Distribution of 14 high risk HPV types in cervical intraepithelial neoplasia detected by a non-radioactive general primer PCR mediated enzyme immunoassay

Ingo Nindl, Beatrix Lotz, Rosemarie Kühne-Heid, Ulrich Endisch, Achim Schneider

Abstract

Aim—To evaluate the presence of high risk human papillomaviruses (HPV) in cervical smears showing intraepithelial neoplasia (CIN).

Methods—The presence of 14 high risk HPV was evaluated in 114 cervical smears with CIN of different degrees, by comparing a non-radioactive polymerase chain reaction (PCR) enzyme immunoassay (EIA) with conventional PCR followed by radioactive Southern blot hybridisation. General primer PCR amplicons detecting low risk and high risk HPV were typed for 14 different high risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) by a non-radioactive PCR-EIA. Virus load of HPV 16 positive CIN was assessed using the semiquantitative PCR-EIA.

Results—Histological evaluation confirmed CIN I in 49 cases (mean age 29.0 years, range 17 to 52), CIN II in 31 cases (mean age 30.8 years, 18 to 54), and CIN III in 34 cases (mean age 31.1 years, 16 to 57). The non-radioactive PCR-EIA showed an overall agreement rate of 90% (κ value 0.75) when compared with conventional general primer PCR followed by radioactive Southern blot hybridisation. High risk HPV types were detected in 47% of CIN I, 77% of CIN II, and 97% of CIN III (p < 0.02). HPV types 39, 51, 56, and 58 were found in CIN I exclusively (between 2% and 8%). HPV 16 and HPV 31 were detected in 12% and 2% of CIN I, 35% and 21% of CIN II, and 74% and 13% of CIN III, respectively (p < 0.03 and p < 0.04). The virus load estimated by the semiquantitative PCR-EIA of HPV 16 was higher in CIN III than in CIN I and II.

Conclusions—The PCR-EIA has high clinical sensitivity for detecting CIN II/III (90%). There was a significantly higher prevalence rate of HPV 16 and 31 in CIN III than in CIN I and II.

Keywords: human papillomavirus; general primer PCR enzyme immunoassay; cervical intraepithelial neoplasia

Epidemiological and molecular biological studies have proved the causal association between certain genital human papillomaviruses (HPV) and anogenital cancer.1 The genital HPV types are divided into low risk and high risk, on the basis of their phylogenetic relations3 and their association with benign or malignant cervical lesions. High risk HPV are associated with high grade cervical intraepithelial neoplasia (CIN II/III) and cancer. HPV types 16, 18, 31, 33, and 45 have been shown to be the most prevalent types in cervical cancers.3–5 Low risk HPV types (such as HPV 6, 11, 42, 43, and 44) occur mainly in benign condylomas or CIN I.3 Prevalence of high risk HPV is more than 90% in CIN II/III or cervical cancers, approximately 50% in CIN I, and between 5% and 10% in women with negative cytology.1 Several studies have shown the relevance of HPV testing in clinical studies. HPV testing may augment cytology in screening programmes.6–10 In addition, women with the diagnosis of atypical squamous cells of undetermined significance (ASCUS) can be triaged,1 11 12 and patients with CIN I may possibly be subdivided into progressors and regressors related to high risk HPV persistence.11 The prevalence rate of HPV 16 is twice as high in CIN II/III as in CIN I.13–16 A higher virus load of HPV 16 was found in CIN III than in CIN I by a semiquantitative polymerase chain reaction (PCR) system.17 18 Recently, Jacobs et al developed a new generation of a general primer (GP) 5+/ biotinylated (bio) GP6+ mediated PCR enzyme immunoassay (EIA) for rapid detection of 14 high risk and six low risk HPV.19 Our study was designed to determine the various high risk HPV types in CIN in German women using this non-radioactive PCR-EIA, to compare its sensitivity with conventional general primer PCR followed by radioactive Southern blot hybridisation, and to estimate the virus load of HPV 16 in smears from CIN by the semiquantitative PCR-EIA.

Methods

Patients

Between March 1996 and April 1997, 368 women, mean age 32.0 years (range 16 to 70), were referred to our colposcopy clinic because of an atypical cytological smear, positive colposcopy, or a positive high risk HPV result. Colposcopically directed biopsies were obtained from all these women. The tissue was fixed in formalin, embedded in paraffin, and haematoxylin and eosin staining was performed for light microscopic analysis. In this study histological classification was in accordance with the WHO recommendations20 and the CIN classification of Richart.21 In addition, we integrated the recently described histological criteria by Crum
and Nuovo, where the expression of nuclear atypia in different grades of CIN is more important than discordance of epithelial maturation. CIN I corresponds to low grade squamous intraepithelial lesions (LSIL), and CIN II and III to high grade squamous intraepithelial lesions (HSIL). CIN lesions were confirmed in 114 of the 368 women. We did not evaluate normal tissue or tissue containing invasive carcinoma or atypical metaplasia. Further analysis is restricted to this cohort. In all women a cervical smear was taken with a cytobrush. Cells were exfoliated on a glass slide for cytology and the brush was then immersed in 5 ml phosphate buffered saline (PBS). Cells were taken from the ectocervix and posterior fornix with a Dacron-tipped swab and suspended in the same PBS solution that was used for HPV DNA detection.

**GENERAL PRIMER PCR**

The cervical specimen were vigorously vortexed and 5 ml of the suspension was divided in two tubes each containing 2.5 ml PBS and both were centrifuged for 10 minutes at 4000 g. The supernatant was discarded and one tube with cells was stored at −70°C. The cell pellet of the second tube was resuspended in 0.5 ml (10 mM Tris HCl pH 8.3, 50 mM KCl); 100 µl of this solution was transferred into a new tube heated at 95°C for 10 minutes and both samples were stored at −70°C until HPV detection was performed.

For HPV analysis 10 µl of the heated 100 µl suspension were used for a general primer PCR. The general primers GP5+ and bioGP6+, which span a region of 140–150 bp from the L1 open reading frame of a broad spectrum of HPV genotypes, were used in the general primer PCR, as described by de Roda Husman et al., with the exception that the GP6+ primer was biotinylated (bioGP6+). The 50 µl PCR reaction mixtures contained 10 µl of pre-treated suspension of cervical cells, 50 mM KCl, 10 mM Tris HCl pH 8.3, 200 µM of each dNTP, 3.5 mM MgCl₂, 1 unit of a thermostable DNA polymerase (Thermus aquaticus; Perkin Elmer-Cetus), and 50 pmol of GP5+ and bioGP6+ primer. The mixtures were incubated for four minutes at 95°C for DNA denaturation, followed by 40 cycles of amplification using a PCR processor (Perkin Elmer). Each cycle included a denaturation step to 95°C for one minute, an annealing step to 40°C for two minutes (non-stringent conditions), and a chain elongation step to 72°C for 1.5 minutes. To ensure a complete extension of the amplified DNA, the final elongation step was prolonged for another four minutes.

Two samples containing distilled water were used as negative controls in each experiment, none of which showed successful amplification; and also two samples of 1 ng and 10 ng genomic DNA from SiHa cells as positive and sensitivity controls, which showed successful amplification in each experiment evaluated.

Ten microlitres of the general primer PCR mixtures were finally analysed by electrophoresis in 1.5% agarose gels. Each agarose gel contained a PCR amplicon of SiHa cells on all four edges (blot control) and two internal negative and two positive controls. The electrophotoretically separated general primer PCR amplicons were blotted onto nylon membranes (Qiagen, Qiagen) in 0.5 M NaOH, 1.5 M NaCl overnight. Thereafter, the membranes were saturated with 2 × SSC (1 × SSC is 0.15 M sodium phosphate (pH 7.4), 7% SDS (sodium dodecyl sulphate), and 1 mM EDTA).

Hybridisation was performed in the same solution supplemented with the denatured probe for 12–16 hours at 55°C. The general probe consisted of a mixture of the GP5+/GP6+ PCR amplicons generated from cloned HPV 6, 11, 16, 18, 31, and 33, which were electrophotoretically separated in NA agarose (Pharmacia), excised from the gel, and purified after freeze squeezing. A total of 2.5 ng of a mixture of these purified DNAs was used to generate an [α-32P]dCTP random primed labelled general probe. The sensitivity of the general probe was between 0.1 and 1 ng SiHa DNA and at the subpicogram level of several cloned HPV plasmids, corresponding to 15 to 200 copies depending on the HPV type.

After hybridisation, the filters were washed three times for 15 minutes in 3 × SSC/0.5% SDS at 55°C. Autoradiography was performed overnight at −70°C with Kodak Royal X-Omat film and intensifying screens. Samples showing a 140–150 bp product after hybridisation with the radioactive HPV general probe were HPV positive (GP+).

The radioactively labelled general probe detects a broad spectrum of at least 19 high risk/low risk genital HPV types. To test the quality of the cervical smear a separate PCR with β globin specific primers PC03 (5′-ACACAACTGTGTTTACATAGC-3′) and PC04 (5′-CAACTTCATCCAGTTTACCG-3′) for each sample was included, and only samples with a positive reaction showing a 100 bp product were used in this study. Positive conditions were the same as described for the general primer PCR except that 25 pmol of each primer, 1.5 mM MgCl₂, and an annealing step of 55°C (stringent conditions) were used.

**DETECTION OF HIGH RISK/LOW RISK HPV BY GENERAL PRIMER PCR-EIA**

For EIA analysis, GP5+/bioGP6+ generated PCR amplicons were captured on streptavidin coated microwells (Boehringer Mannheim), denatured by alkaline treatment, hybridised with cocktails of DIG labelled internal specific oligonucleotide probes, and detected immuno-histochemically by reading optical density values (OD 405 nm) after overnight incubation. To ensure the high quality of type specific probes, we requested more than 90% labelling efficiency of each oligonucleotide, purified by reverse phase chromatography (Eurogentec).

Fourteen different HPV type specific oligonucleotides were used to detect 14 high risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) either as a cocktail or individually. Six type specific oligonucleotides were used as a cocktail to detect the low risk HPV (HPV 6, 11, 40, 42, 43, and 44). Serial dilutions (1:10) of the 20 cloned high risk/low risk HPV types (0.01 fg to 10 pg) were used to
verify the sensitivity while establishing the PCR-EIA. For a detailed description see reports by Jacobs et al.\textsuperscript{19} 25

The PCR-EIA, which measures optical density values, can be used to monitor the amount of the PCR amplicons. In this study we determined the amount of HPV 16 specific PCR products by semiquantitative PCR-EIA. The sensitivity of this HPV 16 PCR-EIA was determined by serial dilutions of cloned HPV 16 plasmid DNA and different amounts of a cell line with only one HPV 16 copy (SiHa). Each experiment was done twice independently. The mean values of each sample were used to estimate the virus load of HPV 16 positive CIN. The total number of cervical cells was not determined. To measure virus load in a more accurate way, a quantitative PCR would have to be applied.

**STATISTICAL ANALYSES**

Statistical analysis was done using $\chi^2$ or Fisher’s exact test, when frequencies were smaller than 5. For estimation of HPV 16 virus load in CIN I, CIN II, or CIN III, the Wilcoxon–Mann–Whitney U test was applied, excluding the high risk HPV negative cases (fig 1). A p value of < 0.05 was considered significant either for $\alpha_i$ or if indicated, for $\alpha$ multiple.

**Results**

Overall, 114 women with a mean age of 30.1 years (range 16 to 57 years) were enrolled in the study. Histological evaluation of the biopsies showed CIN I in 49 cases (mean age 29.0 years, range 17 to 52), CIN II in 31 cases (mean age 30.8 years, range 18 to 54), and CIN III in 34 cases (mean age 31.1 years, range 16 to 57) (table 1). Two double infections were found in each CIN I, CIN II, and CIN III, and an individual HPV type within a double infection was counted as 0.5 (table 1).

Low risk HPV types (HPV 6, 11, 40, 42, 43, and 44) were detected in 6% of CIN I (three of 49) and in no CIN II/III cases, independent of the method used—that is, conventional general primer PCR followed by Southern blot or the PCR-EIA using a cocktail of six specific DIG labelled oligonucleotides (table 2). The sensitivity of the 14 high risk and six low risk HPV types by PCR-EIA was between 10 and 200 HPV copies, depending on the HPV type analysed by dilution experiments of each cloned HPV type (data not shown). A linear logarithm relation between OD values and HPV copy number was found for the HPV types used as a cocktail or individually within the range (OD $>$ 2). Relative OD values (OD minus cut off) between $\geq$ 0 and 2.0 correspond to 10 and 1000 HPV 16 DNA copies, respectively, by the semiquantitative PCR-EIA.

The agreement rate of high risk/low risk HPV positivity in cervical smears by Southern blot of general primer PCR amplicons hybridised with a general radioactively labelled probe and the PCR-EIA was 84% in CIN I, 94% in CIN II, 97% in CIN III, and 90% for all 114 CIN lesions (x value 0.75) (table 2). Seven CIN I and CIN II were positive for genital HPV genotypes; HR, high risk; LR, low risk; SB, Southern blot.

<table>
<thead>
<tr>
<th>Histology (mean age, range)</th>
<th>HR HPV</th>
<th>HPV 16</th>
<th>HPV 18</th>
<th>HPV 31</th>
<th>HPV 33</th>
<th>HPV 39</th>
<th>HPV 45</th>
<th>HPV 51</th>
<th>HPV 56</th>
<th>HPV 58</th>
<th>HPV 66</th>
<th>HPVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN I (n=49) 29.0 years</td>
<td>23</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>3,0añt</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4,0añt</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CIN II (n=31) 30.8 years</td>
<td>24</td>
<td>11</td>
<td>2,0añt</td>
<td>6,5añt</td>
<td>2</td>
<td>0</td>
<td>0,5añt</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CIN III (n=34) 31.1 years</td>
<td>33</td>
<td>25,0añt</td>
<td>0,5añt</td>
<td>4,5añt</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

HPV 35, 52, 59, and 68 were not detected in CIN independently of the grade of the lesion.

High risk HPV positive: CIN I v CIN II, p = 0.03; CIN I v CIN III, p < 0.001; CIN II v CIN III, p = 0.02.

HPV 16 positive: CIN I v CIN II, p = 0.03; CIN I v CIN III, p < 0.001; CIN II v CIN III, p = 0.01.

HPV 31 positive: CIN I v CIN II, p = 0.05; CIN I v CIN III, p = 0.04.

HPVX, HPV types negative for all 14 individually tested high risk HPV types.

The number of double infections is indicated in subscript parentheses.

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<th>Histology (mean age, range)</th>
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<th>HPV 51</th>
<th>HPV 56</th>
<th>HPV 58</th>
<th>HPV 66</th>
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<td>CIN I (n=49) 29.0 years</td>
<td>23</td>
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<td>0</td>
<td>1</td>
<td>3,0añt</td>
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<td>2</td>
<td>2</td>
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<td>24</td>
<td>11</td>
<td>2,0añt</td>
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<td>33</td>
<td>25,0añt</td>
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<td>4,5añt</td>
<td>3</td>
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SD was < 15%. The Wilcoxon–Mann–Whitney U test (significance for experiment was done twice independently, and further analysis was done using the mean general primer polymerase chain reaction enzyme immunoassay (GP-PCR-EIA). Each

Figure 1: Histology vs virus load of HPV 16 DNA amount estimated by semiquantitative PCR-EIA. Each experiment was done twice independently, and further analysis was done using the mean values of each sample. The values were calculated as the mean of duplicate experiments and SD was < 15%. The Wilcoxon–Mann–Whitney U test (significance for multiple = 0.05) was applied, excluding the high risk HPV negative cases. CIN I vs CIN II: NS, p = 0.73; CIN I vs CIN III: NS, p = 0.89; CIN II vs CIN III: NS, p = 1.0. HPV 16−, HPV 16 negative; HPV 16+, HPV 16 positive; OD, optical density.

detected in 47% of CIN I, 77% of CIN II, and 97% of CIN III (p < 0.02) (table 1). HPV types 39, 51, 56, and 58 were found in CIN I, exclusively (between 2% and 8%). HPV 16 and 31 were detected in 12% and 2% of CIN I, 35% and 21% of CIN II, and 74% and 13% of CIN III, respectively (p < 0.03 and p < 0.04).

The virus load of all HPV 16 positive CIN lesions was estimated by the HPV 16 specific semiquantitative PCR-EIA. HPV 16 was found in six of 49 cases of CIN I, 11 of 31 cases of CIN II, and 26 of 34 cases of CIN III (fig 1). The virus load of HPV 16 positive CIN was similar for all grades indicated by the median (0.28, 0.38, and 0.49) and the third quartile (0.76, 0.66, and 0.63) for CIN I, CIN II, and CIN III, respectively.

Discussion

Amplification based technologies, such as PCR, are the most sensitive techniques for detecting HPV DNA in cervical smears. The primary goal of this study was to test the sensitivity of the non-radioactive general primer PCR enzyme immunoassay in cervical smears of histologically confirmed CIN lesions to detect 14 different high risk HPV types. For the radioactive Southern blot a general probe of 150 bp was used to detect at least 19 different high risk/low risk HPV types, compared with 14 different oligonucleotides (30-mers), each specific for one HPV type used to detect the 14 high risk HPV by PCR-EIA. An overall agreement rate of 96% (x = 0.90) between radioactive Southern blot hybridisation, with a general probe developed to distinguish between HPV positive and HPV negative samples, and high risk/low risk HPV PCR-EIA of the same PCR products was recently reported for 417 cytomorphologically abnormal cervical smears. We also found excellent agreement (x = 0.75) between the same radioactive Southern blot hybridisation method and high risk/low risk PCR-EIA with GP5+/bioGP6+ PCR amplicons of two independent reactions in cervical smears of patients with different grades of CIN.

In The Netherlands, high risk HPV positivity of 53% in 34 CIN I, 75% in 32 CIN II, and 96% in 28 CIN III was found by the general GP5/GP6 and type specific (TS) PCR for the high risk HPVs (16, 18, 31, 33). In another study from The Netherlands, in which cervical smears from 1373 patients were analysed by GP5/GP6 and TS PCR, CIN was diagnosed by cytology and the overall HPV positivity rate was 72% in 971 CIN I, 85% in 295 CIN II, and 100% in 107 CIN III. Low risk HPV types (6, and 11) were detected in less than 4%, independently of CIN grade. In a study from The Netherlands using a PCR system, HPV 6/11, 16, 18, 31, and 33 were found in 44% of CIN I, 69% of CIN II, and 86% of CIN III, respectively. Low risk HPV types (6, and 11) were detected in less than 4% of the different CIN grades. We found similar high risk HPV prevalence rates, with 47% in CIN I, 77% in CIN II, and 97% in CIN III. Low risk HPV types (HPV 6, 11, 40, 42, 43, and 43) were only detected in 6% of CIN I (three of 49) in our study. Thus HPV prevalence and HPV distribution in CIN seems to be comparable in the different European countries when similar HPV detection systems are applied.

In a study from Japan, 220 frozen biopsies of CIN lesions were analysed by a restriction fragment polymorphism (RFLP) Southern blot assay. Twenty seven different HPV types were found and the overall HPV prevalence was 94% in CIN I, 100% in CIN II, and 93% in CIN III. The low risk HPV types 6 and 11 were not detected. In our study a similar HPV distribution pattern was found for the HPV types 16, 18, 31, 33, 39, 56, 59, and 66. In contrast, in Japan HPV 51, 52, and 58 were detected in between 2% and 20% of CIN II/III, respectively. In our study these HPV types were detected in CIN I exclusively. This discrepancy may be the result of geographically different prevalence rates for the various HPV genotypes. The overall HPV prevalence in cervical smears of CIN II and CIN III was similar (77% and 97%), but lower in CIN I (47%). This phenomenon may be explained by misclassification—the interobserver and intraobserver agreement for the histological diagnosis of CIN I was poor, in contrast to a good agreement for CIN II/III. CIN I lesions are most likely to be misread in older women. In our study, only 14 of 49 patients (29%) were older than 35 years and a large number of biopsies from elderly women cannot explain...
the low HPV positivity in CIN I. Another explanation may be the differences in the cell material used. Matsukura and Sugase used biopsies of CIN lesions for HPV detection, rather than the cervical smears used in our study.

In a worldwide study, 1035 cervical cancer lesions were investigated for the presence of 26 HPV types by a general primer PCR based method, and 932 were included in the analysis. The most prevalent HPV types in 86 cervical cancers from Europe were HPV 16 (65.6%), HPV 18 (8.0%), HPV 31 (6.5%), HPV 52, 68, 69, 45, 56 (2%), and HPV 33, 35, 58 (1%), whereas HPV 39, 51, and 59 were not detected. We found a similar HPV distribution in CIN III: HPV 16 (74%), HPV 31 (13%), HPV 33 (9%), HPV 18 (1%), HPV 35, 52, 59, and 68 were not detected in CIN, independently of the grade of the lesion, and HPV types 39, 51, 56, and 58 were detected in CIN I exclusively. CIN I is expected to regress spontaneously in about 60% of cases, to persist in 30%, and to progress to invasive cervical cancer in only 1%. Therefore HPV 39, 51, and 59 seem to have a lower oncogenic potential and may play a minor role in the progression of CIN lesions.

Previous studies with semiquantitative PCR or quantitative hybrid capture systems showed an increasing amount of high-risk HPV DNA in cervical smears with increasing severity of the lesions indicating that the severity of the lesion is correlated with the presence of high-risk HPV genomes. In two studies analysing patients with different grades of CIN a significantly higher virus load of HPV 16 was found in CIN III v CIN I by a semiquantitative PCR detection method. However, topological distribution of HPV DNA molecules in cervical tissues is not well explained, and distribution of viral copies may vary for the individual HPV types. For HPV 16 we found a similar virus load in CIN I, CIN II, and CIN III using the semiquantitative PCR-EIA. This discrepancy with respect to previous studies may be caused by the small number of HPV 16 positive CIN I lesions analysed in our study compared with the studies of Cuzick et al. Interestingly, our results are in accordance with a recent study where a quantitative HPV 16 DNA PCR-EIA was used and where the HPV 16 virus load in progressive and regressive precancer lesions did not differ. Thus further studies analysing the virus load in high-risk HPV types with new quantitative assays are warranted to solve some of these inconsistencies.

Our data indicate that the new high risk HPV general primer PCR-EIA has high sensitivity for identifying patients with CIN II/III. Nevertheless, larger trials need to be performed to assess the value of HPV testing in cervical cancer screening programmes.

We thank C J L M Meijer, J M M Walboomers, and MV Jacobs for their supply and low-cost in HPV testing by general primer PCR, and for critical reading of the manuscript. HPV clones of types 6b, 11, 16, 18, 33, 40, 68 were kindly provided by L L van Houdt and E M de Villiers (Heidelberg, Germany); HPV 44 and 52 by J M M Walboomers (Amsterdam, The Netherlands); HPV 31 clones by M A Lorincz (Rockville, Maryland, USA); HPV 58 and 59 by T Matsukura (Tokyo, Japan); HPV 45 by K Shah (Baltimore, Maryland, USA); HPV 51 by G Nuovo (New York, USA); and HPV 52, 31, 33 by G Orth (Paris, France). HPV clones of types 35, 43, and 56 were obtained from the ATCC (Rockville, Maryland, USA). This work was supported by a grant of the DFG, Germany (grant No SCHN 294/5-1).


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doi: 10.1136/jcp.52.1.17

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