LC–MS/MS based determination of basal- and ACTH-stimulated plasma concentrations of 11 steroid hormones: implications for detecting heterozygote CYP21A2 mutation carriers

A E Kulle1, F G Riepe1, J Hedderich2, W G Sippel1, J Schmitz1, L Niermeyer1 and P M Holterhus1

1Division of Pediatric Endocrinology and Diabetes, Department of Pediatrics and 2Institute of Medical Informatics and Statistics, University Hospital Schleswig-Holstein, Christian-Albrechts University Kiel, Schwanenweg 20, D-24105 Kiel, Germany

Abstract

Objective: Heterozygosity in 21-hydroxylase deficiency (21OHD) has been associated with hyperandrogenemic symptoms in children and adults. Moreover, the carrier status is mandatory for genetic counseling. We aimed at defining a hormonal parameter for carrier detection by mass spectrometry.

Design: Eleven basal and ACTH-stimulated steroid hormones of heterozygous carriers of CYP21A2 mutations and control individuals were compared.

Method: Hormones were determined in plasma samples by liquid chromatography tandem mass spectrometry (LC–MS/MS) in 58 carriers (35 males, 23 females, age range 6–78 years) and 44 random controls (25 males, 19 females, age range 8–58 years).

Results: Heterozygotes could be identified best applying the 17-hydroxyprogesterone + 21-deoxycortisol/cortisol × 1000 ((17OHP + 215)/F × 1000) equation 30 min after ACTH injection. An optimal cut-off value of 8.4 provided 89% sensitivity and specificity. Considering this data and a published frequency of heterozygotes of 1/50 to 1/61, the positive predictive value (PPV) of this cut-off is 12%. Of note, the negative predictive value (NPV) excluding heterozygosity in a given patient is 99.8%.

Conclusion: Considering only marginal biochemical effects anticipated from heterozygosity, the stimulated ((17OHP + 215)/F × 1000) identifies and excludes heterozygotes remarkably well. Nevertheless, LC–MS/MS cannot replace genetic testing, since sensitivity and specificity did not reach 100%. However, due to the considerably high NPV of the optimal cut-off and to a specificity of even 100% applying a cut-off higher than 14.7, hormonal assessment of heterozygosity can be of significant aid in conditions with limited access to genetic testing, as in some health care systems. The ((17OHP + 215)/F × 1000) equation can guide diagnostic considerations in the differential diagnosis of hyperandrogenism.

Introduction

21-hydroxylase deficiency (21OHD) is the most common form of congenital adrenal hyperplasia (CAH) (1, 2, 3). Classic 21OHD has an incidence of about 1:10 000–1:15 000 (4, 5, 6). Prevalence rates for non-classic 21OHD are much higher. Little data on heterozygote rates has been published. Based on the data published by Pang & Clark (6), a frequency of heterozygous carriers in Europe could be calculated using the Hardy–Weinberg principle, resulting in a 1:50 to 1:61 ratio.

CYP21A2 mutations may contribute to clinically evident hyperandrogenemia (7, 8). A high prevalence of
heterozygosity of CYP21A2 gene mutations has been documented in premature pubarche (9, 10). Therefore, heterozygosity of CYP21A2 gene mutations has to be considered in the differential diagnosis of hyperandrogenemic symptoms. The carrier status is also mandatory for counselling parents who are themselves affected by CAH and in families where one parent has proven carrier status. At the hormone level, heterozygotes have normal or only slightly elevated basal plasma levels of 17-hydroxyprogesterone (17OHP) (3). Following corticotrophin (ACTH) injection, heterozygotes usually show an increased rise of 17OHP compared with controls, but it is not possible to identify heterozygosity based on stimulated 17OHP alone (11, 12, 13, 14). A different approach to detect heterozygotes is the calculation of substrate-to-product ratios. In an earlier study, in the pre-liquid chromatography tandem mass spectrometry (pre-LC–MS/MS) era, 17OHP/deoxycorticosterone (DOC)-ratios following corticotrophin stimulation had led to the identification of 100% of the carriers investigated (15). Since the determination of steroid hormones is now largely performed by mass spectrometry, the aim of the present study is to analyze 11 steroid hormones measured by LC–MS/MS in controls and in proven heterozygous carriers of CYP21A2 mutations, in order to define hormonal cut-off values for carrier identification.

Subjects and methods

Volunteers

The study was approved by the Ethics Committee of the Christian-Albrechts-University, Kiel, Germany. Subjects provided written informed consent. One cohort of study volunteers, aged 6–78 years, comprised relatives of index patients with proven 21OHD (n = 58; 35 males and 23 females). The second independent cohort consisted of healthy volunteers (n = 44; 25 males and 19 females), aged 8–56 years. Exclusion criteria included any known chronic illness, pregnancy (ruled out by determining β human chorionic gonadotrophin (β-hCG)) and recent use of glucocorticoids.

ACTH test

ACTH stimulation was performed between 0800 and 1000 h on a weekend morning. All women were tested in the early follicular phase. No subjects were in menopause. Blood samples were obtained by venipuncture before, 30 and 60 min after application of 250 µg of synacthen (Novartis Pharma) i.v.

Molecular analysis of the CYP21A2 locus

First we employed multiplex minisequencing to detect the most common CYP21A2 mutations (16) using an automated ABI 310 Sequencer (Applied Biosystems, Inc.). This was followed by sequencing the whole coding CYP21A2 region and all intron exon boundaries (17). In addition, MLPA-multiplex ligation-dependent probe amplification (SALSA MLPA kit CAH, MCR-Holland, Amsterdam, The Netherlands) was performed to identify large gene deletions.

Hormone analysis

Plasma concentrations of 11 steroid hormones, comprising mineralocorticoids, glucocorticoids and androgens, were determined using UPLC Quattro Premier/Xe system (Waters, Milford, MA, USA) as previously described (18, 19). In brief, aliquots of plasma samples, calibrator and controls with a volume of 0.1 ml were combined with the internal standard mixture to monitor recovery. All samples were extracted using Oasis MAX SPE system Plates (Waters). To separate all isobaric substances, a UPLC method was used with a Waters UPLC BEH C18 column (1.7 µm, 100×2.1) at a flow rate of 0.4 ml/min at 50 °C. Water (A) and acetonitrile (B) both with 0.01% formic acid were used. A gradient was used: from 29% to 91% acetonitrile in 2 min, a step gradient to 100% acetonitrile and re-equilibration to initial conditions in 2.5 min. Total running time was 5 min and the injection volume was 20 µl. Electrospray was used and for each hormone two multiple reaction monitoring (MRM) transitions were recorded. The retention time for the isobaric molecules 21-deoxycortisol (21S), corticosterone and 11-deoxycortisol (11S) were 3.49, 3.61 and 3.94 min. The quantifier and qualifier transitions (m/e) for these hormones were: 17OHP 97, 347 and 109; 21S 347, 121 and 121; corticosterone 347 > 121, 347 > 311 (Supplementary Figures 1 and 2, see section on supplementary data given at the end of this article). 21S and corticosterone are not completely baseline-separated, but the calculated resolution (R = 1.85) supports suitability of the detection method for valid quantification of these two steroids. The limit of quantification was for 17OHP 0.03 ng/ml, for 21S 0.04 ng/ml and for cortisol 0.8 ng/ml. The method used is specific to the hormones of interest (18, 19).

Statistical analysis

Statistical evaluation was performed using SigmaStat (Systat Software, Erkrath, Germany) and SPSS (SPSS statistics for Windows V20, IBM Corp., Armonk, NY, USA).
Table 1  Median and range for the adrenal steroid hormones, $P$ values and adjusted $P$ values.

<table>
<thead>
<tr>
<th>Steroid hormone</th>
<th>Time (min)</th>
<th>Controls</th>
<th>Heterozygous CYP21A2</th>
<th>$P$</th>
<th>$P$ adjust</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone ng/ml (nmol/l)</td>
<td>0.03 (0.09)</td>
<td>0.03 (0.09)</td>
<td>0.38 (1.21)</td>
<td>0.66 (0.19)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td></td>
<td>0.38 (0.63)</td>
<td>0.03 (0.09)</td>
<td>0.52 (1.66)</td>
<td>0.49 (1.46)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td>DOC ng/ml (nmol/l)</td>
<td>0.03 (0.09)</td>
<td>0.03 (0.09)</td>
<td>0.31 (0.40)</td>
<td>0.32 (0.07)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td></td>
<td>0.13 (0.36)</td>
<td>0.03 (0.09)</td>
<td>0.29 (0.88)</td>
<td>0.90 (2.79)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td>B ng/ml (nmol/l)</td>
<td>0.36 (0.83)</td>
<td>0.15 (0.43)</td>
<td>1.62 (46.81)</td>
<td>0.53 (1.52)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td></td>
<td>0.20 (0.64)</td>
<td>0.03 (0.09)</td>
<td>0.52 (1.92)</td>
<td>0.29 (0.94)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td>17OHP ng/ml (nmol/l)</td>
<td>0.36 (0.83)</td>
<td>0.15 (0.43)</td>
<td>1.62 (46.81)</td>
<td>0.53 (1.52)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td></td>
<td>0.20 (0.64)</td>
<td>0.03 (0.09)</td>
<td>0.52 (1.92)</td>
<td>0.29 (0.94)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td>11S ng/ml (nmol/l)</td>
<td>0.03 (0.09)</td>
<td>0.03 (0.09)</td>
<td>0.14 (0.40)</td>
<td>0.05 (0.14)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td></td>
<td>0.14 (0.36)</td>
<td>0.03 (0.09)</td>
<td>0.29 (0.88)</td>
<td>0.09 (0.27)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td>21S ng/ml (nmol/l)</td>
<td>0.03 (0.09)</td>
<td>0.03 (0.09)</td>
<td>0.14 (0.40)</td>
<td>0.05 (0.14)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td></td>
<td>0.14 (0.36)</td>
<td>0.03 (0.09)</td>
<td>0.29 (0.88)</td>
<td>0.09 (0.27)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td>F ng/ml (nmol/l)</td>
<td>0.03 (0.09)</td>
<td>0.03 (0.09)</td>
<td>0.14 (0.40)</td>
<td>0.05 (0.14)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td></td>
<td>0.14 (0.36)</td>
<td>0.03 (0.09)</td>
<td>0.29 (0.88)</td>
<td>0.09 (0.27)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td>E ng/ml (nmol/l)</td>
<td>0.03 (0.09)</td>
<td>0.03 (0.09)</td>
<td>0.14 (0.40)</td>
<td>0.05 (0.14)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td></td>
<td>0.14 (0.36)</td>
<td>0.03 (0.09)</td>
<td>0.29 (0.88)</td>
<td>0.09 (0.27)</td>
<td>0.03 (0.09)</td>
</tr>
</tbody>
</table>

DOC, deoxycorticosterone; B, corticosterone; 17OHP, 17-hydroxypregesterone; 11S, 11-deoxycortisol; 21S, 21-deoxycortisol; F, cortisol; E, cortisone and the ratio 17OHP + 21S/F.
Based on the Kolmogorov–Smirnov test, the assumption of normal distribution of the data was rejected for most of the variables, so the median and the reference values 5–95% (90% confidence limits) were calculated using the Harrell-Davis nonparametric quantile estimator (20). The nonparametric Wilcoxon rank sum test was used to evaluate differences between the groups. Adjusted P values were derived from a logistic regression model with age and sex as covariates. Receiver operator characteristics (ROC) were used to define the cut-off value for the ACTH-stimulated ratios \((170\text{HP}+21\text{S})/F\), 170\text{HP}/DOC, and for the delta concentration levels of 170\text{HP} between heterozygotes and controls. The positive and negative predictive values (PPV and NPV) for 17-hydroxyprogesterone + 21-deoxycortisol/cortisol \times 1000 ((170\text{HP}+21\text{S})/F \times 1000) were calculated for an estimated heterozygote frequency of 1/51 to 1/61 for classic mutations and of 1/17 for non-classic mutations, as calculated by the Hardy–Weinberg principle based on the data published by Pang & Clark (6).

Results

Molecular genetics

Following genetic testing, it was necessary to re-categorize some of the study participants. Four individuals in the group of 21OHD patients’ relatives carried neither mutations nor deletions and had to be re-categorized vice versa accordingly.

Hormonal response

Basal and stimulated plasma concentrations of adrenal hormones are shown in Table 1. As expected, heterozygotes showed significantly higher stimulated concentrations of 170\text{HP} after 30 and 60 min (2.55 and 3.07 ng/ml respectively) compared with the controls (1.04 and 1.12 ng/ml) (P value: \(<0.001\) and \(<0.001\)). However, the two groups were not distinctly separate since there was a considerable overlap of stimulated 170\text{HP} concentrations as shown in Fig. 1.

We also found significantly higher concentrations of progesterone in the heterozygous carriers 60 min after ACTH injection (0.49 ng/ml) compared with the controls.
(0.25 ng/ml) (P value: <0.001). As observed for 17OHP, there was a significant overlap between the groups. Another difference between controls and carriers were the baseline concentrations of corticosterone and cortisol, which were significantly higher in the controls (corticosterone: 3.06 ng/ml, cortisol: 161.99 ng/ml) compared with the carriers (corticosterone: 0.53 ng/ml, cortisol: 99.77 ng/ml) (P values: 0.005 and 0.006 respectively). In contrast, there was no significant difference in the stimulated levels of corticosterone and cortisol between the cohorts. For DOC and cortisol, we did not find any significant differences between the groups. For 11S we found weakly significant differences for the stimulated concentrations after 30 and 60 min.

Androstenedione (Δ4), testosterone and dihydrotestosterone (DHT) differed significantly between the sexes at baseline and after ACTH stimulation (Tables 2, 3, and 4), while mineralocorticoids and glucocorticoids did not vary according to the sex of the subjects (Tables 2, 3, and 4). There were no significant increases of Δ4, testosterone or DHT in either sex. The differences reported for mineralocorticoids and glucocorticoids between heterozygotes and controls remained significant after the exclusion of prepubertal and pubertal children with only slightly different P values (Table 1). We therefore kept the children in the cohorts for calculations. In addition to the absolute hormone concentrations, we observed significant differences (= deltas in the ACTH-induced increases of plasma concentrations between controls and heterozygotes. We found significant differences in delta corticosterone between the groups after 30 min (P value: <0.001) and after 60 min (P value: <0.001), in delta 17OHP after 30 min (P value: <0.001) and 60 min (P value: <0.001) and in delta cortisol after 30 min (P value: <0.001) and 60 min (P value: <0.001). All results were used for a ROC analysis to distinguish between controls and carriers. In the male subjects, we observed a cut-off of 1.28% for delta 17OHP after 60 min with a sensitivity of 80% and a specificity of 95%. Since this failed in women, we excluded this approach from further calculations. ROC also failed for deltas of all other hormones (data not shown).

We then tested the ratio 17OHP/DOC previously published by Peter et al. (15). Since Peter et al. performed their RIAs following column extraction, these values were anticipated to be fairly comparable to our LC–MS/MS data. We confirmed significant differences for ACTH-stimulated 17OHP/DOC ratios between the two groups at both time points, after 30 and 60 min (P values: <0.001, <0.001 respectively). ROC analysis revealed 0.849 (30 min) and 0.885 (60 min) for the area under the curve (AUC). After 30 min, we revealed a sensitivity of 76% and a specificity

### Table 3 Median and range for testosterone for males only, adults, P values and adjusted P values.

<table>
<thead>
<tr>
<th>Steroid hormone</th>
<th>Controls n = 25</th>
<th>Heterozygous CYP21A2 n = 27</th>
<th>P adjust</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone ng/dl</td>
<td>Time (min)</td>
<td>Median</td>
<td>5%</td>
</tr>
<tr>
<td>(only adults) (nmol/l)</td>
<td>0</td>
<td>500.80 (17.39)</td>
<td>249.13 (8.65)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>492.91 (17.11)</td>
<td>225.36 (7.83)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>481.75 (16.73)</td>
<td>134.06 (4.65)</td>
</tr>
</tbody>
</table>

### Table 4 Median and range for the androgens for females, P values and adjusted P values.

<table>
<thead>
<tr>
<th>Steroid hormone</th>
<th>Time (min)</th>
<th>Controls n = 19</th>
<th>Heterozygous CYP21A2 n = 23</th>
<th>P adjust</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ4 ng/dl (nmol/l)</td>
<td>0</td>
<td>78.42 (2.74)</td>
<td>15.48 (0.54)</td>
<td>152.36 (5.33)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>90.52 (3.17)</td>
<td>23.41 (0.82)</td>
<td>171.11 (5.98)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>114.06 (3.99)</td>
<td>28.98 (1.01)</td>
<td>190.09 (6.63)</td>
</tr>
<tr>
<td>Testosterone ng/dl (nmol/l)</td>
<td>30</td>
<td>26.81 (0.93)</td>
<td>4.88 (0.17)</td>
<td>75.52 (2.62)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>30.04 (1.04)</td>
<td>10.56 (0.37)</td>
<td>433.20 (15.04)</td>
</tr>
<tr>
<td>DHT ng/dl (nmol/l)</td>
<td>0</td>
<td>15.28 (0.53)</td>
<td>3.56 (0.12)</td>
<td>47.62 (1.64)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>24.16 (0.83)</td>
<td>1.19 (0.04)</td>
<td>79.74 (2.75)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>19.47 (0.67)</td>
<td>2.90 (0.10)</td>
<td>169.63 (5.85)</td>
</tr>
</tbody>
</table>

Δ4, androstenedione; DHT, dihydrotestosterone.
of 56% for correct diagnosis of heterozygosity. After 60 min, respective values were 70 and 63%.

We observed markedly higher values for AUC (0.934 at 30 min and 0.924 at 60 min respectively) when we inserted the ACTH-stimulated concentrations for 17OHP, 21S and cortisol into the equation ((17OHP \(\div\) 21S)/F \(\times\) 1000) (Fig. 2, ROC analysis). This has previously been reported by Janzen et al. (21) in the context of neonatal screening for CAH. A cut-off value of 8.4 (8.0 for adults excluding the prepubertal and pubertal children) for the ACTH-stimulated ((17OHP \(\div\) 21S)/F \(\times\) 1000) equation after 30 min provides 89% sensitivity and 89% specificity. If a specificity of 100% is defined (no false-positive hormonal prediction of a genetically proven carrier status), the cut-off value for the ACTH-stimulated ((17OHP \(\div\) 21S)/F \(\times\) 1000) equation after 30 min is 14.7 with a sensitivity of 42%. Figure 3 shows the calculated ((17OHP \(\div\) 21S)/F \(\times\) 1000) ratios for all heterozygous carriers and all controls, together with the optimal cut-off value of 8.4. Of note, each of the re-categorized heterozygous individuals responded to cosyntropin exactly as predicted from the ((17OHP \(\div\) 21S)/F \(\times\) 1000) equation (i.e., all heterozygotes \(<\) 8.4), as did all the re-categorized homozygous controls (i.e., all homozygotes controls \(<\) 8.4). Based on the data for sensitivity, specificity and heterozygote frequency in the population (Hardy–Weinberg principle) (22), we revealed a PPV of 12% and a NPV of 99.8% for classical mutations and a PPV of 33% and a NPV of 99.2% for non-classical mutations.

**Discussion**

Compared with all other tested parameters, the result of the equation ((17OHP \(\div\) 21S)/F \(\times\) 1000) 30 min after ACTH stimulation proved to be the best for specific and sensitive identification of heterozygotes in our study, with a statistically optimal cut-off value of 8.4. We could not reproduce the high 100% specificity and sensitivity of the 17OHP/DOC ratio that was found by Peter et al. (15) based on RIA following column extraction verified by human leukocyte antigen (HLA) haplotyping. In fact, in our study, the 17OHP/DOC ratio was markedly inferior to the (17OHP \(\div\) 21S)/F equation using the new LC–MS/MS data. Janzen et al. (21) previously applied the (17OHP \(\div\) 21S)/F ratio in newborn screening for classical 21OHD. The much higher cut-off value of 0.53 (530 when using ((17OHP \(\div\) 21S)/F \(\times\) 1000)), combined with much higher sensitivity of 100% and specificity of 99%, is not in contradiction to our present study. Janzen et al. (21) analyzed classical CAH patients with extensive changes in their steroid hormones compared with our cohort, in which heterozygotes are only expected to show very subtle

![Figure 2](image1.png)

**Figure 2**

ROC analysis for the ((17OHP \(\div\) 21S)/F \(\times\) 1000) equation 30 min after ACTH injection. The marked black spot indicates the optimal cut-off of 8.4.

![Figure 3](image2.png)

**Figure 3**

Ratio of 17-hydroxyprogesterone + 21-deoxycortisol/cortisol \(\times\) 1000 ((17OHP \(\div\) 21S)/F \(\times\) 1000) for heterozygous carriers of CYP21A2 mutations and healthy controls. Solid line: the optimal cut-off value (8.4); dotted line: the 100% specificity cut-off value (14.7).
deviations in the steroid metabolome, with hormone concentrations still within the normal ranges. The Janzen et al. (21) study thus supports our present strategy of including 21S and cortisol rather than 17OHP/DOC for sensitive and specific detection of the carrier status. We also did not find the significant correlation between 17OHP and 21S published by Costa-Barbosa et al. (23). This is possibly due to the fact that we only studied asymptomatic heterozygous carriers instead of non-classic CAH patients.

Despite the remarkably high values for specificity and sensitivity of the (17OHP+21S)/F×1000 equation using the optimal cut-off of 8.4, and considering the only marginal metabolic changes anticipated in heterozygotes, both parameters still did not reach 100%. This approach, therefore, is not suitable as a general replacement for genetic testing. Interestingly, though, a ((17OHP+21S)/F×1000) ratio exceeding 14.7 has a specificity of 100% for correct prediction of heterozygosity. However, in this case sensitivity at the cohort level is limited (42%) so this cut-off would miss a significant number of true heterozygotes. The high NPV of the ((17OHP+21S)/F×1000) equation below 8.4 is also clinically relevant because it excludes heterozygosity in 99.8% of tested cases. We conclude that LC–MS/MS-based determination of only the three hormones 17OHP, 21S and cortisol following ACTH stimulation is a straightforward, fast and comparably simple test that can be of significant assistance to the clinician in the suspicion, the identification or exclusion of heterozygosity of CYP21A2 gene mutations. On the one hand, this can be valuable under conditions in which hormone determinations are possible but access to genetic testing is limited due to financial restrictions of health care systems or health insurances. Since heterozygosity of the CYP21A2 gene mutations has to be considered in the differential diagnosis of hyperandrogenism (7, 8, 9, 10) and since the ACTH test is often performed in these clinical situations (24, 7), calculation of ((17OHP+21S)/F×1000) provides important additional information for test interpretation and diagnostic workup.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-14-1084.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
The study was funded by the HaFo grant of the Medical Faculty of the Christian-Albrechts-University of Kiel, Germany.

Acknowledgements
The authors wish to thank Tanja Stampe, Brigitte Karvelies, Gisela Hohmann, Susanne Olin, Sabine Stein and Silke Struve for excellent technical assistance.

References


