Fetal endoscopic myelomeningocele closure preserves segmental neurological function

RENAE J VERBEEK1 | AXEL HEEP2 | NATALIA M MAURITS1 | REINHOLD CREMER3 | EELCO W HOVING4 | OEBELE F BROUWER1 | JOHANNES H VAN DER HOEVEN1 | DEBORAH A SIVAL5

AIM Our aim was to compare the effect of prenatal endoscopic with postnatal myelomeningocele closure (fetally operated spina bifida aperta [fSBA]) versus neonatally operated spina bifida aperta (nSBA) on segmental neurological leg condition.

METHOD Between 2003 and 2009, the fetal surgical team (Department of Obstetrics, University of Bonn, Germany) performed 19 fetal endoscopic procedures. Three procedures resulted in fetal death, three procedures were interrupted by iatrogenic hemorrhages and 13 procedures were successful. We matched each successfully treated fSBA infant with another nSBA infant of the same age and level of lesion, resulting in 13 matched pairs (mean age 14 mo; SD 16 mo; f/m=1.6; female-16, male-10). Matched fSBA and nSBA pairs were compared in terms of segmental neurological function and leg muscle ultrasound density (MUD). We also determined intraindividual difference in MUD (dMUD) between myotomes caudal and cranial to the myelomeningocele (reflecting neuromuscular damage by the myelomeningocele) and compared dMUD between fSBA and nSBA infants. Finally, we correlated dMUD with segmental neurological function.

RESULTS We found that, on average, the fSBA group were born at a lower gestational age than the nSBA group (median 32wks [range 25–34wks] vs 39wks [34–41wks]; p=0.001) and experienced more complications (chorioamnionitis, premature rupture of the amniotic membranes, oligohydramnios, and infant respiratory distress syndrome necessitating intermittent positive-pressure ventilation). Neurological function was better preserved after fSBA than after nSBA (median motor and sensory gain of two segments; better preserved knee-jerk [p=0.006] and anal [p=0.032] reflexes). The dMUD was smaller in fSBA than in nSBA infants (mean difference 24, 95% confidence interval [CI] 15–33; p<0.05), which was associated with better preserved segmental muscle function.

INTERPRETATION Fetal endoscopic surgery is associated with spinal segmental neuroprotection, but it results in more complications. Before considering clinical implementation of fetal endoscopic myelomeningocele closure as standard care, the frequency of complications should be appropriately reduced and results assessed in larger groups over a longer period of time.

In fetuses with spina bifida aperta (SBA), leg movements caudal to the myelomeningocele (MMC) are often present, but they disappear shortly after birth.1–4 Fetal neuroprotection strategies (such as fetal MMC closure) aim to maintain leg motor function by preserving neuromuscular innervation.5–9 In humans, fetal closure of the MMC is performed by open7 or endoscopic surgical techniques.8,9 Recently published results from the Management of Myelomeningocele Study,7,9 a randomized controlled trial, suggest that open fetal surgery can improve neurological outcome. However, open fetal surgery is also associated with fetal and maternal risks, including preterm birth, intraoperative complications, and uterine scar defects.7,9 One of the major goals of fetal endoscopic MMC closure is to preserve neuromuscular integrity and to minimize iatrogenic damage.8 Kohl et al.8 reported that fetal endoscopic treatment resulted in an improved neurological condition, although effects on leg motor function were incompletely assessed. From a theoretical perspective, fetal endoscopic therapy might induce neurological gain by improving both cerebral (brain dependence; Chiari 2 malformation) and spinal (MMC) conditions.10 However, given the presence of many aberrant, readily haemorrhaging, blood vessels in the region of...
the MMC, it remains a matter of debate whether fetal endoscopic MMC closure can protect neuromuscular innervation at and caudal to the MMC. In the present study, we aimed to elucidate the effect of fetal endoscopic MMC closure on segmental neurological function and integrity caudal to the MMC.

Recently advanced magnetic resonance imaging (MRI) techniques have provided clinicians with new diagnostic insights (into muscle structure, function, and metabolism) without the need for invasive biopsy. To avoid anaesthesia in young children, neuropaediatric clinicians can also apply the non-invasive ‘muscle ultrasound’ technique, which measures quantitative muscle ultrasound density (MUD) parameters for neuromuscular assessment and surveillance. MUD parameters are based upon secondary muscle alterations after neural innervation damage (inducing reduced muscle water content, fibrosis, fat deposition, and atrophy), causing increased MUD. From this perspective, we reasoned that muscle ultrasonography could provide a useful, quantitative, non-invasive tool in children with SBA. In particular, the intraindividual difference in MUD (dMUD) between myotomes caudal and cranial to the MMC could quantitatively reveal muscle damage caused by the MMC itself. In the present study, we compared neurological and quantitative MUD parameters in children with SBA treated by fetal endoscopic MMC closure (fetally operated SBA [fSBA]) or neonatal MMC closure (neonatally operated SBA [nSBA]).

METHOD

Participants

The medical ethics committees of Bonn University, Germany, and the University Medical Center Groningen (UMCG), the Netherlands, approved the comparison of age- and lesion-matched fSBA and nSBA children. fSBA children were operated on and delivered at Bonn University; nSBA children were operated on and treated at the UMCG. Both participating university centres provided multidisciplinary care by spina bifida teams. The UMCG SBA team caring for the nSBA control group is associated with similar neonatal predictive parameters and neurological outcomes as reported by Hunt and Poulton. All parents of children with SBA included in the study (mean age 14mo; SD 16mo; mean upper MMC level at L1; SD one segment; female-16, male-10) gave informed consent. Between 2003 and 2009, Kohl’s surgical team performed a total of 19 fetal endoscopic procedures at the German Center for Fetal Surgery and Minimally-Invasive Therapy, Bonn University, Germany. For technical details of the surgical patch coverage, refer to the descriptions published by the operating team. Of the 19 fetuses who underwent endoscopic procedures, 13 infants survived and were successfully treated, and all 13 were included in the study. Three fSBA fetuses died (from complicated anaesthesia, placental haemorrhaging, or oligohydramnios-related lung hypoplasia; for further information see Kohl et al.) and in three cases the endoscopic procedure was interrupted by placental haemorrhaging. These three fetuses survived and underwent postnatal MMC closure. All fetal endoscopic procedures were performed at a gestational age of between 20+5 and 24+3 (median 23+6) weeks.

We matched each fSBA infant with another of the same age and with the same level of lesion, resulting in 13 matched pairs. The level of the lesion was considered to be the upper border of the MMC (determined by fetal ultrasonography and confirmed by postnatal MRI). When more than one lesion-matched nSBA child was available, we selected the infant nearest in age. Matched pairs showed age ranges from 0 to 2 months under 1 year of age and from 0 to 1 year in older children. All 13 nSBA comparison children were born at the UMCG and operated on during the first week of life. The data for each individual are shown in Table I.

All fSBA children were born by Caesarean section (performed after initiation of preterm labour). All nSBA children were born by vaginal delivery. To study the potential influence by Caesarean section, we included another 13 age- and lesion-matched pairs of nSBA children delivered by Caesarean section or vaginal delivery (mean upper MMC level at L4; SD one segment; mean upper MMC level; mean age 29mo; SD 24mo). In pregnancies in which the child with SBA underwent neonatal operation, Caesarean section was performed either electively (n=9) or after initiation of labour (failed delivery progression; n=4). nSBA children delivered by Caesarean section were born and treated at Bonn University and Cologne Children’s Hospital; all nSBA children who were delivered vaginally were born and treated at the UMCG.

Neurological examination

Standardized neurological examinations were performed by the same paediatric neurologist. Neurological examinations were videotaped and scored offline for segmental neurological (motor and sensory) assessment. Motor levels were indicated by the lowest myotomes involved in active motor behaviour. Sensory levels were indicated by the lowest dermatome at which a pinprick elicited an emotional response. In children in whom neurological levels were different on the left and right sides, we obtained the mean segmental level of both legs. For statistical comparison between age- and lesion-matched pairs, we attributed numerical scores to each neurological level ranging from 0 to 8 (i.e. T12=0; L1=1; L2=2; L3=3; L4=4; L5=5; S1=6; S2=7; and no neurological dysfunction=8).

For analysis of leg reflex activity, we examined knee-jerk (L2-3) and anal reflexes (S1-2). Knee-jerk reflexes were evoked in the supine position. The reflex was scored as present when at least five taps upon the tendon evoked a visible contraction of the quadriceps muscle and as absent when no contraction
recordings were performed with fixed ultrasound equipment in the Netherlands, muscle ultrasound were formally calibrated by the GE technician before the muscle ultrasound registrations of biceps, quadriceps, and calf muscles were recorded in the supine position and calf muscles in the prone position. For digital quantification, we stored five ultrasound images per muscle and determined MUD within a well-defined region of interest. MUD outcome is derived by excluding the highest and lowest values and calculating the mean of the three remaining MUD values. To minimize variation and bias, all muscle ultrasound recordings were performed by the same investigators (RJV and JHvdH). In Germany, recordings were performed with portable ultrasound equipment (LOGIQ e; GE Healthcare). Portable and fixed muscle ultrasound machines were compatible GE Healthcare LOGIQ systems, both owned by UMCG. Both machines were formally calibrated by the GE technician before the study. Before assessments, we compared the MUD outcomes of both machines by performing a regression. We therefore assessed MUD in leg myotomes of healthy children and children with SBA (n=32; age range 3–64mo; myotomes of C5–C8 [biceps muscle], L2–L4 [quadriceps muscle], and S1–S2 [calf muscle], i.e. both cranial and caudal to MMC). Thus, the same investigator separately assessed these myotomes twice using each ultrasound machine. The MUD conversion equation is given as:

$$\text{MUD}_{\text{LOGIQ} \text{e}} = 37.262 + 1.368 \cdot \text{MUD}_{\text{LOGIQ} \text{9}} \cdot r^2 = 0.74$$

This conversion equation is reliable, as indicated by the fact that coefficients of variation were similar for both machines (LOGIQ 9, 24%; LOGIQ e, 20%) and the Bland–Altman plot showed no residual correlation. Evaluation of MUD within the bounds of error of the conversion equation revealed similar results.

### Assessment of muscle ultrasound density

Muscle ultrasound registrations of biceps, quadriceps, and calf muscles were assessed with standard muscle ultrasound gain, dynamic range, compression, and time-gain compensation parameters. In accordance with standardized reference points, biceps and quadriceps muscle ultrasound images were recorded in the supine position and calf muscles in the prone position. For digital quantification, we stored five ultrasound images per muscle and determined MUD within a well-defined region of interest. MUD outcome is derived by excluding the highest and lowest values and calculating the mean of the three remaining MUD values. To minimize variation and bias, all muscle ultrasound recordings were performed by the same investigators (RJV and JHvdH). In Germany, recordings were performed with portable ultrasound equipment (LOGIQ e; GE Healthcare). Portable and fixed muscle ultrasound machines were compatible GE Healthcare LOGIQ systems, both owned by UMCG. Both machines were formally calibrated by the GE technician before the study. Before assessments, we compared the MUD outcomes of both machines by performing a regression. We therefore assessed MUD in leg myotomes of healthy children and children with SBA (n=32; age range 3–64mo; myotomes of C5–C8 [biceps muscle], L2–L4 [quadriceps muscle], and S1–S2 [calf muscle], i.e. both cranial and caudal to MMC). Thus, the same investigator separately assessed these myotomes twice using each ultrasound machine. The MUD conversion equation is given as:

$$\text{MUD}_{\text{LOGIQ} \text{e}} = 37.262 + 1.368 \cdot \text{MUD}_{\text{LOGIQ} \text{9}} \cdot r^2 = 0.74$$

This conversion equation is reliable, as indicated by the fact that coefficients of variation were similar for both machines (LOGIQ 9, 24%; LOGIQ e, 20%) and the Bland–Altman plot showed no residual correlation. Evaluation of MUD within the bounds of error of the conversion equation revealed similar results.

### Inter- and intraindividual comparison of muscle ultrasound density parameters

Of the 13 age- and lesion-matched pairs of infants, 12 had a lumbar MMC and one pair had a thoracic MMC. In all 12 pairs with a lumbar MMC (pairs 2–13), the quadriceps muscle...
was innervated at or cranial to the MMC and the calf muscle caudal to the MMC. In these 12 pairs, we assessed the MUD of the quadriceps (innervation L2–L4, i.e. at/cranial to the MMC) and calf muscles (innervation S1–S2, i.e. caudal to the MMC) and determined the intraindividual MUD difference [i.e. \(d\text{MUD} = (M\text{UD}_\text{calf muscle}) - (M\text{UD}_\text{quadriceps muscle})\)]. In the only thoracic MMC pair (pair 1), we computed \(d\text{MUD}\) between biceps (C5–C6, i.e. cranial to the MMC) and calf muscles (S1–S2, i.e. caudal to the MMC) by \(d\text{MUD} = (M\text{UD}_\text{calf muscle}) - (M\text{UD}_\text{biceps muscle})\). We estimated the functional significance of the \(d\text{MUD}\) treatment outcome by associating \(d\text{MUD}\) with neurological segmental S1 function (caudal to the MMC).

Statistical analysis
Statistical analysis was performed using SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). As MUD values were not normally distributed (according to Q–Q plots and the Shapiro–Wilk test), we compared matched pairs by non-parametric Wilcoxon signed-rank test. To obtain an estimate of the functional significance of the quantitative MUD treatment effect, we associated MUD outcomes with segmental neurological function using the Mann–Whitney \(U\) test. The level of significance was \(\alpha=0.05\).

RESULTS
Clinical data
Clinical data are shown in Table I. fSBA children were delivered at a lower gestational age than nSBA children (median 32wks [range 25\(^{+3}\)–34\(^{+3}\)wks] vs 39wks [34\(^{+6}\)–41\(^{+2}\)wks], respectively; \(p=0.001\)). The prevalence of shunt-dependent hydrocephalus was lower in fSBA than in nSBA children (4/13 vs 12/13 respectively; \(p<0.05\)). Complications of fetal endoscopic surgery included amnion infection (3/13 pregnancies), maternal haemorrhaging (3/13 pregnancies), premature rupture of the amniotic membranes (11/13 pregnancies), and oligohydramnios (8/13 pregnancies). All fSBA neonates received respiratory support (10/13 neonates by intermittent positive-pressure ventilation; 13/13 neonates by continuous positive airway pressure). Perinatal complications consisted of asphyxia (2/13 neonates), infant respiratory distress syndrome (12/13 neonates), lung hypoplasia (2/13 neonates), infections (7/13 neonates), and endocrine disturbances (4/13 neonates).

![Graph](image)

**Figure 1:** Segmental motor function in age- and lesion-matched pairs of fetally operated spina bifida aperta (fSBA) and neonatally operated spina bifida aperta (nSBA) children. The \(y\)-axis indicates the highest myotome participating in spontaneous movements. Segmental motor function is better preserved in fSBA than in nSBA children (median difference: two myotomes; \(p=0.008\)). ND, no deficit.
Neurological outcomes
Comparison of age- and lesion-matched fSBA and nSBA children revealed better preserved neuromuscular function in fSBA than in nSBA children (i.e. a median difference of two myotomes [range –0.5 to 4] for motor function and two dermatomes [range –1.5 to 5] for sensory function; \( p=0.008 \) and \( p=0.003 \) respectively; see Figs 1 and 2).

Numerical scores for both knee-jerk and anal reflexes were higher in fSBA than in lesion-matched nSBA infants (knee-jerk reflexes: 22/26 vs 7/26 points respectively; \( p=0.006 \); anal reflexes: 11/26 vs 0/26 points respectively, \( p=0.032 \)).

Inter- and intra-individual comparison of muscle ultrasound density parameters
Comparison of MUD cranial to the MMC (i.e. MUDquadriceps muscle) did not reveal significant differences between age- and lesion-matched fSBA and nSBA children (mean difference 15; 95% CI 5–24). Comparison of MUD caudal to the MMC (i.e. MUDcalf muscle) showed that the lowest outcome group was fSBA children (mean difference 20; 95% CI 7–34; \( p<0.05 \)). To estimate the functional significance of this finding, we subsequently associated MUDcalf muscle with segmental neurological S1 functioning. Lower fSBA MUDcalf muscle outcomes were associated with preserved neurological S1 (motor and sensory) function (MUDcalf muscle in present vs absent plantar flexion: mean difference 31; 95% CI 4–58; and MUDcalf muscle in present vs absent sensory S1 function: mean difference 31; 95% CI 5–58; both \( p<0.05 \); see Fig. 3a,b). As preserved neurological S1 function could theoretically be attributed to better preserved cerebral and spinal conditions, we determined the dMUD in each infant. Mean dMUD was lower in the fSBA group than in the nSBA group (mean difference 24; 95% CI 15–33; \( p<0.05 \)). To estimate the functional significance of this finding, we associated dMUD with neurological S1 function (i.e. caudal to the MMC). Quantitative fSBA dMUD outcomes appeared to be related to segmental neurological S1 functioning (dMUD in present vs absent plantar flexion: mean difference 20; 95% CI –7 to 47; cut-off point 40; \( p<0.05 \); dMUD in present vs absent sensory S1 function: mean difference 32; 95% CI 12–52; both \( p<0.05 \); Fig. 3c).

Effect of delivery mode
Neurological comparison of motor and sensory function between nSBA children born by Caesarean section and nSBA

Figure 2: Segmental sensory function in age- and lesion-matched pairs of fetally operated spina bifida aperta (fSBA) and neonatally operated spina bifida aperta (nSBA) children. The y-axis indicates the highest dermatome with sensory function. Sensory function is better preserved in fSBA than in nSBA children (median difference: two dermatomes; \( p=0.003 \)). ND, no deficit.
children born by vaginal delivery revealed no significant differences. The knee-jerk and anal reflex response pattern was similar in both groups. Age- and lesion-matched MUDcalf muscle (caudal to the MMC) and dMUD did not significantly differ between the two groups (MUD mean difference 26; 95% CI 14–38; dMUD mean difference 21; 95% CI 10–31).

**DISCUSSION**

In the present study, we aimed to elucidate whether fetal endoscopic MMC closure can provide fetal spinal neuroprotection. Comparison of age- and lesion-matched infants revealed that segmental neurological outcomes and muscle ultrasound densities were better after fetal endoscopic MMC closure than after neonatal MMC closure.

Fetal closure of MMC can be performed by open or endoscopic surgical techniques. The recently published results of the Management of Myelomeningocele Study have convincingly shown that open fetal surgery can improve neurological outcome. However, open fetal treatment is also associated with iatrogenic maternal and fetal risks, which should be taken into account. As with open fetal treatment, we observed severe iatrogenic complications after endoscopic fetal treatment, such as premature rupture of the amniotic membranes, amnion infection, oligohydramnios, preterm delivery, pulmonary hypoplasia, and fetal death. Despite iatrogenic complications, fetal endoscopic MMC closure is associated with preserved cerebral condition (i.e. ameliorated Chiari 2 malformation and reduced drain dependence). Accordingly, our results reveal a reduced incidence of drain dependence similar to that reported by the Management of Myelomeningocele Study. However, as the fSBA children were young (median age 14mo), a longer follow-up period might be needed to confirm whether these differences are persistent.

The aim of the present study was to determine whether fetal endoscopic surgery can preserve segmental leg function by providing spinal neuroprotection. Our data indicate that segmental leg function is better preserved after fSBA than after nSBA treatment (median of two segments motor and sensory median). As segmental reflexes cranial and caudal to the MMC (i.e. knee-jerk and anal reflex activity) were also better preserved in fSBA than in nSBA, both cerebral and spinal improvements could (theoretically) contribute to these results. For further differentiation, we assessed dMUD and compared

![Figure 3: Muscle ultrasound density (MUD) parameters.* (a) Association between fSBA MUDcalf muscle and calf muscle function. The calf muscle is innervated caudal to the MMC (S1–S2). The x-axis subdivides motor function into present and absent foot plantar flexion (calf muscle function). The y-axis indicates MUDcalf muscle. In fSBA, MUDcalf muscle is associated with calf muscle function (p=0.047). (b) Association between fSBA MUDcalf muscle and sensory S1 function. The x-axis subdivides sensory function into cranial and caudal to S1. The y-axis indicates MUDcalf muscle. In fSBA, MUDcalf muscle is associated with sensory S1 function (p=0.037). (c) Comparison between fSBA dMUD and segmental neurological (motor and sensory) function caudal to the MMC. The x-axis represents S1 motor function (present or absent plantar flexion) on the left and S1 sensory function (present or absent perception) on the right. The y-axis indicates intraindividual dMUD. Quantitative dMUD appeared associated with segmental neurological (motor and sensory) function caudal to the MMC. *Box plots mark first and third quartiles; whiskers represent data points 1.5 times the interquartile range below and above the first and third quartiles. Dots represent outliers. MUDcalf muscle, muscle ultrasound density of calf muscle; fSBA, fetally operated spina bifida aperta; S, sacral segment; dMUD, intraindividual difference in muscle ultrasound density.](image-url)
the outcomes in age- and lesion-matched fSBA and nSBA children. The results indicate lower dMUD and better preserved segmental neurological (motor and/or sensory) function caudal to the MMC in fSBA than in nSBA children. Thus, these data may indicate that spinal neuroprotection is (at least partly) involved. It could also be argued that results can be attributed to group differences in rehabilitation practice. However, as preserved fSBA outcomes were already present in neonates, and as all nSBA children received multidisciplinary care by a large, well-equipped academic spina bifida team (providing care for the northern to middle eastern part of the Netherlands, reporting outcomes within the expected academic European range), whereas fSBA children received care at different local European centres, this appears less likely. Taken together, the present data appear supportive of the ‘second-hit hypothesis’, suggesting that segmental neurological damage at the MMC is partly ameliorated by fetal endoscopic spinal neuroprotection.

It is known that SBA pregnancies with predefined conditions (i.e. midlumbar cystic MMC) may neurologically benefit from elective Caesarean section (before the onset of uterine contractions). It might therefore be questioned whether group differences in delivery modes (Caesarean section vs vaginal delivery) could explain the present results. However, as Caesarean section of fSBA children was performed after preterm initiation of delivery and because all cystic fSBA MMMCs had been operated on before Caesarean section, this treatment did not correspond with predefined ‘favourable’ Caesarean section conditions. To control for a (theoretically potentially) confounding effect by the delivery mode under ‘unfavourable’ Caesarean section conditions, we assembled a second lesion- and age-matched group of nSBA children delivered by Caesarean section and fSBA children delivered vaginally, regardless of delivery initiation and regardless of MMC type. Under such ‘unfavourable’ conditions, we did not detect a confounding influence by Caesarean section. Although we conclude that Caesarean section (under ‘unfavourable’ conditions) did not confound the current results, one cannot extrapolate forthcoming data to the assumption that Caesarean section is thus ineffective when it is electively performed for midlumbar, cystic MMC.

We realize that the present study has several limitations. First, the number of fetal endoscopic surgeries assessed in this study is small. However, we included all feasible fSBA children and therefore our results could be regarded as indicative. Second, neurological assessments were performed with prior knowledge of the treatment groups. However, all assessments and scorings were performed by the same UMCG team, which has been critical about fetal surgery and never performed, collaborated, or referred children with SBA for fetal MMC closure. Furthermore, this limitation did not influence quantitative MUD outcomes, which substantiated neurological results. Third, the children with SBA were relatively young (median age 14 mo). In this perspective, a longer period of neurological surveillance is needed to control for the potential occurrence of delayed complications (shunt dependency or tethering) and to confirm whether more preserved neurological fSBA outcomes persist.

Fourth, there was still a small difference in age between members of matched pairs. However, as MUD is independent of age, we do not expect that this influenced outcomes. Fifth, as our sample included only one matched pair with thoracic MMC, the present results are mainly representative for individuals with lumbar MMC (which is associated with a distinctly different prognosis and life expectancy from thoracic MMC). However, inclusion or exclusion of the pair with thoracic MMC did not influence results. Finally, fetal endoscopic treatment is associated with considerable iatrogenic risks, including three fetal deaths. These fetal deaths were caused by severe iatrogenic complications of the fetal endoscopic procedure rather than the result of poor fetal neurological integrity. Although this implies that fetal deaths did not influence our neurological analysis, it should be clearly stated that these risks are to be carefully outweighed before rational clinical treatment choices can be made for future clinical practice.

**CONCLUSION**

Fetal endoscopic MMC closure could be a promising technique for segmental preservation of neurological leg parameters, but results are achieved at the cost of complications. Before considering clinical implementation of fetal endoscopic MMC closure as standard care, complications should be adequately decreased and results scrutinized in larger study groups over a longer period of time.

**ACKNOWLEDGEMENTS**

The authors thank Professor T Kohl and Professor U Gembruch for their generous permission in allowing independent evaluation; E Muskens and M Gremmer for sharing clinical ultrasound equipment; and S Korbmacher-Haase, L Kuhl, H Kunst, J Bijmolt, J Sikkema, and G Oosterhof for excellent administrative help.

**REFERENCES**


