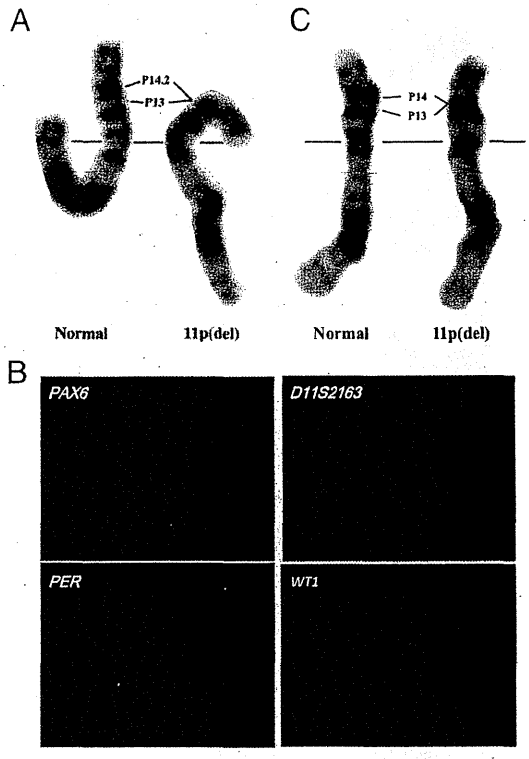


FIGURE 2

High-resolution G-banding of chromosome 11 and fluorescence in situ hybridization (FISH) in patient 2 and G-banding of chromosome 11 in patient 3. A, Patient 2 had deletion of chromosome 11p13-p14.2 in one allele. B, FISH using P1-derived artificial chromosome clones (1083G3 for *PAX6*; 65P5 for *D11S2163*; 685F3 for *PER*; and 104M13 for *WT1*) as probes was performed for patient 2, as previously reported.[6] Each FISH signal for *PAX6, D11S2163, PER, and WT1* was observed in only one chromosome 11 homolog, indicating heterozygous deletion of the WAGR region of 11p. C, Patient 3 had deletion of chromosome 11p13-p14 in one allele.

histology. The possibility of reflux nephropathy, however, could not be ruled out in patient 2. The perihilar variant with glomerular hypertrophy is particularly common in the secondary FSGS such as reduced renal mass–induced FSGS.[8] However, all 3 patients exhibited FSGS (not otherwise specified) without glomerular hypertrophy, suggesting that surgical renal ablation (patient 1) and reflux nephropathy (patient 2) may not have been the main cause of FSGS in these 2 patients. These findings suggest that the complete deletion of one *WT1* allele might have a pathogenetic role in the development of nephropathy.

The spectrum of glomerular diseases associated with *WT1* mutations has been reviewed.[9] *WT1* mutations can cause syndromic and nonsyndromic glomerular disease. The syndromic forms include DDS (early-onset nephrotic syndrome with diffuse mesangial sclerosis [DMS]); 46,XY disorders of sex development and Wilms' tumor; and Frasier syndrome (disorders of sex development, FSGS, and gonadoblastoma), which is caused by a mutation in the intron 9 splice site of *WT1* leading to the loss of the +KTS isoform of the protein. Mutations associated with both syndromic and nonsyndromic glomerular disease tend to cluster in exons 8 and 9 of *WT1*, which encode zinc fingers 2 and 3.[9,10] Orloff et al[11] reported that single-nucleotide polymorphisms in *WT1* may modulate the development of FSGS by altering *WT1* function. The current study suggests that complete deletion of one *WT1* allele may also induce the development of nephropathy.

Reduced expression levels of *Wt1*-induced glomerulopathies (crescentic glomerulonephritis or DMS) depending on gene dosage derived by combining *Wt1*-knockout mice and an inducible *Wt1* yeast artificial chromosome transgenic mouse model.[12] Eleven percent of mice heterozygous for the *Wt1* mutation showed severe proteinuria and DMS with tubular cysts, protein casts, and severe interstitial inflammation, although nephrogenesis was not delayed.[12] These findings indicate that the expression level of *WT1* plays an important role, not only during nephrogenesis but also in the homeostasis of normal kidney function. These findings also support our conclusion that complete deletion of one *WT1* allele in atypical WAGR syndrome could induce glomerulopathy without delayed nephrogenesis, although the reason for the discrepancy in histologic findings between man (FSGS) and mouse (DMS) is unclear.

CONCLUSIONS

Besides dominant-negative missense mutations in the eighth or ninth exon of *WT1* and mutations at the donor splice site of intron 9, complete deletion of one *WT1* allele may induce the development of FSGS. The findings in this study also suggest that haploinsufficiency of *WT1* could be responsible for the development of FSGS.

REFERENCES

1. Miller RW, Fraumeni JF Jr, Manning MD. Association of Wilms' tumor with aniridia, hemihypertrophy and other congenital malformations. *N Engl J Med.* 1964;270:922–927

2. Little MH, Williamson KA, Mannens M, et al. Evidence that WT1 mutations in Denys-

Drash syndrome patients may act in a dominant-negative fashion. *Hum Mol Genet.* 1993;2(3):259–264

3. Huff V. Genotype/phenotype correlations in Wilms' tumor. *Med Pediatr Oncol.* 1996;27 (5):408–414
4. Breslow NE, Takashima JR, Ritchey ML, Strong LC, Green DM. Renal failure in the Denys-Drash and Wilms' tumor-aniridia syndromes. *Cancer Res.* 2000;60(15):4030–4032
5. Kawase E, Tanaka K, Honna T, Azuma N. A case of atypical WAGR syndrome with anterior segment anomaly and microphthalmos. *Arch Ophthalmol.* 2001;119(12): 1855–1856
6. Muto R, Yamamori S, Ohashi H, Osawa M. Prediction by FISH analysis of the occurrence of Wilms tumor in aniridia patients. *Am J Med Genet.* 2002;108(4):285–289
7. Lee VW, Harris DC. Adriamycin nephropathy: a model of focal segmental glomerulosclerosis. *Nephrology (Carlton).* 2011;16 (1):30–38
8. D'Agati VD. The spectrum of focal segmental glomerulosclerosis: new insights. *Curr Opin Nephrol Hypertens.* 2008;17(3):271–281
9. Niaudet P, Gubler MC. WT1 and glomerular diseases. *Pediatr Nephrol.* 2006;21(11): 1653–1660
10. Mucha B, Ozaltin F, Hinkes BG, et al; Members of the APN Study Group. Mutations in the Wilms' tumor 1 gene cause isolated steroid resistant nephrotic syndrome and occur in exons 8 and 9. *Pediatr Res.* 2006; 59(2):325–331
11. Orloff MS, Iyengar SK, Winkler CA, et al. Variants in the Wilms' tumor gene are associated with focal segmental glomerulosclerosis in the African American population. *Physiol Genomics.* 2005;21(2): 212–221
12. Guo JK, Menke AL, Gubler MC, et al. WT1 is a key regulator of podocyte function: reduced expression levels cause crescentic glomerulonephritis and mesangial sclerosis. *Hum Mol Genet.* 2002;11(6): 651–659

Eur J Pediatr
DOI 10.1007/s00431-012-1772-y

SHORT COMMUNICATION

Is the new Schwartz equation derived from serum creatinine and body length suitable for evaluation of renal function in Japanese children?

Osamu Uemura · Masataka Honda · Takeshi Matsuyama · Kenji Ishikura · Hiroshi Hataya · Takuhito Nagai · Yohei Ikezumi · Naoya Fujita · Shuichi Ito · Kazumoto Iijima · The Japanese Society for Pediatric Nephrology, the Committee of Measures for Pediatric CKD

Received: 13 May 2012 / Accepted: 5 June 2012

Abstract The present study was performed to determine whether the new Schwartz "bedside" equation can be used to estimate the glomerular filtration rate (GFR) in Japanese children as there are differences in renal function and muscle mass between Japanese and American individuals. It is also important to determine whether one common equation can be used in children from 1 to 16 years old, including the period of adolescence. Blood samples were collected from a total of 1,074 healthy children (466 males and 608 females) between 1 and 16 years old. The estimated GFR (eGFR) derived by the new Schwartz bedside formula [eGFR (in milliliters per minute per 1.73 m^2)=0.413×body length (in centimeters)/serum Cr value (in milligrams per deciliter)] was calculated in all subjects, and the relationship between age and eGFR was analyzed. The eGFR decreased gradually with age, and the decrease was more marked in males than females, mainly in adolescence. Weak negative but significant correlations were observed in 466 males and 608 females. The median of the eGFR value showed a gradual significant decrease with age. Conclusion: A common coefficient cannot be used in children between 1 and 16 years old, including the period of adolescence, with the Schwartz type formula, and the new Schwartz bedside formula cannot be used when we estimated GFR in Japanese children. It is necessary to establish an eGFR equation specifically for Japanese children.

O. Uemura (✉) · M. Honda · T. Matsuyama · K. Ishikura · H. Hataya · T. Nagai · Y. Ikezumi · N. Fujita · S. Ito · K. Iijima
Department of Pediatric Nephrology,
Aichi Children's Health and Medical Center,
1-2 Osakada Morioka-cho,
Obu, Aichi 474-8710, Japan
e-mail: o_uemura@hkg.odn.ne.jp

Published online: 20 June 2012

Keywords Reference serum creatinine level · Japanese children · Enzymatic method · New Schwartz formula · eGFR

Introduction

Schwartz et al. expressed the relations between body length, glomerular filtration rate (GFR), and serum Cr level as estimated GFR [eGFR (in milliliters per minute per 1.73 m^2)=κ×body length (in centimeters)/serum Cr value (in milligrams per deciliters)] [2]. The coefficient κ is 0.33 in preterm infants under 1 year old, 0.45 in full-term infants under 1 year old, 0.55 in children 2–12 years old, and 0.55 and 0.70 in females and males over 12 years old, respectively [2–5]. This formula is clinically useful as it allows estimation of the patient's GFR from body length and serum Cr level. This equation utilizes the Jaffe method to measure Cr. However, enzymatic methods have recently been used to measure Cr, making the above formula no longer applicable. In 2009, the updated Schwartz formula, the so-called bedside version, was reported as follows: eGFR (in milliliters per minute per 1.73 m^2)=0.413×body length (in centimeters)/serum Cr value (in milligrams per deciliter) by enzymatic Cr determination in children 1–16 years old [6].

We have previously reported reference serum creatinine levels determined by an enzymatic method in Japanese children according to sex and age [7]. The present study was performed to determine whether the new Schwartz "bedside" equation can be used to estimate the GFR in Japanese children as there are differences in renal function and muscle mass between Japanese and American individuals. It is also important to determine whether one common

equation can be used in children from 1 to 16 years old, including the period of adolescence.

Materials and methods

Blood samples were collected from a total of 1,151 children (517 males, 634 females) between 1 month and 18 years old presenting at the facilities of the members for the Committee of Measures for Pediatric Chronic Kidney Disease (CKD) and Tokyo Health Service Association between 2008 and 2009 without renal, urogenital, infectious, inflammatory, muscular, cardiovascular, liver, and pancreas diseases and not being hypertensive, dehydrated, or pregnant [7]. The study was approved by the local ethics boards of all participating institutions, and written informed consent was obtained from the parents of all subjects. Data from children under 1 or over 16 years old were excluded, and the remaining data from 1,074 children (466 males, 608 females) between the ages of 1 and 16 years were used in this study.

Subjects were divided into the following groups based on age. The eGFR derived by the new Schwartz formula [eGFR (in milliliters per minute per 1.73 m^2)=0.413×body length (in centimeters)/serum Cr value (in milligrams per deciliter)] was calculated in all subjects and the median, 2.5 percentile, and 97.5 percentile values of the eGFR in each age and sex. In all subjects, the relationship between age and eGFR was determined by linear regression analysis.

Serum samples were stored at −70 °C until serum Cr was measured at SRL, Inc. (Tokyo, Japan). The serum level of Cr was determined by an enzymatic method using a Bio Majesty automated analyzer (JCA-BM8060; JEOL Ltd., Tokyo, Japan) with Pureauto S CRE-L (Sekisui Medical Co., Ltd., Tokyo, Japan). The coefficient of variation was satisfactory (2.08 %).

All analyses were conducted using Microsoft Excel 2007 (Microsoft, Redmond, WA) and the statistical software package JMP 8 (SAS Institute Inc, Cary, NC). We conducted linear regression analysis to determine whether the new Schwartz formula can be used to evaluate the renal function in Japanese children. We used Wilcoxon's analysis to compare differences in the reference eGFR values between the ages. In all analyses, $P<0.01$ was taken to indicate statistical significance.

Results

We examined the correlations between eGFR derived by new Schwartz formula and age in males and females (Fig. 1). These scattergrams showed that eGFR decreased gradually with age, and the decrease was more marked in males than females mainly in adolescence. Weak correlations were

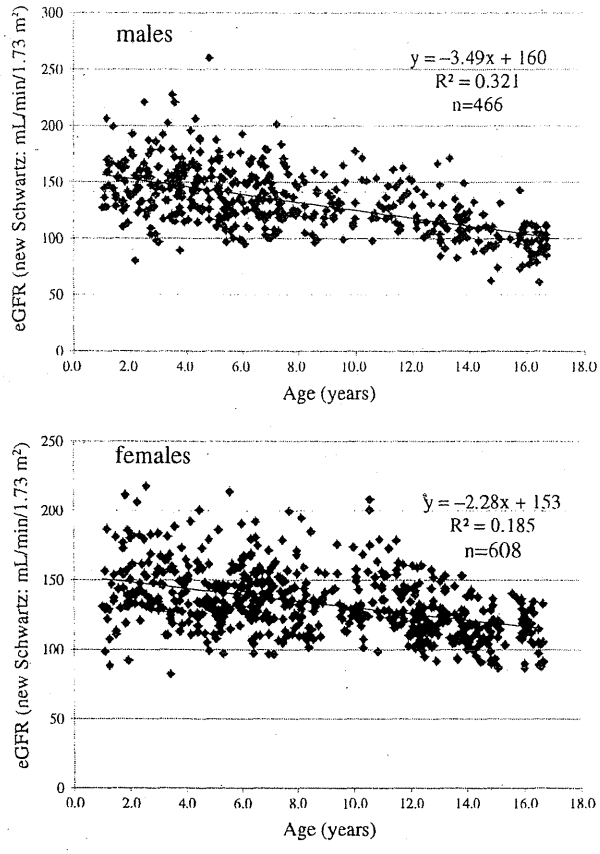


Fig. 1 Correlation between eGFR derived by the new Schwartz bedside formula and age in all male and female subjects, respectively. The scattergram shows that eGFR decreased gradually with age showing a weak but significant negative correlation

observed in 466 males and 608 females, and the correlation coefficients were 0.567 and 0.430 (Fig. 1, $P<0.001$), respectively.

We reviewed the median, 2.5 percentile, and 97.5 percentile of the eGFR value in each age group between 1 and 16 years old in males and females, respectively (Table 1). The median of the eGFR value decreased gradually with age, i.e., 130–150 mL/min/1.73 m^2 between 1 and 11 years old and 95.9 mL/min/1.73 m^2 in males and 112.3 mL/min/1.73 m^2 in females at 16 years old, respectively ($P<0.001$).

Discussion

GFR is used to assess kidney function and is measured by renal clearance. Inulin clearance is the gold standard for evaluation of kidney function but cannot be measured easily. Therefore, various methods have been used to determine GFR. The eGFR (in milliliters per minute per 1.73 m^2)=κ× body length (in centimeters)/serum Cr value (in milligrams per deciliter) by the Jaffe method devised by Schwartz has been used clinically [2]. Recently, however, enzymatic

Table 1 The median, 2.5 percentile, and 97.5 percentile of the eGFR value in each age group between 1 and 16 years old in males and females, respectively

Age (years old)	*n*	2.5 %	50 %	97.5 %
Males				
1	33	113.3	147.5	200.7
2	40	98.0	146.2	193.4
3	48	100.2	148.7	217.6
4	43	116.9	149.6	206.0
5	47	99.2	134.8	177.2
6	43	98.2	134.5	179.7
7	38	103.6	131.4	185.0
8	18	97.8	123.4	159.1
9	18	104.5	130.6	159.0
10	12	103.5	131.3	176.0
11	19	106.9	133.8	162.3
12	15	102.4	116.0	161.4
13	30	84.3	112.5	155.4
14	17	73.1	98.0	129.1
15	15	73.8	103.0	139.2
16	30	73.3	95.9	113.2
Females				
1	36	91.8	136.4	190.1
2	33	128.1	148.7	208.8
3	40	109.8	140.8	184.2
4	38	104.9	136.2	193.4
5	49	108.8	132.7	181.7
6	58	105.4	140.3	186.8
7	47	98.1	137.6	178.1
8	38	106.8	132.7	185.9
9	17	113.5	129.2	167.8
10	32	100.9	132.4	202.7
11	39	110.0	136.9	173.6
12	54	96.6	119.5	160.4
13	38	93.7	121.5	153.5
14	40	91.3	112.3	139.1
15	22	87.3	117.7	138.9
16	27	87.5	112.3	133.9

methods have been used to measure Cr rather than the Jaffe method, so it is not possible to use the formula in this form. Therefore, it was necessary to reevaluate the value of the coefficient κ in the formula. Recently, Zappitelli et al. revised the Schwartz formula relating eGFR to serum creatinine level determined enzymatically and reported that the κ value in the Schwartz equation decreased from 0.55 to 0.47 for children and adolescent girls [8]. Schwartz et al. reported the updated formula, the so-called bedside version, as eGFR=0.413× body length (in centimeters)/serum Cr value (in milligrams per deciliter) by the enzymatic method showing a 25 % reduction in κ value from the previous value of 0.55 generated from Jaffe-based serum Cr measurements [6]. The work was defined from a population of American children with chronic kidney disease, enriched with obstructive uropathy. They concluded that the formula can be used regardless of age or gender in children 1–16 years old. However, the work has been misread and misused to assess eGFR in healthy children.

The present study was performed to determine whether the new Schwartz bedside equation can be used for the evaluation of renal function in Japanese children. Previously, we reported reference serum creatinine levels determined by an enzymatic method in Japanese children according to sex and age [7]. The eGFR derived by the new Schwartz formula [eGFR (in milliliters per minute per 1.73 m^2)= 0.413×body length (in centimeters)/serum Cr value (in milligrams per deciliter)] was calculated for our 1,074 subjects between the ages of 1 and 16 years old and the median, 2.5 percentile, and 97.5 percentile values of the eGFR in each age and sex. The median of the eGFR value showed a gradual significant decrease with age. In addition, the relationship between age and eGFR was determined by linear regression analysis, and weak but significant negative correlations were observed in both male and female subjects. It seems to be a large problem that the ranges of the reference value of boys over 12 years and girls over 14 years old overlap a range of CKD stage 2. In healthy children, normal serum creatinine values are sufficient to define normal kidney function.

Brodehl et al. reported that GFRs derived from inulin clearance approached adult levels within 2 years and were approximately constant between 3 and 15 years old, showing values of 111.2 and 117.2 mL/min/1.73 m^2 at 3–4 years and 13–15 years old, respectively [1]. Our results indicating that eGFR value derived by the new bedside Schwartz formula decreased gradually with age suggest that this formula should not be used for estimating the GFR of Japanese children, at least in those with normal renal function. Weak points of our study are that our materials were healthy not chronic kidney disease children and that they were not actually measured with GFR. In addition, we entrusted the judgment of each coauthor whether each case met our exclusion criteria. We will go ahead through the study of the inulin clearance for patients with Japanese pediatric CKD and intend to review new Schwartz formula in this study.

Schwartz et al. expressed the relationship between body length, GFR, and serum Cr level as eGFR (in milliliters per minute per 1.73 m^2)=κ×body length (in centimeters)/serum Cr value (in milligrams per deciliter) [2], and in this old Schwartz formula, the coefficient κ is 0.55 in children 2–12 years old and 0.70 in males over 12 years old [2, 4]. The new Schwartz formula has an inherent problem with using the same coefficient between the ages of 1 and 16 years old. In addition,

we assume that renal function and muscle mass show ethnic differences.

While indeed it is inappropriate to use the new Schwartz bedside formula in normal Japanese children, it may be inappropriate in all normal children of any ancestry, ethnicity, or national origin. We must realize that it was not defined for these populations of normal children.

In conclusion, the common coefficient cannot be used between 1 and 16 years old, including the adolescent period, in the Schwartz type formula, and the new Schwartz bedside formula cannot be used when we estimated GFR in Japanese children. It is necessary to establish a specialized estimated GFR equation for use in Japanese children and to review new Schwartz formula for patients with pediatric CKD.

References

1. Brodehl J, Gellissen K, Weber HP (1982) Postnatal development of tubular phosphate reabsorption. Clin Nephrol 17:163–171
2. Schwartz GJ, Brion LP, Spitzer A (1987) The use of plasma creatinine concentration for estimating glomerular filtration rate in infants, children, and adolescents. Pediatr Clin N Am 34:571–590
3. Schwartz GJ, Feld LG, Langford DJ (1984) A simple estimate of glomerular filtration rate in full-term infants during the first year of life. J Pediatr 104:849–854
4. Schwartz GJ, Gauthier B (1985) A simple estimate of glomerular filtration rate in adolescent boys. J Pediatr 106:522–526
5. Schwartz GJ, Haycock GB, Edelmann CM Jr, Spitzer A (1976) A simple estimate of glomerular filtration rate in children derived from body length and plasma creatinine. Pediatrics 58:259–263
6. Schwartz GJ, Muñoz A, Schneider MF, Mak RH, Kaskel F, Warady BA, Furth SL (2009) New equations to estimate GFR in children with CKD. J Am Soc Nephrol 20:629–637
7. Uemura O, Honda M, Matsuyama T, Ishikura K, Hataya H, Yata N, Nagai T, Ikezumi Y, Fujita N, Ito S, Iijima K, Kitagawa T (2011) Age, gender, and body length effects on reference serum creatinine levels determined by an enzymatic method in Japanese children: a multicenter study. Clin Exp Nephrol 15:694–699
8. Zappitelli M, Parvex P, Joseph L, Paradis G, Grey V, Lau S, Bell L (2006) Derivation and validation of cystatin C-based prediction equations for GFR in children. Am J Kidney Dis 48:221–230

Clin Exp Nephrol (2013) 17:99–105
DOI 10.1007/s10157-012-0658-7

ORIGINAL ARTICLE

Establishment of a normal reference value for serum β2 microglobulin in Japanese children: reevaluation of its clinical usefulness

Yohei Ikezumi · Masataka Honda · Takeshi Matsuyama · Kenji Ishikura · Hiroshi Hataya · Nahoko Yata · Takuhito Nagai · Naoya Fujita · Shuichi Ito · Kazumoto Iijima · Tetsuji Kaneko · Osamu Uemura

Received: 17 April 2012 / Accepted: 7 June 2012 / Published online: 14 July 2012

Abstract
Objective Serum β2 microglobulin (β2MG) is considered to be a marker of renal function, which is independently associated with age. However, only a few studies have reported the reference values for β2MG in children thus far, particularly in young children. In this study, we evaluated the distribution of serum β2MG values in healthy Japanese children and assessed its clinical usefulness.
Method The normal reference value of serum β2MG was assessed in serum samples from 1131 normal Japanese children (504 boys and 627 girls; age 0–17 years). To test the validity of the reference value, serum samples from children with various kidney diseases were also examined retrospectively.
Results The mean values for β2MG were significantly negatively correlated with age ($r = -0.47$, $P < 0.001$). No significant difference was observed between the values of boys and girls in any age group. The established β2MG reference range covered 99.7 % of patients with decreased kidney function below 75 % based on their serum creatinine (Cr) value and body length.
Conclusion The newly established β2MG reference value in children can be used to detect kidney impairment in children. Serum β2MG in combination with serum Cr used as markers for predicting glomerular function can provide an accurate detection of kidney dysfunction in children.

Keywords β2 microglobulin · Body mass · Children · Chronic kidney disease · Kidney function · Reference value

Introduction

The worldwide increase in the number of patients with chronic kidney disease (CKD) is being recognized as a global public health problem. CKD is not only a cause of end-stage renal disease (ESRD) during childhood but also a key cause of CKD and ESRD in adults. Therefore, the early detection of impaired glomerular function in children, facilitated by routine examinations of kidney function, is essential to inhibit the progression of CKD and reduce the incidence of ESRD. However, this assessment is limited by the lack of markers for impaired kidney function in children. In addition, there are few studies that have established race-based reference values for children.

A multicenter study was recently conducted to establish normal reference values for serum creatinine (Cr), beta 2 microglobulin (β2MG), and cystatin C levels in Japanese children, and a normal serum Cr reference value was established for Japanese children by using an enzymatic detection method [1]. There is a significant correlation between the serum Cr concentration and body length (BL), expressed as BL (m) × 0.30 for children aged 1–12 years, providing a simple formula convenient for estimating glomerular function. A polynomial equation that can predict serum Cr values in children of all ages was also established [1]. Serum Cr is the most widely used marker

Y. Ikezumi · M. Honda · T. Matsuyama · K. Ishikura · H. Hataya · N. Yata · T. Nagai · N. Fujita · S. Ito · K. Iijima · T. Kaneko · O. Uemura
The Japanese Society for Pediatric Nephrology, The Committee of Measures for Pediatric CKD, Tokyo, Japan

Y. Ikezumi (✉)
Department of Pediatrics, Niigata University Medical and Dental Hospital, Asahimachi-dori, Niigata 951-8510, Japan
e-mail: ikezumi@med.niigata-u.ac.jp

for predicting kidney function. The newly established Cr value for Japanese children will further improve the diagnostic accuracy for detecting reduced renal function. However, Cr concentrations are insensitive to mild reduction in the glomerular filtration rate (GFR). In addition, the age and muscle mass dependencies of serum Cr complicate GFR assessment in children; physicians, particularly if they are not nephrologists or pediatricians, often do not take these complications into account [2, 3]. Therefore, additional markers independent of age and sex are preferable to aid the screening of renal function.

β2MG is a well-established marker that is independent of muscle mass and age; therefore, it has better diagnostic sensitivity than serum Cr for the detection of impaired GFR in growing children and children associated with severe loss of body mass [4, 5]. The production of β2MG, however, is known to increase during infection, inflammatory processes, proliferative syndromes, autoimmune diseases, and malignancies [6], which may affect the evaluation of glomerular function in children. Therefore, it is necessary to establish an accurate range of β2MG in healthy children, which can be used as an accurate diagnostic marker of renal dysfunction in children.

Despite the clinical importance of evaluating the renal function independent of age, sex, and race, there are few studies on normal β2MG reference values in children. Therefore, this large-scale study was performed to evaluate the normal reference values of β2MG in healthy Japanese children.

Materials and methods

Collection of blood samples (multicenter study)

Blood samples were collected from a total of 1151 children (517 boys and 634 girls) between the ages of 1 month and 18 years who presented at the member facilities of the Committee of Measures for Pediatric CKD and the Tokyo Health Service Association between 2008 and 2009 [1]. The study was approved by the local ethics boards, and written consent was obtained from the parents of all subjects. Data lacking β2MG values were deleted, and the remaining data from 1131 healthy children (504 boys and 627 girls) with ages between 1 month and 17 years (mean overall age, 7.7 ± 4.7 years; mean age of boys, 7.0 ± 4.8 years; mean age of girls, 8.4 ± 4.6 years) were used in this study. Children with kidney diseases, urogenital diseases, infectious diseases, inflammatory diseases, dehydration, muscular diseases, anomaly syndrome, malignancies, cardiovascular diseases, and liver or pancreas diseases were excluded from this study.

Measurement of B2MG

The serum samples were stored at −70 °C until further measurements were performed at SRL Inc. (Tokyo, Japan). The serum concentrations of both β2MG and Cr were determined by a latex agglutination immunoassay and an enzymatic method, respectively, by using a Bio Majesty automated analyzer (JCA-BM8060; JEOL Ltd, Tokyo, Japan).

Test validity of reference value

The archival serum β2MG and Cr data collected from patients with various kidney diseases hospitalized between 2004 and January 2010 for routine examinations for clinical management were used to test the validity of the established β2MG reference values. The collected data included 345 serum samples from 21 children with various kidney diseases, including hypoplastic or dysplastic kidney ($n = 8$), kidney injury during the neonatal period ($n = 3$), reflux nephropathy ($n = 1$), post-hemolytic uremic syndrome ($n = 1$), focal segmental glomerulosclerosis (FSGS) ($n = 4$), congenital nephrotic syndrome ($n = 1$), IgA nephropathy ($n = 1$), drug-induced renal dysfunction ($n = 1$), and mitochondrial disease ($n = 1$). The patients were aged 0.1–13.6 years (mean 6.0 ± 4.8 years) at the time of diagnosis, and all developed decreased GFR during their disease course. Samples were collected when the patients were 0.6–16.9 years of age (mean 8.3 ± 5.3 years). The mean observation period was 3.1 ± 2.6 years. The male-to-female ratio was 14:7. All samples were confirmed to be C-reactive protein-negative to exclude the possible effect of inflammation on β2MG values. Medical records for the BL and body weight taken during blood tests were also collected. All patients gave their informed consent at the beginning of treatment for the use of the data in addition to that required for diagnostic purposes, i.e., for research purposes.

Individual serum Cr values and the reference value calculated by the recently established polynomial equation formula were used to evaluate the kidney dysfunction, as follows [1]:

$$\text{For boys: } y = -1.259x^5 + 7.815x^4 - 18.57x^3 + 21.39x^2 - 11.71x + 2.628$$

$$\text{For girls: } y = -4.536x^5 + 27.16x^4 - 63.47x^3 + 72.43x^2 - 40.06x + 8.778,$$

where y is the reference serum creatinine (mg/dl) and x is body length (m).

Thus, kidney function was defined as [patient Cr/reference Cr (y) × 100 (%)].

tistical analysis

ɜ statistical analysis was performed with the GraphPad ɜm software package (Ver. 5.0; GraphPad Software, San ɜgo, CA). The reference cohort with β2MG and Cr was ɔdivided into separate age groups for girls and boys. The ɜerences between the groups were tested with Kruskal-Wallis nonparametric analysis of variance NOVA), Mann-Whitney *U* test, or chi-square analyses appropriate. The relationship between age and serum MG concentration was determined by both linear and ynomial regression analyses. The data are expressed as mean ± standard deviation (SD) or 95 % confidence ɜrval (CI). Associations between age, BL, serum Cr, and ney function (%) were assessed with correlation coefents according to Pearson (*r*). $P < 0.05$ was defined as tistically significant in all analyses.

sults

MG reference values in Japanese children

e characteristics of healthy children were as follows: the ɜan age was 7.8 ± 4.7 years (95 % CI 7.5–8.1 years) ɜh a range of 0.1–16.7 years and a median of 6.9 years. e mean BL was 1.21 ± 0.30 m (range 0.54–1.85 m). There were 64 children who were taking cold medicine or antiallergic agents, though no one had fever or any other symptoms of inflammation. The median, 2.5 percentile, and 97.5 percentile serum β2MG reference values in each subgroup of age are summarized in Table 1. Combining these values as a single cohort yielded a mean serum β2MG concentration of 1.45 ± 0.3 mg/l (95 % CI 1.43–1.47 mg/l). There were no differences in β2MG concentrations between boys and girls of any age group; however, the β2MG data varied widely, particularly in younger subjects (Table 1). It appears that there was a significant change in the value of the upper limit (97.5th percentile) between children aged between 1 and 2 years (Table 1).

Scattergrams show the age-dependent distribution of serum β2MG concentrations (Fig. 1) in which the serum β2MG concentration gradually decreases with age. There is a significant negative correlation among the serum β2MG concentration, age, and BL (both $r = -0.47$, $P < 0.0001$), and the regression equations were $y = -0.0341x + 1.72$ and $y = -0.0055x + 2.12$, respectively (Fig. 1a, b). The relationships between serum β2MG level and age (years) or BL (m) were also determined by polynomial regression analysis, and the reference serum β2MG level was expressed as a cubic equation of age or BL (Fig. 1a, b; broken lines). The regression equations were as follows:

ble 1 Median, 2.5th centile, and 97.5th percentile serum β2MG reference ues in each age group ording to sex

Age	All subjects				Boys				Girls			
	n	2.5 %	50 %	97.5 %	*n*	2.5 %	50 %	97.5 %	*n*	2.5 %	50 %	97.5 %
3–5 months	21	1.5	1.8[a]	3.2	17	1.5	1.8	3.2	4	1.6	1.8	2.1
6–8 months	18	1.4	1.8[a]	2.6	14	1.4	1.9	2.6	4	1.6	1.6	2.3
9–11 months	29	1.3	1.7[a]	3.3	15	1.3	1.7	3.3	14	1.3	1.8	3.2
1 years	69	1.4	1.7[a]	3.1	32	1.4	1.7	3.2	37	1.2	1.6	3.0
2 years	73	1.0	1.5	2.5	40	1.0	1.5	2.2	33	1.0	1.5	3.4
3 years	85	1.0	1.5	2.3	46	1.1	1.5	2.3	39	1.0	1.5	2.4
4 years	78	1.1	1.4	2.5	42	1.0	1.4	2.1	36	1.1	1.4	3.1
5 years	94	1.1	1.4	2.3	46	1.1	1.5	2.7	48	1.0	1.4	2.2
6 years	101	1.1	1.4	2.3	43	1.1	1.4	2.4	58	1.0	1.5	2.3
7 years	83	1.0	1.4	2.1	36	0.9	1.3	2.1	47	1.0	1.4	2.2
8 years	55	1.0	1.4	2.5	19	1.0	1.4	1.8	36	1.0	1.4	2.3
9 years	37	1.0	1.4	2.1	18	1.1	1.4	1.8	19	1.0	1.4	2.1
10 years	42	0.9	1.3	1.9	11	1.1	1.4	1.6	31	0.9	1.3	1.9
11 years	58	1.0	1.3	2.3	19	1.1	1.3	2.1	39	1.0	1.2	2.4
12 years	69	1.0	1.3	1.8	14	1.2	1.3	1.5	55	0.9	1.3	1.9
13 years	68	1.0	1.3	1.8	30	1.0	1.4	2.0	38	1.0	1.2	1.5
14 years	57	0.9	1.3	2.0	17	1.1	1.4	2.0	40	0.9	1.2	1.7
15 years	35	0.8	1.2	1.8	15	0.8	1.2	1.8	20	0.8	1.1	1.7
16 years	59	0.8	1.2	1.8	30	0.8	1.2	1.8	29	0.8	1.1	1.4
All ages	1311	1.0	1.4	2.3	504	1.0	1.4	2.3	627	1.0	1.4	2.3

' < 0.0001 in comparison to mean value in all subjects

For age: $y = -0.000472x^3 + 0.0139x^2 - 0.149x + 1.94$

For BL: $y = -0.354x^3 + 1.79x^2 - 3.26x + 3.36$

β2MG exhibited significant correlations with age (correlation coefficient of −0.50) and with BL (correlation coefficient of −0.49), which were slightly improved compared to those in the linear regression analysis.

There was no relationship between the β2MG concentration and age in children less than 2 years of age; however, β2MG levels showed a significant negative correlation with age in children more than 2 years of age (Fig. 1c, d). Statistical analyses revealed that the β2MG levels in age groups of 0–5 months (1.94 ± 0.44 mg/l), 6–8 months (1.92 ± 0.38 mg/l), 9–11 months (1.80 ± 0.48 mg/l), and 1 year (1.80 ± 0.42 mg/l) were significantly higher than the overall mean value of all the subjects (1.45 ± 0.34 mg/l, $P < 0.001$). However, no difference was found in the >2 years age group.

There were 14 outliers of the upper limit of age-specific values (Fig. 2a); however, these were unrelated to the corresponding Cr values, which were within the normal range (Fig. 2b). Out of the 14 children, 6 were taking cold medicine or antiallergic agents, and the number of subjects taking such medicines was significantly high (66 cases) among the total subjects ($P < 0.001$, by the chi-square test).

Assessment of β2MG value in children with CKD

The validity of the reference range of the established reference value for β2MG was tested by reviewing data from children with various kidney diseases during the course of

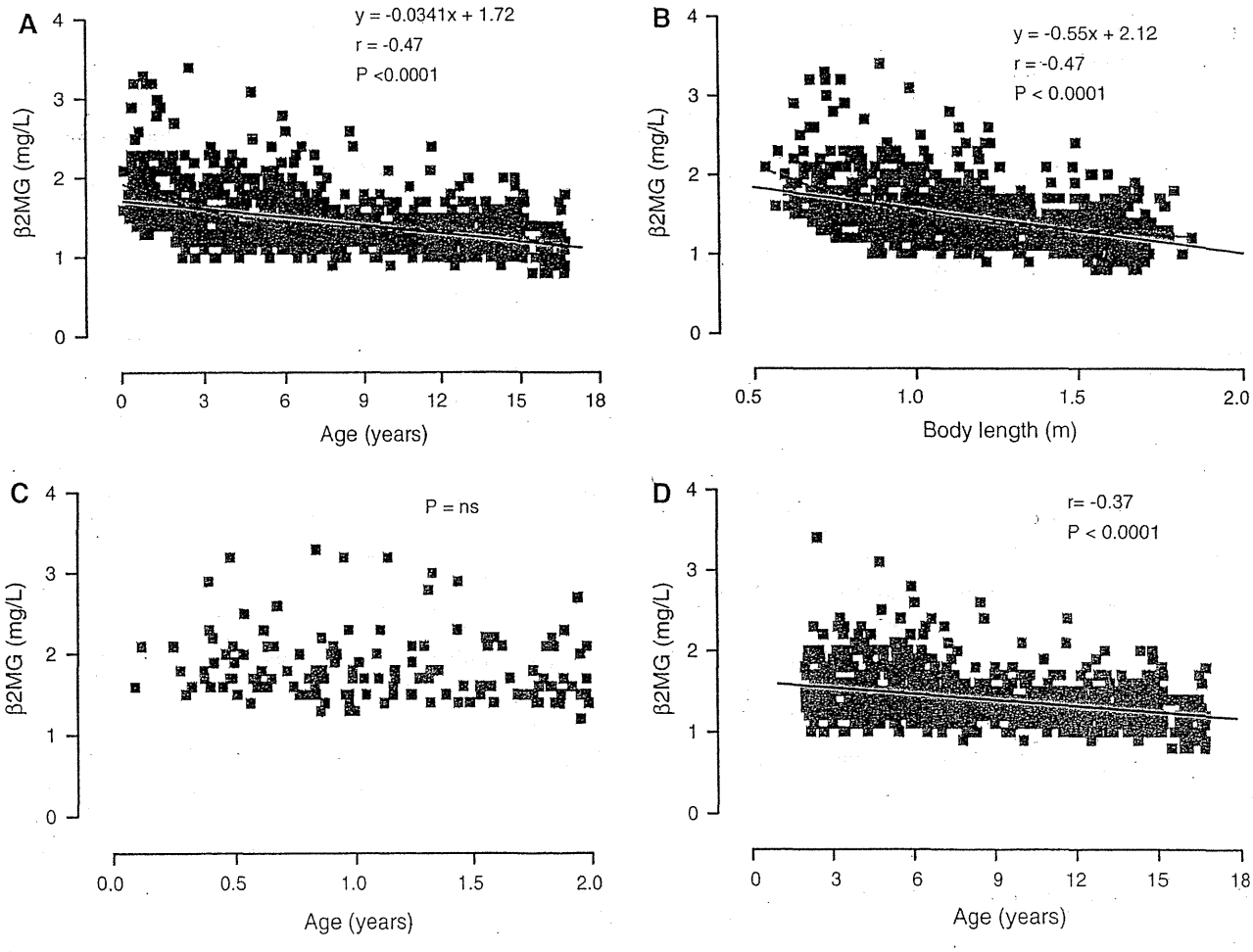
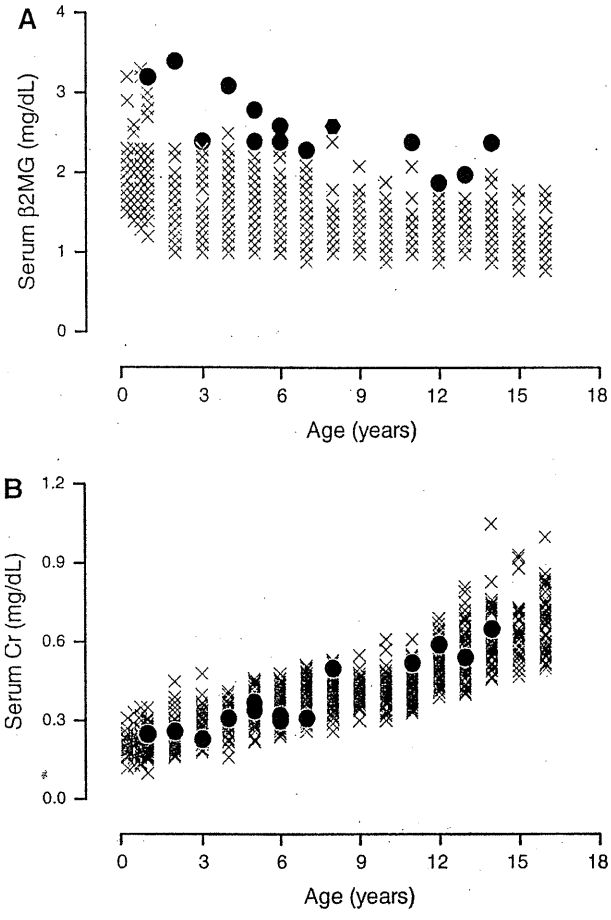


Fig. 1 Serum concentrations of β2MG in relation to age and body length (BL). Linear regression lines between the serum concentration β2MG and age (year) (**a**) or BL (m) (**b**) of all subjects are shown. The regression equations are $y = -0.0341x + 1.72$ and $y = -0.0055x + 2.12$, respectively (*straight lines*). The relationships are also determined by polynomial regression analysis, and the reference serum β2MG level is expressed as a cubic equation of age (**a**) or BL (**b**) (*broken lines*). The regression equations are as follows: $y = -0.000472x^3 + 0.0139x^2 - 0.149x + 1.94$ for age and $y = -0.354x^3 + 1.79x^2 - 3.26x + 3.36$ for BL. β2MG did not correlate with ages less than 2 years (**c**), but it did correlate significantly with ages above 2 years (**d**)



'ig. 2 Age-specific serum concentrations of β2MG (**a**) and Cr (**b**).)utliers beyond the 97.5th percentile range for β2MG reference alues (**a**) and their corresponding Cr values (**b**) are shown as *black 'ots*

heir disease. Most of the serum concentrations of β2MG vere beyond the upper 97.5th percentile of age-appropriate eference values when the Cr level was beyond the 97.5th ercentile of age-appropriate reference values. Out of the 45 samples, 329 indicated reduced kidney function below 5 %, and 344 of these (99.7 %) could be detected using he newly established age-specific β2MG reference range. Iowever, data from 2 patients showed a discrepancy etween the serum β2MG concentrations and serum Cr evel or kidney function (Fig. 3). Their kidney function as valuated based on the serum Cr value and BL was grad- ally decreased from the normal level to below 75 % luring their course, but it was accompanied by a relatively uick increase of β2MG for their age (Fig. 3).

Patient 1 was a 14-year-old boy and was referred to the lepartment of pediatric nephrology for proteinuria and evere emaciation. He had been diagnosed with mito- hondrial disease by a muscle biopsy when he was 11 years ld. His body weight was 21.1 kg (−3.0 SD for mean apanese weight at his age) and body length was 136.5 cm (−3.6 SD). Laboratory data showed proteinuria, 120 mg/dl without kidney insufficiency; serum Cr, 0.42 mg/dl; and β2MG, 1.6 mg/l. His BL gradually increased to 143.8 cm (−4.4 SD) over the next 2 years, but his body weight was stable at 20 kg (−3.9 SD). The serum β2MG level gradually increased with the decrease of kidney function and exceeded the upper limit (97.5th percentile) of the established standard range for his age when he was 15.6 years old (Fig. 3a). At that time, an endogenous Cr clearance (CCr) test revealed his CCr to be 53.0 ml/min/1.73 m^2.

Patient 2 was a boy diagnosed with FSGS when he was 13 years old. At diagnosis, his serum Cr and β2MG levels were 0.5 mg/dl and 1.9 mg/l, respectively. His BL was 144.6 cm (−1.6 SD for the mean Japanese BL at his age) and calculated kidney function was 110.3 %. In addition to FSGS, he had an uncontrolled nephrotic range of proteinuria, and his kidney function decreased below 75 % in the next 9 months (Fig. 3b). His serum levels of both Cr and β2MG were elevated according to his kidney function, and the β2MG level was beyond the upper 97.5th percentile range during the same time that the kidney function decreased below 75 %. In contrast, his serum Cr level was still within the normal range for his age when the calculated kidney function decreased below 75 % (Fig. 3b).

Discussion

Several serum markers, including Cr, β2MG, and cystatin C, have been used to evaluate kidney function [7, 8]. However, for the use of these markers in children, an understanding of their normal reference values and their relationships with age and build according to differences among races is essential. Therefore, we recently conducted an ongoing multicenter large-scale study to examine this point. The reference value for serum Cr in healthy Japanese children has already been established [1]. The present study was aimed at determining the reference range of β2MG in healthy Japanese children as the second step of our study.

In this study, we found a significant correlation between β2MG concentration and age (Fig. 1a), which was different from previous reports [4, 5]. There was also a significant negative correlation between β2MG and BL, and they had the same regression coefficient ($r = -0.47$) (Fig. 1b). Therefore, it can be argued that the independent relation of β2MG with age and body mass, which has been one of the advantages for its use as a marker, is not applicable in studies on children. However, the current study showed that the slope of the regression line for β2MG with age is gradual and reaches a plateau in a short time (Fig. 1). Moreover, β2MG and age are negatively correlated, and therefore, elevations in β2MG concentrations relative to age can be easily detected. Indeed, the retrospective

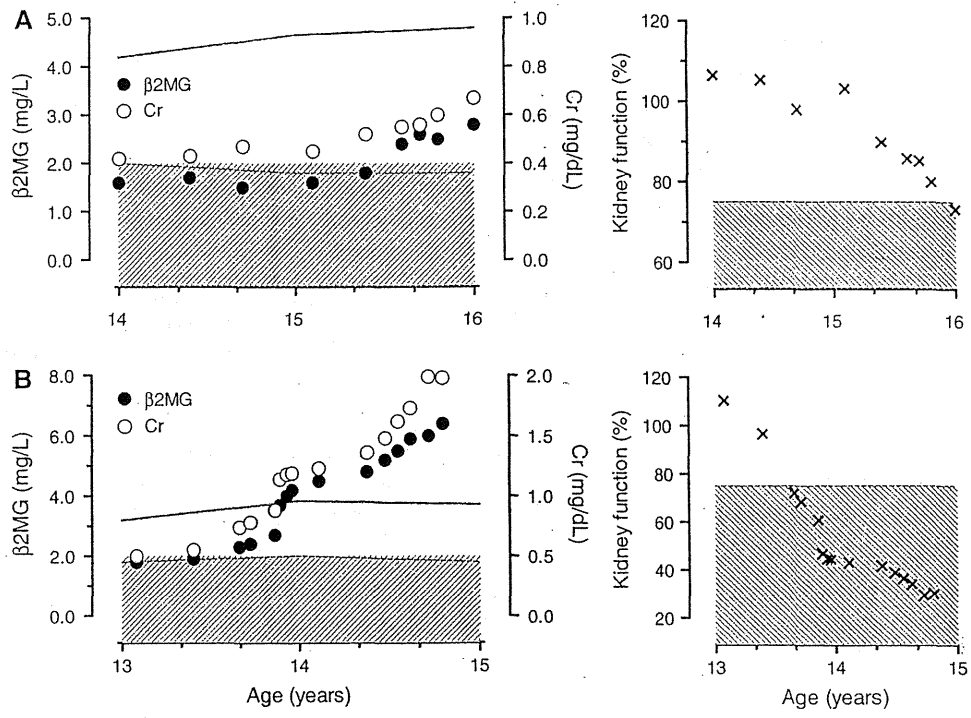


Fig. 3 Time course of kidney function (%) and serum β2MG and Cr concentrations in patient 1 (**a**) and patient 2 (**b**). *Shaded area* and *solid line in the left panel* represent the age-specific reference range (2.5–97.5th percentile) for β2MG and Cr, respectively. The *shaded area in the right panel* represents the age-specific reference range for kidney function under 75 %

assessments tested the clinical validity of the newly established β2MG reference in patients with various kidney diseases, distributed over a wide range of age groups, revealed that β2MG was a highly sensitive marker (99.7 %) for detecting kidney dysfunction below 75 %.

β2MG forms the beta chain of the human leukocyte antigen class I molecule and is present on the surface of most nucleated cells [9]. Although the mechanism of the dependency of β2MG on age is unknown, many immunological features in children, including an immature immune system in infants and lymphocytic predominance of circulating leukocytes in young children, could explain how serum β2MG concentrations change with age. Many of the subjects among the high β2MG outliers were taking cold medicine or antiallergic agents, indicating that some kind of immune reaction caused by the common cold or some allergic diseases, including bronchial asthma and atopic dermatitis, could affect β2MG production. Indeed, such diseases are common among young children. Data from studies examining serum β2MG values in fetuses or neonates reveal that the mean value of β2MG is relatively higher (around 3.5 mg/l) than that of young children with no renal complications in the present study [10, 11].

The current study used the equation for kidney function derived from serum Cr: kidney function (%) = (reference serum Cr/patient's serum Cr) × 100, since the reciprocal of serum Cr is generally correlated with GFR [12, 13]. Assuming 100 % kidney function to be GFR 120 ml/min, 75 % kidney function is comparable to GFR 90 ml/min, which is the borderline between CKD stage 1 and 2 [14]. An advantage of using this method is that since this formula is based on BL rather than age, kidney function can be appropriately estimated for growing children. There are, however, still significant disadvantages of using Cr as a marker for detecting mild impairment of kidney function in children. Herein, we presented a typical case of this situation (Fig. 3). In children with a very low muscle mass, Cr-based estimation of GFR can be misleading. Cr can also be overestimated in children with advanced renal failure, in whom there is reduced Cr production due to malnutrition [13]. Although β2MG has the disadvantage of being increased in patients with inflammatory and infectious diseases and several malignancies [6], detection of increased β2MG concentrations appears to be easier than that of Cr. Therefore, as compared to Cr, β2MG appeared to be a better marker of kidney impairment in children with abnormally low body mass. It also appears to be favorable for children with short stature in mild kidney dysfunction.

In addition to β2MG, recent studies have reported that cystatin C also facilitates the recognition of abnormal renal function in children compared to Cr because its reference range is independent of age, gender, height, and body composition [7, 8]. The applicability of cystatin C, however, remains a matter of debate. A standard value for cystatin C in children has not yet been established; therefore, considering the diagnostic sensitivity of cystatin C for impaired GFR in pediatric patients, particularly in patients with only mildly impaired kidney function, cystatin C may not be a better indicator than the BL/Cr ratio [15]. Furthermore, the measurement of cystatin C is currently too

ensive for routine use in clinical practice. However, atin C will also be a potentially useful marker once a rence value in normal children, according to race, has n established, and the differences between the diagic significance of Cr and β2MG become clear. We eve that our ongoing large-scale study that aims to blish the reference value of cystatin C in Japanese dren will provide a better understanding of this marker clinical use.

n summary, the current study determined a new β2MG rence value for detecting kidney impairment in chil-n. Measurement of the serum β2MG concentrations in ibination with serum Cr concentrations, and perhaps atin C in the near future, as markers for predicting nerular function will provide better accuracy in the ction of reduced kidney function in children.

nowledgments This study was financially supported by the ey Foundation, Japan. We thank Midori Awazu, MD, Takashi ne, MD, Mayumi Sako, MD, Takuji Yamada, MD, Yuko Akioka, and Hirotsugu Kitayama, MD, of the Committee of Measures for atric CKD for their contributions.

erences

Uemura O, Honda M, Matsuyama T, Ishikura K, Hataya H, Yata N, Nagai T, Ikezumi Y, Fujita N, Ito S, Iijima K, Kitagawa T. Age, gender, and body length effects on reference serum creatinine levels determined by an enzymatic method in Japanese children: a multicenter study. Clin Exp Nephrol. 2011;15:694–9.
Schwartz GJ, Haycock GB, Edelmann CM Jr, Spitzer A. A simple estimate of glomerular filtration rate in children derived from body length and plasma creatinine. Pediatrics. 1976;58:259–63.
Kwong MB, Tong TK, Mickell JJ, Chan JC. Lack of evidence that formula-derived creatinine clearance approximates glomerular filtration rate in pediatric intensive care population. Clin Nephrol. 1985;24:285–8.
4. Donadio C, Lucchesi A, Ardini M, Giordani R, Cystatin C. Beta 2-microglobulin, and retinol-binding protein as indicators of glomerular filtration rate: comparison with plasma creatinine. J Pharm Biomed Anal. 2001;24:835–42.
5. Filler G, Priem F, Lepage N, Sinha P, Vollmer I, Clark H, Keely E, Matzinger M, Akbari A, Althaus H, Jung K. Beta-trace protein, cystatin C, beta(2)-microglobulin, and creatinine compared for detecting impaired glomerular filtration rates in children. Clin Chem. 2002;48:729–36.
6. Karlsson FA, Wibell L, Evrin PE. Beta 2-microglobulin in clinical medicine. Scand J Clin Lab Invest Suppl. 1980;154:27–37.
7. Bökenkamp A, Domanetzki M, Zinck R, Schumann G, Byrd D, Brodehl J. Cystatin C—a new marker of glomerular filtration rate in children independent of age and height. Pediatrics. 1998;101:875–81.
8. Sharma AP, Yasin A, Garg AX, Filler G. Diagnostic accuracy of cystatin C-based eGFR equations at different GFR levels in children. Clin J Am Soc Nephrol. 2011;6:1599–608.
9. Vincent C, Revillard J. B2M and HLA-related glycoproteins in human urine and serum. Contr Nephrol. 1981;26:66–88.
10. Assadi FK, John EG, Justice P, Fornell L. Beta 2-microglobulin clearance in neonates: index of tubular maturation. Kidney Int. 1985;28:153–7.
11. Engle WD, Arant BS Jr. Renal handling of beta-2-microglobulin in the human neonate. Kidney Int. 1983;24:358–63.
12. Perrone RD, Madias NE, Levey AS. Serum creatinine as an index of renal function: new insights into old concepts. Clin Chem. 1992;38:1933–53.
13. Hari P, Bagga A, Mahajan P, Lakshmy R. Effect of malnutrition on serum creatinine and cystatin C levels. Pediatr Nephrol. 2007;22:1757–61.
14. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis. 2002;39:S1–266.
15. Filler G, Priem F, Vollmer I, Gellermann J, Jung K. Diagnostic sensitivity of serum cystatin for impaired glomerular filtration rate. Pediatr Nephrol. 1999;13:501–5.

Article

Two-Year Follow-Up of a Prospective Clinical Trial of Cyclosporine for Frequently Relapsing Nephrotic Syndrome in Children

Kenji Ishikura, Norishige Yoshikawa,† Hitoshi Nakazato,‡ Satoshi Sasaki,§ Kazumoto Iijima,‖ Koichi Nakanishi,† Takeshi Matsuyama,¶ Shuichi Ito,** Nahoko Yata,†† Takashi Ando,‡‡ and Masataka Honda,* for the Japanese Study Group of Renal Disease in Children*

Summary

Background and objectives Although the safety and efficacy of cyclosporine in children with frequently relapsing nephrotic syndrome (FRNS) have been confirmed, no prospective follow-up data on relapse after cyclosporine have appeared. This study is a prospective follow-up trial after 2-year treatment with cyclosporine to investigate cyclosporine dependency after its discontinuation.

Design, setting, participants, & measurements Participants who had undergone 2-year protocol treatment with microemulsified cyclosporine for FRNS between January 2000 and December 2005 were followed for an additional 2 years. The primary end point was relapse-free survival after the complete discontinuation of cyclosporine, and the secondary end point was regression-free survival (time to regression to FRNS).

Results After exclusion of 7 patients who showed regression to FRNS during the 2-year treatment period, 49 children (median age, 6.5 years) were followed, and classified as children without (n=32; group A) and with (n=17; group B) relapse during the initial cyclosporine treatment. Overall, relapse-free survival probability at 24 months after cyclosporine discontinuation was 15.3% and regression to FRNS-free survival probability was 40.8%. By group, the probability of relapse-free survival was significantly higher in group A (17.9%) than in group B (8.3%) (P<0.001).

Conclusions Children with FRNS who receive cyclosporine are at high risk of relapse after discontinuation, particularly those who experience relapse during cyclosporine treatment.

Clin J Am Soc Nephrol 7: 1576–1583, 2012. doi: 10.2215/CJN.00110112

Departments of *Nephrology and ††Pediatrics, Tokyo Metropolitan Children's Medical Center, Fuchu, Japan; †Department of Pediatrics, Wakayama Medical University, Wakayama, Japan; ‡Department of Pediatrics, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan; §Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan; ‖Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan; ¶Department of Pediatrics, Fussa Hospital, Fussa, Japan; **Division of Nephrology and Rheumatology, National Center for Child Health and Development, Setagaya, Japan; and ‡‡Japan Clinical Research Support Unit, Tokyo, Japan

Correspondence: Dr. Kenji Ishikura, Department of Nephrology, Tokyo Metropolitan Children's Medical Center, 2-8-29 Musashidai, Fuchu, Tokyo 183-8561, Japan. Email: kenji_ishikura@tmhp.jp

Introduction

The safety and efficacy of cyclosporine in children with frequently relapsing nephrotic syndrome (FRNS) and steroid-dependent nephrotic syndrome (SDNS) have been demonstrated in a number of studies, including a randomized controlled trial (1–8). We previously showed that microemulsified cyclosporine administered according to our treatment protocol is safe and effective in children with FRNS (2). In that study, the probability of relapse-free survival at month 24 was 58.1% and the probability of regression (to frequently relapsing nephrotic syndrome)–free survival at month 24 was 88.5%. Cyclosporine nephrotoxicity was detected in only 8.6% of patients who underwent renal biopsy after 2 years of treatment. Nevertheless, an important limitation of this treatment is cyclosporine dependency, namely the frequent relapse of nephrosis after discontinuation (9–12). Most studies of relapse after cyclosporine to date have been retrospective, however, and no prospective evaluation in these patients has been reported.

Several factors have been linked to a prolonged disease course in children with FRNS, including age at the onset of nephrotic syndrome (13,14) and number of relapses or steroid dependency (13,15,16). However, factors associated with relapse after cyclosporine have not been clearly established. Furthermore, it is unclear whether infrequent relapse during cyclosporine treatment is associated with disease activity. Identification of risk factors would allow better treatment decisions, particularly in predicting patients at highest risk of regression of FRNS.

Here, we conducted a prospective follow-up study of the participants of our previous clinical trial to evaluate the rate of relapse of nephrosis and FRNS after the complete discontinuation of cyclosporine. We also evaluated factors associated with relapse.

Materials and Methods

Previous Trial

This study was a prospective follow-up analysis of a previous multicenter trial that evaluated the efficacy

nd safety of 2-year treatment with cyclosporine in children vith FRNS between January 2000 and December 2005. ntry criteria and protocol have been detailed elsewhere 2). Briefly, children aged 1–18 years with FRNS who had diopathic nephrotic syndrome were eligible. Patients vith a history of cyclosporine treatment were excluded. Microemulsified cyclosporine (Neoral; Novartis, Basel, Switzerland) was administered for 2 years under trough ontrol. For the first 6 months, all patients were dministered a dose that maintained a whole-blood trough evel between 80 and 100 ng/ml of cyclosporine. The dose vas adjusted over the next 18 months to maintain a trough evel between 60 and 80 ng/ml. Maintenance prednisolone vas not prescribed. After 2 years of treatment, all patients vere scheduled to undergo renal biopsy, and cyclosporine vas stopped by dose tapering at a rate of 0.5–1.0 mg/kg per week.

Follow-Up Study

This study was conducted under a prospective, follow-up design in 21 participating institutions. Eligibility was restricted to patients who completed 2-year treatment with cyclosporine in the previous trial. Follow-up was conducted for 2 years, beginning from the time of complete cessation of cyclosporine. Patients who experienced relapse of nephrosis during the study period were administered prednisolone at 2 mg/kg per day in three divided doses (maximum, 80 mg/d) until remission, and then a single dose of prednisolone at 2 mg/kg in the morning on alternate days for 2 weeks, 1 mg/kg on alternate days for the next 2 weeks, and 0.5 mg/kg on alternate days for a final 2 weeks. Children who then met the criteria for FRNS received cyclosporine by the same protocol as in the original trial (2), or cyclophosphamide (2.5 mg/kg per day) for 8–12 weeks if cyclosporine nephrotoxicity was found on renal biopsy. Use of antihypertensive drugs was not restricted, including angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers. Data were collected from the participating institutions for analysis at 1-year intervals, and included body weight and height, BP, medications given, blood analysis (complete blood cell count, blood chemistry), and urine tests (urinalysis, quantitative proteinuria).

The design and execution of this study were in accordance with the ethical standards of the Declaration of Helsinki. The protocol was approved by the ethics committee of Tokyo Metropolitan Kiyose Children's Hospital (predecessor of Tokyo Metropolitan Children's Medical Center). Informed consent was obtained from all patients or their parents. This study was registered in the University Hospital Medical Information Network public trials registry (ID C000000010; http://www.umin.ac.jp/ctr/index.htm).

Criteria and Definitions

The criteria for and definitions of nephrotic syndrome, remission, and relapse were in accordance with the International Study of Kidney Disease in Children (17). FRNS was defined as ≥2 relapses of nephrotic syndrome within 6 months after the initial episode, ≥3 within any 6-month period, or ≥4 within any 12-month period. Furthermore, FRNS was defined as including SDNS and any case requiring the administration of an immunosuppressant (cyclosporine or cyclophosphamide). Steroid dependence was defined as the occurrence of two consecutive relapses during tapering of the steroid dosage or within 14 days after the cessation of administration. Renal function was evaluated as estimated GFR (eGFR) calculated using the Chronic Kidney Disease in Children study equation [0.413 × (height/serum creatinine)] (18).

Statistical Analyses

Kaplan–Meier analyses for relapse from the complete discontinuation of cyclosporine were conducted in children who had discontinued cyclosporine without the occurrence of FRNS during tapering. Analyses included time to first relapse and time to the regression to FRNS. Differences between groups were compared using the log-rank test. Risk ratio and 95% confidence interval (CI) for first recurrence and for regression to FRNS after discontinuation of cyclosporine were evaluated by Poisson regression analysis, using the explanatory variables of group (patients without [group A] and with [group B] relapse during cyclosporine treatment), age (<6 years and ≥6 years), and history of steroid dependence before the start of cyclosporine treatment. Sandwich variance was used to handle the overdispersion problem. Relapses occurring during tapering were excluded from the primary analysis of relapse-free survival, with only those occurring after the complete discontinuation of cyclosporine counted as events in the Kaplan–Meier and Poisson analyses. Patients administered cyclosporine or cyclophosphamide by their attending physician were considered to have regressed to FRNS regardless of whether they fulfilled the criteria of FRNS. All statistical analyses were performed using the SAS software package for Windows (release 9.13; SAS Institute Inc, Cary, NC). A two-sided P value <0.05 was considered statistically significant in all analyses.

Results

Data Set

Of the 62 children with FRNS in our previous study who received cyclosporine for 2 years (2), 7 regressed to FRNS during protocol treatment. Cyclosporine in these children was discontinued and their data were excluded from the present analyses. After exclusion of 6 other children, 4 due to loss to follow-up and 2 due to protocol violation (refusal to discontinue cyclosporine), this study followed and analyzed a total of 49 children (median age at complete discontinuation of cyclosporine, 6.5 years; 39 male patients) (Figure 1). Although follow-up was set at 24 months, one patient each was censored at 16, 19, and 21 months due to loss to follow-up, and six patients completed 2-year follow-up at 23 months. The participants were classified into two groups, with group A consisting of patients who had not experienced relapse during the 2-year cyclosporine treatment (n=32) and group B consisting of those who had (n=17). Basic characteristics of these children are shown in Table 1.

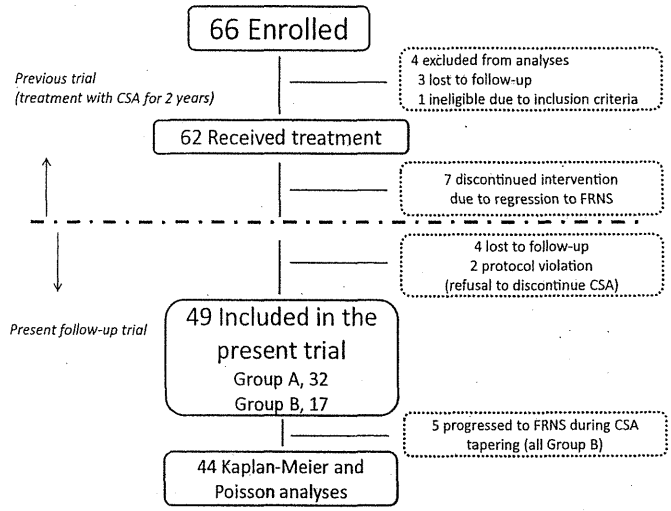


Figure 1. | Flow diagram. Group A included children without relapse during the initial cyclosporine treatment, whereas group B included children with relapse during the initial cyclosporine treatment. CSA, cyclosporine; FRNS, frequently relapsing nephrotic syndrome.

Table 1. Background characteristics of study participants

	All Participants			Participants Succeeding in Discontinuing CSA		
	Group		All	Group		All
	A	B		A	B	
Number of participants	32	17	49	32	12	44
Sex						
Male	24	15	39	24	10	34
Female	8	2	10	8	2	10
Age (yr)						
0<6	12	5	17	12	4	16
6–12	17	6	23	17	6	23
≥12	3	6	9	3	2	5
Median (IQR)	6.4 (5.3–8.5)	7.4 (5.9–12.1)	6.5 (5.5–9.6)	6.4 (5.3–8.5)	6.5 (5.8–10.3)	6.4 (5.4–8.5)
Steroid dependence						
No	16	7	23	16	6	22
Yes	16	10	26	16	6	22
Discontinuation of CSA						
Successful	32	12	44	32	12	44
Failed	0	5	5			
CSA does tapering period (d)						
Median (IQR)				68 (45–94)	77 (63–112)	69 (48–97)
Relapse in the tapering period						
No				31	9	40
Yes				1	3	4

CSA, cyclosporine; IQR, interquartile range.

Probability of Relapse-Free and Regression-Free Survival

The median (interquartile range [IQR]) cyclosporine dose tapering period was 68 (45–94) days in group A and 77 (63–112) days in group B (Table 1). Discontinuation of cyclosporine failed in five participants due to the regression of FRNS during tapering, all of whom belonged to group B. Kaplan–Meier and Poisson analyses were conducted in the remaining 44 children (group A, n=32; group B, n=12).

Thirty-seven children experienced relapse during the follow-up period. The median (IQR) time from the complete cessation of cyclosporine to relapse was 4.3 (1.5–15.6) months for group A and 0.5 (0.0–1.1) months for group B

le 2). In group A, 6 patients (19%) did not experience a ›se, 9 (28%) had infrequent relapses, and 17 (53%) re-ɜed to FRNS after the discontinuation of cyclosporine. ·oup B, one patient (8.3%) did not experience a relapse, (16.7%) had infrequent relapses, and nine (75%) re-ɜed to FRNS after discontinuation. Time to regression RNS was 16.6 months for group A and 3.8 months for ıp B. The probability of relapse-free survival at 24 ths from complete discontinuation was 15.3% in all lren (Figure 2) and that of regression to FRNS-free ival was 40.8% (Figure 3). By group, the probability lapse-free survival was significantly higher in group A)%) than in group B (8.3%) ($P<0.001$, Figure 2).

ors Associated with Relapse

ıe recurrence rates were 0.089 per month (26 of 292.7 ıths) for group A and 0.30 per month (11 of 36.3 months) for group B, whereas the rate of regression to FRNS was 0.034 per month (17 of 506.2 months) and 0.074 per month (9 of 122.4 months), respectively. Results of Poisson regression are shown in Table 3. Controlling for age and steroid dependence at the beginning of cyclosporine treatment, the risk ratio of group A to B for first recurrence was 0.17 (95% CI, 0.04–0.72; $P=0.02$). Similar results were obtained for the risk of regression to FRNS. The risk ratio for group A compared with group B was 0.35 (95% CI, 0.15–0.83; $P=0.02$).

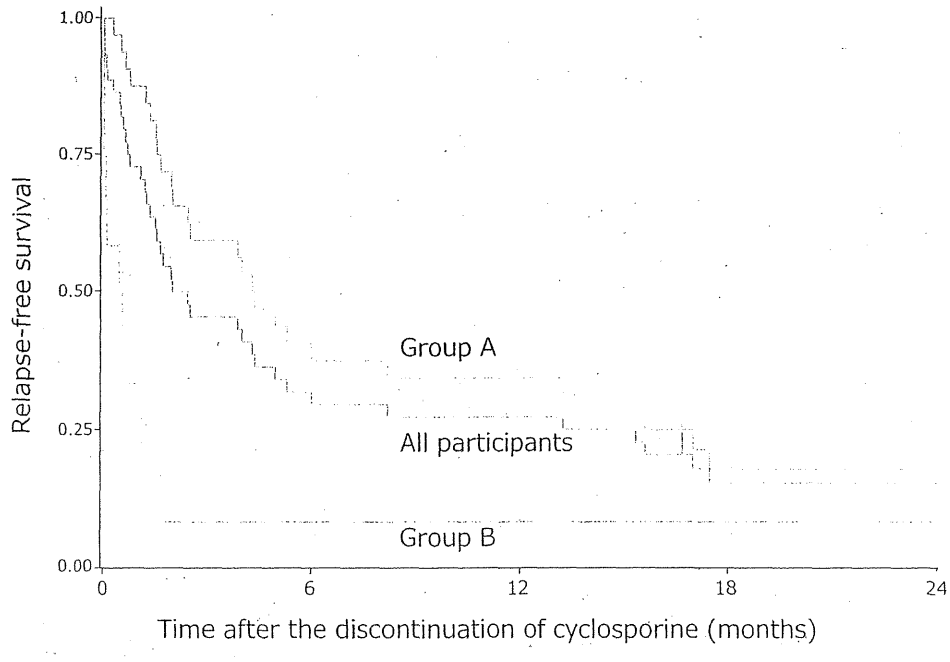
Patient Survival, Renal Survival, and Growth

Information regarding patient survival and renal survival was available for 58 of 62 patients who received protocol treatment with cyclosporine. No lethal event was seen. Mean eGFR was 140.9±31.6 ml/min per 1.73 m^2 ($n=62$, no patient with eGFR <90 ml/min per 1.73 m^2) at

ble 2. Number of participants who experienced relapse and time to relapse

:oup	Total, *n*	Children Who Experienced Relapse, *n* (%)	Time to First Relapse (mo), Median (IQR)	Children Who Regressed to FRNS, *n* (%)	Time to FRNS (mo), Median (IQR)
A	32	26 (81)	4.3 (1.5–15.6)	17 (53)	16.6 (7.5)
B	12	11 (92)	0.5 (0.0–1.1)	9 (75)	3.8 (3.1–11.1)
All	44	37 (84)	2.0 (0.7–13.3)	26 (59)	10.2 (4.4)

RNS, frequently relapsing nephrotic syndrome; IQR, interquartile range.



Number of participants remaining

Group A	32	13	11	5	5
Group B	12	1	1	1	1

ure 2. | Relapse after the discontinuation of cyclosporine. Relapse-free survival probability at 2 years was 15.3% in all participants, and 9% in those without (group A) and 8.3% in those with (group B) relapse during cyclosporine treatment ($P<0.001$, log-rank test).

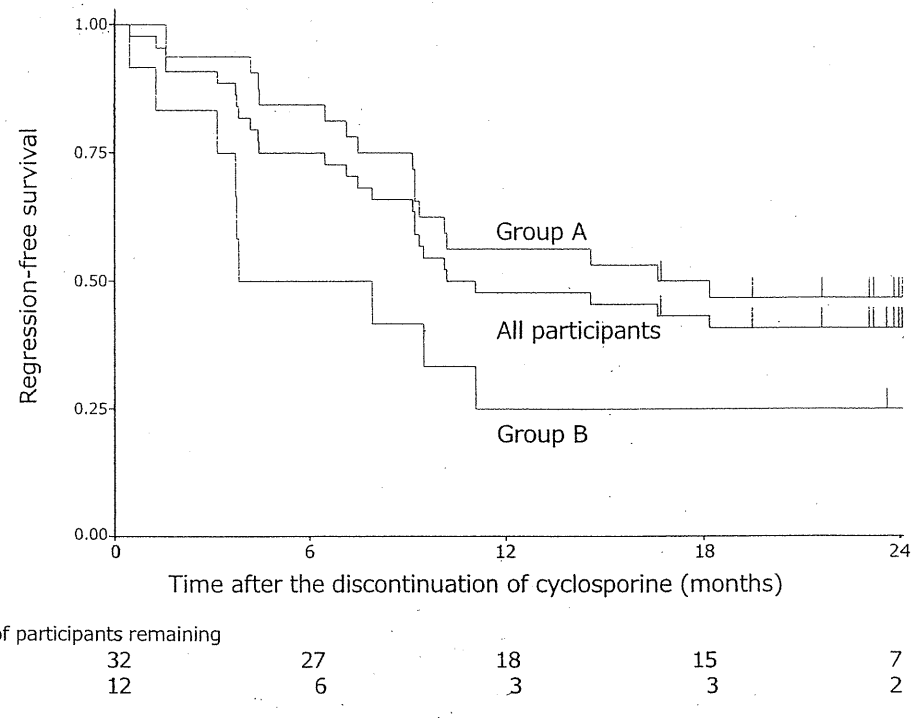


Figure 3. | Regression to FRNS after discontinuation of cyclosporine. Regression (to FRNS)–free survival probability at 2 years was 40.8% in all participants, and 46.7% in those without (group A) and 25.0% in those with (group B) relapse during cyclosporine treatment (P=0.05, log-rank test). FRNS, frequently relapsing nephrotic syndrome.

Table 3. Poisson regression analyses for first recurrence (n=44)

Parameter	Risk Ratio	95% Confidence Interval	P
Group			
A	0.17	0.04–0.72	0.02
B	1.00	—	
Steroid dependence			
Yes	0.31	0.11–0.88	0.03
No	1.00	—	
Age at the start of follow-up (yr)			
≤6	1.23	0.44–3.40	0.70
>6	1.00	—	

initial study entry (beginning of cyclosporine treatment), 143.5±27.7 ml/min per 1.73 m^2 (n=58, again no patient with eGFR <90) at the end of cyclosporine treatment, and 132.1±23.9 ml/min per 1.73 m^2 (n=36, eGFR of one patient in group A was <90 [87.8 ml/min per 1.73 m^2]) in the children analyzed in this study. One patient (group A) progressed to SRNS during the follow-up period, but finally remitted with cyclosporine. Mean (±SD) score for body height was −0.27±1.01 (n=62) at initial study entry, 0.27±0.97 (n=58) at the end of cyclosporine treatment, and 0.15±1.09 (n=42) at the end of follow-up.

Adverse Events of Steroids and Immunosuppressants

Other adverse events attributable to corticosteroids and immunosuppressants at the end of follow-up are shown in Table 4. Hypertension, defined as a requirement for antihypertensive agents during the trial, was seen in 3 of 49 (6.1%) patients but was manageable, and no severe sequelae of hypertension such as encephalopathy, seizures, or cardiac dysfunction were detected.

Discussion

In this follow-up study of patients who had completed 2-year protocol treatment with cyclosporine for the treatment of FRNS, we found that the relapse rate was high, with approximately 60% of patients regressing to FRNS within 2 years of cessation. Risk of relapse of nephrotic syndrome and regression to FRNS were higher in those who had experienced relapse during the 2-year treatment with cyclosporine. These findings indicate that patients experiencing relapse of nephrotic syndrome during treatment with cyclosporine are at high risk of relapse after discontinuation of the drug. To our knowledge, this is the first prospective study to identify the risk of relapse in children with nephrotic syndrome treated with cyclosporine.

The effectiveness and limitations of cyclosporine in children with FRNS have been reported. Several regimens have been tried, which differed with regard to target blood cyclosporine concentration and duration of administration (5–8,19), and although these showed certain efficacy for controlling relapse with varying degrees of adverse events, including nephrotoxicity, these studies did not provide

Table 4. Adverse events

	All Participants			Participants Succeeding in Discontinuing CSA		
	Group		All	Group		All
	A	B		A	B	
Number of participants	32	17	49	32	12	44
Adverse reaction						
Yes	2	4	6	2	4	6
No	30	13	43	30	8	38
Gastrointestinal discomfort	1	2	3	1	2	3
Hypertension	0	3	3	0	3	3
Osteoporosis	0	2	2	0	2	2
Cataract	0	1	1	0	1	1
Obesity	1	0	1	1	0	1
Adrenal suppression	0	1	1	0	1	1

CSA, cyclosporine.

definitive information on treatment dosage or duration. To better define the optimal dosage and duration of treatment with cyclosporine, we conducted a randomized controlled trial with cyclosporine (Sandimmune; Novartis, Basel, Switzerland) (1), followed by a prospective multicenter trial with microemulsified cyclosporine (Neoral) (2), on which the present follow-up study was based. These two trials helped establish that 2-year treatment with cyclosporine under trough control is effective and safe in children with FRNS. Nevertheless, the frequent relapse seen in patients after the cessation of cyclosporine remained of concern.

In this study, most children with FRNS experienced relapse within a few months after the 2-year protocol treatment with cyclosporine was discontinued. These findings strongly suggest that the current 2-year administration period for cyclosporine in the long-term management of children with FRNS is insufficient. The 2-year period was originally established on the basis of previous findings of an increased risk of nephrotoxicity when administration was extended beyond 2 years (20). However, our more recent prospective finding that only 5 of 58 children experienced biopsy-proven nephrotoxicity (mostly mild arteriolar hyalinosis and mild interstitial fibrosis) indicates the good safety of cyclosporine for 2 years (2). In addition, the present high prevalence of relapse after complete discontinuation indicates that the initial treatment period may be extended beyond 2 years, with consideration to the advisability of repeated renal biopsy at 2- to 3-year intervals (9) and relatively lower doses of cyclosporine (8).

Risk factors for relapse after the complete discontinuation of cyclosporine were also evaluated. Comparison of relapse-free survival between those who did (group B) and did not (group A) relapse during the 2-year treatment showed a significantly greater risk in those who did relapse. Similarly, group B had a significantly greater risk of regression to FRNS than group A. These results indicate that the experience of relapse during cyclosporine treatment predicts the course after its discontinuation. Furthermore, they may also suggest the necessity of extending the cyclosporine treatment period beyond 2 years, or a change in immunosuppressive therapy after 2-year treatment with cyclosporine.

We also analyzed other factors with a possible association with relapse, namely age at the beginning of observation and steroid dependency before treatment with cyclosporine. Contrary to our expectation and the results of previous reports (13,14,16), age at the beginning of observation was not significantly associated with either relapse or the regression of FRNS. Similarly, nonsignificant results were also obtained using age at the onset of nephrotic syndrome instead of age at the beginning of observation (data not shown). Moreover, steroid dependency was not a risk factor for relapse or regression to FRNS. The trend is nevertheless consistent with our previous finding that relapse during cyclosporine treatment was unrelated to steroid dependence (2). To note, however, four of five children who were excluded from analysis due to relapse during tapering were steroid dependent; inclusion of these children would have strengthened the association between steroid dependency and risk. In six patients (three each in group A and B), mizoribine, an agent in the same antimetabolite class as mycophenolate mofetil whose efficacy is mild and limited to patients aged ≤10 years (21), was administered during follow-up to prevent regression to FRNS (data not shown). On inclusion in Poisson analyses, administration of mizoribine was positively associated with relapse. Administration was at the discretion of the physician in charge and may have been administered early in those at risk of relapse.

Consistent with a previous report (15), the life expectancy and renal prognosis of our patients were excellent, supporting cyclosporine's role as first-line treatment in children with FRNS. No lethal events occurred during the approximately 4 years from the beginning of cyclosporine treatment in any participant, and only one patient developed a very mild decrease in renal function. Growth in terms of height was also maintained or slightly improved during the 4 years in spite of relapses and the

administration of prednisolone. Other adverse events attributable to corticosteroids and immunosuppressants were also acceptable. This may have been partly due to our prednisolone protocol for relapse, which moves relatively quickly from daily administration to administration every second day.

FRNS has been shown to be a chronic condition (22,23) which requires a long-term strategy with several immunosuppressants and prednisolone (24). Whereas the efficacy of cyclosporine remains clear, we speculate that in addition to prolonged or repeated administration of cyclosporine, the combination of several immunosuppressants given in sequence should also be considered. Recent attention has focused on the efficacy of several emerging immunosuppressants, including tacrolimus (24,25) and rituximab (24,26), in controlling relapse, and both are now under evaluation in children with FRNS in a multicenter, prospective, randomized fashion in Japan. In addition to the control of relapse, long-term management must also consider any adverse effects of therapy as well as the growth and quality of life of children.

Several strengths of this study warrant mention. First, the study is the first multicenter, prospective trial to evaluate the clinical course of children with FRNS after the discontinuation of cyclosporine. Second, the number of participants was relatively large. Several limitations also warrant mention. First, although protocols for the administration of immunosuppressants and prednisolone after treatment with cyclosporine were provided, treatment was at the discretion of the physician in charge. Immunosuppressants were occasionally started before criteria for the diagnosis of FRNS or SDNS had been met, and mizoribine to prevent relapse was sometimes started very early, even before the first relapse, owing to its low incidence of adverse events (21). Second, several data points were missing, including some measurements of height and serum creatinine, particularly among stable children. Third, because of the small sample size, several factors potentially associated with relapse were not examined in Poisson regression analysis. Fourth, five patients in group B who failed in tapering were excluded from analysis. Findings for group B would have been worse if these cases had been included.

In conclusion, we found that children with FRNS who received cyclosporine were at high risk of relapse after its discontinuation. This effect was particularly large in those who experienced relapse during cyclosporine treatment. Given that overall prognosis in terms of life expectancy and renal survival in these children was excellent, long-term strategies should be formulated in consideration of not only relapse, but also adverse effects of treatment, as well as growth and quality of life.

Acknowledgments

The authors thank Drs. Y. Akioka (Tokyo), M. Awazu (Tokyo), A. Furuse (Kumamoto), S. Fujinaga (Saitama), M. Goto (Tokyo), R. Hamada (Tokyo), K. Hamahira (Hyogo), Y. Hamasaki (Tokyo), T. Harada (Kanagawa), K. Hatae (Fukuoka), H. Hataya (Tokyo), M. Hattori (Tokyo), S. Hattori (Kumamoto), M. Hiramatsu (Oita), T. Igarashi (Tokyo), M. Ikeda (Tokyo), S. Kagami (Tokushima), Y. Kaku (Fukuoka), K. Kamei (Tokyo), S. Kodama (Kagoshima), T. Konomoto (Miyazaki), Y. Mishuku (Kanagawa), N. Nakanishi (Osaka), F. Niimura (Kanagawa), K. Nozu (Hyogo), R. Ochiai (Tokyo), Y. Otsuka (Saga), Y. Owada (Tochigi), M. Sako (Tokyo), T. Sato (Saga), H. So (Kagoshima), R. Tanaka (Hyogo), Y. Tanaka (Saitama), H. Wakaki (Tokyo), and K. Yoshidome (Kagoshima) of the Japanese Study Group of Renal Disease in Children for their contributions to this study.

This study was supported in part by the Kidney Foundation, Japan.

The results presented in this article have not been published previously in whole or part, except in abstract form at the 42nd Annual Meeting of the American Society of Nephrology, October 27–November 1, 2009, in San Diego, California.

Disclosures

N.Y. has received research grants from Novartis, Japan.

References

1. Ishikura K, Ikeda M, Hattori S, Yoshikawa N, Sasaki S, Iijima K, Nakanishi K, Yata N, Honda M: Effective and safe treatment with cyclosporine in nephrotic children: A prospective, randomized multicenter trial. *Kidney Int* 73: 1167–1173, 2008
2. Ishikura K, Yoshikawa N, Hattori S, Sasaki S, Iijima K, Nakanishi K, Matsuyama T, Yata N, Ando T, Honda M; for Japanese Study Group of Renal Disease in Children: Treatment with microemulsified cyclosporine in children with frequently relapsing nephrotic syndrome. *Nephrol Dial Transplant* 25: 3956–3962, 2010
3. Niaudet P, Reigneau O, Humbert H: A pharmacokinetic study of Neoral in childhood steroid-dependent nephrotic syndrome. *Pediatr Nephrol* 16: 154–155, 2001
4. Gregory MJ, Smoyer WE, Sedman A, Kershaw DB, Valentini RP, Johnson K, Bunchman TE: Long-term cyclosporine therapy for pediatric nephrotic syndrome: A clinical and histologic analysis. *J Am Soc Nephrol* 7: 543–549, 1996
5. Tanaka R, Yoshikawa N, Kitano Y, Ito H, Nakamura H: Long-term ciclosporin treatment in children with steroid-dependent nephrotic syndrome. *Pediatr Nephrol* 7: 249–252, 1993
6. Inoue Y, Iijima K, Nakamura H, Yoshikawa N: Two-year cyclosporin treatment in children with steroid-dependent nephrotic syndrome. *Pediatr Nephrol* 13: 33–38, 1999
7. Kitano Y, Yoshikawa N, Tanaka R, Nakamura H, Ninomiya M, Ito H: Ciclosporin treatment in children with steroid-dependent nephrotic syndrome. *Pediatr Nephrol* 4: 474–477, 1990
8. El-Husseini A, El-Basuony F, Mahmoud I, Sheashaa H, Sabry A, Hassan R, Taha N, Hassan N, Sayed-Ahmad N, Sobh M: Long-term effects of cyclosporine in children with idiopathic nephrotic syndrome: A single-centre experience. *Nephrol Dial Transplant* 20: 2433–2438, 2005
9. Cattran DC, Alexopoulos E, Heering P, Hoyer PF, Johnston A, Meyrier A, Ponticelli C, Saito T, Choukroun G, Nachman P, Praga M, Yoshikawa N: Cyclosporin in idiopathic glomerular disease associated with the nephrotic syndrome: Workshop recommendations. *Kidney Int* 72: 1429–1447, 2007
10. Hulton SA, Neuhaus TJ, Dillon MJ, Barratt TM: Long-term cyclosporin A treatment of minimal-change nephrotic syndrome of childhood. *Pediatr Nephrol* 8: 401–403, 1994
11. Hino S, Takemura T, Okada M, Murakami K, Yagi K, Fukushima K, Yoshioka K: Follow-up study of children with nephrotic syndrome treated with a long-term moderate dose of cyclosporine. *Am J Kidney Dis* 31: 932–939, 1998
12. Kemper MJ, Kuwertz-Broeking E, Bulla M, Mueller-Wiefel DE, Neuhaus TJ: Recurrence of severe steroid dependency in cyclosporin A-treated childhood idiopathic nephrotic syndrome. *Nephrol Dial Transplant* 19: 1136–1141, 2004
13. Fakhouri F, Bocquet N, Taupin P, Presne C, Gagnadoux MF, Landais P, Lesavre P, Chauveau D, Knebelmann B, Broyer M, Grünfeld JP, Niaudet P: Steroid-sensitive nephrotic syndrome: From childhood to adulthood. *Am J Kidney Dis* 41: 550–557, 2003
14. Kabuki N, Okugawa T, Hayakawa H, Tomizawa S, Kasahara T, Uchiyama M: Influence of age at onset on the outcome of steroid-sensitive nephrotic syndrome. *Pediatr Nephrol* 12: 467–470, 1998
15. Rüth EM, Kemper MJ, Leumann EP, Laube GF, Neuhaus TJ: Children with steroid-sensitive nephrotic syndrome come of age Long-term outcome. *J Pediatr* 147: 202–207, 2005