The 8th EFCC Continuous Postgraduate Course in Clinical Chemistry

Under the Auspices of IFCC

NEW TRENDS IN CLASSIFICATION, DIAGNOSIS AND MANAGEMENT OF KIDNEY DISEASES

Handbook

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Editorial

The Eight EFCC Continuous Postgraduate Course in Clinical Chemistry:
New Trends in Classification, Diagnosis and Management of Kidney Diseases

The Croatian Society of Medical Biochemists and Slovenian Association for Clinical Chemistry, together with the European Federation of Clinical Chemistry and Laboratory Medicine (EFCC), have organized the eight in a series of postgraduate weekend courses under the auspices of IFCC. The Course entitled "New Trends in Classification, Diagnosis and Management of Kidney Diseases" promotes continuing postgraduate education of professionals in clinical chemistry and laboratory medicine, and ensures the laboratory knowledge harmonization, this time on kidney disease in particular.

Renowned experts in different fields try to cover the clinical and laboratory aspects of kidney diseases. The integrated knowledge of the authors and the material prepared by these experts especially for this Course, is intended to provide optimal information to the reader.

The contents of Handbook is divided into three chapters according to the Course program. The chapter Basic Concepts covers topics such as Pathophysiology and classification of kidney diseases, Inflammation, cytokines and chemokines in chronic kidney disease, Podocyte injury in glomerular diseases and Kidneys and autoimmune disease. In the chapter Diagnosis procedure in nephrology, Hereditary kidney disorders, Diabetic nephropathy, Drug-induced kidney injury, Dislipidemia at chronic renal failure, GFR - where are we now? and Cardiovascular risk in chronic kidney disease are presented. The last chapter is dedicated to New approach to diagnosis and management, where Laboratory standards in the diagnosis and monitoring of therapy, The urinary proteomics: a tool to discover new and potent biomarkers for kidney damage, Point-of-care creatinine testing in high-risk patients and Recent approaches to therapy: is there real progress? are presented.

We do hope that the Course program as well as this Course Handbook meets the intended goals by presenting the state-of-the-art, contributing to harmonization of new trends in the classification, diagnosis and management of kidney diseases.

Zagreb, October 2008 *Elizabeta Topić*

1. PATHOPHYSIOLOGY AND CLASSIFICATION OF KIDNEY DISEASES

Mirjana Sabljar Matovinović

1.1 Classification of CKD

Chronic kidney disease (CKD) is far more prevalent worldwide than was previously assumed. It affects 10 - 15% of the adult population in the western countries, many of whom require costly treatments or renal replacement therapy. According to the Third National Health and Nutrition Examination Survey and the National Kidney Foundation Kidney Disease report nearly 26 million persons in the USA fall into this category and another 20 millions are at an increased risk for CKD. Moreover, it has been recognized that CKD is a major risk factor for increased cardiovascular disease and death. This knowledge has been incorporated in the recent cardiologic guidelines as well as in the 2007 European Guidelines for the Management of Arterial Hypertension. At the same time, there is an increasing prevalence of diseases that predispose individuals to CKD, such as hypertension, diabetes, obesity and other, rendering the prevention and early detection of CKD a health-care priority in both developed and developing countries.

In 2002 the Kidney Disease Outcomes Quality Initiative (K/DOQI) of the National Kidney Foundation has published guidelines to define CKD and to classify stages in its progression. This classification system is based on the level of kidney function as estimated by glomerular filtration rate (GFR) regardless of the underlying pathology. Subsequent interventional guidelines, specific to each of these stages, have been published on dyslipidemia, bone mineral metabolism and disease, and blood pressure. In 2004 the international organization Kidney Disease: Improving Global Outcomes (KDIGO), governed by an international board of directors, was formed to address the worldwide epidemic of CKD by facilitating the development and implementation of the guidelines with a stated mission to "improve the care and outcomes of kidney disease patients worldwide through promoting coordination, collaboration and integration of initiatives to develop and implement clinical practice guidelines". KDIGO held the first conference in Amsterdam in November 2004. The recommendations from the conference were ratified by the KDIGO board of directors in Paris in December 2004 offering, as a position statement, a clearer definition of CKD and its classification (Tables 1.1. and 1.2.) and practical advice on its screening and management.

Table 1.1. *Criteria for the definition of chronic kidney disease (CKD)*

Table 1.2. *Definition and classification of chronic kidney disease. Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int 2005;67:2089.*

Treatment by dialysis or transplantation was added in this K/DOQI modified classification. According to Levey, this was deemed necessary to link with clinical care and policy, especially regarding reimbursement. The „T“ was added for all kidney transplant recipient at any level of GFR (CKD stages 1-5) and „D“ for dialysis for CKD stage 5. Irrespective of the level of GFR at which the dialysis was initiated, all patients treated with dialysis were designated as CKD stage 5D. To improve the classification the need for elucidation of the cause of CKD as well as the prognosis was expressed.

In line with these considerations, a growing body of literature is questioning the appropriateness of grouping all patients with similar GFR in the same CKD stage, given the considerable heterogeneity in the CKD population. Studies by Menon, O' Hare and their coworkers have shown that outcomes in the same CKD stage can vary considerably depending on age, background cardiovascular risk, etiology and the rate of CKD progression. There are claims that staging system needs to be modified to reflect the severity and complications of CKD in order to allow identification and treatment of clinically relevant disease and avoidance of what seem exaggerated prevalence estimates. These considerations will probably be taken into account by the next K/DOQI Clinical Practice Guidelines for CKD.

1.2 Pathophysiology of kidney disease

When discussing the pathophysiology of CKD, renal structural and physiological characteristics, as well as the principles of renal tissue injury and repair should be taken into consideration.

Firstly, the rate of renal blood flow of approximately 400 ml/100g of tissue per minute is much greater than that observed in other well perfused vascular beds such as heart, liver and brain. As a consequence, renal tissue might be exposed to a significant quantity of any potentially harmful circulating agents or substances. Secondly, glomerular filtration is dependent on rather high intra- and transglomerular pressure (even under physiologic conditions), rendering the glomerular capillaries vulnerable to hemodynamic injury, in contrast to other capillary beds. In line with this, Brenner and coworkers identified glomerular hypertension and hyperfiltration as major contributors to the progression of chronic renal disease. Thirdly, glomerular filtration membrane has negatively charged molecules which serve as a barrier retarding anionic macromolecules. With disruption in this electrostatic barrier, as is the case in many forms of glomerular injury, plasma protein gains access to the glomerular filtrate. Fourthly, the sequential organization of nephron's microvasculature (glomerular convolute and the peritubular capillary network) and the downstream position of the tubuli with respect to glomeruli, not only maintains the glomerulo-tubular balance but also facilitates the spreading of glomerular injury to tubulointerstitial compartment in disease, exposing tubular epithelial cells to abnormal ultrafiltrate. As peritubular vasculature underlies glomerular circulation, some mediators of glomerular inflammatory reaction may overflow into the peritubular circulation contributing to the interstitial inflammatory reaction frequently recorded in glomerular disease. Moreover, any decrease in preglomerular or glomerular perfusion leads to decrease in peritubular blood flow, which, depending on the degree of hypoxia, entails tubulointerstitial injury and tissue remodeling. Thus, the concept of the nephron as a functional unit applies not only to renal physiology, but also to the pathophysiology of renal diseases. In the fifth place, the glomerulus itself should also be regarded as a functional unit with each of its individual constituents, i.e. endothothelial, mesangial, visceral and parietal epithelial cells - podocytes, and their extracellular matrix representing an integral part of the normal function. Damage to one will in part affect the other through different mechanisms, direct cell-cell connections (e.g., gap junctions), soluble mediators such as chemokines, cytokines, growth factors, and changes in matrix and basement membrane composition.

The main causes of renal injury are based on immunologic reactions (initiated by immune complexes or immune cells), tissue hypoxia and ischaemia, exogenic agents like drugs, endogenous substances like glucose or paraproteins and others, and genetic defects. Irrespective of the underlying cause glomerulosclerosis and tubulointerstitial fibrosis are common to CKD.

An overview of the pathophysiology of CKD should give special consideration to mechanisms of glomerular, tubular and vascular injury.

1.2.1 Mechanism of glomerular impairment

Hereditary defects account for a minority of glomerular disease. A prototype of an inherited glomerular disease is the Alport's syndrome or hereditary nephritis, usually transmitted as an X-linked dominant trait although autosomal dominant and recessive forms have been reported as well. In its classical X-linked form there is a mutation in the COL4A5 gene that encodes the α5 chain of type IV collagen located on the X chromosome. As a consequence, GBM is irregular with longitudinal layering, splitting or thickening, and the patient develops progressive glomerulosclerosis and renal failure. Other types of inherited glomerular disease are thin membrane syndrome, nail-patella syndrome, partial lipodystrophy, and familial lecithin-cholesterol acyltranferase deficiency.

Most **acquired glomerular disease** is triggered by immune mediated injury, metabolic and mechanical stress. From a pathological and pathogenetic point of view glomerular diseases can broadly be divided into three groups:

- nonproliferative (without cell proliferation) glomerular diseases without glomerular inflammation and without deposition of immunoglobulins (minimal change disease, idiopathic focal, and segmental glomerulosclerosis [FSGS]) or with deposition of

immunoglobulins, but without glomerular inflammation, most likely because of subepithelial localization of immunoglobulins (e.g., membranous nephropathy)

- proliferative glomerular diseases with deposition of immunoglobulins leading to increased cellularity (proliferative glomerulonephrites, e.g., lupus nephritis, IgA nephropathy, anti-GBM, postinfectious GN), or with severe glomerular injury and inflammation, but without deposition of immunoglobulins (e.g., pauci-immune glomerulonephritis).

- heterogenous group of glomerular diseases in systemic diseases like glomerular disease in diabetes, amyloidosis and paraproteinemia.

The podocyte seems to occupy the central role in the pathogenesis of the first group of glomerular diseases as well as in diabetic nephropathy. This topic will be elaborated separately.

In the second group of glomerular diseases with cell proliferation, either deposition of immune complexes from the circulation or formed in situ lead to activation of intrinsic renal cells (via Fc receptors and complement cascade activation), resulting in inflammatory cell recruitment. Futhermore, severe glomerular injury and inflammation can occur without discernible immune complexes in the glomeruli, as in ANCA (antineutrophil cytoplasmic antibodies) positive glomerulonephritis. The offending etiologic agents are mainly unknown, with the rare exception of ß hemolytic streptococci in poststreptococcal glomerulonephritis, and hepatitis C virus in type 1 cryoglobulinemic membranoproliferative glomerulonephritis. Most antibody-mediated glomerulonephrites are initiated by the reactivity of circulatory antibodies and glomerular antigens, whereby antigens might be the components of normal glomerular parenchyma as in anti-GBM antibody disease (Goodpasture' syndrome), or the antigens are planted from the circulation within the glomeruli as in poststreptococcal glomerulonephritis (the in situ formation of immune complexes). The immune complexes formed in systemic circulation can be deposited and trapped in glomeruli (in cryoglobulinemic glomerulonephritis). Additional mechanism of antibody-mediated glomerular injury, but without immune complexes in the glomeruli, is represented by circulating autoantibody against neutrophil cytoplasmatic antigens (ANCA). Reactive oxygen species, protease, cytokines, chemokines and other inflammatory mediators originating from recruited and resident inflammatory cells play the key pathogenic roles.

Immune complexes can be deposited in the mesangium (as in IgA nephropathy, Henoch Schonlein purpura, lupus nephritis class II, postinfectious GN), in subendothelial (lupus nephritis class III, membranoproliferative GN), or subepithelial area (idiopatic membranous nephropathy or class V lupus nephritis, postinfectious GN), or along GBM (as in anti-GBM disease). The site of antibody deposition defines the response to injury and clinicopathological presentation. A strong inflammatory reaction occurs only when circulating inflammatory cells can be activated by contact with immunoglobulins or soluble products released by intrinsic renal cells. Thereby, the deposition of antibodies in the subendothelial area, mesangium or membrane elicits a nephritic response, as the position of immune complexes enables activation of endothelial or mesangial cells which release soluble products and rapidly recruit leukocytes and platelets from the blood. Leukocyte-derived products, such as cytokines, lysosomal enzymes, reactive oxygen species, complement components and other, damage the vascular wall and filtration barrier and attract more leukocytes from the circulation. The subepithelial position of immune complexes (as in membranous nephropathy) leads to nephrotic response, as GBM precludes the contact between immune complexes and

inflammatory cells from the circulation. Another reason for this kind of response is that large fluid flow from vascular lumen to Bowman's space does not permit inflammatory mediators formed in the subepithelium to diffuse retrogradely from epithelial to the endothelial layer and vascular lumen.

Tissue injury after IC deposition is mediated through complement activation resulting in the formation of C5-9 membrane attack complex which appears to be the major effector of glomerular injury through release of chemotactic C5a and C3a. C5-9-activated cells release chemokines and oxidant proteases, and upregulate adhesion molecules.

T-cells also act as mediators of glomerular injury and as modulators of the production of nephrite/ogenic antibodies, especially in pauci-immune GN. They interact through their surface receptor/CD3 complex with antigens presented in the clefts of MHC molecules of endothelial, mesangial and epithelial glomerular cells. This process is facilitated by the cell-cell adhesion and costimulatory molecules. Once activated, T-cells release cytokines and other mediators of inflammatory reaction, cytotoxicity and fibrogenesis. Soluble factors from T cells have been implicated in the pathogenesis of minimal change disease and focal and segmental glomerulosclerosis, but their identity has yet to be determined.

TGF-ß and connective tissue growth factor (CTGF) are important in glomerular fibrogenesis, as they stimulate glomerular cells to produce extracellular matrix (ECM), a key event in the progression of kidney disease, inhibiting the synthesis of tissue protease, mostly matrix metalloproteinase, which otherwise degradates matrix proteins.

Glomerular inflammation can either completely recover or resolve with a variable degree of fibrosis. The resolution process requires cessation of further antibodies production and immune complex formation, degradation and removal of deposited and circulating immune complexes, cessation of recruitment and clearing of inflammatory cells, dispersing of inflammatory mediators, normalization of endothelial adhesiveness, permeability and vascular tone, and clearance of proliferating resident glomerular cells.

Nonimmunologic glomerular injury. Hemodynamic, metabolic and toxic injuries can induce glomerular impairment alone or in conjunction with immunological processes.

Systemic hypertension translated to glomeruli and glomerular hypertension resulting from local changes in glomerular hemodynamics may cause glomerular injury. The kidney is normally protected from systemic hypertension by autoregulation which can be overwhelmed by high blood pressure, meaning that systemic hypertension is translated directly to glomerular filtration barrier causing glomerular injury. Chronic hypertension leads to arteriolar vasoconstriction and sclerosis with consequent secondary sclerosis and glomerular and tubulointerstitial atrophy. Different growth factors like angiotensin II, EGF, PDGF, and CSGF, TGF-ß cytokine, activation of stretch-activated ion channels and early response gene are involved in coupling high blood pressure to myointimal proliferation and vessel wall sclerosis.

Glomerular hypertension is normally an adaptive mechanism in remaining nephrons to increased workload resulting from nephron loss, whatever the cause. This sustained intraglomerular hypertension increases mesangial matrix production and leads to glomerulosclerosis by ECM accumulation. The process is mediated by TGF-ß in the first place, with a contribution of angiotensin II, PDGF, CSGF and endothelins.

Systemic and glomerular hypertension are not necessarily associated, as glomerular hypertension may precede systemic hypertension in glomerular disease.

Metabolic injury as that occurring in diabetes is discussed separately.

1.2.2 Mechanism of tubulointerstitial impairment

Regardless of the etiology, chronic kidney disease is characterized by renal fibrosis - glomerulosclerosis and tubulointerstitial fibrosis. The impairment of the tubulointerstitium (tubulointerstitial fibrosis and tubular atrophy) is at least as important as that of the glomeruli (glomerulosclerosis). There is a common consensus that the severity of tubulointerstitial injury correlates closely (and better than glomerular injury) with long-term impairment of renal function. This is not surprising, considering that tubules and interstitium occupy more than 90% of the kidney volume. As very recently summarized by Fine and Norman, tubulointerstitial fibrosis encompasses a number of characteristic features including an inflammatory cell infiltrate which results from both activation of resident inflammatory cells and recruitment of circulating inflammatory cells; an increase in interstitial fibroblasts due to increased proliferation and decreased apoptosis of resident interstitial cells, as well as recruitment of cells to the tubulointerstitium; the appearance of myofibroblasts expressing the cytoskeletal protein α-smooth muscle actin, which arise by differentiation of resident interstitial fibroblasts and infiltrating cells and via transdifferentiation; accumulation of extracellular matrix (ECM) as the net result of increased synthesis of ECM components and decreased ECM degradation, mostly by specific metalloproteinases that are under the control of specific inhibitors; tubular atrophy as a consequence of apoptosis and epithelial–mesenchymal transdifferentiation (EMT); and rarefaction of peritubular capillaries. The development of fibrosis is associated with an increase in the expression of proinflammatory, vasoconstrictive and profibrotic factors.

Renal fibrogenesis. The initial insult leads to inflammatory response with the generation and local release of soluble mediators, an increase in local vascular permeability, activation of endothelial cells, extravasation of leukocytes along the endothelium, subsequent secretion of various mediators by infiltrating leukocytes and tubulointerstitial cells, and activation of profibrotic cells. As a consequence a vicious cycle of cell stress is initiated generating profibrotic and proinflammatory mediators, leukocyte infiltration and fibrosis.

Induction and development of the inflammatory response. Leukocytes migrate from the circulation through postcapillary venules and peritubular capillaries into the interstitium following gradients of chemoattractants and chemokines. All tubular cells can generate soluble mediators when stimulated by hypoxia, ischaemia, infectious agents, drugs, and endogenous toxins like lipids, high glucose, paraproteins or genetic factors as in cystic renal diseases. Glomerular disease is usually associated with a variable degree of tubulointerstitial injury and inflammation because tubular cells are exposed to proteins which are normally not filtered. The factors involved in the formation of tubulointerstitial inflammatory infiltrates are: proteinuria, immune deposits, chemokines, cytokines, calcium phosphate, metabolic acidosis, uric acid, lipids, hypoxia and reactive oxygen species.

The inflammatory infiltrate. Infiltrating inflammatory mononuclear cells are composed of monocytes/macrophages and lymphocytes, particularly T lymphocytes. CD4-positive T cells

and CD3 T cells carrying chemokine receptors CCR5 and CxCR3 are closely associated with renal function. This inflammatory cells secrete profibrotic cytokines.

Profibrotic cytokines. Infiltrating inflammatory cells and resident interstitial macrophages release cytokines which stimulate fibroblasts to become myofibroblasts. The most important profibrotic factors involved in renal fibrogenesis are angiotensin II, TGF-ß1, CTGF, PDGF, FGF-2 (fibroblast growth factor -2), EGF, ET-1, tryptase mast cell. Angiotensin II induces TGF- ß synthesis in tubular epithelial cells and fibroblast. AII induces hypertrophy in tubular epithelial cells together with connective tissue growth factor (CTGF), independently of TGF-ß. It is currently assumed that TGF-ß1 is the key cytokine in renal fibrogenesis.

Fibroblast proliferation and activation. Fibroblasts proliferate and become active following infiltration of inflammatory cells into the tubulointerstitial space. To express α-smooth muscle actin, the fibroblasts must be activated by cytokines (mostly derived from infiltrating macrophages), change their phenotype and transit from fibroblasts to myofibroblasts. The important mitogens for renal fibroblast are PDGF, bFGF-2 and others, but no single profibrotic „master cytokine„ has been identified so far.

Epithelial-mesenchymal transition. Phenotypic conversion of epithelial cells into mesenchymal cells is known as the epithelial-mesenchymal transition. Evidence for EMT in human disease comes from utilization of mesenchymal marker proteins such as vimentin or S100A4, the human analogue of fibroblast-specific protein-1. The expression of these mesenchymal marker proteins in tubular epithelial cells was well correlated with renal function in IgA nephropathy, lupus nephritis and chronic allograft failure. TGF-ß1 is thought to be the most potent inducer of EMT, which may be induced by a variety of factors other than cytokines.

It has been shown lately that hypoxia-inducible factor-1 (HIF-1), considered to be master regulator of the adaptive response controlling expression of hundreds of genes, also stimulates EMT, which explains why hypoxia results in fibrosis and progressive renal failure. Hypoxia as a consequence of peritubular capillaries loss has been frequently observed in chronic kidney disease. It alters proximal tubular epithelial (PTE) matrix metabolism, promoting ECM accumulation, with a switch to production of interstitial collagen and suppression of matrix degradation. Exposure of PTE to hypoxia induces transition to myofibroblastic phenotype, whereas more prolonged exposure leads to mitochondrial injury and apoptosis consistent with the loss of tubular cells *in vivo*. In PTE, hypoxia also induces expression of fibrogenic factors. Reports from biopsies carried out in patients with diabetic nephropathy, IgA nephropathy, polycistic kidney disease, and chronic allograft nephropathy have confirmed increased expression of HIF, supporting the hypothesis that hypoxia is an important contributory factor in the pathogenesis of CKD in humans. Furthermore, changes in HIF expression correlate with the extent of tubulointerstitial injury.

Proteinuria and tubulointerstitial damage. Proteinuria can damage tubulointerstitium through multiple pathways including direct tubular toxicity, changes in tubular epithelial metabolism, induced cytokine and chemokine synthesis, and increased expression of adhesion molecules. (Abbate). Excess protein reabsorption in proximal tubule may exceed lysosomal processing capacity, lead to lysosomal rupture and result in direct tubular toxicity. There is a great variability in tubular toxicity induced by proteinuria. For example, patients with nephrotic range proteinuria exclusively consisting of albuminuria as in minimal change disease, rarely exhibit tubulointerstitial damage. Different experimental models have

demonstrated generation of chemotactic factor for macrophages, secretion of chemokines such as monocyte chemoattractant protein-1 and RANTES, and expression of fractalkine (a chemokine promoting mononuclear cell adhesion). In addition to inducing chemokine secretion proteinuria may induce secretion of TGF-ß as well as that of adhesion intercellular adhesion molecule-1 and vascular adhesion molecule-1. In a study reporting on results from 119 renal biopsies the formation of interstitial infiltrates and the degree of tubulointerstitial fibrosis was associated with the level of expression of adhesion molecules.

The reversibility of renal fibrosis was demonstrated in different animal studies with relatively mild degrees of fibrosis. In this context BMP-7, which offers strategy to prevent the progression of renal disease and possibly even reverse fibrosis, has been extensively studied. However, only Fioretto has given evidence of reversibility of tubulointerstitial fibrosis in humans in a small group of patients with type 1 diabetes who underwent pancreas transplantation.

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2. INFLAMMATION, CYTOKINES AND CHEMOKINES IN CHRONIC KIDNEY DISEASE

Christopher W K Lam

2.1 The burden of CKD: improving global outcomes

2.1.1 CKD is common

The World Kidney Day was proposed by the International Society of Nephrology (ISN) and International Federation of Kidney Foundations (IFKF) for reminding the public, government, and medical and healthcare professionals that kidney disease is common, harmful, and treatable besides being very costly and preventable (1). It has been celebrated on every second Thursday of March from 2006 by an increasing number of countries, including Hong Kong that was among the 66 and 88 participants in the last two years.

This continuous alert is well justified because chronic non-communicable degenerative diseases are now the leading cause of death at least in industrialized countries, accounting for 35 of the 58 million deaths worldwide in 2005 from a WHO survey (2). Besides the four top killers of cardiovascular disease (CVD), cancer, chronic respiratory disease and type 2 diabetes, chronic kidney disease (CKD) is increasingly a global health problem. Currently in the US, 13% (26 million) of non-institutionalized adults are estimated to have CKD (3). About 1.0 million patients are being treated for end-stage renal disease (ESRD) with 0.5 million surviving on renal replacement therapy (RRT), while a worrying higher proportion (15 million) are at earlier stages of CKD that may escape timely diagnosis and intervention. Prevalence rates are similar in Europe, Australia and Asia including Hong Kong, where RRT prevalence and incidence in 2007 were respectively 1026 and 164 per million population (pmp) according to the Hong Kong Renal Registry.

2.1.2 KDOGI and KDIGO definition and classification of CKD

CKD is a heterogeneous condition, whose clinical manifestations, progression and management depend on its cause, pathology and other comorbid conditions. Prevailing causes of CKD in Hong Kong were diabetes (23%), glomerulonephritis (GN, 34%) and hypertension (7%) for existing RRT patients surveyed in 2007, with (i) IgA nephropathy being the most common biopsy-proven GN (45%) and (ii) diabetic nephropathy rising to 40% among newly admitted RRT patients in 2006-2007 (Hong Kong Renal Registry), reflecting escalation of diabetes in our community. In 2002, the Kidney Disease Outcomes Quality Initiative (KDOQI) of the US National Kidney Foundation proposed a simple definition and classification of CKD based on severity that was modified by the Kidney Disease: Improving Global Outcomes (KDIGO) organization in 2004 (4). With such paradigm shift from cause to severity, glomerular filtration rate (GFR) became a central parameter for diagnosis and staging that is comprehensible to nephrology and non-nephrology communities for international development and implementation of clinical practice guidelines. CKD is defined as GFR lower than 60 ml/min/1.73 m^2 or kidney damage for at least 3 months, and staging according to GFR reduction is supplemented by additional classification based on treatment by dialysis (D) or transplantation (T), for examples, stages 3T and 5D (Table 2.1.).

Table 2.1. *Definition and classification of CKD proposed by KDOGI (2002) and modified by KDIGO (2004) (4)*

2.2 Inflammation in CKD: causes and possible outcomes

2.2.1 Renal progression and other causes of inflammation

The most serious adverse outcomes of CKD include not only debilitating metabolic complications of decreased GFR progressing to ESRD (hypertension, anemia, malnutrition, bone and mineral disorders, etc), but also increased risk for CVD, which is 100 times higher than that in the general population, accounting for about half of all deaths in North American patients receiving RRT, although the proportion has been slightly lower in Hong Kong (30-40%) paralleling death from infection (5).

Among risk factors for atherosclerotic vascular disease and cardiac valvular calcification in CKD, inflammation has been identified as epidemiologically most important. Increased circulating inflammatory proteins, such as plasma C-reactive protein (CRP) and amyloid A (SAA), are powerful predictors of all-cause mortality and cardiovascular death in ESRD patients (6, 7). Inflammation involves complex interactions among immune cells and soluble proteins (cytokines, chemokines, adhesion and co-stimulatory molecules) occurring in affected tissues in response to infection, trauma, ischemia or autoimmune injury. Like most immune reactions, inflammation is a two-edged sword. It is an evolutionary advantage that usually leads to recovery from infection or healing (8). However, if the targeted defense or assisted repairs are not properly orchestrated, inflammation can cause progressive tissue damage by leukocytes and collagen resulting in CKD, diabetes, atherosclerosis, allergy and autoimmunity depending on whether the nephron, pancreatic islet, artery, airway or multiple organs are affected (Figure 2.1.). Although various renal injuries may progress at different rates, there are six sequential mechanisms of CKD that may constitute a common pathway building on each other: (i) glomerular hypefiltration, (ii) worsening prorteinuria, (iii) downstream cytokine and chemokine bath, (iv) interstitial nephritogenic inflammation, (v) tublular epithelial-mesenchymal transition (EMT), and (vi) nephron fibrosis and scarring (9). Other contributing causes of inflammation in CKD include loss of residual renal function (10) resulting in impaired clearance and accumulation of pro-inflammatory metabolites and post-synthetically advanced glycation end products (AGE, 11); increased oxidative stress due partly to depletion of antioxidants (Zn, Se, vitamins C and E) consequent to renal failure or dialysis; exposure of blood to bio-incompatible dialysis membranes and endotoxins in dialysate; and infection from vascular access materials in hemodialysis (HD), peritonitis

during long-term peritoneal dialysis (PD), or actively infected graft of transplant recipients (12).

Figure 2.1. *Acute and chronic effects of the inflammatory response. (8)*

As summarized in two continuous reviews over 10 years, the above pro-inflammatory and other cytokines are soluble signaling proteins for intercellular communication amongst immune cells as well as cells of other systems (13, 14). They have been named generically either by their origin such as the interleukins, or according to their biological actions, such as colony stimulating or growth factors, and chemokines, which are a large family of leukocyte chemoattractive cytokines with over > 50 members divisible into 4 groups, the CXC, CC, C and CX3C chemokines depending on the configuration of cysteine residues near the N-terminal end of these chemokine proteins with different specific target cells receptors. For example, the two major chemokine families either have an amino acid between the two N-terminal cysteines, or the two cysteines are next to each other, constituting the CXC and CC chemokines. Similar to all other cytokines and most regulatory molecules, chemokines act on leukocytes via seven transmembrane domain G protein-coupled cell surface receptors that are specific for each of the 4 chemokine families (15). This has led to a logical receptor nomenclature (in 1996) in which each receptor is designated by the name of the chemokine family (like CC) followed by the letter R for receptor, and then a number based on the chronological sequence of discovery. Until recently, chemokines have been named randomly by trivial names such as monocyte chemoattractive protein (MCP) and monokine induced by interferon-γ (MIG). A similar nomenclature was recommended in Year 2000 by the International Union of Immunological Societies and the WHO using the family name (like CXC) followed by an L for ligand preceding the chronological number of discovery (16). Modern laboratory analysis of cytokines and chemokines include the use of immunoassays, molecular biology and proteomic methods such as RT-PCR of mRNA, real-time quantitative PCR of DNA, gene expression and protein expression arrays, and multi-fluorescence flow cytometry for (i) simultaneous assay of a panel of inflammatory cytokines and chemokines and (ii) intracellular staining of T-helper lymphocyte types 1, 2 and 17 signature cytokines (14).

2.2.2 MIAC syndrome

Returning from causes of inflammation in CKD to possible consequences, malnutrition is prevalent in up to 76% ESRD patients, manifesting reduced body weight, depleted energy store (adipose tissue), and loss of somatic protein (muscle mass), with decreased plasma albumin, transferrin, retinol-binding protein, pre-albumin and apolipoprotein (apo) A-I concentrations (17). These anthropometric and serologic derangements generally cannot be reversed by oral nutritional supplementation, and are associated with poor outcomes. The pathophysiolgy of such type 2 malnutrition in renal failure comprises chronic inflammation driven by pro-inflammatory cytokines (IL-1, IL-6, TNF-α, IFN-γ and others) that accelerate muscle protein catabolism, up-regulate hepatic synthesis of positive acute phase proteins (CRP, SAA, and fibrinogen), and suppress production of negative acute phase proteins including albumin, which is also lost additionally from proteinuria or dialysis to reach very low plasma concentrations (< 30 g/L). Such severe hypoalbuminemia cannot be attributed entirely to anorexia in uremia resulting in decreased protein-calorie intake; it is not even attainable by long-term semi-starvation (e.g. 24 weeks of 1500 kcal / 24 h) in normal subjects causing very marked reduction (e.g. 25%) in body weight.

Inflammation plays an even more life-threatening pivotal role in the initiation and progression of atherosclerosis, and is considered a major non-traditional risk factor for accelerated carotid intima-thickening and plague formation in dialysis patients (18). An elevated plasma CRP concentration (> 5 mg/L) is not only associated with greater prevalence of atherosclerotic vascular disease but also more severe cardiac hypertrophy and dilatation (6). Cellular adhesion molecules which are expressed increasingly in inflammation for enhancing leukocyte-endothelial activation have also been associated with carotid atherosclerosis (19). Other inflammatory proteins that are elevated in CKD and become causative of vascular disease include fibrinogen and lipoprotein (a) that are thrombogenic besides atherogenic. During inflammation, hepatic synthesis of apo A-I, the principal structural protein of high-density lipoproteins (HDL), is suppressed. Consequently, apo A-I on HDL is replaced by the positive acute phase protein SSA altering both the structure and function of circulating HDL resulting in these particles being (i) more adherent to the vascular endothelial surface causing arterial damage and (ii) less protective of LDL oxidation facilitating atherogeneis.

Inflammation is also involved in the calcification process as evidenced by the strong link between inflammatory cytokines / proteins (e.g. IL-6 and CRP) and coronary artery, aortic and valvular calcification in ESRD (7). Decreased plasma concentration of fetuin-A, another negative acute phase protein and inhibitor of calcification, has been associated with valvular calcification and cardiovascular events in PD patients (20). The vicious cycle of malnutrition, inflammation and atherosclerosis instigated by pro-inflammatory cytokines and chemokines was originally given the acronym of MIA syndrome. Our research group has expanded the designation from MIA to MIAC syndrome paying due concern to the clinical and pathological significance of the concurrent calcification (20).

2.2.3 Renal anemia and erythropoietin resistance

Anemia is a major complication of stages 2-5 CKD affecting > 50% ESRD patients before treatment, consequent again to the chronic inflammatory state resulting in accelerated erythrocyte destruction, low hematocrit and blood hemoglobin (Hb) level, decreased serum iron, transferrin and transferrin receptor concentrations, and hyperferrintinemia that cannot be normalized by the now reduced erythropoietin (EPO) production from nonfunctional peritubular kidney cells (21). Anemia affects cognitive function, exercise capacity, cardiac

function and other qualities of life, and is associated with increased CVD and all cause mortality in CKD patients as well as the general population (22). Recombinant human erythropoietin (rHuEPO) has been widely used for treatment of renal anemia. However, up to 25% of dialysis patients are relatively resistant to replacement requiring higher doses to reach target Hb concentration (11 g/dL), and 5-10% fail to respond even on high doses of EPO (23). The immunopathology of EPO resistance is that patients with uremia or other chronic inflammatory conditions have enhanced activation of T-helper type 1 (Th1) lymphocytes and monocytes secreting pro-inflammatory cytokines IL-1, IL-6, IL-12, IFN-γ and TNF-α, and chemokines IFN-inducible protein (IP)-10 / CXCL10 and monocyte chemotactic protein (MCP / CCL2) that exert pro-apoptotic activity to suppress erythrocyte stem cell proliferation (24). This antagonizes the anti-apoptotic effect of EPO on erythroid progenitor cells resulting in rHuEPO resistance. Accordingly, early identification of EPO hypo-responsiveness might alert clinicians to some treatable causes of renal anemia. A potential strategy might involve the use of short-term anti-cytokine or anti-lymphocyte therapy.

2.3 Cytokine and chemokine aberrations in CKD

I shall now very quickly illustrate cytokine and chemokine aberrations in CKD with observations in (i) diabetic nephropathy, (2) lupus nephritis, and (iii) renal dialysis.

2.3.1 Diabetic nephropathy

As mentioned in the beginning of this presentation, type 2 diabetes is an increasingly prevalent, morbid and life-threatening chronic degenerative disease with an epidemiological estimation of 60 million patients in China in Year 2000, 194 million worldwide in 2003, and a projected doubling increase in both prevalence and mortality by 2025. Over the past decade there has been a frightening 88% increase in younger age of onset in Asia. Within our community, prevalence rate was 10% in Year 2002 and increasing. Twenty five % of our population will eventually be affected. Many will die of heart disease or stroke preceding or following renal damage eventually requiring dialysis, making diabetic nephropathy a particularly important diabetic complication in Asia. Compared to sex- and age-matched control subjects, the 88 type 2 diabetic patients with nephropathy in our study manifested increased plasma concentrations of pro-inflammatory cytokines TNF-α, IL-6 and IL-18, anti-inflammatory cytokines IL-10 and adiponectin, as well as neutrophil chemokine IL-8 / CXCL8, monocyte chemokine MCP / CCL2, and Th1 chemokines MIG / CXCL9 and IP-10 / CXCL10, all of them correlating positively with urine albumin:creatinine ratio, which is a marker of renal involvement, as expected from a Th1 mediated inflammation (25, 26).Adiponectin is a relatively new adipocyte-derived cytokine with anti-atherogenic and anti-inflammatory activities. Hypoadiponectinemia occurs in obesity, type 2 diabetes and other conditions associated with insulin resistance and hyperinsulinemia (27). Elevation of plasma adiponectin concentration in diabetic nephropathy is postulated to be due to impaired renal clearance despite decreased production.

2.3.2 Lupus nephritis

System lupus erythematosus (SLE) is a severe systemic autoimmune disease characterized by derangements of both T and B lymphocytes causing multiple organ damage including and involving the kidneys. Published studies to-date have documented significant increases in an array of Th1, Th2 and B lymphocyte-related cytokines and chemokines, all correlating positively with SLE disease severity index, alerting that derangements are more complex involving both Th1 and Th2 inflammatory pathways for tissue inflammation and production

of autoantibodies (28, 29). This reminds us that Nature should unlikely be a purist and we must not over-emphasize or over-classify any disease into a rigid or restricted Th1 or Th2 stereotype. In physics, the co-existing particle and wave properties of radiation were recognized in the beginning of the last century.

It is also reasonable to expect that Nature might employ more than two pathways of T-helper lymphocyte activity. Over the last three years, we have contributed to the concept that newly discovered cytokine IL-23 produced by dendritic cells and macrophages can drive a third T-helper lymphocyte subpopulation, Th17, capable of producing IL-17A and IL-17F that are both cytokine-inducing cytokines in initiating and perpetuating autoimmunity (30). In research, we simply must continuously re-examine old concepts based on new findings.

2.3.3 Renal dialysis

Conventionally, CRP, IL-6, TNF-α and INF-γ have been used as markers of systemic inflammation in ESRD that can be caused by the intrinsic CKD, dialysis membrane or technique, or quality of dialysate. However, previous studies have also shown that T lymphocytes from HD patients are dysregulated and characterized by an increase in circulating Th1 cells with normal number of Th2 lymphocytes. This Th1/Th2 imbalance can be induced by IL-18 produced by monocytes and macrophages. Further, most previous studies used healthy non-CKD subjects as controls instead of pre-dialysis ESRD patients. We have recently reported our study of 146 ESRD patients treated or not treated by PD or HD, and found that plasma IL-18, IL-6, TNF-α, CRP and cardiac troponin T concentrations were significantly higher in dialysis patients than low creatinine clearance pre-dialysis controls (31). These elevations should confer increased cardiovascular risk of ESRD patients on dialysis.

2.4 Summary remarks

CKD is increasingly a global public health problem. It can cause great suffering and impose a serious financial burden to patients and / or their society. CKD can be diagnosed and monitored using simple laboratory tests. Early treatments can decelerate the progression of renal dysfunction, prevent or delay metabolic complications, and reduce the risk of CVD. The pathogenesis and pathophysiolgy of CKD involve cytokine and chemokine driven inflammation potentially causing the MIAC syndrome, renal anemia, and other adverse outcomes. Therefore, like acute infections (e.g. SARS) and other chronic illnesses (allergy, diabetes and autoimmunity), CKD can also be regarded as a communication disease initiated by derangements of cytokine and chemokine homeostasis activating leukocytes and disrupting their normal trafficking and apoptosis to result in nephron injury. Laboratory and clinical studies of such messenger and message pathology are firstly a noble academic pursuit elucidating the immunological mechanisms of CKD. They also constitute an applied science for (i) monitoring disease severity, (ii) assessing risk, and (iii) developing therapy. In recent years, pharmacological modulation of biological communication has targeted on the development of cytokine and chemokine antibodies, receptor antagonists, soluble receptors, and low molecular-weight inhibitors of intracellular signaling (32). Examples include anti-TNF-α monoclonal antibody (Infliximab) and EGFR tryrosine kinase inhibitors (Tarceva and Iressa) that are being used increasingly for treating rheumatoid arthritis and metastatic non-small cell lung carcinoma, respectively. The newest promising leukocyte migration inhibitors that were featured last month (September 2008) comprise α_4-integrin monoclonal antibodies Natalizumab (Tysabri) and Efaluzimab (Rativa) for anti-inflammatory therapy (33).

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3. PODOCYTE INJURY IN GLOMERULAR DISEASES

Mirjana Sabljar Matovinović

Podocytes are injured in diabetic and non-diabetic renal diseases (Figure 3.1. The spectrum of podocyte diseases).

Figure 3.1. *The spectrum of podocyte diseases. (6).*

Together with glomerular endothelial cells (GEN) and glomerular basement membrane (GBM), podocytes form the glomerular filtration barrier in the kidney (Figure 3.2.). Podocyte damage or loss is an early symptom of many kidney diseases presenting clinically with proteinuria with or without nephrotic syndrome and renal failure owing to glomerulosclerosis. Injury to other components of the glomerular filtration barrier, such as GEN and GBM, may also present with nephrotic syndrome, proteinuria and renal failure, which suggests podocyte injury is not the only cause of those abnormalities.

Figure 3.2. *Glomerular filtration barrier. 1. Fenestrated endothelium; 2. glomerular basement membrane; 3 podocyte foot processes and slit diaphragm*

The response of podocytes to injury is determined by their structure and function.

3.1 Podocyte structure

Podocytes are terminally differentiated (postmytotic) epithelial visceral cells with a unique and complex cellular organization. With respect to their cytoarchitecture, podocytes consist of three different segments: the voluminous cell body, major processes, and interdigitating foot processes. The foot processes are separated by filtration slits (slit pores), bridged by the thin slit membrane called slit diaphragm, a meshwork of proteins anchored at the sides of the foot processes. The cell body is located in the center of the cell bulging into the urinary space. Like an octopus, the podocyte cell body emits thick extensions. These structures - long primary processes branch to form the foot processes - pedicels that cover the surface of the glomerular capillary loops like the interdigitating fingers of two. The cytoskeleton composed of microfilaments comprises actin, myosin, α actinin, talin, paxillin and vinculin serving as podocyte backbone, not only maintains the shape of podocytes but also enables its continuous forming and adapting. This cytoskeleton supports the glomerular capillary wall and opposes the high hydrostatic pressure necessary for glomerular filtration. There are two actin cytoskeletal networks in foot processes: dense actin bundles above the level of the slit diaphragm running parallel to the longitudinal axis, and a cortical actin network just below the plasma membrane of the foot processes. The actin cytoskeleton is linked with other actin-binding proteins.

According to its molecular structure the podocyte has three domains: junctional, apical and basal (Figure 3.3.). The change in podocyte shape, called effacement, is not simply a passive process following injury, but occurs owing to a complex interplay of proteins that comprise the molecular anatomy of the different protein domains of podocytes.

Figure 3.3. *The three boxes (1-3) in the right half define the three domains of the foot processes: 1. junctional - slit diaphragm complex; 2. basal domain and GBM; 3. apical plasma domain with glycocalyx. Interference with any of these three domains can cause foot process effacement and nephrotic syndrome.*

The *junctional domain* encompasses slit diaphragm a complex of proteins located in the extracellular space, bridging adjacent foot processes. Tryggvason discovered the first slit diaphragm protein, nephrin. Nephrin is a transmembrane protein type I with both structural and signaling function. The cytoplasmic tail of nephrin binds to podocin. Nephrin also interacts with and localizes to CD2AP. The discovery of several proteins and their mutation

analysis, including FAT, Neph-1, which interacts with nephrin, podocin, and CD2-associated protein, Neph-2 and -3, and densin, has emphasized the critical role of the slit diaphragm in maintaining normal function of the glomerular filtration barrier. By forming the only connection between adjacent podocytes, the slit diaphragm limits protein leakage by acting as a size barrier, analogous to a sieve. The slit may also function as a charge barrier, as some of these proteins are phosphorylated. Moreover, certain slit diaphragm proteins actively participate in podocyte signaling, thereby enabling the slit to communicate with other podocyte proteins such as the actin cytoskeleton (Figure 3.4.)

Figure 3.4. *The slit diaphragm of podocytes is a specialized cell junction with signaling properties. It connects interdigitating foot processes serving as an essential part of the glomerular filtration barrier. Slit diaphargm proteins (nephrin and neph1) recruit cytoplasmatic adaptor proteins to initiate signal transduction.*

The *apical membrane domain* located on the podocyte luminal side (facing the urinary space) has a negatively charged surface coat, owing to the presence of the surface anionic proteins podocalyxin, podoplanin, and podoendin. This serves two functions. First, negative charge limits the passage of negatively charged proteins into urinary space and second, it prevents parietal cell adherence to podocytes and keeps adjacent podocytes separated. Another important molecule on the luminal membrane is GLEPP-1, which has a possible receptor function. The *basal domain* is required to anchor podocyte to the underlying GBM. α3ß1 integrin and α and ß -dystroglycans serve this function, and connect podocyte body to certain matrix proteins within GBM.

3.2 Podocyte functions

The podocyte has many functions**:** 1) it acts as a size and charge barrier to proteins; 2) supports the glomerular capillary wall maintaining the capillary loop shape; 3) opposes the high intraglomerular hydrostatic pressure; 4) provides synthesis and maintenance of the GBM; 5) produces and secretes VEGF required for GEN. The impairment of any of these functions following podocyte injury results in proteinuria and possibly renal failure.

3.3 Podocyte reaction to injury

The podocyte reaction to injury or damage in many diseases known as *foot processes effacement* is also called fusion, retraction, or simplification and *changes in podocyte number*. The foot processes effacement is characterized by flattening of foot processes due to gradual simplification of the interdigitating foot process. The whole podocyte looks flat due to retraction, widening, and shortening of the processes of each podocyte. (Figure 3.5.). The

frequency of filtration slits is reduced, giving the appearance of a continuous cytoplasmic sheet covering the GBM. According to experimental data foot processes length decreases up to 70% and the width increases up to 60% compared to normal finding. This process is not a simple passive phenomenon but rather an energy dependent event. It is initiated by rearrangement of the *podocyte actin cytoskeleton.* Normally, actin cytoskeleton determines the shape of podocytes. Therefore, any disarrangement in actin or in actin-regulating proteins might lead to a change in podocyte shape and consequently function.

Figure 3.5. *Foot processes effacement. a) normal; b) foot processes effacement*

The cytoskeleton can be altered by at least four different mechanisms:

1. Systemic or locally produced toxins, viral infection, and local activation of the renin-angiotensin system
2. Abnormalities of cytoskeleton structural proteins α-actinin-4 and synaptopodin can adversely affect cytoskeletal dynamics. For example hereditary autosomal dominant FSGS is caused by mutation in α-actinin-4 increasing the affinity for actin and resulting in a change in cytoskeletal fluidity.
3. Congenital or acquired disorders can injure the slit diaphragm proteins deranging actin and nephrin signaling and leading to cytoskeletal reorganization. Mutation in slit diaphragm protein podocin causes autosomal- recessive steroid resistant nephrotic syndrome and FSGS in children. Benzing *et al*. showed that slit diaphragm proteins send signals regulating the podocyte polarity, survival, and cytoskeleton organization. Several studies have elucidated complicated signaling pathways by which specific slit diaphragm protein regulates the actin cytoskeleton interacting with proteins outside the slit diaphragm.
4. Experimental data (laminin ß deficient mice) point to changes in the GBM structure as a cause of cytoskeletal derangements.

As already mentioned, actin cytoskeleton determines the shape of podocytes. Proteins regulating the actin cytoskeleton are of utmost importance for podocyte function, as any abnormality in actin-regulating proteins or actin itself might alter the shape and consequently the function of podocytes. Each of podocyte domains (junctional, apical and basal) as well as actin-associated proteins, synaptopodin and α-actinin-4 are linked to actin cytoskeleton enabling their interaction which, in a pathological setting, might lead to a rearrangement of the *podocyte actin cytoskeleton* and *foot processes effacement*.

Mutation and abnormalities in the slit diaphragm proteins in *junctional domain* (nephrin, podocin, CD2AP, and others) are associated with the rearrangement of the podocyte actin cytoskeleton and consequently foot processes effacement.

The *basal domain* is also important in maintaining the shape of podocyte. Two basal domain constituents, α3ß1 integrin and α and ß dystroglycans, serve as matrix receptors for podocytes, anchor podocyte to GBM and mediate podocyte cell-matrix interaction. Experimental studies have shown that splitting of dystroglycans - matrix interaction and blocking β1 integrins with an antibody, is associated with foot processes effacement.

Apical domain and foot processes effacement. The anionic apical proteins podocalyxin and podoplanin repel anionic proteins and prevent their passage into Bowman's space. Experimental data show that neutralization of the anionic surface charge leads to foot processes flattening and exposure to hyperglycemia suppresses the levels of podocalyxin. Podocalyxin overexpression inhibits cell-cell adhesion, and maintains an open filtration pathway between neighboring foot processes, i.e. neutralizing the anionic surface charge influences cell-cell adhesion and junctional permeability. There is experimental evidence linking podocalyxin directly to actin cytoskeleton, emphasizing that podocalyxin is needed to maintain foot processes shape. In summary, podocalyxin maintains podocyte shape, and a decrease in levels or loss of anionic surface charge leads to podocyte shape changes - foot processes effacement and distortion of the slit diaphragm, both leading to proteinuria. Similarly, studies have shown that injecting rats with an anti-podoplanin antibody results in podocyte effacement and proteinuria.

*Changes in podocyte number occur in renal disease and with aging***.** A decrease in podocyte number resulting from *apoptosis or detachment* from the GBM is found in diabetic as well as non-diabetic glomerular diseases. This abnormality is associated with proteinuria and glomerulosclerosis. The decreased number of podocytes leaves areas of bare GBM, which are foci for adhesions to parietal epithelial cells and crescent formation. Namely, nude areas of GBM are bulging into urinary space because the podocytes opposing the intraglomerular pressure are lost. A synchial attachment is formed upon the contact of the nude prominent part of GBM with parietal epithelial cells leading to focal segmental glomerulosclerosis. The mechanism of podocyte detachment is not well known. One explanation is that abnormalities in integrins or dystroglycans normally responsible for podocyte adhering to underlying GBM, might be the cause of podocyte detachment. As a consequence of detachment podocytes and podocyte-specific proteins can be found and measured in the urine of patients with proteinuria but not in healthy people. Measuring podocytes and their products in the urine might be a better disease marker than proteinuria.

Apoptosis is the second cause of podocyte depletion. There is evidence that slit diaphragm proteins govern podocyte survival. The research has been focused on CD2AP showing that absence or reduction of CD2AP and mutation in nephrin is associated with increased podocyte apoptosis. To survive, podocytes must be attached to GBM and once they detach the apoptosis increases significantly. This notion directed the research to α3ß1 integrin and α and ß dystroglycans, showing reduced podocyte survival with their alterations. TGF-ß induces podocytes apoptosis as well as angiotensin II. Angiotensin II blockade not only reduces systemic and intraglomerular pressures, but also podocyte apoptosis, thereby minimizing podocyte loss. Reduction of systemic and intraglomerular pressure and podocyte apoptosis reduce proteinuria and glomerulosclerosis. Hyperglycemia induces apoptosis providing a plausible explanation why the number of podocytes is reduced in diabetes.

Podocytes are terminally differentiated cells that normally cannot proliferate. They do not change their phenotype in response to injury, rendering the *inability to proliferate* the third cause of podocyte depletion. However, there are experimental data showing that podocyte can change their phenotype and proliferate in experimental crescentic glomerulonephritis and HIV nephropathy. Nevertheless, the increased and the reduced podocyte numbers are detrimenal to glomerular function. The mechanisms underlying the inability of podocytes to proliferate are being investigated.

In summary, owing to their unique and complex cellular organization and many functions, podocytes are the most vulnerable constituent of the glomerular filtration barrier. Their injury and dysfunction lead to progressive glomerular filtration barrier failure presenting as nephrotic or non-nephrotic proteinuria, glomerulosclerosis, and eventually as renal failure. Podocyte injury leads to rearrangement of the *podocyte actin cytoskeleton* and *foot processes effacement.* The major causes (genetic or acquired) of foot processes effacement are impaired formation of the slit diaphragm complex, abnormality of the GBM or adhesion of podocytes to the GBM, abnormalities in the cytoskeleton or associated proteins, and alterations in the apical membrane domain of the podocyte.

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4. KIDNEYS AND AUTOIMMUNE DISEASE

Maksimiljan Gorenjak

4.1 Autoimmune diseases

The human immune system limits invasion of foreign organisms and eliminates foreign cells. Discrimination between self and foreign structures is essential in this process. Ability to recognize ‘‘self’’ and limit ‘‘auto’’-immune responses against self-antigens is defined as tolerance. In many situations, the mechanisms either inducing or maintaining tolerance are disrupted. This breakdown leads to activation of autoreactive cells which, in turn, may initiate overt autoimmune disease.

In breaking tolerance to self-structures several underlying mechanisms act alone or in combination, including apoptosis, defective clearance of apoptotic cells, molecular mimicry and, certainly, genetics.

In order to develop autoimmune disease, an individual may possess a variety of susceptibility genes which lead to abnormalities in a number of biological pathways.

It is important to appreciate that dysfunction in multiple processes occurs simultaneously. Thus a genetic polymorphisms leading to a variety of immunological abnormalities will be molded by environmental and hormonal factors to produce a particular clinical disease phenotype.

Once an uncontrolled immune response is directed to self-structures, the consequences may be devastating. Approximately 3% of the population suffers from a so far described autoimmune disorder. An additional number of diseases may not yet have characterized autoimmune causes.

Cells of the innate and adaptive immune system participate in the development of autoimmunity. It has been observed that the majority of self-reactive immune cells are normally deleted or inactivated during development. This process has been termed central tolerance. There are also checkpoints that regulate the emergence of autoreactive cells during adult life (e.g., during immune responses versus foreign antigen); this process has been termed peripheral tolerance. Nevertheless, some cells escape both checkpoints, and their activation may lead to autoimmunity.

The generation, maintenance, and proliferation of autoreactive B and T-cells and emergence of autoimmune disease, involves the simultaneous breakdown of multiple central and peripheral checkpoints involved in the maintenance of tolerance. It is well established that the mere presence of autoreactive B or T-cells is insufficient. For example, in lupus patients autoantibodies have been detected long before the onset of clinical disease (e.g., nephritis).

4.2 Kidneys in autoimmune disease

Renal involvement in autoimmunity has many facets. Glomerular, tubular and vascular structures are targeted and damaged as a consequence of autoimmune processes.

Autoimmunity resulting in renal injury occurs as a systemic disturbance of immunity with the central feature being loss of tolerance to normal cellular and/or extracellular proteins. Some of the target autoantigens are now identified in autoimmune diseases where tissue injury includes the kidney .

In most cases, the autoantigens are non-renal and become renal targets because of the physiological properties of the high flow, high-pressure perm-selective filtration function of the glomerulus. Circulating autoantigens can deposit in glomeruli as part of circulating immune complexes or become a ‘‘planted’’ target antigen by their physico-chemical properties that predispose to their glomerular fixation.

A potentially unique model of deposition of a non-renal antigen in the kidney is seen in anti-neutrophil cytoplasmic antibody (ANCA)-associated small vessel vasculitis, where target autoantigens originating in neutrophil cytoplasmic granules and expressed in the cell membrane (including proteinase-3 [PR3] and myeloperoxidase [MPO]) are targeted by ANCA. These ANCA-activated neutrophils have altered flow characteristics resulting in their lodging in small vessels, particularly glomeruli, resulting in renal injury.

Inflammatory renal disease in the context of autoimmunity occurs because the kidney is targeted by effector responses. The effectors of autoimmunity in the kidney are many, but most often disease is initiated either by antibody deposition or infiltration of immune cells. Once antibodies are deposited, their exposed Fc (fragment crystalline) regions activate and recruit inflammatory cells, and initiate complement activation. This process leads to further cellular infiltration, and secretion of inflammatory mediators by both infiltrating and endogenous cells. Infiltrating cells, which include neutrophils, T-cells and macrophages, and platelets also secrete soluble mediators and directly interact with renal cells and each other to perpetuate the disease process.

Within the kidney, the local response of resident cells plays an important role in determining the severity of inflammation. If severe and/or unlimited, these events may lead to fibrosis and organ failure. The intensity and severity of inflammation and fibrosis are also influenced by genetic factors (e.g., that determine the fibrogenic response).

As mentioned, one can envision several ways by which the kidneys become involved. Among the possibilities, renal tissue may harbour a self-antigen (e.g. the ‘‘Goodpasture antigen’’). In addition, the kidneys may become affected by antibody-mediated mechanisms where the autoantigen resides outside the kidney. Deposition of resulting immune-complexes within the kidneys subsequently triggers tissue damaging events (e.g. lupus nephritis). Third, antigen and antibodies are neither derived nor deposited within the kidneys. However, the interaction of antibodies with the antigens, or with antigen-bearing cells, causes the disease (e.g. ANCA vasculitis and glomerulonephritis).

4.2.1 Anti-GBM disease

Anti-glomerular basement membrane (anti-GBM) disease is the best-defined renal organ-specific autoimmune disease. The disease is strongly associated with autoantibody formation to a specific target found in the glomerular and alveolar basement membranes and is characterized by a rapidly progressive glomerulonephritis (RPGN) which is often associated with pulmonary hemorrhage, though either may occur alone.

Collagen IV is a major component of the GBM. Six alpha chains of type IV collagen are known and these chains form triple helical molecules (protomers). The major antigen of the circulating and deposited anti-GBM antibodies is the non-collagenous domain of the type IV collagen alpha-3 chain(a3(IV)NC1).

Diagnosis is based on the demonstration of anti-GBM antibodies, either in the circulation or fixed to basement membrane of affected organs on biopsy.

Probably the best test for anti-GBM is the renal biopsy with the detection of linear IgG depositions along the GBM. However, most patients also have circulating anti-GBM antibodies in their plasma detected by enzyme-linked immunosorbent assay (ELISA) or Western blotting. The majority of these antibodies are of the IgG1 subtype, with only few IgG4 antibodies. Very rarely, patients have no detectable anti-GBM IgG, but IgA or IgM antibodies instead.

4.2.2 Lupus nephritis

Systemic lupus erythematosus (SLE) is the prototypic systemic autoimmune disease with widespread clinical manifestations. The prevalence of renal involvement depends strongly on the definition. Almost 100% of the patients will have renal manifestation if immunoglobulin deposition is the criterion, whereas the percentage is approximately 50% if proteinuria is applied. Renal involvement is one of the most serious complications, since nephritis may progress into end stage renal disease (ESRD) and is associated with increased mortality. Changing classifications were applied over past decades. More recently, the ISN/RPS 2003 classification was introduced. The most severe lesions are found in Class IV, with diffuse proliferative GN.

Several autoantibodies are generated in lupus patients (anti-nuclear antibodies (ANAs) and anti-double stranded DNA antibodies (dsDNA) included in diagnostic criteria).

Not all of these antibodies seem to mediate renal damage or indicate renal involvement. For nephrologists, antibodies to anti-C1q and to nucleosomes are of particular interest. Nucleosomes consist of DNA and histones. Anti-nucleosome antibodies may occur even before the development of anti-DNA antibodies and were found in patients as well as in murine disease models.

Nucleosomes are generated during apoptosis as a consequence of linker DNA cleavage between the nucleosomes. Nucleosomes are then presented in membrane blebs that are characteristic of apoptotic cells. Presentation of nucleosomes within blebs results in T-cell-driven B-cell stimulation. It is suggested that complexes of nucleosomes and the resulting antinucleosome antibodies bind to heparan sulphate-rich glomerular structures and induce the inflammatory reactions leading to glomerulonephritis.

4.2.3 ANCA-associated vasculitis and glomerulonephritis

The most frequent subgroup of primary systemic vasculitis is that associated with circulating autoantibodies to neutrophil cytoplasmic antigens (ANCA), with involvement of microscopic blood vessels without immune deposits in the vessel walls, ‘‘pauci-immune micro-vasculitis’’. They are also the most frequent autoimmune diseases that affect the kidneys in a rapidly progressive manner. Glomerulonephritis, with fibrinoid necrosis and crescent formation, is common.

ANCA are autoantibodies that are directed to neutrophil and monocyte constituents. ANCA are found in sera of patients with Wegener’s granulomatosis (WG), microscopic polyangiitis (MPA), Churg-Strauss syndrome (CSS) or a renal-limited form presenting with necrotizing crescentic glomerulonephritis (ANCA-GN).

ANCA are detected by indirect immunofluorescence on ethanol-permeabilized neutrophil preparations. A fixation artefact actually leads to the fact that a cytoplasmic ANCA pattern (c-ANCA) can be distinguished from a perinuclear pattern (p-ANCA).

Detailed studies identified proteinase 3 (PR3) and myeloperoxidase (MPO) as the major ANCA antigens. ANCA specificity to these antigens is tested by the use of enzyme-linked immunoassays (ELISA). The c-ANCA mainly recognizes PR3,whereas p-ANCA bind to MPO. However, p-ANCA also recognizes non-MPO molecules, including elastase, lactoferrin, lysozyme and cathepsin G. The perinuclear staining pattern results from distribution of cationic MPO along the negatively charged nuclear membrane after ethanol treatment of the neutrophils.

The p-ANCA pattern becomes a cytoplasmic pattern when MPO-ANCA is tested on formalin fixed neutrophil. An ANCA work-up should always include IF and PR3 and MPO ELISA. Over the past two decades, ANCA has become an important diagnostic tool. However several issues need to be considered when employing ANCA testing. These points include pretest patient selection, technical issues and consideration of the clinical context.

In addition to being a clinical tool, ANCA are causal for the disease induction. The central mechanism in inducing vasculitis is the interaction of ANCA with the neutrophil that contains the ANCA antigens. The majority of MPO and PR3 are stored in neutrophil granules. This granule pool is mobilized to the cell membrane during cytokine-mediated neutrophil priming. PR3 and MPO translocation is controlled by p38 MAPK. ANCA bind to cell surface-expressed ANCA antigens, resulting in subsequent neutrophil activation. The activation process involves cross-linking of ANCA antigens on the cell surface and Fc-gamma receptor signals. ANCA-activated neutrophils respond by generation of reactive oxygen species, degranulation of proteolytic enzymes and up-regulation of adhesion molecules. PI3-K/Akt signaling is central to the activation process.

ANCA-activated neutrophils adhere to and damage endothelial cells. Interestingly, this neutrophil-endothelial cell interaction results in suppression of ANCA-stimulated superoxide production, whereas degranulation of toxic molecules is accelerated.

In the most likely scenario, neutrophils, once rolling over the endothelial surface, become primed, express PR3/MPO, and interact with ANCA. This interaction leads to firm adhesion, transmigration, and also local endothelial damage, all compatible with necrotizing vasculitis and glomerulonephritis.

4.3 Conclussions - what the future might hold

Numerous human and animal studies support the hypothesis that for example lupus nephritis is an immune complex disease and signal the potential therapeutic benefit of suppressing autoantibody production.

The clinical utility of testing for autoantibodies is immediately apparent but even robust associations between specific immunoglobulins and particular autoimmune diseases or patterns of organ involvement do not guarantee a causal link.

Anti-double stranded DNA antibodies were first characterized 50 years ago and it is 25 years since anti-neutrophil cytoplasm antibodies were discovered. Anniversaries coincide with a growing enthusiasm for the use of B-cell targeted therapies in proliferative lupus nephritis and systemic ANCA-vasculitis, the diseases with which these autoantibodies are respectively linked.

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5. HEREDITARY KIDNEY DISORDERS

Ana Stavljenić-Rukavina

5.1 Introduction

Hereditary kidney disorders represent significant risk for the development of end stage renal desease (ESRD). Most of them are recognized in childhood, or prenataly particularly those phenotypicaly expressed as anomalies on ultrasound examination (US) during pregnancy. They represent almost 50% of all fetal malformations detected by US (1). Furthermore many of urinary tract malformations are associated with renal dysplasia which leeds to renal failure.

Recent advances in molecular genetics have made a great impact on better understanding of underlying molecular mechanisms in different kidney and urinary tract disorders found in childhood or adults. Even some of clinical syndromes were not recognized earlier as genetic one. In monogenic kidney diseases gene mutations have been identified for Alport syndrome and thin basement membrane disease, autosomal dominant polycystic kidney disease, and tubular transporter disorders. There is evident progress in studies of polygenic renal disorders as glomerulopathies and diabetic nephropathy. The expanded knowledge on renal physiology and pathophysiology by analyzing the phenotypes caused by defected genes might gain to earlier diagnosis and provide new diagnostic and prognostic tool. The global increasing number of patients with ESRD urges the identification of molecular pathways involved in renal pathophysiology in order to serve as targets for either prevention or intervention. Molecular genetics nowadays possess significant tools that can be used to identify genes involved in renal disease including gene expression arrays, linkage analysis and association studies.

5.2 Major monogenic kidney diseases

Alport syndrome is a hereditary progressive nephropathy characterized by lamellation and splitting of glomerular basement membrane (GBM) and associated with sensorineural defect leading to hearing loss and ocular defects (2). It is recognized in early childhood by the hematuria and later progression to renal failure, predominantely in males before the age of six. In 85% families it was confirmed X-linked dominant inheritance. After years of recurrent or persistent hematuria, renal insufficiency is noted to occur, usually in the third or forth decade of life, occasionally before the age of twenty. Nephrotic syndrome may occurs in 30 - 40% of patients. Hearing loss is variable, ranging from complete deafness to high-frequency loss detected by audiometric exam. Associated abnormalities may include megalocornea, lenticlonus, spherophakia, myopia, retinitis pigmentosa, and macrothrombocytopenia. In females, the disorder is usually mild, with only microscopic hematuria, and does not typically progress to renal failure. The disease occurs at a gene frequency of 1/5000 and is transmitted in most families as X-linked dominant trait (2). The variety of mutations in COL4A5 gene is underlying cause (3) (Table 5.1.). The disease is closely connected to other, thin membrane disease, which is associated with COL4A3 and COL4A4 gene, members of gene families responsible for type IV collagen synthesis. Collagen type IV is a major component of basement membranes and different mutations are underlying defect in all Alport syndrome and related diseases.

The term *thin-basement – membrane nephropathy* characterized by diffuse thining of GBM is often associated with urinary abnormalities and correspond not to a single clinical syndrome and should be differentiated from thickened GMB with split lamina densa as most characteristic ultrastructural lesion in Alport syndrome.

Table 5.1. *Genes involved in major kidney disorders*

The most prevalent hereditary kidney disease is autosomal dominant **polycystic kidney disease (APKD)** (1/400- 1/1000) individuals) caused by genetic changes of PKD1 gene located on chromosome 16 encoding membrane protein polycystin.The expression of PKD1 protein was licalized ti the tubular epithelium (podocytes). Polycystin protein contain a large extracellular adhesive component, a series of 13 membrane-spanning domains and at the C terminus a cytoplasmatic tait. The pathophysiological background of clinical symptoms is probably in the function of this protein. Polycystin is responsible for maintaining of renal epithelial differentiation and organization (4-6). Polycystin is involved in the signal conveying. The signal normaly conveyed from the polycystin legends in the extracellular space to the interior of cell is dirupted by mutations in PKD1 which probably leads to abnormal differentiation of tubular cells and cyst formation. Furthermore it was shown that PKD1 gene in some patients is contiguous to one of genes involved in other disease (TSC2 gene) named tuberous sclerosis (7).

PKD2 gene is localized on chromosome 4 and PKD2 protein contain more restricted extracellular domain than PKD1 and the structure is compatible with one ion channel[4]. PKD 2 protein has six transmembrane spans but the N and C- terminal domains has aminoacid similarity with PKD1 protein. It was suggested that PKD2 protein belong to family of

voltage-activated calcium and sodium channel and therefore it is speculated that both PKD1 and PKD2 proteins are involved in a common signal transduction pathway.

The clinical manifestations of APKD as pain, bleeding, infection and stone have been known for decades. The most frequent complication is progressive renal failure which leads to end stege renal disease (ESRD) at age between 40-59. But the large deletions disrupting both PKD1 and TSC2 gene are responsible for early progression of APKD and ESRF in young children.

The determinants of progression are both genetic and non-genetic (infections, comorbidity), the rate of progression is slower in PKD2 families, in females than in males, in whites than in black patients. Hypertension is an early complication leading to ventricular hypertrophy. The earlier clinical intervention might be of benefit for prevention of cardiovascular complications. Potential complications are additionally liver cysts, gastrointestinal manifestations, portal hypertension and fatal intracranial aneurism rupture.

In comparison with autosomal dominant PKD where cysts arise from any tubular segment in autosomal recessive PKD (ARPKD) the renal cysts develop from collecting ducts. Beside renal involvement congenital liver fibrosis and portal hypertension were found in early life.

Further cystic disease complex include **juvenile nephronophthisis** characterized by diffuse interstitial fibrosis with thickened and multilayered tubular basement membranes. The leading finding are medullar cysts. It is an autosomal recessive disease caused by gene located on chromosome 2 (8, 9).

Beside APKD renal cysts sometimes may be found in other patients suffering from **tuberous sclerosis (TSC) and von Hippel Lindau disease (VHL).**

Nephrogenic diabates insipidus as congenital form include X linked recessive and autosomal recessive types.The various mutations of gene ADHRV2 that encodes V2 ADH receptor in the collecting tubular cells (10). or heterozygous gene mutations encoding aquaporin-2, a water channel in the collecting tubule (11, 12) are major genetic background. Clinical symptoms are characterized by insensitivity of renal concentrating system to the effects of antidiuretic hormone arginine vasopressin (ADH).

Other hereditary disorder of tubular transport system is **Liddle's syndrom** caused by gene mutations encoding of β and γ subunits of Na^+ channels.

Hypocalcemic alkalosis associated with hypocalcinuria and hypomagnesemia are biochemical characteristics of other tubular transporter disorder named **Gitelman syndrome.** Other three hereditary disorders of hypercalciuric nephrolithiasis (X-linked recessive nephrolithiasis, Dent' disease and X-linked phosphemic rickets) are caused by mutations in the same CLCN5 gene which encodes kidney Cl^- channel (13). Laboratory findings are characterized by low-molecular weight proteinuria and hypercalciuria. Those disorders might participate in the pathogenesis of essential hypertension. Some of these disorders are complicated with nephrolithiasis. Cystinuria, autosomal recessive disorder, due to defect in dibasic tubular reabsorption liads to stone formation, Dent's disease and X-linked calcium nephrolithiasisare also characterized by proximal tubular dysfunction .

Autosomal recessive **Bartters syndrome** is recently characterized as mutation of gene encoding for burnetanide/furosemide sensitive Na-K-2 C/co-transporter located in the apical membrane of ascending limb of Henleys loop (14). The inhibitory mutations of gene encoding for Na-Cl co-transporter inhibited by thiezide are found and this explains why this syndrome encompasses abnormalities reminiscent of long-term thiazide administration as well as low blood pressure. In the contrary activating mutations of these genes are accompanied with high blood pressure. (Liddle's syndrom).

A number of inherited metabolic disorders have an significant impact on kidney function. Most promiments metabolic disorders with prominent glomerular involvment as Anderson-Fabry's disease, lecitin-cholesterol acyl transferase (LCAT) deficiency, genetic amyloidosis have been identified recently and diagnostic methods improved by new technologies (PCR in real time, microarray). Moreover the improvement of diagnostic methods for prominent extraglomerular metabolic diseases with renal involvment as for hyperoxaluria,uremic nephropathy,cysinosis, APRT deficiency and mitochondrial cytopathies brings to clinicians new potentials for earlier diagnosis and intervention as well.

5.3 Polygenic kidney disorders

The association between glomerulonephritis and some genetic potential background were studied in recent years .An insertion/deletion polymorphism in intron 16 of angiotenzin-converting enzyme (ACE) gene studied in the number of patients with glomerulonephritis as well as other chronic disorders did not bring new data. The DD genotype on the contrary was found to be associated with rapid progression in IgA nephropathy. In addition IgA nephropathy patients with DD genotype respond to ACE inhibition therapy with lisinopril for decresing proteinuria (15, 16).

The association studies concerning diabetic nephropathy gave contradictory results. Several studies have found an association between a trinuleotide repeats in exon 2 of the CNDP1 gene which encodes carnosine and diabetic nephropathy (17).

Renal injuries with monigenic cause represent a small but significant fraction of the total spectrum of renal diseases. The most common types of renal disoreders are the result of complex interplay between multigenic and environmental interplay. But there is no doubt that altered expression of genes that are mutated in monogenic kidney damage are contributing to a great extent to acquired renal damage. Further studies should determine the nature of association between genetic and environmental factors involved in renal injury and progression of disease

5.4 The strategies for research of genes potentially involved in kidney disease

The technologies developed for the Human Genome Project, the recent surge of available DNA sequences resulting from it and the increasing pace of gene discoveries and characterization have all contributed to new technical platforms that have enhanced the spectrum of disorders that can be diagnosed. The importance of determining the disease-causing mutation or the informativeness of linked genetic markers before embarking upon a DNA-based diagnosis is, however, still emphasized.

Fluorescence in situ hybridization (FISH) technologies provide increased resolution for the elucidation of structural chromosome abnormalities that cannot be resolved by more conventional cytogenetic analyses, including microdeletion syndromes, cryptic or subtle duplications and translocations, complex rearrangements involving many chromosomes, and marker chromosomes (Figure 5.1. FISH technologies in molecular cytogenetic studies).

Figure 5.1. *FISH technologies for molecular cytogenetic studies.*

Interphase FISH and the *quantitative fluorescence polymerase chain reaction* are efficient tools for the rapid diagnosis of selected aneuploidies, the latter being considered to be most cost-effective if analyses are performed on a large scale.

Interphase and metaphase FISH, either as a single probe analysis, or using multiple chromosome probes, can give reliable results in different clinical situations. It should be noted that there may be variation in probe signals both between slides (depending on age, quality, etc. of metaphase spreads) and within a slide. Where a deletion or a rearrangement is suspected, the signal on the normal chromosome is the best control of hybridisation efficiency and control probe additionaly provides an internal control for the efficiency of the FISH procedure.

Depending on the sensitivity and specificity of the probe and on the number of cells scored, the possibility of mosaicism should be considered, and comments made where appropriate. By using locus-specific probes at least 5 cells should be scored to confirm or exclude an abnormality. In multiprobe analysis: three cells per probe should be scored to confirm a normal signal pattern. Where an abnormal pattern is detected, confirmation is advisable.

More recently new method for fast identification of chromosomal abnormalities has been developed as high resolution array comparative genomic hybridization (aCGH) which provide genome-wide analysis of chromosome copy number and structural change.

DNA as analyte in genetic testing may be isolated from different biological material as peripheral blood, amnionic fluid, chorion villi, or maternal blood as free DNA. Todays techniques for gene mutation analysis are in general modification of polymerase chain reaction (PCR) technique where small quantity of DNA “in vitro” is multiplicated by under

the activity of specific enzyme, the presence of primers and nucleotide mixture. The quantity of DNA obtained "in vitro" allow further the aplication of other analytical technologies for detection of mutation, deletion or other changes. Single strand conformation polymorphism (SSCP) method is used for detection of small mutations of gene and still is the most convenient method for detection of mutations of particular exons, as step before confirmation of mutation by sequencing. PCR in real time combine PCR and automatic multicolor fluorescence analysis of mutations and deletions allow fast analysis of number of DNA samples (18).

Sequencing of DNA molecule allow determination of subsequent nucleotide A(denin), T(imin), G(uanin) and C(itozin) sequence. Multicolor, multichanell automatic sequencing with fluorescence emission developed in last five years made this procedure fast, efficient and safe.

The high throughput microarray technologies combined with robotics are the newest development in molecular genetic testing. The application of this technology and its different modifications allowed to analyze whole gene or more genes simultaneously which bring to clinicians new tool for rapid and safe diagnostic procedures (18). The advances in automation of analytical procedures and fast growing of test number bring to analyst the need for broad external quality assessment by certified proficiency testing bodies. In general, every laboratory which delivers test results for prenatal care should be recognized by certified referral laboratory for each test performed in this laboratory.

Gene expression array is used for profile gene expression. The advantage of this technique is that it enables innovative study design such as integration with other techniques and comparison between tissues or cell types (19).

Linkage analysis is recommended for identification of a gene or genetic region that has large effect on phenotype. It allows the causal relationship between genotype and phenotype. The limitation is that it requires rare families for evaluation of results (19).

The association analysis enables to identify common susceptibility variants underlying the disease and is suitable for study of complex diseases. Nevertheless it requires large cohorts, higher costs and clinical significance of association is unknown.

Genome-wide association study is recommended for the study of genetic factors that influence common, complex diseases with high throughput covering whole genome. The large number of participants is needed for genes that have not very strong influence.

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6. DIABETIC NEPHROPATHY

Gábor L. Kovács

6.1 Summary

Between 20% and 40% of patients with diabetes ultimately develop diabetic nephropathy, which in the US is the most common cause of endstage renal disease requiring dialysis. Diabetic nephropathy has several distinct phases of development and multiple mechanisms contribute to the development of the disease and its outcomes. This Review provides a summary of the latest published data dealing with these mechanisms; it focuses not only on candidate genes associated with susceptibility to diabetic nephropathy but also on alterations in various cytokines and their interaction with products of advanced glycation and oxidant stress. Additionally, the interactions between fibrotic and hemodynamic cytokines, such as transforming growth factor β1 and angiotensin II, respectively, are discussed in the context of new information concerning nephropathy development. We touch on the expanding clinical data regarding markers of nephropathy, such as microalbuminuria, and put them into context; microalbuminuria reflects cardiovascular and not renal risk. If albuminuria levels continue to increase over time then nephropathy is present. Lastly, we look at advances being made to enable identification of genetically predisposed individuals.

6.2 Introduction

Diabetic nephropathy is the most common cause of end-stage renal disease requiring dialysis in the US (1). The incidence of diabetic nephropathy in this country has increased substantially over the past few years. Advanced diabetic nephropathy is also the leading cause of glomerulosclerosis and end-stage renal disease worldwide (2, 3). Between 20% and 40% of patients with diabetes ultimately develop nephropathy, although the reason why not all patients with diabetes develop this complication is unknown.

The natural history of diabetic nephropathy differs according to the type of diabetes and whether microalbuminuria (defined as > 30 mg but < 300 mg albumin in the urine per day) is present. If untreated, 80% of people who have type 1 diabetes and microalbuminuria will progress to overt nephropathy (i.e. proteinuria characterized by > 300 mg albumin excreted daily), whereas only 20-40% of those with type 2 diabetes over a period of 15 years will progress. As Nielsen *et al*. (4) demonstrated more than a decade ago, a clear, early predictor of disease progression is increasing systolic blood pressure, even within the prehypertensive range. Among patients who have type 1 diabetes with nephropathy and hypertension, 50% will go on to develop end-stage renal disease within a decade (5). Mortality among dialysis patients with diabetes is 22% higher in the first year following the initiation of dialysis and 15% higher at 5 years than that among dialysis patients without diabetes (6).

Diabetic nephropathy has several distinct phases of development. Functional changes occur in the nephron at the level of the glomerulus, including glomerular hyperfiltration and hyperperfusion, before the onset of any measurable clinical changes. Subsequently, thickening of the glomerular basement membrane, glomerular hypertrophy, and mesangial expansion take place. Seminal studies by Mauer and colleagues (3) and Steinke and

colleagues (7) demonstrated that individuals with type 1 diabetes and microalbuminuria in whom these histological alterations were detected were destined to progress to overt nephropathy. Microalbuminuria, however, has a variable course; its progression to macroalbuminuria (> 300 mg per day) is unpredictable and does not always lead to development of nephropathy (7). Moreover, the rate of kidney function decline after the development of nephropathy is highly variable between patients and is influenced by additional factors, including blood pressure and glycemic control.

Multiple mechanisms contribute to the development and outcomes of diabetic nephropathy, such as an interaction between hyperglycemia-induced metabolic and hemodynamic changes and genetic predisposition, which sets the stage for kidney injury (8). Hemodynamic factors are the activation of various vasoactive systems, such as the renin-angiotensin-aldosterone and endothelin systems. In response, secretion of profibrotic cytokines, such as transforming growth factor β1 (TGF-β1), is increased and further hemodynamic changes occur, such as increased systemic and intraglomerular pressure. Metabolic pathway involvement, among other features, leads to nonenzymatic glycosylation, increased protein kinase C (PKC) activity, and abnormal polyol metabolism. Findings from various studies support an association between increased secretion of inflammatory molecules, such as cytokines, growth factors and metalloproteinases, and development of diabetic nephropathy (9). Oxidative stress also seems to play a central part (11). Studies that have used inhibitors of the pathways involved in genesis of diabetic nephropathy have shed light on the pathogenesis of this condition but have not led to expansion of the therapeutic armamentarium to halt the disease process (10). This Review is intended for a clinical audience and we discuss pathological changes to the glomeruli during the development of diabetic nephropathy. Although many factors have been implicated in the pathogenesis of diabetic nephropathy, we have focused on the particular factors outlined above.

6.3 Hemodynamic pathways

The early signs of glomerular hyperperfusion and hyperfiltration result from decreased resistance in both the afferent and efferent arterioles of the glomerulus. The afferent arteriole seems to have a greater decrease in resistance than the efferent. Many factors have been reported to be involved in this defective autoregulation, including prostanoids, nitric oxide, vascular endothelial growth factor (VEGF; now formally known as VEGF-A), TGF-β1, and the renin–angiotensin system, specifically angiotensin II. These early hemodynamic changes facilitate albumin leakage from the glomerular capillaries and overproduction of mesangial cell matrix, as well as thickening of the glomerular basement membrane and injury to podocytes (12). In addition, increased mechanical strain resulting from these hemodynamic changes can induce localized release of certain cytokines and growth factors (13, 14).

The renal hemodynamic changes are mediated partly by the actions of vasoactive hormones, such as angiotensin II and endothelin. Glomerular hypertension and hyperfiltration contribute to the development of diabetic nephropathy because use of renin–angiotensin blockers preserves kidney function and morphology. Blockade of the renin–angiotensin–aldosterone system antagonizes the profibrotic effects of angiotensin II by reducing its stimulation of TGF-β1 (15). Support that such profibrotic effects underlie diabetic nephropathy has also been provided by study of an animal model of diabetic nephropathy (16). Transient blockade of the renin–angiotensin system (for 7 weeks) in prediabetic rats reduced proteinuria and improved glomerular structure. Additionally, the administration of an angiotensin-converting-enzyme inhibitor to patients with type 1 diabetes and nephropathy lowered serum

concentrations of TGF-β1 (17). A correlation exists between decreased levels of TGF-β1 in serum and urine and renoprotection, as determined by changes in the glomerular filtration rate over time. We discuss this effect further in the Cytokines section.

6.4 Hyperglycemia and advanced glycosylation end products

Hyperglycemia is a crucial factor in the development of diabetic nephropathy because of its effects on glomerular and mesangial cells, but alone it is not causative. Mesangial cells are crucial for maintenance of glomerular capillary structure and for the modulation of glomerular filtration via smooth-muscle activity. Hyperglycemia is associated with an increase in mesangial cell proliferation and hypertrophy, as well as increased matrix production and basement membrane thickening. *In vitro* studies have demonstrated that hyperglycemia is associated with increased mesangial cell matrix production (18, 19) and mesangial cell apoptosis (20, 21). Mesangial cell expansion seems to be mediated in part by an increase in the mesangial cell glucose concentration, since similar changes in mesangial function can be induced in a normal glucose milieu by overexpression of glucose transporters, such as GLUT1 and GLUT4, thereby increasing glucose entry into the cells (19).

Hyperglycemia might also upregulate VEGF expression in podocytes (14), which could markedly increases vascular permeability (22, 23). Hyperglycemia, however, does not account fully for the risk of diabetic nephropathy, as shown by studies in which kidneys from nondiabetic donors were transplanted into patients with diabetes and nephropathy developed irrespective of the glucose control (24). Hyperglycemia might, therefore, be necessary for but not sufficient to cause renal damage.

Three mechanisms have been postulated that explain how hyperglycemia causes tissue damage: nonenzymatic glycosylation that generates advanced glycosylation end products, activation of PKC, and acceleration of the aldose reductase pathway (25, 26). Oxidative stress seems to be a theme common to all three pathways (27).

6.5 Glycosylation

Glycosylation of tissue proteins contributes to the development of diabetic nephropathy and other microvascular complications. In chronic hyperglycemia, some of the excess glucose combines with free amino acids on circulating or tissue proteins. This nonenzymatic process affects the glomerular basement membrane and other matrix components in the glomerulus and initially leads to formation of reversible early glycosylation end products and, later, irreversible advanced glycosylation end products. These advanced products can be involved in the pathogenesis of diabetic nephropathy by altering signal transduction via alteration in the level of soluble signals, such as cytokines, hormones and free radicals. Circulating levels of advanced glycosylation end products are raised in people with diabetes, particularly those with renal insufficiency, since they are normally excreted in the urine (28). The net effect is tissue accumulation of advanced glycosylation end products (in part by cross-linking with collagen) that contributes to the associated renal and microvascular complications (29). Moreover, advanced glycosylation end products (AGE) interact with the AGE receptor, and nitric oxide concentrations are reduced in a dose-dependent manner (30).

6.6 Protein kinase C

Other proposed mechanisms by which hyperglycemia promotes the development of diabetic nephropathy include activation of PKC (31). Specifically, activation of this enzyme leads to increased secretion of vasodilatory prostanoids, which contributes to glomerular hyperfiltration. By activation of TGF-β1, PKC might also increase production of extracellular matrix by mesangial cells (32).

The mechanism by which hyperglycemia leads to PKC activation involves *de novo* formation of diacylglycerol and oxidative stress (33). PKC activation induces the activity of mitogen-activated protein kinases (MAPK) in response to extracellular stimuli through dual phosphorylation at conserved threonine and tyrosine residues. The coactivation of PKC and MAPK in the presence of high glucose concentrations indicates that these two families of enzymes are linked (34).

6.7 Aldose reductase pathway

The polyol pathway is implicated in the pathogenesis of diabetic nephropathy. A number of studies have shown a decrease in urinary albumin excretion in animals administered aldose reductase inhibitors (35), but in humans these agents have not been studied widely and the results are inconclusive.

6.8 Prorenin

Initial clinical studies in children and adolescents suggest that increased plasma prorenin activity is a risk factor for the development of diabetic nephropathy (36, 37). The prorenin receptor in the kidney is located in the mesangium and podocytes, and its blockade has a beneficial effect on kidneys in animal models of diabetes. This effect is mediated by intracellular signals that are both dependent on and independent of the renin–angiotensin system. Prorenin binds to a specific tissue receptor that promotes activation of p44/p42 MAPK (38).

A possible pathogenic role for prorenin in the development of diabetic nephropathy was noted in an experimental model of diabetes-mice with streptozotocin-induced diabetes. Sustained prorenin-receptor blockade abolished MAPK activation and prevented the development of nephropathy despite an unaltered increase in angiotensin II activity (39).

If prorenin is a key player in the pathogenesis of this disease, use of renin inhibitors for hypertension that increase prorenin concentrations should demonstrate a harmful effect. To date, no such adverse effects have been reported.

6.9 Cytokines

Activation of cytokines, profibrotic elements, inflammation, and vascular growth factors such as VEGF might be involved in the matrix accumulation that arises in diabetic nephropathy (40-42). Although some evidence suggests that VEGF increases permeability of the glomerular filtration barrier to proteins (22), levels of this growth factor can be low in patients with diabetic nephropathy. Thus, the role of VEGF in the pathophysiology of nephropathy is unclear.

Hyperglycemia is thought to stimulate VEGF expression and, therefore, act as a mediator of endothelial injury in human diabetes (40, 41). Studies showed initially that in patients with diabetic nephropathy the degree of neovascularization was increased and correlated with expression of VEGF and angiopoetin (43-45). Later findings, however, showed that levels of VEGF messenger RNA were actually decreased in patients with diabetic nephropathy (46). Evidence against the roles of VEGF and angiopoetin demonstrates promotion of vessel leakage and reduction in transendothelial electrical resistance; these two growth factors have key roles in development of retinopathy and contribute to nephropathy development in animal models.

Further evidence to support a pathogenic role for VEGF in diabetic nephropathy is the observation that VEGF blockade improves albuminuria in an experimental model of the disorder (41, 42). Animal studies that used a neutralizing antibody to VEGF demonstrated the involvement of this growth factor in glomerular hypertrophy and mesangial matrix accumulation (41, 47). High glucose levels, TGF-β1, and angiotensin II stimulate VEGF expression, which leads to the synthesis of endothelial nitric oxide. This action promotes vasodilatation and hyperfiltration, which are the early processes in diabetic nephropathy. VEGF also stimulates the production of the α3 chain of collagen IV, an important component of the glomerular basement membrane. Indirect evidence suggests that increased production of this collagen chain contributes to the thickening of the glomerular basement membrane observed in diabetic nephropathy. In animal studies, administration of an antibody to VEGF decreased urinary albumin excretion compared with that in untreated diabetic controls (22).

Findings from some studies refute a causative role for high VEGF levels in diabetic nephropathy. Instead, results imply that low levels are harmful. Eremina *et al*. (48) showed in a mouse model that VEGF is produced by podocytes and is necessary for glomerular endothelial cell survival and differentiation as well as for mesangial cell development and differentiation. Gene expression of VEGF is decreased in humans with diabetic nephropathy (49), although whether this effect is due to podocytes loss, leading to reduced production of VEGF, has been questioned. Baelde *et al*. (46) showed that VEGF messenger RNA concentrations were decreased in the glomeruli of patients with diabetic nephropathy and correlated with reduction in the number of podocytes and progression of renal disease.

Hyperglycemia also increases the expression of TGF-β1 in the glomeruli and of matrix proteins specifically stimulated by this cytokine (42). In the glomeruli of rats with streptozotocininduced diabetes, TGF-β1 levels are increased, and use of a neutralizing antibody to TGF-β1 prevents renal changes of diabetic nephropathy in these animals. In addition, connective tissue growth factor and heat shock proteins, which are encoded by TGF-β1-inducible genes, have fibrogenic effects on the kidneys of patients with diabetes. However, diabetes is associated with decreased expression of renal bone morphogenetic protein 7, which in turn seems to counter the profibrogenic actions of TGF-β1 (17). Evidence clearly shows that TGF-β1 contributes to the cellular hypertrophy and increased synthesis of collagen, both of which occur in diabetic nephropathy (17, 42, 50, 51). Further evidence for these actions is provided by studies in which the combination of an antibody to TGF-β1 plus an angiotensin-converting-enzyme inhibitor normalized levels of protein in the urine of rats with diabetic nephropathy; proteinuria was only partly resolved with the use of an angiotensin-converting-enzyme inhibitor alone (52). Glomerulosclerosis and tubulointerstitial injury were also improved by the combined therapy.

The administration of hepatocyte growth factor, which specifically blocks the profibrotic actions of TGF-β1, ameliorates diabetic nephropathy in mice (53).

Inflammatory cytokines also contribute to the development and progression of diabetic nephropathy, specifically interleukin 1 (IL-1), IL-6 and IL-18 and tumor necrosis factor. Concentrations of all these cytokines were increased in models of diabetic nephropathy and seemed to affect the disease via multiple mechanisms. In addition, raised levels of several of these cytokines in serum and urine correlate with progression of nephropathy, indicated by increased urinary albumin excretion (54).

Each cytokines has several different effects. IL-1 alters the expression of chemotactic factors and adhesion molecules, alters intraglomerular hemodynamics (by affecting mesangial cell prostaglandin synthesis), might increase vascular endothelial cell permeability, and increases hyaluron production by renal tubular epithelial cells (which in turn could increase glomerular cellularity) (55). IL-6 has a strong association with the development of glomerular basement membrane thickening as well as possible relationships with increased endothelial permeability and mesangial cell proliferation. IL-18 induces the production of other inflammatory cytokines, such as IL-1, interferon γ and tumor necrosis factor, and might be associated with endothelial cell apoptosis. Tumor necrosis factor has the widest variety of biological activities and effects that contribute to development of diabetic nephropathy - too many to describe here. Importantly, though, it causes direct renal injury as a cytotoxin, as well as affecting apoptosis, glomerular hemodynamics, endothelial permeability, and cell-cell adhesion. It also seems to play an important part in the early hypertrophy and hyperfunction of diabetic nephropathy (54, 56, 57).

6.10 Lipid mediators

Small lipids derived from arachidonic acid have been implicated in the pathogenesis of diabetic nephropathy. Cyclo-oxygenase 2 breaks down arachidonic acid into several different prostanoids. In a rat model of streptozotocin-induced diabetes, levels of inflammatory prostanoids, such as prostaglandins E_2 and I_2, were raised (58). Furthermore, increased expression of cyclooxygenase 2 has been reported in animal studies of diabetes and in the macula densa of kidneys from humans with diabetes (59). In diabetic rats, inhibition of cyclo-oxygenase 2 is associated with decreased glomerular hyperfiltration (60). A more detailed characterization of how the production of prostanoids affects the pathogenesis of diabetic nephropathy is needed.

Arachidonic acid can also be oxidized by lipoxygenases (61). Evidence is accumulating that some of the products derived from the actions of lipoxygenases contribute to diabetic nephropathy. Specifically, levels of lipoxygenases 12 and 15 are increased in diabetic animals. In addition, high glucose levels increase expression of lipoxygenases 12 and 15 in cultured mesangial cells. To conclude, this pathway has a key mediatory role in the critical processes of mesangial cell hypertrophy and extracellular matrix accumulation mediated by TGF-β1 and angiotensin II (61).

6.11 Oxidative stress

Generally, metabolic activity within the nephron produces a large amount of reactive oxygen species that are counterbalanced by a large number of antioxidant enzymes and free radical

scavenging systems. Reactive oxygen species mediate many negative biological effects, including peroxidation of cell membrane lipids, oxidation of proteins, renal vasoconstriction and damage to DNA. Unfortunately, hyperglycemia tips the balance towards production of reactive oxygen species, most of which seem to be generated in the mitochondria (62). The metabolism of glucose through harmful alternate pathways, such as via PKC activation and advanced glycation end-product formation, in the setting of hyperglycemia also seems partly dependent on reactive oxygen species (62-64).

Hyperglycemia specifically induces oxidative stress, even before diabetes becomes clinically apparent. Concentrations of markers of DNA damage induced by reactive oxygen species are higher in patients with more-severe nephropathy (i.e. proteinuria versus microalbuminuria). Furthermore, histological analysis of human kidney biopsy specimens has detected products of glyco-oxidation (combined products of glycation and protein oxidation) and lipoxidation in the mesangial matrix and glomeruli, whereas these lesions are much less common in specimens from individuals without diabetes (64, 65).

6.12 Nephrin

Podocytes (specialized visceral epithelial cells) are important for the maintenance of the dynamic functional barrier (66). Nephrin, a protein found in these cells, is crucial for maintaining the integrity of the intact filtration barrier. The renal expression of nephrin might be impaired in diabetic nephropathy.Patients with diabetic nephropathy have markedly reduced renal nephrin expression and fewer electron-dense slit diaphragms compared with patients without diabetes and minimal nephropathic changes or controls (67). Furthermore, nephrin excretion is raised 17-30% in patients with diabetes (with and without albuminuria) compared with that in individuals without diabetes. Thus, nephrin excretion could be an early finding of podocyte injury, even before the onset of albuminuria (13, 14). Treatment with blockers of the renin-angiotensin-aldosterone system might help protect nephrin expression. In a study of patients with type 2 diabetes, treatment with an angiotensin-converting-enzyme inhibitor for 2 years maintained nephrin expression at control levels compared with that in untreated patients with diabetes (23). By contrast, the expression of two other important podocyte and slit diaphragm proteins, podocin and CD2AP, was similar in the three groups. Comparable decreases in renal nephrin expression were reported in other studies of diabetic nephropathy (68, 69).

6.13 Genetic susceptibility

Genotype seems to be an important determinant of both incidence and severity of diabetic nephropathy (9, 31, 70). The increase in risk cannot be explained by the duration of diabetes or hypertension, or the degree of glycemic control. Environmental and genetic factors must, therefore, have roles in the pathogenesis of diabetic nephropathy. In patients with type 1 or type 2 diabetes, the likelihood of developing diabetic nephropathy is markedly increased in those who have a sibling or parent with diabetic nephropathy (71, 72). One study evaluated Pima Indian families in whom two successive generations had type 2 diabetes (72). The likelihood of the offspring developing overt proteinuria was 14% if neither parent had proteinuria, 23% if one parent had proteinuria and 46% if both parents had proteinuria.

Advances in molecular genetics have led to the development of a system for genotyping single-nucleotide polymorphisms and have enabled exploration of loci involved in diabetic

nephropathy in genome-wide association studies. In the search for susceptibility genes for microvascular complications of diabetes in Pima Indians, four loci on chromosomes 3, 7, 9 and 20 were identified (73). Additional loci are identified as diabetic nephropathy susceptibility genes areas on chromosomes 7q21.3, 10p15.3, 14q23.1 and 18q22.3 (74, 75).

Association studies of candidate genes have been the most common approach to identify genes involved in susceptibility to diabetic nephropathy. The greatest risks seem to be associated with genes encoding angiotensinconverting enzyme, angiotensin II receptor, cytokines, proteins involved in glucose or lipid metabolism, and extracellular matrix proteins.

The angiotensin-converting-enzyme gene (ACE) polymorphism has been explored in several studies. The insertion-deletion polymorphism is responsible for the difference between individuals in plasma levels of angiotensin-converting enzyme. In patients with type 2 diabetes, the DD polymorphism of the ACE gene has been associated with an increased risk of developing diabetic nephropathy, severe proteinuria, progressive renal failure, and of mortality during dialysis (76-78). In addition, an analysis of more than 1,000 white patients with type 1 diabetes showed a strong correlation between genetic variation in the ACE gene and the development of nephropathy (79). Other studies have, however, produced conflicting data. A critical review of 19 studies that examined a possible link between this gene and diabetic nephropathy failed to confirm an association among white people with either type 1 or type 2 diabetes, although a possible association in Asians could not be excluded (78). Ongoing studies are taking a multigene approach, since the likelihood of diabetic nephropathy being a single-gene disease is low.

6.14 Conclusions

While progression to diabetic nephropathy cannot yet be prevented, the pathogenesis is better characterized than a decade ago. The hemodynamic changes of glomerular hyperperfusion and hyperfiltration become evident before the earliest measurable clinical signs of nephropathy but do not predict the demise of kidney function. Various structural changes, including podocyte foot process effacement, decrease in podocyte number, thickening of the glomerular basement membrane and mesangial expansion, all occur with the early changes. These features, when assessed independently, cannot, however, predict disease progression. Hyperglycemia plays a central part in a cascade of damaging effects mediated by cytokines and growth factors that produces oxidative stress, abnormal glycosylation, lipid peroxidation, and the production of further inflammatory elements. With the anticipated completion of current studies that are evaluating the genetics of nephropathy in the next 2-4 years, understanding of how to integrate genetic and environmental susceptibility factors into risk assessment should be improved. This knowledge should give clinicians the ability to predict earlier who will develop nephropathy and, therefore, improve prevention of this devastating disease.

Key points

- Microalbuminuria is not a predictor of nephropathy development in individuals with diabetes
- Multiple mechanisms are operative in diabetes that are related to injury to the kidney and, in susceptible individuals, contribute to nephropathy development
- Defects in nephrin and podocin are central to the development of macroalbuminuria and associated with nephropathy progression

- Abnormally high concentrations of lipids contribute to β-cell injury and development of albuminuria

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7. DRUG-INDUCED KIDNEY INJURY

Mladen Knotek

7.1 Introduction

Acute kidney injury is an independent risk factor for patient mortality, even with small decrements in kidney function. In addition, it increases length of stay in the hospital and increases cost of treatment. Renal injury is often multifactorial, with drugs being only one of the factors in its pathogenesis. Hence, it is often difficult to estimate involvement of drugs as a cause of acute kidney injury. However, some data shows that in almost one quarter of cases of severe acute kidney injury nephrotoxic drugs are signifficant contributors. Renal handling of drugs involves glomerular filtration, excretion through transcellular transport into tubular fluid and reabsorbtion from the tubular fluid. High renal blood flow and process of concentration of drugs and their metabolites during formation of urine predisposes kidneys to toxic drug injury. From the pathogenic (pathophysiologic) perspective drug-induced kidney injury can be devided into hemodynamic, intrinsic (injury to renal tissue) and intrarenal obstruction (obstruction of tubule fluid flow). From didactical point of view kidney histology can be divided into four compartments: glomeruli, tubules, interstitium and vasculature. Each of these compartments can be target of drug-induced injury, with clinical and laboratory manifestations being dependent on which of them is predominantly involved. It is important to appreciate that a single drug renal toxicity can involve multiple pathophysiologic pathways and that predisposing factors are common to virtually all causative agents mediating kidney injury. Dehydration, hypotension, preexisting kidney disease, advanced age, diabetes and simultaneous use of multiple nephrotoxic drugs all greatly increase risk for any nephrotoxic drug to exert its nephrotoxic effect. At an increased risk are particularly patients in intensive care units.

7.2 Hemodynamic kidney injury

7.2.1 Non-steroidal antiinflammatory drugs and drugs that inhibit renin-angiotensin system

Renal blood flow and glomerular filtration normaly depend on renal perfusion pressure (determined by the mean arterial pressure) and on tonus of the afferent and efferent arteriole. In the setting of decreased perfusion pressure glomerular filtration is maintained by the afferent arteriole dilatation, mediated in part by vasodilatory prostaglandins and by the efferent arteriole vasoconstriction mediated partly by angiotensin II. Therefore, it is not surprising that inhibition of prostaglandin synthesis by the non-steroidal antiinflammatory drugs (NSAID) may precipitate kidney dysfunction. Renal microvasculature expresses both isoforms of cyclooxygenase (COX), COX-1 and COX-2. In conditions where renal blood flow is impaired, such as congestive heart failure, liver cirrhosis, dehydration and chronic kidney disease vasodilatory prostaglandins help to maintain renal blood flow and glomerular filtration. Both, selective (COX-2) and non-selective COX inhibitors impair synthesis of vasodilatory prostaglandins in the kidney and are associated with development of intrarenal vasoconstriction and renal function impairment. Other forms of kidney injury by the NSAID

are acute tubulointerstitial nephritis, chronic interstitial nephritis and glomerulopathy (usually minimal change disease).

Similarly, in the setting of effective blood volume depletion (decompensated heart failure, decompenstaed cirrhosis, systemic hypotension), or renal hypoperfusion due to bilateral renal artery stenosis, administration of drugs that block synthesis of angiotensin II (angiotensin-converting enzyme inhibitors), or its binding to type I receptors (AT1 receptor antagonists) reverses efferent arteriole vasoconstriction and decreases intraglomerular pressure, which reduces glomerular filtration rate.

Both NSAID-induced or anti-angiotensin drug-induced kidney injury is functional and quickly resolves upon withdrawal of a causative drug. Diagnosis relies on clinical judgement. Urinalysis reveals blank sediment. Hemodynamic kidney injury is treated by withdrawal of causative drug. Renal replacement therapy is rarely needed.

Other drugs that may cause kidney injury by intrarenal vasoconstriction are vasopressors, calcineurin inhibitors (cyclosporine and tacrolimus) and amphotericin B.

7.2.2 Contrast-induced nephropathy

Contrast-induced nephropathy (CIN) is a form of acute kidney injury that occurs after intravenous administration of iodine-based radiocontrast agents for radiologic examinations. At particular risk for CIN are diabetics, volume-depleted patients, older patients and patients with preexistant kidney injury. Acute worsening of glomerular filtration occurs within several days of radiologic procedure (usually after 48-72 hrs). Decrease in glomerular filtration is usually small or moderate and renal function returns to baseline level within several days. However, sometimes hemodialysis is needed to bridge period to recovery. Even small decrements in kidney function have been linked to increased mortality in patients with CIN, although it is not clear whether CIN is an independent risk factor for mortality. Because of this potential effect on patient survival, and increased costs of care for patients with CIN, great effort should be put to prevention of CIN in patients at risk. Preventive measures include adequate hydration of patients prior to and after procedure, use of low-osmolar or iso-osmolar contrast agents and limiting ammount of agent used. Role of particular agents such as bicarbonate and N-acetyl cystein, as well as continuous venovenous hemofiltration in prevention of CIN is not clearly established.

7.3 Intrinsic kidney injury

7.3.1 Tubulointerstitial injury

Acute tubulointerstitial injury can be caused by two mechanisms: by the hypersensitive idiosyncratic reaction that is dose-independent and is reffered to as acute hypersensitive tubulointerstitial nephritis and by the toxic acute kidney injury characterized by acute tubular necrosis. Acute tubular necrosis is dose-dependent. Chronic form of tubulointerstitial nephritis is seen with long-term use of NSAID, usually in combination and is reffered to as analgesic nephropathy.

7.3.2 Acute hypersensitive interstitial nephritis

It is an idiosyncratic fenomenon, caused by the allergic reaction to variety of drugs. Characteristically, reexposure to the same drug causes recidive of the disease. Many drugs have been implicated in inducing tubulointerstitial nephritis (TIN). Among them are beta-lactam antibiotics (penicillins and cephalosporins), quinolone antibiotics (ciprofloxacin),

NSAID, proton pump inhibitors (e.g. omeprazole), sulfonamides, allopurinol, etc. Histologicaly, interstitial inflammatory infiltrate consisting of T and B lymphocytes, with frequently prominent eosinophils is found in renal tissue obtained by biopsy. Accordingly, sterile leucocyturia with eosinophyluria is found on urinalysis. Acute interstitial nephritis causes acute kidney injury, characterized by an increase in serum creatinine levels, which is reversible upon discontinuation of the offending drug. Corticosteroids may foster resolution of kidney inflammation and recovery of renal function.

7.3.3 Acute tubular necrosis

Prototype class of agents that induces acute tubular necrosis (ATN) are aminoglycoside antibiotics. These drugs are freely filtrable by the glomerulus. Their nephrotoxic potential is dependent on the number of cationic groups on the molecule. Aminoglycosides bind to acidic phospholipids and megalin on the apical membrane of proximal tubule cells, and after uptake into the cells by endocytosis they accumulate in lysosomes causing their rupture. They are also thought to interfere with cellular functions such as protein synthesis and mitochondrial function. Ultimately, proximal tubule cell apoptosis and necrosis occurs, leading to acute kidney injury. In addition, there is some evidence that aminoglycosides may potentiate nephrotoxicity of gramm-negative bacterial endotoxin. Acute kidney injury caused by aminoglycosides is frequently non-oliguric, with increases in serum urea and creatinine within days of initiation of antibiotic therapy. Kidney injury may be severe enough to require renal replacement therapy. Urinalysis shows mild proteinuria with hyaline and granular casts in the sediment. After stopping the drug renal function returns to baseline values usually within weeks. To prevent aminoglycoside-induced acute kidney injury it is important to identify patients at risk, as stipulated in the introduction section. In patients with reduced kidney function, it is of paramount importance to adjust the dose according to glomerular filtration rate. Also, it seems that once-daily dosing of aminoglycosides decreases incidence of acute kidney injury (although this is a metter of some controversy). The role of therapeutic drug monitoring, usually by measuring trough plasma concentration is helpful in determination of appropriate dose, but its role in preventing kidney injury is not clearly established.

Other agents that may cause acute tubular necrosis are chemotherapeutics such as platinum derivatives, amphotericin B, foscarnet, cidofovir and statins (by causing rhabdomyolysis and myoglobinuria).

7.3.4 Osmotic nephrosis

Osmotic nephrosis is a form of acute kidney injury caused by a high-dose intravenous immunoglobuline, or osmotic diuretics such as mannitol and plasma expanders, such as hydroxiethylstarch. Histologicaly, it is characterized by isometric vacuolization of proximal tubules. It is thought that proximal tubule cell injury occurs after uptake of either osmotic agent itself, or its vehicle (such as sucrose in case of intravenous immunoglobuline) with consequent tubule cell swelling and injury.

7.3.5 Analgesic nephropathy

Analgesic nephropathy was a relatively frequent cause of chronic kidney disease in the past. It is characterised by the chronic interstitial nephritis, often with papillary necrosis. First manifestation is mildly decreased glomerular filtration and decreased urinary concentration capability. Later, interstitial fibrosis, especially in the medulla, with papillary necrosis occurs. Unless analgesic abuse is stopped, renal injury is progressive and leads to end-stage kidney

disease. Responsible agents are analgesics in combinations. The most important causative drug was phenacetin, often in mixtures with acethylsalicilic acid, codeine or caffeine. A metabolite of phenacetin, acetaminophen, which is a very frequently used analgesic may be also associated with nephrotoxicity, although the risk is lower compared to phenacetin. Similarly, consummation of other NSAID may be related to development of chronic kidney disease. However, large quantities of these drugs is required over many years to induce chronic kidney disease. Mechanisms by which these drugs induce kidney damage include oxidative stress and chronic inhibition of synthesis of vasodilatory prostaglandins with consequent chronic renal ischemic injury. Diagnosis relies on careful history taking, urinalysis showing sterile leucocyturia and mild or moderate (usually subnephrotic) proteinuria, with or without erythrocyturia. Urinary infections are frequent in patients with analgesic nephropathy. Hallmark of analgesic nephropathy, papillary necrosis can be diagnosed by intravenous urography, CT scan, or by the ultrasound. Other suggestive features on imaging procedures are shrunken kidneys, nephrocalcinosis and kidneys with bumpy contours.

7.4 Intrarenal obstruction

Drug-induced intrarenal obstruction is mainly due to antiviral drug precipitation. It is observed sometimes with use of acyclovir. Risk factors are rapid bolus administration in a volume-depleted patient. Crystaline nephropathy has also been a complication of antiretroviral drugs such as indinavir or tenofovir, especially in patients with high urinary pH values (pH >6). Toxicity of these drugs is potentiated by concomitant use of sulfometoxazole. Another drug which may precipitate in kidney tubules is methotrexate used in high doses, in the setting of dehydration and/or low urine pH (pH < 7). Crystal-induced tubule obstruction is accompanied with crystaluria, which helps establishing diagnosis. Kidney injury caused by drug precipitation may be severe and hemodialysis is frequently needed to treat renal failure and decrease drug burden.

7.5 Conclusion

Drug-induced kidney injury is a frequent, and probably underappreciated causative or contributory event in pathogenesis of acute or chronic kidney injury. At the same time, it is often preventable and easily treatable if diagnosed early. Diagnosis of drug-induced kidney injury requires vigilance and knowledge of drug pharmacokinetics and pharmacodynamics. It is a multidisciplinary task involving clinicians, pharmacists and clinical chemists.

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8. DYSLIPIDEMIA AT CHRONIC RENAL FAILURE

Victor Blaton

8.1 General aspects

The nephrotic syndrome is defined by a urinary protein level exceeding 3.5 g per 1.73 m^2 of body surface area per day. The term is still clinically useful and has persisted, because heavy proteinuria, irrespective of its origin, is associated with a spectrum of clinically important sequelae, sodium retention, hyperlipoproteinemia, infection and thrombo-embolic complications. Coronary heart disease is an important cause of morbidity and mortality in patients with chronic renal disease. The high CHD prevalence in these patients is likely related to their high frequency of hyperlipidemia. The characterization of the degree of type of lipid and lipoprotein abnormalities should therefore be considered important in the management of patients to prevent CHD. It has also been suggested that dyslipidemia may contribute to accelerate development of renal insufficiency. There is a variable increase in the levels of VLDL, IDL (intermediate density lipoprotein) and LDL fractions resulting in elevated serum cholesterol alone or in simultaneous elevation of serum cholesterol and triglycerides. Hypertriglyceridemia are associated with accumulation of chylomicron remnants and VLDL remnants which are also very atherogenic. Hypertriglyceridemia generates small dense LDL particles and is associated with low HDL-c. It is also associated with increased coagulability and decreased fibrinolysis by its association with increased levels of PAI-1 and factor VIIc activation of prothrombin to thrombin. HDL looks the missing link, persons with low HDL particles have a higher risk to lose their renal functions. The higher the ratio of low-density lipoproteins to high-density lipoproteins found in chronic-dialysis and renal-transplant patients may be related to their premature morbidity and mortality from cardiovascular causes. HDL structures and properties are discussed in function of their protective effects. HDL-c is now emerging as a key entity in both determining risk and providing protection although none as yet specifies HDL as target for treatment.

Chronic renal failure (CRF) results in profound lipid disorders, which stem largely from dysregulation of high density lipoproteins (HDL) and triglyceride-rich lipoprotein metabolism. Specifically, maturation of HDL is impaired and its composition is altered in CRF. In addition, clearance of triglyceride-rich lipoproteins and their atherogenic remnants is impaired, their composition is altered, and their plasma concentrations are elevated in CRF. Impaired maturation of HDL in CRF is primarily due to down regulation of lecithin-cholesterol-acyltransferase and, to a lesser extent, increased plasma cholesteryl ester transfer protein (CETP). Triglyceride enrichment of HDL in CRF is primarly due to hepatic lipase deficiency and elevated CETP activity. The CRF induced hypertriglyceridemia, abnormal composition, and impaired clearance of triglyceride-rich lipoproteins and their remnants are primarily due to down regulation of lipoprotein lipase, hepatic lipase, and the very low density lipoprotein receptor, as well as, up regulation of hepatic acyl-CoA cholesterol acyltransferase (ACAT). In addition, impaired HDL metabolism contributes to the disturbance of triglyceride-rich lipoprotein metabolism. These abnormalities are compounded by down regulation of apolipoproteins apoA-I, apoA-II and apoC-II in CRF. Together, these abnormalities may contribute to the risk of atherosclerotic cardiovascular disease and may adversely affect progression of renal disease and energy metabolism in CRF.

Two mechanisms contribute to nephrotic dyslipidemia: overproduction and impaired catabolism of apolipoproteine B-containing lipoproteins, decreased catabolism of chylomicrons and VLDL has been documented in the nephrotic syndrome. It is probable that abnormal lipoprotein catabolism results, at least in part, from urinary loss of some substances.

In view of the effect of dyslipidemia on cardiovascular risk and possibly on the progression of renal disease, treatment seems sensible, although evidence from controlled studies are not available. There is some role for no pharmacologic intervention, although treatment with statins and fibrates in most cases are acceptable

8.2 Plasma lipids and lipoprotein profile in CRF: Abnormalities in metabolism

The common features of dyslipidemia of CRF and their modifications by heavy proteinuria and dialytic modalities are summarized in Table 8.1.

Table 8.1. *Common features of serum lipid/lipoprotein profile in predialysis CKD, patients with or without nephrotic proteinuria and in ESRD patients treated with chronic hemodialysis or peritoneal dialysis.*

Plasma triglyceride concentration is frequently elevated in patients and experimental animals with CRF. However, plasma cholesterol concentration is usually normal, even reduced, and only occasionally elevated in patients with end-stage renal disease (ESRD). Elevation of plasma triglycerides in ESRD patients is accompanied by increased plasma concentration and impaired clearance of VLDL. This is associated with the accumulation of atherogenic VLDL remnants, commonly known as IDL. Similarly, clearance of chylomicrons is impaired and plasma concentration of chylomicron remnants is elevated in CRF patients. In contrast, plasma concentration of LDL is usually normal and only occasionally elevated in ESRD patients. Plasma HDL concentration is consistently reduced, and maturation of cholesterol ester-poor HDL-3 to cholesterol ester-rich cardio protective HDL-2 is impaired in CRF.

As noted earlier, CRF is consistently associated with reduced plasma HDL cholesterol concentration, impaired maturation of cholesterol ester-poor HDL-3 to cholesterol ester-rich HDL-2, increased HDL triglycerides, and depressed plasma apoA-I. These abnormalities are

primarily due to CRF-induced dysregulation of several important proteins, which are briefly described below.(Table 8.2.).

Table 8.2. *Major changes in the key enzyme and receptors in chronic renal failure and their impact on plasma lipid/lipoprotein levels*

LCAT plays an important role in HDL-mediated cholesterol uptake from the extrahepatic tissues and, as such, serves as a main determinant of HDL maturation and plasma HDL cholesterol level. Thus LCAT deficiency can potentially account for diminished plasma HDL cholesterol and impaired HDL maturation in CRF. In fact, plasma LCAT activity is consistently diminished in patients with ESRD. This is accompanied by a significant elevation of plasma-free cholesterol and a marked reduction in plasma esterified cholesterol concentration, providing functional evidence for diminished LCAT-dependent cholesterol esterification.

CETP mediates transfer of cholesterol ester from HDL to IDL in exchange for triglycerides. Thus a potential increase in plasma CETP can contribute to the CRF-associated reduction in HDL cholesterol ester and elevation of HDL triglycerides. In fact, according to a recent study, more than 34% of hemodialysis-dependent patients were found to have high plasma CETP levels. The mechanism responsible for the reported elevation of CETP in ESRD patients is unknown and requires future investigation. The effect of CRF is amplified by proteinuria, which has been shown to increase synthesis and markedly raise plasma concentration of CETP. Thus plasma CETP is expectedly elevated in patients with heavy proteinuria and mild to severe renal insufficiency.

Hepatic lipase. Hepatic lipase catalyzes hydrolysis and removal of the triglyceride content of HDL. Thus hepatic lipase deficiency can potentially contribute to increased HDL triglyceride content. In fact, as described later (abnormalities of lipoprotein remnants), CRF results in pronounced hepatic lipase deficiency in humans and experimental animals.

apoA-I and apoA-II. apoA-1 and apoA-II constitute the main structural constituents of HDL. In addition, apoA-I serves as the LCAT activator as well as ligand for the SRB-1 and HDL

binding protein (ABCA1 transporter), whereas apoA-II serves as the hepatic lipase activator. Plasma concentrations of apoA-I and apoA-II are significantly reduced in patients with ESRD. Studies in animals with experimental CRF have demonstrated that the CRF-induced reduction in plasma apoA-I is due to down regulation of hepatic apoA-I gene expression. The reduction in plasma concentration of these important constituents can, therefore, contribute to both diminished plasma HDL concentration and impaired HDL function in CRF.

SRB-1. Hepatic SRB-1 is the primary pathway for disposal of HDL-borne cholesterol ester and triglycerides. Therefore, potential dysregulation of this protein can impact HDL metabolism. Heavy glomerular proteinuria has been shown to significantly reduce hepatic SRB-1 protein expression in experimental animals. In contrast, CRF per se, without heavy proteinuria, induced by nephrectomy, does not significantly change SRB-1 mRNA or protein abundance in the liver. However, concomitant heavy proteinuria and renal insufficiency may affect SRB-1 expression and hence, HDL-mediated reverse cholesterol transport.

ACAT. HDL-mediated cholesterol uptake from the extrahepatic tissues depends on deesterification of cholesterol esters contained in the intracellular vesicles and the resultant release of free cholesterol. This process is opposed by ACAT, which is the main enzyme for intracellular esterification of cholesterol. Therefore, a relative increase in ACAT activity can potentially limit HDL-mediated cholesterol uptake and, hence, contribute to the reduction in plasma HDL cholesterol and impaired maturation of HDL. Although the effect of CRF on ACAT expression and activity in the extrahepatic tissues is not known, CRF has been recently shown to markedly raise hepatic ACAT-2 mRNA and protein abundance, as well as total ACAT activity. The potential contribution of ACAT to the CRF-induced dysregulation of HDL metabolism was illustrated by a recent study which revealed that pharmacological inhibition of ACAT results in a dramatic shift in plasma cholesterol from apoB-containing lipoproteins to HDL with virtually no change in plasma total cholesterol in CRF animals. Interestingly, the improvement in the lipid profile with an ACAT inhibitor was accompanied by a significantly higher creatinine clearance in the treated than the untreated animals. This phenomenon may be due to amelioration of dyslipidemia and enhanced HDL-mediated reverse cholesterol transport, leading to attenuation of glomerulosclerosis.

8.3 Consequences of dyslipidemia

8.3.1 Progression of renal disease

Hyperlipidemia can potentially accelerate progression of renal disease by several mechanisms. First, reabsorption of fatty acids, phospholipids, and cholesterol contained in the filtered proteins (albumin and lipoproteins) by tubular epithelial cells can stimulate tubulointerstitial inflammation, foam cell formation, and tissue injury. Second, accumulation of lipoproteins in glomerular mesangium can promote matrix production and glomerulosclerosis. In this context, native and oxidized lipoproteins, particularly LDL, stimulate production of matrix proteins by cultured mesangial cells and promote generation of proinflammatory cytokines, which can lead to recruitment and activation of circulating and resident macrophages. In addition, impaired HDL-mediated reverse cholesterol transport can further contribute to tissue injury by limiting the unloading of the excess cellular cholesterol and phospholipid burden. In fact, low plasma HDL has been identified as an independent risk factor for progression of renal disease. Moreover, hereditary LCAT deficiency, which is associated with a marked reduction in HDL cholesterol and impaired HDL-mediated reverse cholesterol transport, results in progressive renal disease. It is of note that both chronic renal

insufficiency and nephrotic syndrome lead to acquired LCAT deficiency and impaired HDL metabolism. Correction of these abnormalities by ACAT inhibitor administration has been shown to reduce proteinuria and retard progression of renal disease in experimental animals.

In addition to the animal studies, a number of clinical studies have provided evidence for the potential contribution of dyslipidemia in progression to renal disease. For instance, the Physicians Health Study demonstrated a significant increase in the risk of deterioration of renal function among individuals with mildly elevated baseline serum creatinine who had elevated serum cholesterol and/or reduced HDL cholesterol concentrations. Similarly, the Modification of Diet in Renal Disease (MDRD) study identified low plasma HDL cholesterol as an independent risk factor for progression of renal disease. Together, these observations have prompted a limited number of clinical trials exploring the effect of lipid-lowering agents in humans with chronic kidney disease (CKD). The value of lipid-lowering therapies on the progression of renal disease in humans remains uncertain and requires further investigation.

8.3.2 Cardiovascular disease

The risk of cardiovascular morbidity and mortality is profoundly increased in patients with CKD. For instance, the majority of patients with CKD die of cardiovascular events before reaching ESRD. Moreover, cardiovascular mortality among dialysis-dependent ESRD patients is 10- to 30-fold greater than in the general population despite stratification for gender, age, race, and the presence of diabetes. Numerous factors contribute to atherogenic diathesis and the high risk of cardiovascular disease in CKD. These include oxidative stress, inflammation, hypertension, and altered metabolism of lipids, carbohydrates, nitric oxide, calcium, and phosphate, among others.

In a group of 135 patients with CRF, containing 58 severe predialysis, 36 on dialysis and 41 renal transplant. The primary causes of the disease has the highest percentage for glomerulonephritis and diabetic nephropathy. In Table 8.3. we have several lipid data and they are significantly increased with major changes in TG, CRP and tHcy, which relate also to the metabolic syndrome. In Table 8.4. we have in the same study the relationship between the plaque scores and CAD risk factors in CRD. There is a significant relationship between the score and LDL-c also to age and duration of hypertension and no relation to tHcy. In Table 8.5. There were data on carotid IMT measurements and plaque scores. There were no differences in the three groups and controls for IMT. There is however a significant change in plaque score against control but similar in the three groups and the score is highest in the pre-dilaysis group.

Table 8.3. Dyslipidemia in CRF

Table 8.4. *Carotid plaque score and CAD risk factors in CRF.*

Table 8.5. *Carotid IMT and plaque score in CRF*

The plasma cholesterol concentration is frequently elevated in patients with nephrotic proteinuria and mild to moderate renal insufficiency, it is frequently normal or reduced and only occasionally elevated in those with ESRD. Accordingly, the high risk of cardiovascular disease in ESRD populations cannot be attributed to hypercholesterolemia. On the contrary, a reduction in plasma cholesterol (which denotes intense inflammation) predicts cardiovascular events, in contrast to the pattern in the general population. However, the paradox of plasma total cholesterol by no means diminishes the participation of lipid disorders as a culprit in this process. Instead, accumulation of oxidation-prone atherogenic lipoprotein remnants and impaired HDL-mediated reverse cholesterol transport (Figure 8.1.) which are the defining features of uremic dyslipidemia, may play a major part in the pathogenesis of atherosclerosis in this population.

8.4 Basic observed facts

CRF results in profound dysregulation of several key enzymes and receptors involved in the metabolism of lipoproteins, particularly those of HDL and triglyceride-rich lipoproteins. Downregulation of LCAT, apoA-1, and hepatic lipase together with upregulation of CETP are largely responsible for the reduction in HDL cholesterol and elevation of HDL triglyceride in CRF. Down regulation of skeletal muscle and adipose tissue LPL, hepatic lipase, and the VLDL receptor and of hepatic LRP is collectively responsible for hypertriglyceridemia, impaired clearance, and elevated plasma levels of VLDL, IDL, and chylomicron remnants despite down regulation of hepatic triglyceride synthetic capacity. Dysregulation of lipid metabolism can contribute to atherogenic diathesis and possibly to progression of renal disease and impaired energy metabolism in CRF.

Figure 8.1. *HDL interactions and interconversion in CRD.*

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9. GFR - WHERE ARE WE NOW?

Joris R. Delanghe

9.1 Abstract

The availability of a worldwide standard for creatinine is an important milestone for the improvement of GFR estimations for adults. However, an unacceptable interlaboratory variation is still observed which is mainly due to differences in calibration. In adults, the MDRD formula allows to obtain a reliable GFR estimation. Systematic reporting of eGFR by clinical laboratories helps to identify patients at risk for developing end stage renal failure. Care has to be taken when using estimated GFR values for drug dose adjustment. The use of enzymatic creatinine assays is recommended. Updating the currently used estimation formulas for calculating GFR in children is far from easy.

Low molecular mass marker proteins like Cystatin C and beta trace protein can be regarded as an attractive practical alternative for assessing GFR since they only require a determination in serum or plasma and are better suited in the blind range of creatinine.

9.2 Introduction

Determination of serum or plasma creatinine concentrations are of importance because of its central role in the assessment of renal function and the use of creatinine values for estimation of glomerular filtration rate (GFR) (1). For adults, estimating equations have been developed from the Modification of Diet in Renal Disease (MDRD) Study (2). The recent availability of the international NIST SRM 967 creatinine standard means an important milestone in the further improvement of GFR estimation (3). For adults, an improved GFR-estimating equation based on serum creatinine values traceable to IDMS reference measurement procedures has been recently presented (4). Clinically validated adaptations of creatinine-based formulas for estimating GFR in children are about to be published.

On the other hand, evidence is growing that serum concentrations of low molecular mass marker proteins can be considered as an interesting alternative for estimating renal function (5). In the present review, the various possibilities for assessing GFR are discussed.

9.3 Exogenous markers

Reference values for GFR are often expressed as a value adjusted to adult ideal body surface area. These values work well for many clinical situations, but in subjects with an atypical body mass, they may not accurately reflect renal function.

The reference method to determine GFR is the urinary clearance of inulin during a continuous intravenous infusion. Alternatively, the plasma clearance of inulin can be determined, which does not require urine collection (6). Similarly, iohexol and iothalamate are radiographic contrast agents that can be used as exogenous GFR markers comparable to inulin and Cr^{51}-EDTA (7). They can be measured by HPLC. Exogenous markers are very accurate but are expensive and rather impractical and therefore mainly restricted to research use.

9.3.1 Creatinine assays

Creatinine is by far the most commonly used biochemical marker of renal function. The commonest principle for assaying creatinine is the so-called Jaffe reaction (11). Since Jaffe only observed a complex formation between picric acid and creatinine in alkaline environment in 1886 and never described an analytical method, variation amongst “Jaffe method” recipes is broad (8). The analytical bias of current creatinine methods is still disappointing: the liquid enzymatic based and the compensated Jaffe method showed a small positive bias, whereas a major positive bias was observed for the creatinine iminohydrolase (9) and the uncompensated Jaffe method (9). This bias is due to the analytical interference by pseudochromogens for the Jaffe group and the calibration used in the dry chemistry method (9). Interlaboratory variation for creatinine is still unacceptably high; which leads to an unacceptable variation in the estimation of kidney function.

9.3.2 Global creatinine restandardization

The NKDEP, CAP, and NIST have collaborated to prepare a human serum-creatinine reference material with acceptable commutability with native clinical specimens. These materials are value-assigned with the GC-IDMS and LC-IDMS reference measurement procedures (3). The materials are designated NIST SRM 967. Implementing traceability of serum creatinine assays to GC- or LC-IDMS will lead to changes in the clinical decision-making criteria currently used for serum creatinine concentrations and creatinine clearance. In 2008 - 2009, the process of implementation of the new ID-MS standardization by the IVD industry is ongoing. Use of serum creatinine concentrations or equations to estimate GFR requires knowledge of the calibration of the serum creatinine assay (10).

9.3.3 Correcting for non-specificity based on average values for adults

In the earliest manual methods, serum creatinine was assayed by the Jaffe reaction after deproteinisation, eliminating the pseudo-chromogen effect of proteins (11). Early automated methods used dialysis membranes to prevent interference from plasma proteins. Today, analyzers use undiluted serum and plasma, making them prone to the "protein error" in the alkaline picrate reaction (11). In the serum of adults, this effect produces a positive difference of about 27 µmol/L creatinine compared with HPLC or enzymatic methods (11). Because urine contains relatively little or no protein, the protein error affects only creatinine determinations in serum or plasma. Therefore, creatinine clearance is underestimated when creatinine methods affected by protein error are used. For calculating GFR, this positive bias is greatly compensated by the overestimation attributable to tubular secretion of creatinine, which is relatively more important in children (11).

In order to comply with new regulations, manufacturers of Jaffe based methods can restandardize their creatinine assays using a compensation, a mathematical correction which compensates for analytical non-specifity due to the protein error. Since children have lower reference ranges for total protein, this protein error is considerably smaller in children (11). In consequence, use of restandardized Jaffe-type assays results in overcompensation when used in children or infants.

The enzymatic methods manage to measure the serum creatinine more correctly (9). Due to the elimination of analytical non-specificity in these methods, the lower enzymatic creatinine result (when the result has not been adjusted to Jaffe-like results) leads to a marked increase of creatinine clearance estimations because of the increased effect of tubular secretion on test

results. Paradoxically the analytical improvement makes creatinine less suited as a GFR marker in pediatric medicine (12).

When creatinine clearance is measured following administration of cimetidine (a blocker of tubular secretion of creatinine), the effect of tubular secretion can be corrected. The cimetidine protocol allows estimating of GFR in a clinical setting. However it cannot be used on a wide scale.

9.3.4 Calculated creatinine clearance in adults

For adults, the currently recommended GFR estimating equation has been developed from the Modification of Diet in Renal Disease (MDRD) study (2, 4). The coefficients of this GFR estimating equation have recently been adapted for the new ID MS creatinine standardization. Clinicians and laboratorians should therefore be very careful: when using these formulas the coefficients used in the MDRD formula should always match with the creatinine calibration used. It should be noted that the MDRD formula measures GFR which is not exactly the same as the earlier Cockroft & Gault formula for creatinine clearance estimation. In contrast to the Cockroft & Gault formula, the MDRD equation does not require the body mass so that it can be preported more easily by clinical laboratories. For estimated GFR values exceeding 60 mL/min, no exact eGFR values should be reported by the laboratories as the uncertainty of the serum creatinine determination is too important in that range. Also in subjects younger 18 or older than 70, the MDRD formula has not been validated. The MDRD formula is excellently suited for detecting patients at risk for developing end - stage renal disease. However, for adjusting drug dose in patients with a reduced renal clearance, the MDRD formula is to be handled with care since the vast majority of available pharmacokinetical data collected during the last three decades have been based upon the Cockroft & Gault formula (dating from 1976). Relative differences between Cockroft & Gault and MDRD results are most pronounced in the elderly.

9.3.5 Calculated creatinine clearance in children

The bias in serum creatinine concentration in the lower range is a major concern in pediatrics due to the lower reference ranges for serum or plasma creatinine in infants and children (12). For estimating GFR in children and infants, the Schwartz and the Counahan-Barratt equations are recommended (12, 13). Both provide GFR estimates based on a constant multiplied by the child’s height divided by the serum creatinine concentration. The values for the constant used in both equations differ considerably (12). Since these formulas have been validated 30 years ago, reassessment of formulas for estimating GFR using enzymatic creatinine assays is ongoing. Enzymatic creatinine methods are recommended (14). However, it is clear that it will be difficult to develop reliable formulas for compensated Jaffe results.

9.3.6 Cystatin C, a promising alternative

Serum concentrations of low-molecular mass marker proteins are primarily determined by GFR. An ideal marker has to have a constant production rate and should not vary in its concentration in situations with an acute-phase reaction**.** Cystatin C (Cys C) shares these properties. It is a 13 kDa cysteine protease and is produced by all nucleated cells. In normal conditions, serum Cys C is almost completely filtered by the glomerulus and largely catabolized by the tubules. Since serum Cys C concentration is closely correlated with the GFR, serum Cys C has been introduced as a GFR marker (5). Studies comparing Cys C and creatinine as marker of GFR generally showed diagnostic superiority of serum Cys C vs.

serum creatinine concentrations. In the blind range of creatinine, Cys C proves to be a superior marker. Formulas have been developed allowing reliable estimation of GFR based on Cys C (12). Unlike creatinine, serum Cys C reflects GFR independent of age, gender, height, and body composition. Because of its low individuality, Cys C has fewer inherent limitations as a screening test for detecting deteriorating GFR than serum creatinine. However, clinicians should be cognizant of extrarenal conditions (upregulation in certain tumours) and pharmacological factors (e.g. glucocorticoid treatment) that can influence the results of serum Cys C assays (12). Also thyroid dysfunction affects serum Cys C concentration by influencing the production rate of the protein (12). Serum creatinine concentrations are lower in malnutrition and lead to overestimation of GFR, while Cys C levels are unaffected (12).

Cys C-based GFR estimates show significantly less bias and serves as a better estimate for GFR (12). Cys C can be measured using immunochemical methods in a highly reproducible manner. Validation of a candidate primary recombinant reference material by an IFCC working group is ongoing.

9.4 Other protein markers

Beta trace protein (BTP) or prostaglandin D synthase is a glycoprotein with a molecular mass of 23 000–29 000, depending on the degree of glycosylation (5). BTP has been introduced for the measurement of kidney function in the creatinine-blind range. International standardization of BTP is still lacking.

Beta 2 microglobulin (11.3 kDa) has been advocated as a GFR marker (12), but its serum concentration can increase as an acute-phase reactant (5). Beta 2 microglobulin has the disadvantage of being increased in patients with several malignancies, particularly lymphoproliferative disorders (5, 12). Like Cys C and BTP, beta 2 microglobulin has the advantages of age and muscle mass independence (5).

9.5 Conclusions

Despite the stricter regulations and the technical progress in laboratory automation, between-laboratory variation of Jaffe based methods has not decreased over the last decade,. Analytical bias in creatinine assays needs to be reduced and non-specificity bias should be improved (9). The creatinine standardization issue has major clinical consequences which are far beyond the significance of the parameter itself. Apart from the conventional calculation of the creatinine clearance, also the calculation of the clearance using derived formulas is a key element in the assessment of renal function and the calculation of the correct dose of many drugs which are characterized by a narrow therapeutic index and a renal elimination mechanism. The MDRD formula is recommended for identifying individuals at risk for developing renal insufficiency. However, care should be taken when estimated GFR values are used for dose calculation of drugs since literature data are still mostly based on the older Cockroft & Gault formula. Data obtrained by Cockroft&Gault and MDRD equations are not per se interchangeable.

When introducing revised serum creatinine calibration to be traceable to IDMS, laboratories will need to communicate the following to clinicians: the serum creatinine reference interval will change to lower values, calculations of estimated GFR used to adjust drug dosages will be affected by the decreased creatinine values, measured and calculated creatinine clearance values will increase, and the corresponding reference interval will be different.

In view of the difficulties in adapting creatinine assays to the new calibrators in the pediatric concentration range in a uniform way, the low molecular mass proteins Cys C and BTP offer promising alternatives for calculating GFR in children. In comparison with serum creatinine, these proteins have a better diagnostic sensitivity for detection of impaired GFR (12). Although some caveats have to be taken into account when interpreting test results, protein-based GFR calculations only require serum values. The progress in the standardization of these protein assays will enable the wide-scale use of these methods.

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10. CARDIOVASCULAR RISK IN CHRONIC KIDNEY DISEASE

Mitja Lainščak

10.1 Introduction

Burden of chronic disease throughout the world is steadily increasing. Cardiovascular disease (CVD) and chronic kidney disease (CKD) frequently coexist and represent a major challenge in today's medicine. Although exact pathophysiological mechanisms are not fully understood, it seems that CVD and CKD can initiate, enhance, and perpetuate each other, eventually leading to vicious circle and premature death. Current evidence suggests there may be additional non-conventional risk factors for CVD. The evidence for prognostic management is less robust than in patients with preserved or mildly impaired renal function.

10.2 Cardiovascular co-morbidity burden in chronic kidney disease

Prevalence of CVD, including stroke, peripheral vascular disease, sudden death, coronary artery disease, and congestive heart failure is about twice of that observed in general population and is increased over the entire span of CKD. In addition, the onset of CVD frequently is premature when compare to general population. The Cardiovascular Health Study analysis demonstrated that per every 10 mL/min per 1.73 m^2 decrease in glomerular filtration rate (GFR) the risk of CVD and all-cause mortality increased by 5% and 6%, respectively. Similar observations for decrease in renal function were reported in general population and in patients suffering from myocardial infarction, enrolled to VALIANT trial. Indeed, in end-stage renal failure, the CVD is by far leading cause of morbidity and mortality, causing 40-50% of hospitalizations and deaths. This is likely due to a combination of factors, including high prevalence, an increased risk for adverse outcomes after coronary revascularisation or valve interventions, and under use of established primary and secondary prevention strategies.

Patients with CKD may present with CVD limited to heart or to vessels. Myocardial damage has clinical correlates in left ventricular hypertrophy and/or dilatation, which are associated with systolic and diastolic dysfunction. Right ventricle is affected in advanced stages of the disease. Arterial remodelling due to atherosclerosis or structural changes in arterial wall may be independent from myocardial damage. However, and since patophysiological processes are interrelated, most of patients have complex CVD. It is therefore not surprising that patients with CKD are entering the cardiovascular continuum early. When arterial hypertension and atherosclerosis develop, they pick up the pace in patients with CKD and lead to development of ischemic heart disease, degenerative valve disease, and chronic heart failure.

10.3 Risk factors and biomarkers

Traditional risk factors for development of CVD include hypertension, diabetes, dyslipidemia, smoking, increased body mass index, older age, male gender, physical inactivity, stress, and positive family history. As CVD in patients with CKD occurs frequently and prematurely, it

seems plausible that other risk factors are involved in the pathogenesis (Figure 10.1.). Some of those are specific to CKD and include haemodynamic overload, anaemia, chronic inflammation, oxidative stress, hypercatabolic state, uremia, calcium-phosphate imbalance, hyperhomocysteinaemia, endothelial dysfunction, increased sympathetic activity, insulin resistance, thrombogenic disorders, and metabolic syndrome. Both traditionaland non-traditional factors promote cardiomyopathy, aterosclerosis, and/or arteriosclerosis. In patients treated with dialysis, fluctuations in blood pressure, electrolytes, and cardiac filling can further aggravate the condition. From clinical perspective, it is important that risk factors can be identified and monitored by means of biomarkers. Whilst some are biomarkers per se (e.g. cholesterol), other risk factors are reflected by measurable biomarkers. Widely available and established laboratory biomarkers are lipids, blood glucose and glycated haemoglobin, haemoglobin, and C-reactive protein which are mainstay of regular patient follow-up. With identification of novel risk markers, battery is expanding to chronic (sub-clinical) inflammation, endothelial dysfunction, oxidative stress, and vascular ossification. Each of these is not only highly prevalent in CKD but also more strongly linked to CVD than in the general population. However, a causal relationship remains to be established. The biomarkers like IL-6, TNF-α, and asymmetric dimethly-arginine are therefore not ready for prime time and clinical use. Whilst needed evidence is pending, it may be worthwhile to consider experience from other chronic disease and to test whether it is applicable to patients with CKD. Insulin resistance, catecholamine, uric acid, albumin, TSH, natriuretic peptides, matrix metaloproteinases, high sensitivity troponin, testosterone are associated with poor outcome in patients with chronic heart failure. If those associations could be replicated in patients with CKD, we would be able to better stratify their risk and to adjust the pharmacological management accordingly.

Figure 10.1. *Relationship between cardiovascular disease, chronic kidney disease, and risk factors.*

10.4 Evidence based management and clinical practice

There is robust evidence for beneficial effects of renin-angiotensin-aldosterone system inhibitors, hypolipemic drugs, and beta-blockers in patients with CVD and normal renal function. However, patients with advanced CKD were usually excluded from randomized trials and only limited data is available on this topic. Most evidence comes from observational studies, subgroup and post hoc analyses of earlier trials. The benefit observed has to be interpreted cautiously in order to avoid early enthusiasm. To date, the randomized, placebo-controlled trials have been disappointing and unable to show a survival benefit of various treatment strategies, including lipid-lowering, increased dialysis dose and normalization of haemoglobin. The contradictory findings in CKD compared with the general population are not completely understood but may be attributed to different risk factor profile (see above). Indeed, seemingly paradoxical associations between traditional risk factors and cardiovascular outcome in patients with advanced CKD have complicated our efforts to identify the real cardiovascular culprits. Findings are further diluted by reverse epidemiology, which be discussed below.

Renin-angiotensin-aldosterone system inhibitors, hypolipemic drugs, and beta-blockers are associated with a variety of side effects and some of those are more frequent in patients with CKD. Hyperkalaemia is main concern for treatment with aldosterone antagonists, angiotensin converting enzyme inhibitors and angiotensin receptor blockers. In patients with chronic heart failure, the use of aldosterone antagonists in patients with GFR < 60ml/min should be cautious and in patients with GFR < 30ml/min those agents should generally be withheld. Hypolipemic drugs may also cause concern of side effects in patients with CKD stage 3-5. Clinicians have to be familiar with their pharmacokinetic properties as renal excretion of statins varies from < 2% in atorvastatin to 20% in pravastatin. Fibrates can increase serum creatinien concentrations which had led to recommendations for cautious use of fibrates in patients with CKD. Combination of different drugs increases propensity of side effects which poses an important limitation to use in clinical practice.

With increased risk profile associated with several drugs, the lack of mortality benefit may be due to side effects rather than to lack of clinical efficacy. Same concern may cause lower use of specific treatment in patients with coexisting CKD and CVD. When evidence-based cardiovascular therapies are used in patients with CKD, their clinical effect is not as big as in patients with preserved renal function. A recent study in 7884 patients (1766 had CKD with GFR < 60ml/min) showed that targets for blood pressure and glycosilated haemoglobin were achieved in 39% and 44% of patients with CKD, which was significantly lower than in patients without CKD (65% and 53%, respectively).

10.5 Reverse epidemiology

In a variety of chronic disease, including CKD, the so called “reverse epidemiology” is described. Conventional risk factors for CVD such as obesity, increased body fat, and cholesterol are paradoxically associated with lower long term mortality. Whether this applies to all CKD patients or only to those with chronic cardiac condition currently remains unknown. The reverse epidemiology could contribute to inconclusive findings of specific treatments in patients with CKD. Patients with lower BMI, fat tissue content, and cholesterol

have increased activation of inflammatory system and more pronounced metabolic disturbances. It is therefore very likely that therapeutic targets differ over span of chronic disease and that all patients do not benefit from same treatment.

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11. LABORATORY STANDARDS IN THE DIAGNOSIS AND MONITORING OF THERAPY

Draško Pavlović

11.1 Abstract

Albuminuria is a powerful marker of kidney disease and predictive factor of cardiovascular disease. Early detection and treatment of albuminuria in patients with diabetic and nondiabetic kidney disease, hypertension and cardiovascular disease improves overall survival. Nephrologists and clinical chemistries should be aware of screening, monitoring and treatment of albuminuria.

11.2 Introduction

Chronic kidney disease (CKD) defined as either kidney damage or decreased kidney function for three or more months is a worldwide public health problem. It affects approximately 10% of adult population in western world (1).

CKD could be simplified classified in two major groups: diabetic and nondiabetic chronic kidney disease. (Table 11.1.) (2). The diagnosis of CKD is based on level of glomerular filtration rate (GFR) and by some of the markers of kidney damage. Differential diagnosis of CKD is based on the history, physical examination and laboratory evaluation. Proteinuria is the principal marker of kidney damage. Moreover proteinuria, i.e. albuminuria is a powerful marker of progressive kidney function decline (3). There are also some other markers of kidney damage like hematuria, abnormalities in urine sediment, abnormal findings on imaging studies, e.g. ultrasound etc.

Table 11.1. *Classification of chronic kidney disease*

In this paper we will briefly review the mechanism of proteinuria, particularly albuminuria, the clinical importance of albuminuria and clinical approach in the diagnosis of albuminuria and monitoring of the therapy.

Since 1817, when Richard Bright described proteinuria in patients with kidney disease detection of proteinuria remains one of the major indicator of kidney disease. At the late sixties of last century increased urinary albumin excretion was observed in new diabetic patients. In 1981 the term microalbuminuria was used for the first time to describe urinary albumin excretion not detected by a standard dipstick. Today it is well known that microalbuminuria and albuminuria, i.e. proteinuria are predictors of progression of renal disease and also marker and risk factor of cardiovascular disease: myocardial infarction, stroke and premature death. Sir Robert Hutchinson' s words from the beginning of 20th century are still appropriate today at the beginning of 21st century: „…the ghosts of dead patients that haunt us do not ask why we did not employ the latest fad of clinical investigation. They ask us, why did you not test my urine?" (4).

11.3 Albuminuria

A healthy adults excretes in urine less than 150 mg of protein per day. It is well known that kidney, i.e. glomerular capillary wall has high permeability to water, small solutes, low molecular proteins (< 40000 Da and radius < 30 A) but very low permeability to plasma proteins of the size of albumin (~65000 Da) and larger. Normal composition of urine is: ~40% albumin, ~10% immunoglobulin G, light chains ~5%, and ~42% other low molecular proteins. There are four mechanism of excessive (> 150 mg/24 hours) protein excretion: increased glomerular filtration (glomerular proteinuria), inadequate tubular reabsorption or increased tubular secretion (tubular proteinuria) and overflow proteinuria (5).

Albuminuria is of major interest because it is well known determinant of renal as well as cardiovascular disease.

Albumin is the most abundant plasma protein. It has diverse functions: carrier of hormones, metabolites, drugs, vitamins, ions, maintenance of the oncotic pressure and blood volume, acid-base buffer functions etc. It is well known that the size and the charge of the protein determine the amount of filtered protein (6). For many years it was thought that amount of filtered albumin is very low and that tubular reabsorption of albumin is of no clinical relevance. Recently it was recognized that mechanism regulating tubular uptake of albumin is very important and probably derangement of tubular reabsortion determine the amount of albuminuria (6). Even more increased tubular reabosption of albumin could be a cause of kidney interstitial inflammation and fibrosis. There is no secretion of albumin in tubular apparatus of the kidney, therefore glomerular filtration and tubular reabsorption of albumin determines the amount of albuminuria. The amount of filtered albumin was detected by several techniques and despite some controversial it is clear that a significant of albumin is filtered through glomerular capillary wall (7). In proximal tubule albumin is reabsorbed by a receptor mediated endocytosis. Several receptor of albumin have been identified, but most important are megalin and cubulin (6). Why is this process important? The excess of albumin in the tubular lumen due to increased filtration through glomerular capillary wall leads to the induction of inflammation and interstitial fibrosis. Several studies in vitro has shown that in excess of albumin there is increased expression of inflammatory and fibrogenic mediators in tubular cells and it is important factor in progression in number of renal disease. Therefore,

albuminuria is marker but also a pathogenic factor in progression of renal disease. The relation between albuminuria and cardiovascular disease is still poorly understood, but albuminuria is strong and independent indicator of increased cardiovascular risk, i.e. it is a marker of generalized vascular endothelial damage.

11.4 How to detect and measure albuminuria?

Urine protein testing involves a screening test to detect excess of protein, a test to detect the amount of protein and sometimes an assay to detect specific proteins. We will briefly described how to measure albuminuria because it is a central component in screening and management of patients with kidney disease and could be of great value in patients with cardiovascular disease.

It is important to know that albumin excretion could be, and usually is increased after exercise, after a meal and in young people erect posture can also increase albumin excretion (4, 8). There is day-to-day variation in albumin excretion , and what is very important, there is a circadian rhythm of urinary albumin excretion. Therefore, measurement of 24-hour urine albumin excretion is the "gold standard" to assess albuminuria. Unfortunately, collecting urine during 24 hours is time-consuming and inconvenient, it is also subject to error due to inaccurate timing and incompleteness. It is widely accepted to use dipstick test to detect protein, i.e. albumin in the urine. The test is semiqantitative and is insensitive to detect small amounts of albumin, i.e. < 30 mg/dl. The test has specificity of > 95% but very low sensitivity ~40%. It can give false-positive results (concentrated urine, hematuria, contrast agents etc) and false - negative results (dilute urine). Dipsticks test for microalbuminuria (very low level of albumin in urine) are also available, with good sensitivity of 88% and a specificity of 80%. At present various antibody - based methods are used to measure urinary albumin (RIA, ELISA, nephelometry etc). Recently, a new method, i.e. high-performance liquid chromatography (HPLC) was developed. By this method immunoreactive and immunononreactive albumin could be measured (8, 9, 10). It is beyond the scope of this lecture to evaluate these techniques.

From clinical point of view more important is which sample of urine should be collected and how should be albuminuria expressed.

There is no doubt that the reference method to measure urinary albumin excretion is a 24-hour urine collection. But it is impractical, and we need more simple and less costly methods, et least in screening and in epidemiological studies. There are another reliable methods in evaluation of albuminuria: timed overnight collections, spot urine, i.e. first morning samples and random samples. Last two methods are untimed and results are expressed as albumin concentration or as albumin-creatinine ratio (Table 11.2.) To avoid influence of circadian variation, physical activity and hydration status the best sample is first-morning sample. More studies have been published and suggest that expression albuminuria as albumin-creatinine ratio is acceptable method in evaluation of albuminuria with good correlation with gold standard, i.e. 24-hour albumin excretion. Creatinine excretion in the urine depends on muscle mass, i.e. on gender, therefore we need different definitions for albuminuria for women and men. (Table 11.2.) (8, 9).

Table11.2. *Classification of urinary albumin excretion*

11.5 When and how often to evalaute albuminuria?

Once again albuminuria is an early sign of progressive kidney and cardiovascular disease in persons with and without diabetes (8, 11, 12). Unfortunately, despite many data of clinical value of screening and treatment of albuminuria, many diabetic and much more non diabetics are not screened for albuminuria. Any clinical screening program should fulfil some criteria, e.g. the disease for which the screening test should be used is an important health problem, the course of the disease is well described, the disease could be detectable in an early phase, there is suitable test to indicate the early phase of the disease, etc. Early detection of albuminuria in diabetics but also in general population fulfils these criteria.

It is now imperative to test for albuminuria in every day practice in persons with increased risk for chronic kidney disease, persons with increased risk for cardiovascular disease and to monitor therapy. There is no doubt that screening albuminuria is of great value in diabetics. Besides them it is reasonable to screen for albuminuria in individuals with obesity, hyperlipidemia, metabolic syndrome and with hypertension could (13, 14). At this moment we do not have enough date to start with screening in general population. We need to have in our mind that many individuals are not aware that they have diabetes or hypertension but they have albuminuria. Moreover in the PREVEND study has been shown that albuminuria gradually increases with increasing blood pleasure or plasma glucose level even within normal range. In other words, persons with higher but normal range of blood pressure or glucose level are et risk to have albuminuria, i.e. it means that albuminuria may precede manifest hypertension or diabetes.

Another important issue is treatment of albuminuria. A lot of studies (IRMA, BENEDICT, PREVEND IT etc.) have shown that lowering of albuminuria by either an angiotensin-converting enzyme (ACE) inhibitors or an angiotensin II receptor blocker (ARB) are associated with a better renal and cardiovascular outcome (15, 16, 17, 18). In fact there is suggestion by some authors that albuminuria reduction should be a clinical treatment target, like blood pressure changes in hypertension or glucose level in diabetes. Some observational studies showed that reduction of albuminuria strongly predict improve cardiovascular and kidney outcomes and that this prediction could be largely dissociated from blood pressure changes. Unfortunately we do not have enough randomized controlled trials to support

albuminuria as an independent therapeutic target. Currently renoprotective drugs (ACE or ARB drugs) are primarily antihypertensive drug and reduction of albuminuria is “side effect”.

At the end, how often should be albuminuria tested? First, every positive result should be repeated in next two weeks. Authors opinion is that if both tests are positive treatment to lower albuminuria should be started in diabetics, individuals with hypertension and cardiovascular disease. If only one test is positive it should be repeated after three months (4, 8). During treatment once per year detection of albuminuria could be performed (author’s opinion).

11.6 Conclusion

There is a lot of evidence that screening for albuminuria should be carried out in individuals with diabetes, but also in individuals with hypertension and cardiovascular disease. At this moment we need more date to support screening for albuminuria in general population.

At the end, the author did not use in this review the term microalbuminuria. As it is stated in article by Ruggenenti P and Remuzzi G, it is time that term microalbuminuria should be eliminated from our lexicon as there are data to suggest that albuminuria in “normal” range carries significant risk of cardiovascular risk. In other words there are no “cut off” values of normoalbuminuria and microalbuminuria (19). In addition in urine could be find the intact molecule but also albumin fragment. Therefore the term microalbuminuria was not used in this article.

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12. THE URINARY PROTEOMICS: A TOOL TO DISCOVER NEW AND POTENT BIOMARKERS FOR KIDNEY DAMAGE

Hassan Dihazi

12.1 Introduction

The increasing number of patients suffering from chronic renal failure represents one of the major challenges to which nephrologists are faced worldwide today. For a better therapeutic outcome of this disease earlier detection is urgently warranted in routine clinical practice. Urine is a kind of messenger of the urinary system function. Kidney damage or dysfunction results in release of peptides and proteins in urine (Figure 12.1.), this renders urine analyses of wide clinical interest for evaluation of kidney and urinary tract disorders. Urinary diagnostic can help to detect diseases that do not produce striking signs or symptoms at an earlier stage. Following parameter are routinely analysed for urine: method of collection, urine specific gravity, colour, turbidity, pH, glucose, ketones, bilirubin ictotest, blood and epithelial cells, and detection of proteins. Urinary proteins are of particular importance as their amount and composition reflect renal function and disorder (1). The estimation of protein amount in urine is of big importance for diagnostic as proteinuria is a marker for renal disfunction (2) and responsible for the progression of renal failure (3). Different methods were established to estimate the protein amount in urine, several of them found their way in routine diagnostic for evaluation of proteinuria. However, all these assays still not fulfil the conditions required for an adequate diagnostics. New techniques such as the analysis of the diseased renal proteome are highly promising to overcome some of these problems (4-9). Proteomics has enormous potential to improve the quality of urinproteins based diagnostic, as well as providing practical insights that will impact medical practice and therapy. Beside direct analysis of renal tissue, mass spectrometric approaches to urinary peptide/protein profiling are promising to have potential value in the none-invasive diagnosis, monitoring or prediction of renal and urinary tract diseases.

Figure 12.1. *Origin of urine proteins: in the urinary system high molecular weight proteins (> 40 kDa) are hold back in the glomerular part, whereas the low molecular weight proteins are absorbed in tubulus. Glomerular proteinuria led to increased release of high molecular proteins and tubular proteinuria is characterized by high excretion of low molecular weight proteins. Illustrated is the ratio of small and large proteins release in urine depending on the origin of the proteinuria. GFR: glomerular filtration rate.*

12.2 Defining proteomics and clinical proteomics

Proteomics is the systematic study of proteomes, which describes the entire protein content of one or all cells of an organism as ell as of bodily fluids such as blood, urine and sweat. While the genome of an organism is considered to be mostly static, the proteome shows dynamic properties with protein profiles changing in dependence of a variety of extra- and intracellular stimuli (i.e. cell cycle, temperature, differentiation, stress, apoptotic signals). Proteomics can be divided into three main areas: primarily, protein micro-characterization for large-scale identification of proteins and their post-translational modifications; secondly, differential display proteomics for comparisons of protein levels with potential application to a wide range of diseases; and thirdly studies of protein-protein interactions. Clinical proteomics is the part of proteomics that aims to characterize the interconnection between different tissues in organs or between organ and circulatory systems together, with clinical applications for diagnosis and therapy as ultimate target. Clinical proteomics include a large number of areas e.g. cancer proteomics, biomarker discovery, toxicoproteomics, pharmacoproteomics, stem cells proteomics, fluids proteomics… In clinical application, a comparative approach of normal and abnormal status of cells, tissues or bodily fluids is employed to identify proteins that exhibit quantitative changes in a disease-specific manner for use as diagnostic markers or therapeutic targets. Clinical proteomics still is a new promising analytic discipline with the following main aims: a) discovery of biomarkers allowing an early detection, risk management or therapeutic monitoring of diseases for the establishment of individualized treatment procedures, b) identification of protein targets for the development of new mechanistic intervention therapies with the promise of an improved clinical outcome.

12.3 Urinary proteomics and the advantages for clinical applications

Proteomics offer a new technology platform for identification and quantification of novel urinary biomarkers that may lead to the development of simple and more personalized diagnostic tests to be used in clinical practice for earlier disease detection and/or better therapeutic outcome (10). The proteomics techniques used to characterize urine can be divided in two groups: gel based urine proteome analysis and gel free urine proteome analysis (Figure 12.1., Table 12.1.) (11, 12).

The gel based techniques use two-dimensional gel electrophoresis. This method is powerful and widely used for the analysis of complex protein mixtures extracted from cells, tissues or biological fluids (13). The two-dimensional gel electrophoresis separates and characterizes proteins according to their charge/ion strength and molecular weight in two consecutive gel electrophoresis steps: Proteins are first separated by isoelectric focusing according to their isoelectric points and then distinguished according to their molecular weights in SDS-polyacrylamide gel electrophoresis. 2-D gel-electrophoresis is generally labour- and time-intensive and without strict standardization in the applied reagents, apparatus and software for the analysis usually not routinely applicable in clinical settings.

The gel free urine proteome analyses offer important conditions for the integration of proteomics in routine laboratories because of the reduced sample requirement and the high throughput and automation scale. For this reason, different methods have been developed which effectively couple high-end mass spectrometry to array formats, to capillary electrophoresis or to chromatography. The surface-enhanced laser desorption/ionization

(SELDI) technique offers such an opportunity for urine analysis. Small amounts of native urine samples can be applied to the surface of a SELDI ProteinChip without prior concentration or precipitation of the urinary proteins (8, 14). The bound proteins may then be directly analysed by MALDI-TOF-MS (Figure 12.2.) (15, 16). Also CE-MS coupled to the high-resolution properties of capillary electrophoresis (CE) can be used combined with the powerful identification ability of the electrospray time-of-flight MS to profile and sequence urinary proteins. Liquid chromatography coupled to mass spectrometry (LC-MS) offers also a gel free alternative for sensitive urine proteome analysis. Thus, protein profiles or single identified proteins may be characterized as disease specific protein pattern or biomarkers which, however, have to be validated in controlled retro- and prospective clinical studies.

Figure 12.2. *Gel based and gel free proteomics methods in urinary proteome analyses: Gel based urine analysis using 2D gel electrophoresis proteins will be separated according to their masses and pIs. After in-gel enzymatic digestion of the proteins the tryptic product can be analyzed by mass spectrometry. The identification can be performed by data bank search. Gel-free urinary proteome analysis. ProteinChip coupled to MALDI-TOF-MS (SELDI-TOF-MS) technology. Different types of ProteinChip surfaces are available. The chips are spotted with different chromatographic surfaces for urine protein binding. Bound proteins are then ionized with mass spectrometry resulting in protein profiles. CE-MS coupled the high-resolution properties of capillary electrophoresis (CE) and the powerful identification ability of the electrospray time-of-flight MS to profile urinary proteins. The resulting protein pattern can be used for diseases discrimination. Liquid chromatography coupled to mass spectrometry (LC-MS) offers also a gel free alternative for urine proteome analysis. Dihazi et al. (11)*

Table 12.1. *Summary of the proteomic platforms used for urine analysis, their advantages in disadvantages.*

Diagnostic tools using urine and non-invasive proteomic methods are particularly promising for the detection and differentiation of renal deterioration early before overt clinical symptoms during the various kidney specific or associated diseases. Furthermore proteomics methods have the potential advantage of lower costs and higher efficiency of patients care. Nevertheless, robustness, sensitivity, reliability and consistency of the test systems for the detection of changes in protein expression are crucial parameters in addition to labour and cost expenses for the acceptance of proteomics studies in specific clinical settings such as renal diagnostics. At present many proteomics techniques still suffer from insufficient standardization and only a few have the potential to fulfil essential criteria for future practical clinical application.

12.4 Trends in urine proteome analysis and biomarker discovery

Non-invasive accessibility of urine makes it attractive for the clinical proteomics. Different studies have already applied clinical proteomics to analyze the urinary proteome and tried to identify markers associated with renal diseases. The majority of these studies were carried out with a small number of individuals. Moreover these studies reported a peptide pattern or peptide/protein masse to charge (8, 9, 17-22). The identity of the discovered protein or peptide markers that discriminate renal disease is still lacking in most of this studies. Since the function of the protein marker can be very important for understanding the pathophysiology of the disease and might shed light on the involved pathways in the disease development. Regardless of the great promise of urine proteome analysis, the identification of urinary biomarkers by mass spectrometry technologies for an earlier diagnosis, prognosis or prediction of therapeutic responses in renal diseases has still many obstacles to cross. Additional to the technical aspects, handling conditions for urine are critical. The standardisation of urine collection is the first problem to be solved (Table 12.2.). In our days the midstream of the second morning urine was found to be optimal and was used with success in several studies (4, 23, 24). Urine collecting tubes should always include appropriate amount and composition of protease inhibitors to avoid protein degradation. After urine collection delays in analyzing the samples can result in artefacts, the interval of time between collection and analysis should be kept as short as possible. The delay in this handling step could have a high impact on the urine status and protein pattern. Protein degradation caused by proteases in urine, decreased clarity due to crystallisation of solutes, rising pH, loss of ketone bodies, loss of bilirubin, cell lysis leading to additional proteins in samples, overgrowth of contaminating microorganisms all these factors could be a source of artefacts in urine proteome analysis. The fragility of urine proteome renders the standardization of sample collection one of the main challenges facing the clinical proteomics and biomarker discovery. Recently published papers presented optimized protocols for urine handling for proteomics analysis (24-27). However, more intensive investigations are needed in this area to deliver optimal protocols for handling the fragile urinary proteome.

Important protein candidates for the therapy and for the understanding of the pathophysiology of renal disease are mostly in low amount in urine. Using depletion methods e.g., albumin/globulin depletion prior to proteome analysis make the access to low abundance proteins possible. Urine prefractionation can also be very helpful to prevent the complexity of the samples and to increase the analysis outcomes.

Additional to the biomarker identification, the quantification represent the next challenge to overcome. Traditionally urinary proteomics used gel based or mass spectrometry based methods (SELDI-TOF, LC-MS, CE-MS) for relative quantification. These approaches have their disadvantages. Quantification methods based on stable-isotope labeling coupled with mass spectrometry as the readout could offer promising alternatives. These alternatives are either peptide or protein based. The peptide based methods like the global internal standard technology (GIST) (28), or isobaric tags for relative and absolute quantification (iTRAQ) (29) have their drawback in the protein quantification, detection of posttranslational modifications, in detection of protein degradation, and in the reproducibility in the yield of the digestion which can result in errors in quantification. Among the protein based approaches the isotope-coded affinity tags (ICAT) (30) was the first established mass spectrometry based quantification method. The ICAT have cysteine as target amino acid for labelling. The low abundance of cysteine in proteins results in decrease of the quantification output. In in-gel stable-isotope labeling (ISIL) (31), protein samples are labeled with stable isotopes in the gel

matrix. The labeled proteins are digested, and analyzed by LC-MS. Isotope Coded Protein Label (ICPL) (32) is based on isotopic labelling of all free amino groups in proteins. Although these methods show their ability the perform relative and absolute peptide/protein quantification, most if not all are far from being applicable as a routine methods and it will be very challenging to implement effectively in routine urine analysis. In addition, information about the accuracy of these techniques in practice across multiple laboratories having various levels of expertise is still missing.

Table 12.2. *Urine collection methods advantages and disadvantages for urine proteome analysis*

12.5 Conclusion

There is a strong need for inter-laboratory standardization of the techniques and of the interpretation of the results at the first place. These challenges can only be overcome by intensively collaborating teams of researcher scientists, clinicians and statisticians also with the support of HUPO (Human Proteome Organisation http://www.hupo.org/) and HKUPP (Website of the International Human Kidney & Urine Proteome Project http://hkupp.kir.jp/), which try to provide organized platforms of all information available on normal and diseased human proteomes at the international level.

The adequate diagnosis of complex diseases e.g., renal disease with a single biomarker seems to be an illusion. A multiple biomarker assay could deliver a better and a more individualized diagnosis and allow therapeutic strategies that delay or prevent the progression of the disease. Due the above named limitations and uncertainties, urinary proteomics at present cannot replace invasive standardized diagnostic procedures such as the renal biopsy, but holds great promise and potential for future highly improved diagnosis and care of the patient in nephrology (12).

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13. POINT-OF-CARE CREATININE TESTING IN HIGH-RISK PATIENTS

Yolanda B. de Rijke

13.1 Introduction

In some circumstances, a creatinine check at home instead of a complete clinical control could be sufficient for patients with renal disease. Home monitoring of serum creatinine concentrations could, for instance, be useful during insecure periods after kidney transplantation, or to keep a close watch on possible disease recurrences. Below we describe a pediatric case of recurrent hemolytic-uremic syndrome (HUS), in which we would have liked to monitor creatinine levels at home in order to restrict the number of hospital visits.

Point-of-care testing (POCT) for creatinine concentrations offers the advantage of providing a result within minutes outside the hospital, which potentially enables faster diagnosis and management of HUS and other conditions threatening kidney function. Obviously, POCT devices employed at home must be easy to use in daily practice by the patient or his/her parents. Therefore, it was considered essential that tests could be carried out with a small volume of capillary blood. Notably, POCT systems used at home should produce results comparable to reference testing in the laboratory. To that purpose, we evaluated two different POCT systems for creatinine measurement (Roche Reflotron Plus and Abbott i-STAT) versus the routine test used in the laboratory of the Erasmus MC-Sophia Children's Hospital, in 20 children displaying an extensive range of creatinine blood values.

Children with atypical HUS have frequently been referred to the pediatric clinic in Rotterdam. This syndrome is characterised by microangiopathic hemolytic anemia, thrombocytopenia, and renal failure (1). A representative case described below gave rise to the present data.

13.1.1 Case history

At the age of four months, a boy presented with atypical HUS for the first time. Plasma exchange resulted in temporary clinical improvement, but the HUS recurred after a few months, and plasmapheresis could not prevent him from progression to end-stage renal failure. Peritoneal dialysis was started, yet after two years this was switched to hemodialysis due to a devastating peritonitis. Bilateral nephrectomy was performed at four years of age because of therapy-resistant hypertension.

Finding a suitable deceased kidney donor was difficult due to the patient's homozygosity on HLA-A, -B and -DR alleles. At the age of five years, the boy received an adult deceased donor kidney graft. Post-transplant, serum creatinine values quickly normalised to circa 25 µmol/L (see Figure 13.1.). Ten days post-transplant he showed perforation of the colon ascendens, for which a temporary stoma was applied. One month post-transplant, an acute rejection occurred, which was successfully treated with methylprednisolone. Subsequently, with monthly intervals, two episodes of bacterial gastroenteritis with increased serum creatinine concentrations due to dehydration, and a primo Epstein-Barr virus infection occurred. A quiet period of ten months ensued.

At the age of 6 1/2 years the patient displayed a sudden increase in serum creatinine concentration, along with hematuria. A graft biopsy was suspect for recurrence of HUS. The boy was treated by plasma exchange, daily during the first two weeks, followed by tapering in frequency, and after three months he was switched to weekly plasma infusions. During this treatment, serum creatinine values suddenly deteriorated, after which hemodialysis was restarted.

Figure 13.1. *Case history: patient serum creatinine concentrations in time. Each data point represents a single serum creatinine concentration obtained by routine laboratory measurement (Y-axis) at the age indicated (X-axis). Bold horizontal lines represent the reference range.*

At seven years of age, a second transplantation was performed using a kidney from the patient's mother. After problems with urinary drainage followed by reimplantation of the graft ureter, serum creatinine concentration settled at about 120 µmol/L. However, eleven months post-transplant another sudden, biopsy-proven recurrence of HUS occurred, with a rise in serum creatinine to approximately 700 µmol/L. Daily plasma exchanges resulted in reversal of the creatinine concentration to pre-recurrence level, and the frequency was very slowly tapered to twice every week. Two further episodes of rising serum creatinine concentration were seen, due to gastroenteritis and dehydration.

Ideally, the patient would have been checked by the physician on an almost daily basis. However, as the boy lived at a distance of about 50 km from our clinic and went to school normally, this was not feasible for him and his parents. To save the family anxiety and several trips to the hospital, we intended to optimise care by checking the boy's blood creatinine value at home.

13.1.2 Patients

We randomly included 20 outpatients of the Division of Pediatric Nephrology and dialysis patients, aged two months to 17.8 years, with varying creatinine serum levels. The parents were informed about the study and granted informed consent, provided that children resisting additional finger pricking would not be enrolled. In addition, samples were taken from six healthy adult volunteers, in order to obtain creatinine concentrations in the normal range. Capillary blood for regular laboratory testing and for the two point-of-care tests was collected at the same time. Collection of capillary blood was performed according to the NCCLS

protocol (2). Blood was collected in heparinised capillaries and immediately used to perform the Reflotron Creatinine and i-STAT Creatinine/Crea tests. Another 400 µL of blood was drawn into a heparin microtainer. Subsequently, tubes were centrifuged and plasma was stored at room temperature for up to one hour until routine creatinine measurement.

13.1.3 Primary outcome measure

The primary outcome measure was the difference between the creatinine concentration obtained by the routine automated assay (reference test) and creatinine POCT, in µmol/L. In addition, ease of use in daily practice was extensively assessed.

13.1.4 Creatinine tests

The Reflotron and i-STAT test were performed near the patient, whereas the routine assay was carried out in the laboratory.

The Reflotron Plus (Roche Diagnostics, Almere, The Netherlands) is a table model diagnostic POCT device working on the principle of reflectance photometry with approximate dimensions 21 × 30 × 35 cm, which uses reagent strips to measure a variety of clinical chemistry parameters. For the Reflotron creatinine test, 32 µL of whole blood was collected in a dedicated lithium heparine capillary to determine the creatinine concentration in µmol/L using a Reflotron Test Creatinine strip (Roche Diagnostics, Almere, The Netherlands) on the Reflotron Plus apparatus.

The i-STAT (Abbott Point-of-Care, Hoofddorp, The Netherlands) is a hand-held diagnostic analyser of approximate dimensions 5 × 6 × 21 cm, which uses cartridges to perform a range of clinical chemistry tests. In our hands, the i-STAT creatinine test required a minimum volume of 65 µL. Therefore, 125-µL heparinised capillaries were used to collect whole blood for the enzymatic amperometric measurement of the creatinine concentration (in µmol/L) employing the i-STAT device with Creatinine/Crea cartridges (Abbott Point-of-Care, Hoofddorp, The Netherlands). Two highly qualified laboratory technicians of the Department of Clinical Chemistry, who were blinded to the results of the routine test, performed all POCT measurements.

For the routine automated creatinine test, the enzymatic colorimetric CREA plus assay (which has been calibrated against HPLC) was performed on plasma in the Hitachi 912 analyser (Roche Diagnostics, Penzberg, Germany).

13.1.5 Statistical analysis

The linear association between the results from the near-patient creatinine tests and the routine test was analysed employing Pearson's correlation coefficient. The differences between the POCT and the reference creatinine concentrations were analysed using the methods of Bland and Altman (3) and Passing and Bablok (4). Over the concentration range between 50 and 500 µmol/L, creatinine POCT was considered clinically valid when values did not differ from the results of the routine test by more than 20 µmol/L (see below), at a biological within-subject coefficient of variation of 4.3% (5).

13.2 Results

For all patients, parental consent was given. Additional finger pricking was resisted by none of the patients, and sufficient material could be obtained from all of them. Reflotron, i-STAT and routine creatinine measurements were available for 20 children and six healthy adult volunteers. For the 20 children enrolled, the median age was 14.8 years (range, 0.2-17.8). Ten patients (50%) were male and ten (50%) were female. The median age for the boys and the girls was 14.6 years (range, 0.2-17.8) and 14.8 years (range, 6.8-17.7), respectively.

13.2.1 Method agreement

For the Reflotron and i-STAT test, the correlation coefficient versus the routine automated Hitachi 912 creatinine test was 0.99 and 1.00, respectively.

The Reflotron test generally produced lower creatinine values than the routine test, both overall and for the two outliers displaying a difference > 50 µmol/L (109 versus 167 and 167 versus 246 µmol/L) in the creatinine concentration range < 500 µmol/L (Bland-Altman plot, see Figure 13.2.A).

The overall mean difference between Reflotron and routine creatinine measurement was -16 µmol/L (n=26, 95% CI -30 to -3). In the concentration range of up to 500 µmol/L and for concentrations > 500 µmol/L creatinine, the mean difference was -11 µmol/L (n=23, 95% CI -21 to -2) and -55 µmol/L (n=3, 95% CI -149 to 39), respectively. By contrast, the i-STAT test mainly generated slightly higher values in the creatinine concentration range < 500 µmol/L, with a smaller distribution as compared to the Reflotron test and no outliers showing differences > 50 µmol/L between i-STAT and Hitachi 912 creatinine values. However, above 500 µmol/L creatinine the i-STAT produced substantially lower values than the routine test (Bland-Altman plot, see Figure 13.2.B). The overall mean difference between i-STAT and routine measurement was 4 µmol/L (n=26, 95% CI -5 to 13). In the concentration range of up to 500 µmol/L and for concentrations > 500 µmol/L creatinine, the mean difference was 11 µmol/L (n=23, 95% CI 6 to 15) and -52 µmol/L (n=3, 95% CI -68 to -35), respectively.

With regard to the Reflotron test, 16 out of 26 results (62%) fell within ± 20 µmol/L of the routine test results. In the creatinine concentration range above 500 µmol/L, three values were outside this window: differences between Reflotron and routine measurement were -138, -54 and 28 µmol/L. In the creatinine concentration range below 500 µmol/L, the maximum difference was surpassed for 7 out of 23 (30%) pairs of data, with deviations between -79 and 41 µmol/L.

With regard to the i-STAT test, 20 out of 26 (77%) of the results fell within ± 20 µmol/L of the routine test results. In parallel to the Reflotron data, three values were also outside this window in the creatinine concentration range above 500 µmol/L: differences between i-STAT and routine measurement were -61, -59 and -35 µmol/L. In the concentration range below 500 µmol/L, the maximum difference was surpassed for 3 out of 23 (13%) pairs of data, with deviations of 21, 24 and 40 µmol/L.

When Reflotron and Hitachi 912 test were compared according to Passing and Bablok (4), the slope was 0.95 (95% CI 0.87 to 1.06), and the intercept was -2.8 (95% CI -13.5 to 7.4; y = 0.95x - 2.8). When the i-STAT and Hitachi 912 test were compared, the slope was 0.96 (95%

CI 0.90 to 1.00), and the intercept was 16.3 (95% CI 9.2 to 24.6; y = 0.96x + 16.3), indicating a constant bias. P-values from cusum tests for linearity were > 0.1.

Figure 13.2. *Bland-Altman comparison plots of creatinine concentrations measured by (A) the Roche Reflotron versus the Hitachi 912 routine automated test, and (B) the Abbott i-STAT versus the Hitachi 912 test. A dotted horizontal line represents the mean difference between the values obtained by either POCT system and the routine test, and the bold solid horizontal lines represent a -20 µmol/L and 20 µmol/L difference.*

13.2.2 Ease of use

Next to the difference between the creatinine concentration measured by creatinine POCT and the routine test, we extensively assessed the ease of use in daily practice for both POCT systems. Although the Reflotron test required slightly less material than the i-STAT test (32 versus 65 µL whole blood, respectively), it turned out that measurement could be carried out using only a drop of blood and yielding a result within 2-3 min employing either apparatus. Obviously, we consider it essential that the intended users receive the appropriate instructions from laboratory personnel, before any POCT device can be adequately applied at home. Although the i-STAT cartridge system may require a little more training, we feel that Reflotron and i-STAT could both perform well in this situation, provided that proper instruction is given. However, the i-STAT system will be easier to use at home: as it seems to have been developed for use in smaller laboratories, it can be applied as a hand-held instrument. By contrast, the Reflotron is a table model apparatus; moreover, even in the hands of our experienced laboratory workers, air bubbles present in the blood capillary tended to be more problematic (by yielding markedly decreased creatinine values) using the Reflotron device.

13.3 Discussion

In the present study, two creatinine POCT devices were compared to a routinely used laboratory method for clinical use in children at risk for sudden decrease of renal function, such as those with recurrent disease, as in non-infectious HUS, or instable kidney transplant patients. In healthy children, serum creatinine concentrations are between 18 and 88 µmol/L (18-35 µmol/L between one and four years of age, 31-68 µmol/L between four and 13 years of age, and 37-88 µmol/L between 13 and 17 years of age). At a creatinine value of 500-600

µmol/L, end-stage renal failure will have been reached in all children. To detect significant changes in creatinine blood concentrations, a home monitoring device should be able to detect an increase in concentration exceeding 20 µmol/L in the range between 50 and 500 µmol/L creatinine.

Both the Reflotron and the i-STAT test correlated well with the routine automated test, especially in the creatinine concentration range up to 500 µmol/L. The Bland-Altman plot for the Reflotron test demonstrates good correlation with the routine test up to a concentration of 500 µmol/L creatinine, displaying only two outliers with deviations > 50 µmol/L. However, above a concentration of 500 µmol/L, it shows a considerable increase in distribution with deviations of -138, -54 and 28 µmol/L. The Bland-Altman plot for the i-STAT test displays a smaller distribution up to a creatinine concentration of 500 µmol/L. However, the i-STAT also shows an increasing distribution above 500 µmol/L, with deviations of -61, -59 and -35 µmol/L.

Both near-patient tests thus show less precise measurement of creatinine values in the highest concentration range (> 500 µmol/L). In the abovementioned clinically relevant creatinine range between 50 and 500 µmol/L, however, the maximum difference was surpassed in only 30% and 13% of cases for the Reflotron and i-STAT test, respectively.

The Reflotron and i-STAT POCT creatinine tests can both be performed using only a drop of blood for measurement within 2-3 min employing either. Although we did not explicitly evaluate the use of both POCT systems outside our hospital, the use of the i-STAT test at home seems more feasible because of its greater ease of use. The i-STAT device was evaluated to everyone's satisfaction by two inexperienced medical students at the outpatient clinic. At present, it is already frequently used for near-patient measurements in hospital settings, with the main purpose of blood gas and glucose monitoring at the Intensive Care Unit. To our knowledge, this is the first study in literature, in which a creatinine point-of-care test has been evaluated for its possible use at home. Hence, financial support has been given by the Social Policy unit of the Dutch Kidney Foundation in order to cover the reagents' expenses. Taking into account the general underestimation of creatinine values and its somewhat smaller ease of use, we feel that, although not tested in daily practice, the Reflotron test is less suitable for creatinine testing at home and more appropriate for a laboratory setting.

Our eventual aim is to be provided with predictive information by measuring the patient's blood creatinine concentration at home. Although the distribution with both POCT devices suggests that they would perform well in daily practice, it can not be ruled out that they will achieve worse when employed by less experienced users. For both systems, we therefore consider it essential that the patient and/or his/her parents receive the appropriate instructions from laboratory personnel before such devices can be adequately used at home.

Regarding both the patient's quality of life and the expenses of POCT systems versus plain routine creatinine testing, it should be considered that faster diagnosis and management of serious conditions such as HUS might be attained employing near-patient testing at home on a daily basis. The additional cost of the device plus the reagents might thus be outweighed by both the patient's eventual benefit and the reduction in extra expenses that might have arisen otherwise (e.g., the higher cost of hospital treatment upon leaving such conditions untreated for a longer time).

At the moment, patients who are suspected for changes in creatinine values have to visit the hospital for measurement of their serum creatinine concentration. A few transplant patients have learned to draw capillary blood, collect it in a microtainer and send it to the hospital by regular mail, as previously described (6). However, this takes a few days between drawing blood and test conclusion, resulting in cumbersome communication. Direct measurement at home provides a creatinine value within a few minutes, after which the result may directly be communicated to the treating physician in the hospital by email. For the patient, this may allow restriction of hospital visits and fine-tuning of his or her treatment.

In conclusion, the use of point-of care creatinine tests is a promising tool for the early diagnosis and management of conditions threatening kidney function. The Roche Reflotron Plus with Creatinine strips and the Abbott i-STAT with Creatinine/Crea cartridges are good candidates for near-patient creatinine testing in blood, yet the i-STAT system seems best for physiologic monitoring at home given its greater ease of use.

Early recognition and treatment of declining kidney function may lead to a better prognosis in renal patients.

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14. RECENT APPROACHES TO THERAPY: IS THERE REAL PROGRESS?

Markus Ketteler

14.1 Introduction

"Real progress" must be considered a subjective term with regard to therapeutic advances in any field of medicine. Novel approaches may be of significance because of introducing new treatment targets, representing completely new mechanistic strategies or reaching major success rates when compared to standard approaches, respectively. Labelling strategies as "real progress" must however be done with caution in any case, because favorably influencing outcomes on short or intermediate term may still not change or even unfavorably alter long-term perspectives. Nevertheless, this brief article intends to address a couple of recent developments in the field of nephrology which either unexpectedly discovered a new avenue in an established therapeutic field, i.e. the use of active vitamin D analogues, or opens new perspectives in a disastrous disease, calciphylaxis (= calcific uremic arteriolopathy [CUA]), which previously presented with mortality rates of 50 to 80%.

14.2 Vitamin D and survival

In each year's last issue of the TIME magazine, the ranking of the 10 most important breakthroughs of the current year is published in fields of culture, politics, music, economy, medicine etc. Surprisingly, in the year 2007 the realization of the importance of vitamin D, an old player in physiology and pathophysiology of the human body, was ranked among the 10 medical breakthroughs of the year. This was due to increasing awareness of the fact that vitamin D does not just play a role in calcium homeostasis and bone turnover, but acts as a pleiotropic steroid hormone controlling cell growth (anticancer effects), the immune system (antiautoimmune and antiinfectious properties) and cardiovascular functions (myocardial integrity). The vitamin D receptor (VDR) is virtually expressed in most tissues indicating a wide-spread biological importance of vitamin D signalling throughout the body (Table 14.1.)

Table 14.1. *Vitamin D receptor distribution (from: Andress DL. Vitamin D in chronic kidney disease: a systemic role for selective vitamin D receptor activation. Kidney Int 2006;69:33-43).*

Recent studies indicated that vitamin D deficiency was associated with impaired survival in all patient cohorts under investigation, i.e. in normal populations, in patients with chronic kidney disease (CKD) but not on dialysis as well as in CKD patients on dialysis. Moreover, treatment with active vitamin D analogues was consistently correlated with improved survival in CKD patients, while one recent study showed a beneficial relationship between vitamin D supplementation and survival in normal individuals. The caveat of these treatment reports, despite their consistency, was that they were all observational and thus not prospective interventional studies. This means that some degree of decision bias may have influenced final observations, and that these studies therefore do not prove any cause-and-effect relationships.

14.2.1 Biology of cardiovascular vitamin D actions

One factor in order to judge such associations is biological plausibility, and these biological insights are mostly gained by interventional experimental research. One of the keys towards the understanding of vitamin D-related actions was the creation of VDR knockout ($VDR^{-/-}$) mice. These animals showed a significant up-regulation of both renin gene expression and activation of angiotensin II. As a central pathophysiological readout, these $VDR^{-/-}$ mice developed severe left ventricular hypertrophy (LVH) associated with myocardial upregulation of renin gene expression (1). This observation deserves particular attention given the clinical fact that most dialysis patients develop into being calcitriol "knockouts" and show a high incidence of suffering from LVH. Bodyak *et al*. extended the experimental results by demonstrating that salt-induced myocardial hypertrophy could be completely prevented by treatment with the novel vitamin D receptor activator paricalcitol, independent on its influence on hypertension (2). These findings initiated the design of two clinical studies (PRIMO I in CKD stages 3b-4, and PRIMO II in CKD 5D) investigating the influence of paricalcitol on the potential of regressing LVH in patients evaluated by both echocardiography as well as MR scan. Both PRIMO studies just started recruitment.

From the nephrology perspective, the foremost indication of vitamin D treatment still remains secondary hyperparathyroidism (sHPT). In this regard, there is still uncertainty to which degree the potential of active vitamin D analogues to induce hypercalcemia and hyperphosphatemia in higher doses may incur unfavorable effects on CKD patients. While observational studies again do not point to a relevant risk association even in the highest quintiles of calcium, phosphate and iPTH levels, it can not be entirely excluded that individual elevations of the calcium x phosphate product under treatment may indicate overtreatment and risk. In experimental studies, paricalcitol is by far less calcitropic than the first and second generation active vitamin D analogues (calcitriol, 1-alpha, doxercalciferol), while in clinical trials this benefit seems somewhat less pronounced. Nevertheless, in uremic rats following 5/6-nephrectomy, it was recently shown that paricalcitol did not cause any medial calcification in the aortic wall, in dramatic contrast to animals treated with calcitriol or doxercalciferol, respectively. Lopez *et al*. presented data that the combination of a calcimimetic with paricalcitol was particular effective to prevent vascular calcification, while the combination with calcitriol showed an intermediate effect. Low-dose calcimimetic combined with low-dose active vitamin D treatment may thus become the mainstay of effective sHPT treatment in the future.

14.2.2 Vitamin D analogues and all-cause mortality

In the largest observational trials on the impact of active vitamin D treatment on survival in CKD 5D patients, it has to be noted that the survival benefits stretched well beyond cardiovascular outcomes (3). There are meanwhile numerous experimental reports as well as preliminary clinical studies in cancer patients demonstrating inhibitory effects on tumor cell growth (prostate, leukemia, colon). As one out of several mechanistic example, metabolism and thus detoxification of the colon cell carcinogen lithocholic acid is facilitated through the vitamin D-dependent enzyme CYP3A9, thus genuine vitamin D deficiency may induce a specific risk for developing neoplasms of the colon.

Vitamin D deficiency is also linked to autoimmunity and as such to diseases including rheumatoid arthritis, multiple sclerosis and type I diabetes mellitus. Potentially even more interesting was the recent observation that availability 25-OH-vitamin D may be a key defense mechanism against intracellular pathogens such as mycobacterium tuberculosis. Macrophages endogenously “turn on” their 1-alpha-hydroxylase as well as their VDR following contact with mycobacteria, and 25-OH-vitamin D levels then determine whether the tuberculocidic protein cathelicidin is expressed in sufficient amounts (4). This breakthrough finding may be a protoptypic for some anti-infectious properties related to vitamin D metabolism on a cellular level. The new therapeutic paradigm might be low to moderate “hormone replacement” instead of high-dose PTH suppression by active vitamin D analogues in CKD patients, in addition to the correction of insufficient 25-OH-vitamin D levels.

14.3 Definition of calciphylaxis

Calciphylaxis is a rare, but potentially life-threatening syndrome characterized by progressive and painful skin ulcerations associated with media calcification of medium-size and small cutaneous arterial vessels (5). Calciphylaxis primarily affects patients on dialysis or after renal transplantation, however, exceptions have been reported in patients with normal renal function and in association with chronic-inflammatory disease, malignancy or primary hyperparathyroidism. Clinical manifestation of calciphylaxis is associated with high mortality of up to 80%, superinfection of necrotic skin lesions with subsequent sepsis significantly contributing to this dramatic outcome. However, many calciphylaxis patients also suffer from advanced cardiovascular disease characterized by severe calcifications of larger arterial vessels. There are currently no exact numbers on the incidence of calciphylaxis available. Based on small international surveys, incidence is estimated to be in the range of 1:1.000 to 1:1.500 cases in patients on chronic renal replacement therapy per year, but there is good reason to suspect underrecognition caused by mild cases or misdiagnosis in a relevant percentage of patients.

14.3.1 Therapeutic options: old and new

Therapeutic approaches are limited in calciphylaxis. As pointed out above, the available data is restricted to case reports and small case-control studies, while prospective studies are not available. Once calciphylaxis is suspected or diagnosed in a uremic patient, the first therapeutic aim must be normalization of the calcium x phosphate product, i.e. by intensifying dialysis treatment, by using a low dialysate calcium and by high-dose treatment with (preferably calcium-free) phosphate binders. Reduction or withdrawal of active vitamin D treatment must be considered depending on the corresponding levels of PTH and calcium x phosphate product. In calciphylaxis patients with hyperparathyroidism and signs of high bone

turnover, „emergency“ parathyroidectomy must be considered immediately. However, in such patients administration of calcimimetics may represent an effective therapeutic alternative - promising case reports on this conservative intervention have been published recently. Once progressive ulcerations and necrosis are observed, early broad-spectrum antibiotics should probably be initiated.

Some data are available concerning the use of sodium thiosulfate and of bisphosphonates in the treatment of calciphylaxis. Thiosulfate is available as a chelating agent indicated for the treatment of cyanide intoxication. On the one hand, it possesses a high affinity to calcium ions, which may interfere with calcium and phosphate precipitation producing soluble calcium thiosulfate which can potentially be removed by dialysis. On the other hand, thiosulfate may also interfere with the local inflammation process by antioxidant properties. Both concepts currently lack proof.

It is currently unclear, whether bisphosphonates interact with extraosseous calcification processes via their antiresorptive bone effects or via direct peripheral pyrophosphate-like effects at the tissue sites. Pyrophosphates are small molecules acting as potent inhibitors of calcification at local tissue sites, while pyrophosphate deficiency causes severe soft-tissue calcifications in experimental animals as well as in humans (“Idiopathic Infantile Arterial Calcification”) (6). Although case reports on beneficial effects of pamidronate in calciphylaxis patients have recently been published, caution is advised concerning uncritical use of bisphosphonates in this patient group unless adynamic bone disease (ABD) is excluded or highly unlikely, since ABD will be aggravated by these compounds, especially in renal failure patients.

14.3.2 Vitamin K and calciphylaxis: a novel pathomechanistic concept

Matrix Gla protein (MGP) is a 10 kD protein exclusively expressed in vascular smooth muscle cells (VSMC) and chondrocytes (6). This protein requires post-translational vitamin K-dependent γ-carboxylation for activation. Accordingly, warfarin treatment suppresses MGP activation. Knockout of the MGP gene in mice ($MGP^{-/-}$) causes severe media calcification of large arteries with subsequent rupture of the ossified aorta -$MGP^{-/-}$ mice actually die of internal arterial hemorrhage at the age of 6 -8 weeks. MGP acts purely as local inhibitor, systemic overexpression is not capable of counteracting arterial calcification induced by $MGP^{-/-}$. Analogously, media calcification can also be induced by treatment with vitamin K antagonists. In rats, warfarin-induced vascular calcification can be partially reversed by feeding supraphysiological doses of vitamin K1 or K2 following withdrawal of warfarin, whereas calcification progresses when only low doses of vitamin K are fed (7).

Case reports already suggested a relatively high coincidence between warfarin treatment and calciphylaxis. The German registry branch of the “International Cooperative Calciophylaxis Network” (ICCN) collected 50 cases of calciphylaxis during the least 1.5 years and found that 42% of these patients had been on warfarin treatment when calciphylaxis developed (Ketteler M, Brandenburg VM, unpublished). Therefore, and based on the biological plausibility related to MGP inactivation, warfarin withdrawal and switch to heparin use is most probably warranted and urgently recommended, despite a lack of clear-cut prospective clinical evidence. Subsequent high-dose vitamin K supplementation may have to be addressed by future studies in this patient group and may even develop into a protective therapeutic means. Current and emerging treatment strategies of calciphylaxis are listed in Table 14.2.

Table 14.2. *Current and future therapeutic strategies for calciphylaxis (adapted from: Ketteler M and Biggar P. Calciphylaxis: Epidemiology, Pathophysiology and Therapeutic Options. BANTAO J 2008;6:1-5).*

14.4 Summary

Among many recent developments of therapeutics in the nephrology field, e.g. new phosphate binders such as sevelamer carbonate and lanthanum carbonate, long-acting ESA's such as C.E.R.A., novel therapeutics in the transplant field, new indications for powerful biologicals such as rituximab etc., the biology of vitamin D and the new promise of successfully counteracting calciphylaxis appear to be this reviewer's "personal highlights". Still, even these perspectives will have to prove their reliability and validity in the future.

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