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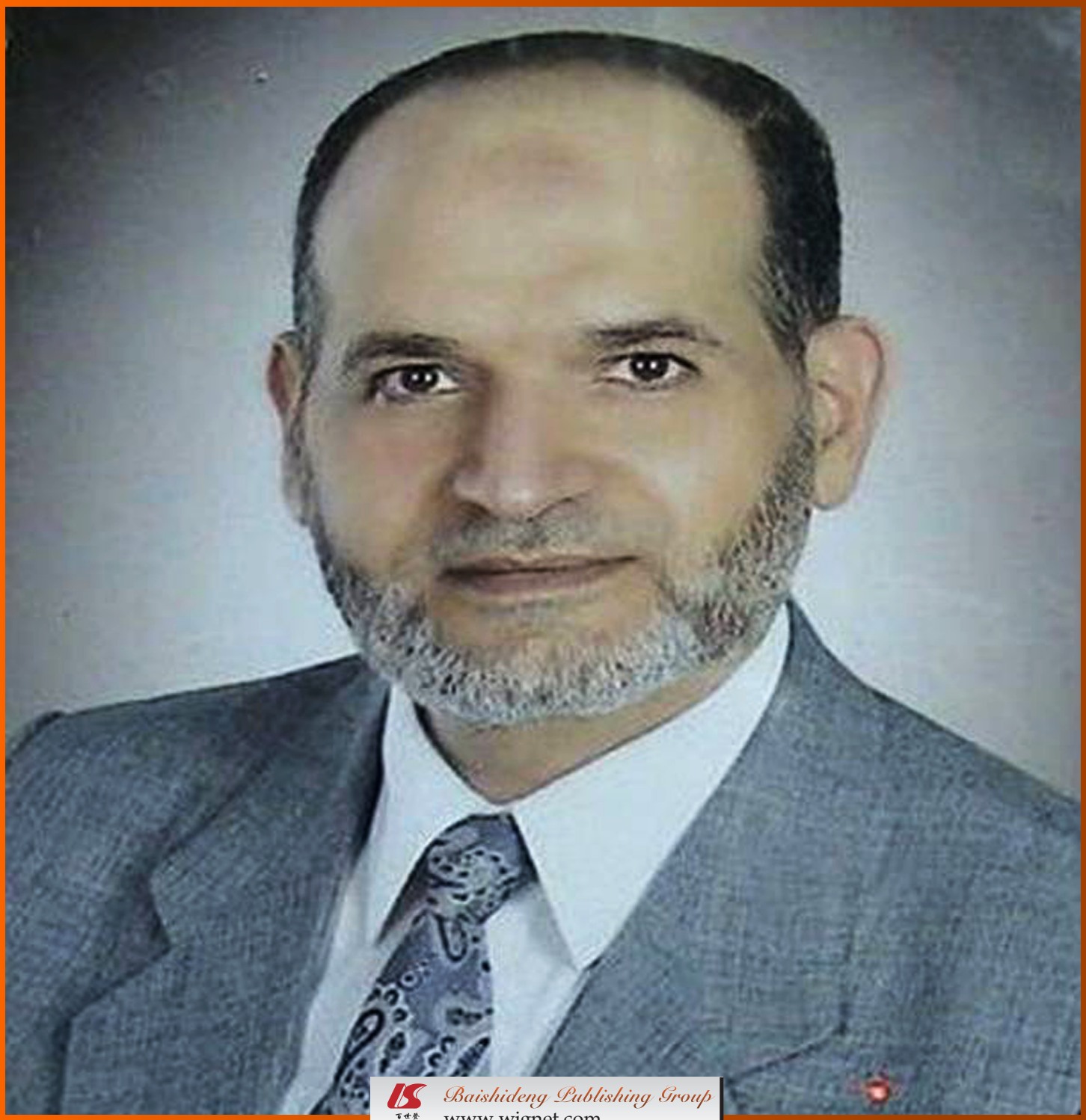

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World Journal of Nephrology (*World J Nephrol*, *WJN*, online ISSN 2220-6124, DOI: 10.5527) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Nephrology
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: wjnephrol@wjgnet.com
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EDITORIAL

Cystinosis as a lysosomal storage disease with multiple mutant alleles: Phenotypic-genotypic correlations

Mohammad Al-Haggar

Mohammad Al-Haggar, Pediatrics Department, Genetics Unit, Mansoura University Children's Hospital (MUCH), 35516 Mansoura, Egypt
Author contributions: Al-Haggar M solely contributed to this paper.
Correspondence to: Mohammad Al-Haggar, MD, Professor, Pediatrics Department, Genetics Unit, Mansoura University Children's Hospital (MUCH), 60 El Gomhoureya St., 35516 Mansoura, Egypt. m.alhaggar@yahoo.co.uk
Telephone: +20-50-2310661 Fax: +20-50-2234092
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Abstract

Cystinosis is an autosomal recessive lysosomal storage disease with an unclear enzymatic defect causing lysosomal cystine accumulation with no corresponding elevation of plasma cystine levels leading to multisystemic dysfunction. The systemic manifestations include a proximal renal tubular defect (Fanconi-like), endocrinal disturbances, eye involvements, with corneal, conjunctival and retinal depositions, and neurological manifestations in the form of brain and muscle dysfunction. Most of the long-term ill effects of cystinosis are observed particularly in patients with long survival as a result of a renal transplant. Its responsible *CTNS* gene that encodes the lysosomal cystine carrier protein (cystinosin) has been mapped on the short arm of chromosome 17 (Ch17 p13). There are three clinical forms based on the onset of main symptoms: nephropathic infantile form, nephropathic juvenile form and non-nephropathic adult form with predominant ocular manifestations. Avoidance of eye damage from sun exposure, use of cystine chelators (cysteamine) and finally renal transplantation are the main treatment lines. Pre-implantation genetic diagnosis for carrier parents is pivotal in the prevention of recurrence.

Key words: Cystinosis; *CTNS* gene; Phenotypic-genotypic correlation

Core tip: Cystinosis is an autosomal recessive lysosomal storage disease of cystine manifested primarily in the eye and kidneys; corneal cystine deposition detected by slit lamp and a proximal renal tubular defect (Fanconi-like) are the main clinical features. Its responsible gene, called *CTNS*, encodes the lysosomal cystine carrier protein (cystinosin) and has been mapped on the short arm of chromosome 17. Clinical forms of cystinosis depend upon age of onset of main symptoms. Besides cystine chelation, treatment includes eye protection from sun exposure and renal support up to transplantation. Carrier detection among parents and prenatal genetic diagnosis is the mainstay of prevention.

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CLASSIFICATIONS OF INHERITED FORMS OF RENAL TUBULAR ACIDOSIS

Inherited forms of proximal renal tubular acidosis

Proximal renal tubular acidosis (RTA) resulting from Fanconi syndrome is a frequent part of systemic syndromes. Among systemic disorders that result in RTA, the inheritance pattern is usually autosomal recessive. Some of these disorders are cystinosis, tyrosinemia, galactosemia, Fanconi Bickel Syndrome and others. These syndromes are heterogeneous groups of disorders. Their genes are mapped in many chromosome regions[1]. These inherited forms are demonstrated in Table 1[2].

Proximal RTA unrelated to Fanconi syndrome is a rare disorder and might be sporadic, autosomal dominant

Table 1 Chromosomal mapping of some inherited proximal renal tubular acidosis

Inherited proximal RTA	Gene	Chromosomal mapping
Autosomal recessive	*SLC4A4*	4q21
Dent´s syndrome	*CLCN5*	Xp11.22
Cystinosis	*CTNS*	17p13.2
Tyrosinemia type 1	*FAH*	15q23-q25
Galactosemia	*GALT*	9p13
Wilson´s disease	*ATP7B*	13q14.3-q21.1
Fanconi Bickel Syndrome	*SLC2A2*	3q26.1-26.3

RTA: Renal tubular acidosis.

or autosomal recessive. The autosomal recessive disorder is associated with ocular abnormalities and frequent coursing with mental retardation. Other clinical features are short stature, dental enamel defects, pancreatitis and basal ganglia calcification[3]. Loss-of-function mutations in the gene that encodes the NBC-1 protein (*SLC4A4* gene) were first identified in two Japanese patients with proximal RTA associated with cataracts, glaucoma and band keratopathy[4].

NBC-1 is formed by 1035 amino acids and contains ten transmembrane domains and two cytoplasmic termini. It is present in the kidney, brain, eye, pancreas, heart, prostate, epididymis, stomach and intestine. In the kidney, NBC-1 is expressed mainly at the basolateral membrane of the proximal tubule. At least two genes encode the NBC-1 proteins. Mutations were identified in the human NBC-1 gene (*SLC4A4*) mapped at chromosome 4q21[5,6]. Another interesting candidate gene for proximal RTA is the *TASK* gene. Its expression is located in the pancreas, placenta, lung, small intestine, colon and kidney. *TASK* seems to be important to HCO_3^- reabsorption in renal proximal tubules[7]. Another inherited form of proximal RTA is the one resulting from mutations in the gene *CA2* that encodes CA Ⅱ. The carbonic anhydrases are members of a family of zinc metalloenzymes that catalyze the hydration of CO_2. The human CA Ⅱ maps to the chromosome region 8q22. In the kidney, the majority of CA activity is attributable to CA Ⅱ, which is localized in the proximal tubular cells and in α-intercalated cells of the cortical and outer medullary collecting tubules[8]. Due to their localization, this RTA courses with some proximal and distal components. In terms of clinical aspects, this form of RTA presents with osteopetrosis, cerebral calcification and different levels of mental retardation.

The autosomal dominant proximal RTA was originally described in a large Costa Rican family[9], consisting of nine individuals presenting with growth retardation and osteomalacia. No gene was found to be associated with this clinical presentation. Later on, another family with isolated proximal RTA inherited as an autosomal dominant disease was described[10].

Inherited forms of type III RTA

Type Ⅲ RTA is a mixed type that exhibits both impaired proximal HCO_3^- reabsorption and distal acidification. The condition is due to an inherited deficiency of CA Ⅱ caused by a recessive mutation in the CA2 gene (*SLC26A6*) on chromosome 8q22, which encodes this widely expressed enzyme. The expression of CA Ⅱ is affected in bone, kidney (in both proximal and distal nephron segments, explaining the mixed acidosis) and brain[11,12]. The mechanisms that underlie the clinical course in type Ⅲ RTA, apart from much slower conversion of carbonic acid to and from HCO_3^-, apparently also involve a direct interaction between CA Ⅱ and the kidney NBC1 or Cl^-/HCO_3^- exchanger[13]. Mutations of the identified CA Ⅱ binding site reduce SLC26A6 activity, demonstrating the importance of this interaction[11,14]. Patients with this deficiency exhibit osteopetrosis and cerebral calcification, as well as mixed RTA with proximal and distal components[15]. There is a considerable degree of heterogeneity, both in the predominance of proximal or distal acidosis and in the osteopetrotic phenotype. In different kindred, mild or severe mental retardation has also been described[12]. Different mutations in CA2 gene have been described; for example, the common "Arabic" mutation, consisting of loss of the splice donor site at the 5' end of intron 2[12,15].

Inherited forms of Type IV RTA

Type IV RTA is a heterogeneous group of disorders associated with hyperkalemia due to aldosterone deficiency or impairment in aldosterone molecular signaling. The inheritance might be autosomal dominant or autosomal recessive. The autosomal dominant form is a frequent and milder form without other organ involvement. It seems to be associated with loss-of-function mutations in the mineralocorticoid receptor gene, the *MRL* gene. MRL-knockout mice develop symptoms of pseudohypoaldosteronism. In humans, clinical presentation varies from non-symptomatic to important neonatal Na^+ loss. The autosomal recessive form is associated with Na^+ transport defects in all aldosterone target tissues, not only kidney, but also colon, lungs, salivary and sweat glands. The recessive form is more severe with more pronounced salt wasting. However, both types of inheritance might result in the same degree of natriuresis, hyperkalemia and metabolic acidosis[16].

Other inherited causes of type IV RTA include hyperkalemia associated with hypertension and low or normal levels of plasma aldosterone[17,18]. This syndrome is called pseudohypoaldosteronism type 2 (PHA2) or Gordon's syndrome, which results in renal aldosterone resistance. It is inherited as an autosomal dominant pattern. Mutations in the gene of two isoforms of WNK serine-threonine kinases (*WNK4* and *WNK1* genes) were identified in patients with PHA2[19]. WNKs are serine kinase proteins lacking a lysine residue at the active site, being the WNK type 1, a regulatory protein from WNK 4. WNK4 is found in the distal nephron and controls Na^+ and Cl^- re-uptake and inhibits K^+ efflux[12].

CLINICAL BACKGROUNDS OF CYSTINOSIS

Cystinosis has been classified as a lysosomal storage disorder based on the intralysosomal localization of stored cystine; however, it differs from the other lysosomal diseases in that the principal lysosomal enzyme of acid hydrolysis is not known to play a role in the metabolic disposition of cystine. Moreover, plasma cystine levels are well below saturation, indicating that the defect is a cellular one. With electron microscopy of cystine-laden cells, cystine is compartmentalized with acid phosphatase and is membrane-bound, as demonstrated by electron microscopy.

In heterozygotes, concentration of free cystine was found to be several times the normal in the leukocytes of parents of patients[20], proximal renal tubular deposition of cystine results in Fanconi-like syndrome[2,21]. Teree *et al*[22] studied 2 male sibs physiologically and anatomically with cystinosis. Microdissection of the kidney tubules suggested that the morphological abnormality of the proximal tubule is "acquired" and progressive; however, these changes did not develop in renal transplants among four cystinosis children[23]. Endocrinal disturbances in the form of hypothyroidism due to extensive cystine deposition have been reported and represents one of the factors that could explain growth retardation of cystinosis patients[24-26]. Jonas *et al*[27] described a cystinosis patient who started at the age of one year, in end-stage renal failure at the age 7 years and at the age of 24 years, she was very dwarf (her height was 123 cm). She had marked photophobia, corneas and conjunctiva laden with refractile material, and a patchy retinopathy. There were signs of ovarian failure, intermittent confusion, short-term memory loss and cerebral atrophy on computerized axial tomography. Autopsy examination at the age of 25 showed cystine storage in multiple tissues, including pancreatic islet cells, the aorta, the atrophic ovaries and brain[27]. Myopathy with generalized muscle weakness and wasting due to accumulation of cystine in and around muscle fibers has been reported in a 22-year-old man who had a renal allograft at the age of 10 years[28]. In a patient who underwent renal transplantation aged 30 mo, cystinosis was the only detected cause for progressive renal failure[29]. The long-term ill effects of cystinosis, observed particularly in patients with long survival as a result of renal transplant, include pancreatic endocrine and exocrine insufficiency[30,31] and, as mentioned earlier, recurrent corneal erosions, CNS involvement and severe myopathy. Oral motor function was assessed in 43 cystinosis patients aged 3-30 years, 24 of whom had received a renal transplant. Approximately half of them were slow eaters and the marked oral motor dysfunction increased with age[32]. In studies of intelligence in 14 families of children with infantile nephropathic cystinosis, Williams *et al*[33] found that the IQs of 15 children with cystinosis were significantly lower on average than those of their sibs and parents. Even although the mean IQ of the children with cystinosis (94.4 ± 10) was within the average range, there was evidence that they had a mild global intellectual deficit compared to their expected IQ based upon the IQs of their relatives. Several have commented that patients with cystinosis have skin and hair pigmentation noticeably lighter than that of their unaffected sibs. It has been speculated that pigment formation may be impaired in the melanosomes, which are the melanocyte counterparts of lysosomes[34]. Most children with nephropathic cystinosis display an inability to produce the normal volume of sweat, resulting in heat intolerance and avoidance, flushing, hyperthermia and vomiting in small children, although sweat electrolyte concentrations are normal[35].

CLINICAL VARIANTS OF CYSTINOSIS

Accumulation of the amino acid cystine in lysosomes occurs throughout the body. Depending on the age at presentation and the degree of disease severity, three clinical forms of cystinosis are distinguished: (1) Nephropathic infantile form (OMIM #219800), the most frequent and severe form of the disease; (2) Nephropathic juvenile form (OMIM #219900); synonyms: intermediate cystinosis, late-onset form, adolescent form; and (3) Non-nephropathic adult form (OMIM #219750); synonyms: benign non-nephropathic cystinosis, ocular non-nephropathic cystinosis.

All three forms of the disease are autosomal recessive and caused by mutations of the *CTNS* gene and have phenotypic overlap[36].

Nephropathic infantile cystinosis

Patients with infantile cystinosis are generally born from uneventful pregnancies and have normal birth weight and length. Despite cystine accumulation starting in utero, clinical symptoms are absent at birth and gradually develop during the first months of life. The kidneys are the first affected organs and progressively lose function of their proximal tubular transporters. This results in urinary loss of water, Na^+, K^+, HCO_3^-, Ca^{2+}, Mg^{2+}, phosphate, amino acids, glucose, proteins and many other solutes reabsorbed in this nephron segment. Asymptomatic aminoaciduria can appear during the first weeks of life and is followed by glucosuria, phosphaturia and urinary HCO_3^- losses during the first months of infancy. In one sibling of a known patient with cystinosis longitudinally followed from birth, the excretion of low molecular weight protein (α-1 microglobulin) was only increased at the age of 6 mo. This observation indicates that diverse proximal tubular transporters have a differential sensitivity to the cystinosin dysfunction and that the diagnosis of cystinosis can be missed during the first months of life, especially when only a limited number of urinary markers are used to identify renal Fanconi syndrome[36,37]. At the age of 6 mo, a full-blown Fanconi syndrome is usually present and causes clinical symptoms of polyuria, thirst, failure to thrive, growth retardation, vomiting, periods of dehydration, constipation, developmental delay and rick-

ets in some patients. Biochemically, patients present with hypokalemia, hypophosphatemia, metabolic acidosis, low serum uric acid, low serum carnitine and sometimes hyponatremia[38]. Occasionally, hypokalemia in combination with hypochloremic metabolic alkalosis and an elevated plasma rennin activity can mimic Bartter syndrome[39,40]. Proteinuria can reach grams per day and consists of LMW proteins, albumin and high molecular weight proteins[41]. Excessive losses of calcium and phosphate can cause the development of nephrocalcinosis and the formation of renal stones[42]. In most untreated patients, glomerular filtration rate remains normal for up to two years and then progressively deteriorates towards end stage renal disease (ESRD) at the end of the first decade[43]. Renal transplantation is the treatment of choice in patients with ESRD as the disease does not recur in the grafted organ. Cystine crystals can be observed in graft biopsies but originate from the host mononuclear cells and are of no pathological value[44].

Nephropathic juvenile form

It is diagnosed in a minority of the patients (about 5%) and manifests with a spectrum of symptoms, varying from milder (compared with the infantile form) proximal tubulopathy to an apparent nephrotic syndrome. Most of the patients described were older than ten years. The deterioration of renal function also occurs in the late-onset form but the rate of renal disease progression is mostly slower compared with the infantile form of cystinosis[38].

Non-nephropathic adult form

Ocular non-nephropathic cystinosis manifests only with complaints of photophobia due to cystine accumulation in the cornea of the eye, which is also present in nephropathic cystinosis. The kidney, retina and other organs are spared in these patients[45]. The coexistence of juvenile and ocular forms of cystinosis was described in one family, suggesting that there might be a continuum between mild forms of cystinosis and thus warranting the follow-up of renal functions in patients with adult cystinosis[46].

EXTRA-RENAL SYMPTOMS OF CYSTINOSIS

Cystinosis is accompanied by other organ involvement. Untreated teenagers may develop painful corneal erosions, peripheral corneal neovascularization, punctate, filamentous or band keratopathy, iris crystals and retinal degeneration[47,48]. In addition, impairment of endocrinal glands is reported, including hypothyroidism, IDDM and hypogonadism. Cystinosis could be accompanied by encephalopathy, stroke-like episodes, benign intracranial hypertension and myopathy[36]. A novel truncating mutation has been described with recognizable heart malformations in Egyptian families[49].

DIAGNOSIS OF CYSTINOSIS

Cystinosis is an autosomal recessive disease that should be suspected in all patients with failure to thrive and signs of renal Fanconi syndrome. After one year of age, the observation of cystine crystals in the cornea is pathognomonic for cystinosis; however, the absence of the crystals beyond the age of 2 years excludes the diagnosis. Detection of elevated intracellular cystine content is the cornerstone for the diagnosis. The methods for cystine determination differ depending on the cell type: mixed leukocyte preparation or polymorphonuclear leukocytes. Furthermore, several biochemical methods are currently used for cystine measurement, such as a cystine-binding assay, amino acid chromatography or high performance liquid chromatography, making it difficult to compare the results of different laboratories[50].

The cystine-binding assay has been used as a standard method for cystine measurements for years. However, at present, most laboratories have switched to other methods of detection because of the lower price and avoidance of radioactivity. In this respect, tandem mass spectrometry is the most sensitive method and is currently widely used for cystine determination in cystinosis. Each laboratory performing cystine measurements should provide their own reference values for patients at the time of diagnosis and also for heterozygotes and healthy subjects[51]. Prenatal diagnosis of cystinosis can also be made by measuring 35S-labeled cystine accumulation in cultured amniocytes or chorionic villi samples (CVS) and by a direct measurement of cystine in uncultured CVS[52].

TREATMENT

All patients with cystinosis should avoid sun exposure because of photophobia and the risk of dehydration. The specific therapy for cystinosis is cysteamine. It depletes lysosomal cystine content by a disulfide exchange reaction with cystine. The administration of cysteamine at 1.3-1.9 g/m^2 in four daily doses dramatically lowers the cystine content of the lysosomes, postpones or even prevents the deterioration of renal functions and the development of extra-renal complications. Furthermore, cysteamine treatment improves growth[53,54]. It could be used also topically for the eye[55]. Cysteamine should be administered as soon as the diagnosis of cystinosis is made and continued for life, even after renal transplantation, to protect the extra-renal organs. Recently, a prodrug long acting cysteamine has been introduced to overcome some side effects of the old preparations[56].

In addition, symptomatic therapy, including good nutrition, fluid and electrolyte balance and treatment of rickets, is indicated. A follow up schedule is mandatory, including growth monitoring, slit lamp and fundus examination. White blood cell cystine measurements are also needed for adapting the cysteamine dose. In addition, yearly check-ups by an endocrinologist and neurologist are required to monitor the extra-renal complications of the disease[36].

An experimental study using *CTNS*-knockout mice demonstrated a beneficial effect of syngeneic bone marrow and hematopoietic stem cell transplantation on cys-

Table 2 Summary of mutation variants of *CTNS* gene and with its phenotypic correlation

Phenotype	Mutation
Five nephropathic: 3 by Town *et al*[58]	*CTNS*, GLY95TER and *CTNS*, 2-BP DEL, 397TG[58], *CTNS*, TRP138TER[58,73].
2 forms by Shotelersuk *et al*[66]	*CTNS*, GLY169ASP and *CTNS*, 5-BP DEL, NT545[66].
Two nephropathic forms in adolescents	*CTNS*, 4-BP DEL, 18GACT[58,65], *CTNS*, 57-KB DEL[61], originally reported by Town *et al*[58] as a 65-kb deletion
Two atypical nephropathic	*CTNS*, VAL42ILE, *CTNS*, IV-S7AS, C-G, -10[67]
Two ocular non-nephropathic	*CTNS*, IVS10AS, C-G, -3, *CTNS*, GLY197ARG[45]
Three forms by Phornphutkul *et al*[68]	*CTNS*, -295G-C[68]
Nephropathic	*CTNS*, -303G-T, *CTNS*, 1-BP INS, 303T[68]
Ocular non-nephropathic	
Nephropathic	*CTNS*, GLY339ARG[69]
Adolescent nephropathic	*CTNS*, ASN323LYS[70]
Atypical nephropathic	*CTNS*, GLY110VAL[62]
Adolescent nephropathic Atypical nephropathic	*CTNS*, SER139PHE[65]

tine accumulation in various organs and on renal function survival, emphasizing the novel potential therapeutic possibilities for cystinosis patients[57].

MOLECULAR VARIANTS

The responsible gene (*CTNS* gene) that encodes the lysosomal cystine carrier cystinosin was cloned in 1998 and is located on the short arm of chromosome 17 (p13)[58]. Molecular analysis of the *CTNS* gene allows early diagnosis and can be used for prenatal diagnosis of the disease. Since the cloning of *CTNS* in 1998, over 90 mutations have been reported, with a detection ratio close to 100%[59,60]. The most common mutation accounting for approximately 75% of the affected alleles in Northern Europe is a 57-kb deletion, affecting the first 10 exons of *CTNS*[61]. A genotype-phenotype correlation related to the clinical forms of cystinosis was observed, with severe truncating mutations mostly found in patients with the infantile form of the disease and at least one mutation in patients with intermediate or adult cystinosis. However, several unexplained exceptions were reported[62].

Selected examples of allelic variants of the *CTNS* gene are listed in Table 2, with some phenotypic correlations. Recently, we described a novel G > A substitution in exon 10 of the *CTNS* gene (c.734 G > A) causing a nonsense truncating mutation (TGG > TAG) due to premature stop codon at position 245 of cystinosin protein (p.W245X). Two patients were diagnosed as homozygous for this mutation, whereas their parents were heterozygous. The patients ran a severe infantile nephropathic course and have recognizable heart malformations in the form of ventricular and atrial septal defects in one case and mild mitral and aortic incompetence in the other[21].

Five different forms of *CTNS* gene mutation were associated with nephropathic cystinosis[58]: (1) a gly95-to-ter mutation; (2) a 2-bp deletion of the *CTNS* gene: a deletion of TG at 397/399 resulted in a stop codon at the site of the mutation; (3) a (TGG-to-TGA) transition was detected at nucleotide 753 resulting in a trp138-to-ter non-sense mutation; (4) a deletion of four nucleotides (GACT) was found at nucleotide 357 of the *CTNS* gene. This resulted in a frameshift and premature termination; and (5) a 65-kb deletion that removes the first 10 exons of *CTNS* gene[58]. The fifth mutation (65-kb del) was originally reported by Town *et al*[58] to be the most common form of *CTNS* gene responsible for nephropathic cystinosis. This deletion was modified to be 57-kb rather than 65-kb after sequencing 200kb surrounding the *CTNS* gene[61]. This mutation was found in a homozygous state later[38,63]. A FISH method was developed, permitting cytogenetic laboratories to test for the 57-kb deletion[64]. A compound heterozygosity for the 57-kb deletion was found with a (928G-A) transition, resulting in a glycine to arginine substitution at codon 197 and with a (416C-T) transition in the *CTNS* gene, resulting in a ser139-to-phe respectively[45,65].

Among seven missense mutations, two mutations were linked to the nephropathic form: (1) a gly169-to-asp substitution; and (2) a 5-bp deletion starting at nucleotide 545 resulting in an (I69R) amino acid substitution and a stop codon at position 73 of the *CTNS* gene[66].

Attard *et al*[67] found different forms of mutations with different presentations. They found a (G > A) transition in the *CTNS* gene, resulting in a val42-to-ile substitution in the non conserved region toward the N terminus. This mutation was consistent with a milder phenotype. In addition, they identified an intronic mutation of the *CTNS* gene, a (C > G) transversion at nucleotide (801-10) in a patient with adolescent cystinosis.

For ocular non-nephropathic type, a (G > A) transition at nucleotide 928 was found, resulting in a glycine to arginine substitution at codon 197 (G197R). In addition, a (C > G) transversion was reported at the -3 position of the acceptor splice site of IVS10 of the *CTNS* gene[45]. Phornphutkul *et al*[68] identified heterozygosity for a (G197R) mutation and a promoter mutation, a (G > T) transversion at nucleotide 303 in the *CTNS* gene. In addition, they identified heterozygosity for a (G197R) mutation and a promoter mutation, an insertion of a single base (T) after position -303 in the *CTNS* gene. Phornphutkul *et al*[68] and Rupar *et al*[69] found mutations linked to the classic form of nephropathic cystinosis. The former identified heterozygosity for a 57-kb deletion and a promoter mutation, a (G > C) change at nucleotide 295 involving the Sp-1 regulatory element in the *CTNS* gene. The latter identified a (G > A) transition at nucleotide 1354. This transition resulted in a glycine-to-arginine substitution at residue 339 (G339R)[68,69].

For adolescent nephropathic variant, Thoene *et al*[70] identified homozygosity for a (1308C-G) mutation in the *CTNS* gene, resulting in the substitution of lysine for the conserved asparagine at position 323 (N323K). In two unrelated Spanish patients with juvenile-onset

nephropathic cystinosis, Macías-Vidal *et al*[65] identified a compound heterozygosity for a (416C-T) transition in the *CTNS* gene and a 4-bp deletion, resulting in a ser139-to-phe (S139F) substitution and a 57-kb deletion respectively.

In a patient who had atypical nephropathic cystinosis (presenting with Fanconi syndrome and end-stage renal disease but surprisingly without extra renal symptoms even late in life), a gly110-to-val (G110V) mutation was detected in the N-terminal region of the *CTNS* gene[62].

GENETIC COUNSELING FOR A PREVENTIVE STRATEGY

As cystinosis is an autosomal recessive disease, parents of a proband are obligate heterozygotes and thus carry one mutant allele, the heterozygotes (carriers) are asymptomatic. Genetic counseling is a method of prevention of recurrence. Recurrence risk is estimated for each sib of an affected individual to be a 25% chance of being affected, a 50% chance of being an asymptomatic carrier and a 25% chance of being unaffected and not a carrier. Once an at-risk sib is known to be unaffected, the chance of him/her being a carrier is 2/3. However, offspring of a proband are obligate heterozygotes for a mutant allele for *CTNS* gene. Carrier detection has been done in two Egyptian families with reported cases of cystinosis, both biochemically, using freshly prepared leukocytes, and molecularly, defining disease-causing mutations. Marriage of obligate carriers was prevented in one family to prevent the overall disease incidence among these suffering families. With the definition of mutation-causing cystinosis in a target family, we succeeded in preventing recurrence of the disease through the use of pre-implantation genetic diagnosis[49].

The optimal time for determination of the genetic risk, clarification of carrier status and discussion of the availability of prenatal testing is before pregnancy. It is appropriate to offer genetic counseling (including discussion of potential risks to offspring and reproductive options) to young adults who are affected, carriers or at risk of being carriers. Women with cystinosis have had successful pregnancies resulting in healthy newborns; however, the potential teratogenic effects of cysteamine on fetuses have not been studied in humans. No data on fertility in males with cystinosis exists; however, spermatogenesis in testicular biopsies was sufficient. Cryopreservation of sperm could be considered in affected males[71].

Prenatal diagnosis allows for early detection of diseases and early cysteamine treatment. This is very important in delaying the onset of renal failure and other complications in cystinosis. In addition, it allows for an early link to cystinosis supporting groups that play important roles in supplying them with unavailable medications and tests[54,72]. Prenatal testing includes both biochemical and molecular tools: (1) Biochemical testing based upon the measurement of cystine concentrations in either chorionic villi obtained at approximately 10-12 wk gestation by CVS or amniocytes obtained by amniocentesis usually performed at approximately 15-18 wk gestation[34]; and (2) Molecular genetic testing is possible by analysis of DNA extracted from fetal cells obtained either by amniocentesis usually performed at approximately 15-18 wk gestation or CVS at approximately 10-12 wk gestation. Both disease-causing alleles of an affected family member must be identified before prenatal molecular testing[54,72]. To sum up, using these advanced optimal methods, obligate carrier parents of *CTNS* gene mutant alleles could be helped to have normal asymptomatic offspring.

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REVIEW

Primary focal and segmental glomerulosclerosis and soluble factor urokinase-type plasminogen activator receptor

Hernán Trimarchi

Hernán Trimarchi, Servicio de Nefrología, Hospital Británico de Buenos Aires, Buenos Aires 1280, Argentina
Author contributions: Trimarchi H contributed to the conception, design, analysis, and collection of data; to the drafting and revision of the article for its content; and to the final approval of the version to be published.
Correspondence to: Hernán Trimarchi, MD, Servicio de Nefrología, Hospital Británico de Buenos Aires, Perdriel 74, Buenos Aires 1280, Argentina. htrimarchi@hotmail.com
Telephone: +54-11-43096400 Fax: +54-11-43093393
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Abstract

Primary focal and segmental glomerulosclerosis (FSGS) may be due to genetic or acquired etiologies and is a common cause of nephrotic syndrome with high morbidity that often leads to end-stage renal failure. The different available therapeutic approaches are unsuccessful, in part due to partially deciphered heterogeneous and complex pathophysiological mechanisms. Moreover, the term FSGS, even in its primary form, comprises a histological description shared by a number of different causes with completely different molecular pathways of disease. This review focuses on the latest developments regarding the pathophysiology of primary acquired FSGS caused by soluble factor urokinase type plasminogen activator receptor, a circulating permeability factor involved in proteinuria and edema formation, and describes recent advances with potential success in therapy.

Key words: Primary acquired focal and segmental glomerulosclerosis; Soluble factor urokinase type plasminogen activator receptor; Proteinuria; Podocyte; Plasmin

Core tip: Primary acquired focal and segmental glomerulosclerosis is a frequent cause of nephrotic syndrome with no specific treatment. New discoveries in its pathophysiolohy have revealed that a podocyte permeability factor named soluble urokinase plasminogen activator receptor (suPAR) may be involved in the development of proteinuria and edema formation. This effect is supposed to be achieved by its interaction with podocyte integrins and subsequent cell contraction. Moreover, suPAR also activates water and sodium retention in this disease. Interestingly, plasmin mediates both effects. Amiloride is postulated to interfere with suPAR proteinuric actions.

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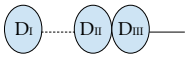
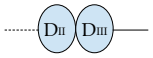
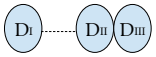
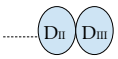
INTRODUCTION

Focal and segmental glomerulosclerosis (FSGS) is a major cause of chronic kidney disease in children and adults[1-3]. It can occur as a primary disorder (called primary acquired FSGS), as a consequence of genetic mutations in podocyte-specific proteins (also called primary genetic FSGS) or as a secondary disorder[4,5]. In recent years, much of the progress obtained in unraveling the pathophysiological events in FSGS has been focused primarily on the identification of genetic mutations of membrane and podocyte slit diaphragm proteins and on immune factors, but the real identity of the primary acquired variant apparently caused by circulating permeability factors remains elusive. In this regard, the role of these permeability factors in the pathogenesis of proteinuria has also shown progress in recent years.

Molecule	Structure	Molecular weight (kDa)	Location	Action
uPA		Approximately 54-57	Blood Urine	Vitronectin Plasminogen Urokinase Chymotrypsin
uPAR$_{I-III}$ (CD87)		Approximately 55-60	Bound to membrane	Adhesion Migration Convesion of plasmin
uPAR$_{II-III}$		Approximately 45-50	Bound to membrane	?
suPAR$_{I-III}$		Approximately 55-60	Soluble	Bind to α5β3 integrin Bind to uPAR
suPAR$_{II-III}$		Approximately 40-45	Soluble	?
suPAR$_{I}$		Approximately 16	Soluble	?

····· : Linking part; ○ : Domain; —— : Anchoring glycosylphosphatidylinositol.

Figure 1 Differences in urokinase-type plasminogen activator, urokinase-type plasminogen activator receptor and soluble urokinase plasminogen activator receptor (modified from reference number 18). D$_{I}$: Domain 1; D$_{II}$: Domain 2; D$_{III}$: Domain 3. uPA: Urokinase-type plasminogen activator; suPAR: Soluble urokinase plasminogen activator receptor.

The soluble factor urokinase type plasminogen activator receptor (suPAR) has become one of the most studied permeability factors with potential involvements in FSGS. It is supposed to be responsible for the contraction of podocytes and its eventual detachment from the glomerular basement membrane, denuding it and causing proteinuria in the majority of primary acquired cases of FSGS[6]. However, this phenomenon is not shared by others, who question whether elevated levels of suPAR are indeed pathogenic, or just a mere marker of a split urokinase-type plasminogen activator (uPAR) (CD87) molecule. Moreover, in other clinical situations in which suPAR is elevated, proteinuria does not occur[7-9]. It is not a specific marker of FSGS, as in other glomerulopathies suPAR levels are also high; in addition, after FSGS post-transplant recurrence elevated suPAR levels are not always encountered[7-9]. Finally some authors state that is not the plasmatic but the urinary presence of suPAR the real culprit of primary acquired FSGS[9].

Biological aspects of uPAR and suPAR

Urokinase receptors, expressed on the cell surface of various cells, are committed to the pericellular proteolysis of plasminogen, are essential for the remodeling of the extracellular matrix, and are involved in vasculogenesis and cell migration processes[10]. The urokinase receptor, also known as uPAR (urokinase-type plasminogen activator) is a membrane bound protein linked to glycosylphosphatidylinositol (GPI) of about 45-55 kDa (Figure 1)[10,11]. UPAR consists of three domains (D Ⅰ, D Ⅱ and D Ⅲ) and is present in various immunologically active cells, including monocytes, macrophages and activated T cells, and also in endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, megakaryocytes, certain cells tumor, podocytes and renal tubular cells[12-18]. It therefore follows that suPAR is not a specific marker, although in the context of high circulating levels in a nephrotic patient with FSGS, it suggests a leading role as a permeability factor[8]. UPAR can be cleaved not only at the portion of the GPI-anchored protein to the cell membrane, but also in the inner part of the receptor itself (for example, in the connection region between DI and D Ⅱ-Ⅲ), giving rise to various soluble forms of suPAR with different molecular weights (Figure 1). The most common form of soluble suPAR originates from the cleavage and release of membrane-bound uPAR, detaching the membrane anchoring compound GPI, and is present in plasma, urine and cerebrospinal fluid in different concentrations depending on the level of activation of the immune system[19-22] (Figure 1). It has also been documented the existence of the whole molecule of suPAR in serum from healthy individuals and of two truncated soluble forms of the entire molecule (suPAR$_{I}$ and suPAR$_{II-III}$) in the urine[23] (Figure 1).

Physiology of uPAR and suPAR

UPAR can be activated by various molecules, such as uPA (urokinase-type plasminogen activator, or simply urokinase), plasminogen, chymotrypsin, various metalloproteinases and some elastases[24-27]. Studies are generally based on the action of these molecules on the uPAR, but as SuPAR barely shares the same structure as uPAR, these proteases are also likely to cleave suPAR fragments. Furthermore, once activated, suPAR or uPAR are capable of catalyzing the conversion of plasminogen to plasmin, an important molecule in fibrinolytic processes and in the activation of several matrix metalloproteinases, in the recycling and degradation of the extracellular matrix, in cell activation, migration, contraction, vasculogenesis and in vitronectin degradation[10,28-32]. This phenomenon may occur in plasma, on the podocyte surface or in renal distal tubular

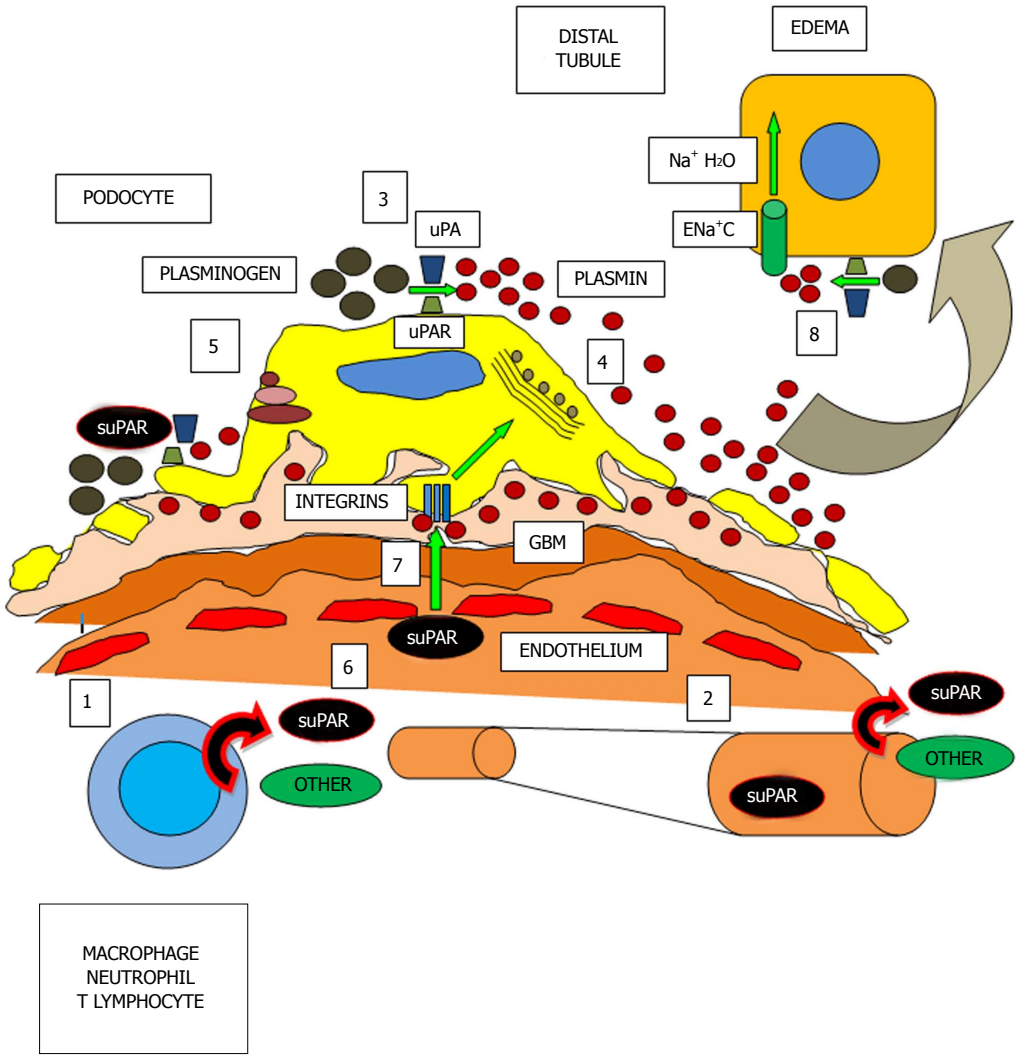


Figure 2 Potential pharmacological strategies in primary acquired focal and segmental glomerulosclerosis. Potential strategies. 1: Inhibition of soluble urokinase plasminogen activator receptor (suPAR) or other permeability factors secretion onto circulation or a decrease in the pool of suPAR secreting cells (immunosuppression); 2: suPAR or other permeability factors removal from the circulation (plasmapheresis, immunoadsorption); 3: Inhibition of uPAR activation; 4: Plasmin antagonists 5: Stabilization of podocyte and slit diaphragm proteins (immunosuppression, angiotensin converting enzyme inhibitors, angiotensin receptor blockers); 6: Endothelial protectors; 7: Plasmin-integrin coupling inhibitors (monoclonal antibodies, amiloride); 8: Plasmin-tubular ENa+C inhibitors (amiloride). GBM: Glomerular basement membrane.

cells[16,17] (Figure 2).

SuPAR whole molecule (suPAR$_{I-III}$) consists of three domains (D$_{I}$, D$_{II}$ and D$_{III}$) of uPAR , as mentioned previously but lacks the GPI anchor protein; however, the I-III portion of suPAR can compete with uPAR$_{I-III}$ for Upa[33] (Figure 1). Another agonist of UPAR is vitronectin, the main antagonist of plasminogen activator inhibitor type-1 (PAI-1), the most important physiological inhibitor of tissue plasminogen activator and urokinase (uPA)[10]. Thus, vitronectin can accomplish its adherent and fibrinolytic actions increasing plasminogen and plasmin levels by two independent pathways: blocking PAI-1 and activating uPAR. Furthermore, vitronectin achieves its adherent action to the cell matrix through integrins, particularly those that possess the α5 domain[10]. While this point will be addressed below, it is worth to mention that patients with nephrotic syndrome present elevated serum levels of plasminogen and plasmin[34]. In turn, after being filtered, urinary plasminogen is converted to plasmin by podocyte or distal renal tubular epithelial uPA/uPAR; at this distal location, plasmin has been reported to function as a regulator of water and sodium absorption, a key event in the pathogenesis of edema in nephrotic syndrome, and also as a mediator in calcium tubular transport[17,35,36].

Cell migration across the endothelium and into tissues is a critical component in inflammation, in immune responses against infections, and in tissue repair and remodeling after injury. The UPA/uPAR system is directly involved in these mechanisms of adhesion, migration and chemotaxis[18,31]. For example, the adhesion and migration of monocytes involves a functional interaction between cellular uPAR and matrix integrins[37] and in uPAR-dependent changes in integrin-mediated adhesion to fibrinogen, collagen and vitronectin[10,38,39]. It is known that uPAR is needed to activate the integrin α5β3 in podocytes, which promotes cell motility and activation of small GTPases that control cell division, as Cdc4240. If α5β3 integrin is activated, the podocyte contracts and proteinuria ensues.

However, it is believed that suPAR has inhibitory properties on adhesion uPAR dependent migration but not on cell contraction. Thus, it would be able to interact with α5β3 integrin, vitronectin or plasmin[18,40]. Finally, it has been shown that suPARⅡ-Ⅲ is a chemotactic agent[41,42], and its circulating levels reflect the activation status of the immune system[18].

suPAR and the pathophysiology of FSGS

Abnormally high circulating levels of suPAR have been associated with the pathogenesis of acquired primary FSGS, since approximately two thirds of patients with acquired FSGS have increased circulating levels of suPAR[6]; suPAR would then bind to and activate α5β3 integrin in podocytes by a lipid-dependent mechanism[16], leading to alterations in the morphology and dynamics of the metabolism of podocytes and foot process effacement, detachment and podocyturia, finally resulting in proteinuria and the beginning of glomerulosclerosis, nephrotic syndrome and renal insufficiency[16,43].

What is the cellular origin of this increased membrane uPAR and circulating suPAR in FSGS? Wei *et al*[16] suggest that neutrophils and monocytes may be culprits, but another possibility lies in circulating lymphocyte T cells, since there is an association between T-cell activation and systemic proteinuria. In turn, as mentioned previously, not in all cases of idiopathic acquired FSGS circulating levels of suPAR have been increased. This is another confirmation that the mere histologic FSGS description is not a disease but a form of kidney damage characterized by common histopathological features but with completely different pathophysiological pathways. Even within the primary FSGS scenario, and even more, within the primary FSGS circulating factors, more than one peptide may cause damage to the glomerular basement membrane. In this regard, other described permeability factors are angiopoetin-4 and vascular endothelial growth factor (VEGF), both secreted by the podocyte, operating in autocrine or paracrine fashions[43-45]. In addition, plasma and urinary levels of CD80 from T cells due to a lymphocyte-podocyte interaction, are elevated in primary acquired FSGS; CD80 could potentially contribute to the diagnosis and serve as a potential marker of damage in FSGS, being another potential tool to help distinguish clinically primary FSGS from minimal change nephropathy at the initial steps of the disease. In minimal change nephropathy, hemopexin may be the main permeability factor[46,47]. Another molecule that has been identified in primary acquired recurrent FSGS is CLC-1 (cardiotrophin type-1 cytokine), a member of the family of interleukin (IL)-6, and which is present in the plasma of patients with active disease. CLC-1 decreases the expression of nephrin in glomeruli and cultured podocytes, and CLC-1 concentration in the circulation of patients with recurrent FSGS can be up to 100 times higher than in normal subjects[48]. To make matters more difficult to understand in primary acquired FSGS, suPAR activity has been identified in recurrence after kidney transplantation in some patients with concomitant genetic mutations in podocyte proteins[49,50]. One can only speculate on the relationship between mutations and the coexistence of podocyte permeability circulating factors in this setting. It may be that the occurrence of both phenomena is attributable just to mere coincidence, or that genetic abnormalities in podocytes may cause subsequent structural local damage and inflammation inducing leukocyte stimulation via the uPAR, ending with the secretion of molecules with permeability actions, giving rise to severe kidney recurrent disease[48].

Is there any relationship between the etiology of minimal change nephropathy and that of primary focal segmental sclerosis? If FSGS is of genetic origin, the link would be none. If chronic minimal change nephropathy leads to an inflammatory state that induces focal sclerosis histological changes, this morphology would be of secondary origin and have no connotation with primary acquired FSGS. If a causal factor it is to be established as a primary permeability factor in minimal change nephropathy, hemopexin would be the first candidate. Hemopexin is a protease which activates protein kinase B and the small GTPase RhoA (ras homolog gene family, member A) and induces a nephrin-dependent reorganization of the actin cytoskeleton in cultured podocytes[51]; reduces endothelial glycocalyx and increases the albumin diffusion through glomerular endothelial cell monolayers[51]. Hemopexin injection in rats causes proteinuria and glomerular changes characteristic of minimal change nephropathy[48,52]. Another candidate is vascular permeability factor (VPF). VPF is a lymphokine that is produced by T lymphocytes stimulated by concanavalin A of patients with idiopathic nephrotic syndrome. VPF acts on systemic capillary glomerular basement membrane[53]. Its secretion is enhanced by IL-2, IL-15, IL-12, and IL-18 is inhibited by transforming growth factor-β_1[54] and causes a histological damage identical with minimal change nephropathy[48]. Whether two or more factors such as the suPAR permeability can coexist in these situations has not been reported. Finally, in the early course of idiopathic nephrotic syndrome, histological changes may not be present even at the ultrastructural level, in turn making more problematic and difficult the distinction between minimal change nephropathy and primary FSGS. The histology of primary FSGS caused by a permeability factor compared to that caused by a mutation (podocytopathy) is indistinguishable at early stages, although in the latter focal ultrastructural microscopic damage may be seen first. Moreover, none of the permeability factors mentioned in the case of minimal change nephropathy or FSGS are currently measured in clinical grounds. In the future, samples of blood or urine may be part of a diagnostic panel.

Treatment of FSGS

As to treatment, to date no randomized controlled trials of sufficient numbers of patients are available to provide robust information so as to guide us in the treatment of primary FSGS in native kidneys or in renal allografts. Current treatment results in complete or

partial remissions in approximately 50% of cases. Treatment approaches that have been used to date include corticosteroids with or without cyclophosphamide[55,56], cyclosporine[57], mycophenolate[58], rituximab[59,60] and plasmaphresis[61,62]. When proteinuria is reduced by these agents or by non-specific drugs as angiotensin converting enzyme inhibitors, angiotensin Ⅱ receptor blockers, statins, antiaggregants and/or reduction of salt intake, the progression of renal dysfunction is slowed[63,64]. Regardless of the debates that arise about the true etiology of nephrotic syndrome in primary FSGS, current and proposed therapies include strategies such as the identification and reversal of the primary cause of renal injury (usually not possible), the decrease in proteinuria by interventions related to hemodynamic factors, and retarding renal fibrosis by the action of nonspecific agents (Figure 2).

In a study by Wei *et al*[6] in which blood samples of 164 pediatric and adult patients with primary steroid-resistant FSGS were analyzed and suPAR concentrations were measured, the main conclusions arrived by the authors were that circulating suPAR levels were significantly elevated in most patients with primary FSGS in both groups; 84.3% of patients in the American cohort (CT) and 55.3% of those belonging to the European group (PodoNet) had elevated suPAR levels; high suPAR levels were not associated with systemic inflammatory phenomena according to C-Reactive Protein titers as did not differ from controls, treatment with mycophenolate/dexamethasone was associated with lower circulating suPAR levels in comparison to those treated with cyclosporine A; a sustained decrease in suPAR levels over the course of 26 wk of treatment was associated with a reduction in proteinuria and more likely to accomplish complete remission; suPAR serum levels were higher in the familial cases, including those with a genetic disorder, as in the diagnosis of a podocin mutation (podocytopathy in coexistence with elevated suPAR levels)[6]. The fact that in 15%-45% of patients in both groups had normal levels of suPAR shows that primary FSGS is a heterogeneous disorder which additional factors contributing to the renal damage and to proteinuria. It is possible that patients with primary FSGS express higher levels of suPAR in response to a certain pathological stimulus with independent features or related to a primary inflammatory instigator[6].

An agreed cut-off level of suPAR is another controversial issue. Gao *et al*[65] proposed 3000 pg/mL as the cut-off level for the population with primary FSGS, since in a previous study of a normal population the cut-offs level was set at 2710 pg/mL. As therapies, chronic plasmapheresis and plasma adsorption of suPAR are supporting treatments that can help maintain normal suPAR blood levels, which would lead to lower podocyte damage and partial resolution of nephrotic syndrome with a possible slowing of progression to renal failure[61,62]. Cyclosporine may be useful to stabilize the podocyte by inhibiting synaptopodin dephosphorylation; therefore, synaptopodin interaction with actin would be blocked, and the podocyte contraction abrogated[66]. Salomon et al found cyclosporine trough levels between 250 and 300 ng/mL suffice to obtain a rapid remission in proteinuria (average intravenous dose 3 mg/kg per day)[67], although the treatment is generally cyclosporine-dependent and may lead to chronic renal damage[68-70]. Rituximab may be another option in refractory cases, not only due to its action by decreasing the population of CD20 lymphocytes, but also because it would bind other podocyte molecules as protein SMPDL-3b (sphingomyelin phosphodiesterase acid-like 3b). In primary FSGS, this molecule (acting on the remodeling of podocyte actin) is decreased. Rituximab levels would increase SMPDL-3b concentrations, stabilizing the podocyte[71].

Novel aspects with potential targets

A recent study has shown that podocyte uPAR expression can be reduced using amiloride. Amiloride plays a significant role in reducing podocyte cell motility in vitro and proteinuria in mice[72]. Amiloride inhibits the synthesis of uPAR and uPAR mRNA and consequently the $\alpha5\beta3$ integrin activation mediated by uPAR. The reduced uPAR pool would translate in a lower suPAR concentration. Amiloride capacity to inhibit uPAR synthesis and suPAR secretion by T lymphocytes should be of particular interest in FSGS, because blocking their activation would inhibit $\alpha5\beta3$ integrin activation and the development of proteinuria with final renal dysfunction[16,73]. Furthermore, amiloride may further decrease proteinuria by acting on the distal nephron in ENaC channels, as nephrotic range proteinuria stimulates the activity of these channels by promoting the reabsorption of sodium and water[17]. Tubular plasmin, already high in patients with nephrotic syndrome, would act as the mediator in sodium and water reabsorption and amiloride may inhibit its action by blocking uPAR[17,34,72,74] (Figure 2). This would be another additional and relevant non-immunosuppressive strategy contributing to the fall in proteinuria, if tolerated hemodynamically and no hyperkalemia ensues.

CONCLUSION

In summary, these observations aim to explain the possibility that circulating suPAR is the most prominent factor in the pathophysiology of primary acquired FSGS due to the encountered high levels in blood and urine, activating $\alpha5\beta3$ integrin, contracting the podocyte and causing the proteinuria, and acting on the water and sodium reabsorption at the distal tubular. Moreover, it explains the importance of urokinase and its receptor uPAR play in cell adhesion and migration, being plasmin the final effector. Whether suPAR causes a rise in plasminogen and plasmin levels, and the consequent final action on the podocyte integrins and renal distal tubular cell, in primary acquired FSGS both proteinuria and edema would have suPAR as the trigger for plasmin activation, a final effector, and amiloride as a potential novel adjunct antiproteinuric agent in this complex nephropathy.

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MINIREVIEWS

Vitamin E and diabetic nephropathy in mice model and humans

Nakhoul Farid, Dahan Inbal, Nakhoul Nakhoul, Farber Evgeny, Rachel Miller-Lotan, Andrew P Levy, Asleh Rabea

Nakhoul Farid, Dahan Inbal, Farber Evgeny, Department of Nephrology and Hypertension, Baruch-Padeh Poriya Medical Center, Faculty of Medicine, Bar-Ilan University, Lower Galilee 15208, Israel
Nakhoul Nakhoul, Ophtalmology Unit, Baruch-Padeh Poriya Medical Center, Lower Galilee 15208, Israel
Rachel Miller-Lotan, Andrew P Levy, Asleh Rabea, The Vascular Biology Lab, the Technion Faculty of Medicine, Haifa 31096, Israel
Author contributions: Farid N, Nakhoul N, Miller-Lotan R designed research; Farid N, Inbal D and Rabea A performed research; Miller-Lotan R contributed new reagents or analytic tools; Evgeny F, Farid N and Levy AP analyzed data; Farid N and Nakhoul N wrote the paper.
Correspondence to: Nakhoul Farid, MD, Department of Nephrology and Hypertension, Baruch-Padeh Poriya Medical Center, Faculty of Medicine, Bar Ilan University Galilee, Max ve-Anna Webb, Ramat Gan, Lower Galilee 15208, Israel. fnakhoul@poria.health.gov.il
Telephone: +97-24-6652587 Fax: +97-24-6652587
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Abstract

Diabetes mellitus (DM) is associated with increased oxidative stress due to elevated glucose levels in the plasma. Glucose promotes glycosylation of both plasma and cellular proteins with increased risk for vascular events. Diabetic patients suffer from a higher incidence of cardiovascular complications such as diabetic nephropathy. Haptoglobin (Hp) is an antioxidant plasma protein which binds free hemoglobin, thus preventing heme-iron mediated oxidation. Two alleles exist at the *Hp* gene locus (1 and 2) encoding three possible Hp genotypes that differ in their antioxidant ability, and may respond differently to vitamin E treatment. Several clinical studies to have shown that Hp 1-1 genotype is a superior antioxidant to the Hp 2-2 genotype and Hp 2-2 genotype is associated with a higher incidence of cardiovascular disease. Vitamin E was found to have beneficial effect in patient and mice with Hp 2-2 genotype. In this review we have summarized the results of our studies in patients with diabetic nephropathy treated with vitamin E and in diabetic mice with different haptoglobin genotypes.

Key words: Haptoglobin; Cardio-vascular complications; Diabetic nephropathy; Vitamin E

Core tip: In diabetes mellitus there is an increase in oxygen radical formation due to glucose auto oxidation, the formation of advanced glycosylation end products, and metabolic stress. Epidemiologic studies suggest that vitamin E supplementation might decrease the risk of developing cardiovascular disease, others showed increased risk of cardiac death with the vitamin E treatment. To the contradictory results in the literature regarding the beneficial role of vitamin E in protecting against cardiovascular complications, high dose vitamin E supplementation has not been recommended by the medical community. In fact, a meta-analysis of over 135000 individuals treated with vitamin E concluded that high dose vitamin E (greater than 400 mg/d) slightly increases the risk of mortality. However, recent investigations into the polymorphic serum protein haptoglobin (Hp) indicate that vitamin E may be beneficial in a genetically defined subgroup of patients, namely, diabetic patients of the Hp 2-2 genotype. The role of Hp as an antioxidant, its importance in diabetes, and the therapeutic role of vitamin E will be discussed in this review.

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INTRODUCTION

Diabetic Nephropathy (DN) is the leading cause of end stage renal disease and accounts for approximately 40% of all patients who require replacement therapy. The well known risk factors for DN are uncontrolled diabetes mellitus and genetic factors[1,2]. The inter-individual variability in the probability for developing DN and its clustering within families, suggest a substantial genetic predisposition. Reactive oxygen species, particularly those derived from iron, have been implicated in the progression of DN and other vascular complications of diabetes. Therefore, polymorphic genetic loci, encoding variants in enzymes protecting against iron-induced oxidative stress, serve as potential susceptibility determinants for the development of DN[3,4]. Diabetes is accompanied by severe oxidative stress (especially lipid per-oxidation) which is caused by increased oxygen free radical production. Toxic oxygen free radicals have been implicated in the pathogenesis of diabetes mellitus, and its micro- and macro vascular complications. An imbalance resulting from the increased production and/or reduced scavenging of these free radicals, leads to a metabolic state of oxidative stress, which consequently leads to tissue damage.

One of these protecting factors is the haptoglobin (Hp). Hp is an acute phase protein synthesized in the liver by the hepatocytes. It acts as an antioxidant by virtue of its ability to prevent hemoglobin (Hb) induced oxidative tissue damage[4-6]. Its synthesis is rapidly and dramatically increased in response to numerous inflammation stimuli due to a transcriptional activation of the *Hp* gene. Whenever Hb is released into the circulation, its binds immediately to Hp to form an Hp-Hb complex and this complex is rapidly removed predominately by the monocyte/macrophage CD 163 Hp-Hb receptor expressed on Kupfer cells in the liver (Figure 1). When Hp is depleted, as a result of hemolysis or in Hp Knockout mice, Hb accumulates in the kidney and us secreted in the urine. Therefore, a major role of Hp is to prevent renal damage[6-9]. Two classes of Hp alleles are known in humans (1 and 2) with homozygous (1-1 or 2-2) and heterozygous (2-1) possible genotypes. The Hp 1 allele contains 5 exons and is found in all animal species while the Hp 2 allele contains 7 exons and exists only in humans, with polymorphic expression using the two classes of alleles. Our group has revealed profound differences in the antioxidant capacity of the protein product of the two Hp alleles and has demonstrated that these differences are exaggerated in the diabetic state. Studies, both *in vivo* and *in vitro*, have shown that the Hp 1 protein has superior antioxidant capacity compared to the Hp 2 protein[9,10] (Figure 2).

Vitamin E is a fat-soluble vitamin with antioxidant properties. Vitamin E exists in eight different forms (isomers): alpha-, beta-, gamma-, and delta-tocopherol; and alpha-, beta-, gamma-, and delta-tocotrienol. Alpha-tocopherol is the most active form in humans. Dosing and daily allowance recommendations for vitamin E are often provided in alpha-tocopherol equivalents to account for the different biological activities of the various forms of vitamin E, or in international units (IU), which food and supplement labels may use. Due to its antioxidant properties, Vitamin E has been proposed to have a role in preventing or treating numerous health conditions, often by its antioxidant properties. When highly-reactive species attack the membranes lipids or the lipoproteins, it sets off a chain reaction of lipid per oxidation. Vitamin E halts this chain reaction, *e.g.*, it thereby acting as a chain breaking inhibitor of lipid per oxidation[11,12].

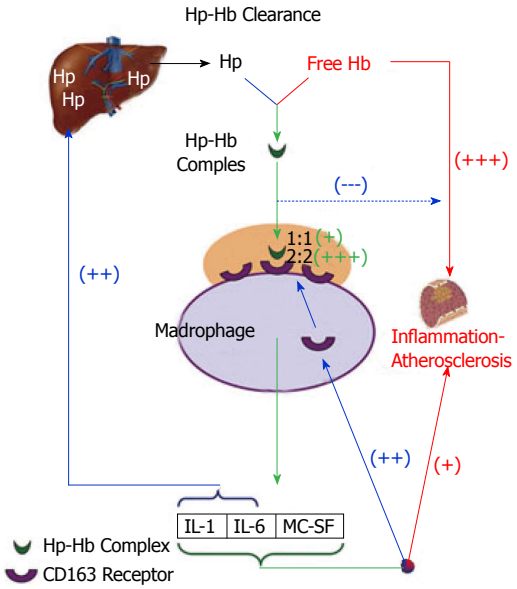


Figure 1 Hemoglobin-haptoglobin complex clearance by macrophage CD163 receptor. Hp: Haptoglobin; Hb: Hemoglobin; MC-SF: Macrophage colony-stimulating factor; IL: Interleukin.

In patients with type 1 diabetes mellitus (DM), the most important renal structure changes occur in the glomeruli: In these patients, diabetic glomerulopathy is characterized by increased glomerular basement membrane (GBM) width and mesangial expansion with reduction in the glomerular filtration surface area. Concomitantly, the renal arterioles, tubules and interstitium also develop lesions. Early stage of diabetic nephropathy is associated with the development of glomerular hyperfiltration, hyperalbuminuria, thickening of the GBM, mesangial expansion, and progressive decline in glomerular filtration rate. Fioretto *et al*[13] also described proximal tubular basement thickening with atubular glomerular junction. When renal insufficiency ensues with proteinuria and hypertension, glomerulosclerosis and fibrosis develops. Although animal models with diabetes mellitus type 1 and 2 are exist, no single animal model develops glomerular and tubular changes identical to those seen in humans. While the field of diabetic nephropathy has made much progress in understanding the disease process and its progression, only limited success has been attained. One reason for this is the inability to develop a murine model of diabetic nephropathy with the spectrum of micro- and macrovascular complications similar to human disease.

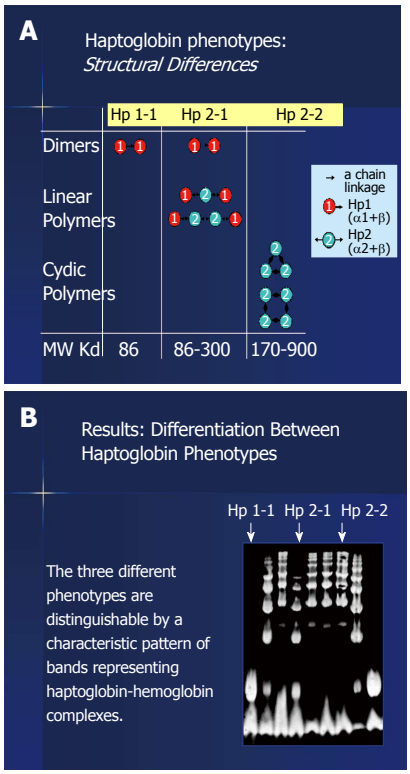


Figure 2 Haptoglobin structure and gel electrophoresis. A: Haptoglobin phenoytpes; B: Results. Hp: Haptoglobin.

Antioxidants have been shown to play a beneficial role in the prevention of the diabetic complications. Diabetes is a good model of chronic oxidative damage and it is a particularly suitable disease for antioxidant supplementation. It was found that there is a significant correlation between the increased blood sugar levels and the depletion of the antioxidants has been found. This depletion was a major risk factor for developing diabetic complications, and antioxidant supplementation (vitamin E, C) could decrease this risk. Nevertheless, only a few studies have shown the impact of the antioxidant therapy in diabetic patients. According to these facts, the present review will evaluate the role of antioxidant supplementation along with the standard diabetic therapy in the prevention of diabetic complications[14-16].

AIMS OF THE REVIEW

Aims of the review: (1) to evaluate the role of vitamin E therapy in preventing the development of complications in diabetic patients (primary prophylaxis); (2) to evaluate the role of the vitamin E supplementation in controlling the progression of the complications in diabetic patients (secondary intervention); and (3) to evaluate the role of the vitamin E supplementation in controlling the progression of diabetic nephropathy in diabetic mice with different Hp genotype.

CLINICAL STUDIES OF VITAMIN E

Diabetes mellitus is associated with increased oxygen radical formation due to glucose auto-oxidation, the formation of advanced glycosylation end products and metabolic stress. Therefore the influence of different antioxidants has been the subject of many studies over the years. Due to the involvement of low-density lipoprotein (LDL) oxidation in the pathology of atherosclerosis, numerous studies have been made carried out on vitamin E, as an antioxidant that has the ability to react with lipid peroxyl radicals and terminate the propagation of free radicals. Several observational epidemiologic studies suggest that vitamin E supplementation might decrease the risk of developing cardiovascular disease (CVD). These studies have been divided into two categories. The first includes primary prevention studies involving healthy people, and the second category comprises secondary prevention studies involving people with known cardiovascular complications[17,18].

Extensive preclinical and observational studies have shown the apparent benefit of vitamin E supplementation in preventing cardiovascular events, created an atmosphere in which more than 40% of cardiologists were routinely prescribing high doses of vitamin E[19,20]. Over the past 10 years, several prospective randomized clinical trials including the first randomized controlled trial (RCT) published by Virtamo *et al*[21], have investigated whether vitamin E supplementation provides cardiovascular protection. The overwhelming consensus from these studies was that vitamin E supplementation does not provide cardiovascular benefit.

In the physicians health study, 14641 males over age 50 years were randomized to receive vitamin E (400 IU/d) and C for 8 years. In this study, no effect was found on CV death, nonfatal stroke or CVD[22]. The meta-analysis of these studies suggests that high doses of vitamin E and C supplementation may increase mortality, and several opinion articles have called for a moratorium on the prescription of high dose vitamin E supplements. A possible explanation for the failure of these studies in spite of solid preclinical data is the inadequate nature of patient selection in these studies.

High-dose antioxidant therapy may provide benefit only to individuals who suffer from particularly high levels of oxidative stress. Hence, the Hp genotype may help identify patients with high levels of oxidative stress that may benefit from antioxidant therapy with vitamin E. The *Hp* gene is polymorphic with 2 common classes of alleles denoted 1 and 2. We and others have demonstrated that the Hp 2 allele protein product is an inferior antioxidant compared with the Hp 1 allele protein product. These differences in antioxidant protection are profoundly accentuated in the diabetic state resulting in a marked relative increase in oxidative stress in Hp 2 individuals with DM (the distribution of the 3 Hp genotypes in Western societies is approximately 16% Hp 1-1, 36% Hp 2-2, and 48% Hp 2-1)[23-27].

Our groups, at the Technion-Faculty of Medicine, have demonstrated an interaction between the Hp genotype and DM with regard to the development of cardiovascular events. In multiple longitudinal studies Hp 2-2

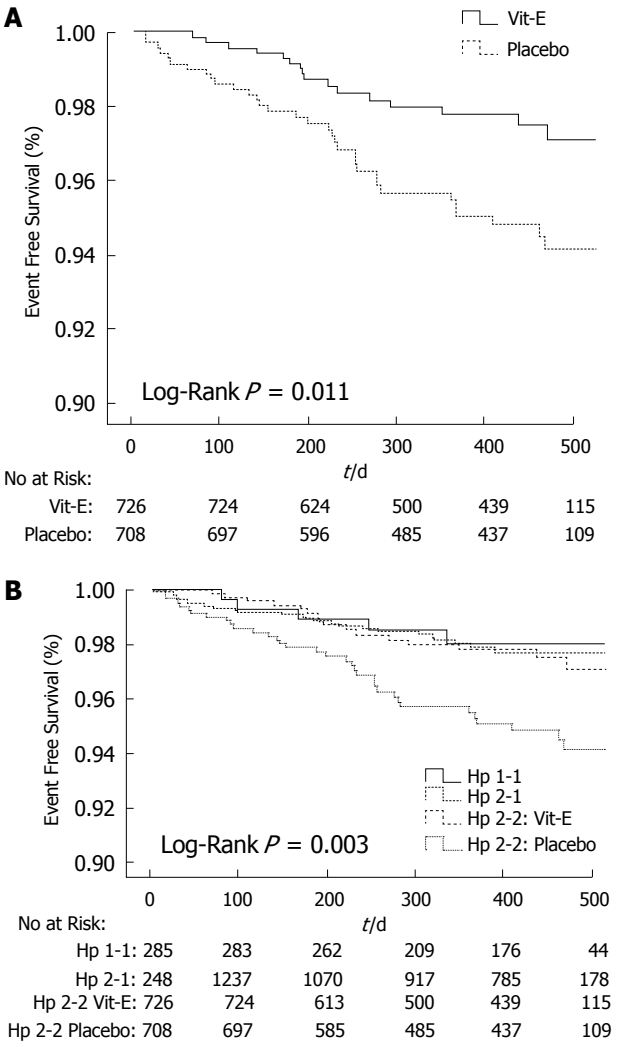


Figure 3 Kaplan-Meier plot. A: The composite end point in haptoglobin (Hp) 2-2 Hp diabetes mellitus (DM) individuals allocated to vitamin (Vit) E or Placebo. Events are cardiovascular death, myocardial infarction or stroke. There was a significant decrease in the composite end point in the vitamin E group compared with placebo group (P = 0.01 by Log-Rank); B: The composit end point in Hp 1-1 and Hp 2-1 DM individuals compared with Hp 2-2 DM individuals receiving vitamin E or placebo.

DM individuals have shown 2- to 5-fold increase in cardiovascular events as compared with Hp 1-1 and Hp 2-1 DM individuals. According to our data we next examined whether antioxidant therapy with vitamin E may reduce cardiovascular events in Hp 2-2 DM individuals in the heart and outcome prevention evaluation (HOPE) study. For this purpose we have assessed the Hp genotype in stored blood samples from HOPE and found that in Hp 2-2 DM individual's vitamin E significantly reduced myocardial infarction and cardiovascular death by 43% and 55%, respectively. However, these data were interpreted with considerable caution because of the retrospective nature of this analysis, as well as the inability to demonstrate a statistical interaction between vitamin E and Hp genotype for either the HOPE composite outcome (stroke, CVD death, myocardial infarction, MI) or any of its components. Then, we sought to test the validity of these findings in Hp 2-2 DM individuals in a prospective, double-blind, placebo-controlled trial of vitamin E[28-33] (Figure 3).

According to our results vitamin E provides cardiovascular protection to individuals with diabetes and the haptoglobin 2-2 genotype but appears to increase cardiovascular risk in individuals with diabetes and the haptoglobin 2-1 genotype. We have previously demonstrated that the haptoglobin protein is associated with high-density lipoprotein (HDL) and HDL function and its oxidative modification are haptoglobin genotype dependent. Hence, we set out to test the hypothesis that the pharmacogenetic interaction between the haptoglobin genotype on cardiovascular risk might be secondary to a parallel interaction between the haptoglobin genotype and vitamin E on HDL function.

Oxidative modification has been proposed to be the mechanism by which HDL is rendered dysfunctional, and antioxidant therapy appears to restore HDL functionality. We therefore sought to determine whether the interaction between vitamin E and Hp genotype on RCT can be explained by a differential effect of vitamin E on HDL oxidative modification in Hp 2-1 and Hp 2-2. We have found that vitamin E supplementation resulted in a 50% reduction in HDL associated lipid peroxides in Hp 2-2 (0.55 ± 0.10 nmol vitamin E *vs* 1.07 ± 0.19 nmol placebo; P = 0.003) but had no effect in Hp 2-1.

In order to determine why vitamin E reduced HDL lipid peroxidation in Hp 2-2 but not Hp 2-1 we have investigated the effect of vitamin E on the mass or activity of the antioxidant proteins glutathione peroxidase and paraoxonase known to be associated with HDL, as well as the amount of redox-active non-transferrin-bound iron which has previously been implicated in the oxidation of HDL. While the effect of vitamin E did not reach statistical significance for any of these measurements it was associated with an approximately 50% increase in HDL associated glutathione peroxidase and a 25% reduction in redox active iron in Hp 2-2, while there was a 3-4 fold decrease in HDL associated glutathione peroxidase (P = 0.06) and no change in redox active iron in Hp 2-1[34-39].

The increase in redox-active iron in Hp 2-2 DM has been attributed to the impaired clearance of Hp 2-2-Hb by the CD163 Hp-Hb receptor. The surface expression of CD163 is regulated by oxidative stress and hyperglycemia. Thus we sought to determine whether the decrease in redox-active iron in Hp 2-2 DM individuals who received vitamin E, may be associated with an increase in CD163 expression on peripheral blood mononuclear cells (PBMs). We have observed a trend showing a greater than 50% increase in CD163 expression in PBMs of Hp 2-2 individuals receiving vitamin E. Vitamin E appeared to be associated with a 50% reduction in CD163 expression in Hp 2-1 individuals. In addition to functioning in the promotion of RCT and preventing the oxidation of LDL, HDL has been described as having an anti-inflammatory function. However, pro-inflammatory biomarkers such as C3 have been associated with dysfunctional HDL

in individuals with CVD. We next sought to determine whether vitamin E would decrease the association of C3 with HDL in Hp 2-2. We have found that vitamin E treatment was associated with a borderline significant decrease in C3 associated HDL in Hp 2-2 individuals (1.07 ± 0.09 vitamin E *vs* 1.34 ± 0.20 placebo; $n = 25$; $P = 0.09$) but had no effect on the association of C3 with HDL in Hp 2-1 (1.08 ± 0.07 *vs* 1.08 ± 0.07; $n = 30$; $P = 0.99$). This effect of vitamin E on a HDL associated inflammatory marker was not associated with an overall change in serum markers of inflammation such as CRP and adiponectin.

Collectively, we have provided a plausible mechanism for the divergent effects of vitamin E therapy on cardiovascular risk in DM individuals with the *Hp 2-1* and *Hp 2-2* genotypes. While vitamin E improves HDL function in Hp 2-2 DM individuals it decreases HDL function in Hp 2-1 DM individuals. Structural analysis of HDL in study participants suggested a similar interaction in which vitamin E appeared to result in a favorable although non-statistically significant change was found in a number of HDL associated oxidative and inflammatory markers in Hp 2-2 individuals (the decrease in HDL associated lipid peroxides was statistically significant) while it was produced no beneficial effect on these markers in Hp 2-1 individuals. Therefore, this study supports the concept that there is a pharmacogenetic interaction between the *Hp* genotype and vitamin E in individuals with DM.

The favorable effects of vitamin E on HDL structure and lipid peroxidation described here are most likely due to the inhibition of oxidative modifications mediated by Hp 2-2-Hb associated with apolipoprotein A-I ($ApoA_1$). Hp has been demonstrated to binds $ApoA_1$ and this Hp can tether Hb to HDL. Hp 2-2 is inefficient in blocking the redox activity of Hb derived iron and therefore in Hp 2-2 individuals, HDL becomes the carrier of a cargo which is pro-oxidative. The detrimental effects of vitamin E on HDL structure in Hp 2-1 individuals may be due to an overshoot in the suppression of oxidative stress by vitamin E. Excessive suppression of oxidative stress may be deleterious. Several groups have demonstrated, both in animals and in humans, a down-regulation of protective antioxidant enzymes in response to high dose antioxidant supplementation. This down-regulation may paradoxically increase the susceptibility of these individuals to acute increases in oxidative stress (as with wide swings in hyperglycemia). Therefore, it may be possible to demonstrate that in Hp 2-1 individuals a lower dose of vitamin E may have beneficial effects on CVD.

The public health and economic implications of the pharmacogenetic interaction between the Hp type and vitamin E on CVD are profound. Implementation of a pharmacogenetic algorithm for DM patients in which all individuals with DM and the *Hp 2-2* genotype would receive vitamin E cannot be achieved without an additional clinical trial testing this hypothesis. We hope that the mechanistic data presented here will help to increase the interest for such a trial. The notion of pharmacogenomics is that not all individuals with a given disease may benefit from the same drug treatment. We have demonstrated that vitamin E provides renal protection to Hp 2-2 DM mice but does not have any effect on Hp 1-1 DM mice. The pharmacogenomic implications of these findings are significant. Large-scale clinical trials of vitamin E to prevent macrovascular complications of diabetes have failed to show that vitamin E provided any clinical benefit. Studies assessing the effect of vitamin E on the progression of DN in humans with DM have yielded inconsistent findings. Moreover, recent meta-analysis suggested that there is an increased risk of all cause mortality with high-dose vitamin E supplementation. One explanation for the failure of vitamin E to provide benefit in human studies may be due to the inadequate nature of patient selection in these studies. We have recently provided concrete evidence in humans for a pharmacogenomic interaction between the Hp genotype and vitamin E supplementation in relation to the development of atherosclerotic cardiovascular disease. We have found by analyzing stored blood samples from the HOPE study that individuals with DM and the Hp 2-2 genotype received significant clinical benefit from vitamin E. Moreover, we have recently demonstrated in a prospective double blind clinical trial that vitamin E dramatically reduces cardiovascular disease in Hp 2-2 DM individuals. The ability of vitamin E to reduce features of renal disease characteristic of early human DN in Hp 2-2 DM mice but not in Hp 1-1 DM mice, suggests that there may also be an interaction between Hp genotype and vitamin E therapy in diabetic renal disease.

In the other arm of the studies of secondary prevention, we can mention the Gruppo Italiano per lo Studio della Sopravvivenzane Ⅱ'Infartomiocardico-Prevenzione study, in which 11324 patients who had a recent MI were randomized to vitamin E (300 mg/d), polyunsaturated fatty acids, both or neither of them. They were followed for 3.5 years after which no effect of treatment with vitamin E was observed. In the Cambridge heart antioxidant study, treatment with vitamin E decreased the risk of developing non-fatal MI, but increased the risk of CV death. Similar results were obtained from the HOPE in 2000. The patients, men and women, were followed for about 4.5 years during which they received Vitamin E (400 mg/d) or angiotensin converting enzyme (ACE) inhibitor or a placebo. The results showed that vitamin E did not influence the risk for developing CVD. In 2005 an extension of this study was performed (HOPE-TOO study). In this analysis the incidence of CVD, cancer and cancer death was investigated in patients who received vitamin E or a placebo. Patients who received vitamin E had a higher risk of heart failure and required hospitalization.

ANIMAL STUDIES OF VITAMIN E

As we mentioned above, hyperglycemia is accompanied by severe oxidative stress (especially lipid per-oxidation) which is caused by increased oxygen free radical produc-

tion. Toxic oxygen free radicals have been implicated in the pathogenesis of diabetes mellitus, and its micro and macro vascular complications. An imbalance resulting from the increased production and/or reduced scavenging of these free radicals leads to a metabolic state of oxidative stress, which consequently leads to tissue damage. Auto glycosylation reactions, alterations in the sorbitol pathway and hyperglycemia have been proposed as some of the mechanisms which are responsible for this increased oxidative stress.

Antioxidants have been shown to play a beneficial role in preventing diabetic complications. Diabetes is a good model for chronic oxidative damage and it is a particularly suitable disease for antioxidant supplementation. It was found that there is a significant correlation has been found between the increased blood sugar levels and the depletion of the antioxidants. This depletion was a major risk factor for developing diabetes complications, and antioxidant supplementation (such as vitamin E) could decrease this risk.

Genetically modified mice offer the most direct means to demonstrate a gene-disease association. Such mice are inbred allowing one to study the effect of a change in one single gene. The Hp 2 allele is found only in humans. All other animals including higher primates have only the Hp 1 allele and therefore the *Hp 1-1* genotype. One approach to mimic the Hp polymorphism in mice is to introduce the human Hp 2 allele as a transgene. Human Hp 2 transgenic mice in an Hp knockout background have been used to study mice only expressing the Hp 2 allelic protein product[41,42]. The wild-type murine *C57Bl/6 Hp* gene is a type 1 Hp allele with over 90% homology to the human Hp 1 allele. The construction of C57Bl/6 mice with targeted insertion of a murine Hp 2 allele has generated mice with the *Hp 2-2* genotype. It was created by genetically engineering an Hp 2 allele by duplication of exons 3 and 4 in the genomic sequence of the murine Hp 1 allele. Then this murine Hp 2 allele was inserted at the endogenous Hp locus using a targeting strategy that is specifically selected for a homologous recombination event between the murine Hp 2 allele and the endogenous murine Hp 1 allele.

STREPTOZOTOCIN-INDUCED DIABETES

Streptozotocin was administered at 6 wk of age in a low-dose 5-d protocol as recently described by the National Institutes of Health sponsored Diabetes Consortium (50 mg/kg for 5 d). For all studies, a group of littermates who were not injected with streptozotocin was followed in parallel so that the only difference between the groups was the presence or absence of DM. Therefore, the parameters described below were measured for four groups of animals: Hp 1-1, Hp 1-1 DM and Hp 2-2, Hp 2-2 DM. There was no difference in spot glucose or HbA1c between Hp 1-1 and Hp 2-2 DM mice. For all analysis measurements were made when mice were 4 mo of age.

VITAMIN E SUPPLEMENTATION

Vitamin E was administered in the drinking water, for 6 wk, beginning 1 mo after onset of DM until the mice were killed at 4 mo of age. We used vitamin E from Merck which is water miscible as documented by the manufacturer (Merck cat. No. 500862). This is DL alpha tocophorol acetate which enters easily into water. We made up a stock solution of vitamin E 1 mL in 50 mL of water and then used 5 mL of this stock solution in a 250-mL bottle of water for the mice. Each DM mouse received 600 mg/kg per day during the course of treatment.

RESULTS IN HP 2-2 DM MICE

Vitamin E and morphometry: Increased renal hypertrophy in Hp 2-2 DM mice

Gross kidney size which expressed as the kidney index (kidney mass/body mass) was significantly elevated in Hp 2-2 DM mice compared with their non-DM littermates and with Hp 1-1 DM mice (15.5 ± 0.97 g/kg for Hp 2-2 DM *vs* 11.9 ± 1.1 g/kg for Hp 1-1 DM and 10.1 ± 0.4 g/kg for Hp 2-2 non-DM; $P < 0.05$ comparing Hp 2-2 DM mice with Hp 1-1 DM or Hp 2-2 non-DM mice). There was a significant increase in both total glomerular area and proximal tubule area in Hp 2-2 DM mice compared with Hp 1-1 DM mice glomerular area: 4852.9 ± 308.7 for Hp 2-2 DM *vs* 3176.8 ± 99.3 for Hp 1-1 DM, $P < 0.001$ (Figure 4); Proximal tubular area: 1152.6 ± 42.4 for Hp 2-2 DM *vs* 818.0 ± 7.2 for Hp 1-1 DM, $P < 0.05$. We observed no significant difference in the cellularity of Hp 1-1 *vs* Hp 2-2 glomeruli or tubules suggesting that the glomerular expansion seen in Hp 2-2 DM mice was more likely to be due to hypertrophy than hyperplasia. There was a significant decrease in total glomerular area in Hp 2-2 DM with vitamin E ($P < 0.05$)[40-42].

Histology: Increased collagen, smooth muscle actin, and iron in Hp 2-2 DM mice

Collagen type IV (Figure 5A) and smooth muscle cell actin (Figure 5B), which are proteins known to be increased in human DN glomeruli, were significantly increased in Hp 2-2 DM mice (0.10 ± 0.07 for Hp 2-2 DM and 0.030 ± 0.003 for Hp 1-1 DM, $P < 0.001$ and 0.14 ± 0.01 for Hp 2-2 DM and 0.04 ± 0.01 for 1-1 DM, $P < 0.001$ respectively). There was a significant decrease in collagen IV immunostaining area and actin staining in Hp 2-2 DM mice with Vit-E ($P < 0.05$, $P < 0.001$ respectively). Furthermore, significantly greater amounts of iron were found in the renal tissue (localized to the proximal tubular cells) of Hp 2-2 DM mice compared with Hp 1-1 DM mice(1.34 ± 0.19 for Hp 2-2 DM and 0.56 ± 0.12 for Hp 1-1 DM, $P < 0.01$).

PREVENTION OF DN IN HP 2-2 DM MICE WITH VITAMIN E SUPPLEMENTATION

As we mentioned above, the Hp 2-2 mice have increased

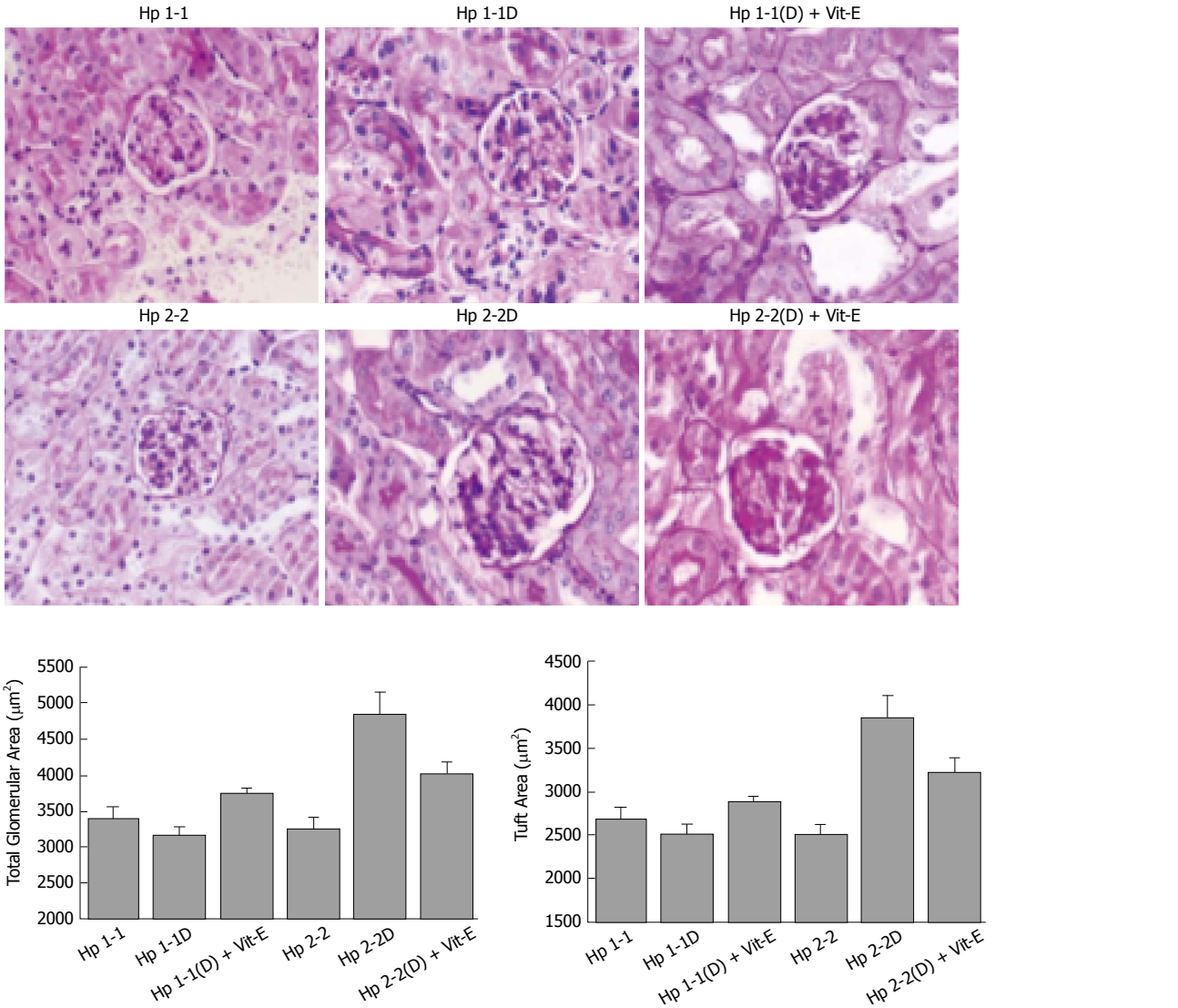
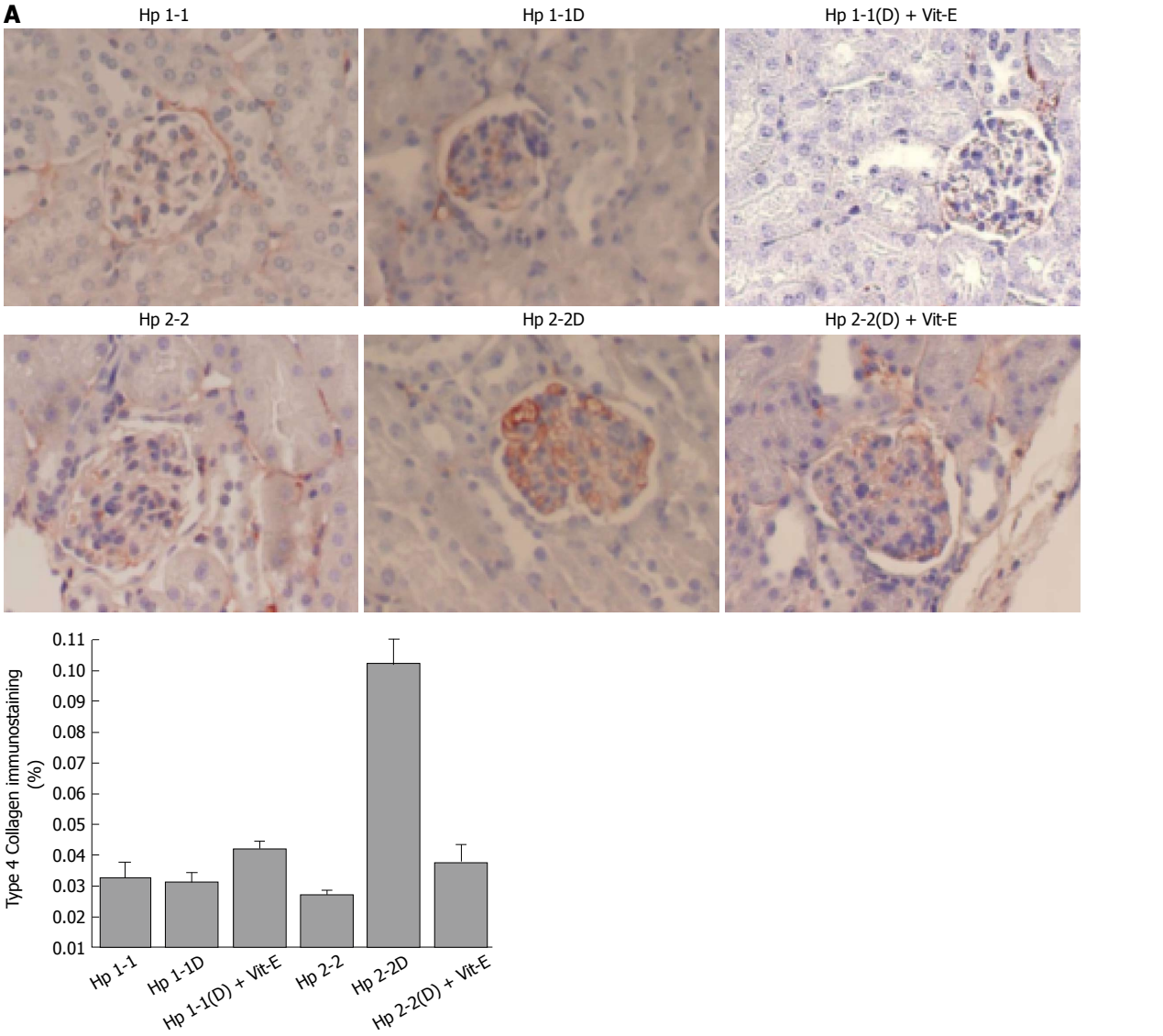


Figure 4 Total glomerular and glomerular tuft area is increased in haptoglobin 2-2 diabetes mellitus mice. Periodic acid-Schiff stained, paraffin-embedded sections from kidneys of haptoglobin (Hp) 1-1 and Hp 2-2 mice with D or without diabetes mellitus (DM). Areas were measured using Image Pro software. Values are expressed as mean ± SE (30 glomeruli measured for each animal). There was a significant increase in total glomerular area in Hp 2-2 DM mice *vs* Hp 1-1 DM mice ($P < 0.001$) as well as between Hp 2-2 DM and Hp 2-2 non-DM mice ($P < 0.001$). There was a significant decrease in total glomerular area in Hp 2-2 DM mice with vitamin E (Vit-E; $P < 0.05$).

renal hypertrophy together with increased levels of Collagen, Smooth Muscle Actin, and Iron. We found in the Hp 2-2 DM mice which received vitamin E, a significant reduction in total glomerular area ($P < 0.05$), proximal tubule area ($P < 0.05$), glomerular collagen content ($P < 0.001$), glomerular actin content ($P < 0.001$). Creatinine clearance (CCT) and albuminuria are increased in Hp 2-2 DM mice (Figure 6). We have found a significant increase in CCT in Hp 2-2 DM mice compared with Hp 1-1 DM mice ($P < 0.05$) and Hp 2-2 non-DM mice ($P < 0.05$). There was a significant decrease in CCT in Hp 2-2 DM mice treated with vitamin E ($P < 0.05$). We have also found a non significant reduction in albuminuria in Hp 2-2 DM mice receiving vitamin E (18.5 ± 7.2 *vs* 95.3 ± 38.0; $P < 0.16$). Vitamin E supplementation to Hp 2-2 DM mice also resulted in a significant 50% reduction in global oxidative stress in renal tissue slices assessed as lipid peroxidation ($P < 0.01$). In contrast, in Hp 1-1 DM mice, vitamin E did not affect any morphometric or functional parameter as demonstrated in Figures 4-7.

In our last publication[43], we have studied the protective effect of vitamin E against the toxic effects of free radicals in diabetic mice with Hp 2-2 phenotype. The primary objective of this study was to determine the intracellular localization of this iron in the proximal tubule cells and to assess its potential toxicity. Transmission electron microscopy demonstrated a marked accumulation of electron-dense deposits in the lysosomes of proximal tubules cells in Hp 2-2 DM mice. Energy-dispersive X-ray spectroscopy and electron energy loss spectroscopy were used to perform elemental analysis of these deposits and demonstrated that these deposits were iron rich. These deposits were associated with lysosomal membrane lipid peroxidation and loss of lysosomal membrane integrity. Vitamin E administration to Hp 2-2 DM mice resulted in a significant decrease in both intralysosomal iron-induced


A
Hp 1-1
Hp 1-1D
Hp 1-1(D) + Vit-E
Hp 2-2
Hp 2-2D
Hp 2-2(D) + Vit-E
Type 4 Collagen immunostaining (%)
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Hp 1-1
Hp 1-1D
Hp 1-1(D) + Vit-E
Hp 2-2
Hp 2-2D
Hp 2-2(D) + Vit-E
B
Hp 1-1
Hp 1-1D
Hp 1-1(D) + Vit-E
Hp 2-2
Hp 2-2D
Hp 2-2(D) + Vit-E

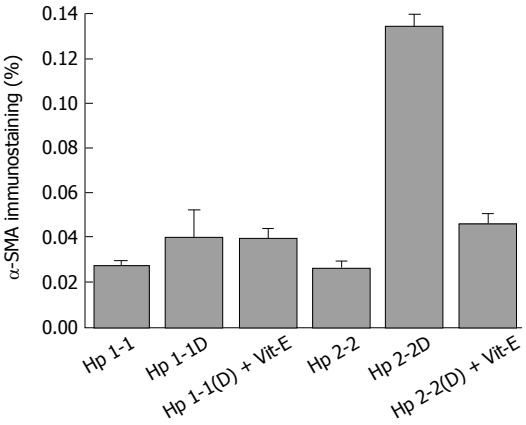


Figure 5 Increased mesangial collagen IV and smooth muscle actin in haptoglobin 2-2 diabetes mellitus mice. A: Increased mesangial collagen IV in haptoglobin (Hp) 2-2 diabetes mellitus (DM) mice. Quantitation of the immunostaining area was reported in % area of the glomeruli. All values are expressed as means SE (30 glomeruli for each animal). There was a significant increase in collagen IV immunostaining (%) in Hp 2-2 DM *vs* Hp 1-1 DM mice ($P < 0.001$) and Hp 2-2 non-DM mice ($P < 0.001$). There was a significant decrease in collagen IV immunostaining area in Hp 2-2 DM mice with vitamin (Vit)-E ($P < 0.05$); B: Increased smooth muscle actin in Hp 2-2 DM mice. Immunohistochemical identification of smooth muscle actin (orange-red) was performed using a monoclonal antibody to mouse smooth muscle actin . All values are expressed as mean ± SE. Quantitation of actin staining was performed similar to collagen (as% of glomerular area) with a highly significant increase in actin staining in Hp 2-2 DM mice *vs* Hp 1-1 DM mice ($P < 0.001$) and Hp 2-2 non-DM mice ($P < 0.001$). There was a significant decrease in actin staining in Hp 2-2 DM mice treated with vitamin E (Vit-E) ($P < 0.001$).

oxidation and lysosomal destabilization. Therefore, Iron-induced renal tubular injury may play a major role in the development of diabetic nephropathy and may be a target for slowing the progression of renal disease.

Proof of concept that the loss of lysosomal membrane integrity in Hp 2-2 DM proximal tubule cells is due to oxidative damage

We sought to evaluate the role of lipid peroxidation in the maintenance of lysosomal membrane integrity by showing that chronic administration of the lipid-soluble antioxidant, vitamin E, could decrease lysosomal membrane oxidation and maintain lysosomal membrane integrity. Vitamin E supplementation resulted in a significant 45% reduction in lysosomal redox-active iron in Hp 2-2 DM mice ($P < 0.05$) with no significant effect on lysosomal redox-active iron in Hp 1-1 DM mice. Moreover, we have found that vitamin E supplementation significantly decreased lysosomal lipid peroxides in Hp 2-2 DM kidneys as compared with lysosomal preparations of Hp 2-2 DM mice treated with placebo (75.7 ± 8.7 nmol lipid peroxides/mg protein for Hp 2-2 DM with vitamin E *vs* 109.2 ± 8.8 nmol lipid peroxides/mg protein for Hp 2-2 DM without vitamin E; $P = 0.03$). There was no significant reduction in lysosomal lipid peroxides in Hp 1-1 DM mice treated with vitamin E (Figure 8). Moreover, there was a significant correlation between lysosome membrane α-tocopherol concentrations and the degree of lysosomal membrane oxidation in Hp 2-2 DM mice but not in Hp 1-1 DM mice. Finally, we have found a significant reduction in the loss of lysosomal membrane integrity in lysosomes purified from kidneys of Hp 2-2 DM mice treated with vitamin E as compared with those treated with placebo (24.1% ± 2.3% for vitamin E group *vs* 30.7% ± 1.7% for placebo group; $n = 6$ per group; $P = 0.03$). No significant differences in lysosomal membrane integrity were found after vitamin E administration to Hp 1-1 DM mice as compared to those treated with placebo (19.9% ± 2.7% for vitamin E group *vs* 22.1% ± 2.3% for placebo group; $n = 6$; $P = 0.24$) . There was a significant correlation in Hp 2-2 DM mice, but not in Hp 1-1 DM mice, between the concentration of vitamin E in the lysosomal membrane and the lysosomal membrane integrity (Figure 9).

DIABETIC RETINOPATHY

An early morphological characteristic of the microangiopathy seen in diabetic retinal disease is retinal capillary basement membrane (RCBM) thickening. RCBM thickness as assessed by electron microscopy was performed on a total of 12 eyes taken from three mice in each of the four study groups (three eyes from C57Bl/6 Hp 1 and C57Bl/6 Hp 2 mice with and without streptozotocin-induced diabetes). Diabetes was produced by intraperitoneal injection at 6 wk of age with streptozotocin at a concentration of 200 mg/kg dissolved in 50 mmol/L citrate buffer, pH 4.5. Glucose levels were monitored with a glucometer. Animals were sacrificed at 6 mo of age. For these studies involving diabetes, a group of non-diabetic mice was followed in parallel so that the only difference between the groups was the presence or absence of diabetes. We found no difference in the degree of glucose control between mice with the different Hp genotypes.

Electron microscopy was performed on a total of 12 eyes from the four groups (three eyes from Hp 1 and Hp 2 animals with and without diabetes) for the determination of the retinal basement membrane thickness. Mice were sacrificed with intraperitoneal injection of pentabarbitone sodium. The eyes were enucleated, opened at the equator, fixed in 3.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 1 h, and then post-fixed in 2%

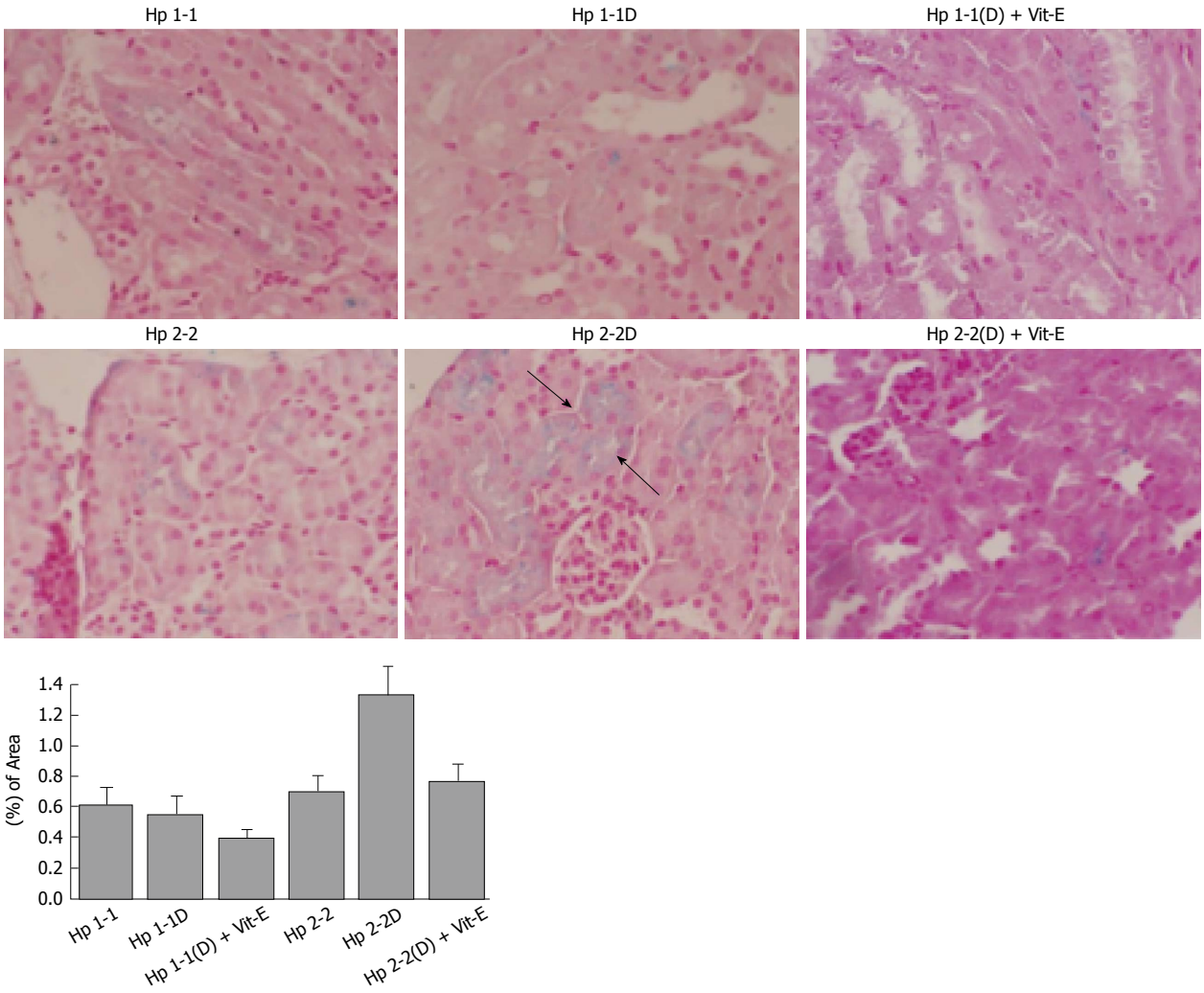


Figure 6 Increased renal iron deposition in the proximal tubule of haptoglobin 2-2 diabetes mellitus mice. Perl's iron stain was used to localize iron in paraffin-embedded kidney sections in haptoglobin (Hp) 1-1 and Hp 2-2 mice with and without diabetes mellitus (DM). Arrow indicates iron-induced stain in blue (× 400 magnification) located within proximal tubular cells. There was a significant increase in iron staining in the renal tissue of Hp 2-2 DM (D) *vs* Hp 1-1 DM (D) and Hp 2-2 non-DM mice ($P < 0.001$).

osmium tetroxide. Semithin sections (1 μm) were stained with toluidine for orientation and identification of the capillary. Thin sections (60 nm) were produced with a diamond knife, placed on 300-mesh copper grids, and stained with uranyl acetate and lead citrate. The sections were viewed and photographed with a JEOL JEM 100SX electron microscope.

ASSESSMENT OF RETINAL CAPILLARY BASEMENT MEMBRANE THICKNESS

Sections mounted on copper grids and treated with the tannic acid solution prepared as described above were analysed using Image Pro software analysis. Basement membrane thickness was measured on five distinct capillaries for each eye and 5-10 measurements were taken per capillary with a minimum of 40 independent measurements from each eye. One reader scored all eyes in the study and was blinded to the genotype of the mice.

RETINAL CAPILLARY BASEMENT MEMBRANE THICKNESS

Retinal capillary basement membrane thickness was assessed from electron microscope photographs from three different mice with either Hp 1 or Hp 2 with or without streptozotocin-induced diabetes. For each animal, a minimum of 40 separate measurements of the RCBM thickness were obtained. This analysis demonstrated that there was no significant difference in retinal basement membrane thickness between non-diabetic Hp 1 and non-diabetic Hp 2 mice (Mann-Whitney $P = 0.70$; difference in median 2.6 nm). Diabetic Hp 1 mice did not demonstrate a significant increase in basement membrane thickness as compared to non-diabetic Hp 1 mice (Mann-Whitney $P = 0.42$; difference in median 5.2 nm). However, induction of diabetes resulted in a marked increase in basement membrane thickness in Hp 2 mice compared to non-diabetic Hp 2 mice (Mann-Whitney $P = 0.0004$; difference in median 32.1 nm), and to diabetic Hp 1 mice

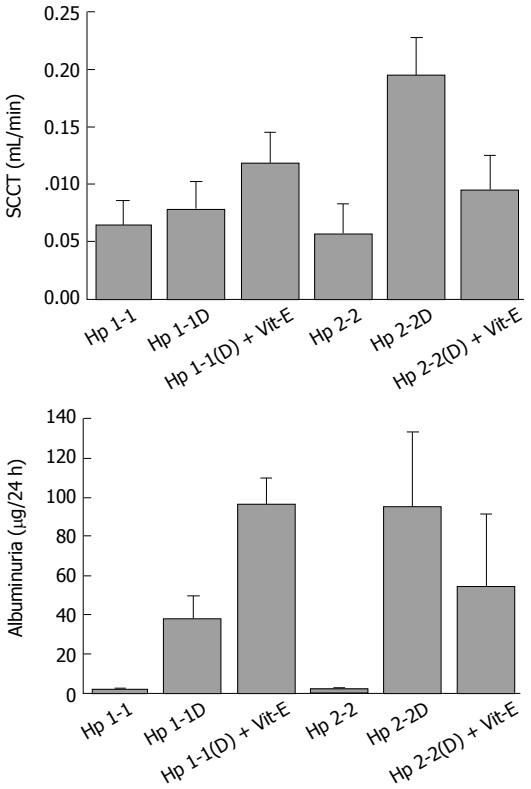


Figure 7 Creatinine clearance and albuminuria are increased in haptoglobin 2-2 diabetes mellitus mice. Creatinine clearance time (CCT). Values are reported as mean ± SE in mL/min of a minimum of 5 animals from each group. There was a significant increase in CCT in haptoglobin (Hp) 2-2 diabetes mellitus (DM) mice compared with Hp 1-1 DM mice ($P < 0.05$) and Hp 2-2 non-DM mice ($P < 0.05$). There was a significant decrease in CCT in Hp 2-2 DM mice treated with vitamin (Vit)-E ($P < 0.05$). We found a marked increase in albuminuria in both Hp 1-1 DM and Hp 2-2 DM mice compared with their non-DM littermates and a nonsignificant 2- to 3-fold increase in albuminuria in Hp 2-2 DM mice compared with Hp 1-1 DM mice (95.3 ± 38.0 *vs* 37.9 ± 11.9, $P < 0.16$).

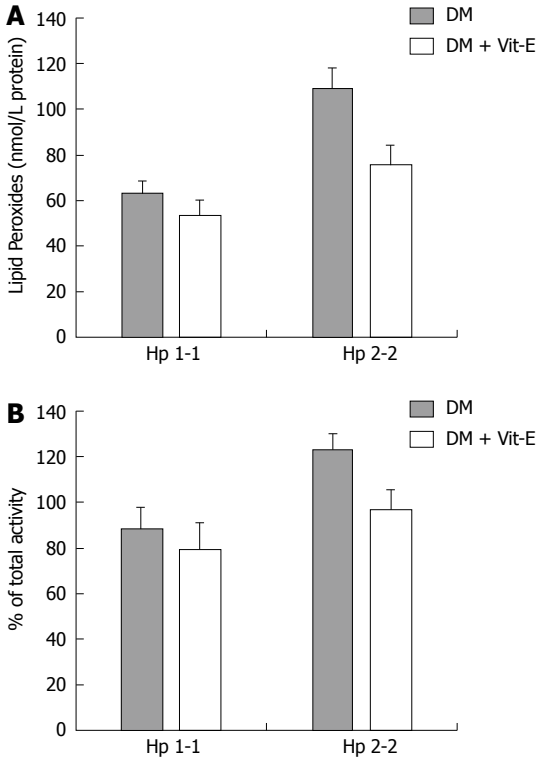


Figure 8 Effect of vitamin E supplementation on lysosomal membrane lipid peroxides and membrane integrity. A: Lysosomal membrane lipid peroxides were significantly reduced in haptoglobin (Hp) 2-2 diabetes mellitus (DM) mice which received vitamin E supplementation as compared to Hp 2-2 DM mice receiving placebo ($P = 0.03$); B: Lysosomal membrane activity was reduced in Hp 2-2 DM mice received vitamin E (Vit-E) supplementation as compared to Hp 2-2 DM mice receiving placebo ($P = 0.03$).

(Mann-Whitney $P = 0.0005$; difference in median 24.3 nm). Thus, the effect of diabetes in increasing basement membrane thickness occurred only in the Hp 2 group[43].

Fardoun *et al*[17] described the protective effect of vitamin E in patients with diabetic retinopathy in a prospective clinical study. Diabetic patients of either sex, above the age 45 years old, with or without diabetic complications were studied. The recruited patients were categorized into two groups: the primary and the secondary prevention groups. Type I group were divided in two groups, which consisted of the patients who received insulin and the vitamin E supplementation and the patients who received only insulin. The type Ⅱ patients were further divided into the test and the control groups which consisted of those who received oral hypoglycemic and the vitamin E supplementation and those who were on oral hypoglycemic only. The number of the patients who developed cardiovascular complications and diabetic retinopathy in the test group (vitamin E) was significantly low in both type I and type Ⅱ DM, as compared to those in the control groups. This study suggests that a long term vitamin E supplementation was beneficial for the cardiovascular complications.

DISCUSSION

The pharmacogenomic implications of these findings are significant. Large-scale clinical trials of vitamin E to prevent macrovascular complications of diabetes, have failed to show that vitamin E provided any clinical benefit. Studies assessing the effect of vitamin E on the progression of DN in humans with DM have yielded inconsistent findings. Moreover, recent meta-analysis has suggested that there is an increased risk of all causes of mortality with high-dose vitamin E supplementation. One explanation for the failure of vitamin E in providing benefit in human studies may be due to the inadequate nature of patient selection in these studies. We have recently provided concrete evidence in humans for a pharmacogenomic interaction between the *Hp* genotype and vitamin E supplementation in relation to development of atherosclerotic cardiovascular disease. We have found by analyzing stored blood samples from the HOPE study that individuals with DM and the *Hp 2-2* genotype showed significant clinical benefit from vitamin E[17]. Moreover, we recently demonstrated in a prospective double blind clinical trial that vitamin E dramatically reduces cardiovascular disease in Hp 2-2DM individuals[24]. The ability of vitamin E to reduce features of renal disease characteristic of early human DN in Hp 2-2 DM mice but not in Hp 1-1 DM mice, suggests that there may

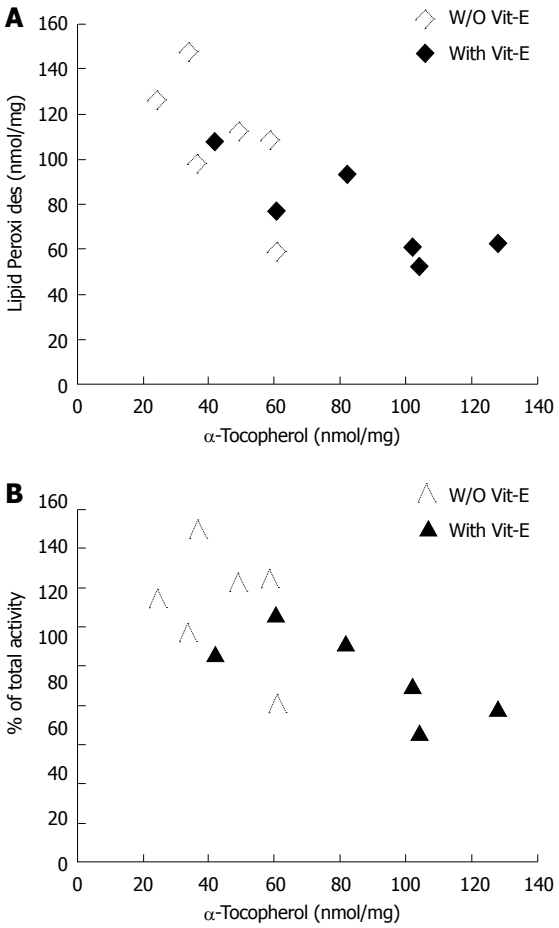


Figure 9 Lysosomal vitamin E. A: Lysosomal vitamin E (Vit-E) concentration is correlated with lysosomal membrane lipid peroxidation and lysosomal membrane integrity. Filled symbols are data points for mice receiving Vit-E supplementation while empty symbols are data points for mice who received placebo; B: Lysosomal Vit-E and lysosomal membrane integrity.

also be an interaction between Hp genotype and vitamin E therapy in diabetic renal disease.

Different studies have shown that the vitamin E supplemented diabetics had a lesser incidence (a 25% lower risk) of the cardiovascular complications after 24 mo. This suggested that a long term vitamin E supplementation was beneficial for the cardiovascular complications. This is in accordance with the findings of the Cambridge Heart Antioxidant Study[9-11]. The Cambridge Heart Antioxidant Study showed that tocopherol treatment significantly reduced the risk of cardiovascular death and nonfatal myocardial infarction after 1 year of the treatment. An improvement was observed in the retinopathy in the test group treated with vitamin E. There were no significant differences between antioxidant vitamin supplementation and placebo in the relative risk for major cardiovascular outcome. In our last study[44], we have demonstrated that increased lysosomal redox-active iron results in lysosomal membrane injury in renal cells of Hp 2-2 DM mice. Therefore, this data provide a novel pathophysiological mechanism explaining why the progression to end-stage renal disease is increased in DM individuals with the Hp 2-2 DM genotype. Moreover, the interaction between the vitamin E and the Hp genotype on lysosomal injury suggests that a pharmacogenomic paradigm of selective administration of vitamin E to Hp 2-2 DM individuals may offer considerable renal protection similar to that recently demonstrated for cardiovascular disease.

Multiple studies blocking the course of diabetic retinopathy and nephropathy based on studies in rodents found the blocking agent under trial to be without value in humans, especially blockers of advanced glycation end-products. Diabetes induces the formation of advanced glycation end products (AGEs), which can alter the function of proteins and stimulate pathological cellular responses *via* AGE receptors. Increasing levels of AGEs, and their deposition in diabetic kidneys, correlate with the development of DN. Of the pathophysiologic mechanisms that have been identified in the development and progression of DN, oxidative stress is of major importance.

Pyridoxamine was introduced as an inhibitor of AGE formation from Amadori products[39-41]. The effects of pyridoxamine include: (1) the inhibition of AGE formation by blocking the oxidative degradation of the Amadori intermediate of the Millard reaction; (2) the scavenging of toxic carbonyl products of glucose and lipid degradation; and (3) the trapping of reactive oxygen species[42]. We demonstrated that pyridoxamine (K-163) ameliorates the levels of urinary albumin creatinine ratio (ACR) and serum 3-deoxyglucosone (3DG) in KK-A^{y} mice without changing systemic blood pressure. Furthermore, pyridoxamine prevented accumulations of Nq-(carboxymethyl)-lysine (CML), nitrotyrosine, transforming growth factor-β (TGF-β1), and laminin-β1 in the kidney tissues[41]. AGEs and oxidative stress might activate autocrine Ang Ⅱ signaling and subsequently induce TGF-β1-Smad signaling in mesangial cells[24,43]. Our findings suggested that the amelioration of urinary ACR was related to the improvement of TGF-β1 and laminin-β1 expressions in the kidney because CML and nitrotyrosine accumulations were improved and the levels of serum 3DG were reduced by anti-AGE and/or the antioxidant effects of pyridoxamine.

Despite the successful use of lifestyle changes, metabolic control, and blood pressure control, including ACE inhibitors and angiotensin receptor blocker therapy, residual renal risk remains very high, leaving the diabetic population with a clear unmet need for novel treatment options. As outlined in this review, various drugs are in development. It is anticipated that some of the newer agents that are currently the focus of clinical trials will ultimately lead to improvements in slowing the progression and eventually improving the prognosis of this devastating disease.

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MINIREVIEWS

Vascular response to vasodilator treatment in microalbuminuric diabetic kidney disease

Narisa Futrakul, Prasit Futrakul

Narisa Futrakul, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok 10330, Thailand
Prasit Futrakul, Bhumirajanagarindra Kidney Institute, Bangkok 10400, Thailand
Prasit Futrakul, Academy of Science, the Royal Institute of Thailand, Dusit District 10300, Thailand
Author contributions: Futrakul N is the principle investigator; Futrakul N and Futrakul P contributed equally to the work.
Supported by Thailand Research-Fund, Bhumirajanagarindra Kidney Institute and National Research Council Fund of Thailand
Correspondence to: Narisa Futrakul, MD, PhD, Professor, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330, Thailand. fmednft@yahoo.com
Telephone: +66-2-81351214 Fax: +66-2-2564911
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Abstract

Under common practice, the conventional diagnostic marker such as microalbuminuria determination does not recognized early stage of diabetic kidney disease (normoalbuminuria, chronic kidney disease stage 1, 2); due to the insensitiveness of the available marker. Treatment at later stage (microalbuminuria) simply slows the renal disease progression, but is rather difficult to restore the renal perfusion. Intrarenal hemodynamic study in these patients revealed an impaired renal perfusion and abnormally elevated renal arteriolar resistances. Treatment with vasodilators such as angiotensin converting enzyme inhibitor and angiotensin receptor blocker fails to correct the renal ischemia. Recent study on vascular homeostasis revealed a defective mechanism associated with an impaired nitric oxide production which would explain the therapeutic resistance to vasodilator treatment in microalbuminuric diabetic kidney disease. This study implies that the appropriate therapeutic strategy should be implemented at earlier stage before the appearance of microalbuminuria.

Key words: Microalbuminuria; Diabetic kidney disease; Renal hemodynamics; Fractional excretion of magnesium; Renal function

Core tip: This manuscript demonstrates the therapeutic resistance to vasodilator treatment in restoring the renal functions in microalbuminuric diabetic nephropathy. It is supported by the intrarenal hemodynamic study which reveals a decline in renal plasma flow, peritubular capillary flow and glomerular filtration rate following vasodilator treatment. The above finding concerns with the recent study on vascular homeostasis which reveals a defective angiogenesis associated with an impaired nitric oxide production, which explains the therapeutic resistance to vasodilator and clinical failure in restoring renal perfusion in late stage diabetic nephropathy.

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INTRODUCTION

Diabetic kidney disease has been the public health threat which is the most common cause of end-stage renal failure[1-3]. Under common practice, it is recognized when there is presence of microalbuminuria (albumin/creatinine ratio is greater than 30 microgram/milligram creatinine[4-6]. In this regard, microalbuminuria cannot recognize early stage diabetic kidney disease (normoalbuminuria). Such practice would allow these early stage

Table 1 Renal function and intrarenal hemodyamic study in microalbuminuric type 2 diabetic kidney disease

	Healthy Subject	Initial value in Microal-buminuric patients	*P* value
Renal function			
Microalbumin/creatinine ratio, μg/mg	< 30	170 ± 193	0.010
Creatinine clearance, mL/min per 1.73 m^2	117 ± 13	73 ± 28	0.001
Fractional excretion of magnesium, %	1.6 ± 2.2	4.1 ± 1	0.050
Mean arterial pressure, mmHg	79	99 ± 5	0.001
Renal hemodynamics			
Renal plasma flow, mL/min per 1.73 m^2	585 ± 30	505 ± 120	NS
Peritubular capillary flow, mL/min per 1.73 m^2	479 ± 26	423 ± 120	NS
Glomerular filtration rate, mL/min per 1.73 m^2	116 ± 14	82 ± 6	0.010
Afferent arteriolar resistance, dyne.s.cm^{-5}	2331 ± 108	2841 ± 299	0.050
Efferent arteriolar resistance, dyne.s.cm^{-5}	3012 ± 130	4045 ± 1168	NS

NS: Not significant.

diabetic kidney disease patients to progress without therapeutic interruption. Intrarenal hemodynamic study in this stage reveals reduction in renal perfusion indicating renal ischemia[7-10]. Treatment with vasodilators such as angioconverting enzyme inhibitor (ACEI), angiotensin receptor blocker (ARB) during microalbuminuria or macroalbuminuria simply slows the renal disease progression determined by creatinine clearance, but is unable to restore all of the renal abnormalities[11-13]. This information concurs with the progressive increment in number of diabetic kidney disease patients entering end-stage renal disease. Recent study on vascular homeostasis in these patients revealed a defective angiogenesis namely vascular endothelial growth factor receptor 1, angiopoietin 1 leading to impairing the nitric oxide production as well as impairing the vascular repair. In addition, the abnormally elevated level of antiangiogenic factors namely vascular endothelial growth factor receptor 2, and angiopoietin 2 would induce the progression of renal microvascular disease and the progressive reduction in renal perfusion[14-18]. The altered vascular homeostasis observed in late stage diabetic kidney disease is believed to be the crucial mechanism of renal disease progression. In contrast to the altered vascular homeostasis observed in late stage diabetic kidney disease, the study on vascular homeostasis in early stage associated with normoalbuminuria has recently been demonstrated to be normal or mildly impaired values of both angiogenic and antiangiogenic factors[19].

RENAL FUNCTION IN MICROALBUMINURIC DIABETIC KIDNEY DISEASE

In microalbuminuric diabetic kidney disease, recognition of its status can be made through the conventional marker such as microalbuminuria (Table 1). In addition, fractional excretion of magnesium (FE Mg) appears to be more sensitive than the conventional markers and becomes abnormally elevated even in the stage of normoalbuminuria and recognizes chronic kidney disease (CKD) stage 1 and early stage 2[20]. FE Mg has been earlier demonstrated to correlate directly with the magnitude of tubulointerstitial fibrosis reflecting the presence of diabetic kidney disease[21,22]. It is noted that this group of diabetic kidney disease is associated with systemic hypertension. Creatinine clearance or estimated glomerular filtration rate is also a sensitive diagnostic marker for early stage diabetic kidney disease.

INTRARENAL HEMODYNAMIC STUDY IN MICROALBUMINURIC DIABETIC KIDNEY DISEASE

Altered renal hemodynamics has already been documented in normoalbuminuric diabetic kidney disease[20,23]. In microalbuminuric stage, renal plasma flow, peritubular capillary flow and glomerular filtration rate were depleted, whereas afferent and efferent arteriolar resistances were abnormally elevated. As indicated in Table 1, efferent arteriolar resistance was greater than the resistance of afferent arteriole - a phenomenon indicating a preferential constriction of the efferent arteriole. This phenomenon in turn, would induce intraglomerular hyperfiltration and therefore increase glomerular filtration rate. Subsequently, there is a greater degree of reduction in peritubular capillary flow. A longitudinal study on intrarenal hemodynamics along the clinical course of diabetic kidney disease has revealed a greater increase in degree of efferent arteriolar resistance indicating a further reduction in peritubular capillary flow as the disease severity progresses[18]. This finding implies that the sustained and progressive elevation of efferent arteriolar resistance would be capable of inducing a chronic renal ischemia to the tubulointerstitial structure, which is the crucial determinant of renal disease progression in diabetic kidney disease.

THERAPEUTIC RESPONSE TO VASODILATORS IN MICROALBUMINURIC DIABETIC KIDNEY DISEASE

It has been a general consensus that treatment of diabetic kidney disease with vasodilators, under common practice, does not cover all of the diabetic kidney disease patients, but infact excludes the group of early stage diabetic kidney disease (normoalbuminuria). Such practice would stabilize temporarily the renal function, or simply slow the renal disease progression, which is due to the defective angiogenesis and an impaired nitric oxide production induced by a variety of circulating toxins namely oxidative stress lipid, cytokines and glycation end-products[18,19]. The preceding information of altered vascular homeostasis concurs with the therapeutic resistance to vasodilators, as well as the progression of renal disease toward end-

Table 2 Follow-up value of intrarenal hemodynamic study in microalbuminuric diabetic kidney disease

	Pre-treatment	Post-treatment	*P* value
Renal function			
Creatinine clearance, mL/min per 1.73 m^2	73 ± 28	80 ± 37	NS
Fractional excretion of magnesium, %	4.1 ± 1	4.2 ± 2	NS
Microalbumin/creatinine ratio, μg/mg	170 ± 193	109 ± 148	NS
Mean arterial pressure, mmHg	99 ± 5	85 ± 14	< 0.05
Hemodynamics			
Renal plasma flow , mL/min per 1.73 m^2	505 ± 120	416 ± 9	NS
Peritubular capillary flow, mL/min per 1.73 m^2	423 ± 120	350 ± 13	NS
Glomerular filtration rate, mL/min per 1.73 m^2	82 ± 6	75 ± 9	NS
Afferent arteriolar resistance, dyne.s.cm^{-5}	2842 ± 299	3359 ± 1587	NS
Efferent arteriolar resistance, dyne.s.cm^{-5}	4045 ± 1168	4093 ± 53	NS

NS: Not significant.

stage renal disease in late stage diabetic kidney disease.

Recently, we had performed intrarenal hemodynamic study during pre-treatment and post-treatment period with vasodilators containing ACEI Enalapril 10-20 mg/d, ARB Telmisartan 40-80 mg/d ± calcium channel blocker in 29 microalbuminuric diabetic kidney disease patients. Following vasodilator treatment, progressive reductions in renal plasma flow, peritubular capillary flow and glomerular filtration rate were noted. In addition, a progressive increase in both afferent and efferent arteriolar resistances was also noted (Table 2). Such progressive change in intrarenal hemodynamics confirms the therapeutic resistance to vasodilators, and is in accordance with the altered vascular homeostasis observed in microalbuminuric diabetic kidney disease[17,18].

The preceding information of intrarenal hemodynamics observed in microalbuminuric diabetic kidney disease renders support that it would be appropriate to change the conceptual view of therapeutic strategy towards an early treatment of diabetic kidney disease during normoalbuminuria. Recent study of treatment with vasodilators during normoalbuminuric diabetic kidney disease has successfully restore renal perfusion and function indicating such therapeutic strategy at this early stage is under environment favourable for vascular repair and renal regeneration[8,18-23].

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BRIEF ARTICLE

A retrospective Aliskiren and Losartan study in non-diabetic chronic kidney disease

Keng-Thye Woo, Hui-Lin Choong, Kok-Seng Wong, Han-Kim Tan, Marjorie Foo, Fook-Chong Stephanie, Evan JC Lee, Vathsala Anantharaman, Grace SL Lee, Choong-Meng Chan

Keng-Thye Woo, Hui-Lin Choong, Kok-Seng Wong, Han-Kim Tan, Marjorie Foo, Grace SL Lee, Choong-Meng Chan, Department of Renal Medicine, Singapore General Hospital, 169608, Singapore
Fook-Chong Stephanie, Department of Clinical Research, Singapore General Hospital, 169608, Singapore
Evan JC Lee, Vathsala Anantharaman, Department of Nephrology, National University of Singapore, 169608, Singapore
Author contributions: Woo KT, Main author, coordinated study, recruited patients and preparation of manuscript; Choong HL helped in designing data bases and strategy, recruited patients and helped in writing; Wong KS involved in recruiting and participation in trial and helped in editing paper; Tan HK helped in recruiting, participation of trial , preparation of references and tables; Foo M helped in recruitment of patients and their treatment, preparation of paper; Stephanie FC, Statistician involved in design and analysis of data, writing of relevant part of paper; Lee EJC contributed patients and participated in treatment and follow of patients; Anantharaman V helped to recruit and treat patients, advised on paper; Lee GSL, Co-author and Co ordinator for patient recruitment, helped in editing of paper; Chan CM recruited and treatment of trial patients, editing manuscript.
Supported by Singhealth Cluster with IRB approval, CIRB Ref: 569E
Correspondence to: Keng-Thye Woo, Professor, Department of Renal Medicine Singapore General Hospital, Outram Road, 169608, Singapore. woo.keng.thye@sgh.com.sg
Telephone: +65-63266049 Fax: +65-62202308
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Abstract

AIM: To assess the efficacy of combined Aliskiren and Losartan *vs* high dose Losartan and Aliskiren alone in chronic kidney disease (CKD).

METHODS: This is a retrospective study of 143 patients with non-diabetic CKD comparing combined Aliskiren (150 mg/d) with Losartan (100 mg/d) therapy *vs* High dose Angiotensin receptor blockers (ARB) (Losartan 200 mg/d) and the third group Aliskiren (150 mg/d) alone. This study involved only patient medical records. Entry criteria included those patients who had been treated with the above drugs for at least 36 mo within the 5 years period; other criteria included proteinuria of 1 g or more and or CKD Stage 3 at the start of the 36 mo period. The study utilised primary renal end points of estimated Glomerular Filtration Rate (eGFR) < 15 mL/min or end stage renal failure.

RESULTS: Patients treated with high dose ARB compared to the other two treatment groups had significantly less proteinuria at the end of 36 mo ($P < 0.007$). All 3 groups had significant reduction of proteinuria ($P < 0.043$, $P < 0.001$). Total urinary protein was significantly different between the 3 groups over the 3-year study period ($P = 0.008$), but not eGFR. The changes in eGFR from baseline to each year were not significantly different between the 3 therapeutic groups ($P < 0.119$). There were no significant differences in the systolic and diastolic blood pressure between the 3 drug groups throughout the 3 years. The incidence of hyperkalemia (> 5.5 mmol/L) was 14.2% (7/49) in the Combined Aliskiren and ARB group, 8.7% (4/46) in the Aliskiren alone group and 6.3% (3/48) in the High dose ARB group ($P < 0.001$).

CONCLUSION: This study in non-diabetic CKD patients showed that Combination therapy with Aliskiren and ARB was effective but was not safe as it was associated with a high prevalence of hyperkalaemia.

Key words: Aliskiren; Chronic kidney disease disease; Clinical trial

Core tip: The Aliskiren trial in type 2 diabetes using Cardio-Renal Endpoints (ALTITUDE) study was able

to unmask serious adverse events like ischemic heart disease and strokes because it had included Cardio-Renal Endpoints among its primary end points. It may be advisable to require future trials on drugs which could impact on the kidneys, heart and brain to have similar Cardio-Renal Endpoints or Cardio-Neuro-Renal End points to further ensure therapeutic safety of the trial drug. Our modest study compared to the magnitude of the ALTITUDE study still managed to detect the problem of hyperkalaemia in the group treated with Combination therapy with Aliskiren and ARB. Based on our study it would appear that the findings of the ALTITUDE study would also apply to non-diabetic Chronic Kidney Disease patients.

Woo KT, Choong HL, Wong KS, Tan HK, Foo M, Stephanie FC, Lee EJC, Anantharaman V, Lee GSL, Chan CM. A retrospective Aliskiren and Losartan study in non-diabetic chronic kidney disease. *World J Nephrol* 2013; 2(4): 129-135 Available from: URL: http://www.wjgnet.com/2220-6124/full/v2/i4/129.htm DOI: http://dx.doi.org/10.5527/wjn.v2.i4.129

INTRODUCTION

One of the important strategies in the treatment of chronic kidney disease (CKD) is the use of Angiotensin converting enzyme inhibitors Angiotensin-converting enzyme inhibitors (ACEI) and Angiotensin receptor blockers (ARBs) to reduce proteinuria as well as to retard the progression to end stage renal failure[1,2]. ACEI and ARBs compete with the receptor for angiotensin and therefore inhibit the action of angiotensin. However one of the concerns in the use of these agents is the long term side effects of progressive renal fibrosis with worsening of estimated Glomerular Filtration Rate (eGFR)[3]. ACEI and ARBs indirectly cause renal fibrosis as they also promote the increase of aldosterone which causes renal fibrosis[4]. Because renin is not converted to angiotensin, there is a build- up of renin in patients on ACEI and ARB.

Aliskiren is a new renal protective agent that inhibits renin, the rate limiting step in the renin angiotensin aldosterone system[5]. In both healthy volunteers and disease states, aliskiren reduces angiotensin Ⅱ levels and plasma renin activity (PRA), without stimulating compensatory increases in PRA, angiotensin I and angiotensin Ⅱ as seen with ACEI and ARB. Aliskiren allows for total blockade of the renin angiotensin system and its beneficial effect is independent of blood pressure (Bp) control[6].

In our recent paper on the beneficial effects of long term high dose ARB therapy in patients with IgA nephritis over a period of 6 years[7], we showed that high dose ARB is more efficacious in reducing proteinuria and preserving renal function in terms of earlier and more effective improvement in eGFR in those treated with high dose ARB compared to those on normal dose ARB and ACEI. We believe that high dose ARB does so by causing regression of glomerular sclerosis[8]. Busch *et al*[9] have also reported the beneficial effects of high dose Irbesartan in patients with diabetic nephropathy.

The present study examines the effects of ARB (Losartan 100 mg/d) combined with Aliskiren (150 mg/d), Aliskiren (150 mg/d) alone and High dose ARB (Losartan 200 mg/d) in patients with Chronic Kidney Disease (Chronic Glomerulonephritis). We compared the therapeutic efficacy between High dose ARB (Losartan 200 mg/d) versus Combined Aliskiren (150 mg/d) with Losartan (100 mg/d) and the third group Aliskiren alone (150 mg/d to determine whether a Combined dose of Aliskiren and Losartan is as effective as High dose Losartan therapy and whether it confers additional renoprotective effects.

MATERIALS AND METHODS

In a database comprising 312 patients with Chronic Kidney Disease, 143 patients with CKD due to Chronic Glomerulonephritis and not due to diabetic nephropathy, hypertensive nephrosclerosis, lupus nephritis or Henoch Schonlein nephritis were recruited for the study. Data of these 312 patients from 2007 to 2012 were examined for the purpose of a retrospective study. Non biopsied CKD patients formed the bulk of our clinical practice and were more readily recruited. For purposes of standardisation of the study, we decided to recruit only non-biopsied patients into the study. In this new database for the purpose of this study, the database of 143 patients were selected, among which 49 patients were treated with combination therapy using Aliskiren and Losartan, 46 patients were treated with Aliskiren alone and the remaining 48 patients were treated with High Dose Losartan alone. This was a retrospective study involving only patient medical records. Entry criteria included those patients who had been treated on the above drugs for at least 36 mo within the 5 years period; other criteria included proteinuria of 1 gram or more and or CKD Stage 3 at the start of the 36 mo period. There were no significant differences in the various parameters between the 3 groups on entry into the study (Table 1). All selected patients had adequate control of Bp control which was achieved with addition of atenolol, amlodipine or nifedipine.

Study design

All 143 patients in the database had the following investigations documented at six monthly intervals: serum creatinine, eGFR and total urinary protein (TUP). Serum creatinine was quantitated with alkaline picrate and TUP was quantitated by biuret agent. Glomerular Filtration Rate was estimated using the Cockcroft Gault formula for eGFR[10]. Decrease in eGFR was expressed as mililiter of eGFR loss per year over the 3 year duration from time of entry to exit of the trial. Improvement in eGFR was taken as the positive difference between the entry eGFR and the exit eGFR over the study period. End stage renal failure was equated with decline of eGFR to CKD stage

Table 1 Comparing demographic and clinical profile of patients treated with combined dose Aliskerin and Angiotensin receptor blockers, Aliskerin alone and high dose Angiotensin receptor blockers

	Combined Aliskerin and ARB	Aliskerin	High dose ARB	[1]*P* value
	n = 49	*n* = 46	*n* = 48	
Sex (F : M)	16:33	22:24	23:25	NS
Age at biopsy (yr)	58 ± 12	57 ± 12	60 ± 11	NS
Duration of Trial (mo)	37 ± 1	36 ± 1	36 ± 1	NS
Hypertension (Yes : No)	16:15	15:31	17:31	NS
Serum Creatinine (μmol/L)				
Baseline	142 ± 39	142 ± 40	139 ± 32	NS
Year 3	159 ± 53	161 ± 54	150 ± 47	NS
	(*P* = 0.001)	(*P* < 0.001)	(*P* = 0.015)	
eGFR (mL/min)				
Baseline	49 ± 19	48 ± 14	48 ± 14	NS
Year 3	44 ± 18	42 ± 15	45 ± 15	NS
	(*P* < 0.001)	(*P* < 0.001)	(*P* = 0.002)	
Decrease in eGFR (mL/min per year)	1.8 ± 2.5	2.0 ± 3.0	1.0 ± 2.1	*P* = 0.119
Urinary Protein (gm/d)				
Baseline	0.7 ± 0.6	0.8 ± 0.9	0.6 ± 0.7	NS
Year 3	0.5 ± 0.8	0.6 ± 0.7	0.3 ± 0.3	*P* = 0.007
	(*P* = 0.002)	(*P* = 0.043)	(*P* < 0.001)	
Blood Pressure (mmHg)				
Systolic before	132 ± 12	135 ± 10	132 ± 12	NS
Systolic after	127 ± 10	130 ± 9	128 ± 11	NS
	(*P* < 0.001)	(*P* < 0.001)	(*P* < 0.001)	
Diastolic before	84 ± 7	85 ± 6	84 ± 7	NS
Diastolic after	82 ± 5	83 ± 6	83 ± 6	NS
	P = 0.040	*P* = 0.084	*P* = 0.361	
Distribution of CKD at baseline				
CKD 1	1	0	0	0.465
CKD 2	8	5	4	
CKD 3	40	41	44	
Distribution of CKD at year 3				0.606
CKD 1	1	0	0	
CKD 2	8	7	5	
CKD 3	28	26	34	
CKD 4	12	13	9	
Non-ESRF	49	46	48	NS
ESRF	0	0	0	
Improvement in eGFR				
Yes	12	15	15	0.645
No	37	31	33	

[1]*P* value either from chi-square test or analysis of variance test comparing the 3 groups. M: Male; F: Female; IQR: Interquartile Range, 25th percentile-75th percentile, continuous data are presented as mean ± SD or median (IQR) and categorical data as count (%); eGFR: estimated Glomerular Filtration Rate; CKD: Chronic Kidney Disease; ARB: Angiotensin receptor Blockers; ESRF: End-stage renal failure; NS: No significant.

5 with eGFR less than 15 mL/min per year. The primary end points were stage 5 CKD or end stage renal failure. The secondary end points were reduction of proteinuria by 50% and change in eGFR. Of the 143 patients, 49 patients were treated with combination therapy using Aliskiren and Losartan, 46 patients were treated with Aliskiren alone and the remaining 48 patients were treated with High dose ARB (Losartan alone). Entry criteria for the study included proteinuria of 1 gram or more and or CKD Stage 3. There were no significant differences in the various parameters between the 3 groups on entry into the study. The records showed that additional Bp control was achieved with atenolol, amlodipine and nifedipine

Sample size

Sample size calculation was based on the proportion of patients achieving 30% decrease in TUP. A second sample size calculation was done to compare the rate of 30% TUP decrease between High dose ARB and Aliskiren alone. Assuming that the rate of TUP decrease to be 30% in the Normal dose ARB and Normal dose Aliskiren and 60% in the High dose ARB, the number of patients required in each group was 49 for a 2-sided test with $\alpha = 0.05$ and power of 80%. As we expected High dose ARB to be even more efficacious, 50% reduction of TUP was chosen. We expected the effects of combination dose of ARB plus Aliskiren to be about the same as that of High dose ARB.

Statistical analysis

SPSS 10.1 for Windows was used for all analysis. Results were expressed as mean ± SD or median (range) or count (%). For univariate analysis, Pearson's χ^2 test was used for comparing categorical data and analysis of variance (ANOVA) for comparing numeric data between the 3 treatment arms. ANOVA was followed by multiple comparison with Student-Newman-Keuls (SNK) range test whenever statistical significance was found between the 3 arms. Next, Multivariate Analysis of Variance (MANOVA) with repeated measures was used to test the effect of drug treatment on both eGFR and total urine proteinuria (TUP). The dependent variables were eGFR and TUP measured at 4 time points, namely baseline and thereafter every year of the 3 years of the study. The between-subject factor was treatment group with 3 levels corresponding to Combination dose of ARB and Aliskiren, Aliskiren alone and high dose ARB. Adjustment was made for the covariates of average systolic Bp and average diastolic Bp. Average blood pressures were calculated by taking the mean of all blood pressures while on medication (mean of blood pressures from 1-3 year). Within MANOVA, the effect of high dose ARB on the outcomes of eGFR and TUP was compared with each of the other drug dosage groups by simple contrast comparison testing. Similarly, repeated contrast testing was done to obtain and compare the loss in eGFR in each year between the various drug groups.

Plots of mean values of eGFR and TUP adjusted for covariates of systolic Bp and diastolic Bp were presented. So were the contrast estimates, their corresponding 95%CI and *P* values for the comparison of eGFR and TUP between the levels of interest of the treatment group. Results of 2 types of comparison, change in eGFR and TUP from baseline to each time point compared between the 3 drug groups and loss in eGFR compared between the 3 drug groups were presented in a table with the *F* statistics and *P* values.

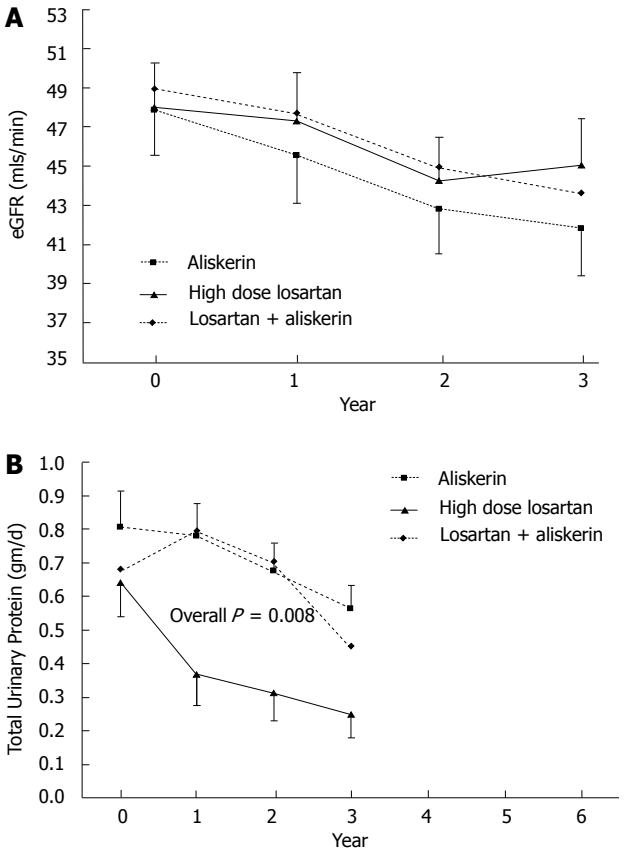

Figure 1 Mean estimated glomerular filtration rate and mean total urinary protein with its standard error for the 3 treatment groups. A: Mean estimated Glomerular Filtration Rate; B: Mean Total Urinary Protein. eGFR: Estimated glomerular filtration rate; TUP: Total urinary protein; SE: Standard error.

RESULTS

Table 1 compares the eGFR, proteinuria and decrease in eGFR between Combination dose of Aliskiren and ARB, Aliskiren alone, and high dose ARB before and after the trial. High dose ARB had significantly higher eGFR and less proteinuria at the end of the trial compared to the other 2 treatment groups by *post-hoc* Student-Neuman-Keuls range test. However, there was no significance difference in these parameters between the 2 other groups by the same SNK range test. The decrease in eGFR was 0.98 mL/min per year in the high dose ARB group compared to 1.77 and 2.02 mL/min per year in the Combined Aliskiren and ARB group and the group on Aliskiren alone respectively ($P < 0.119$). There were 15 patients out of 48 (31.3%) who had improved eGFR at the end of the study in the high dose ARB group compared to 12 out of 49 (24.4%) in Combined Aliskiren and ARB group and 15 out of 46 patients (32.6%) in the group on Aliskiren alone ($P < 0.645, \chi^2 = 645$). There were no patients with ESRF at the end of the study in all the 3 groups. There tended to be fewer patients with CKD4 in the high Dose ARB compared to the other 2 treatment arms at the end of the study (High dose ARB *vs* Combined Aliskiren and ARB *vs* Aliskiren alone: 18.8% *vs* 24.5% *vs* 28.3%, $P = 0.55, \chi^2 = 1.199$, Table 1).

Table 2 Test of within-subject contrast (change from baseline and change from previous year) for estimated glomerular filtration rate and total urinary protein

Variable	Measure	Year	*F*	*P* value
Change from baseline Year1 treatment	eGFR	year 1 *vs* baseline[1]	0.63	0.530
		year 2 *vs* baseline	0.46	0.632
		year 3 *vs* baseline	2.16	0.119
	TUP	year 1 *vs* baseline	6.00	0.003
		year 2 *vs* baseline	4.16	0.018
		year 3 *vs* baseline	0.90	0.409
Change from previous year Year1 treatment	eGFR	year 1 *vs* baseline[1]	0.63	0.536
		year 2 *vs* year 1	0.03	0.967
		year 3 *vs* year 2	2.93	0.057
	TUP	year 1 *vs* baseline	6.01	0.003
		year 2 *vs* year 1	0.16	0.851
		year 3 *vs* year 2	3.11	0.048

[1]Each row of the table tests the change from baseline to that time between the 4 groups. eGFR: Estimated glomerular filtration rate; TUP: Total urinary protein.

From the results of the multivariate ANOVA with repeated measures, TUP ($F_{2140} = 5.041$, $P = 0.008$, Figure 1A) was significantly different between the 3 groups over the 3-year study period, but not eGFR ($F_{2140} = 0.194$, $P = 0.824$, Figure1B). Over the whole study period, TUP for combined Aliskiren and ARB group (contrast estimate = 0.26, 95%CI = 0.06, 0.47, $P = 0.013$) and Aliskiren alone group (contrast estimate = 0.31, 95%CI = 0.10, 0.52, $P = 0.004$) were significantly higher than for high dose ARB (Table 2).

The changes in eGFR from baseline to each year were not significantly differently between the 3 therapeutic groups (Figure 1A, Table 2). For TUP, ARB high dose distinctively showed a bigger drop from baseline value, compared to the rest at years 1 and 2 (Figure 1B, Table 2). There was a trend towards improvement in eGFR from year 2 to year 3 in High dose ARB group as opposed to Combined Aliskiren and ARB group, and Aliskiren alone group which both showed a drop in eGFR (Figure 1A, $P = 0.057$ as seen in Table 2). This is also shown in Figure 2 which depicted the yearly loss of eGFR (loss from previous year) in the various groups. For TUP the yearly changes were significantly higher in high dose ARB group compared to Combined Aliskiren and ARB group and Aliskiren alone group (Figure 1B, Table 2). The Bp levels, Systolic and Diastolic over the 3 years for the 3 drug groups are displayed graphically in Figure 3A and 3B respectively. There were no significant differences between the 3 drug groups throughout the 3 years.

The incidence of hyperkalaemia (> 5.5 mmol/L) was 14.2% (7/49) in the combined Aliskiren and ARB group, 8.7% (4/46) in the Aliskiren alone group and 6.3% (3/48) in the high dose ARB group ($P < 0.001$). For hyperkalaemia ⩾ 6.0 mmol/L it was 4.1% (2/49) in the Combined Aliskiren and ARB group, 0% (0/46) in the Aliskiren alone group and 2.1% (1/48) in the High dose ARB group ($P < 0.07$).

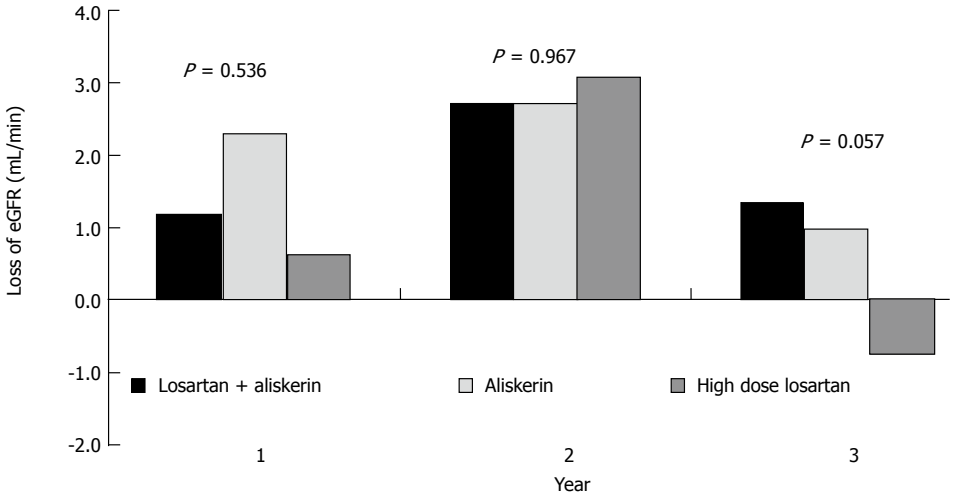

Figure 2 Loss of estimated glomerular filtration rate in each year. eGFR: Estimated Glomerular Filtration Rate.

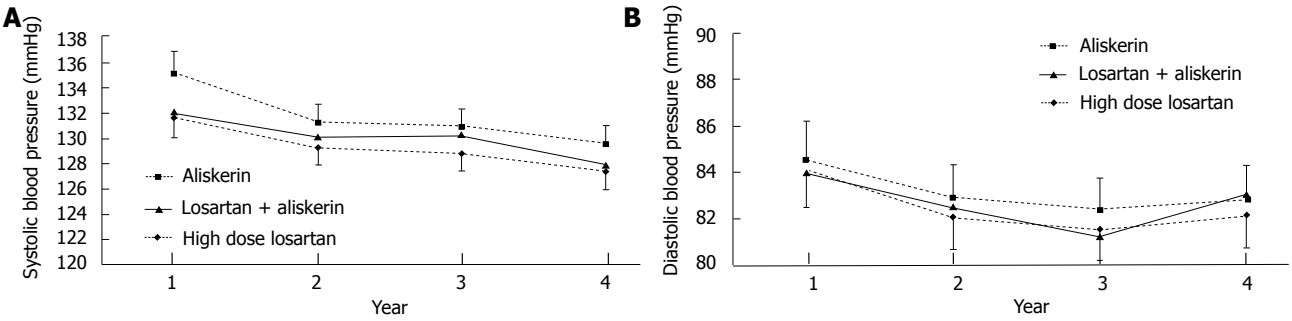

Figure 3 Mean systolic blood pressure and diastolic blood pressure with its standard error for the 3 treatment groups. A: Systolic Bp; B: Diastolic Bp. SE: Standard error; Bp: Blood pressure.

DISCUSSION

The above data from our study showed that among patients with non-diabetic CKD, those treated with High dose ARB tended to have higher eGFR and less proteinuria at the end of the trial compared to the other 2 treatment groups on Combined Aliskiren and ARB and those on Aliskiren alone. The decrease in eGFR in the high dose ARB group tended to be less compared to the Combined Aliskiren and ARB group and the group on Aliskiren alone respectively though this difference was not significant ($P < 0.119$). Multivariate analyses showed no confounding factors to account for the above differences. The Bp of all 3 drug groups showed no significant differences and did not influence any of the above data.

This retrospective study was initiated because of the recent early termination of the Aliskiren trial in type 2 diabetes using Cardio-Renal Endpoints (ALTITUDE)[11] as the results showed that there was no benefit with Aliskiren and that there were more cases of stroke, renal complications, hyperkalaemia and hypotension in patients who received Aliskiren compared with patients who received a placebo.

In view of futility of meeting the final endpoint and the substantial safety concerns through the preliminary analyses of the interim results, the Data Monitoring Committee recommended that all subjects cease treatment with Aliskiren. Additional analyses from ALTITUDE by Norvatis are ongoing[12].

To date, the Health Science Authority (HSA) of Singapore[13] has received 14 suspected adverse reaction reports associated with the use of Aliskiren, of which 4 involved CV events (1 case of hypotension, myocardial infarction and stroke and 3 cases of hypotension). HSA has recommended that Aliskiren or Aliskiren combination with ACE Inhibitors (ACEI) or ARBs should not be used in Diabetics or patients with severe renal failure (eGFR < 30 mL/min)[13].

Parving *et al*[14] in 2008, published the results of a double blind randomised controlled trial of Aliskiren combined with Losartan in 599 patients with type 2 Diabetes with nephropathy (AVOID Study) over a 6 mo period. Patients on the Aliskiren arm ($n = 301$) were prescribed a dose of Aliskiren of 150 mg/d for 3 mo and then increased to 300 mg/d for the next 3 mo in combination with Losartan 100 mg/d. There were 298 patients in the placebo arm. The primary outcome was a reduction in the ratio of albumin to creatinine, measured in an early morning urine sample at 6 mo. The results of the study showed that the decline in eGFR was the same in the treatment

and placebo group but the decline in the treatment group tended to be less than in the placebo group at 6 mo. The reduction of albuminuria by 50% occurred twice as often in the treatment group compared to the placebo group. The authors concluded that Aliskiren appeared to have a renoprotective effect independent of its Bp lowering effect in patients with type 2 diabetes who were receiving maximal renoprotective treatment and optimal antihypertensive therapy. Hyperkalamia, based on a single measurement of serum potassium > 5.5 mmol/L was more frequent in the Aliskiren treated group (41/301 or 13.7%) compared with the placebo group (32/298 or 10.8%) (P = 0.07). Severe hyperkalaemia (serum potassium > 6.0 mmol/L) occurred in 14 patients in the Aliskiren treated group (4.7%) compared to 5 placebo treated patients (1.7%) (P = 0.113). Symptoms of hypotension were not a frequent adverse event, with no difference in the 2 groups. Parving *et al*[14] in a post hoc analysis of Parving's AVOID trial[14] concluded that Aliskiren added to Losartan reduced albuminuria and renal dysfunction and was well tolerated, except for hyperkalaemia in stage 3 CKD patients, independent of baseline CKD stage in patients with type 2 diabetes, hypertension and nephropathy[15].

In an open labelled pilot study by Tang *et al*[16] in 25 consecutive patients where Aliskiren (300 mg/d) was prescribed despite being on maximum ARB therapy with Losartan (100 mg/d) for 3 mo in patients with IgA nephropathy (stage 3 CKD with proteinuria > 1 mg/d) over a 12 mo period, there was a 22% reduction in proteinuria at 6 mo and 26% reduction at 12 mo. This was associated with significant reductions in plasma renin activity, serum interlukin-6 and transforming growth factor β levels compared to baseline levels. Two patients developed mild allergic reactions and 6 (24%) patients had transient hyperkalaemia (serum K^+ > 5.5 mmol/L). The authors concluded that Aliskiren conferred an antiproteinuric effect in patients with IgA nephropathy with significant residual proteinuria, despite receiving the recommended renoprotective treatment.

In a systemic review and meta-analyses of aliskiren and angiotensin receptor blockers in the management of essential hypertension, 7 randomised controlled trials, duration of follow up for at least 4 wk by Zheng *et al*[17] , no differences were found between the two groups.

The trials of Uresin *et al*[18] and that of Oparil *et al*[19] , gave no indication regarding the renoprotective efficacy of Aliskiren, the initial trials of Aliskiren involved patients with hypertension, being first developed as an antihypertensive agent. They were short trials in hypertensive patients[20,21] lasting 8 wk and did not address the question of renoprotection.

The ALTITUDE study was able to unmask serious adverse events like ischemic heart disease and strokes because it had included Cardio-Renal Endpoints among its primary end points.

It would be advisable to require future trials on drugs which could impact on the kidneys, heart and brain to have similar Cardio-Renal Endpoints or Cardio-Neuro-Renal End points to further ensure therapeutic safety of the trial drug.

In conclusion, our present study in 143 patients with CKD over 3 years showed that the use of Combination therapy of Aliskiren with ARB Losartan or Aliskiren alone were efficacious as an antiproteinuric drug when compared to High dose Losartan. But like Parving[14], our study showed that the incidence of hyperkalaemia (> 5.5 mmol/L) was 14.2% in the Combined Aliskiren and ARB group, 8.7% in the Aliskiren alone group and 6.3% in the High dose ARB group (P < 0.001).

The problem of hyperkalaemia was 36.9% in the Aliskiren group versus 27.1% in the placebo group in the ALTITUDE study. Parving's AVOID study[14] showed that patients on combined Aliskiren and ARB had 13.7% with Hyperkalaemia > 5.5 mmol/L compared to 10.8% for Placebo group and 4.7% and 1.7% respectively for serum $K^+ \geqslant$ 6mmol/L (P < 0.113). Like Parving[14], all our patients had stage 3 CKD but we were only using 150 mg/d of Alisikiren compared to 300mg/d in Parving's study[14].

Our modest study compared to the magnitude of the ALTITUDE study still managed to detect the problem of hyperkalaemia in the group treated with Combination therapy with Aliskiren and ARB like those of Parving[14] and Tang's[16]. Based on our study it would appear that the findings of the ALTITUDE study would also apply to non-diabetic CKD patients.

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COMMENTS

Background

For the future, Aldosterone blockade and the mineralocorticoid receptor antagonist would probably be the new emerging therapy in the management of Chronic Kidney Disease (CKD). Hitherto, the major therapeutic intervention to delay progression of CKD and the risk of endstagerenaldisease has been the use of Angiotensin-Converting Enzyme Inhibitors/Angiotensin receptor Blockers and more recently the use of direct renin inhibitors. But in The authors' opinion, the release of the Trial in Type 2 Diabetes using Cardio-Renal Endpoints report may have effectively tolled the bell for the use of Combination therapy involving Aliskiren as renin inhibitors for now.

Research frontiers

The authors have used Aliskiren in a dose of 150 mg/d for the treatment of CKD patients with proteinuria. The maximum therapeutic dose is 300 mg/d. Perhaps, if the authors had used Aliskiren in a dose of 300 mg/d, the effects would have been better and comparable to high dose Angiotensin receptor blockers (ARB) therapy. However, the authors would have to contend with the major side effect of hyperkalaemia which is a disadvantage of combination therapy of Aliskiren and ARB.

Applications

Treatment of Patients with CKD with proteinuria. The authors believe that Aliskiren, by itself is still an effective and innovative therapy for treatment of hypertension. It was first introduced as an anti- hypertensive drug and still is widely used as such.

Terminology

Aliskiren, a direct renin inhibitor, hypertensive drug. Also renoprotective as it reduces proteinuria, but major side effect of hyperkalaemia.

Peer review

This retrospective study has its limitations. It is not a randomised controlled trial and though the statistics show that it is adequately powered, the number of patients entered in the study are small. The dose of Aliskiren employed in most studies is 300 mg/d. Here the investigators have chosen a dose of 150 mg/d. Perhaps with a dose of 300 mg/d, Aliskiren may prove to be more efficacious.

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and position-

ing tool assembly. United States patent US 20020103498. 2002 Aug 1

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