The pathogenesis of developmental and acquired renal abnormalities in paediatric refluxive and obstructive disease

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THE PATHOGENESIS OF DEVELOPMENTAL AND ACQUIRED RENAL ABNORMALITIES IN PAEDIATRIC REFLUXIVE AND OBSTRUCTIVE DISEASE

DE PATHOGENESE VAN PRIMAIRE EN VERWORVEN NIERAANDOENINGEN IN REFLUXIEVE EN OBSTRUCTIEVE AANDOENINGEN OP DE KINDERLEEFTIJD

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To my mother and my father

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chapter 1 Introduction

1.1 The Kidney, its Normal Development and Pathology in Refluxive and Obstructive Disease of the Urinary Tract.

1.1.1. The development of the kidney and its relation to the urinary tract as a whole

The nephron, the functional unit of the human excretory system, is similar in its essential features in all vertebrate classes from cyclostomes to mammals (Torrey, 1965). Differences between classes are created mainly by the spatial organisation of nephrons within the organism and it has therefore become customary to distinguish three spatially and temporally different excretory organs: the pronephros, the mesonephros, and the metanephros.

1.1.1.1. The pronephros

In man the pronephros, which appears around the 22nd day of development (Hoar & Monie, 1981), is a transitory, nonfunctional structure resulting from the canalization of the 7th to 14th nephrotomes. Development of the pronephros into a functional organ is restricted to the lower vertebrates, however this structure is of significance in man as the proximal pronephric tubules fuse to form the mesonephric duct. This duct plays a pivotal role in subsequent development of the excretory system since, in addition to draining the mesonephros and giving rise to the ureteric bud of the metanephros, the epithelium of the nephric duct and its derivatives determine the differentiation and morphogenesis of the mesonephric and metanephric mesenchymal blastema by acting upon them as an "inductor" (1.1.1.6.) (Saxen, 1987a).

1.1.1.2. The mesonephros

The mesonephros in man appears around the 24th day (Hoar & Monie, 1981), caudal to the rudimentary pronephros (Toivonen, 1945). Although larger and consisting of more numerous and complicated tubules than the pronephros, there is no direct proof of the functional maturity of human mesonephric nephrons. By the end of the 16th week (Maizels, 1986) the mesonephros has involuted and disappeared, except for its duct and a few tubules which persist as genital ducts in males or form vestigial remnants in females.

1.1.1.3. The metanephros

The metanephros, the definitive human kidney, is derived from the mesenchymal nephrogenic blastema and the epithelial, originally mesoderm-derived, nephric or Wolffian duct (Jokelainen, 1963; Kazimierczak, 1971). Development commences around day 28 (Hoar & Monie, 1981) when the ureteric bud emerges from the most caudal part of the Wolffian duct and grows dorsally toward the caudal portion of the nephric cord. Having invaded the mesenchymal blastema and induced a longitudinal condensate of mesenchyme, the ureteric bud starts to divide in a dichotomous fashion. Ramification of the ureteric ampullae is associated with concomitant splitting of the mesenchymal condensate and this continuous branching and mesenchymal fractionation ultimately results in the formation of distinct nephric vesicles, the first indication of metanephric nephrons. In this regard, recent observations of time-lapse motion pictures obtained from mouse metanephric kidney "anlagen" cultured in vitro as three-dimensional whole-organ explants (Saxen et al., 1965a) have contributed greatly to an increased understanding of the first developmental changes in the metanephrogenic mesenchyme following the invasion of the ureteric bud.

The elucidation of metanephric development was greatly facilitated by the microdissection work of Osathanondh and Potter (1963a, 1963b, 1963c). Their technique, an improvement of previous methods (Peter, 1927; Oliver, 1939; Darmady & Stranack, 1957), permitted the microscopical

examination of individual nephrons obtained from the fractionated microdissection of complete (acid-macerated) embryonic kidneys. From these extensive studies it became apparent that the ultimate architecture of the collecting system reflects the balance between elongation and further ramification of individual ampullae. Indeed, the division of metanephric development into four periods (1.1.1.4.) is based on the differing temporal characteristics of the ampullary portions of the ureteral bud branches.

1.1.1.4. The four periods of nephron formation within the metanephros
(after Osathanondh and Potter, 1963c)

Period One (5th - 14/15th week)

During this period, successive dichotomous ramification of the ampullae produces those branches which elaborate into renal pelvis, calyces, and most of the collecting tubules. This first stage of branching leads to separate cells of blastema, merging later, with some remaining fetal lobulation, into a single kidney. The first renal vesicles, the prospective secretory nephrons, arise next to the ampullae and connect to the collecting epithelium in its zone of active growth. With subsequent development of the collecting system these nephrons are carried deeper into the nephric mesenchyme, always remaining attached to the ampullae of the terminal branches of the collecting tubules (Figure 1-1). Urine production commences around the 9th week of gestation (Maizels, 1986) and continues actively throughout fetal life. As no animal models of urinary obstruction in this early phase are available, the precise relation between production and deficient drainage of urine and the different forms of (cystic) renal dysplasia, of both hereditary and nonhereditary kind, is not fully understood.

Period Two (14/15th - 20/22nd week)

During this period the ampullae, although only seldom branching, become capable of inducing new nephrons even though already having one nephron attached. New nephrons thus become arranged in arcades with only the most recently formed being in direct communication with the ampullae (Figure 1-2). Individual arcades consist of 4-7 nephrons and contribute the glomeruli of the inner half of the cortex in the fully developed kidney.

Period Three (20/22nd - 32/36th week)

During this period the ampullae almost never branch. They advance beyond the point of arcade attachment, inducing a set of 5-7 terminal, subcapsular nephrons which are directly attached to the collecting duct. This period is therefore responsible for the formation of the terminal, unbranched portion of the collecting tubules and the associated nephrons whose glomeruli lie within the outer half of the cortex.

Period Four (32/36th - adult life)

The fourth period commences with the disappearance of the ampullae. No further branching of collecting tubules or induction of nephrons occurs; changes in this period solely reflect interstitial growth and differentiation.

The most common arrangement of nephrons at birth is shown in Figure 1-3.

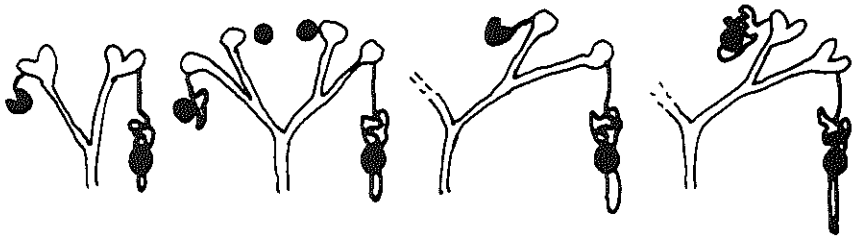

Figure 1-1. Figure showing formation of nephrons during Period One. As the ampullae divide and form successive generations of collecting tubules, the attached nephrons advance with the ampullae.

1.1.1.5. Nephron formation

In marked contrast to the temporally-dependent characteristic of the ampulla and its associated development of the collecting system, formation of individual nephrons follows a remarkably constant pattern. Accessible literature reviews on this topic include those by Potter (1965), Evan et al. (1984), and Saxen (1987b).

Early tubulogenesis has been extensively documented from light microscopy of semi-thin sections. During the early stage of vesicle formation, the polarised nephrogenic cells proliferate vigorously (Jokelainen, 1963). Following this "growth phase", the first diversification of cells occurs resulting in the "comma-shaped anlage" (Jokelainen, 1963; Dorup & Maunsbach, 1982; Saxen, 1984a). Formation of two slits at opposite poles precedes development of the classical S-shaped structure and its connection to the collecting system. Although formation of the S-shaped body has generally been considered the result of differential growth of the tubular renal vesicle, observations using time-lapse cinematography strongly suggest formation of the slit by cell detachment in situ (Saxen et al., 1965a). To this end, a hypothesis on the development of the S-shaped body, based on an assumption of a postinductory, gradually increasing intercellular affinity of the pretubular mesenchymal cells has been proposed (Saxen & Wartiovaara, 1966; Saxen, 1970a).

After establishing the communication between the lumen of the ampulla and the lumen of the developing nephron, the upper and middle limbs of the S-shape form the tubular portion of the nephron. In contrast, the lower limb broadens and becomes concave with its lower component developing into Bowman's capsule (Osathanondh & Potter, 1963c). The implications of the temporal dissociation between the development of filtration capability and the other nephron functions have not been fully elucidated.

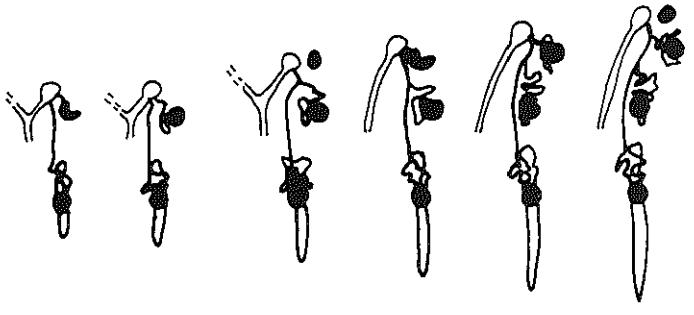

Figure 1-2. Figure showing formation of nephron arcade during Period Two. The ampullae are now capable of inducing new nephrons despite already having one nephron attached.

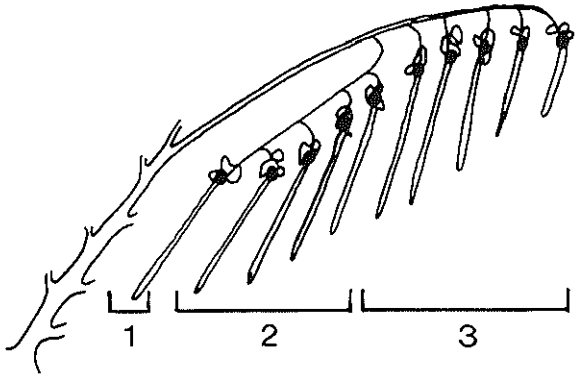

Figure 1-3. Figure showing the most common arrangement of nephrons at birth. 1: Nephron formed during Period One. 2: Arcade of nephrons formed during Period Two. 3: Nephrons formed during Period Three.

1.1.1.6. Control of morphogenesis

Development of the two major cell lineages of the metanephros, the branching epithelium of the ureter and the mesenchyme converted into epithelial elements, occurs in a strictly controlled, temporally and spatially synchronous manner (Saxen, 1987c). This is believed to result from two interacting mechanisms: an organismal control system directing development of both components, and local interaction of these components by their exchanging of signals. Although embryology has long postulated that the ureteric bud induces the formation of nephrons, studies have suggested that competent metanephrogenic tissue may be important for ureteric bud branching (Saxen & Lehtonen, 1978; Cunha et al., 1983). A detailed description is beyond the scope of this thesis, however reviews are available (Lehtonen & Saxen, 1986; Saxen & Lehtonen, 1986; Bard, 1992; Bard & Woolf, 1992). In essence, the changes within nephrogenic mesenchymal cells occurring in response to an inductive stimulus can be divided into three main types (Saxen, 1987d):

(1) stimulation of the DNA synthesis and proliferation of the target cells,

(2) degradation of the interstitial-type proteins from the extracellular matrix, and

(3) enhanced (or neo-) synthesis of epithelial-type proteins of the extracellular matrix and the cytoskeleton.

Indeed recent data suggests that postinductive nephrogenesis may be regulated by the overall balance of a number of local autocrine and/or paracrine growth factor systems (Fouser & Avner, 1993); platelet-derived growth factor and insulin-like growth factor have been specifically implicated (Chin & Bondy, 1992; Daniel & Kumjian, 1993).

However, the kidney does not develop in isolation and a close relation exists with the development of the lung, perhaps based on the role of the kidney as a producer of proline (Clemmons, 1977; Hislop et al., 1979).

Table 1-1. **Syndromes associated with cystic dysplasia.**

Autosomal recessive	**Autosomal dominant**
Meckel syndrome	tuberous sclerosis
Jeune syndrome	v. Hippel-Lindau syndrome
Zellweger syndrome	Branchio-oto-renal syndrome
Short rib polydactyly syndromes	
Retina renal dysplasia syndromes	
Ivemark syndrome	
Fryns syndrome	
Asphyxiating thoracic dystrophy syndrome	
Laurence-Moon-Bardet-Biedl syndrome	
Kaufman-Mckusick syndrome	
Roberts syndrome	
Smith-Lemli-Opitz syndrome	
Variable	**X-linked**
DiGeorge syndrome	Oral-facial-digital syndrome I
Ehlers-Danlos syndrome	
Lissencephaly syndrome	
Questionable / not inherited	
VATER association	
Hypothalamic hamartoma syndrome	
Prune belly syndrome	

At present knowledge on renal development is mainly based on (microscopical) structural analysis. With the exception of Adult and Infantile Type Polycystic Kidney Disease, no (single) gene defects or defects of expression are known which result primarily in renal developmental abnormalities. Many renal malformations are associated with or occur within syndromes (Crawfurd, 1988).

The syndromes which are associated with cystic dysplasia can be summarised as above (Table 1-1) (after Zerres, 1987;1989; Zerres et al., 1984).

Although renal dysplasia has occasionally been described in defined forms of chromosomal disorder (Carter et al., 1969; Mulcahy et al., 1974; Shokier et al., 1975; Ying et al., 1982), the association is not strong (Crawfurd, 1988).

The forms of dysplasia seen in developmental disorders can, using a histopathological approach, be roughly separated into the two forms of autosomal disorder on the one hand, each with their own characteristic, highly reproducible forms of architectural abnormality, and the disturbances found in all other syndromes on the other. The latter vary to a considerable extent in severity, often displaying asymmetrical characteristics. Within the overlapping groups of Potter Type IIa, IIb and IV individual patients often combine a Type IIa or IIb kidney on one side with a Type IV or alternatively renal agenesis/aplasia on the other.

Such forms of developmental disorder are rare in patients who are investigated clinically for primary refluxing or obstructive disease. Often unilaterally affected the nephrectomy specimens, when systematically assessed, do not commonly demonstrate signs of primary dysplasia comparable to those described under Potter Type II or IV abnormalities. As such these kidneys are not automatically assessed from the perspective of a developmental pathologist, and segmental defects or global reductions of the parenchymal mass may go unnoticed.

The development of the kidney and other components of the urinary tract are closely temporally related. Initially the assumption was made that only limited interdependence existed. Thus all abnormal development of more cranial components was considered due to abnormal *induction* by the immediately preceding component.

A subsequent concept considered that pathology could be caused later in intrauterine life, mediated for example by *obstruction* to urinary flow (Osathanondh & Potter, 1964). This would result in (local) developmental disturbance of the segments cranial to the obstruction and forms the rationale behind in utero (surgical) intervention in abnormally developing urinary tracts. Such intervention aims to maintain normal development of the most cranial component of the urinary tract, i.e. the kidney, by correcting abnormalities of more caudal segments.

However, if the Ureteral Bud concept (1.1.4.) adequately explains the pathogenesis of renal abnormalities associated with lateral ureteric ectopia (Mackie & Stephens, 1975; Henneberry & Stephens, 1980; Sommer & Stephens, 1981), a rationale for antenatal intervention is absent (although the possibility of additional, "sequential", disturbance of cortical development, due to the often associated obstructive abnormalities of lower segments, has not been excluded from this concept).

The existence of kidneys with isolated numerical deficiencies of the primary branches of the ampullary system, but normally developed parenchyma of the segments present, is recognised although they have not received a separate classification. Although not uncommon and in many ways related, these cases, which may represent localised mesenchymal defects rather than deficiencies of branching, are perhaps not strictly comparable to the abnormalities discussed above and below, and as such will not be further discussed.

1.1.2. Developmental pathology of the kidney in obstructive and refluxive abnormalities of the urinary tract

1.1.2.1. Different pathogenetic mechanisms of renal developmental abnormality

In each condition described below (1.1.2.2.) the review will concentrate on the documented presence of:

1.developmental arrest i.e. hypoplasia

2. developmental disturbance
i.e. dysplasia
3. globoid/dilatation pressure-related atrophy
4. (segmental) intracortical reflux of (sterile) urine

Hypoplasia, associated with antenatal urinary obstruction, is expressed homogenously throughout the kidney. When it is not associated with dilated pelvises, hypoplasia must reflect a retardation or arrest of renal development due to mechanisms other than global ischaemia. If retardation of glomerulo-/nephrogenesis is considered to result from the intracortical reflux of urine, then this must be occurring in a diffuse manner, affecting all calyx/papilla systems equally. Alternatively, when the ureteric orifice is positioned ectopically, hypoplasia may reflect a poor quality of interaction between ureteric bud and metanephric blastema (1.1.4.).

Dysplasia, associated with antenatal urinary obstruction, is also typically diffuse in nature (Risdon, 1981a; Williams & Risdon, 1982). Once again, poor-quality development of metanephric blastema by an abnormally-positioned ureteral bud or field defect may be responsible. Similarly if intracortical urinary reflux is a causal mechanism, this must occur globally throughout the developing calyx/papilla systems. Forms of diffuse dysplasia (limited to the outermost cortex), which are associated with incomplete obstruction and more medial, though still ectopic origins of the ureteric bud are considered to be induced, or at least arise, after the (normal) formation of the inner cortex (Bernstein & Gardner, 1989), i.e. later in intrauterine life.

In globoid atrophy, a dilated pelvis with inverted papillae and thin parenchyma is characteristically seen. This is thought not to be a direct effect of the retained urine, but rather the increased interstitial pressure exerted by the urine on the envelope of renal parenchyma, through compromising the blood and nutrient supply.

Intracortical reflux in obstructive abnormalities of the lower urinary tract may occur as a consequence of the immature shape of the renal papillae with, as yet, insufficient valve mechanism. This has been described in some papillae of developing kidneys (Risdon, 1981b). Intracortical reflux of sterile fetal urine may disturb parenchymal development in several ways (Risdon, 1981b), including local disturbance of concentrations of growth controlling substances. However, as not all papillae are abnormal, such pathology would have segmental characteristics.

1.1.2.2. Renal developmental abnormalities in obstructive malformations of the urinary tract

Obstructive lesions may occur anywhere from the distal urethra to the renal pelvis. In infants and children, urinary obstruction is most commonly caused by congenital anomalies such as posterior urethral valves or vesicoureteral obstruction, but may result from acquired lesions such as urolithiasis, traumatic urethral strictures or a neurogenic bladder associated with, for example, spina bifida (Risdon, 1981c). Ureteric stenosis is the usual cause of dilatation of the renal pelvis and calyces, and is typically found as a localised narrowing at the pelvicoureteric or ureterovesical junction. Stenosis may occur elsewhere in the ureter, albeit less commonly.

The relation between some of the lesions capable of causing urinary obstruction and developmental abnormalities of the kidney will be systematically described according to the literature:

THE URETHRA

Urethral Atresia

This is very rare and usually associated with urinary fistula in other segments of the urinary tract. Although the literature is limited, renal developmental abnormalities are uncommonly reported and usually not directly related.

Meatal Stenosis

This was once considered the most common congenital anomaly of the urethra. However most cases are now considered as acquired after traumatic circumcision, resulting in meatal ulceration and generalised ammoniacal dermatitis (Williams, 1982a; Duckett & Snow, 1986).

Although meatal stenosis often allows the passage of only a very thin stream of urine, retention is rarely a complication and hydronephrosis is very uncommon. It is interesting that there is poor correlation between functional abnormality and visual estimation of meatal diameter (Murphy, 1989). Renal developmental abnormalities are rare and usually not directly related.

Urethral Stricture

Urethral stricture is, in the majority of instances, an acquired lesion of males, most often traumatic in origin (Duckett & Snow, 1986). In the congenital form the junction of the anterior and posterior urethra is narrowed without a discernable diaphragm or valve. When present in the absence of infection, high pressure reflux may result in globoid atrophy. Generally some degree of infection of the more proximal system exists. Hypo- and/or dysplasia are not common.

Dyssynergia

Unexplained detrusor-sphincter dyssynergia may be the diagnosis entertained in that minority of boys with periodic urethral obstruction without evidence of an organic obstructive lesion (Williams, 1982a). Renal developmental abnormalities are not over-represented in this group.

Urethral Polyps

The majority of urethral polyps are congenital anomalies (Nesbit, 1962; Downs, 1970). Although the majority have been described in males between 3 and 10 years, polyps have been reported in neonates (Madden et al., 1986). They represent outgrowths of the prostatic tissue lining the male urethra in the region of the verumontanum (Murphy, 1989). Renal developmental abnormalities are not usually seen.

Urethral Cysts

In contrast to urethral diverticula and duplications, urethral cysts are distinguished by a lack of communication with the main urethral channel. Renal developmental abnormalities are not usually seen.

Urethral Diverticula

Urethral diverticula, saccular evaginations of urethral mucosa (Herbut, 1952a), exist in both congenital and acquired forms. They are predominantly seen in males although they may be found in women, most notably parous women as a result of trauma. Rarely seen, renal developmental abnormalities mirror those seen with cysts.

Urethral Membranes

These congenital anomalies consist of transverse mucosal diaphragms usually located in the male membranous or bulbous urethra. Developmental abnormalities in the kidney are described under:

Posterior Urethral Valves

Urethral valves are the commonest obstructive anomaly of male children (Williams, 1982a). Found almost exclusively in males, cusp-like mucosal infoldings are seen just distal to the verumontanum (Young et al., 1919; Herbut, 1952a; Duckett & Snow, 1986). Valves can present at any stage in childhood, a few even come to light during early adult life. Attempts at classification according to location and multiplicity did not improve our understanding and their pathogenesis remains unclear; although there is good evidence for a multifactorial form of inheritance and some association with unilateral cystic dysplasia of the obstructive type (Crawfurd, 1988). The most common subtype is characterised by fusion of the cusps with their apices directed towards the bladder. The obstructive lesion thus created usually presents early in life.

At diagnosis, in most patients, vesicoureteral reflux is (as yet) limited and little pressure-induced globoid atrophy occurs. Other developmental renal abnormalities, other than in the so-called prune-belly cases, are only seldomly reported.

THE URINARY BLADDER

Exstrophy

This group of congenital anomalies, of which a malformed urinary bladder opening to the ventro-caudal abdominal wall is the most prominent (Herbut, 1952b), is considered to reflect abnormal differentiation of the cloacal membrane (Muecke, 1986). Although ectopia of the ureter is typical in this condition,

developmental renal abnormalities are not commonly found.

Bladder Diverticula

The majority of these are encountered in males (Kretschmer, 1940; Forsythe & Smythe, 1959; Livne & Gonzales, 1985) and usually arise from the base of the bladder superior to the ureteral orifices. The extent of the obstruction to urinary outflow is proportional to their size. Unless the ureter is involved, developmental renal abnormalities are rarely reported (Livne & Gonzales, 1985; Murphy, 1989).

Bladder Hyperplasia

In this condition the bladder is most commonly abnormal in both structure and function. Bladder hyperplasia, most commonly found in males, is usually associated with urethral outflow obstruction (Inamdar et al., 1984). As a consequence of their long survival, renal pathology in these patients is poorly described.

URETERS AND RENAL PELVIS

Ureteral Strictures

Ureteral strictures are found most commonly at the pelvicoureteric junction as a unilateral anomaly (Marshall et al., 1980; Herbut, 1952c). If they occur at the ureterovesical junction a ureterocele may result.

The lumen of the ureter may also be narrowed by Ureteral Valves, which are usually found distally (Seitzman et al., 1969), or Ureteral "Kinks" (Redundancies), characteristically in the upper ureter (Antonakopoulos et al., 1985).

Associated abnormalities of the kidney have been described (Sommer & Stephens, 1981), however most authors do not clearly separate developmental pathology from abnormalities which could have been the result of postnatal, for example infectious, damage. Globoid atrophy is not as commonly described as varying degrees of hypoplasia (Murphy, 1989). Dysplastic features, even segmentally distributed, have been reported (Newman et al., 1974; Perrin et al., 1974) but these are discussed by others in relation to (postnatal) renal tissue inflammation (Ericsson & Ivemark, 1958; Taxy and Filmer, 1975). The degree of dysplasia is reported to be related to the extent of obstruction, with milder forms resulting in diffuse dysplasia of the outer cortex, arising after the formation of the inner cortex (Bernstein & Gardner, 1989). Segmental abnormalities (infectious/ inflammatory, with or without focal dysplastic features) are extensively reported, but not in antenatally diagnosed cases studied after termination of pregnancy.

Ureteral Polyps

In contrast to those of the urethra, these are generally held as an acquired condition. Although they can result in ureteral obstruction, very few have been detected in children (Crum et al., 1969; Davides & King, 1976; Wolgel et al., 1982). Information on renal pathology, as most patients show very limited clinical problems, is limited.

Megaureter

In this condition there is an association between megacystis, megaureter and megaduodenum, which is known to have an autosomal dominant pattern of inheritance (Faulk et al., 1978). In children megaureter, in which there is an obstruction to the flow of urine associated with normal diameter of the intravesical and intramural ureteral segments (Johnston, 1982), is most often seen in boys. The precise pathogenesis resulting in the failure to conduct the peristaltic wave from the dilated cranial portion to the non-dilated caudal portion, remains unclear. Megaureter may be uni- or bilateral and can coexist with vesicoureteral reflux, urethral valves and ureteral strictures (Pfister & Hendren, 1978; Lockhart et al., 1979).

In these conditions a certain degree of pressure-related, globoid atrophy is always present. The presence and degree of underdevelopment (hypoplasia) and maldevelopment (dysplasia), often interpreted as secondary atrophy and acquired dysplasia-like change, is poorly defined.

Ureteral Diverticula

With and without mural smooth muscle abnormalities these vary in diameter from 0.2 to 7.0 cm with the largest diverticula found in the pelvis rather than the ureter. There is some association with other lesions such as Ask-Upmark kidney (Abeshouse & Abeshouse, 1963).

Ureteric Duplications

Ureteric duplication is one of the most common anomalies of the upper urinary tract (Herbut, 1952c). It is considered to reflect abnormal duplication of the metanephric bud and has been documented in 0.5-4% of necropsies (Nation, 1944; Campbell, 1951). In the majority of children, except for the dysplasia described by Mackie and Stephens (1975), there are no pathological complications which, when they do occur, are predominantly inflammatory associated with the often present reflux and/or obstruction at the ectopic origin of the ureter draining the upper system.

Commoner than complete duplication is incomplete duplication resulting in a bifid ureter. This may be associated with pelviureteric obstruction, characteristically affecting the lower component (Williams, 1982b).

Ectopic Ureteral Orifice

In single systems ectopic ureteral orifices involve an association with the remnants of the mesonephric duct (Herbut, 1952c), and the different possible relations between the caudal ureter and the other components of the distal urogenital tract are dependent on gender. Aside from functional disturbances resulting in reflux and obstructive features, both duplicated and ectopic ureters are usually structurally unremarkable, i.e. show little localised pathology. Most forms, other than ectopia in the perineum, may be associated with hypo- and/or dysplasia.

Retroperitoneal Fibrosis

Idiopathic retroperitoneal fibrosis, although essentially a disorder of adulthood, has been reported as a cause of obstruction in children (Farrer & Peterson, 1962; Peterson et al., 1974).

Nonidiopathic retroperitoneal fibrosis has similarly been reported associated with a variety of pathology e.g. rheumatoid arthritis, systemic lupus erythematosus or Henoch-Schoenlein purpura (Cerny & Scott, 1971; Lloyd et al., 1974).

As a rule this condition leads to a dilated calyceal system with globoid atrophy as the major form of pathology.

VASCULAR ANOMALIES

Vascular anomalies, for example retrocaval ureter or anomalous inferior polar renal arteries (White & Wyatt, 1942), may be associated with ureteral dysfunction as a consequence of the extrinsic obstruction. These may occur in a familial pattern with an, as yet, unclear pattern of inheritance.

In the condition of anomalous inferior polar renal arteries, which is often associated with a high ureteric insertion, the inferior branch of the anterior division of the renal artery causes pelviureteric angulation by crossing anterior to the ureter before entering the hilum. Aberrant vessels have been reported to be involved in approximately 20% of cases of hydronephrosis, with or without globoid atrophy, in children (Uson et al., 1968; Johnston et al., 1977).

1.1.3. Different pathogenetic mechanisms of postnatally-acquired renal pathology in refluxive and obstructive abnormalities of the urinary tract

The processes leading to abnormality of renal tissue after birth may be considered to result in either loss or impaired growth of the parenchyma present at birth.

These abnormalities can be summarised as follows:

1. Segmental scarring, associated with the intracortical reflux of
   a. sterile urine
   b. infected urine
2. Focal and segmental glomerulosclerosis

Globoid atrophy may continue to contribute to renal damage after birth, however the mechanism of action and the patterns of consequential damage are the same as described above (1.1.2.1.). As such, this contributing factor will be mentioned when relevant, but no further explanation is required.

1.1.3.1. Segmental scarring

Segmental scarring, which is well defined in standard texts (Farrow, 1989) and summarily described in Figures 1-4 - 1-7, is very

common in children with vesicoureteral reflux.

The hypothesis that sterile intrarenal reflux may damage the kidney was first advanced following the observation that experimentally-created high pressure urinary reflux resulted in the scarring typically seen in children with vesicoureteral reflux (Hodson et al., 1975). Immunologically-mediated damage was proposed, specifically involving the Tamm-Horsfall protein (Marier et al., 1978). However, evidence of circulating immune complexes or immunoglobulin deposition within the kidney has not been forthcoming (Jones et al., 1980; Wenk et al., 1981). Similarly, in experiments on young pigs, Ransley and Risdon (1978, 1979) demonstrated scarring only in the presence of infection: sterile reflux, even when of high pressure, failed to produce such changes.

Although some segmental scarring may result from sterile intrarenal reflux, the significance of such reflux becomes more apparent when it coexists with severe outflow obstruction, for example in children with bladder outlet problems or posterior urethral valves (Mendoza & Roberts, 1983; Jorgensen et al., 1984; Ransley et al., 1984). In these circumstances, secondary renal damage will occur although the pathological changes are those of obstructive (globoid) atrophy rather than those of pyelonephritic (segmental) scarring.

Intrarenal reflux of infected urine is considered to play a central role in the development of parenchymal scarring (Smellie & Normand, 1985; Jacobsen et al., 1989; Risdon, 1993). In this regard, scarring has been

Figure 1-4. Photograph (5 micron paraffin section, H&E stain) of histological section taken from a nephrectomy specimen of a 5 year old patient with reflux nephropathy. Note the zones of normal renal (cortical) parenchyma alternating with zones dense with inflammatory infiltrate, creating the typical segmental pattern.

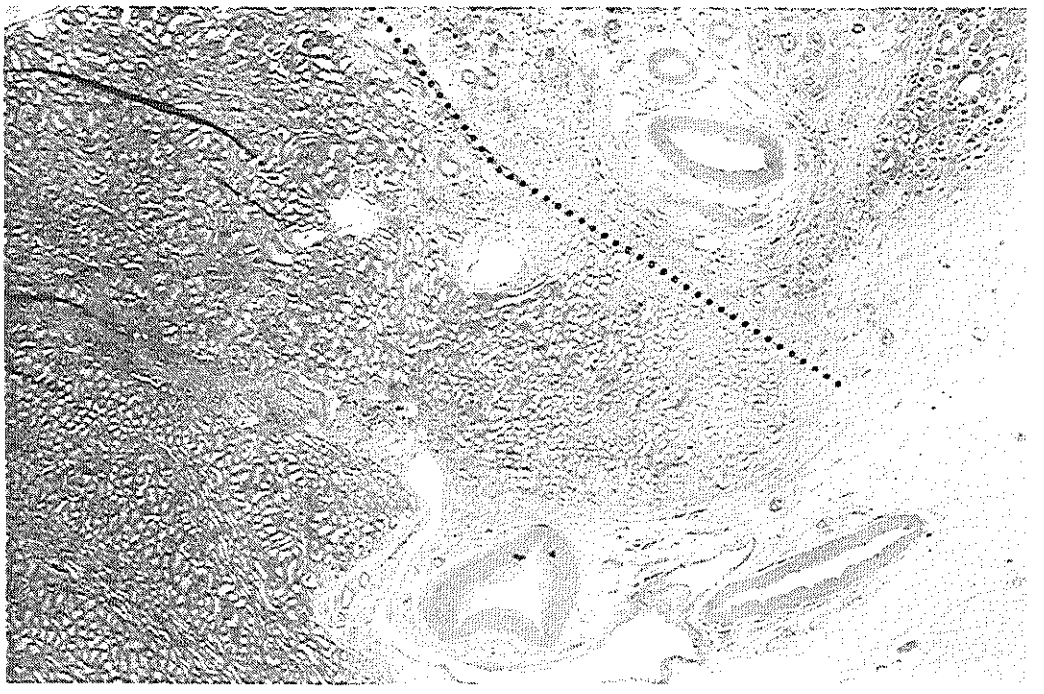

Figure 1-5. Photomicrograph (5 micron paraffin section, H&E stain) of border zone between cortex unaffected by intracortical reflux (one left) and scarred cortex (one right). Note the very sharp delineation running, in radial fashion, from peripelvar fat (dotted line) to cortex. Microscopical magnification x 10.

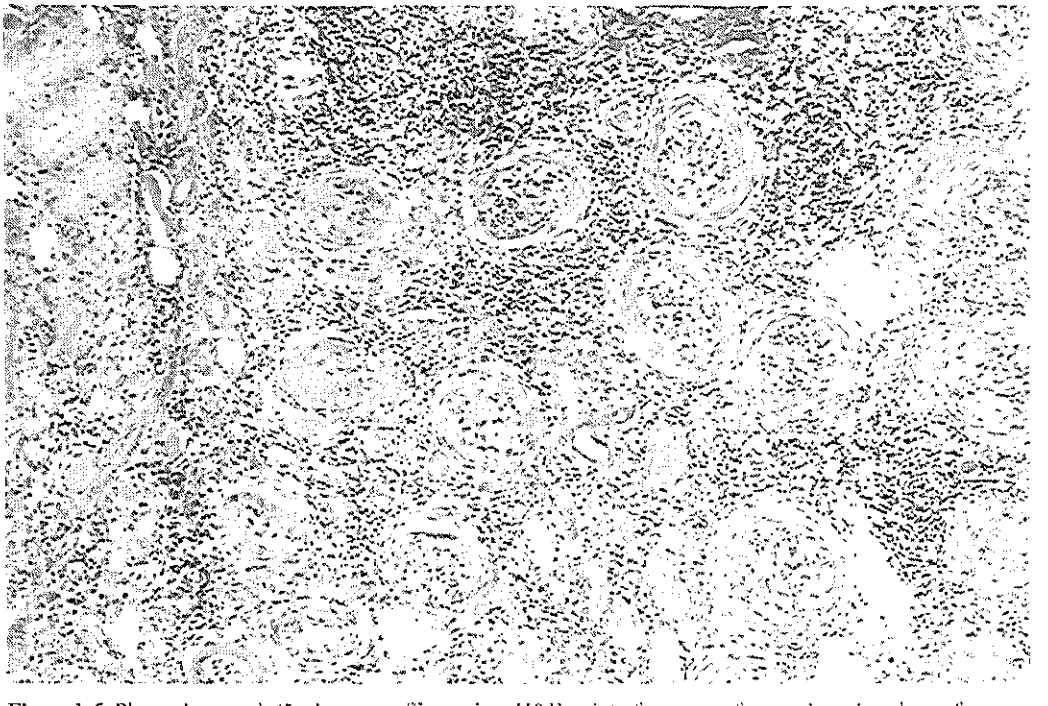

Figure 1-6. Photomicrograph (5 micron paraffin section, H&E stain) of segment of cortex in active phase of intrarenal reflux-associated inflammation. The dense infiltrate obscures the cortical structures other than glomeruli. Note the concentric thickening of the basement membrane of Bowman's capsule, without (as yet) secondary change in most glomeruli (compare to the sclerosis of focal segmental glomerulosclerosis, Figures 1-9 -1-11). Microscopical magnification x 50.

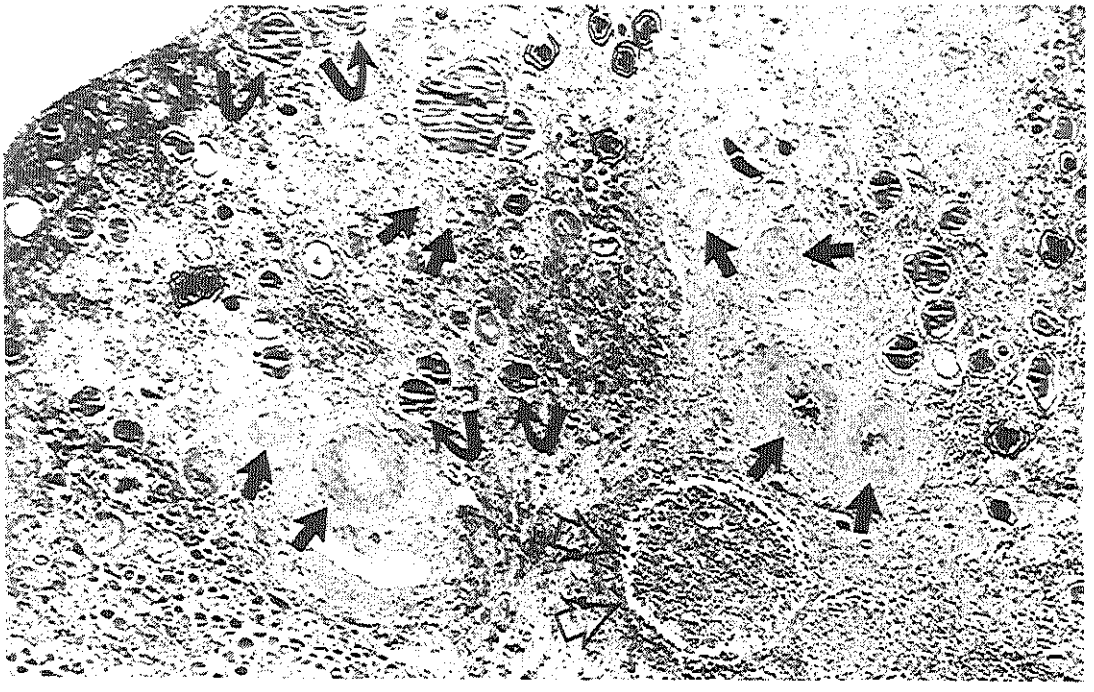

Figure 1-7. Photomicrograph (5 micron paraffin section, H&E stain) of renal cortex in late stage of ontrarenal reflux-associated inflammatory damage. Note lymphoid follicle (open arrows) and dilated duct systems filled with protein resulting in a thyroid-like appearance (curved arrows). Disproportionably thick-walled abnormal arteries indicate previously greater tissue volume and regressive changes after cortical loss (closed arrows). Microscopical magnification x 20.

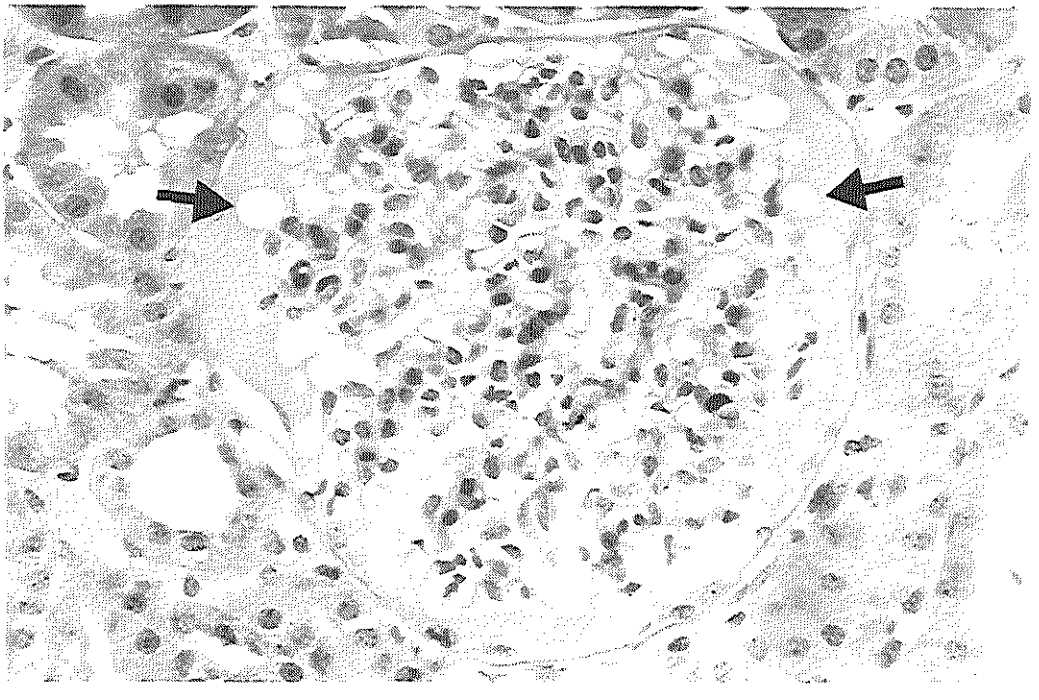

Figure 1-8. Photomicrograph (5 micron paraffin section, H&E stain) of normal appearing, perhaps slightly hypercellular, glomerulus in a patient with proteinuria. Note distended Bowman's capsule space filled with dense eosinophilic protein cast, with occasional retraction cavities (arrows). Microscopical magnification x 200.

convincingly demonstrated to occur following the reflux of infected urine into compound (concave) papillae (Ransley & Risdon, 1978, 1979). These papillae, derived from the fusion of simple papillae (Risdon, 1981b), have concave or deeply indented tips with widened ducts of Bellini opening into the calyx. Found predominantly in the polar regions, these papillae are seen in approximately two-thirds of normal kidneys (Ransley & Risdon, 1975a, 1975b).

The rapidity with which scarring occurs following introduction of bacteria into the urinary tract led to the so-called "Big Bang" theory (Ransley & Risdon, 1979), i.e. renal damage occurs at an early age following the intrarenal reflux of infected urine into susceptible (compound) papillae. Indeed the experimental evidence of the rapidity of infection-related damage, together with the abundance of young children who have demonstrable renal scarring following only a short history of urinary tract infections (Birmingham Reflux Study Group, 1983), led Scott to describe reflux nephropathy (vide infra) as a "once for all" phenomenon initiated by the first infection (Scott, 1985). One important aspect of the care of these patients has recently been emphasised, with the reports of difficulties associated with management of urinary tract infections by general practitioners in the community and the potential consequences of this for the development of renal disease (Smellie et al., 1985; Jacobsen et al., 1989; South Bedfordshire Practitioner's Group, 1990a, 1990b).

Whether or not progressive scar formation occurs with subsequent infections has been debated (Steinhardt, 1985; Scott, 1987). In agreement with Scott, who suggested that "new scars" detected radiologically may represent only "old" ones that have become apparent due to a differential growth of normal, as distinct from scarred, renal tissue (Scott, 1975, 1984), several investigators have concluded that scarring is usually established when the child first presents and after this only infrequently progresses (Smellie & Normand, 1975; Jones et al., 1984). Where new scars develop, this may be explained by the conversion of simple, nonrefluxing papillae into refluxing structures as a consequence of scar contracture (Smellie et al., 1981a; Huland & Busch, 1984; Steinhardt, 1985).

In addition to renal scar formation, urinary infection may interfere with renal growth (Smellie et al., 1981a; Hellstrom et al., 1987; Aggarwal et al., 1991).

In a minority of children, segmental renal scarring may occur in the absence of vesicoureteral reflux. This questions the pressure role attributed to intracortical reflux in segmental inflammation and scarring. Such damage may reflect additional, subtle, bacterial and host factors (Torres et al, 1985). In this regard the interplay between *Escherichia coli* of differing virulence, and the possession by the host of specific blood group antigens and urothelial receptors, has been described (Roberts et al., 1984; Lomberg et al., 1983).

1.1.3.2. Focal and segmental glomerulosclerosis (renal overload nephropathy)

In children with reflux nephropathy, renal scarring most commonly results from the intracortical reflux, at an early age, of infected urine through compound papillae. As discussed above, further parenchymal scarring rarely occurs during adult life. However, although scarring is associated with renal functional impairment and the development of hypertension (Wallace et al., 1978; Bachmann, 1982; Torres et al., 1983), some patients undergo progressive functional deterioration in later life independently of ongoing reflux, hypertension or urinary infection (Bailey, 1973; Salvatierra et al., 1973; Kincaid-Smith, 1975a; Kincaid-Smith & Becker, 1979; Senekjian et al., 1979). This progressive renal failure is associated with proteinuria and a characteristic glomerular lesion in nonscarred areas of parenchyma: focal and segmental glomerulosclerosis (FSGS) (Figures 1-8 - 1-11).

The association between focal segmental glomerulosclerosis, proteinuria and reflux nephropathy was first described in 1973 (Zimmerman et al., 1973). Subsequent studies have reported the almost universal presence of FSGS in patients with endstage renal disease secondary to reflux nephropathy (Kincaid-Smith, 1975b; Bhathena et al., 1980). However, even in patients with VUR, FSGS is not specific for endstage nephropathy, as it has been found in kidneys prior to this phase (Fairley et al., 1975;

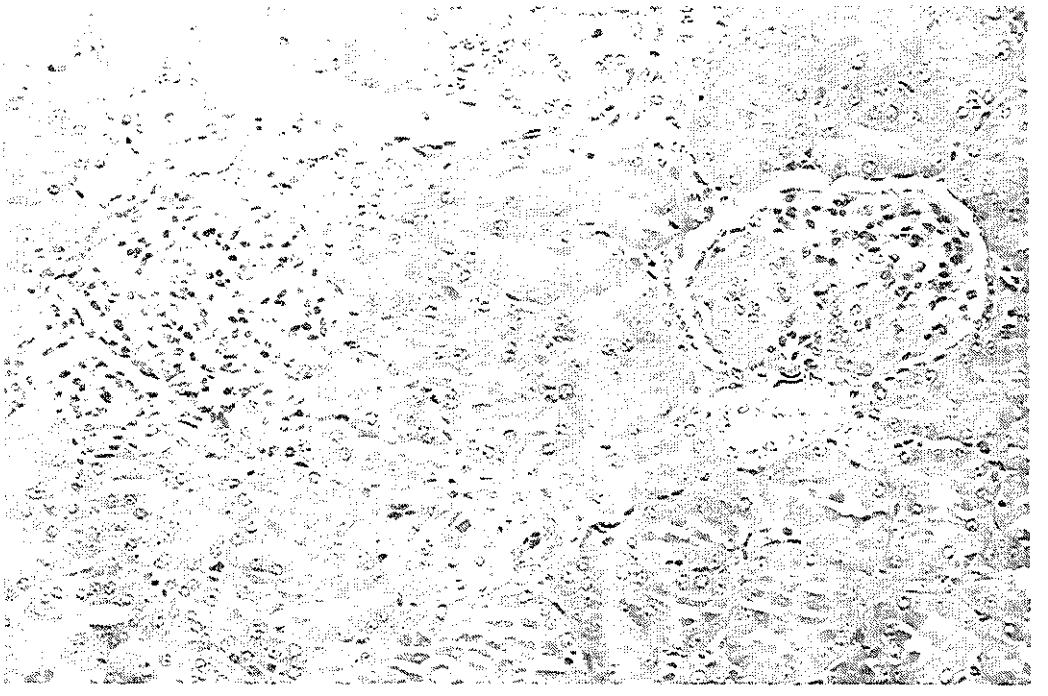

Figure 1-9. Photomicrograph (5 micron paraffin section, H&E stain) of renal cortex in early stage of FSGS. Note the absence of changes associated with intracortical reflux. Glomerulus on right displays 1 fully sclerosed tuft and the beginnings of sclerotic change in the tuft hilus. The remainder of the glomerulus shows no damage and Bowman's capsule basement membrane shows no increase of width. Microscopical magnification x 100.

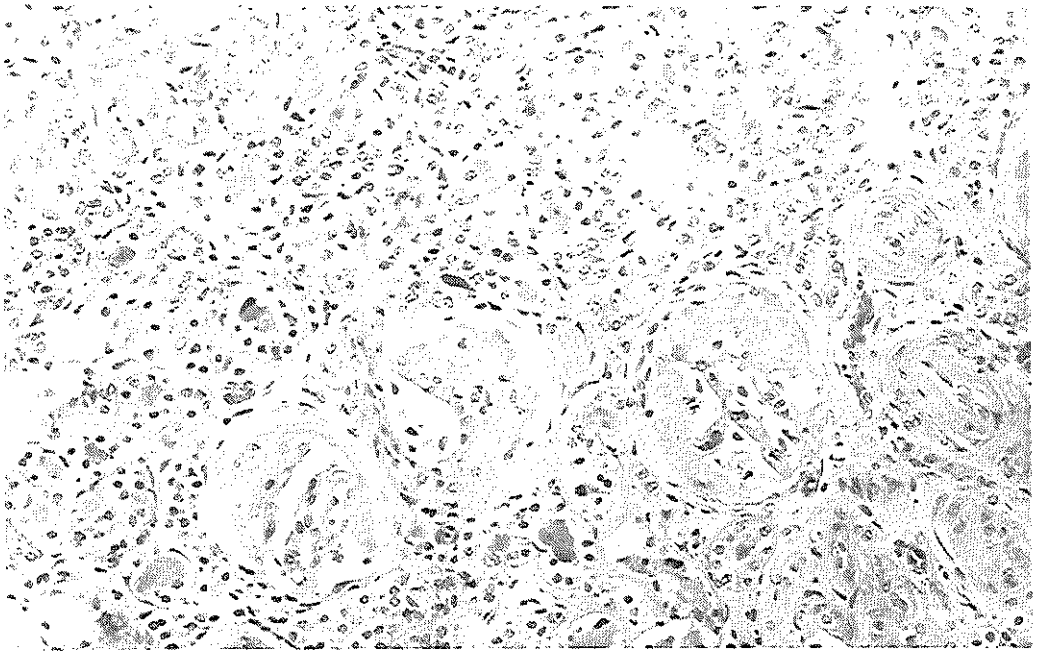

Figure 1-10. Photomicrograph (5 micron paraffin section, H&E stain) of renal cortex in later stage of FSGS. Note the absence in the interstitium of changes associated with intracortical reflux. All 4 glomeruli in lower half of the picture display sclerosed tufts. The glomerulus on the far left still has part of a tuft intact. No glomerulus shows increase in width of the basement membrane of Bowman's capsule. Proteinuria is revealed by small cylinders visible in the upper left. Microscopical magnification x 100.

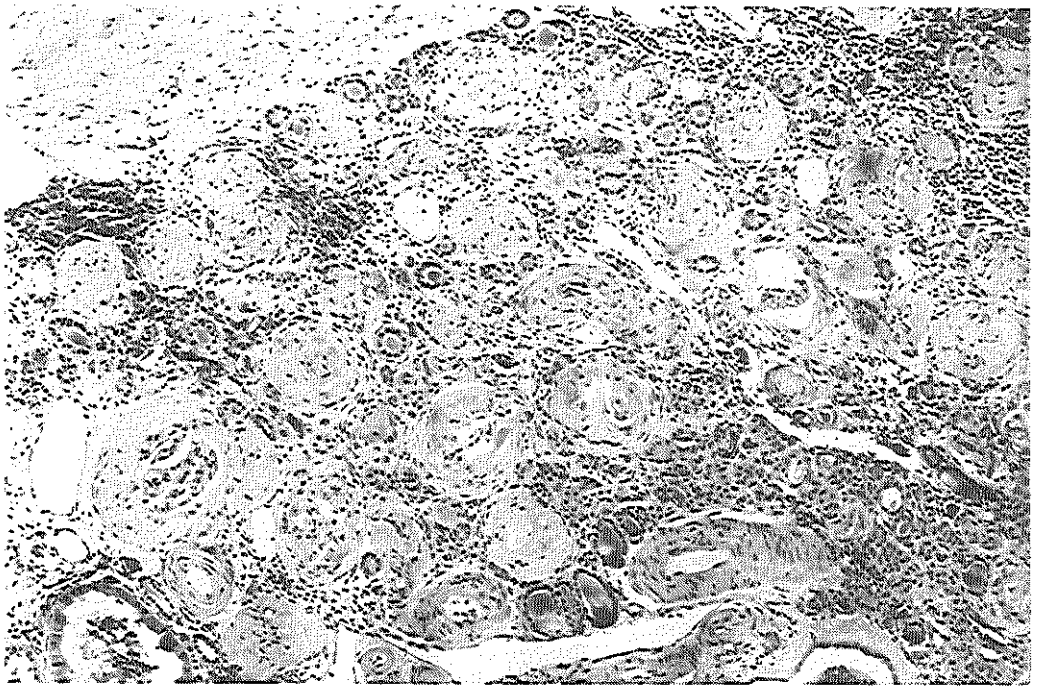

Figure 1-11. Photomicrograph (5 micron paraffin section, H&E stain) of renal cortex in final stage of FSGS. All glomeruli display sclerosed tufts. No glomerulus shows increased width of the basement membrane of Bowman's capsule. Microscopical magnification x 50.

Senekjian et al., 1979; Morita et al., 1990). Although the association of FSGS and reflux nephropathy is well established, the pathogenesis of the glomerular lesion remains incompletely understood (Nagata & Kriz, 1992; Nagata et al., 1992; Becker & Kincaid-Smith, 1993; Howie, 1993; Howie et al., 1993; Yoshiara et al., 1993).

In addition to vesicoureteral reflux, focal segmental glomerulosclerosis may be encountered in a number of different conditions. As well as being one of the most common causes in childhood of the idiopathic nephrotic syndrome (Habib & Kleinknecht, 1971), FSGS may be secondary to Alport's syndrome (Gaboardi et al., 1974), heroin abuse (Grishman & Churg, 1975) and the Acquired Immune Deficiency Syndrome (Rennke & Klein, 1989). More interestingly, when one is considering the pathogenesis of this lesion in reflux nephropathy (see below), FSGS has been reported in a number of other conditions in which the number of nephrons is reduced, e.g. unilateral renal agenesis (Kiprov et al., 1982; Thorner et al., 1984; Gutierrez-Millet et al., 1986), unilateral nephrectomy (Zucchelli et al., 1983; Celsi et al., 1987), oligomeganephronia (Brenner et al., 1982; Kaneko et al., 1990; Nomura & Osawa, 1990) and segmental renal hypoplasia (Habib, 1979). The occurrence of FSGS in the remaining kidney of patients undergoing unilateral nephrectomy seems in marked contrast to the, in general, good outcome for (unilateral) kidney donors. One explanation is perhaps that, in the reported literature, developmental defects of the remaining kidney in non-transplant related resection cannot be excluded, and may be underestimated.

Numerous studies have attempted to clarify the process of progressive deterioration of renal function that is characterised histologically by FSGS (reviews include Steinhardt, 1985; Remuzzi & Bertani, 1990; Becker & Kincaid-Smith 1993; Yoshiara et al., 1993). In the context of reflux nephropathy, parenchymal scarring destroys nephrons and such a decrease in functioning renal mass has been shown in several studies to result in "hyperfiltration" of the remnant nephron population (Deen et al., 1974; Claesson et al., 1981). Once the reduction in the number of nephron units reaches a critical value,

it has been hypothesised that the increased single nephron glomerular filtration rate, glomerular plasma flow and mean net glomerular transcapillary hydrostatic pressure result in structural damage to the glomerulus i.e. sclerosis of the glomerular capillary tuft (Hostetter et al., 1981; Hostetter et al., 1982; Brenner et al., 1982; Anderson et al., 1985; Olson et al., 1985). However, although this haemodynamic pathway is attractive and equally applicable to other conditions associated with a reduced number of nephrons (see above), the precise mechanism for glomerular damage continues to be debated.

Indeed, after measurement of glomerular capillary pressure by several investigators produced equivocal findings (Fogo et al., 1988; Remuzzi et al., 1988; Scholey et al., 1989), a haemodynamic pathogenesis was questioned and alternative hypotheses proposed. These included glomerular hypertrophy (Yoshida et al., 1989), activation of coagulation processes in glomerular microvessels with intraglomerular thrombosis (Klahr et al., 1986) and disorders of lipid metabolism associated with atherosclerosis (Diamond & Karnovsky, 1988).

Recently, in view of the generally unconvincing explanations of renal disease progression, the possibility was raised that, at least in the rat, abnormal transit of plasma proteins across the glomerular capillary membrane might be the principle factor in FSGS (Remuzzi & Bertani, 1990). The sustained increase in glomerular permeability, documented in rat models as a consequence of surgery, aging or certain toxins, might be the (common) determinant, rather than a consequence, of glomerular injury. According to Remuzzi and Bertani, glomerular permeability may be disrupted mechanically by the increased glomerular capillary pressures encountered for example in ("hyperfiltrating") reflux nephropathy, or by toxins or immune reactants which may act even in the absence of increased glomerular pressures. Subsequently, endstage renal failure ensues associated with glomerular epithelial cell damage (Marks & Drummond, 1970; Davies et al., 1978; Davies et al., 1985), increased production of extracellular matrix (Grond et al., 1982; Yoshioka et al., 1987) and tubular protein overload (Eddy, 1989).
In this hypothesis, therefore, the progressive functional deterioration is triggered by the exposure of the renal cells to the abnormal protein load and does not depend on the initial injury. Whether alteration in membrane permeability represents the explanation in man for the development of FSGS and the concomitant progression of renal disease requires further investigation. However Remuzzi and Bertani's hypothesis helps to explain observations demonstrating the significance of protein, as distinct from increased glomerular filtration rate per se, in the pathogenesis of glomerulosclerosis. For example, FSGS and renal functional deterioration, although documented in rats on a normal protein diet after right nephrectomy and segmental infarction of five sixths of the left kidney, were not seen in similarly nephrectomized rats maintained on a low protein diet (Hostetter et al., 1981). Kleinknect et al. (1979) also found that a similar reduction of renal mass resulted in death from renal failure sooner, and was associated with a greater degree of focal segmental glomerulosclerosis, in rats on a high rather than a low protein diet.

1.1.4. Vesicoureteral refluxive - obstructive disease and the associated pathology of the kidney

Although postnatal pathology such as described above (1.1.3.) may affect kidneys in many of the forms of obstruction discussed in 1.1.2.2., the largest group of patients requiring medical care after birth in the endeavour to prevent progressive renal functional loss, has what is defined as vesicoureteral reflux (VUR). As the kidney in VUR often displays features (1.1.2.1.) associated with congenital urinary tract obstruction (Risdon, 1987; Peters et al., 1992), and children with reflux often express some degree of functional obstruction at the ureterovesical junction, nephrectomy specimens associated with this clinical entity are an ideal model for studying the interaction between pathology defined as occurring before and after birth. As such the material used for this thesis was limited to specimens derived from this group of patients.

Vesicoureteral reflux

Vesicoureteral reflux is said to occur when urine can, or is forced to, flow in a

retrograde direction from the bladder into the ureter and kidney. The normal ureterovesical junction allows the free anterograde passage of urine whilst preventing flow in the reverse direction, even when the bladder is contracting (Ransley, 1982). Vesicoureteral reflux results when this valvular mechanism is deficient from whatever cause, e.g. abnormal position of the intramural ureteric component, weakness of vesical musculature surrounding the ureters, bladder outlet narrowing, or impaired ureteral peristalsis (Murphy, 1989), in addition to the temporary reflux found commonly in children with cystitis where it is thought to reflect mural and mucosal oedema. Primary reflux is considered to represent a congenital anomaly in which there is a failure of development of the ureterovesical junction. However, the spectrum of functional disturbance associated with this condition is well documented: the defect is certainly not "all or none" (Scott, 1985).

The first recognition of the antireflux mechanism of the normal ureterovesical junction is attributed to Galen (150 AD) (Ransley, 1982). Although experiments confirming VUR were performed in the late 19th century, development of contrast cystograms and review of these in a variety of urological conditions was required before reflux could be profitably investigated (Bumpus, 1924; Gruber, 1929).

Despite development of surgical antireflux procedures during the 1950s, it was not until the seminal paper of Hodson and Edwards in 1960 that the association between VUR and a small, scarred, chronic pyelonephritic kidney was established (Hodson & Edwards, 1960). Subsequent work documented the importance of urinary infection, renal papillary morphology and intrarenal reflux for the development of renal damage (1.1.3.1.) (Ransley & Risdon, 1978, 1979; Hodson et al., 1976).

At this juncture VUR-associated renal damage was thus regarded largely as an acquired lesion, in which scarring resulted from sterile or, more probably, infected intrarenal reflux into a normally developed kidney. However histological demonstration of developmental abnormalities in affected kidneys, together with clinical evidence of impaired renal function in VUR both in children in whom focal scars could not be shown (Stutley & Gordon, 1992) and in neonates with no history of urinary infection but small kidneys with smooth (non-segmentally scarred) outlines on imaging (Anderson & Rickwood, 1991; Elder, 1992), focused attention on the effects of VUR in utero on renal development. As such the term "Reflux Nephropathy" was discussed by Steinhardt (1985) as a replacement for chronic atrophic pyelonephritis, which does not highlight the possibility of renal abnormalities arising during intrauterine life.

To explain these findings it was suggested that the renal morphology associated with reflux nephropathy reflects abnormal embryological development as distinct from an acquired lesion secondary to intracortical reflux (Mackie & Stephens, 1975; Henneberry & Stephens, 1980; Sommer & Stephens, 1981).

It is well established that VUR is often associated with an ectopic position of the ureteral orifice (Cook & King, 1979): increasing degrees of cranio-lateral ectopia are associated with more severe degrees of reflux (Lyon et al., 1969).

Stephens and colleagues proposed that lateral ureteric ectopia with a short or absent submucosal ureteral segment results from the ureteric bud arising from the most caudal end of the mesonephric duct. This ectopic bud induces development of that region of the nephrogenic cord caudal to that predestined for the metanephros, which is reflected in renal hypoplasia and or dysplasia (Sommer & Stephens, 1981; Bouton, 1984).

As is discussed by Steinhardt (1985), this Ureteral Bud theory correlates well with the clinical observation that parenchymal scars are often present in small children with reflux but no history of infection (Rolleston et al., 1975; Scott, 1987). Although an obstructive component at the ureterovesical junction has been discussed, a final assessment of its clinical relevance is not available. However, certainty that infection has never occurred in these infants may be difficult to accept unequivocally as clinical symptomatology is not infrequently absent (Ransley & Risdon, 1978; Risdon, 1981c).

On balance the bud theory seems to be most applicable to the complete duplex system: where one or other renal segment is dysplastic and associated with an ectopic orifice while the other is normal (Mackie & Stephens, 1975). However, the correlation between dysplasia and

ectopia in single ureter systems is less conclusive (Duckett, 1980; Sommer & Stephens, 1981).

In the above hypothesis both VUR and renal maldevelopment are separate expressions of a malformed urinary tract. Alternatively a more direct relationship has been proposed, in which VUR in utero, particularly if of sufficient severity to produce functional urinary obstruction, may of itself interfere with nephrogenesis (Bialestock, 1965). Others have wondered whether both processes may be operating, with dysplasia resulting from the first and hypoplasia from the second (Anderson & Rickwood, 1991).

1.1.5. Clinical aspects of vesicoureteral refluxive - obstructive disease

1.1.5.1. Introduction

VUR is a complex clinical problem. An indication of the intense interest in this contentious subject was given in 1987 by Scott, who revealed that in the previous fourteen years, in addition to several books and numerous chapters within books, more than 1500 papers appeared in journals referring to various aspects of vesicoureteral reflux (Scott, 1987). Indeed, complete discussion of VUR is beyond the scope of this thesis and readers are referred to reviews (Edwards et al., 1977; Smellie & Normand, 1979; Smellie et al., 1981b; Ransley & Risdon, 1981; Risdon, 1987; Bailey, 1993; Risdon, 1993).

Clinical significance of VUR stems from the associated renal parenchymal damage. Renal impairment progressing to endstage renal failure occurs in about 4% of children with VUR (Berger et al., 1981; Haycock, 1986); vesicoureteral reflux is responsible for 10-30% of patients with endstage renal disease (Huland et al., 1979; Bakshandeh et al., 1976; Bailey, 1981; Donckerwolcke & Brunner, 1981; Senekjian & Suki, 1982; Disney, 1991). Indeed, reflux nephropathy is the commonest cause of endstage renal failure in children (Chantler, 1987). Children thus affected are usually over 5 years of age and a rapid deterioration of renal function, often associated with proteinuria, is typically heralded by the development of hypertension (Bailey, 1979; Ransley, 1982).

1.1.5.2. Aetiology of vesicoureteral reflux

Reflux is thought to affect around 1% of all children (Newcastle Asymptomatic Bacteriuria Research Group, 1975; Cardiff-Oxford Bacteriuria Study Group, 1978). However, as reflux often resolves with increasing age (1.1.5.3.), this figure must be cautiously interpreted. Similarly, although an increased prevalence of VUR in girls is widely reported, this may merely reflect its decreased detection in boys as a consequence of their lower incidence of urinary tract infections (Scott, 1987). Indeed, with the increased detection of VUR in (asymptomatic) infants as a consequence of their investigation following antenatal demonstration of hydronephrosis (see below), any evidence of a sex bias is diminishing - in this group boys predominate (Paltiel & Lebowitz, 1989; Gordon et al., 1990; Anderson & Rickwood, 1991). Similarly endstage reflux nephropathy has a more or less equal sex incidence (Bailey, 1988; Disney, 1991; Bailey, 1992).

The large majority of cases of VUR are sporadic although family studies have revealed strong hereditary and familial connections (Jenkins & Noe, 1982; Hayden & Koff, 1984), and associations with the HLA major histocompatibility antigens are reported (Torres et al., 1980a). Such evidence suggests a multifactorial aetiology for reflux. However the high rate of transmission from parent to child recently reported (Noe et al., 1992) would now appear to favour an autosomal dominant mode of inheritance or a multifactorial pattern with a major single dominant gene with varying penetrants. Vesicoureteral reflux has also been documented within the autosomal dominantly inherited imperforate anus, hand, foot and ear (Townes) syndrome (Townes & Brocks, 1972; Kurnit et al., 1978), and in association with partial trisomy for the long arm of chromosome 4 (Surana & Conen, 1972; Schrott et al., 1974).

1.1.5.3. Natural history of vesicoureteral reflux

A well recognised feature of VUR is the general tendency for it to disappear with increasing age. This is considered to result from the natural lengthening of the intravesical ureter

and the maturation of the ureteric and trigonal musculature (Ransley, 1982). Overall, spontaneous resolution may occur in around 80% of ureters in 70% of children (Normand & Smellie, 1979). As may be expected, resolution is less likely the more gross the derangement of the ureterovesical junction.

The overall pattern of improvement with time is of great import when considering the management of these children. Both the risks and benefits from surgical intervention, the need to prevent urinary infection in the presence of ongoing reflux if antireflux procedures are not performed, and the natural ureteral maturation process must be carefully weighed.

1.1.5.4. Management of vesicoureteral refluxive - obstructive disease

Vesicoureteral refluxive - obstructive disease presents a complex management problem, which varies between children and with time. In addition, the strategy of care varies between clinicians (Cavanagh & Sherwood, 1983; Gordon, 1990; Elder et al., 1992) despite both national and international studies designed to provide a more rational basis for their practice (International Reflux Study Committee, 1981; Birmingham Reflux study Group, 1987; Burge et al., 1992; Scholtmeijer, 1993). As management continues to evolve only a brief discussion will be presented here.

Objective

As discussed above, in reflux nephropathy progressive renal deterioration is associated with hyperfiltration of the remaining functional nephrons and the development of focal segmental glomerulosclerosis (1.1.3.2.). Once a certain population of remnant nephrons is established, hypertension and proteinuria may be inevitable. The objective of management must therefore be to prevent renal damage or minimise its extent, in order to preserve as much normal renal parenchyma as possible. From what has been discussed above it is apparent that the decrease in renal function will reflect the proportion of initial scarring or developmental pathology. We should thus aim to specifically prevent the early destruction of nephrons (Steinhardt, 1985).

Diagnosis and Investigations

1. Antenatal

Routine antenatal ultrasonography has been widely employed since 1975 to screen for neonatal abnormalities, of which urological malformations form approximately 50% (Helin & Persson, 1986). Ultrasound has considerable efficacy for the detection of several structural anomalies of the urinary tract, including the pelvis dilatation resulting from pelviureteric junction obstruction, posterior urethral valves or megaureter. Similarly, dysplastic or polycystic kidneys may be efficiently recognised. In contrast, this technique has unfortunately limited success diagnosing the functional disturbance of vesicoureteral reflux (Livera et al., 1989; Chitty et al., 1991; Paduano, 1991a, 1991b). VUR is usually only (indirectly) detected in those cases where it is associated with hydroureteronephrosis (Grade III, IV or V, International Reflux Study Committee, 1981) and a prenatal diagnosis of obstructive uropathy is reached (Paduano, 1991b) (indeed it is possible that prenatal ultrasound is failing to detect large numbers of fetuses with lower grades of reflux (Gordon et al., 1990)). Although a correct diagnosis of VUR will result from early postnatal investigation and appropriate chemoprophylaxis may be then commenced (Livera et al., 1989), this lack of specificity in the (antenatal) differential diagnosis of disease may be problematic now that in utero surgical intervention is being considered (Harrison et al., 1981a, 1981b, 1982; Berkowitz et al., 1982; Golbus et al., 1982; Turnock et al, 1984). In this regard Paltiel and Lebowitz (1989) reported a series of 14 neonates with primary vesicoureteral reflux diagnosed by micturating cystourethrogram (MCUG) following antenatal ultrasound demonstration of hydronephrosis. In 3 cases inappropriate (anti-obstructive) maternal/fetal intervention occurred following an incorrect diagnosis in utero of posterior urethral valves. Similarly Elder (1992) urged perinatologists considering prenatal drainage of a dilated urinary tract to understand that "vesicoureteral reflux is more common than posterior urethral valves as a cause of bilateral hydronephrosis and a distended bladder in the male fetus.

Moreover because of the inability of

(antenatal) ultrasound to detect reflux per se, prevention of renal developmental abnormality may, in a large number of cases, prove impossible (Scott, 1985). Indeed, despite the widespread use of antenatal ultrasonography (Helin & Persson, 1986; Scott & Renwick, 1988; Livera et al., 1989; Chitty et al., 1991; Paduano, 1991a, 1991b), VUR continues to be largely detected following the diagnosis of urinary tract infection (Ring & Zobel, 1988). In this regard Paduano and colleagues concluded that "the principal disorder of the urinary tract that may fail prenatal investigation is vesicoureteral reflux" (Paduano et al., 1991b), whilst others predicted that even prenatal evaluation in all pregnancies will probably not significantly reduce the number of cases of vesicoureteral reflux detected after urinary tract infection (Ring & Zobel, 1988).

2. Postnatal

In the majority of children, VUR is detected as a result of investigation of urinary tract infection (UTI), where it is the commonest abnormality demonstrated. In one study, the results of which have been more recently confirmed (Blickman et al., 1985; Gleeson & Gordon, 1991) approximately 50% of infants and about 30% of older children with UTI were shown to have reflux (Shannon, 1970). Similarly, Smellie and Normand (1966) found VUR in between 30 to 50% of affected girls. Even when investigating schoolchildren with asymptomatic bacteriuria, VUR was found in 25-33% (Savage et al., 1973; McLachlan et al., 1975).

With the increased detection of fetal hydronephrosis by prenatal ultrasound (see above) a significant number of neonates with reflux is being found. In approximately 10% of cases in which renal pelvic dilatation is assumed to be secondary to obstructive uropathy, primary vesicoureteral reflux is responsible (Elder, 1992). In contrast to the group detected following investigation for UTI, neonates with reflux are typically male with often bilateral and (perhaps not unexpectedly) relatively severe reflux (Gordon et al., 1990; Najmaldin et al., 1990; Anderson & Rickwood, 1991; Scott, 1993).

For both presentations VUR will generally be diagnosed (postnatally) by MCUG, which remains the most sensitive and selective investigation (Scott, 1987). Together with intravenous urography this remains a standard technique for demonstrating renal size and shape in addition to the grade of reflux, location of parenchymal scars and, from serial examinations, renal growth (Scott, 1985).

Ultrasound and ^{99m}Tc dimercapto-succinic acid (DMSA) radioisotope scanning have been increasingly employed in the assessment of renal scars (Handmaker et al., 1975; Leonard et al., 1979; Merrick et al., 1980; Stoller & Korgan, 1986; Verber et al., 1988; Goldraich et al., 1989). Indeed, DMSA scanning is the most sensitive technique for their detection and has particular use in estimating the mass of residual functional parenchyma (Crabbe et al., 1992; Stutley & Gordon, 1992). Furthermore, recent work has suggested that DMSA scanning should be the initial imaging procedure for children over 1 year of age presenting for investigation of a urinary tract infection, since a kidney associated with reflux in this age group is more likely to be normal than scarred (Gleeson & Gordon, 1991): the presence of VUR is predicted following the DMSA-demonstration of renal scars and only in these circumstances is reflux specifically investigated by MCUG.

^{99m}Tc Diethylenetriaminepentaacetic acid (DTPA) radioisotope scanning allows measurement of the differential glomerular filtration rate and is therefore important when considering nephrectomy of a damaged kidney (Barratt, 1982; Gordon, 1982). In addition, DTPA can be used to follow the progress of a kidney in a refluxing system, independently of the presence of scarring (Heelman et al., 1978; Ransley, 1982).

Treatment

In essence, prevention of renal scarring or (perhaps more realistically) minimalisation of parenchymal damage may be approached from two perspectives, i.e. eradicate reflux by a surgical anti-reflux (ureteral reimplantation) procedure or maintain urinary sterility whilst awaiting spontaneous resolution of the refluxing system.

Following the recognition of the largely subjective approach to the management of VUR, based "on limited personal experience and often on bias instead of scientific evidence" (International Reflux Study Committee, 1981) several prospective studies aimed to define the

optimum management of reflux (Birmingham Reflux Study group, 1987; Jodal et al, 1992; Smellie, 1992; Olbing et al, 1992; Weiss et al., 1992).

Although questions remain unanswered and studies continue, present treatment of VUR can be summarised (after Scholtmeijer, 1993):

Children with Grade I, II or III reflux (International Reflux Study Committee, 1981) should be managed conservatively with antibacterial therapy, although breakthrough urinary infections or worsening reflux indicate the need for surgery.

Grades IV and V require surgical intervention, after it has been excluded that reflux is not caused or exacerbated by active infection or urodynamic dysfunction of the bladder i.e. detrusor instability (Koff, 1992; Allen, 1992; van Gool et al., 1992).

Therefore, although micturating cystourethrography is regarded as the gold standard for the detection and grading of VUR, a videourodynamic study is mandatory to decide which treatment should be given (Scholtmeijer & Griffiths, 1990; Scholtmeijer & van Mastrigt, 1991). In the event of bladder instability being demonstrated, anticholinergic and antibacterial drugs should be prescribed regardless of the grade of reflux. If reflux of grade IV or V persists after six months therapy, surgery may be required.

The postnatal management of fetal ureteric reflux, i.e. VUR diagnosed in the first few months of life which was likely to have been present antenatally, is the subject of much interest at present. Some workers propose a conservative approach (Burge et al., 1992), although Scott (1993) reports that surgery ought to be considered for fetal ureteric reflux of "advanced grades" if there is no improvement after two years.

Clearly the above is only capable of addressing the problem of postnatally-acquired renal damage. Any renal impairment related to VUR in utero is less easily prevented - not least from the lack of a technique for its accurate diagnosis (see above).

Where the pathology is predominantly obstructive, early postnatal surgical intervention may be of significant benefit (Johnston, 1982). Similarly there is at present interest in the role of antenatal surgical intervention for the decompression of (severe, bilateral) urinary tract obstruction, via the use of vesicoamniotic shunts. However this issue is far from resolved (Kramer, 1983; Turnock & Shawis, 1984; Elder et al., 1987), not least from the ethical standpoint (Barclay et al., 1981; Fletcher, 1981). Moreover data suggests that only in a very small number of highly selected cases, in which bilateral fetal hydronephrosis is due to urethral obstruction **and** in which oligohydramnios develops after 20 weeks' gestation, can in utero decompression sometimes prevent ongoing damage to the developing kidneys (Glick et al., 1985; Paltiel & Lebowitz, 1989). Similarly Paltiel and Lebowitz (1989) concluded that "there are no data to indicate that fetal intervention, cesarean section, or early induction of labour are beneficial for the fetus with hydronephrosis due to primary reflux, especially if the volume of amniotic fluid is normal". Furthermore a rationale for intervention in utero is lacking if the Ureteral Bud concept adequately explains renal developmental pathology in VUR (Mackie & Stephens, 1975; Henneberry & Stephens, 1980; Sommer & Stephens, 1981). Whatever the pathogenesis it is evident that, even after intervention as early as the 21st to 24th week of gestation, prevention of hypo/dysplasia is far from assured (Harrison et al., 1982; Berkowitz et al., 1982). It is to be hoped that on-going studies will clarify this issue, although whether development of this approach will in the long term diminish the incidence of chronic renal failure is impossible to predict.

1.2. Quantitation of development and acquired change of the kidney.

1.2.1. Quantitative assessment of renal development

Various methods of differing complexity have been employed to quantify renal development.

1.2.1.1. Renal weight

Renal weight, obtained at post-mortem investigation and fetal examination, has long been used as a parameter of development.

Normally there is a close relation between bodysize, surface area, age and renal weight, i.e. renal growth is allometric (Gruenwald & Minh, 1960; Risdon, 1975; Risdon, 1981a). Indeed different reports documenting the mean normal kidney weights throughout childhood are in close agreement (Coppoletta & Wolbach, 1933; Landing & Hughes, 1962). However it is obvious that renal weight may be influenced by mechanisms such as oedema and passive venous engorgement and, together with the large biological variation, these confounding variables limit the usefulness of renal weight. In addition in most studies renal weight shows a skewed distribution further limiting the use of simple standard deviations for description of populations and comparison of group data with a given individual kidney weight. For example, even without taking into account skewness of deviation properly, a confident diagnosis of renal hypoplasia, defined as a kidney that is congenitally small (i.e. less than 2 standard deviations below the expected mean) when correlated with age or the usual parameters of somatic growth (Risdon, 1987; Farrow, 1989), requires a combined renal weight less than half the normal value for age (Landing & Hughes, 1962; Kissane, 1983; Landing et al., 1989). At the age of nephrectomy, diagnosis of hypoplasia on the basis of renal weight is, in practice, even more difficult since kidneys may be shrunken as a result of acquired disease. Similarly, congenitally small kidneys may be more prone to acquired injuries, e.g. pyelonephritis or hydronephrosis, which may mask the underlying developmental abnormality (Risdon, 1981a).

1.2.1.2. Total nephron number

In order to circumvent the problems inherent to the use of renal weight, and because of its more direct relevance to the functional capacity of the organ, the parameter of total nephron or glomerular number has long interested renal pathologists. Estimates of glomerular number have been derived from a variety of methods. These include physical isolation of glomeruli by acid maceration and their subsequent enumeration (1.2.4.) and indirect number estimation on independent or serial histological sections (in which assessments of glomerular profile densities - obtained by counting the number of glomerular transections in a fixed area of a histological section - are combined with those of renal weight or volume to give an estimate of total glomerular number) (1.2.2.1.). Unfortunately, these methods have been shown to have some degree of bias q.v. (Bendtsen & Nyengaard, 1989).

In contrast, recent development of design-based stereological procedures has enabled the unbiased estimation of total renal nephron number, reported in the literature by ourselves (Hinchliffe et al., 1991; this thesis, Chapter 3). However, these techniques, although unbiased, are as yet not routinely applicable in the histopathology laboratory as they are both (relatively) time-consuming and labour intensive. Furthermore the complete kidney is required for analysis, precluding their use on tissue biopsies.

1.2.1.3. Glomerular generation counting

Ever since the microdissection work of Osathanondh and Potter (1963a, 1963b, 1963c), when the relation of glomeruli to the collecting ducts was established, estimation of the number of layers of glomeruli that exist between the capsule and the corticomedullary junction has been considered a possible technique for the assessment of renal developmental status (Emery, 1982). Indeed this technique has been used to describe hypoplasia in children with vesicoureteral reflux by comparing the mean number of glomerular generations in the renal cortex with that of nonrefluxing controls (Sommer & Stephens, 1981). This technique has, in theory, perhaps the greatest potential for the routine (and research-based) assessment of renal developmental status, since it may be performed on individual histological sections and requires a (relatively) short amount of time.

However several problems exist. Although it is possible to compare mean counts of different groups and ascribe the label of hypoplasia to those kidneys whose count is significantly less than that of the (supposedly normal) controls, there are no papers in the literature which present validated protocols for the application of this technique. Furthermore, no data is as yet available on the mean glomerular generation counts at differing stages in gestation. Perhaps of greatest importance, the relation between glomerular count and total renal

glomerular number is also unknown, and thus the possible clinical relevance of a particular (reduced) glomerular count to renal function is largely a matter for speculation.

In conclusion, although renal weight remains the most commonly used parameter for the simple estimation of renal growth (Schulz et al., 1962; Tanimura et al., 1971; Potter & Craig, 1975; Burdi et al., 1981; Risdon, 1981a; Wigglesworth, 1984a; Keeling, 1987; Shepard et al., 1988) its sensitivity for detecting deviations from normality, e.g. hypoplasia, is restricted. Counting the number of glomerular generations associated with parallel bundles of collecting ducts in the cortex has, potentially, considerable advantages. However this technique needs to be validated against an unbiased estimate of total nephron number, and expected values and prediction intervals for its progressive increase through gestation reported. Only with a comprehensive evaluation of this technique will it be possible to increase the resolution of the histopathologist for the detection of subtle degrees of hypoplasia which may be predicted to occur (Scott, 1987), but have not been consistently recognised, in antenatal obstruction of the urinary tract.

1.2.2. Stereology - a brief overview

Stereology may be defined as "that body of techniques which is capable of turning measurements made on two-dimensional, histological sections into unbiased, quantitative information about three-dimensional, microscopical structures" (Cruz-Orive, 1990) or alternatively as "three-dimensional interpretation of flat images or extrapolation from two to three-dimensional space" (Elias, 1967). Recent development of techniques such as Confocal microscopy (1.2.3.4.) has additionally enabled direct three-dimensional measurement.

1.2.2.1. Model-based techniques

In the past, three-dimensional measurements have been made using a number of methods which require very strict model assumptions about the size and geometrical shape of the structures under examination (for a review see Cruz-Orive, 1983). In addition to being extremely inefficient, usually requiring many hundreds or even thousands of measurements before obtaining a stable estimate, estimates thus produced tend to be biased i.e. deviate systematically from the true value.

Such bias results from assumptions about particle shape, truncation (the actual or apparent disappearance of particle segments from the section, "lost caps"), overprojection (the positive thickness of "transparent" sections), and the approximations inherent to the mathematical transformations (De Groot & Bierman, 1983; Cruz-Orive & Hunziker, 1986).

These so-called "model-based" or "unfolding" procedures (Wicksell, 1925, 1926; Weibel & Gomez, 1962; Elias & Hennig, 1967; Saltikov, 1967; Cruz-Orive, 1978; Weibel, 1980a, 1980b) have now been largely assigned to the history books by the development, during the last decade, of techniques which require no assumptions about feature microstructural geometry, orientation or spatial distribution: design-based methods.

1.2.2.2. Design-based techniques

These methods rely entirely on the design of the sampling scheme and have thus been termed "design-based" (for a review see Gundersen, 1986). In marked contrast to model-based methods, design-based stereology produces unbiased information i.e. an accurate estimation of what is actually happening in reality. In addition to their unbiasedness, design-based estimators are extremely efficient, usually requiring around one-tenth of the effort spent in measuring micrographs associated with the earlier model-based techniques (Cruz-Orive & Hunziker, 1986; Gundersen & Jensen, 1987; Howard, 1987).

Using design-based, unbiased estimators it is now possible to assess several useful parameters of microscopical structures or particles on histological sections (Howard, 1986):

1 On single sections of isotropic uniform random orientation - particle volume density, surface density and length density, and mean particle volume in the volume-weighted distribution.

2. On single sections of a preferred orientation

- particle surface, and mean particle volume in the volume-weighted distribution.

3. On pairs of fixed uniform random orientation a known distance apart

- mean number of particles and their mean sizes in the number-weighted distribution.

4. On pairs of sections an unknown distance apart

- total number of particles in a containing space, mean particle volume in both the number- and volume-weighted distributions, and the spread in the particle volume distribution.

It is to be hoped that the availability and ease of application of these novel techniques will facilitate the quantification of pathology, leading, through the accumulation of unbiased and therefore accurate information, to an increased understanding.

Unbiasedness

Considerable emphasis has been placed upon the notion of unbiasedness. But what does it mean? An estimator that is unbiased will, after an infinite number of trials, give the mean of the expectation, i.e. the true answer. A biased estimator will converge (q.v.) on a value which is not the true answer.

Unbiasedness should not be confused with efficiency. It is possible to have a biased estimator which is efficient in that it converges onto a stable ("incorrect") value quickly, just as it is possible to have an inefficient unbiased estimator which eventually converges upon the stable ("correct") value. The only thing that can be said about biased estimators is that by measuring them precisely one guarantees that the correct answer is not found (and, by the same argument, if one measures a biased estimator imprecisely the true answer might, by chance, be stumbled across, although this would not be realised, as bias is invisible and undetectable within any one experiment).

The concept of unbiasedness can be elusive to the non-statistician. Suppose we wish to estimate the number of glomeruli, N, in a kidney with a given sampling procedure. We apply the sampling procedure once and obtain an estimate N^*_1 of N. Suppose now that we could reassemble and 'glue' together the various sections sampled to regain the original kidney. We could now resample the same kidney and arrive at a second estimate N^*_2 of N. Repeating this process would give N^*_3, N^*_4 ... *ad infinitum*. If we collect all these estimates of the fixed number N into a histogram, in the limit we shall obtain the *sampling distribution* corresponding to the chosen sampling procedure, as applied to kidney glomeruli. Like most common distributions, this sampling distribution has a mean and a standard deviation. If the mean of the sampling distribution always coincides with the number N of glomeruli in the kidney, irrespective of the size and shape of the latter, we say that the estimator of N obtained with the chosen sampling procedure is *unbiased* for N.

If a population of kidneys is of interest the property of unbiasedness is invaluable, for the sample mean can be made arbitrarily close to the true population mean by increasing the number of (properly) sampled kidneys. This convergence property is called *convergency* and it is guaranteed (i) if the glomerulus number estimator for each kidney is unbiased, and (ii) if the sample of kidneys is uniform random from the population, and (iii) if the variance among glomerular number is finite (which is always warranted in the present context). If condition (i) fails, then even if the remaining conditions (ii) and (iii) hold, increasing the sample size does not improve the accuracy of the mean, it only increases our confidence in the wrong information.

The concept of unbiasedness has traditionally been ignored in biomedicine. New quantitative methods tend to be adopted only when judged 'reproducible' on large sets of trials in different laboratories, or when performing well in *ad hoc* synthetic phantoms. The underlying belief is: "A new method cannot be adopted by the scientific community until it has been sufficiently validated", without realising that *an unbiased method, unlike an empirical one, neither can, nor need be qualified with data*. Unbiasedness is guaranteed only via mathematical-statistical argument, and an unbiased estimation procedure will act as such on a kidney, a potato or a dish of vermicelli.

1.2.3. Stereological estimation of the total number of structures within an object, with particular reference to glomeruli

From first principles it is evident that an estimate of the total number of any particular structure in a given object, N(structures), may be obtained as the product of the numerical density of those structures within that object (the number of structures per unit volume of object), N_V(struct/object), and the total volume of the object, V(object). Hence:

$$N(\text{structures}) = N_V(\text{struct/object}) \bullet V(\text{object})$$

In the context of estimation of the total number of glomeruli in the cortex of the kidney:

$$N(\text{glom}) = N_V(\text{glom/cortex}) \bullet V(\text{cortex})$$

where N(glom) is the total number of glomeruli in the cortex of the kidney, V(cortex) is the total volume of the kidney, and N_V(glom/cortex) is the numerical density of glomeruli in the cortex.

1.2.3.1. Estimation of object volume by Cavalieri's principle

Unlike an estimate of total kidney volume which may be obtained by displacement, the estimation of renal cortical volume requires an indirect approach. Fortunately Cavalieri (1598-1647), an Italian mathematician, was able to show that the volume of any object, V(object), may be estimated from parallel sections through the object separated by a known distance, d, by summating the areas of all cross-sections of the object, a(cross-sect), and multiplying this by the distance d:

$$V(\text{object}) = \Sigma\ a(\text{cross-sect}) \bullet d$$

This method is completely independent of the orientation of the sections and shape of the object, the only stipulation being that the first section must be randomly positioned within the object (Gundersen & Jensen, 1987).

Application of this principle to the estimation of renal cortical volume is described in detail below (3.3.2., 3.5.4.1.).

1.2.3.2. Estimation of the numerical density of structures within an object: an introduction to the Disector principle

Stereological techniques can be easily understood by direct analogy with statistical methods used in public opinion polls (Hinchliffe et al., 1990). Such polls are conducted in two stages that are independent in a statistical sense. As it is impractical to interrogate every member of the population, the first is to select a sample that is usually uniformly random; that is to say for unbiasedness each member of the population should have an equal chance of being included in the sample. The second stage is to make a measurement on each member of the sample. In a poll, for example, opinion is measured by asking questions. To maintain unbiasedness the questions must be unbiased and not be posed in a biased way. With these procedures it is possible, by sampling a minute proportion of the total population, to obtain a remarkably precise measure of public opinion.

Similarly it is not practical for stereologists to exhaustively assess the entire population of structures contained within an object, e.g. count the total number of glomeruli contained within the total volume of the renal cortex. We need to obtain a reliable estimate of the numerical density of particles by sampling the object and measuring the numerical density of structures within these selected components. In stereology such sampling is almost invariably uniformly (systematically) random. The first step therefore ensures that every unit volume in the object has an equal chance of being in the final sample. On this sample we then take the second step of making measurements, i.e. determine the numerical density of the particles of interest.

Unbiased estimation of the numerical density of structures within objects has, until recently, proved to be a most difficult stereological problem viz. the need to select, i.e. count, particles with a uniform probability which is independent of their size.

Stereologists obtain information about the geometry of a microscopical structure by interrogating it with probes of differing geometries. In this regard, consider throwing zero-dimensional points at random into three-dimensional space. Such point probes will interact with particles contained within that

space in relation to their volume: the larger the structure the greater the probability of a point being found within it. Points thus sample particles in proportion to their volume. Similarly one-dimensional lines sample structures in proportion to their surface, and probes of two-dimensional surfaces (e.g. histological sections) sample in proportion to the length of structures; the dimensions of the probe and the feature selected by the probe sum to three, the dimensions of Euclidean space (Howard, 1990). It is apparent therefore that in order to sample particles within an object with equal probability determined solely by their presence or absence, i.e. proportional to their number (a zero-dimensional property), one requires a three-dimensional probe: volume.

Prior to 1984 and the development of the Disector technique (Sterio, 1984), stereologists were limited to probes of less than three-dimensions and therefore unable to sample structures with a constant probability independent of their size. Thus with a biased selection procedure any subsequent estimations of numerical density, or indeed of any parameter, were doomed to being inaccurate, non-representative indications of the population sampled. However the Disector principle has now, for the first time, provided us with the chance to sample microscopical structures of differing size, e.g. glomeruli, with uniform probability. In essence Sterio's technique compares profiles of the structures of interest in two parallel sections, separated by a distance of less than the minimum structural diameter, thereby sampling the volume contained between those sections. Provided that, as described above, the sampling of tissue, and hence volume, is random (preferably for minimal estimator variance, systematically random) within the object, e.g. renal cortex, the estimate of particle numerical density thus obtained will be an unbiased estimate for the whole population of particles, e.g. glomeruli.

A more detailed explanation of the Disector principle is given below (3.2.3., 3.2.4.) together with practical aspects for the estimation of numerical density of glomeruli within renal cortex (3.3.1., 3.5.3. and 3.5.4.2).

1.2.3.3. The evolution of the Disector principle

As discussed by Bendtsen and Nyengaard (1989) discovery of the Disector technique may be considered more of a "re-discovery", since a similar approach was described by several different investigators during the last one hundred years (Miller & Carlton, 1895; Boycott, 1911; Kittelson, 1917; Thompson, 1932; Rhines, 1967). It is interesting that development of essentially the same counting principle by different individuals occurred largely independently. Bendtsen and Nyengaard (1989) consider this to reflect the lack of emphasis placed on the method and its description by the authors. Furthermore, only in Thompson's paper (1932) is the counting method per se the main issue.

Although Thompson published a detailed description of his methodology (Thompson, 1932), his approach was bedeviled by the necessary requisite of unbiased selection of particle profiles in histological sections. Although he endeavoured to circumvent this problem by measuring the whole section, it was not until 1977 that an unbiased 2D-selecting rule was described (Gundersen, 1977).

1.2.3.4. Other stereological methods for the unbiased estimation of particle number

Sterio's Disector technique (1984) is easily applied to the task of numerical density estimation of convex glomeruli. If, however, structure shape is very irregular, ambiguities may arise about the provenance of profiles in the histological section. The General Requirement (Gundersen, 1986), that structures to be counted can be unambiguously identified as either present or absent in a histological section (3.2.3.), cannot thus be satisfied and it may be necessary to resort to serial sectioning without reconstruction of the object of interest (Cruz-Orive, 1980). An interesting application of this technique, which contrasts well with the relatively simplistic situation inherent to glomerular counting, is the estimation of numerical density of neural synapses (De Groot & Bierman, 1983).

Although the Disector is the procedure of choice if the density of particles within the object is roughly homogenous and tissue

shrinkage is negligible or measurable, variations on the technique have been developed to overcome specific potential problems. If knowledge of section thickness proves particularly inaccessible, for example in the field of electron microscopy, the Selector method may be usefully employed (Cruz-Orive, 1987). This estimator of particle volume, estimates number indirectly by combining Disector-based particle sampling and unbiased estimation of the volume of particles thus selected by the point-sampled intercepts method of Gundersen and Jensen (1985). Similarly, the Fractionator is capable of estimating the total number of particles in a containing space and requires no information on the distance between sections, their thickness or the volume of the object (Gundersen, 1986). This is of particular use if differential tissue shrinkage is a major problem.

Other techniques used in number estimation include Tandem Scanning Light Reflected Microscopy and Confocal Scanning Light Microscopy. Application of the former to the study of bone is reported by Howard et al. (1985). Confocal Microscopy, and its suitability for the examination of both fixed and living tissue, is discussed by White et al. (1987).

1.2.4. Estimation of total glomerular number by acid maceration

As a consequence of the resistance of Bowman's capsule to acid (Rytand, 1938), glomeruli can be isolated by acid maceration of renal tissue. This procedure was first used to estimate the number of glomeruli in a weighted-, as distinct from a volume-, fraction of kidney by Schweigger-Seidel (1865). As a refinement of technique, staining the glomeruli, both during life (Nelson, 1922) and after death (Vimtrup, 1928), was performed. Further endeavours to improve estimation included the sampling of volume-fractions (Kunkel, 1950) and consideration of those glomeruli that do not take up the stain, by estimating the proportion of unstained glomeruli on histological sections (Moritz & Hayman, 1934; Larsson et al., 1980). A comprehensive discussion of the evolution of acid maceration in the context of glomerular number estimation (Bendtsen & Nyengaard, 1989) illustrates well the historical continuity of the methods employed.

Unfortunately, because of the spread of stain when glomeruli are cut (Larsson et al., 1980) and the problem of excessive maceration (Moore, 1931; Larsson et al., 1980), these techniques are biased. As discussed by Bendtsen and Nyengaard (1989), this is clearly demonstrated by comparing the results of Moore (1931) and Moritz and Hayman (1934). Despite using near identical protocols to estimate total glomerular number in normal human kidneys obtained at necropsy, results differ by "a factor of two with negligible overlap between the two groups".

chapter 2 Aims of the study

Management of the child with congenital urinary tract obstruction or vesicoureteral reflux is complex, varies between clinicians and continues to be debated. Indeed, considerable variation in the progression of disease and its relation to therapy exists between patients. This variation could be explained, in part, if kidneys in these conditions were not necessarily uniform at birth but rather showed a spectrum of developmental abnormality, for example hypoplasia and dysplasia.

As a consequence of the lack of simple, objective, histological parameters for quantifying the state of renal development, the pathologist can provide, at present, only limited information about the contribution of any hypoplasia to renal functional impairment in these patients. However, stereological techniques have recently become available which may provide a gold standard against which simpler and more routinely applicable parameters may be analyzed and developed. Such work may facilitate study of the relation between treatment and progression of disease and, through an increased understanding of the pathology, ultimately lead, perhaps, to an improvement in management.

The aims of the studies presented in this thesis may be summarised:

I. Design a method for the accurate, unbiased quantification of renal growth and development, and apply this to the normal process of organogenesis.

II. Quantify renal development under the abnormal circumstances associated with Type II Intrauterine Growth Retardation.

III. Establish histological parameters for renal growth applicable to the study of renal developmental abnormality and postnatally-acquired damage in children with vesicoureteral reflux (with or without associated obstruction).

IV. Quantify the presence and extent of developmental abnormality in nephrectomy specimens from children with vesicoureteral reflux (with or without associated obstruction).

V. Quantify the presence and extent of acquired renal parenchymal loss due to postnatal disease in nephrectomy specimens from children with vesicoureteral reflux (with or without associated obstruction).

VI. Investigate the presence and extent of focal and segmental glomerulosclerosis in relation to developmental abnormalities and postnatally-acquired renal cortical loss.

chapter 3 Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the "disector" method and cavalieri principle.

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3.1 Summary

The "Disector" method, a stereological procedure unbiased by feature size, shape or tissue processing methods, for the estimation of total glomerular number was performed on pairs of human kidneys from 11 normal spontaneous second trimester abortions and stillbirths (15-40 weeks' gestation). In addition, gestational age-dependent patterns of change in the average volume of the nephron and its cortical and medullary segments were analyzed. Mean glomerular number, plateauing at 36 weeks, increased from 15,000 at 15 weeks to 740,000 at 40 weeks. Average volume of the medullary nephron segment (Henle's loop) increased throughout pregnancy. Average volume of the cortical nephron segment (Tubuli Contorti) decreased from 15 weeks to 25 weeks, then increased after 36 weeks. Fractional volume of the renal cortex decreased from 15 weeks to 40 weeks. 3-4 hours of microscopical analysis time were required per kidney on routinely processed 5 micron H&E stained paraffin sections. Average coefficient of error for number estimation was 8.02%. Average intra- and interobserver reproducibilities were 96.8% and 93.7% respectively. The demonstrated temporal differences in the development of the cortical and medullary nephron components may result in a dissociation of function, which may explain the increased incidence of Fetal Hydrops in the second trimester of pregnancy, and which must be taken into account in the treatment of (very) premature infants. Although the number of kidneys included in this study is limited, as they reflect the whole period of antenatal development relevant to Neonatal Intensive Care, the Disector method of glomerular number estimation shows significant potential for the analysis and increased understanding of the development of renal function. The method appears to be more sensitive in detecting small and early deviations from normal renal growth and development than previously available parameters e.g., renal weight and (cortical) volume.

3.2. Introduction

3.2.1. Background

Renal growth and functional development, which play an important role in the neonatal intensive care of preterm infants, were first analyzed in depth by Osathanondh and Potter (1963a, 1963b, 1963c). However, due to the labour intensiveness of their microdissection procedure, renal weight has remained the parameter in common use for clinical assessment of renal growth (Wigglesworth, 1984a; Keeling, 1987). That renal weight is often compromised by oedema and passive venous engorgement is reflected in reference tables for this parameter, dating from 1960 (Gruenwald & Minh, 1960) but still in use today (Wigglesworth, 1984a; Keeling, 1987), which feature coefficients of variation as large as 50%. Even recently published growth curves for renal development reflect this problem (Shepard et al., 1988). Data of Landing and Hughes, for example, suggests that a statistically confident diagnosis of hypoplasia requires a combined renal weight of less than half the average value for age (Landing & Hughes, 1962).

3.2.2. Glomerular development

Renal (spare) functional capacity depends on the number of nephrons. Nephron, i.e. glomerular number may be considered therefore a primary parameter for following renal growth and predicting function. No known relation exists between renal weight and the number of glomeruli, and estimates of glomerular number have therefore for many years been derived from a variety of other methods e.g. physical isolation of glomeruli by acid maceration and indirect number estimation on independent

or serial sections. Unfortunately these methods have been shown to have some degree of bias (Bendtsen & Nyengaard, 1989).

Information on the increase in length or size of individual nephrons is also of interest as glomerular induction precedes the subsequent growth of the other nephronic components. Initially the nephron grows by descent and lengthening of Henle's loop alone, after which in a second phase growth continues by increase in glomerular size, development of the cortical convoluted tubules and continued lengthening of Henle's loop (Osathanondh & Potter, 1963c). The relative timing of these events will influence the balance of renal function, as filtration, concentration and secretion are localised to these different nephron segments. The dissection methods of Potter do not allow for extensive quantitative studies of these phenomena. However changes in total renal, cortical and medullary volumes, combined with an analysis of total glomerular number would allow for the study of, at least, the average volume of the complete nephron and its cortical and medullary components.

3.2.3. Stereology

Bendtsen and Nyengaard's account of the development of modern stereological counting methods has shown that the techniques specified above for assessment of glomerular number have some degree of bias, the extent of which is usually "impossible to determine" (Bendtsen & Nyengaard, 1989).

In marked contrast the recently described "Disector" stereological technique, when used together with Cavalieri's principle, permits a direct, unbiased estimation of particle (e.g. glomerular) number - with one absolute condition: that the particles to be counted can be unambiguously identified as either present or absent in a histological section (Sterio, 1984; Gundersen, 1986). In this regard, additional to the general uncertainty accompanying such decisions on marginal transsections of particles, discrimination is required in renal cortex between nephrogenic mesenchyme and structures evidently a developing glomerulus. However, provided objective criteria are used the method remains applicable (3.5.4.2.).

Using this two-stage technique an estimate of the total number of glomeruli in the cortex of a kidney, N(glom), may be obtained as the product of the estimates of cortical volume, V(cortex), and numerical density of glomeruli in the cortex, N_V(glom/cortex) (Sterio, 1984; Gundersen, 1986):

$$N(glom) = N_V(glom/cortex) \bullet V(cortex)$$

This estimate of glomerular number is unaffected by the shrinkage associated with formalin fixation and paraffin embedding, and by other factors e.g. oedema, passive engorgement and maceration, provided that the individual parameters N_V(glom/cortex) and V(cortex) are themselves unbiased, and that both estimations refer to the same, identical (e.g. postparaffin embedding) volume (Sterio, 1984; Gundersen, 1986; Pakkenberg & Gundersen, 1988).

The quotients V(total)/N(glom), V(cortex)/N(glom) and V(medulla)/N(glom) (where V(total) and V(medulla) are the total renal and medullary volumes respectively) can be derived using the same technique, although these parameters of average total nephron-, cortical nephron segment- and medullary nephron segment volume will be biased by shrinkage-affected volume estimates (Pakkenberg & Gundersen, 1988). However assuming no relevant, gestational age variation in the proportion of tissue shrinkage, trends in these parameters may still be usefully analyzed.

3.2.4. The Disector method

The Disector method, in essence, uses two parallel section planes (a "disector pair") as a three-dimensional probe for the estimation of zero-dimensional cardinality.

Because larger particles have a greater chance of being hit by a single section than smaller particles, the number of (glomerular) profiles seen in a single section has no straightforward relationship to their real number in the 3-D reference space (kidney) (Pakkenberg & Gundersen, 1988). However, the probability that a particle is hit by one section but not by a parallel section is the same for both large and

small particles - if a given particle is present in one, but not in the parallel section, it must "begin" in the sample volume enclosed by the two sections. As all particles have only one "beginning" in relation to any specific direction of parallel sectioning, the number of particles, large or small, related to a sample volume is identical to the number of "beginnings". Particles of arbitrary size can thus be counted unambiguously by comparing two parallel sections: "reference" and "look-up". Such sections should be separated by a distance smaller than the minimum particle height to avoid "missing" particles in the space between the two facing planes of the disector pair. Thus the "disector height", h, must be smaller than the minimum particle height, H'(Figure 3-1). This issue is not relevant if a translucent matrix allows for the use of thicker, adjacent sections with an adequate "disector height", as no particles can then be "missed". Using either variant particles are sampled with a uniform probability in 3-D space and the set of particles obtained constitutes a representative, uniform sample of all particles.

Glomerular profiles are therefore compared in two parallel sections of the kidney. Counting the glomerular profiles seen in the first (reference) but not in the second (look-up) section (Figure 3-1, profiles F and G) identifies the number of glomeruli which have their "beginnings" within the sample volume contained between the bottom faces of the two sections. Glomerular profiles seen in both sections of the disector pair (C,D and E) or profiles present in the look-up but not in the reference section (A and B) are not counted. The number of glomeruli within a single calculable sample volume, i.e. the glomerular numerical density, is thus established. To compensate for regional inhomogeneity of glomerular distribution and therefore obtain a value for this parameter, N_V(glom/cortex), representative of the whole kidney, more sample volumes need to be assessed. This is best carried out (Gundersen & Jensen, 1987; Pakkenberg & Gundersen, 1988) by repeating the above procedure over a systematically randomised, i.e. parallel and equidistant, series of disector pairs throughout the kidney (Figure 3-2). Although sectioning perpendicular to the short axis reduces the sectioning workload, if development of both medulla and cortex is to be studied, adequate representation of both components is best achieved by sectioning perpendicular to the longitudinal axis. Renal cortical volume, V(cortex), is estimated using Cavalieri's principle. A point-counted estimate of cortical trans-sectional area, conveniently obtained from the first section of each disector pair, is multiplied by the (perpendicular) disector distance, m, between the bottom surfaces of the first sections of adjacent disector pairs (Figure 3-2).

This estimator of particle number is, in common with other modern stereological

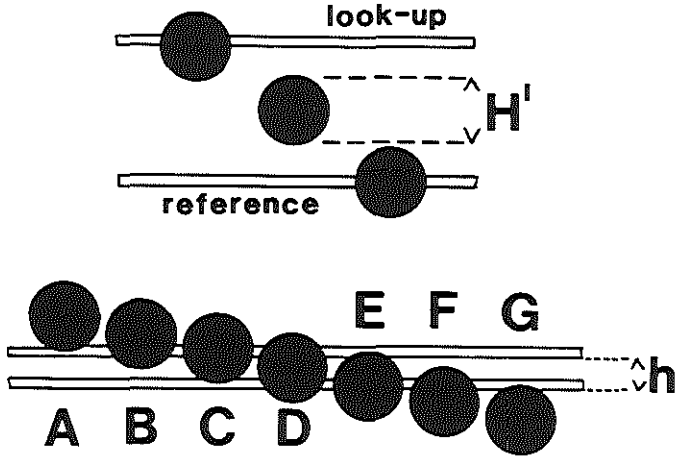

Figure 3-1. Figure showing major principles involved in "Disector" method. Top half illustrates phenomenon of "missed" particles if disector height, h is greater than minimum particle height, H'. Bottom half illustrates the possible combinations of profiles which can be observed in disector pairs. See text for explanation.

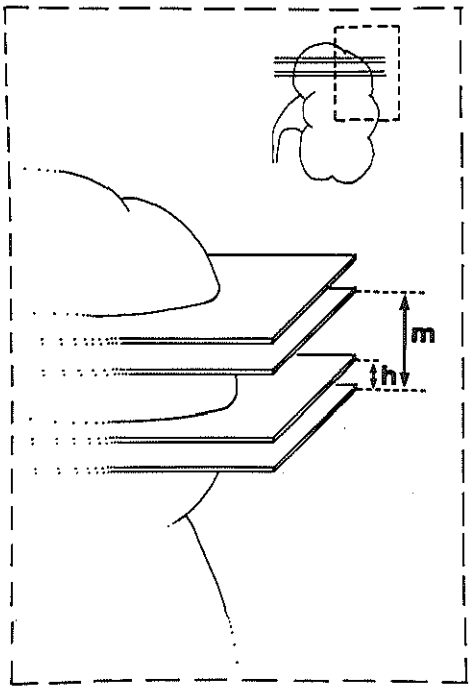

Figure 3-2. Figure showing sectioning procedure for construction of consecutive disector pairs. Sectioning direction is perpendicular to longitudinal axis of kidney (upper half), beginning at inferior pole. Lower half of figure shows 2 consecutive disector pairs, with indication of disector height, h, and disector distance, m. See text for explanation.

techniques, extremely efficient (Gundersen et al., 1988a, 1988b). It has been repeatedly shown that, to obtain a coefficient of error for the number estimate of less than 15%, less than 200 particles (glomeruli) need to be counted in an object (kidney). The procedure can be efficiently performed on as few as ten disector pairs per object (Gundersen & Jensen, 1987; Pakkenberg & Gundersen, 1988).

In view of the potential advantages of the Disector technique, we applied this method to a representative sample of 11 pairs of human kidneys obtained from spontaneous second trimester abortions and stillbirths, without congenital abnormalities or evidence of intrauterine growth retardation, referred for routine pathological examination.

3.3. Experimental Design

3.3.1. N_V(glom/cortex) estimation

The coefficient of error for this parameter is determined by the (in)homogeneity of glomerular distribution, number of microscopical fields analyzed and total number of glomeruli counted (Gundersen & Jensen, 1987; Pakkenberg & Gundersen, 1988). Based on a pilot study, it was decided to estimate N_V(glom/cortex) on roughly 30 cortical fields per kidney. A minimum number of n = 10 disector pairs was chosen. The optimal disector height, which depends on both minimal size and numerical density of particles to be counted (Sterio, 1984; Gundersen, 1986), was found to be 10 microns. At this separation every 6th to 9th glomerulus in the reference frame was counted i.e. a minimum total of 150 glomeruli per kidney. Immature glomeruli included in the sample (see 3.5.4.2. for selection criteria) were always larger than 10 microns in diameter, eliminating any possible bias in their selection.

3.3.2. V(total), V(cortex) and V(medulla) estimation

Although the Cavalieri volume procedure is completely independent of object shape and orientation of the set of transsections (Gundersen et al., 1988a), the associated

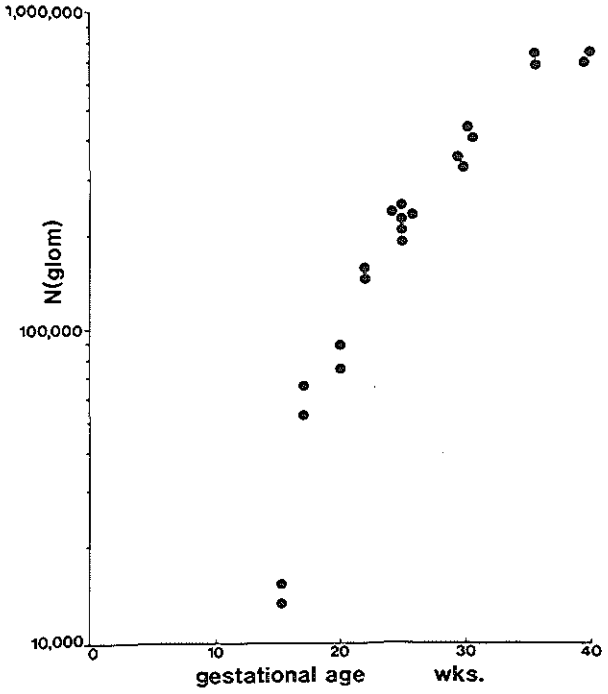

Figure 3-3. Figure showing the relation between total glomerular number, N(glom) (Y-axis, logarithmic plot) and gestational age (X-axis, linear plot).

coefficient of error is influenced by the irregularity of the object and density of grid points on the projection area (Gundersen & Jensen, 1987). Based on a pilot study, it was decided to estimate volumes on all n reference sections in each kidney.

3.4. Results and Discussion

3.4.1. Methodology

Estimation of total renal glomerular number, with a coefficient of error <10%, required approximately 3.5 hours of analysis per kidney.

Calculated coefficients of error (Table 3-1), were in agreement with those predicted by Gundersen and Jensen (1987). Average intraobserver reproducibility of the two methods (area point-counting and numerical density estimation) for both observers was 96.8% (range: 94-99%). Average interobserver reproducibility of the two methods for both observers was 93.7% (range: 90-95%). Reproducibility was least good, 94% and 90% for intra- and interobserver respectively in smaller kidneys (<22 weeks' gestation).

In part this may be explained by the development, with gestation, of a more regular renal shape which increases the reproducibility of Cavalieri's method. In addition, the immature glomeruli in the nephrogenic zone are prone to subjective interpretation and reproducibility of numerical density estimation may be reduced. The width of this zone decreases with gestation. With advancing gestational age and the concomitant increase in width of mature cortex, the number of sample areas which include nephrogenic zone tissue will decrease, and if immature glomeruli are included they will form a smaller part of the area sampled, both factors contributing towards increased reproducibility.

Table 3-1. Coefficients of error for stereological estimates.

	mean %	range %
CE[V]	3.32	2.36-4.50
CE[N_V(glom/cortex)]	7.00	3.22-9.76
CE[N(glom)]	8.01	4.78-10.5

3.4.2. Glomerular number

Results of glomerular number estimation are given in Table 3-2. N(glom) increases from 15,000 at 15 weeks' gestation to 740,000 by 40 weeks (Figure 3-3). The growth curve shows a gradually decreasing slope with increasing gestation. Rate of increase of glomerular number is greatest at 15-17 weeks, corresponding to the start of Osathanondh and Potter's "second period". This explosive increase in N(glom) reflects the pattern of ampullary induction of glomerular arcades peculiar to this period, where several glomerular generations develop from their connecting tubules at the same time (Osathanondh & Potter, 1963c).

Table 3-2. Results of stereological analysis of renal growth.

gest. age	renal weight	V (total)	V (cortex)	V (medulla)	N_V (glom/ cortex)	N (glom)
weeks	grams	mm^3	mm^3	mm^3	mm^{-3}	10^3
15 L	0.253	122	97	25	161	15.5
R	0.223	98	80	17	167	13.9
17 L	0.895	431	343	88	157	53.9
R	0.795	460	342	119	197	67.2
20 L	1.46	563	386	177	197	76.1
R	1.38	609	411	198	223	91.7
22 L	1.97	1270	854	413	183	156
R	1.76	1210	819	387	187	152
25 L tw1	2.72	1450	978	475	226	229
R	2.60	1440	954	489	242	230
L tw2	2.75	1370	903	472	243	219
R	2.73	1490	962	524	237	228
25 L	3.35	1760	1230	527	201	248
R	2.97	1490	1090	404	214	232
30 L	4.77	2970	1760	1200	196	345
R	3.81	2740	1700	1020	207	352
30.5 L	5.00	2740	1890	851	233	439
R	5.36	2790	1960	835	223	436
36 L	11.0	5640	3400	2240	210	714
R	12.2	6080	3760	2320	199	748
40 L	16.2	8030	4600	3430	159	731
R	15.8	8150	4270	3880	175	747

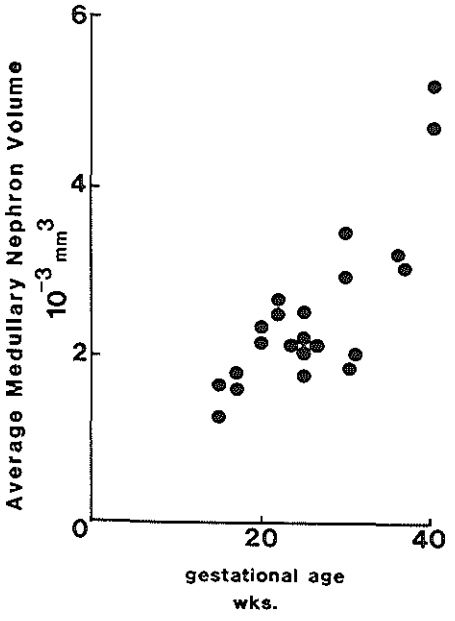

Figure 3-4. Figure showing the relation between average volume of the medullary component of the nephron, AMNV (Y-axis) and gestational age (X-axis).

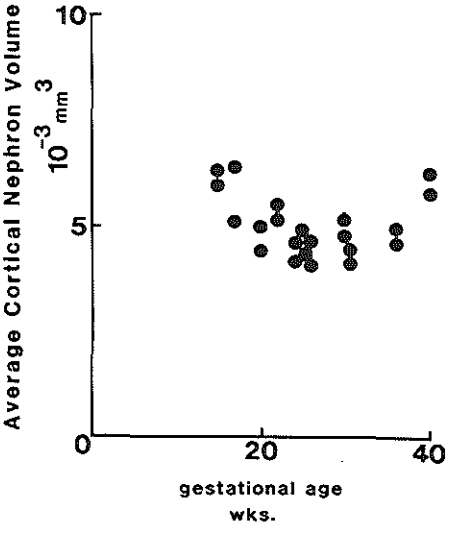

Figure 3-5. Figure showing the relation between average volume of the cortical component of the nephron, ACNV (Y-axis) and gestational age (X-axis).

It is interesting that the mean glomerular number estimate of the 4 kidneys from the homozygous twins (25 weeks) is only 5.4% less than that of the singleton of the same age (Table 3-2). This contrasts with a difference in mean renal weight of 14.6% and does not indicate twinning-associated retardation of renal growth in this respect, although a difference in nephron outgrowth, analogous to the reduced bodyweight generally encountered in twins, may be present.

A deceleration in the rate of increase of glomerular number, seemingly into a plateau, is found at approximately 36 weeks' gestation. This change to a plateau should correspond to the beginning of Potter's "fourth period", defined as commencing when the ampullae, the growth centres of the nephron arcade systems, disappear. Osathanondh and Potter (1963a, 1963b, 1963c) reported this to occur between 32-36 weeks. Thus, even though our dating of this change is based on only 2 cases, there may be a 4 week discrepancy in the timing of this event and the precise beginning of this period remains uncertain. However the dating of Osathanondh and Potter is based on microdissection, establishing the disappearance of ampullae in a limited number of arcade systems per kidney. This results in an estimated period within which this change occurs and may reflect the potential subjectiveness of this assessment. An unbiased glomerular number estimate, representative of the whole kidney, may be considered a more sensitive parameter with which to detect this moment in renal development. We suggest therefore that this alteration in the pattern of growth more probably occurs at 36 weeks' gestation.

It is interesting that the left-right (L-R) difference in glomerular number of specimens over 22 weeks is systematically less than that of renal weight (2.93, 8.90 respectively). This interrenal (L-R) reproducibility may be partially explained by the resistance of the number estimation to confounders such as oedema, which may affect both kidneys in an individual differently. In younger kidneys, probably reflecting the reduced reproducibility in classifying immature glomeruli, differences in number and weight are comparable (13.9, 10.9 respectively).

3.4.3. Nephron volume

As can be seen by comparing the values of preprocessing renal weight and postprocessing total renal volume, processing incurs a shrinkage of approximately 50% (Table 3-1). However there is only a small variation, and thus changes in nephron volume with time may be analyzed.

The average volume of the medullary nephron segment, AMNV (Figure 3-4), increases from $1.46 \times 10^{-3} mm^3$ at 15 weeks' gestation to $4.96 \times 10^{-3} mm^3$ at 40 weeks ($p<0.05$ for 15-25 weeks' gestation versus 30-40 weeks' gestation), reflecting continuous lengthening of the ever increasing number of Henle's loops descending down into the papilla.

The average volume of the cortical nephron segment, ACNV (Figure 3-5), decreases from $6.12 \times 10^{-3} mm^3$ at 15 weeks' gestation to $4.46 \times 10^{-3} mm^3$ at 25 weeks ($p<0.05$ for 15-22 weeks' gestation versus 25-36 weeks' gestation), then increases to $6.02 \times 10^{-3} mm^3$ at 40 weeks (not statistically analyzed in view of limited numbers). The initial decrease in this biphasic curve, corresponding to the period of greatest glomerular induction (Figure 3-3), suggests that the decrease in ACNV due to the induction of glomeruli is greater than the increase in volume associated with (subsequent) development of the cortical convoluted tubules of those nephrons already present.

The average volume of the whole nephron, ANV, shows no significant change until 36 weeks (range 6.23-$8.57 \times 10^{-3} mm^3$, Figure 3-6). After this an increase in ANV is suggested, coinciding with the abrupt plateau for glomerular number (Figure 3-3).

Interpretation of the relationship between Figures 3-4, 3-5 and 3-6, is facilitated by analysis of (cortical) glomerular numerical density estimates (Table 3-1, and Figure 3-7). This parameter may be affected by oedema, which may explain the wider range of values observed than for glomerular number. N_V(glom/cortex) increases to a maximum at 25 ($p<0.05$ for 15-22 weeks' gestation versus 25-36 weeks' gestation), with a subsequent decrease (not statistically analyzed in view of limited numbers). This decrease is in agreement with the experience of perinatal pathologists who generally see kidneys from this latter period, and

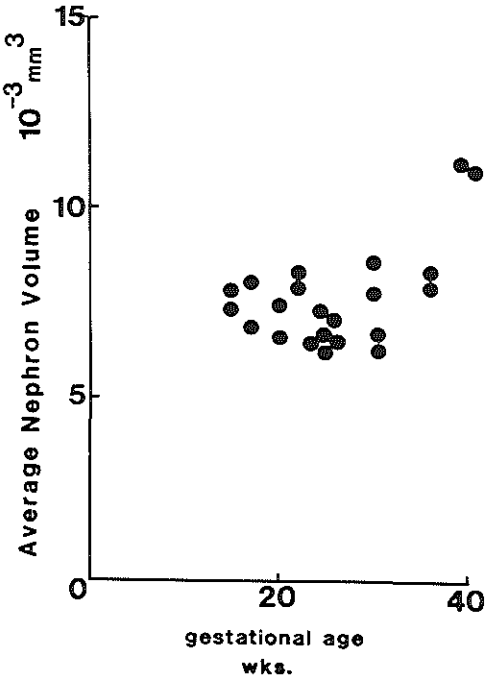

Figure 3-6. Figure showing the relation between average total nephron volume, ANV (Y-axis) and gestational age (X-axis).

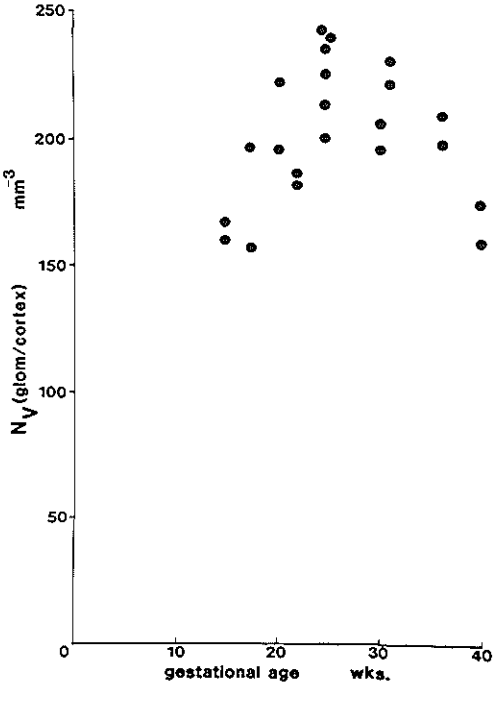

Figure 3-7. Figure showing the relation between glomerular numerical density, N_V(glom/cortex) (Y-axis) and gestational age (X-axis).

to whom a continuous increase in the proportion of nonglomerular cortical tissue is evident on sections. In contrast the initial increase in numerical density has not been described, although this feature may be less apparent than the other features for which these very immature kidneys are examined. As ACNV is calculated from cortical volume and glomerular number, it is not surprising that ACNV and N_V exhibit reciprocal relations (compare Figures 3-5 and 3-7). Subjective pathologist appraisal relates better to N_V. However these results illustrate nicely the improved statistical stability of volume assessment (ACNV) over N_V, and demonstrate why such an objective quantitative parameter should be used in preference.

The changes in cortical and medullary volume are also relevant. To minimise bias due to oedema, cortical volume was expressed as a percentage of total renal volume (Figure 3-8). The percentage of cortical volume decreases in a linear fashion with advancing gestation ($p<0.05$ for 15-22 weeks' gestation versus 25-40 weeks' gestation), suggesting once again a dissociation between the development of the cortical and medullary components of the nephron.

In view of the above we should like to propose the following phases in human (post-15 week) renal development, although for the last phase we presently have only limited information:

1. Population Period (15-25 weeks)
   Generation of new nephrons i.e. glomeruli is the dominant feature and the average cortical structure becomes progressively less mature.
2. Lag Period (25-36 weeks)
   The effects of immature nephron formation are becoming balanced by the increasing growth of the (cortical component of the) nephron.
3. Growth Period (36-40 weeks)
   Cortical nephron segment growth becomes dominant to the induction of nephrons which has reached a plateau.

The Population and Lag phases coincide in

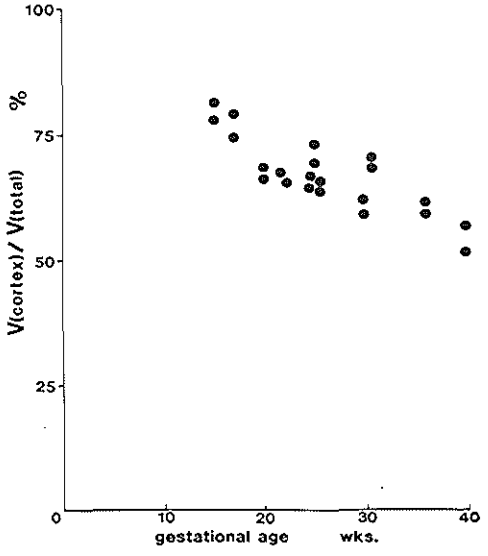

Figure 3-8. Figure showing the relation between renal cortical volume expressed as a percentage of total renal volume (Y-axis) and gestational age (X-axis).

general with Potter's "second" and "third periods", although we time changes in the pattern of renal growth to occur slightly later, at approximately 25 and 36 weeks.

Oedema will positively bias analysis of the average volume of the nephron and its cortical and medullary segments. However, assumption of an equal effect on cortical and medullary tissues allows study of the relative growth of the separate nephron components, especially if trends are followed or groups of kidneys of a given age are compared. Within these limitations, an interesting temporal relation exists between the lowest value for the percentage cortical volume in this study, reached at 40 weeks, and the maximum value for numerical density at 25 weeks. This suggests that it requires at least 15 weeks for the development of the cortical nephron segment to catch up with that of the medullary segment.

This may have interesting consequences for renal function as a whole. Filtration is related to glomerular number. Volume control and electrolyte retention are mainly dependent on Henle's loop, whereas filtration, selective secretion and reabsorption are dependent on the cortical components of the nephron.

From Figures 3-4, 3-5 and 3-6 it may be speculated that directly before 25 weeks there is a strong increase in filtration capacity which in the following 15 weeks is succeeded by an increase in concentration and volume control capability. The increase in reabsorption takes place much later. A relative limiting effect on retention of colloids in the "Lag period" may be related to the preponderance of cases of Fetal Hydrops in the second trimester of pregnancy.

In summary we may conclude that although the number of kidneys included in this study is limited, as they reflect the whole period of antenatal development relevant to Neonatal Intensive Care specialists, the Disector method of absolute glomerular number estimation has shown significant potential for the analysis and increased understanding of the development of renal function.

Assessment of coefficients of error and analysis of reproducibility suggests that the method is more sensitive in detecting small and early deviations from normal renal growth and development than previously available parameters e.g. renal weight and (cortical) volume.

The method should perhaps be applied with caution to very young kidneys until the reproducibility of classifying immature glomeruli is increased, perhaps by the use of more objective criteria such as immune detection of development-associated antigenic determinants (Brown et al., 1989).

3.5. Materials and Methods

3.5.1. Specimen selection

Both kidneys from 11 cases (gestational age 15-40 weeks) of nongrowth retarded, noncongenitally malformed, spontaneous second trimester abortions and stillbirths were collected. 1 pair of nongrowth retarded homozygous twins, gestational age 25 weeks, were included.

3.5.2. Tissue processing

Kidneys were fixed in 0.1 M sodium phosphate-buffered formaldehyde (pH 7.4, 4% formaldehyde) for a minimum of 48 hours, before being embedded whole in paraffin using routine procedures in a Histomat tissue processor.

3.5.3. Section preparation

Specimens were serially sectioned in a transverse plane to generate n ($n \geq 10$) disector pairs per kidney (Table 3-3). Disector height was 10 microns. A systematic random selection of n disector pairs was obtained as follows:
A random number R, $1 \leq R \leq 50$, was looked up in a random number table to provide the (identical) 5 micron section number, into the specimen block, of the first section of the first disector pair in every kidney. Remaining pairs of first and third consecutive 5 micron sections were then selected at regular intervals through the block, the disector distance, m, dependent on the kidney length (Table 3-3). Sections were stained routinely using Haematoxylin and Eosin. On both sections of each pair the corticomedullary junction was delineated using a preparation microscope and pen.

Table 3-3. Stereological sampling scheme data.

gest. age		no. of disector pairs (n)	disector distance (m)	no. of cortical fields (w)	distance between fields
weeks			mm		mm
15	L	11	.75	29	2.12
	R	11	.75	24	2.12
17	L	11	1.21	31	4.24
	R	11	1.33	28	4.24
20	L	11	1.67	25	4.24
	R	11	1.50	30	4.24
22	L	11	1.83	34	6.36
	R	11	1.83	33	6.36
25	L tw1	11	2.08	35	6.36
	R	10	1.95	35	6.36
	L tw2	10	2.00	33	6.36
	R	10	1.95	27	8.48
25	L	11	2.25	30	8.48
	R	10	2.16	28	8.48
30	L	10	2.58	30	10.6
	R	10	2.50	30	10.6
30.5	L	10	2.79	30	10.6
	R	10	2.66	27	12.7
36	L	10	3.08	30	16.9
	R	10	3.25	28	19.1
40	L	10	3.92	29	19.1
	R	10	4.16	28	16.9

3.5.4. Stereological procedures

3.5.4.1. *Estimation of the total/cortical/medullary renal volume*

The total, cortical and medullary volumes, V(total), V(cortex), V(medulla) of each kidney were estimated using Cavalieri's principle (Cavalieri, 1966; Gundersen et al., 1988a). The reference section of each disector pair was projected onto a table using a slide projector with a 45° mirror. The final linear magnification was 13x. A systematic set of points (square-point grid, point distance 11.6 mm) was randomly placed on the projected image and the total sectional area, $\sum^{n} a_i(\text{cortex})$, of cortex in all n reference sections, i=1,2,...,n, was estimated:

$$\sum^{n} a_i(\text{cortex}) = a.(p).\sum^{n} P_i$$

where a(p) was the area corresponding to each point = $(11.6/13)^2 mm^2 = 0.796\ mm^2$ and P_i was the number of points hitting renal cortex on the i th reference section. The cortical volume was estimated:

$$V(\text{cortex}) = m.\sum^{n} a_i(\text{cortex})$$

where m was the disector distance (Table 3-3). Estimates of the total renal and medullary volumes were similarly obtained.

The coefficient of error, CE[V], for each independent volume estimate was calculated (Gundersen & Jensen, 1987; Matheron, 1965):

$$CE[V] = \frac{\sqrt{(3\sum^{n} P_i P_i + \sum^{n-2} P_i P_{i+2} - 4\sum^{n-1} P_i P_{i+1}) / 12}}{\sum^{n} P_i}$$

3.5.4.2. *Estimation of the numerical density of glomeruli in the cortex*

The numerical density of glomeruli in the cortex of each kidney, N_V(glom/cortex), was estimated using the Disector technique (Sterio, 1984). Identical cortical fields from the two sections of each disector pair were viewed simultaneously using two projection microscopes arranged in parallel with a coupled stage mechanism (Howard et al., submitted for publication). The final linear magnification was 170x. An unbiased sampling frame (Gundersen, 1977) of area a(fra) = $(257/170)^2 mm^2 = 2.29\ mm^2$ was randomly placed on the reference field. The number of glomerular tuft profiles sampled in the frame ("disector"), but not present in the look-up field, was counted, Q-. N.B. Transsections of "empty" Bowman's capsule spaces were not included in the sample. This operation was then performed by sampling in the field of the other section and using the first field as the look-up. Developing glomeruli, arising from the nephrogenic zone were counted if the tuft profile demonstrated perfusion by the presence of erythrocytes.

In each kidney a systematic random selection of roughly 30 cortical fields (Table 3-3) was obtained as follows:
The total area of the field of vision, A, was $(360/170)^2 mm^2 = 4.48\ mm^2$ and the total number of non-overlapping cortical fields, M, available for sampling in a kidney was therefore:

$$M = \frac{\sum^{n} a_i(cortex)}{A}$$

The first field sampled had the random number R ($1 \leq R \leq k$) and thereafter every k th field, where k was the nearest integer of M/30, was taken in an two-dimensional systematic random selection as described by Pakkenberg and Gundersen (1988).

The numerical density was:

$$N_V(glom/cortex) = \frac{\Sigma Q-}{v(dis).\ \Sigma D}$$

$\Sigma Q-$ was the total number of glomeruli counted in all disectors in a kidney. ΣD was the total number of disectors sampled in the kidney. v(dis) was the volume of the disector, v(dis) = h.a(fra), where h was the disector height = 0.01 mm.

The coefficient of error, $CE[N_V(glom/cortex)]$, for each numerical density estimate was calculated (Cochran, 1977):

$$CE[N_V(glom/cortex)] = \frac{w}{(w-1).(D)^2} = \sqrt{[\Sigma(Q-)^2 + R\Sigma(D)^2 - 2R\Sigma(Q-.D)]}$$

were w is the number of fields analyzed and $R = \Sigma Q-/\Sigma D$.

3.5.4.3. *Estimation of the total number of glomeruli in the cortex*

An unbiased estimate of the total number of glomeruli in the cortex of each kidney, N(glom), was calculated (Sterio, 1984; Gundersen, 1986):

$$N(glom) = N_V(glom/cortex) \cdot V(cortex)$$

The coefficient of error, CE[N(glom)], of each glomerular number estimate was calculated (Braendgaard et al., 1990):

$$CE[N(glom)] = \sqrt{CE^2[V(cortex)] + CE^2[N_V(glom/cortex)]}$$

3.5.4.4. *Estimation of the average volume of the*
i. (whole) nephron including its glomerulus, ANV
ii. cortical segment of the nephron including its glomerulus, ACNV
iii. medullary segment of the nephron, AMNV

Estimates of the average total nephron, cortical nephron segment, and medullary nephron segment volumes were calculated:

ANV = V(total)/N(glom)
ACNV = V(cortex)/N(glom)
AMNV = V(medulla)/N(glom)

3.5.5. Statistical analysis

Age-related changes in the average total, cortical and medullary nephron segment volumes were analyzed using Wilcoxon's two sided rank-sum test.

3.5.6. Reproducibility control

Intra- and interobserver reproducibility were studied by repeated, blind point-counting of cortical area and Disector estimation of numerical density by two different observers.

chapter 4 The effect of intrauterine growth retardation on the development of renal nephrons

British Journal of Obstetrics and Gynaecology 1992; 99:296-301
S A Hinchliffe, M R J Lynch, P H Sargent, C V Howard, D van Velzen.

4.1. Summary

Objective To investigate the effect of Type II (asymmetrical) Intrauterine Growth Retardation on renal development.
Design Kidneys from a group of (severely) affected stillbirths (n=6) and liveborn infants (postnatal survival < 1 year, n=8) were analyzed using unbiased, reproducible and objective design-based stereological techniques.
Results Nephron number estimates lay below the control group's 5% prediction limit in 5 out of 6 retarded stillbirths, and were significantly ($p<0.01$, IUGR at 65% of the control mean) reduced in the postnatal group. Estimates of nephron (segment) volume did not differ between control and IUGR groups.
Conclusions Type II Intrauterine Growth Retardation may exert a profound effect on renal development. The reduced nephron number at birth, together with the lack of any early postnatal compensation in either nephron number or nephron size, emphasises the need for vigorous antenatal surveillance for IUGR and consideration of elective preterm delivery of affected fetuses. A systematic review of other organs, which develop in a similarly rapid fashion during the late intrauterine period, is indicated by this work. With one exception, retarded cases had a birthweight < 3rd percentile, thus the precise quantitative relation between progressive IUGR and renal function requires further evaluation.

4.2. Introduction

Intrauterine Growth Retardation (IUGR), defined as a birthweight below the 10th percentile for gestational age (Battaglia & Lubchenco, 1967), is associated with a perinatal mortality of up to twelve times (Usher & McLean, 1974; Koops et al., 1982; Callan & Witter, 1990; Kramer et al., 1990) greater than that observed when a birthweight is appropriate - for - gestational - age. Perinatal morbidity is similarly increased by a factor of three (Lin, 1985). Approximately 7% of stillbirths (Magani et al, 1990) and from 2-8% of livebirths (Hobbins & Berkowitz, 1977) are affected, and 80% of all cases are classified as Type II IUGR (Kurjak et al., 1978).

The infant with Type II (asymmetrical) IUGR is characterised by a disproportionately large head, small abdominal viscera and a lack of subcutaneous fat (Gruenwald, 1966; Bruyne, 1966; Winick, 1970; Leeuw, 1973; Usher & McLean, 1974; Kramer et al., 1989). As this form of retardation is unusual before the third trimester (Campbell, 1976), when fetal growth has shifted from a phase of cellular hyperplasia and organogenesis to one of predominantly cellular hypertrophy (Winick & Noble, 1965), individual organ deficits are thought to represent a reduction in cell size rather than in cell number (Usher & McLean, 1974).

However, "organogenesis" of the kidney, in the context of the development of functional units i.e. nephrons, continues into the third trimester with the induction of approximately 60% of the normal complement of nephrons occurring during this period (Hinchliffe et al., 1991). One may hypothesise, therefore, not only qualitative but perhaps also quantitative nephron defects in the kidney in Type II IUGR. Furthermore, as induction of nephrons ceases under normal circumstances by about the 36th week of gestation (Osathanondh & Potter, 1963c; Hinchliffe et al., 1991), the postnatal compensatory growth phase, commonly seen in infants with this form of growth retardation and presumably reflecting (delayed) cellular hypertrophy, may produce only a limited improvement in renal function, based on increased cellular or nephron size rather than increased nephron number. Although no prolonged presence of nephrogenic tissue has been seen in IUGR, suggesting arrest rather than delay of glomerular development, some aspects

of retained immaturity i.e "immature" or "fetal-appearing" glomeruli, have been reported (Wigglesworth, 1984b) in cases of established antenatally-disturbed development. Thus the precise manner in which IUGR may affect renal development requires further clarification.

Recent advances in design-based stereology have permitted, for the first time, the unbiased estimation of total renal nephron number (Sterio, 1984; Gundersen, 1986; Howard, 1990). As we have demonstrated (Hinchliffe et al., 1991) one may also obtain an indication of the average volume of the developing cortical and medullary nephron segments and hence attempt to quantify the development and maturation of the nephron. To analyze the effects of Type II growth retardation on the development of the kidney, in view of a possible contribution of renal insufficiency to IUGR-associated mortality and morbidity, we applied these novel stereological techniques to a group of stillbirths and liveborn infants who demonstrated features of asymmetric IUGR.

4.3. Materials and Methods

4.3.1. The Disector method for the unbiased estimation of total renal nephron (glomerular) number

The Disector method (Sterio, 1984) uses two parallel histological sections (a "disector pair"), separated by a distance less than the minimum glomerular diameter, as a three-dimensional probe for the estimation of zero-dimensional cardinality. By counting those glomerular profiles seen in the first (reference) but not in the second (look-up) section, the number of glomeruli related to the sample volume between the two sections is established. Repeating this procedure over a systematically randomised series of disector pairs throughout the kidney, provides the cortical glomerular numerical density, N_V(glom/cortex). The product of this and the total cortical volume, V(cortex), estimated by the classic point-counting principle of Cavalieri (Sterio, 1984; Gundersen & Jensen, 1987), is the total renal glomerular number:

$$N(glom) = N_V(glom/cortex) \cdot V(cortex)$$

Using the same stereological methods, the quotients V(total)/N(glom), V(cortex)/N(glom) and V(medulla)/N(glom) (where V(total) and V(medulla) are the total renal and medullary volumes respectively) can be derived. These estimates of average total nephron-, cortical nephron segment- and medullary nephron segment volume (more precisely the average "domains" associated with the nephron and its cortical and medullary segments), will be biased by the shrinkage accompanying histological processing. However trend differences between groups of these parameters may be usefully analyzed (Hinchliffe et al., 1991) as approximately the same degree of shrinkage will occur in all specimens, in view of the similar size/volume range and the identical fixation and processing methods.

4.3.2. Case selection

The following case groups were collected consecutively:

4.3.2.1. *Prenatal period*

i. *Type II IUGR* Nonmacerated stillbirths, of accurately determined gestational age, with birthweights below the 10th percentile and displaying features of Type II IUGR (n=6).

ii. *Controls* Nonmacerated, third trimester stillbirths of accurately determined gestational age, with birthweights above the 10th percentile and without congenital malformations. This group was expanded anteriorly with similarly selected spontaneous second trimester abortions (n=11), again with certain gestational age.

4.3.2.2. *Postnatal period*

i. *Type II IUGR Cases* of postnatal deaths in which the birthweight had been below the 10th percentile for the accurately determined gestational age and which displayed features of Type II IUGR at birth and at postmortem examination (n=8).

ii. *Controls* Cases of postnatal deaths in which the birthweight had been above the 10th percentile for the accurately determined gestational age and which were without congenital malformations (n=7).

4.3.3. Specimen selection and tissue processing

On the basis of the stereological results of the prenatal control group, in which both left and right kidneys were analyzed and which are discussed elsewhere (Hinchliffe et al., 1991), it was considered sufficient to study only one kidney, randomly selected (throwing a die: odd number selecting left kidney, even number selecting right kidney), in the postnatal control group. Similarly, and assuming a symmetric effect of IUGR, only one kidney, randomly selected, was used in both pre- and postnatal IUGR groups.

Kidneys of the prenatal groups were fixed in 0.1 M sodium phosphate-buffered formaldehyde (pH 7.4, 4% formaldehyde) for a minimum of 48 hours, before being embedded whole in paraffin using routine procedures in a Histomat tissue processor.

Kidneys of the postnatal groups were fixed in 0.1 M sodium phosphate-buffered formaldehyde (pH 7.4, 4% formaldehyde) for a minimum of 48 hours, before being sectioned into n (n≥10) slices of equal thickness. Slices were embedded separately in paraffin using routine procedures in a Histomat tissue processor.

4.3.4. Section preparation

4.3.4.1. *Prenatal period*

Specimens were serially sectioned in a transverse plane to generate n (n≥10) disector pairs per kidney. Disector height (distance between the same surfaces of the two sections of a disector pair) was 10 microns. A systematic random selection of n disector pairs was obtained as follows: A random number R, 1≤R≤50, was looked up in a random number table to provide the 5 micron section number, into the specimen block, of the first section of the first disector pair in every kidney. Remaining pairs of first and third consecutive 5 micron sections were then selected at regular intervals through the block, the distance dependent on the kidney length after processing.

4.3.4.2. *Postnatal period*

Individual slices were serially sectioned in the same transverse plane to generate one disector pair, per slice. Disector height was 15 microns, reflecting the increased size and decreased numerical density of glomeruli in postnatal, as distinct from prenatal, kidneys. A systematic random selection of n disector pairs (of first and fourth consecutive 5 micron sections) through the kidney was obtained as follows:
A random number R', 1≤R'≤1000, was looked up in a random number table to provide the 5 micron section number, into the specimen block, of the first section of the disector pair in every slice.

Each section was stained routinely with Haematoxylin and Eosin, and the corticomedullary junction was delineated on the slide using a pen and preparation microscope.

4.3.5. Stereological procedures

4.3.5.1. *Estimation of the total/cortical/medullary renal volume*

The total, cortical and medullary volumes, V(total), V(cortex), V(medulla) of each kidney were estimated using Cavalieri's principle (Sterio, 1984; Gundersen & Jensen, 1987). The reference section of each disector pair was projected onto a table using a slide projector with a 45° mirror. The final linear magnification was x13. A systematic set of points (square-point grid, point distance 11.6mm) was randomly placed on the projected image and the total sectional area,

$\sum_{i}^{n} a_i(\text{cortex})$, of cortex in all n reference sections, i=1,2,....,n, was estimated:

$$\sum_{i}^{n} a_i(\text{cortex}) = a.(p).\sum_{i}^{n} P_i$$

where a(p) was the area corresponding to each point = $(11.6/13)^2 mm^2 = 0.796 mm^2$ and P_i was the number of points hitting renal cortex on the i th reference section. The cortical volume was estimated:

$$V(\text{cortex}) = m. \sum_{i}^{n} a_i(\text{cortex})$$

where m was the distance between the bottom surfaces of the first sections of adjacent disector pairs. To ensure the unbiasedness of the estimates of cortical volume and hence glomerular number, this distance was determined, for both pre- and postnatal kidneys, after paraffin embedding. Estimates of the total renal and medullary volumes were similarly obtained.

The coefficient of error for each independent volume estimate was calculated according to Gundersen and Jensen (1987) and found to be ≤5% for every estimate.

4.3.5.2. *Estimation of the numerical density of glomeruli in the cortex*

The numerical density of glomeruli in the cortex of each kidney, N_V(glom/cortex), was estimated using the Disector technique (Sterio, 1984). Identical cortical fields from the two sections of each disector pair were viewed simultaneously using two projection microscopes arranged in parallel with a coupled stage mechanism (Howard et al., submitted for publication). The final linear magnification was x170. An unbiased sampling frame (Gundersen, 1977) of area a(fra) = $(257/170)^2$mm^2 = 2.29mm^2 was randomly placed on the reference field. The number of glomerular tuft profiles sampled in the frame ("disector"), but not present in the look-up field, was counted, Q-. N.B. Trans-sections of "empty" Bowman's capsule spaces were thus not included in the sample. This operation was then performed by sampling in the field of the other section and using the first field as the look-up. Developing glomeruli, arising from the nephrogenic zone were counted if the tuft profile demonstrated perfusion by the presence of erythrocytes.

In each kidney a systematic random selection of roughly 30 cortical fields was obtained as follows: The total area of the field of vision, A, was $(360/170)^2$mm^2 = 4.48mm^2 and the total number of nonoverlapping cortical fields, M, available for sampling in a kidney was therefore:

$$M = \frac{\sum^{n} a_i(\text{cortex})}{A}$$

The first field sampled had the random number R", 1¬≤R"≤k, and thereafter every k th field, where k was the nearest integer of M/30, was taken in an two-dimensional systematic random selection as described by Pakkenberg and Gundersen (1988).

The numerical density was:

$$N_V(\text{glom/cortex}) = \frac{\Sigma Q^-}{v(\text{dis}).\ \Sigma D}$$

where ΣQ^-, the total number of glomeruli counted in all disectors in a kidney, was >150 glomeruli in every case. ΣD was the total number of disectors sampled in the kidney. v(dis) was the volume of the disector, v(dis) = h.a(fra), where h was the disector height.

The coefficient of error for each numerical density estimate was calculated according to Cochran (1977) and found to be <7% for every estimate.

4.3.5.3. *Estimation of the total number of glomeruli in the cortex*

An unbiased estimate of the total number of glomeruli in the cortex of each kidney, N(glom), was calculated (Sterio, 1984; Gundersen, 1986):

$$N(\text{glom}) = N_V(\text{glom/cortex}) \ .\ V(\text{cortex})$$

The coefficient of error of each glomerular number estimate was calculated according to Braendgaard et al. (1990) and found to be <10% for every estimate.

4.3.5.4. *Estimation of the average volume of the*
i. (whole) nephron including its glomerulus, ANV
ii. cortical segment of the nephron including its glomerulus, ACNV
iii. medullary segment of the nephron, AMNV

Estimates of the average total nephron, cortical nephron segment, and medullary nephron segment volumes were calculated:

ANV = V(total) / N(glom)
ACNV = V(cortex) / N(glom)
AMNV = V(medulla) / N(glom)

4.3.6. Statistical analysis

The difference between the mean values of the total glomerular number estimates in the postnatal control and IUGR groups was analyzed using the two-tailed, two-sample t test (Bland, 1987).

The relations between N(glom) and gestational age for the control group, and between ANV, ACNV and AMNV and postnatal age for control and IUGR groups were analyzed by multiple regression using least squares procedures. The techniques are described by Bland (1987) and were performed using the SAS statistical package (SAS Institute Inc., 1985).

4.4. Results

The birthweights of the male and female prenatal IUGR cases are shown in Figure 4-1a and 4-1b respectively. Weights at birth and death of the male and female postnatal IUGR cases are given in Figure 4-2a and 4-2b respectively.

The renal weights of the prenatal cases are given in Figure 4-3. Renal weights of the male and female postnatal cases are given in Figure 4-4a and 4-4b respectively.

The relations between the total glomerular number, N(glom), and age for both pre- and postnatal cases are shown in Figure 4-5a and 4-5b respectively. In the postnatal group, the mean glomerular number of the IUGR cases is highly significantly ($p<0.01$) lower (65% of the control mean) than that of the controls.

The relations between the average total nephron volume, ANV, and age for both pre- and postnatal cases are shown in Figure 4-6a and 4-6b respectively. The relations between the average cortical nephron segment volume, ACNV, and age for both pre- and postnatal cases are shown in Figure 4-7a and 4-7b respectively. The relations between the average medullary nephron segment volume, AMNV, and age for both pre- and postnatal cases are shown in Figure 4-8a and 4-8b respectively. The probabilities of these results, if there is no difference in slope and intercept between postnatal control and IUGR groups, are: $p=0.85$ and 0.88 respectively for ANV, $p=0.71$ and 0.68 respectively for ACNV, and $p=0.72$ and 0.63 respectively for AMNV. In all three cases the regression lines and 95% prediction limits for the control populations are given.

4.5. Discussion

The chief finding of the present study is a reduction of total glomerular, i.e. nephron, number in five out of six stillbirths with Type II IUGR (Figure 4-5a). This may contribute to the increased perinatal morbidity and mortality seen in liveborn infants with IUGR, as a limitation of renal functional reserve may compromise the homeostasis of the individual. Indeed, a reduced glomerular filtration capacity, in addition to helping explain the intrauterine renal failure reported (Steele et al., 1988) in fetuses with severe Type II IUGR, may facilitate understanding of cases where the degree of postnatal renal failure was not fully explained by the severity of perinatal asphyxia. The situation is in some aspects comparable to that of unilateral renal agenesis in which the limitation of renal capacity does not, under normal physiological circumstances, result in increased plasma concentrations of urea or creatinine. However during periods of acute, severe homeostatic stress, such as encountered in perinatal intensive care situations, a limited functional reserve may be expected to be of clinical relevance. Similarly, combined viral and bacterial infection with septicaemia and shock may manifest a reduced homeostatic capacity, especially during the first few months of life when compensatory nephron changes have as yet not occurred (Figures 4-6b, 4-7b and 4-8b).

The magnitude of the difference in total nephron number between control and IUGR groups highlights the sensitivity of the developing kidney to impaired nutritional supply during the period of nephron induction, if one accepts deficient nutrition of the renal blastema as a final common pathway for under-development. In this regard, one may hypothesise that the growth retarded fetus with a normal complement of nephrons (Figure 4-5a, gestational age 36 weeks) had been subject to acute, severe but late-onset IUGR; a process which could result in a similar (reduced) birthweight to that associated with a more insidious insult occurring prior to the loss of the

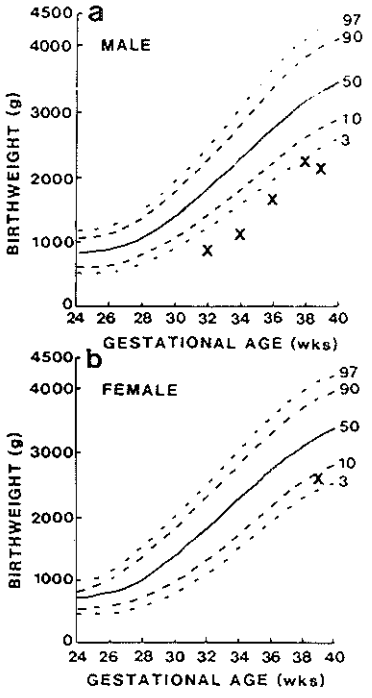

Figure 4-1. Smoothed percentiles (adapted from Yudkin et al., 1987) of birthweight and gestational age, showing the birthweights (crosses) of the male (a), n=5, and female (b), n=1, prenatal IUGR cases.

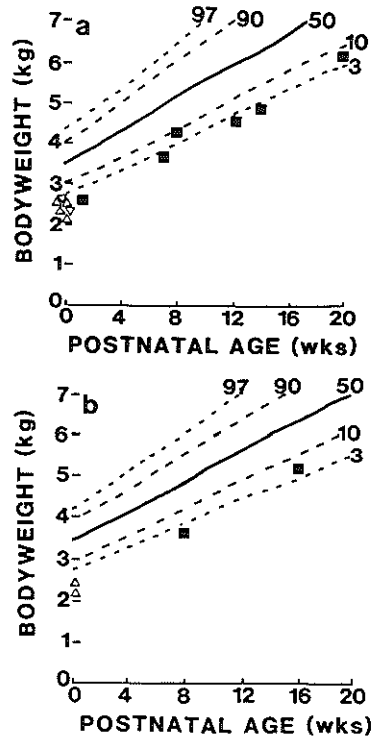

Figure 4-2. Smoothed percentiles (adapted from Tanner et al., 1987) of birthweight and postnatal growth, showing the weights at birth and death of the male (a), n=6, and female (b), n=2, postnatal IUGR cases. Open triangles = weight at birth; closed squares = weight at death.

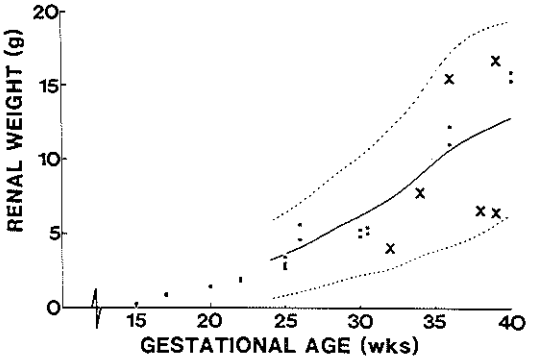

Figure 4-3. Smoothed percentile (adapted from Gruenwald & Minh, 1960) of renal weight and gestational age, showing the renal weights of the prenatal control and IUGR cases. Dots = control; crosses = IUGR.

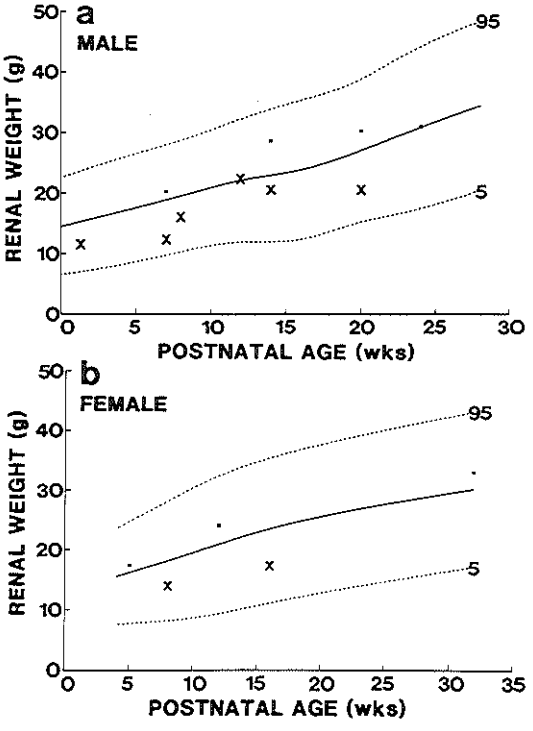

Figure 4-4. Smoothed percentiles (adapted from Schulz et al., 1962) of renal weight and postnatal age, showing the renal weights of the male (a) and female (b) postnatal control and IUGR cases. Dots = control; crosses = IUGR.

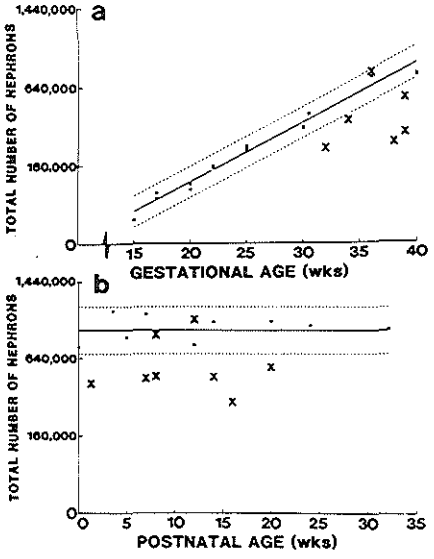

Figure 4-5. (a) Relation between the total glomerular i.e. nephron number, N(glom) (y-axis, square root plot), and gestational age (x-axis) for control and IUGR cases. The regression line and 95% prediction intervals for the control group are plotted. (b) Relation between the total glomerular i.e. nephron number, N(glom) (y-axis, square root plot), and postnatal age (x-axis) for control and IUGR cases. The mean (2SD) for the control group is shown. The difference in number between control and IUGR groups was highly significant: $p<0.01$ (two-tailed, two-sample t test). Dots = control; crosses = IUGR.

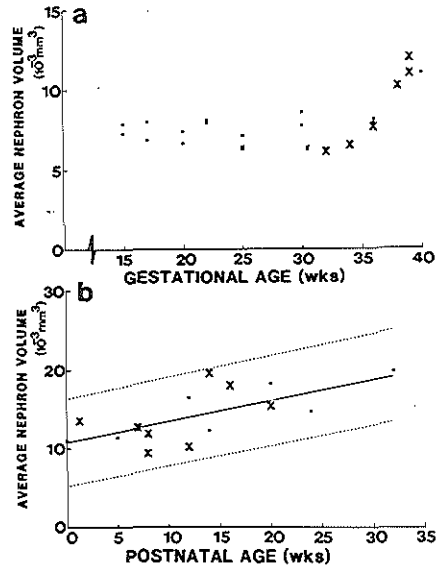

Figure 4-6. (a) Relation between the average total nephron volume (y-axis), ANV, and gestational age (x-axis) for control and IUGR cases. (b) Relation between ANV (y-axis) and postnatal age (x-axis) for control and IUGR cases. Probability of these results if there is no difference in slope and intercept between postnatal control and IUGR groups is p=0.85 and 0.88 respectively (multiple regression). The regression line and 95% prediction intervals for the control population are plotted. Dots = control; crosses = IUGR.

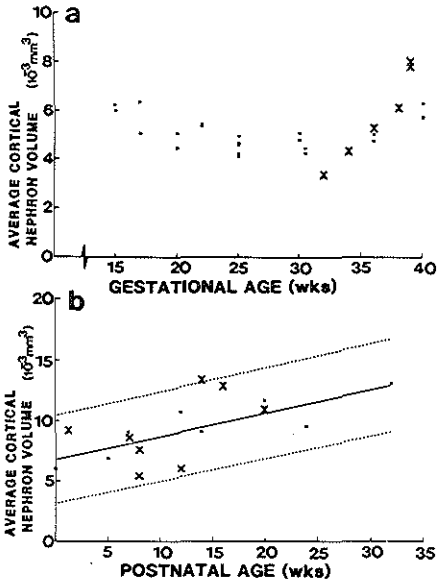

Figure 4-7. (a) Relation between the average cortical nephron segment volume (y-axis), ACNV, and gestational age (x-axis) for control and IUGR cases. (b) Relation between ACNV (y-axis) and postnatal age (x-axis) for control and IUGR cases. Probability of these results if there is no difference in slope and intercept between postnatal control and IUGR groups is p=0.71 and 0.68 respectively (multiple regression). The regression line and 95% prediction intervals for the control population are plotted. Dots = control; crosses = IUGR.

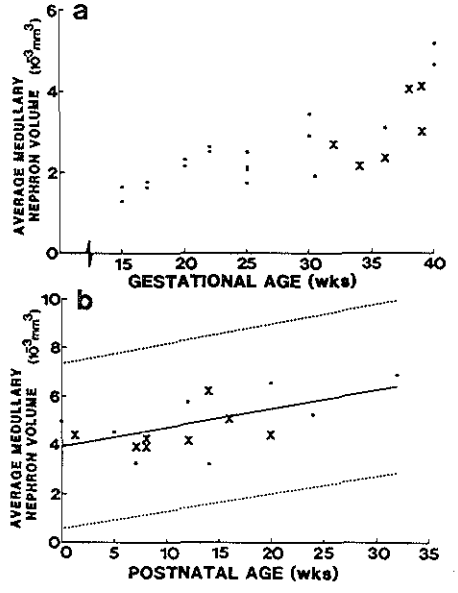

Figure 4-8. (a) Relation between the average medullary nephron segment volume (y-axis), AMNV, and gestational age (x-axis) for control and IUGR cases. (b) Relation between AMNV (y-axis) and postnatal age (x-axis) for control and IUGR cases. Probability of these results if there is no difference in slope and intercept between postnatal control and IUGR groups is p=0.72 and 0.63 respectively (multiple regression). The regression line and 95% prediction intervals for the control population are plotted. Dots = control; crosses = IUGR.

nephrogenic zone and reflected, therefore, in a low number of nephrons.

The absence, postnatally, of a significant compensatory nephron number increase in Type II IUGR (Figure 4-5b) causes concern as it contradicts the general opinion that, when peri-and postnatal complications can be avoided, IUGR is not associated with permanent damage (Fitzhardinge & Steven, 1972; Holmes et al., 1977; Harvey et al., 1982; Ounsted et al., 1982; Low et al., 1982; Westwood et al., 1983).

We feel that these findings emphasise the clinical importance of early prenatal diagnosis in this condition, and provide additional support for the view of other investigators (Chiswick, 1985; Neligan et al., 1976; Vohr et al., 1979) that prenatal therapy or elective preterm delivery of affected fetuses should be considered, to facilitate a resumption of renal development by improving the general fetal condition prior to the loss of the nephrogenic zone. The apparent lack of any compensatory intrauterine hypertrophy in the individual nephron segments (Figures 4-6a, 4-7a and 4-8a) should be interpreted similarly.

As the average volumes of the individual nephron components do not differ significantly between control and IUGR kidneys over the period studied (Figures 4-6, 4-7 and 4-8), the spatial distribution of glomerular profiles seen in histological sections would be similar. This may explain Wigglesworth's conclusions (1984b), based in part on glomerular profile patterns, that IUGR seems not to affect renal development. Furthermore, as the last 400,000 nephrons develop with the emergence of the two most sub-capsular generations of glomeruli, a considerable reduction in nephron number may go unnoticed using the technique of glomerular generation counting, since both intra- and interobserver reproducibility of the method during this period of renal development are limited (Hinchliffe et al., 1992a). It is of interest that the kidneys in growth retarded individuals were of "low normal" weight defined against current tables for normal values; illustrating, as discussed elsewhere (Hinchliffe et al., 1991), the limited potential of renal weight as a parameter for the detection of abnormal development, whilst emphasising the potential of modern stereology for elucidating quantitative aspects of both normal and abnormal development.

In summary we conclude that, at least when of a severe nature, Type II IUGR may exert a profoundly deleterious effect on the development of the kidney. The lack of any significant compensatory increase in either nephron number or nephron size, during the early phases of postnatal life, highlights the need for vigorous antenatal surveillance for IUGR (Sabbagha & Tamura, 1983; Seeds, 1984; Neilson et al., 1984; Hadlock et al., 1984; Ott, 1985). One may predict renal development in affected fetuses to benefit from elective preterm delivery. A systematic review of other organs such as the lung and Central Nervous System, which develop in a similarly rapid fashion during the late intrauterine period, is indicated by this work. The abnormalities demonstrated were found, with one exception, in cases of severe Type II IUGR (birthweight < 3rd percentile), and the precise quantitative relation between a progressive reduction in nephron number and its encroachment on renal reserve capacity similarly requires further elucidation in clinical studies. Early results on comparing quantitative parameters of renal growth by ultrasound with parameters of renal function (Deutinger et al., 1987) indicate the feasibility of in utero assessment of functional limitations of the renal system, which, even when not essential to intrauterine life, may then be used to modulate therapy in the neonatal situation.

chapter 5 "Medullary Ray Glomerular Counting" as a method of assessment of human nephrogenesis

Pathology Research and Practice 1992; 188:775-782

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5.1. Summary

Renal weight (left-right combined), as a parameter of renal development, is required to be less than half the normal value for age for a statistically confident diagnosis of hypoplasia. "Medullary ray glomerular counting" (MRGC), counting cortical glomerular generations, has been proposed as a simple technique of possibly greater sensitivity. Recent development of the Disector method for the unbiased stereological estimation of total glomerular number has provided a, hitherto unavailable, "golden standard" with which to determine the diagnostic potential of MRGC. Both "true" (actual number of generations seen) and "assumed" (a subjective "guess" of the total number of generations) MRGC counts were determined in 11 pairs of kidneys from spontaneously aborted, normally developed, non-malformed fetuses (gestational age: 15-40 weeks). Each kidney was randomly analyzed blind and on two separate occasions by two paediatric pathologists using a written protocol. Results were compared with unbiased stereological estimates of glomerular number. Intra- and interobserver and intra- and inter-(left-right) renal reproducibility were analyzed. In conclusion, MRGC, using "real" counts, is a highly reproducible parameter of renal development from 15-36 weeks' gestation. Sensitivity for detection of both hypoplasia and maturation delay increases with gestational age and generally exceeds that of renal weight.

5.2. Introduction

The presently available parameter for simple estimation of renal growth is total renal weight, obtained at post-mortem and from fetal examination (Wigglesworth, 1984a; Keeling, 1987), which is based on data obtained in 1960 (Gruenwald & Minh, 1960). A more modern analysis of correlation between body weight and renal weight (Shepard et al., 1988) suggests, as does data of Landing and Hughes (1962)[1] that a confident diagnosis of hypoplasia statistically requires a combined renal weight less than half the normal value for age. This parameter of renal weight, which thus seems to have a large biological variation, may additionally be biased by general mechanisms such as oedema and passive venous engorgement. Due in part to the poor quality of the available reference data it is possible that many cases of renal hypo- and hyperplasia remain undetected.

The obvious parameters for assessment of renal growth are nephron (glomerular) number and nephron "quality", expressed for example in nephron length or volume. Techniques used in the past for the estimation of glomerular number, including glomerular isolation by acid maceration, are discussed by Bendtsen and Nyengaard (1989) and are all shown to be biased to varying degrees. Recently, a stereological technique for the unbiased analysis of glomerular number became available (Sterio, 1984) and has been used to study normal renal growth (Hinchliffe et al., 1991)[2]. However, this technique is labour intensive and as yet not easily applied in routine diagnosis. A simple and accurate method for following renal growth thus remains to be identified.

A simple technique has been suggested by Emery (1982) for the assessment of renal age prior to the loss of the nephrogenic zone: "Medullary Ray Glomerular Counting" (MRGC). This method is based on the results of extensive microdissection work by Osathanondh and Potter (1963a, 1963b, 1963c)[3] in which glomeruli were found to develop in a centrifugal manner in association with collecting ducts; the induction of nephrons reflecting the growth of the ampulla. As immature glomeruli are localised to the region immediately adjacent to the kidney capsule - the nephrogenic zone, presence of this region indicates ongoing nephron induction. Therefore, since the induction of nephrons, i.e. glomeruli, ceases at approximately 36 weeks'

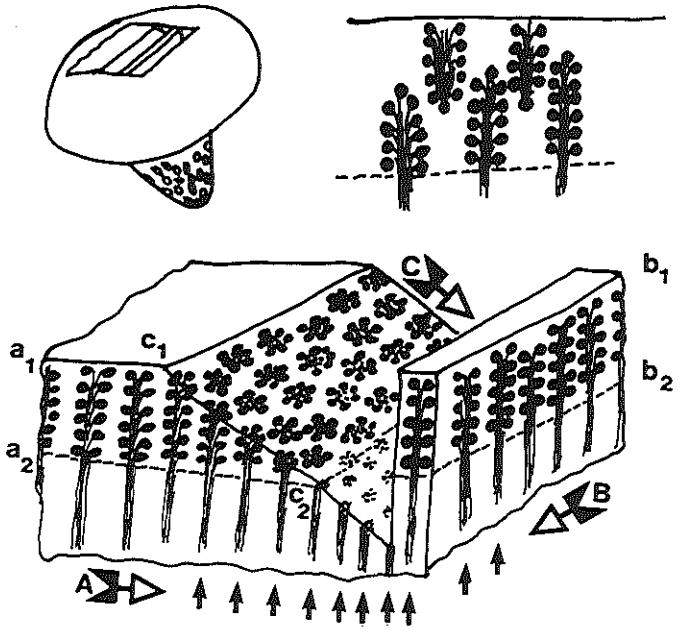


Figure 5-1. Diagram in upper left hand corner shows location of the block of cortex, enlarged below, within a renal lobule. Lower part of the figure shows the arrangement of parallel bundles of collecting ducts ("medullary rays") and their associated glomeruli in a region of cortex in a developing kidney. A and B represent orthogonal, and C sloped sections through the cortex. Medullary rays are seen to traverse the cortex completely in sections orthogonal to the cortex (A,B). In sloped sections (C) no medullary rays are seen, but groups of collecting duct transects are found surrounded by circles of glomeruli. Sections intermediate in inclination between A,B and C (upper right) show incomplete medullary rays that do not span the full width of the cortex. Note dependence of virtual cortical width (a_1a_2, b_1b_2, c_1c_2) on slope of section.

gestation, the absence of the nephrogenic zone is a useful guide to the maturational state of the kidney in this period (Potter & Thierstein, 1943; Valdes-Dapena, 1979).

The method of MRGC assesses renal growth by estimating the number of layers, "generations", of glomeruli that exist between the capsule and the corticomedullary junction, by counting the number of glomeruli associated with individual bundles of collecting ducts, "medullary rays", in the cortex.

Quantifying the number of glomerular generations may be achieved, stereologically, by either completely randomised or systematic sampling of the cortex. To obtain a high Signal-to-Noise ratio in analysis of this anisotropic tissue, systematic sampling is to be preferred. This is best defined in relation to the medullary ray architecture on which glomerular distribution depends. Fortuitously, in addition to this theoretical argument, Emery (1982) had, for practical reasons, already suggested that MRGC use glomeruli arranged along medullary rays as these are easily identifiable histologically.

Figure 5-1 depicts the arrangement of medullary rays and their associated glomeruli in a region of cortex in a developing kidney. Note that only in sections orthogonal to the cortex are medullary rays seen to extend from the medulla through the whole thickness of the cortex. Sloped sections, by comparison, result in incomplete medullary rays and an increase in both the virtual cortical width (from a_1a_2 and b_1b_2 to c_1c_2) and in the number of glomeruli visible between corticomedullary junction and capsule. To obtain a reproducible estimate of the number of glomeruli associated with a medullary ray it is necessary, therefore, to have an orthogonal section i.e. rays must be seen to extend from the corticomedullary junction to the capsule.

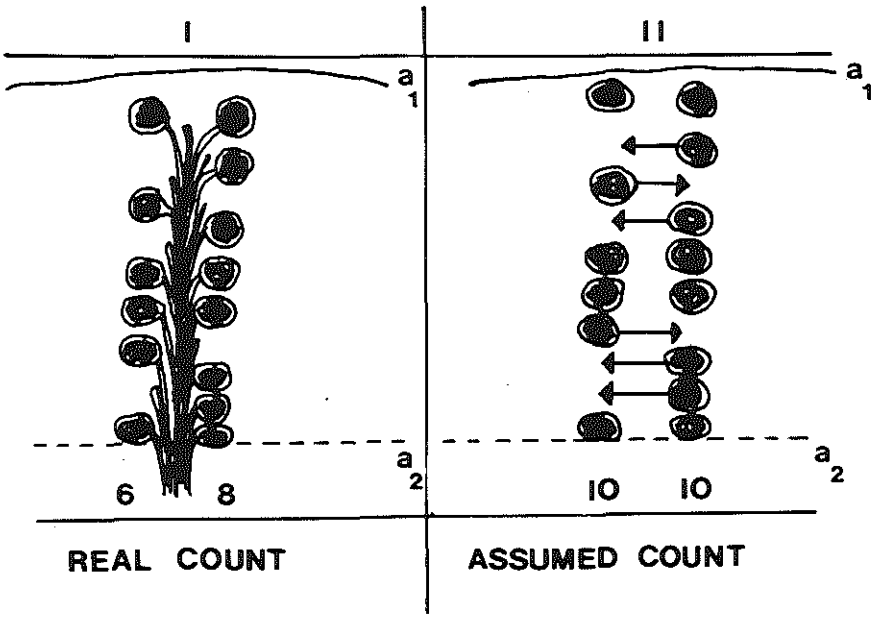


Figure 5-2. Figure showing two alternative methods of assessing the number of glomeruli associated with a complete medullary ray. On left [I] "real count" is achieved by only counting glomeruli actually present to one (6) or other (8) side of a medullary ray. On right [II] "assumed count" (10) is achieved by adding glomeruli obviously missed by chance, to the number of glomeruli actually present.

Figure 5-2, on the left, schematically shows a typical medullary ray and associated glomeruli in an orthogonal section. Gaps along rays where glomeruli were present, but have not been included in the section due to chance, are commonly seen; such gaps usually extend over no more than two "lost" glomeruli. Figure 5-2, on the right, shows a ray where "lost" glomeruli have been artificially superimposed. One therefore count the actual number of glomeruli associated with a ray ("real count") or perform a count which includes an assessment of "lost" glomeruli ("assumed count"). Although the latter probably reflects with greater accuracy the in vivo structure of the kidney, this procedure is necessarily subjective and therefore probably less reproducible than a "real count".

No data is as yet available on the accuracy or reproducibility of MRGC - partly because of the lack, hitherto, of a method for the unbiased estimation of total renal glomerular number, i.e. a "gold standard", with which to correlate the results of "ray counting". As the absolute number of glomeruli for kidneys of varying gestational age is now available (Hinchliffe et al., 1991)' it is now opportune to readdress MRGC.

Aim of the study

To compare, over a representative period of intra-uterine growth of the kidney, the absolute number of glomeruli (determined by Disector-Cavalieri technique (Sterio, 1984; Cavalieri, 1966) with the results of "medullary ray glomerular counting" (MRGC) to determine:

a. the accuracy of MRGC as a parameter of renal growth and development,

b. the period of renal growth for which MRGC is accurate,

c. the intra- and interobserver reproducibility of MRGC,

d. the intra- and inter-(left-right) renal reproducibility of MRGC.

5.3. Materials and Methods

5.3.1. Specimen selection

Both kidneys from 11 cases (gestational

age 15-40 weeks') of nongrowth retarded, noncongenitally malformed, spontaneous second trimester abortions and stillbirths, with acute pathology as the cause of death, were collected. 1 pair of nongrowth retarded homozygous twins, gestational age 25 weeks, were included.

5.3.2. Tissue processing

Kidneys were fixed in 0.1 M sodium phosphate buffered formaldehyde (pH 7.4, 4% formaldehyde) for a minimum of 48 hours, before being embedded whole in paraffin using routine procedures in a Histomat tissue processor.

5.3.3. Section preparation

Specimens were serially sectioned in a transverse plane as previously described elsewhere (Hinchliffe et al., 1991). 5 micron sections were stained routinely with Haematoxylin and Eosin.

5.3.4. Stereological procedures

The total number of glomeruli in the cortex of each kidney was estimated as described by Hinchliffe et al. (1991). The renal cortical volume, V(cortex), was estimated using Cavalieri's principle (Cavalieri, 1966; Gundersen et al., 1988a). The numerical density of glomeruli in the cortex, N_V(glom/cortex), was estimated using the Disector technique of Sterio (1984). An unbiased estimate of the total number of glomeruli in the cortex of each kidney, N(glom), was obtained as the product of the estimates of cortical volume and cortical glomerular numerical density (Sterio, 1984; Gundersen, 1986):

$$N(glom) = N_V(glom/cortex) \cdot V(cortex)$$

5.3.5. MRGC Protocol

5.3.5.1. *Section selection and analysis*

For each kidney, two nonconsecutive 5 micron sections (Sec.1 and 2) with complete medullary rays were selected and randomised. Each section was randomly analyzed blind and twice by two paediatric pathologists (A and B).

5.3.5.2. *Counting protocol*

On each section the number of glomeruli associated with medullary rays was determined using both "real" and "assumed" count techniques, on two separate occasions (Ct. no.1 and no.2) to give the Mean Real and Assumed Counts: a total of 8 counts per kidney per observer. Only medullary rays which completely traversed the cortex were used. In any one section, glomeruli to the same side of the medullary rays were counted for both real and assumed counts.

5.3.5.3. *Sample size assessment*

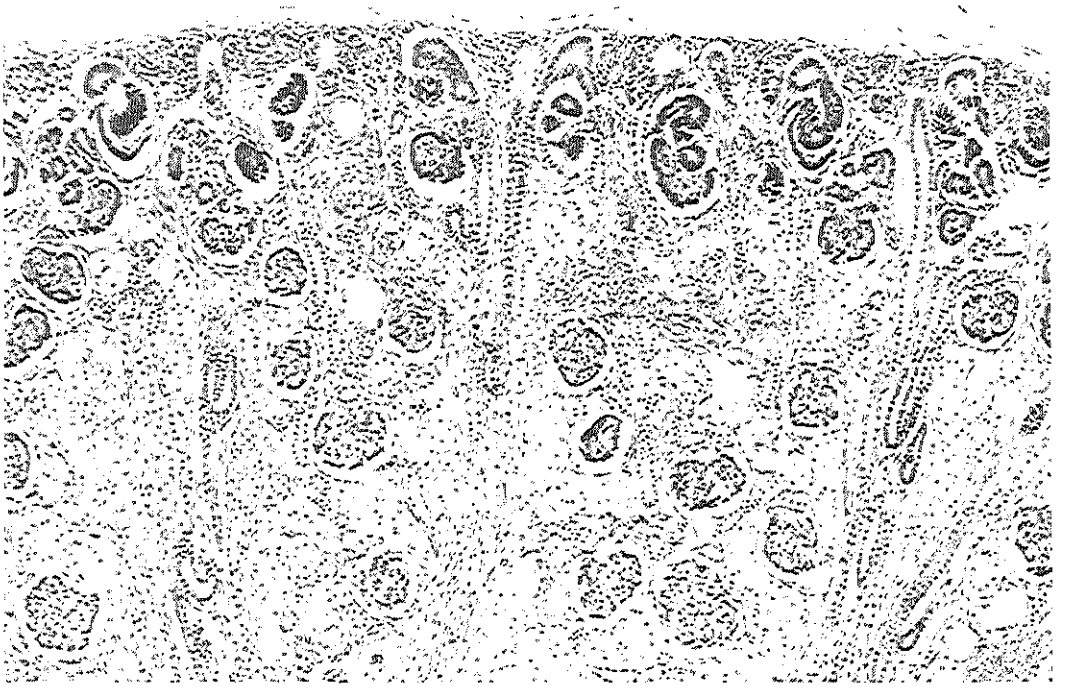
On the basis of a "running mean" analysis (Aherne & Dunhill, 1982) it was decided to count the number of glomeruli associated with 10 medullary rays per section.

5.3.5.4. *Glomerular selection*

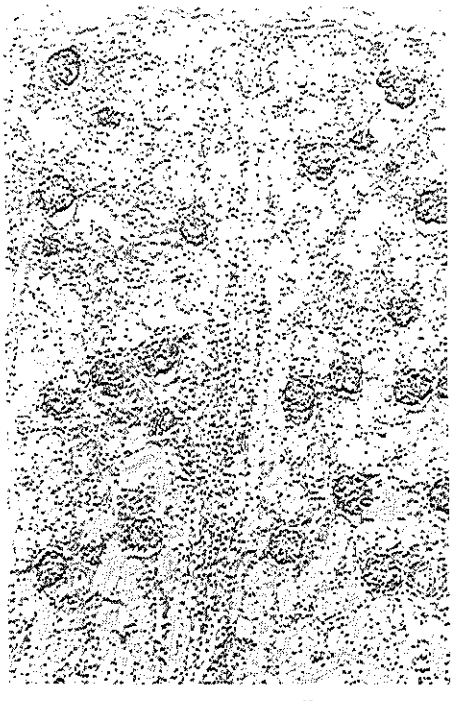
Only "glomeruli" with epithelium of non-fetal type around the glomerular tuft and showing evidence of perfusion were considered as glomeruli for the purpose of this study. In this regard S-shaped neck curves of developing nephrons were not counted.

5.3.6. Reproducibility studies

Reproducibility of MRGC was analyzed by measurements of "agreement" as described by Altman and Bland (1983), thereby avoiding the "inappropriate analysis of correlation" (Bland & Altman, 1986) where a high correlation coefficient may give a false impression of comparability (Serfontein & Jaroszewicz, 1978). In order to assess the reproducibility of a particular parameter, half of the differences between paired observations of the parameter, e.g. Mean Real Ct. of obs.A and Mean Real Ct. of obs.B, were plotted against the means of the paired observations. If there was no obvious relation between the difference and the mean, the "degree of agreement" (Bland & Altman, 1986) between the two observations was assessed by calculating the mean and standard deviation of the differences. If a relation was suggested by the plot, linear regression analysis was performed (Bland, 1987).



Photomicrograph (5 micron paraffin section, H&E stain) of obliquely sectioned renal cortex. Nots incompletaly visible medullary rays either ending or beginning in the middle of the cortex. Microscopical magnification x 50.



Photomicrograph (5 micron paraffin section, H&E stain) of cortex with 10 to 11 generations of glomeruli. Directly adjacent to the medullary ray present in the centre of the image, which runs the full widt of the cortex, 4 glomerular profiles on the left and 3 on the right can be seen. Note that the outermost profiles of either side clearly originate from different glomerular generations. Microscopical magnification x 20.

The following paired observations were analyzed:

a. Intraobserver reproducibility

Real Ct., A:	Real Ct. no.1 and no.2 [RC1(A), RC2(A)]
Real Ct., B:	Real Ct. no.1 and no.2 [RC1(B), RC2(B)]
Assd Ct., A:	Assd Ct. no.1 and no.2 [AC1(A), AC2(A)]
Assd Ct., B:	Assd Ct. no.1 and no.2 [AC1(B), AC2(B)]

b. Interobserver reproducibility

Real Ct.:	Mean Real Ct. of obs.A and obs.B [MRC(A), MRC(B)]
Assd Ct.:	Mean Assd Ct. of obs.A and obs.B [MAC(A), MAC(B)]

c. Intrarenal reproducibility

Real Ct., A:	Real Ct. no.1 on Sec.1 and Sec.2 [RC1S1(A), RC1S2(A)]
Real Ct., B:	Real Ct. no.1 on Sec.1 and Sec.2 [RC1S1(B), RC1S2(B)]
Assd Ct., A:	Assd Ct. no.1 on Sec.1 and Sec.2 [AC1S1(A), AC1S2(A)]
Assd Ct., B:	Assd Ct. no.1 on Sec.1 and Sec.2 [AC1S1(B), AC1S2(B)]

d. Interrenal reproducibility

Real Ct., A:	Left and Right Mean Real Ct. [LMRC(A), RMRC(A)]
Real Ct., B:	Left and Right Mean Real Ct. [LMRC(B), RMRC(B)]
Assd Ct., A:	Left and Right Mean Assd Ct. [LMAC(A), RMAC(A)]
Assd Ct., B:	Left and Right Mean Assd Ct. [LMAC(B), RMAC(B)]

5.3.7. Statistical analysis

The relations between total glomerular number and real and assumed medullary ray counts were analyzed by multiple regression using least squares procedures. The techniques are described by Bland (1987) and were performed using the SAS statistical package (SAS Institute Inc., 1985).
The following models were examined:

$$\log. N(glom) = a_1 + b_1(MRC) + c_1(Obs.A) + d_1(MRCxObs.A)$$

$$\log. N(glom) = a_2 + b_2(MAC) + c_2(Obs.A) + d_2(MACxObs.A)$$

where MRC and MAC were the Mean Real and Assumed Counts respectively. If any term was not significant at the 5% level, the regression was recalculated omitting this variable. For each regression the 95% prediction intervals were derived (Bland, 1987). As explained by Shepard et al.)1988) these differ from confidence intervals and are used, "when one is primarily interested in possible futere single observations, to give an idea of the possible range of single observations from a distribution". Prediction intervals are thus much wider than confidence intervals.

5.4. Results

Table 5-1 gives the Mean Real and Assumed Counts (MRC, MAC) for both observers. These represent the means of 40 observations since Count. no.1 and no.2 are the means of 20 (10 on each of Sec.1 and 2) observations.

Table 5-2 gives the results of the multiple regression analysis. Only those variables significant at the 5% level are included.

Table 5-1. Results of Medullary Ray Glomerular Counts.

gest. age		N(glom)	Mean Real Count		Mean Assumed Count	
weeks		10^3	MRC(A)	MRC(B)	MAC(A)	MAC(B)
15	L	15.5	1.75	1.05	3.05	1.83
	R	13.9	1.90	1.35	2.78	1.86
17	L	53.9	2.35	1.91	4.72	4.25
	R	67.2	2.63	1.92	4.46	3.83
20	L	76.1	2.48	2.62	5.10	3.41
	R	91.7	2.65	2.53	5.13	3.26
22	L	156	3.55	2.60	6.43	4.66
	R	152	3.20	2.56	6.23	5.16
25	L tw1	229	3.35	2.58	6.55	4.14
	R	230	3.41	2.94	6.62	4.42
	L tw2	219	4.18	2.94	7.64	4.86
	R	228	3.93	2.90	6.70	4.62
25	L	248	3.45	3.33	8.04	5.94
	R	232	4.29	3.45	8.18	6.34
30	L	345	4.68	4.22	8.65	6.30
	R	352	4.70	3.93	9.03	6.60
30.5	L	439	4.77	4.32	8.90	6.48
	R	436	4.23	3.76	8.30	5.65
36	L	714	5.68	6.24	10.3	7.26
	R	748	5.25	7.11	10.8	7.99
40	L	731	3.68	4.84	9.95	6.03
	R	747	3.60	4.87	8.85	6.36

Table 5-2. Multiple regression analysis of MRGC and glom. number.

coefficient	coefficient estimate	standard error	p<
a_1	4.15	0.12	0.0001
b_1	0.32	0.03	0.0001
a_2	3.88	0.13	0.0001
b_2	0.28	0.02	0.0001
c_2	-0.10	0.18	0.5893
d_2	-0.07	0.03	0.0309

Log._{10} N(glom) = 4.15 + 0.32(MRC) [Obs.A, Obs.B]

Log._{10} N(glom) = 3.78 + 0.21(MAC) [Obs.A]
Log._{10} N(glom) = 3.88 + 0.28(MAC) [Obs.B]

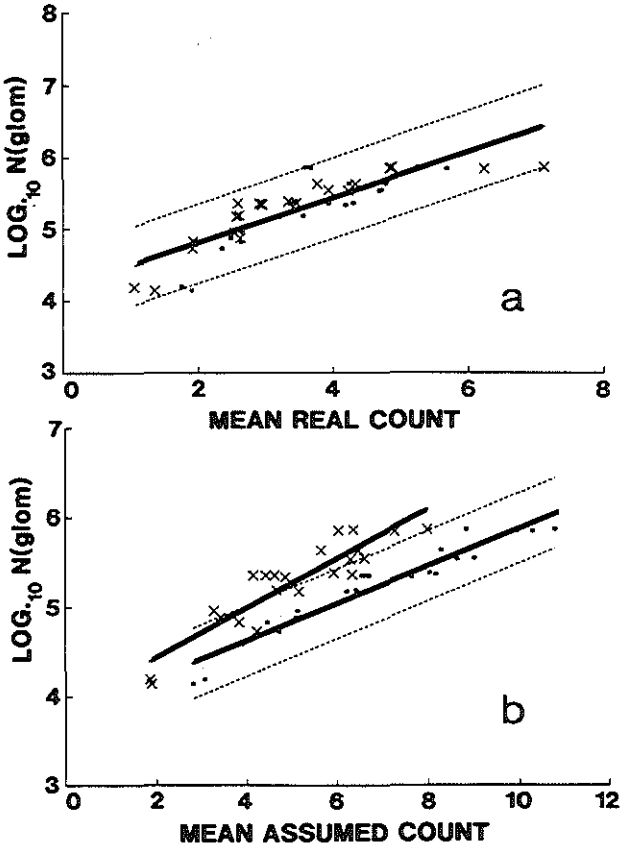


Figure 5-3. Regression lines and 95% prediction intervals describing the relations between total glomerular number (logarithmic plot, y) and Mean Real (a) and Mean Assumed (b) Counts (x) for both observers. Dots = obs.A; crosses = obs.B.

The regression lines describing the relations between the stereological estimate of the total number of glomeruli, N(glom), and the Mean Real and Assumed Counts for both observers, together with their 95% prediction intervals, are shown in Figure 5-3a and 5-3b respectively. Glomerular number may be predicted from real or assumed counts and increases with increasing count. For MRC (Figure 5-3a) there is no significant difference ($p>0.05$) between observers, however for MAC (Figure 5-3b) such a difference is seen.

Results of the reproducibility study are given in Figures 5-4, 5-5, 5-6, and 5-7. In all four intraobserver comparisons of MRGC (Figure 5-4) the means of the differences do not differ noticeably from zero (<0.2 counts).

In analysis of the interobserver reproducibility of real counts (Figure 5-5a) no obvious relation exists between the difference between the mean real counts of the two observers and their mean. However, the mean of the differences is 0.26 counts, suggesting a (small) consistent bias between observers. In analysis of interobserver reproducibility of assumed counts (Figure 5-5b) a clear relation exists between the difference and the mean counts of the observers. The bias between observers increases with increasing assumed count.

In all intra- and interrenal comparisons of MRGC (Figures 5-6 and 5-7) the means of the differences do not differ noticeably from zero (<0.2 counts).

5.5. Discussion

5.5.1. Reproducibility of MRGC

In the analysis of intraobserver "agreement" or reproducibility (Figure 5-4) a difference between individual pairs of counts,

increasing with gestational age to a maximum of approximately 3 glomeruli, may be present for both real and assumed counts of both observers. However, the means of the differences between the paired observations are virtually zero. Reproducibility of MRGC will therefore be increased by performing two counts and using their mean.

Real counts in MRGC analysis are perhaps less attractive than assumed, as they do not directly refer to the "generations" actually present. However in the analysis of interobserver "agreement" or reproducibility (Figure 5-5), real counts lack the considerable systematic bias that is apparent between observers for assumed counts. Therefore assumed counts have no value in comparative studies employing different observers and real counts are the method of choice for clinical use.

In the analysis of intrarenal reproducibility (Figure 5-6) a difference between individual pairs of counts, increasing with gestational age to a maximum of approximately 3 glomeruli, may again be present for both real and assumed counts of both observers. Some increase over the differences found in the intraobserver reproducibility study was to be expected as the difference between analysis of two different locations is added to that caused by repeated analysis by the same observer. The effect seen in this study however is minimal and, once again, the means of the differences between the paired

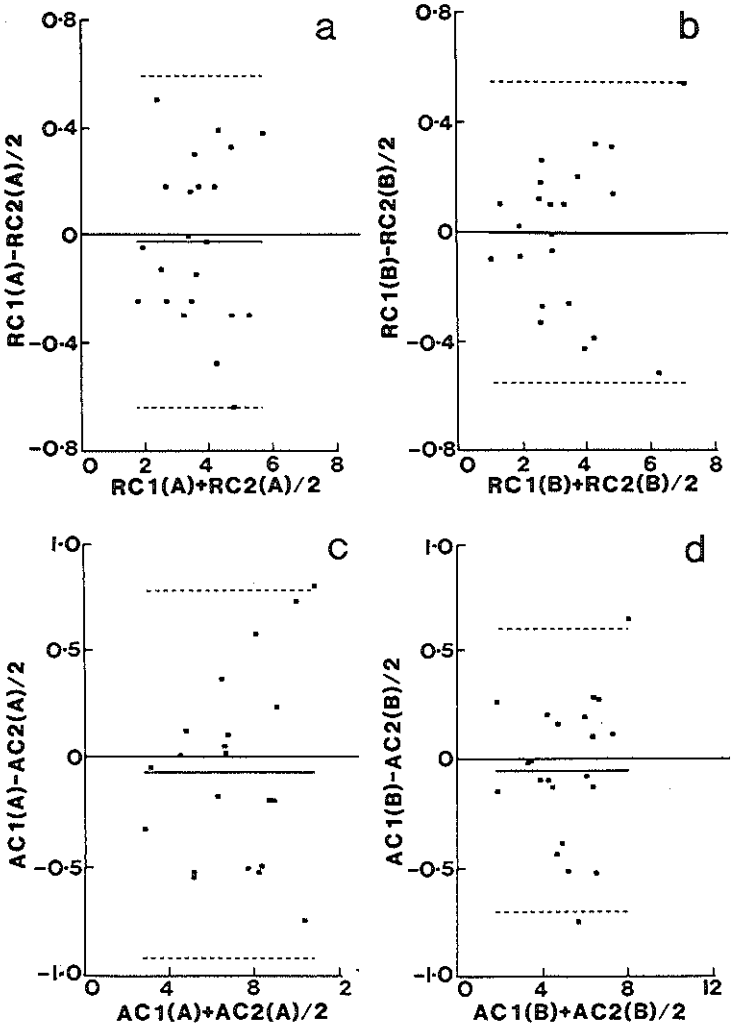


Figure 5-4. Results of the intraobserver reproducibility analysis: real counts, obs.A (a); real counts, obs.B (b); assumed counts, obs.A (c); assumed counts, obs.B (d). Means (2SD) are plotted.

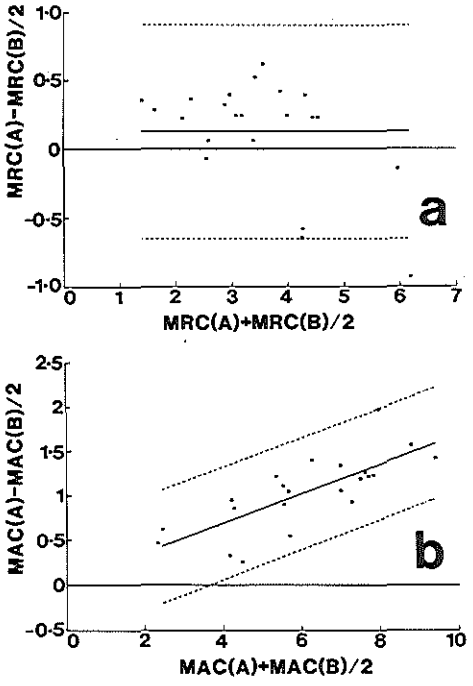


Figure 5-5. Results of the interobserver reproducibility analysis: real counts, mean (2SD) is given (a); assumed counts, regression line and 95% prediction intervals are given (b).

observations remain virtually zero. Reproducibility of MRGC will therefore be increased by performing counts on two sections per kidney and using the mean of these.

The results of interrenal (left-right) reproducibility studies show an expected range of differences, within that observed for the intraobserver and intrarenal analysis. Again the means of the differences are virtually zero for both real and assumed counts.

In summary, comparisons between fetuses are best based on analysis of both kidneys. Each kidney is analyzed by performing real counts (minimum of 10 medullary rays) on two slides per kidney. This procedure is repeated and the mean of the 4 values may be considered a representative parameter for an individual kidney. The mean of both kidneys, or the actual values obtained for each, may be used as representative of the renal development of a given individual.

5.5.2. Application of MRGC

The relations found between MRGC and the "golden standard" of glomerular number (Figure 5-3), indicate that (in agreement with the interobserver reproducibility analysis. Figure 5-5) results obtained by two different observers may be directly compared for real, but not assumed, counts. This was to be expected since, as stated in the Introduction, real counts lack subjectivity. However, the width of the 95% prediction intervals for assumed counts (Figure 5-3) is less than that for real, probably reflecting a strong subjective bias. As this bias is different for the two observers, there is a statistically significant difference ($p<0.05$, Table 5-2) between the two regression lines for mean assumed counts.

Clinical use of parameter sets of this type (gestational age and MRGC) is, in general, bidirectional. MRGC, as an indication of glomerular number, may be predicted from the known gestational age and comparison between the actual and predicted MRGC would allow for the detection of renal hypoplasia. In theory, gestational age may similarly be predicted from the determined MRGC and comparison between the actual and predicted gestational age would allow for the detection of maturation delay (although this is not a commonly assessed parameter, it may be of use in recognising pathogenetically important moments in the

patient's medical history).

In order to investigate the sensitivity of MRGC in this regard, the relations between both real and assumed counts and gestational age were analyzed using the same techniques as for the comparison between glomerular number and MRGC (see 5.3.7. and Table 5-3 for the models and results of multiple regression analysis). The regression lines describing the relations between gestational age and MRGC together with their 95% prediction intervals, are shown in Figure 5-8. A positive correlation is seen between medullary ray counts and gestational age; for MRC (Figure 5-8a) there is no significant difference ($p>0.05$) between observers, however for MAC (Figure 5-8b) such a difference is seen. In contrast to Figure 5-3, the observer bias results in a constant difference between the two (parallel) curves. This could be explained by a systematic difference between observers of inclusion criteria for immature glomeruli, on the inner aspect of the nephrogenic zone.

Using Figure 5-8a, MRC may be predicted from gestational age. At 20 weeks' gestation the lower 95% prediction limit of MRC is <30% of the predicted value, at 40 weeks it is 70%. When the gestational age >28 weeks, the lower limit is >50% of the predicted value. Thus, after 28 weeks' gestation, hypoplasia can be detected by MRGC with greater sensitivity than by renal weight or the index of renal-to-body weight. Similarly, maturation delay may be assessed by prediction of gestational age from MRGC. At the lowest value of MRC the lower 95% prediction limit of gestational age is 30% of the predicted value. However at the highest MRC value the lower limit is 79%.

MRGC thus allows for the diagnosis of both maturation delay and hypoplasia with similar sensitivity in individual cases. When groups of patients are compared, the sensitivity of detection of hypoplasia or maturation delay may be expected to increase considerably.

If higher sensitivity is required, stereological estimation of glomerular number by the Disector-Cavalieri method might be a more appropriate procedure. In this regard, the relation between glomerular number and gestational age is nonlinear (Figure 5-9a) whereas bidirectional predictions are more easily made on linear dependencies. This nonlinear relation may be changed to a more linear form by a logarithmic transformation, by which 92.7% of the total variance can be attributed to the model (Figure 5-9a). A better result is achieved by the,

Table 5-3. Multiple regression analysis of MRGC and gest. age.

coefficient	coefficient estimate	standard error	p<
a_3	9.12	1.84	0.0001
b_3	4.82	0.50	0.0001
a_4	8.95	1.63	0.0001
b_4	3.36	0.29	0.0001
c_4	-6.91	1.24	0.0001

Gest. Age = 9.12 + 4.82(MRC) [Obs.A, Obs.B]

Gest. Age = 2.04 + 3.36(MAC) [Obs.A]
Gest. Age = 8.95 + 3.36(MAC) [Obs.B]

Models:

Gest. Age $= a_3 + b_3(MRC) + c_3(Obs.A) + d_3(MRCxObs.A)$
Gest. Age $= a_4 + b_4(MAC) + c_4(Obs.A) + d_4(MACxObs.A)$

less extreme, square root transformation (97.9% of the total variance is attributable to the model, Figure 5-9b): at the lowest value of gestational age in the study (15 weeks) the lower 95% prediction limit of glomerular number is 53% of the predicted value, at 40 weeks the lower limit is 89%. In predicting gestational age from observed glomerular number (data not shown), the lower 95% prediction limit is 85% of the predicted value at the lower end of the range, 94% at the higher end. Thus, for detection of both hypoplasia and maturation delay, estimation of the absolute glomerular number is a more sensitive method.

In conclusion, provided real counts are performed according to a simple protocol, MRGC is a highly reproducible parameter of renal growth and development. The sensitivity for detection of both hypoplasia and maturation delay increases with gestational age and generally exceeds that of renal weight. The validity of the method has been established for the period of 15 to 36 weeks' gestation. Prediction of glomerular number from MRGC is to be based on real counts only.

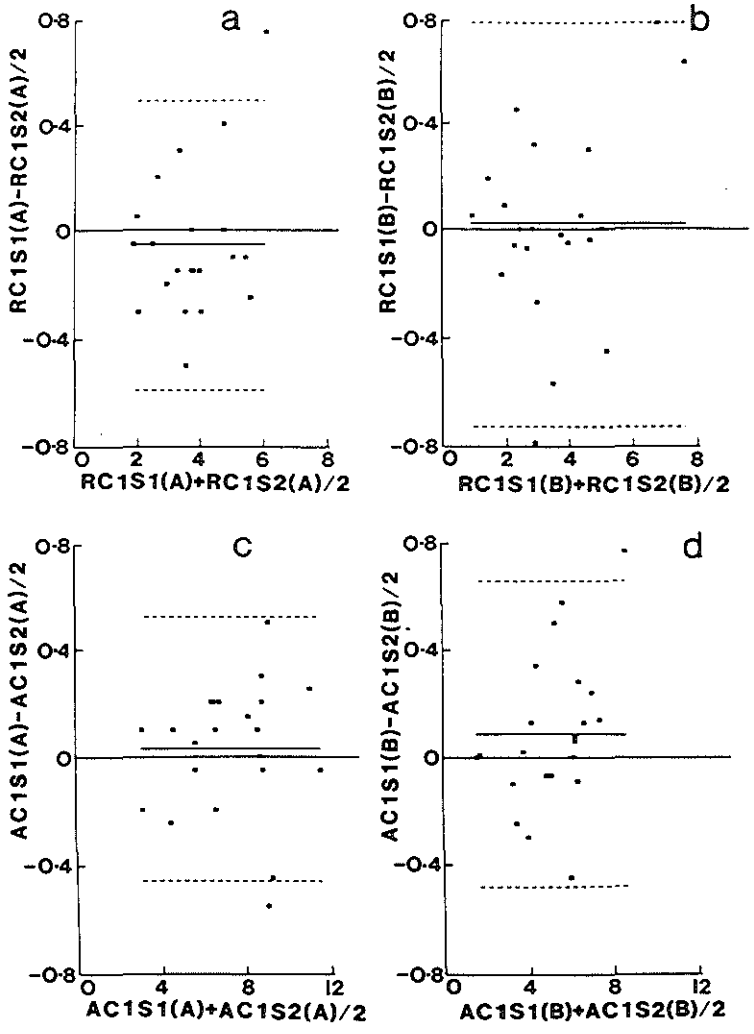


Figure 5-6. Results of the intrarenal reproducibility analysis: real counts, obs.A (a); real counts, obs.B (b); assumed counts, obs.A(c); assumed counts, obs.B (d). Means (2SD) are plotted.

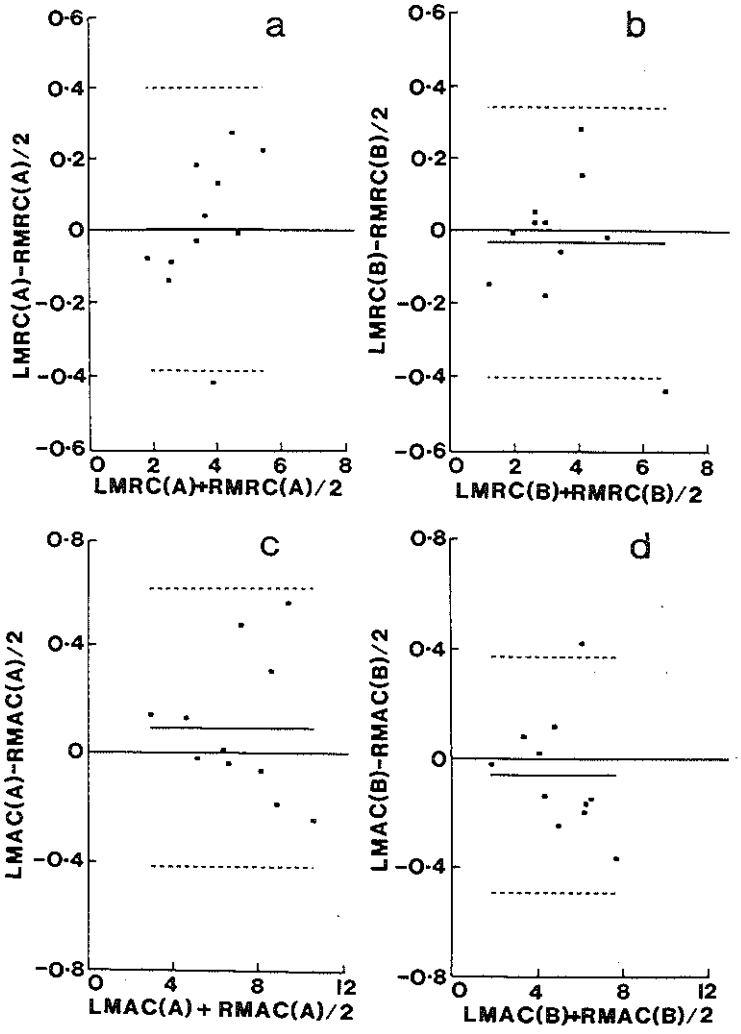


Figure 5-7. Results of the interrenal reproducibility analysis: real counts, obs.A (a); real counts, obs.B (b); assumed counts, obs.A (c); assumed counts, obs.B (d). Means (2SD) are plotted.

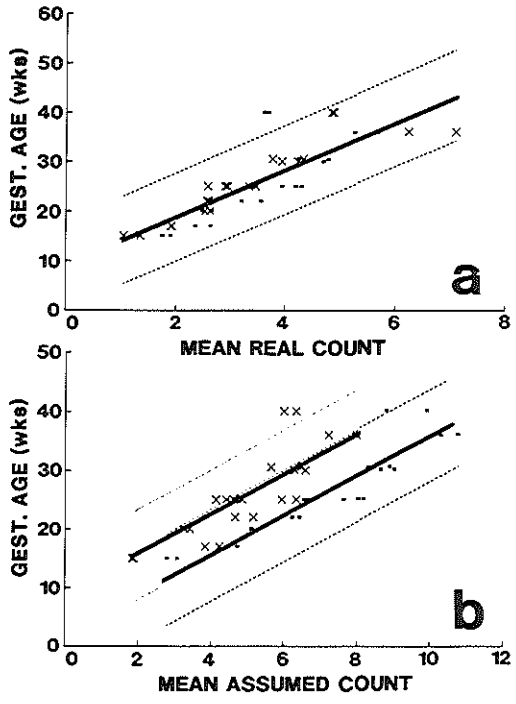


Figure 5-8. Regression lines and 95% prediction intervals describing the relations between gestational age (y) and Mean Real (a) and Mean Assumed (b) Counts (x) for both observers. Dots = obs.A; crosses = obs.B.

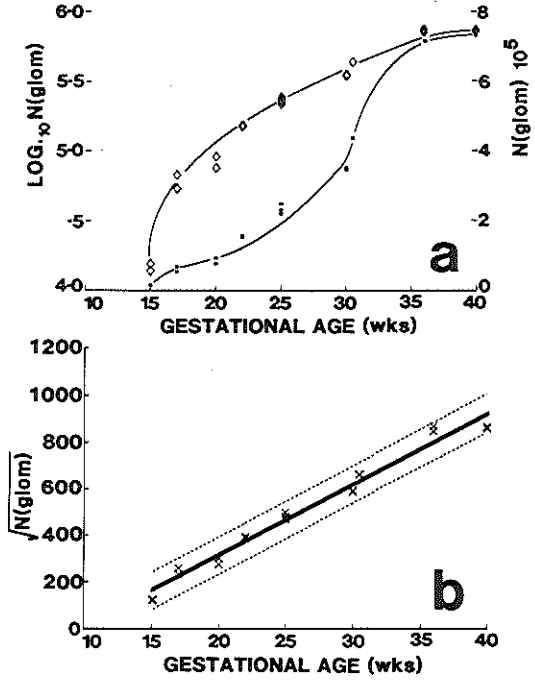


Figure 5-9. (a) Relation between Log.10N(glom) (y1, diamonds), N(glom) (y2, dots) and gestational age (x), best-fit lines are shown. (b) Relation between N(glom) (y) and gestational age (x), regression line and 95% prediction intervals are plotted.

chapter 6 Renal hypoplasia and postnatally-acquired cortical loss in children with vesicoureteral reflux

Pediatric Nephrology 1992; 6:439-444
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6.1. Summary

We reviewed histologically 86 nephrectomy specimens from patients with vesicoureteral reflux (with or without ureterovesical obstruction) to investigate the relationship between coexisting hypoplasia and postnatally-acquired cortical damage. Hypoplasia was assessed independently of the acquired cortical loss using Medullary Ray Glomerular Counting. Severe hypoplasia, glomerular number < 25% of normal, was detected in 47 of 86 patients. These patients underwent nephrectomy at a significantly younger age than those with minimal or no hypoplasia ($p<0.01$). There was no significant relationship between the severity of hypoplasia and the presence or absence of obstruction. Severe acquired cortical loss was found in 68 of 86 patients. There was no significant association between the severity of cortical loss and the presence or absence of obstruction, age at nephrectomy or degree of coexisting hypoplasia. The findings suggest a strong association of hypoplasia and vesicoureteral reflux. Therefore, early postnatal presentation with minimal renal function need not necessarily reflect a failure of management but rather a pre-existing limitation of renal capacity. Furthermore, in a significant proportion of fetuses with ultrasonographic evidence of urinary tract abnormality, renal pathology may be present prior to the time at which in utero surgical intervention may be considered.

6.2. Introduction

Renal hypoplasia, a reduction in the number of glomerular generations in the cortex, has been described in children with vesicoureteral reflux (VUR) (Sommer & Stephens, 1981) and incomplete urethral obstruction due to posterior urethral valves (Henneberry & Stephens, 1980). This developmental abnormality, which is often associated with an ectopically positioned ureteral orifice (Mackie & Stephens, 1975; Wickramasinghe & Stephens, 1977; Henneberry & Stephens, 1980; Sommer & Stephens, 1981), is considered to be expressed homogenously throughout the kidney (Henneberry & Stephens, 1980; Sommer & Stephens, 1981).

In contrast postnatal renal scarring, resulting from the intrarenal reflux of (usually) infected urine into susceptible compound papillae, is typically segmental (Figure 6-1) (Ransley & Risdon, 1975a; Ransley & Risdon, 1979; Scott, 1987). Immediately adjacent areas of cortex remain undamaged for longer periods and, in their regular architecture of glomerular generations, display the developmental status achieved in utero.

A separate histological assessment of the severity of postnatally-acquired cortical loss and any hypoplasia is thus possible, by counting the number of glomerular generations associated with corticomedullary rays in these distinct areas (Henneberry & Stephens, 1980; Sommer & Stephens, 1981; Hinchliffe et al., 1992a). An estimate of the relative contributions of these two pathologies to the functional impairment associated with nephron reduction may thus be obtained. The importance of this may be illustrated: in a kidney with, for example, severe hypoplasia, a minimal volume of "functional cortex" on dimercaptosuccinic acid scan need not necessarily reflect severe, rapidly progressive disease or failure of therapy, but a pre-existing cortical limitation associated with a developmental abnormality.

However the clinical relevance of hypoplasia in children with, for example, vesicoureteral reflux is unclear, since until recently the significance of any specific reduction in glomerular count was largely unknown. Fortunately the recent description of the relationship, during nephrogenesis, between the progressive increase in number of glomerular

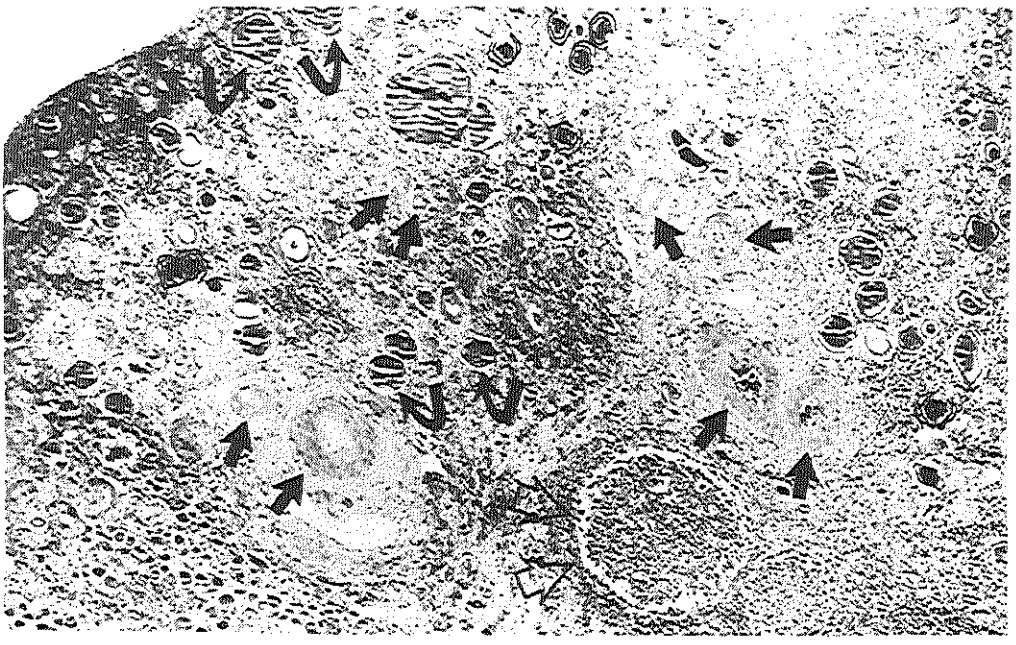

Figure 6-1. Photomicrograph (5 micron paraffin section, H&E stain) of renal cortex affected by intrarenal reflux-associated inflammation. Note lymphoid follicle (open arrows) and dilated duct systems filled with protein resulting in a thyroid-like appearance (curved arrows). Disproportionably thick-walled abnormal arteries indicate previously greater tissue volume and regressive changes after cortical loss (closed arrows) (Scott, 1987; Kincaid-Smith & Hodson, 1979). Microscopical magnification x 20.

generations and the total number of nephrons in the kidney has provided this information (Hinchliffe et al., 1991, 1992a).

The aim of this study was to investigate separately the presence and degree of renal hypoplasia and postnatally-acquired cortical loss associated with vesicoureteral reflux (VUR), with or without obstruction at the ureterovesical junction, using medullary ray glomerular counts.

6.3. Material and Methods

6.3.1. Patient material

From a total of 158 nephrectomy specimens received between January 1980 and December 1990 at the Royal Liverpool Children's Hospital Alder Hey, 86 consecutive specimens were used for the study. These were obtained from patients with a clinical diagnosis of end-stage renal disease due to VUR. Forty-two patients had at least one period of obstruction at the ureterovesical junction. Obstruction was of differing degrees and was often temporary and of varying duration. For the purpose of this study no further stratification was carried out. Cases of prune belly syndrome, those associated with a neurogenic bladder and duplex kidney were not included. Nephrectomies where the only pre-existing abnormality was posterior urethral valves or pelviureteric junction obstruction were similarly excluded.

For the purpose of statistical analysis patients were grouped as:

Group I: < 5 years of age at resection: n = 42
Ia: without obstruction: n = 20
Ib: with obstruction: n = 22
Group II: > 5 years of age at resection: n = 36
IIa: without obstruction: n = 17
IIb: with obstruction: n = 19
nonclassifiable: n = 8
of which 1 patient had obstruction.

(For 8 patients information was conflicting and did not allow for confident allocation to either Group I or II.)

For each specimen, between two and ten histological sections, prepared from formalin-

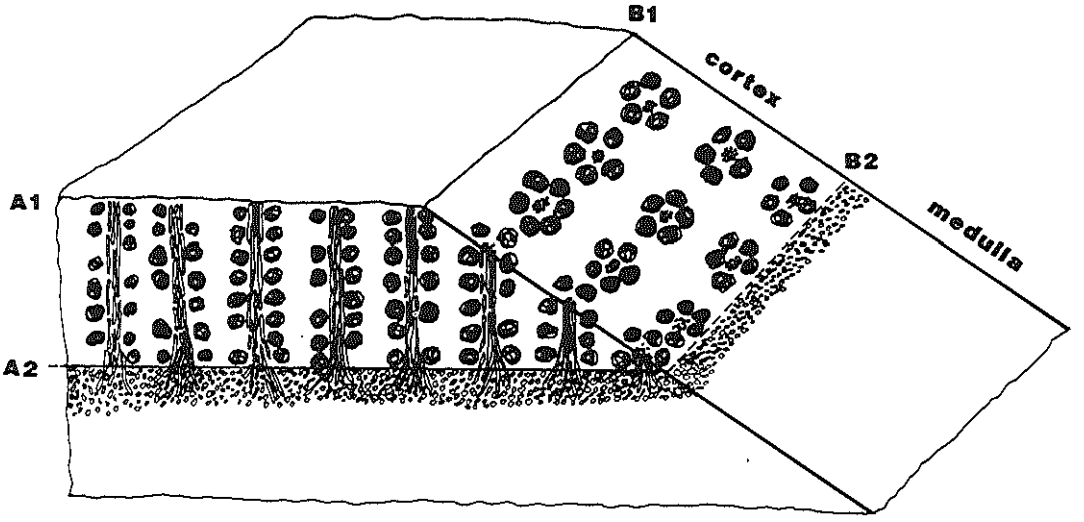

Figure 6-2. (after Hinchliffe et al., 1992a)

The arrangement of medullary rays and their associated glomeruli in a region of renal cortex. Note that only in sections orthogonal to the cortex (A1A2) are medullary rays seen to extend from the medulla through the whole thickness of the cortex. In the sloped section (B1B2) no medullary rays are seen, but groups of collecting duct transects surrounded by circles of glomeruli.

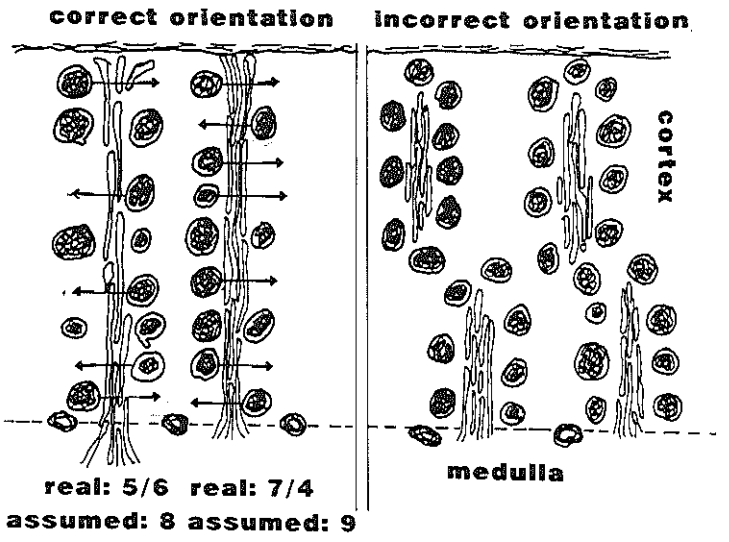

Figure 6-3. (after Hinchliffe et al., 1992a)

On the left (section with correct orientation: orthogonal to the cortex), two alternative methods of assessing the number of glomeruli associated with complete medullary rays are shown. "Real count" is achieved by only counting glomeruli actually present to one or other side of a medullary ray. "Assumed count" is achieved by adding glomeruli obviously missed by chance, to the number of glomeruli actually present.

On the right (section of inclination between A1A2 and B1B2 (Figure 6-2), a pattern of incomplete medullary rays that do not span the full width of the cortex is schematised.

fixed paraffin-embedded tissue and stained with haematoxylin and eosin, were available. In all cases, in addition to sections of cortex evidently affected by segmental inflammation or scarring, areas of unaffected cortex were available.

6.3.2. Assessment of the number of glomerular generations in the renal cortex

For each specimen, the number of glomerular generations present in areas of cortex affected by scarring and/or inflammation and in unaffected areas was determined separately using Medullary Ray Glomerular Counting (Hinchliffe et al., 1992a).

In essence, the number of glomerular generations that exist between the capsule and the corticomedullary junction was estimated by counting the number of glomeruli lying alongside parallel bundles of collecting ducts, "medullary rays" (Figure 6-2). To avoid introducing bias by oblique sectioning, glomerular generations were only counted along those medullary rays present throughout their complete length from capsule to medulla (Hinchliffe et al., 1992a. Incorrectly orientated sections were easily recognised (Figures 6-2 and 6-3). As discussed elsewhere (Hinchliffe et al., 1992a), one may count glomerular generations along medullary rays in two ways (Figure 6-3). A "real count" is obtained by counting only those glomeruli actually seen in the histological section. An "assumed count" also includes an assessment of those "lost" glomeruli which, although obviously associated with the ray during life, have not been included in the section due to chance. Although real counts should be used when one wishes to compare results of different pathologists, assumed counting has a greater intraobserver reproducibility (Hinchliffe et al., 1992a). In this study one histopathologist performed all the analysis and therefore the assumed count technique was used.

Each specimen was analysed blind and twice by a histopathologist who had not reported the original diagnosis. Glomerular generations along different medullary rays were analysed consecutively until the running mean of the estimated number of generations per ray reached a stable value (coefficient of variation <10%). Ten medullary rays were counted in every analysis, with a maximum of six rays being required for a stable value.

6.3.3. Renal hypoplasia

Renal hypoplasia was graded according to the number of glomerular generations present in areas of unscarred, non-inflamed cortex (Figure 6-4):

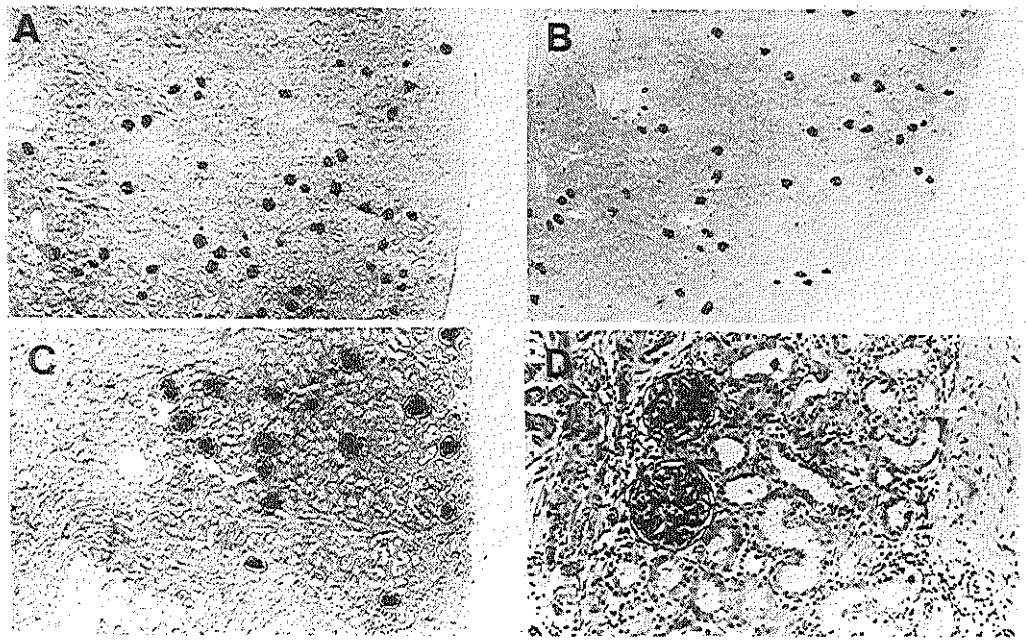

Figure 6-4. Photomicrographs (5 micron paraffin sections, H&E stain) showing different degrees of renal hypoplasia. Medulla to the left, cortex to the right.

A: No hypoplasia, all 12 glomerular generations are evident. Note greater interglomerular distance nearer medulla than in outer cortex. Note medulla with arcuate artery on left. Microscopical magnification 10 x.

B: Minimal hypoplasia, only 8-9 generations have been formed. Interglomerular spacing in outer cortex is increased. Microscopical magnification 20 x.

C: Moderate hypoplasia, only 6 generations have been formed. Spacing in outer cortex is irregular and increased. Note limited development of most latterly developed nephrons, evident by position of glomeruli directly beneath the renal capsule without interposed tubules.
Microscopical magnification 40 x.

D: Severe hypoplasia, 2 glomeruli are shown, directly adjacent to poorly developed medulla. Normal tubules and absence of abnormal vasculature indicate developmental nature. One further generation of glomeruli is occasionally evident directly beneath very fibrous renal capsule (on right running to the margin of the illustration). There is significant nephron development, evident by the number of tubular profiles visible between the glomeruli.
Microscopical magnification 100 x.

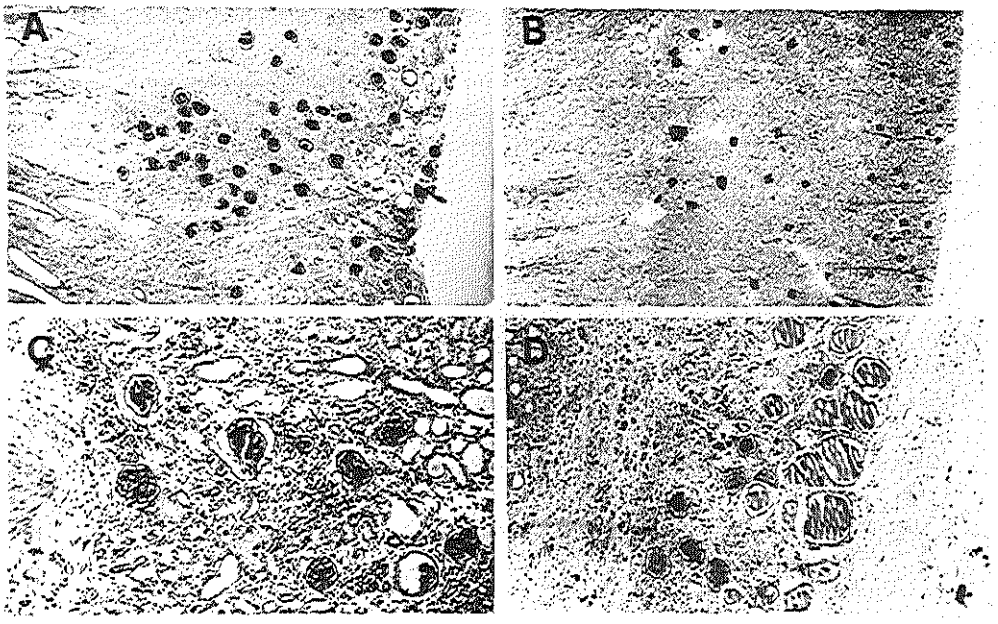

Figure 6-5. Photomicrographs (5 micron paraffin sections, H&E stain) showing different degrees of cortical loss. Medulla to the left, cortex to the right.

A: No loss evident, early secondary change with dilatation of tubules and fibrous perivascular change in the corticomedullary junction. Up to 12 generations of glomeruli are present, with some glomeruli directly beneath the renal capsule suggestive of limited nephron development. Microscopical magnification 20 x.

B: Minimal loss, early loss of generations in comparison to adjacent areas (not shown) which feature 12 generations. 8-9 generations present in this area. Note the "crowding" of glomeruli on right, directly beneath the renal capsule.
Microscopical magnification 10 x.

C: Moderate loss, only 6 generations of glomeruli remaining with active inflammatory infiltrate and dilated tubules containing protein. The hypercellular renal capsule is just visible in the lower right corner.
Microscopical magnification 50 x.

D: Severe, virtually total, loss. Remaining generations of glomeruli are totally sclerosed and remain only as ghosts. Note the through-sections of dilated, protein-filled tubules, especially directly beneath the fibrous renal capsule which is clearly visible on the right.
Microscopical magnification 40 x.

Grade of hypoplasia	*Number of generations*
severe hypoplasia	$n < 4$
moderate hypoplasia	$4 < n < 8$
minimal hypoplasia	$8 < n < 10$
no hypoplasia	$n > 10$

When more than one histological section of unscarred, non-inflamed tissue was available, the mean number of generations in each section was calculated.

6.3.4. Postnatally-acquired renal cortical loss

Renal cortical loss was graded in scarred and/or inflamed areas as the reduction from the number of glomerular generations present in unaffected areas (Figure 6-5):

Grade of loss	*Number of generations lost*
severe loss	$n > 7$
moderate loss	$4 < n < 7$
minimal loss	$2 < n < 4$
no loss	$n < 2$

When more than one histological section of scarred and/or inflamed tissue was available, the mean number of generations lost in each section was calculated.

6.3.5. Other histological features

All specimens were assessed for the presence of renal dysplasia as defined by common histopathological criteria (Bernstein, 1968).

6.3.6. Statistical analysis

In view of the non-random distribution of the results and size of the groups in the study, the findings were compared using Wilcoxon's two-tailed rank-sum test.

6.4. Results

The results are summarised in Tables 6-1 and 6-2. Only 9 of 86 patients showed no evidence of renal hypoplasia (Table 6-1). There is no significant relationship between the severity of hypoplasia and the presence or absence of obstruction. The degree of hypoplasia did not vary significantly between the sections of any 1 kidney. Hypoplasia was severe in 17 patients, moderate in 30 and minimal in 30 patients. Of the 42 patients < 5 years of age, 29 (69%) had severe or moderate hypoplasia, compared with 15 (42%) of the 36 patients > 5 years. In contrast, 21 (58%) of the 36 patients > 5 years had minimal or no hypoplasia compared with 13 (31%) of the 42 patients < 5 years. The probability of non-difference in the incidence of severe/moderate hypoplasia and minimal/no hypoplasia between the two age groups (age at nephrectomy < or > 5 years) was less than 0.01 for both comparisons (Wilcoxon's two-tailed rank sum test).

Severe cortical loss was found in 68 patients (Table 6-2). There is no significant association between the severity of cortical loss and the presence or absence of obstruction, age at nephrectomy or degree of coexisting hypoplasia.

Table 6-1. Relationship between renal hypoplasia and age at nephrectomy in 86 patients with vesicoureteral reflux (with or without obstruction at the ureterovesical junction).

age at operation (years)	grade of hypoplasia			
	severe	moderate	minimal	none
0-1	7	13	3	-
1-2	-	3	1	1
2-3	-	1	2	-
3-4	-	4	4	2
4-5	1	-	-	-
5-10	3	3	5	3
10-15	3	3	8	1
15-20	2	1	3	1
unknown	1	2	4	1
TOTALS	17	30	30	9

Table 6-2. Relationship between postnatally-acquired renal cortical loss and age at nephrectomy in 86 patients with vesicoureteral reflux (with or without obstruction at the ureterovesical junction).

age at operation (years)	grade of cortical loss severe	moderate	minimal	none
0-1	13	6	1	3
1-2	4	-	-	1
2-3	3	-	-	-
3-4	10	-	-	-
4-5	1	-	-	-
5-10	11	1	2	-
10-15	13	1	1	-
15-20	6	1	-	-
unknown	7	-	1	-
TOTALS	68	9	5	4

Renal dysplastic features were noted to a greater or lesser extent in 6 patients. These were strongly associated with severe (5 patients) or moderate (1 patient) hypoplasia. In 4 patients these were limited to cartilage and minor dysplastic change only. In 2 patients, subcortical (dilated collecting duct) cysts were also noted.

6.5. Discussion

Evidence of hypoplasia was found in 77 of 86 nephrectomy specimens removed in the final stages of renal disease. Even when hypoplasia was defined as the presence of fewer than 8 glomerular generations, 47 kidneys were thus affected. The hypoplasia was diffuse, resembling that associated with intrauterine obstruction. This may indicate the presence of an obstructive phase in utero. If intrarenal reflux is responsible then it would be of a less segmental nature than in the postnatal period. Alternatively the diffuse nature of the hypoplasia may reflect a global defect of mesenchymal induction associated with ureteric bud ectopia (Sommer & Stephens, 1981).

There is no significant relationship between the severity of cortical loss and age at nephrectomy; at any particular age, kidneys demonstrated a similar spectrum of acquired injury. The main determinant of age at nephrectomy was therefore the degree of hypoplasia. This may be explained by the extent of nephron loss: in contrast to the normal glomerular complement of 700,000 - 1,000,000 (Hinchliffe et al., 1991), the kidney with severe or moderate hypoplasia may be predicted to contain a maximum of only 40,000 or 220,000 glomeruli, respectively (the relationship between the number of glomerular generations and total glomerular number is non-linear and a reduction in the former by 50% represents a much greater reduction in the latter) (Hinchliffe et al., 1992a). Furthermore, in addition to the low number of glomerular generations, the most recently developed nephrons may remain immature and relatively limited in functional capacity (Figure 6-4C). Alternatively, the more severely underdeveloped kidneys may be more susceptible to infection. This may reflect the presence of abnormal molecular structures, not only associated with cartilaginous differentiation but perhaps also on the surface of epithelial cells lining the tubules.

The lack of a significant relationship between the severity of cortical loss and age at nephrectomy was an unexpected finding. If cortical loss results from independent infections, one would expect severe cortical loss to occur at all ages, whereas if infection is dependent upon reflux per se one would expect to find more severe disease in older children. However, if the damage associated with infection requires several years to develop, one would still expect to find more severe disease in older patients. Our results suggest that the time-scale for infection-related destruction is much shorter and can be interpreted as supporting the "Big-Bang" theory postulated by Ransley and Risdon (1979, 1980).

Clearly, postnatal surgery to resolve VUR, while expected to prevent further cortical loss, will not influence the developmental abnormality. The benefits of such intervention in a kidney with significant hypoplasia may thus be limited. Indeed we now feel that many children presenting early with minimal renal function, rather than reflecting failure of management, have a severely limited intrinsic renal capacity.

In utero intervention to decompress obstructed urinary tracts may facilitate normal renal development if hypoplasia is secondary to antenatal obstruction (Harrison et al., 1982). The developmental stages reached in our patients equate to those found between 14 and 36 weeks' gestation (Hinchliffe et al., 1991). In principle surgical intervention is possible during this period (Harrison, 1981a, 1981b; Berkowitz et al., 1982; Golbus et al., 1982; Turnock & Shawis, 1984). However, although the findings suggest developmental abnormality occurring at this time, delayed rather than arrested development may place the original cause much earlier, effectively beyond the reach of intervention. Furthermore, a rationale for intervention is absent if hypoplasia results from a "poor quality" of interaction between metanephric blastema and an abnormally positioned ureteric bud (Sommer & Stephens, 1981)

The range of dysplastic features noted in our patients is similar to that reported by Sommer and Stephens (1981). The frequency found (6/86) is however lower (35%) which may reflect different inclusion criteria.

chapter 7 Focal and segmental glomerulosclerosis in children with reflux nephropathy

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Submitted for publication

7.1. Summary

A histological review of 86 paediatric nephrectomy specimens from patients with vesicoureteric reflux (with or without apparent obstruction at the vesicoureteric junction) investigated the relationship between the presence and extent of focal and segmental glomerulosclerosis (FSGS) and coexisting renal hypoplasia and postnatally-acquired cortical damage. FSGS was found in 18 patients, 9 of whom were less than 5 years old. Although restricted to those index kidneys with at least minimal degrees of hypoplasia, there was no significant association between the presence (or grade) or absence of FSGS and age at nephrectomy, gender, presence or absence of obstruction and the severity of hypoplasia and/or postnatally-acquired cortical loss. FSGS was absent from 18 hypoplastic kidneys without vesicoureteric reflux (although of relatively young age and limited hypoplasia), 40 normally developed kidneys age-matched with the index population and 72 nephrectomy specimens without vesicoureteric reflux (except in 2 known cases of focal segmental glomerulonephritis). Within the index population FSGS was significantly ($p<0.01$) associated with hypertension, and hypertension was significantly associated with proteinuria ($p<0.001$) but not with an abnormal contralateral kidney. There was no significant association between FSGS, proteinuria and an abnormal contralateral kidney. Our results were unexpected when interpreted within a pathogenesis for FSGS of glomerular "hyperfiltration". They may, at least in the paediatric age group, indicate a possible role for other mechanisms in the development of FSGS.

7.2. Introduction

In patients with vesicoureteric reflux, renal scarring is frequently felt to be a consequence of the intracortical reflux of infected urine (Ransley & Risdon, 1979; Scott 1987). Occurring mainly in childhood, further parenchymal scarring in adult life develops only rarely (Smellie & Normand, 1985; Jones et al., 1984). In addition to the loss of renal function associated with such scarring, some patients show a progressive deterioration in function which is independent of hypertension or infection and which can occur even despite the spontaneous or surgical resolution of vesicoureteric reflux (Salvatierra et al., 1973; Kincaid-Smith & Becker, 1979; Senekjian et al., 1979; El-Khatib et al., 1987). This deterioration is characterised clinically by proteinuria and histologically by focal and segmental glomerulosclerosis (Kincaid-Smith, 1975b; Bhathena et al., 1980; Torres et al., 1980b). The age of onset of this condition shows considerable variation.

In reflux nephropathy, focal segmental glomerulosclerosis (FSGS) is widely thought to reflect "hyperfiltration" of a (reduced) remnant population of nephrons (Deen et al., 1974; Claesson et al., 1981, Brenner et al., 1982; Olson et al., 1985; Anderson et al., 1985). In this regard FSGS has been reported in several other conditions in which the number of nephrons is reduced, e.g. unilateral renal agenesis (Kiprov et al., 1982; Thormer et al., 1984; Gutierrez-Millet et al., 1986), unilateral nephrectomy (Zucchelli et al., 1983; Celsi et al., 1987) and oligomeganephronia (Brenner et al., 1982; Bhathena et al., 1985; Nomura & Osawa, 1990; Kaneko et al., 1990). However, whether glomerular pathology is a direct consequence of a sustained elevation of glomerular capillary pressure, or results indirectly from an associated abnormality of glomerular permeability and exposure of renal cells to an abnormal protein load, remains controversial (Remuzzi & Bertani, 1990). With both mechanisms FSGS may, in any disease process leading to a reduction of functional renal cortex, be considered to occur

later rather than sooner.

Recently we reported the relation between renal hypoplasia and postnatally-acquired cortical loss in nephrectomy specimens received from children with vesicoureteral reflux (Hinchliffe et al., 1992b). In this study approximately half of the specimens had severe hypoplasia with less than 25% of the total number of glomeruli normally present at birth (Hinchliffe et al., 1991). In these children such limitation of the nephron population may be expected to facilitate development of a hyperfiltrating state and concomitant progression of renal impairment earlier than if the deficiency of functional cortex was of a secondary, acquired nature only.

At present information on the occurrence of FSGS in children with vesicoureteral reflux, and its relation to developmental and acquired renal pathology, is limited. The reported incidence varies from less than 2% in all renal biopsies (Velosa et al., 1983) to 90-100% in patients with endstage, uraemic disease associated with vesicoureteral reflux (Kincaid-Smith, 1975b; Bhathena et al., 1980).

The aim of this study was to investigate the relation between focal segmental glomerulosclerosis, age at nephrectomy and previously established renal hypoplasia and postnatal cortical loss in a consecutive, nonselected series of nephrectomy specimens received from children with reflux nephropathy, with or without vesicoureteric obstruction.

7.3. Materials and Methods

7.3.1. Patient material

Index population

From a total of 158 nephrectomy specimens received between January 1980 - December 1990 at the Royal Liverpool Children's Hospital Alder Hey, 86 consecutive specimens were used as the index population for the study. These were obtained from all the patients with renal disease due to vesicoureteral reflux requiring nephrectomy for protracted complicated urinary tract infection and/or hypertension.

On review of the case notes of the 86 index patients, 42 were recorded to have had at least one period of obstruction at the vesicoureteric junction. Obstruction, often based on radiological documentation only, was noted to be of differing degrees and often recorded as temporary and of varying duration. For the purpose of this study, no further stratification with respect to this parameter was carried out.

For the purpose of statistical analysis the index patients were grouped as:

Group I: < 5 years of age at resection: n = 42
Ia: without obstruction: n = 20
Ib: with obstruction: n = 22
Group II: > 5 years of age at resection: n = 36
IIa: without obstruction: n = 17
IIb: with obstruction: n = 19
non-classifiable: n = 8
of which 1 patient had obstruction.
(For 8 patients information available was inconsistent and did not allow for confident grouping within either Group I or II.)

Control / comparison populations

Three groups of kidneys were used for control or comparison.

1. 40 normally developed kidneys (glomerular number estimated unbiasedly by the Disector-Cavalieri technique (Hinchliffe et al., 1991) from a series of non-natural (road traffic accidents) and acute natural (infection, haemorrhage etc.) deaths, age-matched with the index population.
2. 18 hypoplastic kidneys without reflux nephropathy (glomerular number, estimated unbiasedly by the Disector-Cavalieri technique (Hinchliffe et al., 1991), <50% normal (Hinchliffe et al., 1992c; 1993). All the affected children died within the first 2 years of life (7-45 weeks).
3. 72 (i.e. the remaining) nephrectomy specimens resected between January 1980 - December 1990 at the Royal Liverpool Children's Hospital Alder Hey from children without vesicoureteral reflux. 40 specimens were resected for primary or secondary neoplastic disease, 17 for primary cystic dysplasia and 15 for a miscellany of conditions including trauma. Age at nephrectomy was 0.26-16 years.

For each specimen, 2 - 10 Haematoxylin and Eosin stained histological sections, prepared from formalin-fixed paraffin-embedded tissue, were available.

7.3.2. Histological review for focal segmental glomerulosclerosis

All sections of all specimens were reviewed twice, in blind and random fashion, by two paediatric pathologists who had not provided the original diagnostic report. Diagnosis of FSGS required a positive decision in at least three of the four reviews.

In order to discriminate between focal segmental glomerulosclerosis and the (inflammatory) glomerulosclerosis found in areas of interstitial inflammation (Kimmelstein & Wilson, 1936; Steinhardt et al., 1988), a classification of FSGS required appropriately orientated (right angle to cortex) histological sections and the absence of any interstitial inflammatory infiltrate and/or dilatation of collecting duct systems in the area of cortex assessed.

A diagnosis of FSGS required the presence of:

1. more than 5% of glomeruli affected by either segmental or global sclerosis
2. intact glomeruli in addition to affected glomeruli
3. glomeruli with "hyaline" sclerosis of (segments of) tufts or whole glomeruli
4. a diffuse distribution of diseased glomeruli, which must especially be present in areas of cortex not affected by intracortical reflux.

The degree of FSGS was graded as follows:

Grade Ia. Affected glomeruli readily recognised, most affected glomeruli showing segmental lesions, up to 10% of glomerular profiles in a given section affected (Figure 1-9, Chapter 1).

Grade Ib. Affected glomeruli readily recognisable, variable extent of damage, many glomeruli now totally sclerosed, almost all glomeruli affected (Figure 1-10, Chapter 1).

Grade II. Many affected glomeruli already beginning to scar and be less easily recognised, affected glomeruli showing at least segmental lesions, many or most totally sclerosed (Figure 1-11, Chapter 1).

7.3.3. Grading of renal hypoplasia and postnatally-acquired cortical loss

For each specimen the degree of hypoplasia and postnatal loss reported elsewhere was used for this study (Hinchliffe at al., 1992b).

The degrees of hypoplasia and postnatal loss were assessed using the technique of "medullary ray glomerular counting" (Hinchliffe et al., 1992a), in which the number of glomerular generations between the renal capsule and the corticomedullary junction is estimated by counting the number of glomeruli lying alongside parallel bundles of collecting ducts, "medullary rays". This technique of total renal nephron number estimation has been validated using an unbiased stereological "gold standard" and is both reproducible and of greater sensitivity for the detection of non-normality than the traditional parameter of (reduced) renal weight (Hinchliffe et al., 1992a).

Renal hypoplasia was graded (severe, moderate, minimal, none) according to the number of glomerular generations present in areas of unscarred, non-inflamed cortex. Postnatally-acquired cortical loss was graded (severe, moderate, minimal, none) in scarred and/or inflamed areas as the reduction from the number of glomerular generations present in unaffected areas.

7.3.4. Hypertension, proteinuria and the contralateral kidney

Hypertension

Medical records were assessed for the presence of hypertension at nephrectomy. Assessment of the duration of the hypertension and its severity prior to nephrectomy was attempted, but the available data was often inconsistent. In view of the limited number (9, see Results) of hypertensive children, no further stratification of this parameter was attempted.

Proteinuria

Proteinuria was considered to be present at nephrectomy when random "Dipstick" analysis of urine resulted in ++ or +++ protein concentration.

Contralateral kidney assessment

The contralateral kidney was classified as normal or abnormal based on the combined DMSA scan, contrast radiography and

ultrasound assessment of renal size and character. In addition to kidneys classified as normal or even compensatedly hypertrophic, kidneys with normal size and isotope uptake but "minimal scarring" were classified as normal for the purpose of this study. All other defects resulted in classification as abnormal.

7.3.5. Statistical analysis

The relations between the various parameters (age, hypoplasia, acquired cortical loss, presence and grade of FSGS, hypertension, proteinuria, contralateral renal function) were studied (taking into account the nonrandom distribution of patient age at nephrectomy and the size of the groups in the study) using Wilcoxon's two-tailed rank-sum test, the Mann-Whitney U-test and the Chi-squared test.

7.4. Results

The results of the study are summarised in Tables 7-1 - 7-5.

Establishing FSGS proved straightforward with absolute agreement (4 out of 4 positive reviews) in 16 cases and good agreement (3 out of 4 positive reviews) in the remaining 2 cases. 2 Patients diagnosed once by each pathologist and 5 patients diagnosed once by either pathologist were all primarily graded as grade Ia but confirmed negative on joint revision.

From the total of 86 index specimens, 18 cases with FSGS were recognised. There was no significant association between the presence (or grade) or absence of FSGS and gender (Table 7-1). Patients with FSGS were distributed throughout the age range of the study population and 9 patients were younger than 5 years (Table 7-2). There was no significant association between the presence (or grade) or absence of FSGS and the age at nephrectomy (Table 7-1). There was no significant association between the presence (or grade) or absence of FSGS and the presence or absence of obstruction (data therefore not stratified further).

11 of the 18 cases with FSGS had either severe or moderate hypoplasia (Table 7-2). Although FSGS was found only in kidneys with, at least, minimal hypoplasia, there was no significant association between the presence or absence of FSGS and the severity of hypoplasia (Table 7-2), as the proportion of patients with each grade of hypoplasia was the same for both FSGS and non-FSGS groups. Similarly, there was no significant association between the grade of FSGS and the severity of hypoplasia (Table 7-2).

14 of the 18 cases with FSGS had severe postnatally-acquired cortical loss (Table 7-3). There was no significant association between the presence or absence of FSGS and the severity of cortical loss (Table 7-3), as the proportion of patients with each grade of cortical loss was the same for both FSGS and non-FSGS groups. Similarly, there was no significant association between the grade of FSGS and the severity of cortical loss (Table 7-3).

Within the 86 patients with obstructive / refluxive disease there was no significant association between the presence or absence of FSGS and the severity of the product of hypoplasia and cortical loss (Table 7-4), as the proportion of patients within each product category was the same for both FSGS and non-FSGS groups. Similarly, there was no significant association between the grade of FSGS and the product of hypoplasia and cortical loss (Table 7-4).

FSGS was not noticed in any of the control groups (neither the normal or hypoplastic kidneys studied at postmortem, nor the nephrectomy specimens received for reasons other than vesicoureteral reflux).

Of the three additional parameters studied within the index population (hypertension, proteinuria and the contralateral kidney) only hypertension was associated with the presence of FSGS at a statistically significant level ($p<0.01$, Table 7-5). Furthermore there was no relation between the presence of hypertension or proteinuria and abnormality of the contralateral kidney (Table 7-5). All patients with hypertension (9/9) had proteinuria, contrasting with the incidence of proteinuria in non-hypertensive patients (11/72, $p<0.001$).

Table 7-1. Relation between gender, age at nephrectomy and the presence or absence of focal segmental glomerulosclerosis in 86 patients with vesicoureteral reflux (with or without obstruction at the vesicoureteric junction).

	sex	age at nephrectomy		
	m/f/unknown	mean (years)	median (years)	range (years)
FSGS (n=18)		5.59	5.50	0.08 - 16
Ia (n=9)	5/3/1	6.05	5.50	0.42 - 16
Ib (n=4)	2/1/1	4.75	0.15	0.08 - 14
II (n=5)	1/4/0	5.44	3.00	0.25 - 12
non-FSGS(n=68)	26/36/6	5.65	5.50	0.17 - 17

Table 7-2. Relation between renal hypoplasia and age at nephrectomy in 86 patients with vesicoureteral reflux (with or without obstruction at the vesicoureteric junction). Focal segmental glomerulosclerosis was identified in 18 (data in parentheses) of the 86 patients.

age at operation (years)	grade of hypoplasia			
	severe	moderate	minimal	none
0-1	6(Ib)	10(Ia,Ib,II)	3	
1-2		3	(II)	1
2-3		(Ia)	2	
3-4		3(II)	2(Ia,Ia)	2
4-5	1			
5-10	2(Ia)	3	4(Ia)	3
10-15	2(II)	2(II)	6(Ia,Ib)	1
15-20	2	(Ia)	3	1
unknown	(Ib)	2	3(Ia)	1
TOTALS	13(4)	23(7)	23(7)	9

Table 7-3. Relation between postnatally-acquired renal cortical loss and age at nephrectomy in 86 patients with vesicoureteral reflux (with or without obstruction at the vesicoureteric junction). Focal segmental glomerulosclerosis was identified in 18 (data in parentheses) of the 86 patients.

age at operation (years)	grade of cortical loss			
	severe	moderate	minimal	none
0-1	12(Ib)	4(Ib,II)	1	2(Ia)
1-2	3(II)			1
2-3	2(Ia)			
3-4	7(Ia,Ia,II)			
4-5	1			
5-10	10(Ia)	(Ia)	2	
10-15	9(Ia,Ib, II,II)	1	1	
15-20	5(Ia)	1		
unknown	5(Ia,Ib)		1	
TOTALS	54(14)	6(3)	5	3(1)

Table 7-4. Relation between renal hypoplasia and postnatally-acquired cortical loss in 86 patients with vesicoureteral reflux (with or without obstruction at the vesicoureteric junction). Focal segmental glomerulosclerosis was identified in 18 (data in parentheses) of the 86 patients.

	grade of hypoplasia			
	severe	moderate	minimal	none
grade of cortical loss				
severe	7(Ia,Ib,II)	19(Ia,Ia,Ib, II,II)	18(Ia,Ia,Ia, Ia,Ib,II)	10
moderate	2(Ib)	2(II)	2(Ia)	
minimal	2		3	
none	1	2(Ia)		

Table 7-5. Relationship between FSGS, contralateral kidney status, proteinuria and hypertension in 81 patients with vesicoureteral reflux (with or without obstruction at the vesicoureteric junction, for 5 of the 86 index patients data was incomplete).

	Contralateral Kidney	Proteinuria	Hypertension
non-FSGS	Normal: 56	13	4
(n=68)	Abnormal: 8	1	0
FSGS	Normal: 14	5	5
(n=18)	Abnormal: 3	1	0

7.5. Discussion

Information on the incidence of focal and segmental glomerulosclerosis in patients with reflux nephropathy is largely confined to those with endstage, uraemic disease, where incidences approaching 100% are often reported (Kincaid-Smith, 1975b; Bhathena et al., 1980). However, FSGS in patients with vesicoureteral reflux is not confined to those with endstage nephropathy (Fairley et al., 1975; Senekjian et al., 1979). In this regard Kincaid-Smith found the lesion in 21 out of 28 patients with vesicoureteral reflux but preserved renal function (Kincaid-Smith, 1975a). That FSGS shows some association with vesicoureteral reflux is also assumed by Curtiss and colleagues, although they recorded this abnormality in only 4 out of 25 patients with endstage renal disease of undetermined aetiology (Curtiss et al., 1976).

In our study, focal and segmental glomerulosclerosis was found in 18 (21%) cases from a consecutive series of 86 children with vesicoureteral reflux. This frequency is at the lower end of the range reported in nephrectomy specimens from adults, perhaps reflecting the need in children to consider nephrectomy earlier as growth, which is adversely affected by repeated urinary tract infections, is brought into the equation. However, in our index cases, FSGS certainly occurred more frequently than in any of the control populations, supporting the concept that there is an increased incidence of FSGS in reflux nephropathy.

Although the precise pathogenesis remains to be elucidated, FSGS in reflux nephropathy is largely considered to result from glomerular hyperfiltration subsequent to an imbalance between the capacity of the filtration units, i.e. glomeruli and the functional demands thereof (Remuzzi & Bertani, 1990). Therefore in our index group, an association between FSGS, age and developmental deficiency, especially when compounded by an acquired loss of filtration area, may have been expected. Although limited to kidneys with at least minimal degrees of hypoplasia, within those affected no relation was found between FSGS and the severity of hypoplasia. Furthermore, if total glomerular filtration area is the parameter of import, the ipsilateral pathology may be expected to be highly influenced by the functional capacity of the contralateral unit. However in this study FSGS was not found to be significantly associated with an abnormal contralateral kidney. Interestingly FSGS was absent from the comparison group of 18 hypoplastic kidneys without obstructive / refluxive disease. However these were of relatively young age and, as a group, or relatively limited (moderate - minimal) hypoplasia. Furthermore, a direct comparison for hypoplasia between the index and comparison groups is complicated by the additional reduction in glomerular population resulting from the coexisting postnatally-acquired cortical loss in the former. In summary the data, whilst supporting the concept of an association between FSGS and renal hypoplasia, would seem to suggest that this nephron deficit is not, per se, of prime significance in FSGS.

Our findings show a strong relation between the presence of FSGS and hypertension. Proteinuria, not directly associated with FSGS, was however very significantly associated with hypertension per se. One of the explanations may be that proteinuria results from leakage of

proteins into infected or inflamed tissue and from there into the urine, thereby confounding any statistical relationship between FSGS and proteinuria. This may also explain the absence of a demonstrable association between proteinuria and an abnormal contralateral kidney.

There is no simple explanation for the absence of an association between hypertension and the presence of an abnormal contralateral kidney. Hypertension, strongly related to both FSGS and proteinuria, remains a central issue in any proffered mode of pathogenesis. The relative frequencies (18 children with FSGS, 9 with hypertension and 20 with proteinuria) in themselves provide no immediate insight. As all hypertensive patients had proteinuria, taking into account the possible effect of inflammatory processes, at least in this context proteinuria may follow hypertension rather than the reverse. However, as only half (5/9) of the hypertensive children had FSGS, and only 5 of the 18 with FSGS were hypertensive, the correct sequence, at least on the basis of these findings, is not directly evident and both directions may be argued.

An autoimmune process leading to the morphological change of FSGS, with onset independent of hypertension, may explain the above unclarity. As has been suggested, such an event may be related to antigens within the Tam-Horsefall protein group (Cotran, 1982). Within this hypothesis, initiation of glomerulosclerosis may be independent of the magnitude of protein leakage and depend more on a patient's susceptibility, offering an alternative explanation for the absence of a direct relation between FSGS and proteinuria. A similar mechanism of leakage of antigens from the distal tubular system into the renal interstitium as a consequence of "the high backpressure during reflux" has been proposed for the tubulointerstitial injury in vesicoureteral reflux (Yoshioka et al., 1987). However, the same authors question the relevance of this hypothesis to glomerular damage as Tam-Horsefall protein could not be demonstrated in the glomeruli of patients in early or advanced stages of reflux nephropathy (Torres et al., 1980b; Yoshioka et al., 1987). Similarly the possibilities of direct glomerular damage by circulating antibodies, cross reacting with determinants in the glomerulus, or indirect damage by circulating immune complexes, are at present not excluded (Fukatsu et al., 1988; Wardle 1988). An abnormal male-female ratio in patients, suggestive of an autoimmune process, was however not found in our study.

In conclusion our findings suggest that, at least in children, the explanation of FSGS by glomerular "overload" alone may be too limited a concept and that further (experimental) studies in animals are required.

chapter 8 General discussion

Management of the child with congenital urinary tract obstruction or vesicoureteral reflux is complex, varies between clinicians and continues to be debated. Indeed, considerable variation in the progression of disease and its relation to therapy exists between patients. Although this variation may be in part explained by differences in modes of treatment and the differing susceptibility of microorganisms to antibiotics, an alternative explanation, i.e. that there is an intrinsic variability in the "quality" of the kidney at birth, has been investigated in this thesis.

The study population comprised a consecutive series of nephrectomy specimens obtained in a single centre from children with vesicoureteral reflux, with or without features of urinary obstruction. These kidneys were histologically assessed to determine separately the extent of developmental abnormality and postnatally-acquired cortical damage, and to study the relation between these different pathologies. This assessment was made possible by the development of a simple, objective technique for the counting of glomerular generations which was validated against an unbiased stereological "gold standard" for total glomerular number estimation.

In Chapter 3 the Disector method, a stereological procedure unbiased by feature size, shape, or tissue-processing methods, for the estimation of total glomerular number was applied to pairs of human kidneys from 11 normal spontaneous second trimester abortions and stillbirths. Although application of the technique was in essence simple, it was necessary to define inclusion criteria in order to discriminate "glomeruli" from immature structures within the nephrogenic zone. Normal antenatal renal growth in the human was found to be quasi-exponential with the total number of glomeruli in a kidney increasing from approximately 15,000 at 15 weeks gestation to approximately 740,000 at birth. The method used was shown to have a coefficient of error of < 10% and thus to be more sensitive than renal weight. In addition, by combining estimates of glomerular number with those of renal cortical and medullary segment volumes, gestational age-dependent patterns of change in the average volume of the nephron and its cortical and medullary segments were analyzed. Preliminary data of these parameters was established for the first time. One of the advantages of the Disector method when used, as in this thesis, in combination with Cavalieri's principle, is its resistance to the effects of (differential) tissue shrinkage. Thus the possibility may be entertained for multicentre collaborative studies, where data from renal specimens may be centralised over considerable distances without requiring complex and expensive standardisation of tissue conservation protocols.

In Chapter 4 the effect of Type II (asymmetrical) intrauterine growth retardation (IUGR), which not uncommonly accompanies congenital urinary tract abnormalities, on the development of renal nephrons was investigated. The Disector technique was applied to kidneys from a group of stillbirths and liveborn infants (who subsequently died within the first year of life) who displayed features of Type II IUGR. These cases were all "asymmetrically" growth retarded, and were not associated with chromosomal or syndromal pathology. In the kidneys from the latter, liveborn infant group it was necessary to fractionate the kidneys prior to their sectioning to produce disector pairs. However, this additional phase of processing did not diminish the accuracy of the stereological technique or jeopardise its unbiasedness. Intrauterine growth retardation was associated with a significant reduction in the number of nephrons present at birth. No significant early, postnatal compensation in either nephron number or nephron domain was seen in infants

thus affected. Such pathology may contribute to the increased perinatal morbidity and mortality seen in liveborn infants with IUGR, as a limitation of renal functional reserve may compromise the homeostasis of the individual. It should be emphasised that the studies reported here relate to a developmental deficit in only asymmetric IUGR, and that as yet no data exists on the possibility of a similar deficit in symmetric IUGR. As nephrogenesis continues until the 36th week of gestation, IUGR-associated renal developmental delay may be considered, at least from a theoretical perspective, to be a candidate for remedial intervention by early delivery until late in pregnancy.

In Chapter 5 the "gold standard" of renal growth established in Chapter 3 was used to refine the method of medullary ray glomerular (generation) counting. Although this simple assessment had been suggested previously for the estimation of renal development, no data was available on the reproducibility of this technique or the significance of any particular generation count thereby obtained. Similarly, standard protocols for implementation of this procedure were lacking. Two different variants of the method were studied: "real counting" was shown to have greater reproducibility when comparing results of different pathologists, whereas "assumed counting" was seen to be the assessment of choice when data was derived from a single examiner. It was found that medullary ray glomerular counting could be applied to limited areas of renal cortex and this technique may therefore be utilised, in principle, on those small cortical segments undamaged by intracortical reflux in nephrectomy specimens derived from children with, for example, vesicoureteral reflux.

In Chapter 6 the method of medullary ray counting was applied to a consecutive series of 86 nephrectomy specimens obtained from children with vesicoureteral reflux, with or without urinary obstruction at the ureterovesical junction. None of these children showed refluxive pathology as part of a congenital (hereditary) syndrome or recognised chromosomal disorder. Renal hypoplasia was assessed separately from postnatally-acquired cortical loss, and the relation between these two abnormalities and age of the child at nephrectomy was analyzed. Severe hypoplasia was found in approximately half of the specimens and was associated with earlier nephrectomy. Renal dysplasia such as found in congenital (cystic) dysplasia was not noted. The results suggest that the early presentation of a child with vesicoureteral reflux in whom only minimal renal function may be demonstrated, may reflect an intrinsic limitation of renal parenchyma. From the analysis of the relation between postnatal cortical loss and age at nephrectomy, it was found that cortex may be damaged by intracortical reflux in a very short time supporting the "Big Bang" theory of Ransley and Risdon, which until now had not been further substantiated in human specimens. As preexistant renal dysplastic-like changes, often reported in the literature, were found only occasionally within the specimens studied, it may seem possible that the disturbance in renal development may have occurred relatively late in nephrogenesis. However the degree of hypoplasia found in a majority of the kidneys would seem to indicate a significant disturbance occurring early in gestation. In any case, with the present inability to detect functional renal abnormalities (i.e. reflux) prior to the development of structural damage (i.e. hypo- or dysplasia), it can be argued that any intervention to prevent or minimalise this, when directed by the recognition of such "damage", will be (too?) late. These factors may fundamentally limit the benefit of (surgical) intervention, in contrast to the case of delayed development in asymmetric IUGR. Furthermore it cannot be excluded that surgical intervention per se will not add another negative local stimulus to a system already impaired, and as such a positive effect of any intervention should not be expected in itself. In this regard, the Dutch National Health Council recently concluded that fetal surgical intervention after opening the maternal uterus was still mainly at the stage of experimentation, and considered such ex utero intervention in the human fetus not justified at the present time (Gezondheidstraad, 1990).

In Chapter 7 the presence and pathogenesis of focal and segmental glomerulosclerosis (FSGS) was studied in the same nephrectomy specimens. FSGS was found

in 21% of the 86 cases and showed no relation to the age at nephrectomy or the gender of the child. In 9 cases the children were less than 5 years of age at nephrectomy. Considered to be mainly caused by glomerular hyperfiltration as a consequence of a reduction in the population of nephrons, FSGS was expected to be strongly associated with not only age at resection but also hypoplasia and acquired cortical loss. However there was no significant association between the presence or absence of FSGS and age at nephrectomy and the severity of hypoplasia, postnatal cortical damage or the presence of an abnormal contralateral kidney. However there was a significant relation with the existence of hypertension. Although the latter was in itself strongly related to proteinuria, this was not directly related to FSGS. Therefore, at least in paediatric patients, alternative (e.g. autoimmune) pathways for the development of FSGS warrant continued investigation.

SUMMARY

Management of the child with congenital urinary tract obstruction or vesicoureteral reflux continues to be debated. It has been argued that antenatal, developmental pathology of the kidney may define the postnatal prognosis in such children to a considerable extent. However, only limited information is available about this pathology, its relation to postnatally-acquired damage and its possible significance in any particular child, since at present histopathological analysis of nephrectomy specimens is hampered by a lack of objective and reproducible parameters for quantification of renal growth and development.

In this thesis novel, unbiased, design-based stereological techniques were applied to a series of kidneys of differing gestation and postnatal age to determine the progressive increase, with age, in the total number of constituent glomeruli. In addition, gestational age-dependent patterns of change in the average volume of the nephron and its cortical and medullary segments were analyzed. A "gold standard" of renal growth was thus established.

The influence of intrauterine growth retardation, which not uncommonly accompanies congenital urinary tract abnormalities, on the development of the kidney was investigated separately.

The histological procedure of glomerular generation counting, a technique for quantification of development applicable to limited samples of renal tissue, was validated against the gold standard of total nephron number and the reproducibility of this routinely-applicable technique analyzed.

Thus validated, this simple method was applied to a consecutive series of 86 nephrectomy specimens obtained from children with vesicoureteral reflux, with or without obstruction at the ureterovesical junction, to separately assess and study the relation between renal developmental hypoplasia and postnatally-acquired renal cortical loss. A strong association of hypoplasia and vesicoureteral reflux was found in a majority of the cases. The results suggest that early postnatal presentation with minimal renal function need not necessarily represent failure of management of such children, but rather a preexistant limitation of renal capacity.

Focal and segmental glomerulosclerosis was found in 18 of the nephrectomy specimens. There was no significant association between the presence (or grade) or absence of glomerulosclerosis and age at nephrectomy and the severity of hypoplasia, postnatally-acquired cortical loss and or the presence of an abnormal contralateral kidney. FSGS was however strongly related to hypertension, albeit not with the associated proteinuria. The results were unexpected when interpreted within a pathogenesis, for focal segmental glomerulosclerosis, of glomerular "hyperfiltration".

SAMENVATTING

De behandeling van het kind met congenitale afwijkingen aan de urinewegen staat nog steeds ter discussie. Men neemt aan dat prenatale pathologie in de nier ontwikkeling het postnatale beloop in aanzienlijke mate kan beinvloeden. Er zijn echter slechts beperkte objectieve gegevens beschikbaar ten aanzien van ontwikkelingsstoornissen en hun relatie tot postnatale, verworven pathologie. De betekenis van prenatale ontwikkelingsstoornissen in de

evaluatie van de behandeling van het individuele kind wordt aldus beperkt door een gebrek aan objectieve patholoog-anatomische groeiparameters.

In dit proefschrift worden nieuwe, objectieve "design based" stereologische methoden aangewend om met behulp van een serie normale nieren de prenatale ontwikkeling objectief te quantificeren door middel van het bepalen van het absolute aantal glomeruli voor iedere periode van de zwangerschap. Daarnaast werd het gemiddelde volume van het foetale nephron, en de corticale en medullaire componenten daarvan in relatie tot de zwangerschapsduur bepaald. Op deze wijze werd een "gouden standaard" voor renale ontwikkeling gecreerd.

Vervolgens werd separaat de invloed van intrauteriene groeivertraging, een conditie die geassocieerd is met congenitale afwijkingen van de urinewegen, bepaald.

De histologische analyse van nier ontwikkeling door middel van het vaststellen van het aantal glomerulaire generaties, een quantificeringstechniek die toepasbaar is op beperkte hoeveelheden nierschors, werd gevalideerd door vergelijk met de gecreerde "gouden standaard" van het absolute totaal aantal glomeruli. De reproduceerbaarheid van de eenvoudige en in de routine diagnostiek toepasbare methode werd bepaald en ruim voldoende bevonden.

De gevalideerde methode werd vervolgens toegepast op een serie van 86 opeenvolgende, niet geselecteerde nier resectie prepraten, verkregen bij kinderen met vesico-ureterale reflux al of niet met obstructieve component. De relatie tussen beloop, prenatale ontwikkelingsstoornis en postnataal verworven nier verlies werd geanalyseerd. In een meerderheid van de gevallen kon, onafhankelijk van de postnatale schade, een beduidende mate van hypoplasie worden vastgesteld.

De resultaten geven aan dat in veel kinderen bij wie (vroeg) na de geboorte een niet functionele nier aangetoond wordt, dit niet duid op een falen van de (postnatale) behandeling maar het gevolg is van ernstige prenatale ontwikkelings stoornis.

Focale segmentale glomerulosclerose werd in 18 nieren aangetroffen. Een relatie tussen leeftijd bij resectie, mate van hypoplasie en of verworven schorsverlies en het optreden of ernst van focale segmentale glomerulosclerose kon niet worden vastgesteld. Evenmin was een relatie aantoonbar met het bestaan van een afwijkende contralaterale nier. FSGS is sterk gecorreleerd met het bestaan van hypertensie alhoewel er geen aantoonbare relatie bestonf met het voorkomen van proteinurie.

Deze bevindingen zijn onverwacht binnen het kader van een eenvoudige hypothese van glomerulaire overbelasting en hyperfiltratie voor het ontstaan van deze aandoening.

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