (10). This has been a challenging disorder to diagnose by alternative and less invasive means in so far as it has a multi-factorial aetiology (11). As in the case of acute renal transplant rejection, there is growing consensus that such complex and heterogeneous processes could best be fingerprinted using a pattern of collectively and individually informative biomarkers (12).

Proteomic approaches have recently been employed towards this end. Surfaced-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) is a technique that addresses some of the limitations of both two-dimensional gel electrophoresis (2-DE) and liquid chromatography mass spectrometry (LC-MS). This SELDI approach allows for high-throughput profiling of multiple clinical samples. Multiple studies have revealed the feasibility of developing this technology for biomarker discovery (13) for the early detection of diseases including ovarian cancer (14,15), breast cancer (16) and prostate cancer (17,18). Three separate investigative groups have recently described changes in urinary protein excretion in transplant patients with biopsy-proven acute rejection (19,20,21). However, to our knowledge there have been no published studies investigating CAN using SELDI-TOF-MS (22,23).

Although any biologic fluid may be examined in the search for biomarkers by SELDI-TOF-MS (24,25), the overriding principle is the simplicity of obtaining such a material for diagnostic purposes. In the case of CAN, the obvious choice of diagnostic

material is the urine, a biologic fluid that potentially is most reflective of pathologic events that take place in the kidney. A non-invasive biomarker of rejection may benefit the kidney allograft recipient by allowing frequent monitoring to optimise immunosuppressive therapy.

Using SELDI to determine the urinary protein profiles associated with CAN, it could be possible to predict individuals susceptible to its development by non-invasive means compared to the very invasive current investigative tool of the renal biopsy (26). Therefore, the overall purpose of this study was to examine urine from renal transplant patients with documented CAN and also normal renal function using proteomic techniques in order to identify potential novel biomarker patterns for detecting CAN. Identification of non-invasive biomarkers or a biomarker pattern of CAN would benefit the kidney allograft population by allowing frequent monitoring to optimise immunosuppressive therapy thus preventing disease progression.

## 2 Materials and methods

### 2.1 *Study population and sample collection:*

This study was approved by the Beaumont hospital ethics in medical research committee for studies involving human subjects. All renal transplant patients attending the renal transplant clinic at Beaumont Hospital, Dublin between July 2004 and April 2005, who complied with the inclusion criteria detailed below, were asked to enrol in the study with informed consent. Inclusion criteria included first kidney transplant patients,  $\geq 18$  years, transplanted for at least one year. Patients with a urinary tract infection were excluded from the study. The patients were selected by a combination of hospital chart review; histology reports review and then by interviewing each patient in the clinic setting at the National Renal Transplant Centre, Beaumont Hospital. Protocol biopsies were not carried out at our centre and therefore renal biopsies were carried out 'for cause' only. For this reason a surrogate 'renal function' was required for the control group. Thus there were no Banff scores for the normal patients. The 'control' group included 36 renal transplant patients with normal renal function (serum creatinine  $< 140 \mu\text{Mol/L}$ ; glomerular filtration rate (GFR)  $> 50 \text{ ml/minute}$ ) and the 'disease' group included 34 renal transplant patients with histologically proven CAN (according to Banff criteria, serum creatinine  $> 140 \mu\text{Mol/L}$ ; glomerular filtration rate (GFR)  $< 45 \text{ ml/minute}$ ) more than one year after transplantation. An extensive search was carried out on the results of renal transplant biopsies performed at Beaumont Hospital between 1987 and 2005 and those patients diagnosed with CAN as a result of a renal transplant biopsy (by Banff 1997 criteria (10)) were identified and asked to participate in the study if they met the inclusion criteria.

Clinical and historical data were documented for each patient including age, gender and immunosuppression regimen which typically included a calcineurin inhibitor (cyclosporine with trough level 50 to 150 ng/ml or tacrolimus with trough level 5 to 10 ng/ml or sirolimus with trough level 10 to 15 ng/ml), an antimetabolite (either azothioprine or mycophenolate mofetil) and prednisolone.

All patients completed a consent form for urine collection and for release of clinical data in accordance with the Control of Clinical Trials Act 1987 and 1990 and the European Guidelines of Good Clinical Practice and the protocols. Mid-stream urine

specimens were collected from each patient after urinalysis confirmed the absence of a urinary tract infection. Samples were immediately placed on ice and transported to the laboratory in less than 4 hours (19,27). The urine samples were centrifuged at 4000G for 10 minutes at 4 °C and supernatants were immediately frozen in liquid nitrogen prior to storage at -80 °C until further analysis.

## 2.2 *Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS):*

Urine samples were thawed on ice, centrifuged for 10 minutes at 4000G and protein concentrations were estimated by the bicinchoninic acid kit (BCA) method (Pierce). 160 µl of the urine sample was added to 60 µl of U9 buffer (9M urea, 2% CHAPS, and 50 mM Tris (pH9)) to denature the proteins. The samples were applied in duplicate to weak cation exchange (CM10 ProteinChip™), immobilised metal affinity (IMAC30 ProteinChip™) and reverse phase ProteinChip arrays (H50 ProteinChip™) (CIPHERGEN Biosystems, Fremont, CA) according to the manufacturer's protocol. This process has been described previously (19,28, 29).

Peak detection was performed using ProteinChip Software version 3.2 (CIPHERGEN Biosystems); peaks were identified using a signal-to-noise ratio of >5. Baseline subtraction was performed on all spectra, and the data were normalised using total ion current.

## 2.3 *Data analysis:*

The data obtained from the ProteinChip Software was exported into CIPHERGEN Express Software version 3.0 (CIPHERGEN Biosystems). This software generated clustered peaks and analyzed the peak clusters using P-value calculation, receiver operator characteristic (ROC) plots, and principal component analysis (PCA). This cluster data was then exported to Biomarker Pattern Software (BPS) version 5.0.2 (CIPHERGEN Biosystems).

BPS used Classification and Regression Trees (CART) to analyze the proteins and samples with respect to disease background. BPS analysis split each node into a classification tree and assigning each terminal node a predicted value. The splitting decisions were based on the normalised intensity levels (height) of peaks from the SELDI

protein expression profile. The data was analyzed using 10-fold cross-validation, i.e., a model was constructed on 90% of the data and then tested on the remaining 10%. This analysis was performed 10 times.

#### *2.4 Purification of the protein peak clusters:*

Identification of significant proteins of interest in the biomarker pattern required additional protein purification, in order to generate a protein sample of sufficient quantity and purity. The CAN and control samples were separately pooled. The pooled samples then underwent SELDI-assisted anionic fractionation (HyperQ F resin (Biospera)), followed by hydrophobic fractionation (PRC Poly-Bio beads (Biospera)) to separate out the proteins with successively lower pH buffers (increasingly anionic proteins) or successively increasing concentrations of acetonitrile buffers (increasingly hydrophobic proteins). The fraction with the peak of interest of highest intensity was then used for subsequent analysis.

Protein identification involved concentrating and then running the optimum urine fraction on 1-D sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) together with a molecular weight marker and stained with Coomassie Blue stain (Invitrogen). NuPAGE 4-12% Bis Tris prefixed gels (Invitrogen) were used with NuPAGE MES SDS running buffer (MW range 2.5 – 200KDa) (Invitrogen). The band of interest at the appropriate molecular weight was then excised, trypsinised and prepared for further characterisation by mass spectrometry.

#### *2.5 Identification of the protein peak clusters by Liquid Chromatography (LC)*

##### *Tandem mass spectrometry:*

Mass spectrometry was performed on the selected trypsinised gel fragments using a LTQ mass spectrometer (ThermoFinnegan) which was described previously (29). The identified peptides were compared to amino acid sequences from the human IPI protein database (<http://www.ebi.ac.uk/IPI/IPIhelp.html>) (30,31,32).

#### *2.6 Verification of protein peak cluster identity:*

Further confirmation was needed to verify that the purified protein peak identified in the initial study CAN urine samples had exactly the same protein peak by SELDI-TOF-MS as the purified protein. This was performed by comparing a commercially available purified protein and the CAN urine sample on a CM10 ProteinChip.

### *2.7 Quantification of the identified protein:*

Quantification of the identified protein was performed using enzyme immunoassay (ELISA) test kit according to the manufacturer's protocol. Each urine sample was diluted 10-fold with the provided 'sample diluent' before commencement of the ELISA. Statistical analysis of the protein levels in CAN urine and normal renal function urine was performed again using GraphPad Prism 4. An un-paired non-parametric Mann-Whitney test (rank sum test) which did not assume a Gaussian distribution was conducted. Two-tailed p values were calculated with 95% confidence intervals.

### 3 Results

#### 3.1 Patient characteristics:

In this retrospective study, the ‘disease’ group included 34 renal transplant patients with histologically proven chronic allograft nephropathy (CAN) and the ‘control’ group included 36 renal transplant patients with normal renal function (as reflected by the glomerular filtration rate (GFR) > 50 ml/minute and serum creatinine <140 µmol/l). The development of CAN, for the purposes of this study, was represented by a histological diagnosis of CAN from a previous renal biopsy, thus no Banff scores were allocated to the control patients. The demographic details of the patients participating in the study are outlined in Table 1.

As expected, there was a significantly lower glomerular filtration rate (GFR) and a significantly higher serum creatinine in the CAN patients compared to the control patients. The mean serum creatinine in the CAN group was 247.9 µmol/l compared to 115.3 µmol/l in the control group of patients ( $p < 0.0001$ ). The mean GFR in the CAN patients was 37.18 ml/minute as opposed to 74.22 ml/minute in the control group of patients ( $p < 0.0001$ ). The acute rejection rate (biopsy-proven) in the CAN group was 38.25% compared to 5.56% in the control group of patients.

Histologically, 2 out of 34 (5.88%) CAN patients had Banff Grade I on transplant biopsy, 4 out of 34 (14.7%) had Banff Grade II and 27 out of 34 (79.41%) had Banff Grade III diagnosed on transplant biopsy.

#### 3.2 Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS):

A total of 344 protein peaks were identified after SELDI analysis of all samples. The SELDI-TOF-MS spectra of protein peaks, obtained with CM10 ProteinChips at the three molecular weight ranges (described in Figure 1A), showed differential regulation of multiple protein peaks in both the control and CAN patient samples (Figure 1 B-D). Consequently, statistical and bioinformatics analyses were used to determine whether potential biomarkers of CAN were present in the spectra.

### 3.3 *Data analysis:*

Spectra generated by SELDI-TOF-MS were exported to Ciphergen Express Software for normalization, calibration, peak identification and clustering of related peaks. This clustered data was then exported to Biomarker Patterns Software (BPS), which was used to search for significant relationships using Classification and Regression Trees (CART). The classification tree with the greatest specificity and sensitivity was tree number 2 which was chosen for further analysis (Figure 2). This tree contained 7 nodes or proteins, which together made up a biomarker pattern for distinguishing CAN compared from normal transplant controls. This particular tree had a high sensitivity (80.30%) and specificity (67.65%) for predicting CAN compared to normal transplant controls (receiver operating characteristic (ROC) = 0.996). The primary node identified in this tree had a m/z: 11724.0 Da (primary node, and therefore the key protein of the tree).

### 3.4 *Identification of the protein peak clusters:*

Due to the relative importance of this tree in differentiating between CAN and control patients, the 11.7 kDa primary node protein of this tree was chosen as a target for identification. The optimum urine fraction underwent 1-D SDS-PAGE and was stained with Coomassie Blue stain. The band of interest (at 11.7 kDa) was then excised, trypsinised and prepared for mass spectrometry.

This 11.7 kDa band was identified as  $\beta$ 2 microglobulin with an overall probability score using the ProteinProphet software tool of 1. Two unique peptides were identified from the human  $\beta$ 2 microglobulin sequence. The mass spectrum data obtained, together with the peptide sequences and the full protein sequence for  $\beta$ 2 microglobulin are displayed in Figure 3. Attempts were made to identify the other members of the biomarker pattern but isolating sufficient quantity and quality of these proteins proved to be too difficult.

### 3.5 *Verification and quantification of the identified protein:*

Commercially available purified human  $\beta$ 2 microglobulin protein (Serotec) was used to verify that the protein peak identified was indeed  $\beta$ 2 microglobulin. Purified

human  $\beta$ 2 microglobulin applied to a CM10 ProteinChip together with pooled CAN urine and the resultant spectra were compared after SELDI-TOF-MS was performed. Figure 4 A displays that the 11.7 kDa peak identified as  $\beta$ 2 microglobulin by tandem MS, was the 11.7 kDa protein peak seen in the original urine samples.

Using a  $\beta$ 2 microglobulin ELISA, a significant difference in the  $\beta$ 2 microglobulin concentrations was demonstrated between the CAN group and the normal transplant control group as shown in Figure 4 B. The mean  $\beta$ 2 microglobulin concentration for the CAN group was 0.420  $\mu$ g/ml and 0.051  $\mu$ g/ml for the normal transplant control group ( $p < 0.0001$  using the Mann-Whitney test). This almost tenfold increase in  $\beta$ 2 microglobulin levels between patients with normal graft function and patients with CAN is statistically relevant and hence suggests that  $\beta$ 2 microglobulin would be a potentially powerful biomarker for the development of CAN.

## 4 Discussion

Despite improvements in immunosuppressive therapy, long-term allograft survival after kidney transplantation remains low. CAN is a major cause of graft loss in renal transplant recipients. The histopathological indicators of CAN are non-specific. The pathogenesis of CAN is complex and incompletely understood, and involves several immunological and non-immunological factors. The prevention and treatment of CAN need multidisciplinary strategies. To address this fundamental issue this study has identified a novel potential biomarker pattern that can with a high degree of specificity and sensitivity differentiate between CAN in renal transplant recipients from normal transplant controls.

Numerous studies have shown that SELDI-TOF-MS is an accurate reproducible method for high throughput detection of novel proteins in various diseases. Specifically urinary protein biomarkers and biomarker patterns for the diagnosis of acute renal transplant rejection have been demonstrated (33,19,20,21). In fact, Schaub *et al* identified  $\beta$ 2 microglobulin as a potential biomarker for acute renal transplant rejection (33). Several potential biomarkers for CAN have been reported in the past including retinol binding protein (34), transforming growth factor beta (35) and alpha-1 microglobulin (36) but there have not been any urinary biomarker patterns or biomarker proteins identified with SELDI-TOF-MS analysis for CAN.

The optimal biomarker pattern resulted from the CM10 ProteinChip had the highest sensitivity and specificity (ROC 0.996) compared to the biomarker patterns obtained from IMAC30 or H50 ProteinChips (ROC 0.974 and 0.937 respectively). For this reason, the CM10 ProteinChip biomarker pattern was chosen for further characterisation in this study. Importantly even without identifying all of the protein peaks, the classification tree can be used to distinguish CAN from controls with a relatively high degree of certainty. However the identification of these proteins would undoubtedly be advantageous and could potentially provide a better insight into CAN and may be of diagnostic use in a clinical setting. As the principle node of the biomarker pattern tree, the 11.7 kDa M/Z protein was of particular interest. Individually, this protein was a significant disease marker ( $p=0.001$ ; ROC 0.774). By employing several confirmatory experiments including SELDI-TOF-MS of purified  $\beta$ 2 microglobulin which

had an identical protein peak at 11.7 kDa when compared to the urine of CAN patients, the 11.7 kDa protein was identified as  $\beta$ 2 microglobulin. Significantly higher concentrations of  $\beta$ 2 microglobulin were observed in the urine of patients with CAN compared to controls.

$\beta$ 2 microglobulin is a 99 amino acid protein and is found on the cell surface of all nucleated cells. On average, 150-250 mg/day of  $\beta$ 2 microglobulin is produced in normal individuals (37). Normally,  $\beta$ 2 microglobulin is shed from the cell surface, circulates in the serum (98% as a free form) and this serum level depends on glomerular function. Most free  $\beta$ 2 microglobulin is filtered by the glomeruli and more than 99.9% is reabsorbed by the proximal tubular cells where it is degraded into peptides or amino acids by lysosomes before reuptake into the circulation (38). In healthy people, <0.2 mg/l of  $\beta$ 2 microglobulin is excreted daily in the urine in the presence of normal proximal tubular function (39).

Thus,  $\beta$ 2 microglobulin is a good urinary biomarker to assess proximal tubular function.  $\beta$ 2 microglobulin has been identified previously as a marker of other diseases e.g. tubulointerstitial disease (40), acute renal transplant rejection (33), drug toxicity (41), autoimmune diseases (42,43) and lymphoproliferative diseases (44,45). As  $\beta$ 2 microglobulin is a good urinary biomarker to assess proximal tubular function, this may explain why along with the accompanying biomarker tree it is a significant biomarker in this study due to the tubulointerstitial injury present in CAN. However, this study has some limitations, protocol renal transplant biopsies were not carried out at our centre and therefore the number of CAN patients in the study with mild (Banff Grade 1) CAN was very small. A limitation of this study is that more control groups were not included, e.g. patients with urinary tract infections, acute tubular necrosis (20). However such comparison groups have transient or acute urinary changes which would not have been captured with the retrospective methodology of our study. A larger cohort of patients would be required to confirm whether  $\beta$ 2 microglobulin is specific for CAN or simply indicates tubular injury. An expanded study would provide insight into whether the detection of biomarkers such as  $\beta$ 2 microglobulin would be clinically relevant in the diagnosis of CAN.

To summarise, this study has identified a novel urinary biomarker,  $\beta$ 2 microglobulin, that can differentiate between transplant patients with CAN and those with normal renal function. We have also identified a key protein of this classification tree as  $\beta$ 2 microglobulin. Further work is required to validate these markers in a more diverse patient population, in order to determine their true clinical diagnostic and prognostic value.

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**Conflict of Interest:**

The authors have no conflict of interest to declare.

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### **Table and figure legends**

**Table 1:** Demographical data of patients in the study. Both CAN (disease) and control patient demographic data including age, sex, disease aetiology, and transplantation drug regimen is represented in this table. Characteristics represented as whole numbers and as a percentage of the respective sub-populations (disease or control). FSGS= focal segmental glomerulosclerosis; SLE= systemic lupus erythematosus; ANCA= anti-neutrophil cytoplasmic antibody; cyclosporine → sirolimus means that these patients were initially taking cyclosporine and then were changed to sirolimus.

**Figure 1:** Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry conditions including laser intensity and optimised MW range (Figure 1 A).

Representative SELDI-TOF mass spectra comparing CAN urine to control urine are shown at three different molecular weight (MW) ranges on CM10 ProteinChips (Figure 1 B-D). Mass-to-charge ratio ( $m/z$ ) is along x – axis and relative intensity is along y – axis. The corresponding computer-generated gels are views of the same spectra (right).

**Figure 2:** Optimal biomarker pattern tree for urine on **CM10** ProteinChip™ type and Receiver Operator Characteristic curve. C011724 indicates a protein/peptide of M/Z of 11724 Da in the low intensity range. C0 = low intensity; C1 = mid intensity and C2 = high intensity. The trees are made up of a primary node (node one) and child nodes that are descendants of the primary node. The primary node of this tree is a protein cluster of molecular weight 11.7 KDa. The receiver operator characteristic for this patten is highly significant in that it predicts CAN (0.996).

**Figure 3:** Identification of  $\beta 2$  microglobulin. Two high-scoring tryptic peptides (A) were matched to the human sequence of  $\beta 2$  microglobulin (B) using proteomic and mass spectrometry analysis platforms SEQUEST and Protein Prophet. A representative spectrum of the  $[M+2H]^{2+}$  ion from peptide VNHVTLSPK is shown (C).

**Figure 4:** (A) SELDI-TOF-MS spectra of 11.7 kDa protein peak in chronic allograft nephropathy (CAN) urine versus purified  $\beta 2$  microglobulin. A repeat SELDI analysis as was performed in the original urine samples and a comparative examination of commercially available  $\beta 2$  microglobulin and the protein band excised from the CAN urine revealed identical bands. (B) Urinary  $\beta 2$  microglobulin concentrations in chronic allograft nephropathy patients versus control patients. A quantitative ELISA was performed to determine the concentration of urinary  $\beta 2$  microglobulin in transplant patients with CAN and those with normal renal function. (\*\*\*)  $p < 0.001$ .



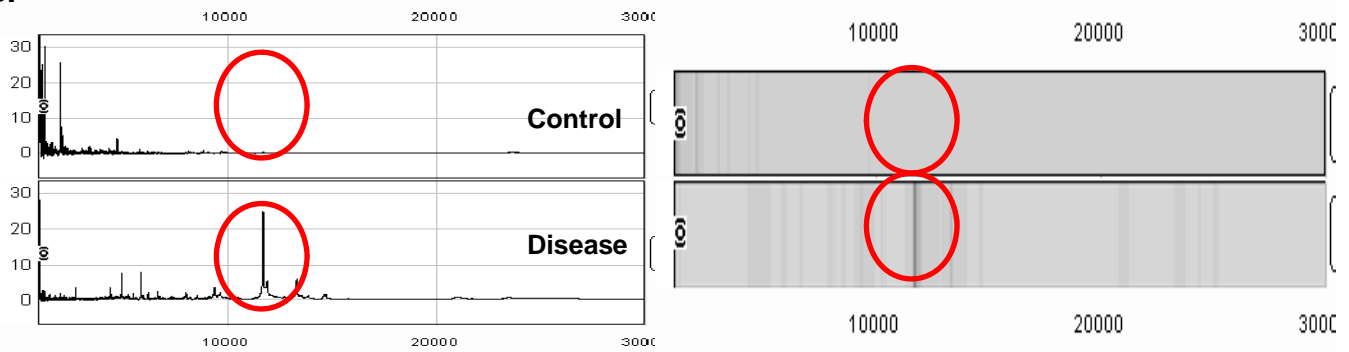
Variable	Disease patients	Control Patients
<b>Mean age (y)</b>	42.91	49.03
<b>Sex male</b>	18 (52.94%)	24 (66.67%)
female	16 (47.06%)	12 (33.33%)
<b>Serum creatinine (mean)</b>	247.9 µmol/l	115.3 µmol/l
<b>Glomerular filtration rate (GFR) (mean)</b>	37.18 ml/min	74.22 ml/min
<b>Acute rejection rate</b>	38.25%	5.56%
<b>CAN Grade (Banff classification)</b>		
I	2 (5.88%)	
II	4 (14.7%)	
III	27 (79.41%)	
Interstitial Nephritis	3 (8.82%)	4 (11.11%)
Hypertensive nephropathy	4 (11.76%)	4 (11.11%)
Reflux nephropathy	6 (17.65%)	5 (13.89%)
Membrano-proliferative glomerulonephritis (MPGN)	2 (5.88%)	0
Alports	2 (5.88%)	1 (2.78%)
Diabetic nephropathy	0	0
Membranous nephropathy	1 (2.94%)	0
Oligomeganephrosis	0	1 (2.78%)
Nephrocalcinosis	0	2 (5.56%)
FSGS	0	1 (2.78%)
Unknown	4 (11.76%)	2 (5.56%)
Goodpastures	1 (2.94%)	0
Obstructive	0	2 (5.56%)
SLE	2 (5.88%)	0
ANCA positive vasculitis	1 (2.94%)	0
<b>Dialysis prior to transplant</b>	97%	94.4%
Peritoneal dialysis (PD) (%)	8 (23.53%)	8 (22.22%)
Haemodialysis (HD) (%)	25 (73.53%)	26 (72.22%)
Pre-emptive (%)	1 (2.94%)	2 (5.56%)
<b>Transplant date range</b>		
Earliest	12/1987	10/1988
Latest	3/2003	1/2002
<b>Calcineurin inhibitor</b>		
Cyclosporine	17/34 (50%)	24/36 (66.67%)
Tacrolimus	10/34(29.41%)	10/36 (27.78%)
Cyclosporine → Sirolimus	5/34 (14.71%)	2/36 (5.56%)
Tacrolimus → Sirolimus	2/34 (5.88%)	0/36
<b>Antimetabolite</b>		
Mycophenolate mofetil (MMF)	17/34 (50%)	8/36 (22.22%)
Azothioprine	12/34 (35.29%)	25/36 (69.44%)
Nil	5/34 (14.71%)	3/36 (8.33%)

**Table 1:** Demographical data of patients in the study

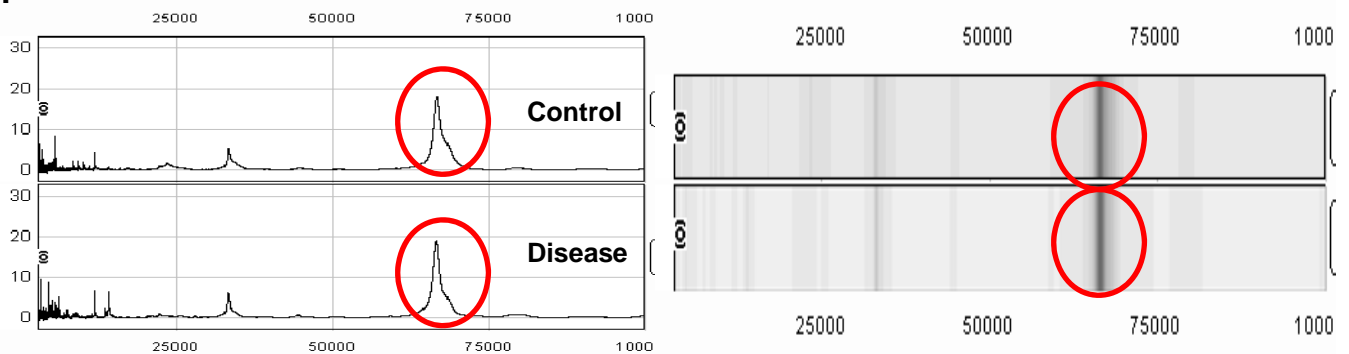
**A.**

	Low molecular weight range	Mid molecular weight range	High molecular weight range
Maximum MW achieved	50 kDa	150 kDa	250 kDa
Optimized range	1-30 kDa	3 – 100 kDa	30 – 200 kDa
Laser intensity	195	205	210
Detector sensitivity	7	8	9
Focus mass	8000	16,000	40,000

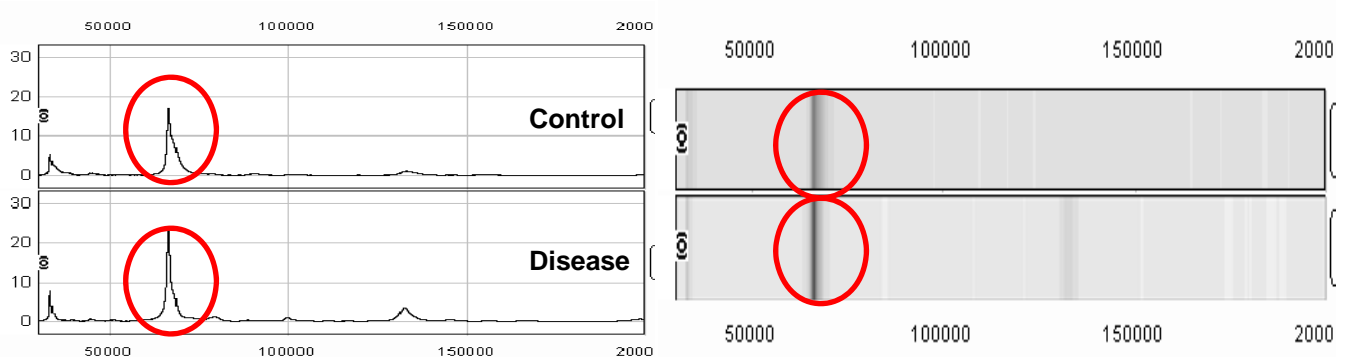
**B.**



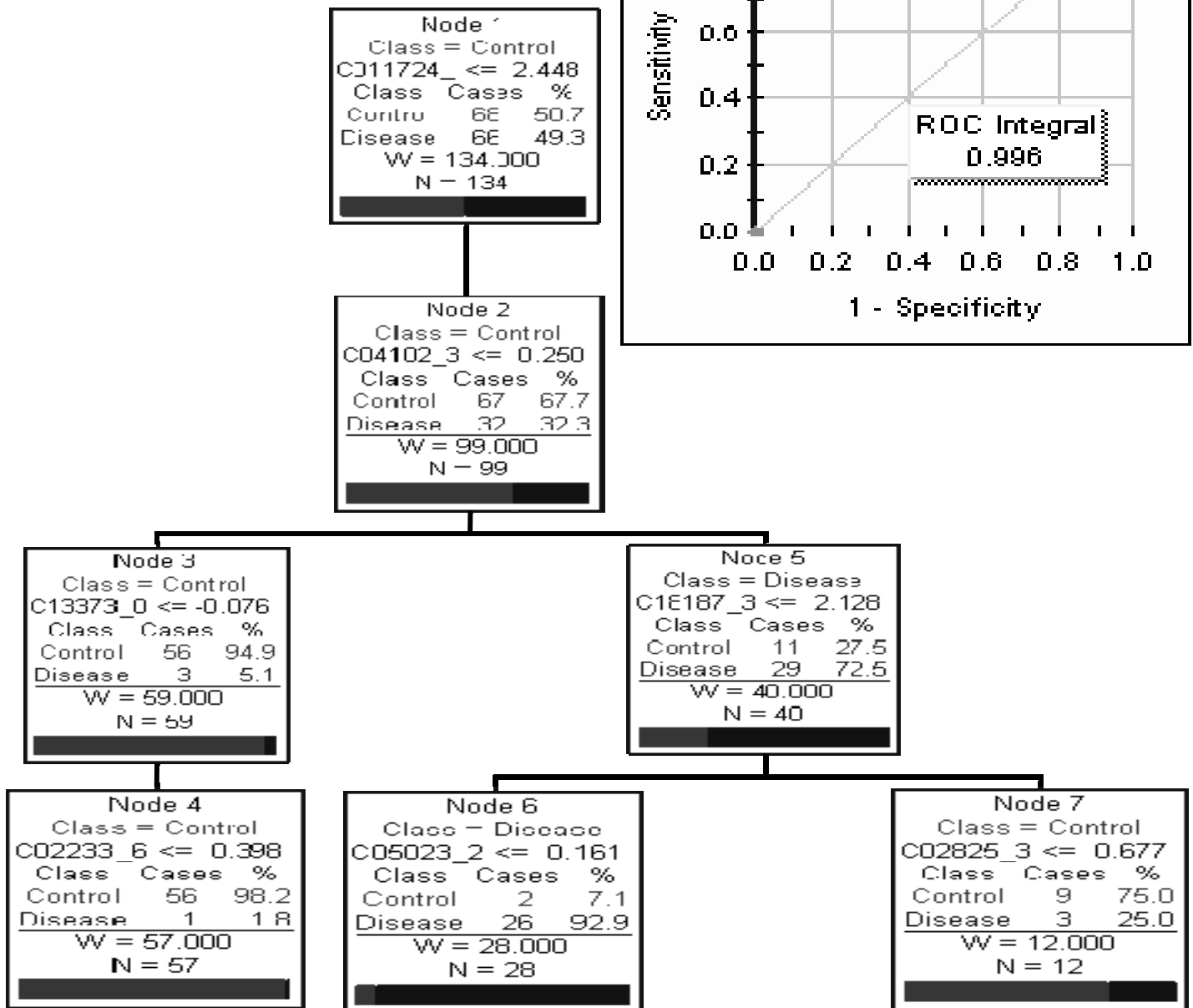
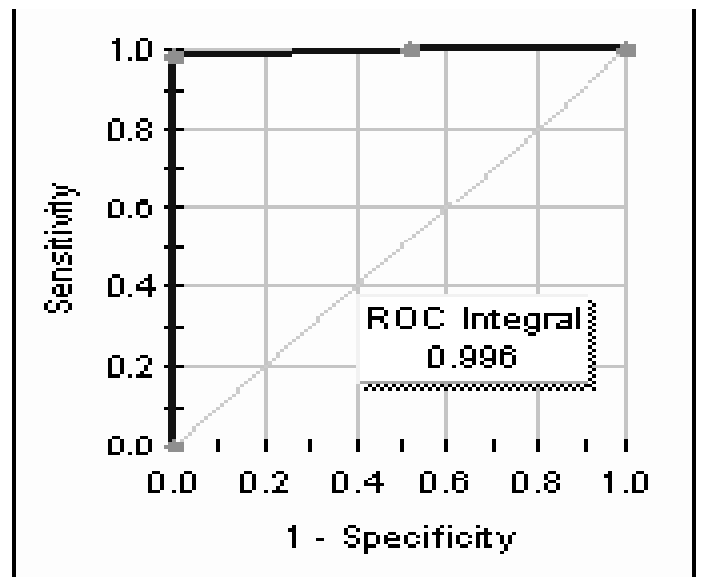
**C.**



**D.**



**Figure 1:** Urinary surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) spectra in three molecular weight ranges of chronic allograft nephropathy



**Figure 2:** Optimal biomarker pattern tree for detecting CAN in transplant patient urine and Receiver Operator Characteristic curve for this tree using a **CM10** ProteinChip™.

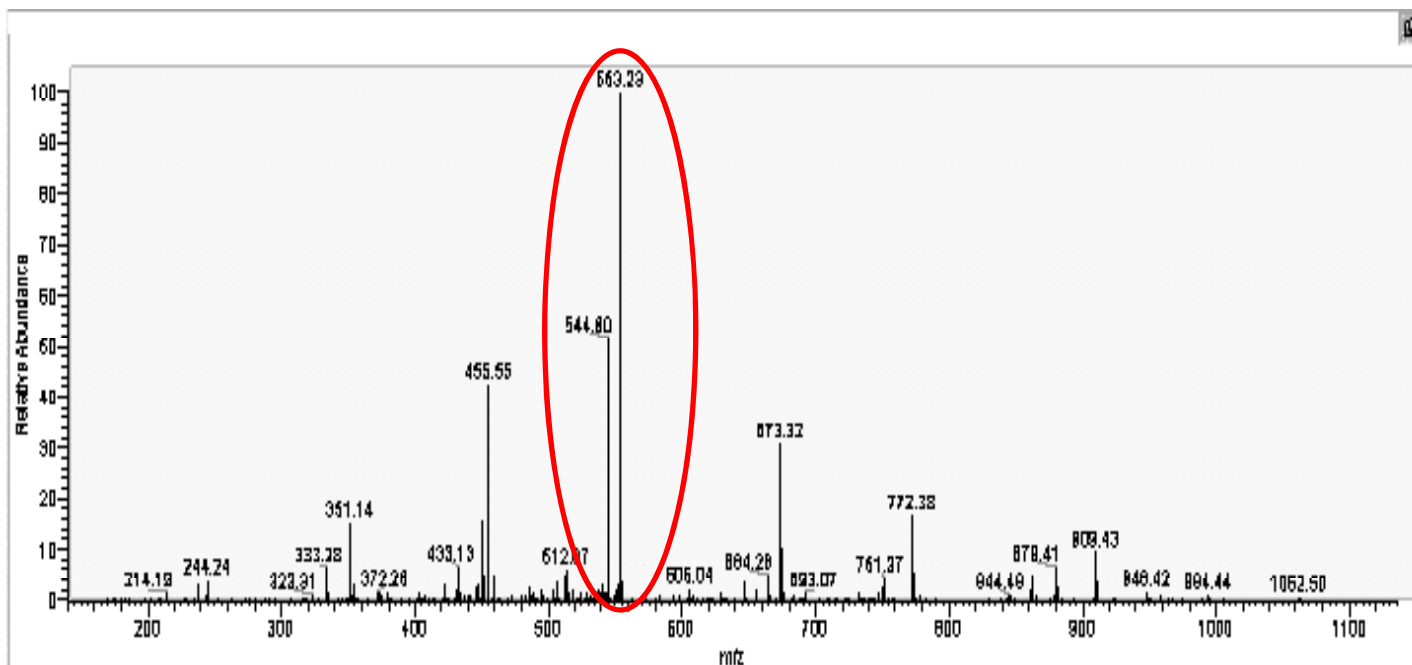
A.

IPI Accession No.	Protein	Peptide Sequence	[M+H] <sup>+</sup>	Protein prophet score	X Corr	DCn	z	Scan
IPI:IPI00004656.1	Beta-2 microglobulin	VNHVTL <b>SQPK</b>	561.7255	1.00	2.646	0.498	2	4786
IPI:IPI00004656.1	Beta-2 microglobulin	VEHSDLS <b>FSK</b>	%&%.23	0.88	1.586	0.158	2	5325

B.

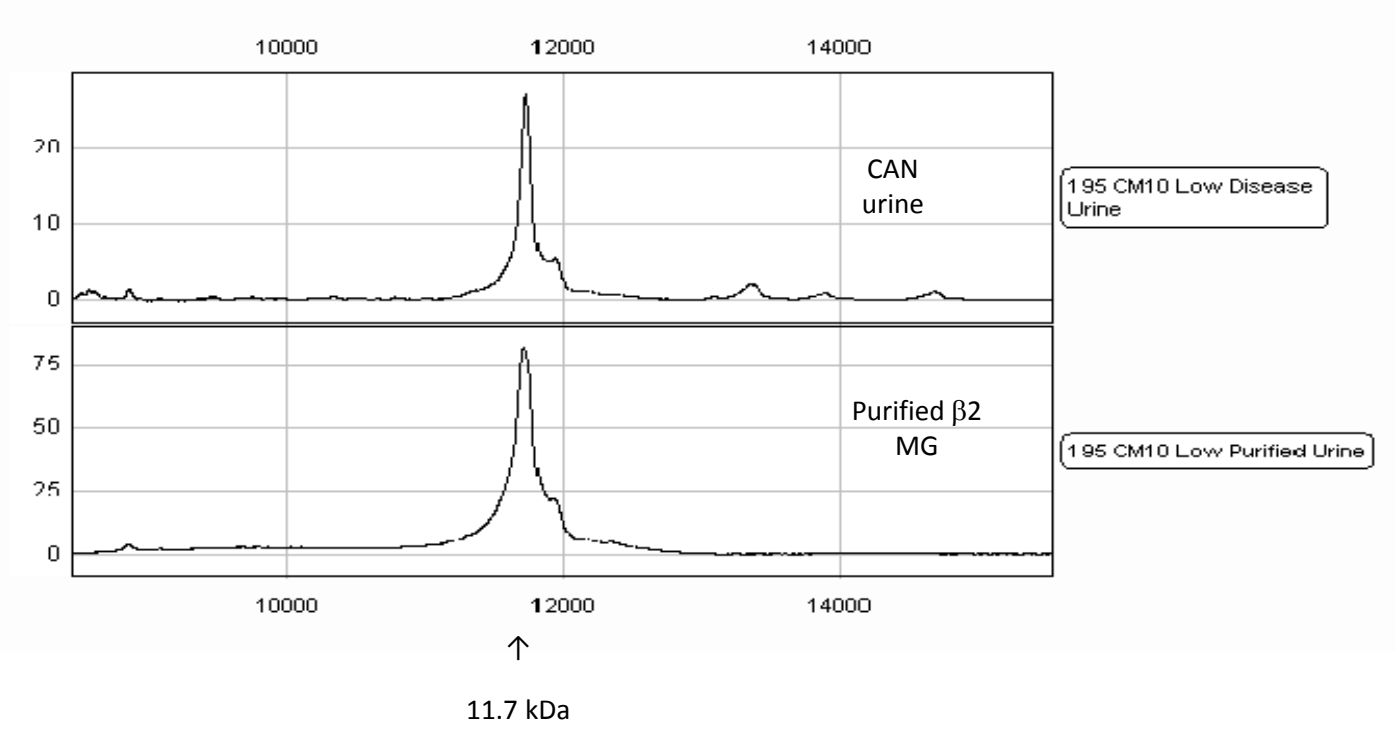
IPI:IPI00004656.1|SWISSPROT:P61769|Beta-2-microglobulin precursor  
 MSRSVALAVLALLSLSGLEAIQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPDIEVDLLKNGERI  
 EK**VEHSDLSFSK**DWSFYLLYYTEFTPTKDEYACR**VNHVTL**SQPK****IWKWDRDM

C.

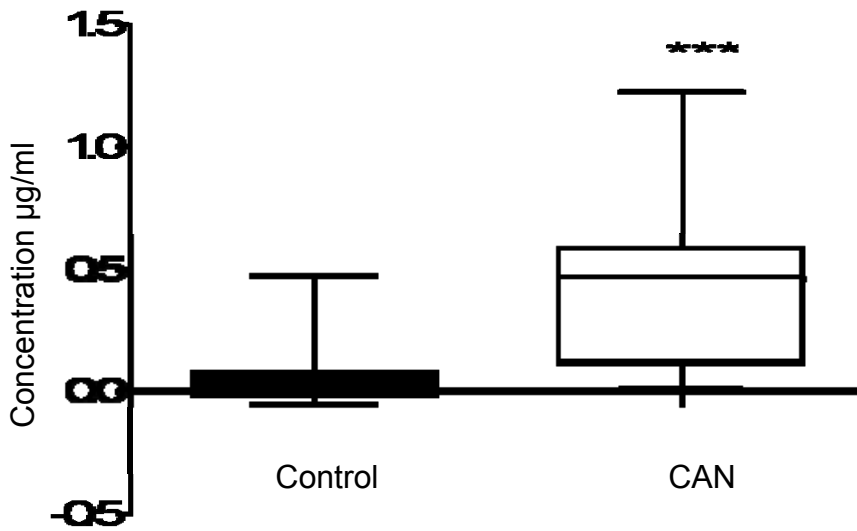


**Figure 3:** Identification of  $\beta 2$  microglobulin. Two high-scoring tryptic peptides (A) were matched to the sequence of  $\beta 2$  microglobulin (B). A representative spectrum of the  $[M+2H]^{2+}$  ion from peptide VNHVTL**SQPK** is shown (C).

A.



B.



**Figure 4:** (A) SELDI-TOF-MS spectra of 11.7 kDa protein peak in chronic allograft nephropathy (CAN) urine versus purified β2 microglobulin. (B) Urine β2 microglobulin quantification by ELISA in chronic allograft nephropathy patients. \*\*\*  $p < 0.001$ .