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The putative role of MALDI-MSI in the study of Membranous Nephropathy☆

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ABSTRACT

Membranous Nephropathy (MN) is an immunocomplex mediated renal disease that represents one of the most frequent glomerulopathies worldwide. This glomerular disease can manifest as primary (idiopathic) or secondary and this distinction is crucial when choosing the most appropriate course of treatment. In secondary cases, the best strategy involves treating the underlying disease, whereas in primary forms, the identification of confirmatory markers of the idiopathic etiology underlining the process is requested by clinicians. Among those currently reported, the positivity to circulating antigens (PLA2R, IgG4 and THSD7A) was demonstrated in approximately 75% of iMN patients, while approximately 1 in 4 patients with iMN still lack a putative diagnostic marker. Ultimately, the discovery of biomarkers to help further stratify these two different forms of glomerulopathy seems mandatory.

Here, MALDI-MSI was applied to FFPE renal biopsies from histologically diagnosed primary and secondary MN patients ($n = 20$) in order to detect alterations in their tissue proteome. MALDI-MSI was able to generate molecular signatures of primary and secondary MN, with one particular signal (m/z 1459), identified as Serine/threonine-protein kinase MRCK gamma, being over-expressed in the glomeruli of primary MN patients with respect to secondary MN. Furthermore, a number of signals that could differentiate the different forms of iMN that were positive to PLA2R or IgG4 were detected, as well as a further set of signals (m/z 1094, 1116, 1381 and 1459) that could distinguish these patients from those who were negative to both. These signals could potentially represent future targets for the further stratification of iMN patients. This article is part of a Special Issue entitled: MALDI Imaging, edited by Dr. Corinna Henkel and Prof. Peter Hoffmann.

1. Introduction

Membranous Nephropathy (MN) is an immunocomplex mediated renal disease that represents the leading cause of nephrotic syndrome in adults and is one of the most frequent glomerulopathies worldwide [1,2]. Notwithstanding its relatively low incidence rate, many cases of MN progress to end-stage renal disease (ESRD), having a clear negative impact upon the patients' quality of life and healthcare costs [3–5]. This glomerular disease can manifest as primary (idiopathic) [6] or secondary [7–10] and in the latter occurs as a result of underlying systemic conditions which can be brought about due to treatment with therapeutic agents (eg. NSAIDs), malignancies or autoimmune rheumatologic conditions. This distinction is crucial when choosing the most appropriate approach for MN patients. In secondary cases, the best strategy consists in treating the underlying disease [11,12], which results in a consequent improvement of the patient's renal condition. On the contrary, in primary forms clinicians would desire a final confirmation of the idiopathic origin of the disease to avoid improper medical investigations and to manage the more correct protocols [11–17]. For these reasons, the discovery of useful biomarkers seems mandatory [19–21]. An

Abbreviations: CSV, Comma separated value; CV, Coefficient of variation; ESRD, End-stage renal disease; FFPE, Formalin-fixed paraffin-embedded; FSGS, Focal segmental glomerulosclerosis; GN, Glomerulonephritis; IgAN, IgA nephropathy; IgG4, Immunoglobulin G4; iMN, Idiopathic membranous nephropathy; MCD, Minimal change disease; MN, Membranous nephropathy; PCA, Principal component analysis; PLA2R, M-type phospholipase A2 receptor; PLS, Partial least square; ROC, Receiver operative curve; RFE, Recursive feature elimination; SVM, Sample vector machine; THSD7A, Thrombospondin type-1 domain-containing 7A.

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important step in this field has been reached by Beck et al. with the identification of circulating auto-antibodies directed against a normally expressed podocyte membrane antigen, the M-type phospholipase A2 receptor (PLA2R), which is present in at least 70% of patients with idiopathic (iMN) [18]. Recently, a further contribution to this field was provided by Tomas et al. with the description of another podocyte membrane antigen, termed thrombospondin type-1 domain-containing 7A (TSHD7A), which was able to account for a further 5% of iMN cases [19]. Alternatively, the evidence of restriction to a particular subtype of immunoglobulins (IgG4) in the context of iMN immunocomplexes and the relative absence of these antibodies in secondary forms may represent another crucial element for the discrimination of iMN subtypes [20]. Such multidisciplinary investigations into MN were traditionally based on immunological, serological and histological tools while proteomic approaches, involving LC-MS, were more recently employed. Furthermore, Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry Imaging (MALDI-MSI) has been demonstrated on renal biopsies to represent a technique capable of detecting changes within the proteome of glomeruli and tubules affected by different types of glomerulonephritis (GN) such as MN and minimal change disease (MCD) or IgA nephropathy (IgAN) [21–23]. This technique is now feasible with formalin-fixed paraffin-embedded (FFPE) renal tissue [24], making the collection of a greater number of patients possible.

In this preliminary study, we analysed FFPE bioptic renal tissue of MN patients aimed at investigating the potentiality of this technique to explore the proteomic alterations that may occur within the glomeruli of primary and secondary forms and to detect preliminary proteomic signatures of this frequently occurring glomerulopathy.

2. Materials and Methods

2.1. Patient selection

Twenty patients with a biopsy proven diagnosis of MN, provided between February 2011 and February 2015 at University Milano-Bicocca, San Gerardo Hospital, Monza, Italy, were included in the study that was approved by the ethical board. MN diagnosis was performed after careful histopathological evaluation of renal biopsies. For adequacy purposes, at least 2 core biopsies were taken from each patient, containing an average of at least 10 glomeruli. The routine histological staining procedures were performed, including immunofluorescence and electron microscopy, with all these techniques being considered for the diagnosis. As inclusion criteria for the study, a strong granular parietal staining for IgG and c3 was needed as the ultrastructural confirmation of subepithelial deposits. Cases were staged in a 4-tiered scheme according to the current guidelines [22]. Patients were classified as secondary according to clinical characteristics. The remaining cases were considered to be idiopathic. Moreover, for comparative purposes, one case of focal segmental glomerulosclerosis (FSGS), one case of IgA nephropathy (IgAN), one case of Minimal Change Disease (MCD) and one normal control taken from a region of normal kidney following total nephrectomy for renal cell carcinoma were enrolled in the study for the MALDI-MSI analysis.

2.2. Materials

Renal biopsies collected for the study underwent standard histological protocols; in particular, core biopsies were fixed for an average time of 12 h. After the fixation phase, inclusion was performed using an automatic Tissue Processing Centre (TPC 15 Duo/Trio, Medite, MeBurgdorf, German). For every patient of the study, a 4-μm thick section from the corresponding FFPE block was mounted onto an ITO slide (Bruker Daltonik, Bremen, Germany) for MALDI-MSI analysis. 3-μm thick sections were also recruited on polarized slides for immunohistochemistry (IHC).

2.3. Immunohistochemistry

Blank sections were deparaffinised, rehydrated and then stained by the polyclonal mouse antibodies against PLA2R (0.4 mg/ml, dilution 1:300, Atlas Antibodies, AlbaNova University Center, Stockholm, Sweden) and IgG4 (0.4 mg/ml, dilution 1:300, AbNova) on a Dako Autostainer (DAKO, Glostrup, Denmark) using a 3.3′-diaminobenzidine (DAB) for PLA2R and 3-amino 9-ethyl carbazole (3 AC, Vector Labs, DBA ITALIA S.R.L. Segrate, Italy) for IgG4 as previously described [23]. The incubation with the primary antibody in the Autostainer Link 48 (DAKO) was performed after endogenous peroxidase blockage and antigen retrieval. Cases were analysed in blind by three different nephropathologists and scored for the positivity/negativity according to the interpretation criteria shown in Fig. 1. IHC results are recorded in Table 1.

2.4. MALDI-MSI sample preparation

The slides were stocked at room temperature until the day of the analysis. Paraffin removal and antigen retrieval was performed as previously described [24]. Then, trypsin deposition (Sigma-Aldrich, Chemie Gmbh, Steinheim, Germany, 100 ng/μl) was performed using the iMatrix Spray (Tardo Gmbh, Subingen, Switzerland) automated spraying system and then left in a humid chamber overnight at 40 °C. Finally, matrix deposition for MALDI analysis was performed by spraying α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50:50 ACN:H_20 w/0.4% TFA) using the iMatrix Spray (Tardo Gmbh, Subingen, Switzerland) and an optimised method.

2.5. Mass spectrometric analysis

All the mass spectra were acquired in reflectron positive mode in the mass range of *m/z* 750 to 3500 with the UltrafleXtreme (Bruker Daltonik GmbH) MALDI-TOF/TOF MS equipped with a Smartbeam laser operating at 2 kHz frequency. External calibration was performed using a mixture of standard peptides within the mass range of *m/z* 750 to 3500 (PepMix I, Bruker Daltonics). Images were acquired with a laser diameter of 50 μm and a rastering of 50 μm. For MALDI-MS/MS, representative mass spectra were acquired in reflectron positive mode in the mass range of *m/z* 750 to 3500, using the "Random Walk" feature. This was performed in order to confirm whether the ion of interest was still present and of sufficient abundance and in order to obtain the correct mass value to be selected for dissociation. A single precursor ion was selected by using the smallest PCIS (Precursor Ion Selector) window possible and dissociated using laser-induced dissociation (LID) and LIFT™ technology, with the laser energy being set within a range of 40–70%. This process was performed until an MS/MS spectra was obtained from the accumulation of ~100,000 laser shots.

After MALDI analysis, the matrix was removed by washing the tissue sections with increasing concentrations of EtOH (70% and 100%) and the slides stained with Periodic acid-Schiff. The slides were converted to digital format using a ScanScope CS digital scanner (Aperio, Park Center Dr., Vista, CA, USA), thus allowing the direct overlap of images and the integration of proteomic and morphologic data.

2.6. Data analysis

The raw MALDI-MSI data were elaborated as previously described [23]. Briefly, FlexImaging 3.0 (Bruker Daltonics, Bremen, Germany) data, containing spectra of each entire measurement region, were imported into SCiLS Lab 2014 software (http://scils.de/; Bremen, Germany) to perform pre-processing: baseline subtraction (TopHat algorithm), normalisation (Total Ion Current algorithm). Average (avg) spectrum representative of the whole measurement region was obtained by peak picking, alignment and spatial denoising. Reproducibility of our method was evaluated based on number of peaks and Coefficient