# Proteomic Approach For Identification of Stage Specific Biomarkers in Streptozotocin Induced Diabetic Nephropathy


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## Introduction

- Diabetic nephropathy (DN) is a serious and common complication in approximately 30-40% of patients with type I and in 15% with type II diabetes (Schrijvers *et al.*, 2004)
- Till date, albuminuria has been the existing marker for detection of diabetic nephropathy
- Albumin is also found in the urine samples from healthy subjects. This poses a major hindrance in the diagnosis of DN
- There is dire need for the novel protein biomarkers which can lay foundation for the development of new diagnostic tests and drug discovery
- In this work we focus on the relevant proteins which are specific for a particular stage and are characteristic signatures of inherent biological outcomes
- Proteomics has been applied to hunt for the differentially excreted proteins in the urine of Streptozotocin induced diabetic rats. Renal proteomics emphasizes on the qualitative changes in the protein expression

## Materials and Methods

- Animals were made diabetic by single intraperitoneal injection of STZ (55 mg/kg, *i.p.*)
- Urine was collected overnight by placing animals in metabolic cages after 12 hours of fasting followed by centrifugation at 1000g to dispel contaminants/cell debris
- Proteins were isolated by TCA precipitation and subsequently dissolved in urea containing rehydration buffer
- For renal proteomics, kidneys were snap frozen, grinded with mortar pestle and solublised in Urea/ thiourea and Chaps buffer with 2% of ampholyte (pI 3-11NL)
- Proteins were focused on IPG strips (GE healthcare) with range 3-10pI NL and 3-11 pI NL for renal proteins
- Isoelectric focusing was performed, after 14 hours of passive rehydration, for a total of 30,000VhT with a constant current of 50 µA throughout the run
- Finally the focused strips were overlaid on a 12.5 % SDS PAGE followed by silver staining
- Gels were analyzed and spots were detected by Image Master 6 Platinum software (GE healthcare)
- Ingel digestion using porcine trypsin (promega) was carried out prior to MALDI– TOF/TOF analysis (Bruker Daltonics)

## Results

- Albumin and immunoglobulins form a major component of the rat urinary proteome
- Our time scan analysis of urine protein changes revealed the existence of proteins which exhibit a temporal pattern
- Calgranulin A and B isoforms appear after the fourth week of induction of diabetes. Both the isoforms disappear by the 8th week
- The expression of E– Cadherin gradually increases as a function of time (weeks) in the diabetic rat urine (2.5 folds as compared to control)
- Control Urine is characterized by the presence of Alpha 2U Globulin along with Pyruvate kinase M2 and ZAP36
- Urinary Proteome of eight week diabetic rats is characterized by the appearance of Casein Kinase, Tropomyosin and guanylate cyclase
- There is conspicuous absence of Alpha 2u Globulin from the diabetic rat urine which is accompanied by a stark and gradual decrease in the expression of Alpha 2u globulin from the kidneys of diabetic rats

## URINE PROTEOMICS

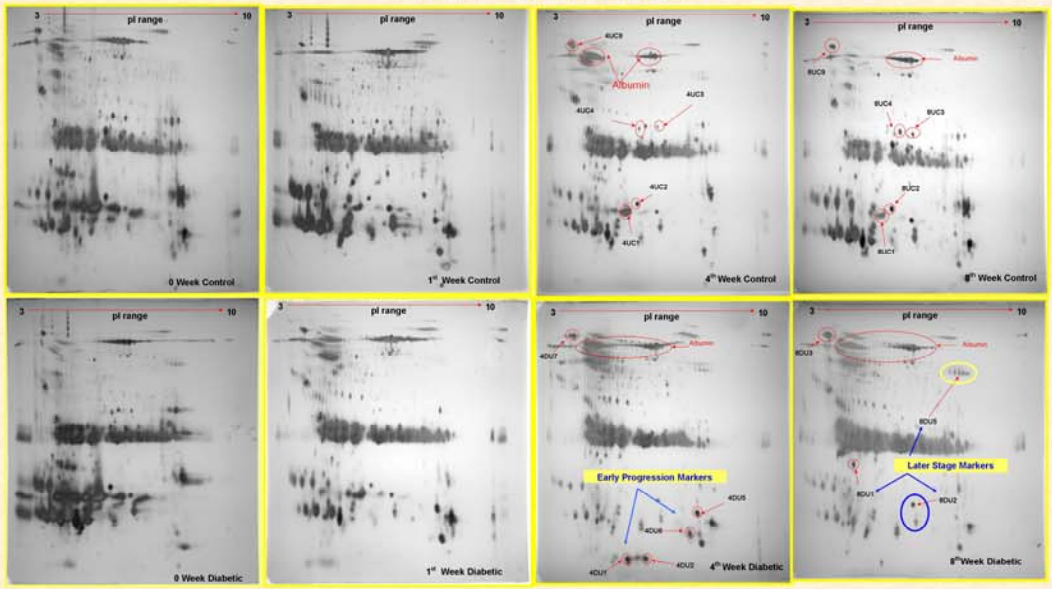



Figure1. This panel demonstrates the appearance/disappearance of proteins in the diabetic rat urine along with the respective controls. 200µg of proteins were loaded in each case followed by a separation on 12.5% SDS PAGE. Software analysis was done with a total of 270 spots and 180 matches in each group. Spots represented as biomarkers were analyzed by Image Master 2D platinum Algorithm.

## RENAL PROTEOMICS

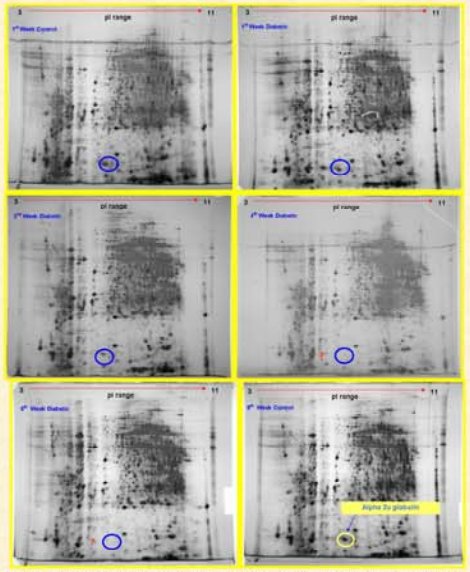
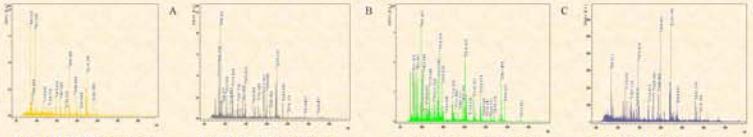



Figure2. This panel demonstrates the appearance/disappearance of Alpha 2u globulin in the diabetic rat kidneys along with the respective controls. 200µg of proteins were loaded in each case followed by a separation on 12.5% SDS PAGE. Software analysis was done with a total of 2100 spots and 1891 matches in each group. Spots represented as biomarkers were analyzed by Image Master 2D platinum Algorithm.




Panel A: MALDI Mass Spectra of Calgranulin A

Panel B: MALDI Mass Spectra of Calgranulin B

Panel C: MALDI Mass Spectra of E-Cadherin

Panel D: MALDI Mass Spectra of Casein Kinase delta

| Spot Number | Protein | Score/ pI | Accession Number |
|---|---|---|---|
| 4UC1 | Alpha 2u Globulin | 129/5.8 | UART |
| 4UC2 | Rab GDP dissociation factor beta | 62/5.6 | B54091 |
| 4UC3 | Pyruvate Kinase,M2 | 60/7 | KPY2_RAT |
| 4UC4 | Annexin like zymogen granule associated protein | 70/5.3 | BAA07399 |
| 4UC9 | E-Cadherin | 130/4.6 | BAA84920 |

Table:1 Putative Proteins specific to control urine identified by MALDI

| Spot Number | Protein | Score/pI | Accession Number |
|---|---|---|---|
| 8dU1 | Tropomyosin | 98/4.52 | 088440 |
| 8dU2 | Guanylate Cyclase | 54/7.3 | A55915 |
| 8dU3 | E-Cadherin | 113/ 4.67 | BAA84920 |
| 8dU5 | Casein Kinase1 delta | 79/9.5 | Q06486 |

Table:2 Putative Later Stage markers (Proteins) in 8th week diabetic rat urine identified by MALDI

| Spot Number | Protein | Score/pI | Accession Number |
|---|---|---|---|
| 4dU1 | Calganulin A | 56/5.6 | S108_RAT |
| 4dU2 | Calgranulin A | 60/5.6 | S108_RAT |
| 4dU6 | Calgranulin B | 75/5.69 | S109_RAT |
| 4dU7 | E- Cadherin | 121/4.6 | BAA84920 |
| 4dU5 | Haptoglobin | 69/7.1 | AAA41349 |

Table:3 Putative Early Stage markers (Proteins) in 4th week diabetic rat urine identified by MALDI

## Discussion &Conclusions

- Our time scanned analysis reveals the existence of proteins in the diabetic and control urine which can be projected as putative biomarkers
- Calgranulin A and B isoforms have been reported to be present in the serum of Type 1 diabetic patients. These isoforms are involved in chemotaxis and are secreted by neutrophils. Existence of these proteins indicate increment in the immune responses during the early phase of progression of Diabetic Nephropathy
- Calgranulin and haptoglobin appearance coincides with the fourth week of the induction of diabetes and gets decreased dramatically by the eighth week, pointing towards the time and "Stage Specific" nature of these proteins. Expression of E-Cadherin (2.5 fold by 8th week) is directly proportional to the stage of disease progression
- Tropomyosin and Casein Kinase appear in a Stage Specific fashion. Their appearance coincides with the development of diabetic nephropathy
- Pyruvate kinase M2 isoform is involved in pyruvate metabolism, deficiency of which is associated with liver disease (Raphael et.,2007). Its absence depicts a gross change in the liver physiology as a feature of diabetic nephropathy
- The presence of Alpha 2u globulin has been reported in the urine of normal rats with liver as a primary source (Roy and Leonard,1973). The absence of this protein in the urine of diabetic rats corroborates well with its absence from renal proteome from the same group
- A cumulative look at the differential changes in the urine/renal samples of diabetic rats indicts towards a greater need to validate the changes by western analysis and/or ELISA along with a study in the human samples. This will foster the diagnostic facilities to provide a battery of tests for pre-diagnosis leading to a timely/tailored treatment of Diabetic Nephropathy


**References:** SCHRIJVERS, B.F., DE VRIESE, A.S. & FLYVBJERG, A. **(2004).** From hyperglycemia to diabetic kidney disease: the role of metabolic, hemodynamic, intracellular factors and growth factors/cytokines. *Endocr Rev*, 25, 971-1010
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