J Clin Periodontol 2011; 38: 984–991 doi: 10.1111/j.1600-051X.2011.01773.x

Journal of Clinical Periodontology

Cyclosporine-induced gingival overgrowth correlates with NFAT-regulated gene expression: a pilot study

Bettina Dannewitz[1,2], Eva-Maria Kruck[1], Hans Jörg Staehle[1], Peter Eickholz[2], Thomas Giese[3], Stefan Meuer[3], Volkhard Kaever[4], Martin Zeier[5] and Claudia Sommerer[5]

[1]Department of Conservative Dentistry, Clinic for Oral, Dental and Maxillofacial Diseases, University Hospital Heidelberg, Heidelberg, Germany; [2]Department of Periodontology, Centre for Dental, Oral and Maxillofacial Medicine (Carolinum), Johann Wolfgang Goethe-University Frankfurt/Main, Frankfurt, Germany; [3]Institute of Immunology, University of Heidelberg, Heidelberg, Germany; [4]Institute of Pharmacology, Hannover Medical School, Hannover, Germany; [5]Department of Nephrology, University Hospital Heidelberg, Heidelberg, Germany

Dannewitz B, Kruck E-M, Staehle HJ, Eickholz P, Giese T, Meuer S, Kaever V, Zeier M, Sommerer C. Cyclosporine-induced gingival overgrowth correlates with NFAT-regulated gene expression: a pilot study. J Clin Periodontol 2011; 38: 984–991. doi: 10.1111/j.1600-051X.2011.01773.x.

Abstract

Objective: To determine whether incidence and severity of cyclosporine A (CsA)-induced gingival overgrowth (GO) is related to expression nuclear factor of activated T cells-regulated genes (NFAT-regulated genes).

Material and Methods: Expression of NFAT-regulated genes was determined in 36 transplant patients medicated with CsA by real-time PCR before and 2 h after drug intake and residual NFAT activity was estimated as ratio of both measurements. Demographic, periodontal and pharmacologic parameters were recorded and GO assessed from models. Subjects were divided into two groups according to the degree of GO (responders: GO score $\geqslant 10\%$). Groups were compared using parametric and non-parametric tests. The association of various CsA-specific and periodontal parameters on incidence and extent of GO were determined using regression analysis.

Results: Responders had a more than twofold lower residual NFAT activity than non-responders (7.9% and 18.1%, respectively; $p < 0.001$). Multiple regression analysis revealed gingival inflammation, salivary CsA concentration, and residual NFAT activity to be significant factors influencing the expression of GO. Seventy-seven percent of the variability of GO could be explained by these parameters.

Conclusions: This study showed that pharmacodynamic parameters such as residual NFAT activity may be promising prognostic indicators to identify patients with increased risk for GO.

Key words: cyclosporine; gene expression; gingival overgrowth; NFAT; pharmacodynamic; pharmacokinetic; prognostic indicator; saliva

Accepted for publication: 29 June 2011

Cyclosporine A (CsA) is one of the basic immuno-suppressants for prevention of transplant rejection as well as for management of various autoimmune conditions. CsA inhibits the activity of calcineurin, which is an upstream regulator of the transcription factor nuclear factor of activated T cells (NFAT) (Rao et al. 1997).

The first case series about CsA-induced gingival overgrowth (GO) was published just one year after its approval (Rateitschak-Plüss et al. 1983). The aetiology of CsA-induced GO is multifactorial and a variety of risk factors have been identified including age, drug variables, concomitant medication, periodontal variables and genetic factors (Thomas et al. 2000, Seymour 2006). A range of pharmacokinetic parameters has been studied including peak and trough blood/serum concentration, drug dosage and drug concentration in saliva. However, the results are conflicting and indicate that these parameters are no useful prognostic indicators for GO in patients treated with CsA (Thomas et al. 2000, Seymour 2006).

Pharmacodynamic monitoring displaying biologic drug efficacy is a promising tool to estimate the individual immunosuppressive responsiveness to CsA (Hartel et al. 2002, Giese et al. 2004a, Sommerer

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interest. The study has been self-supported by the Section of Periodontology, the Department of Nephrology, and the Institute of Immunology, University Clinic of Heidelberg and the Institute of Pharmacology, Hannover Medical School.

et al. 2009). A real-time polymerase chain reaction (PCR) based assay for the quantitative analysis of calcineurin activity by determination of the NFAT-regulated gene expression has been recently established (Giese et al. 2004a, b). However, to date there is no study investigating the correlation of pharmacodynamic parameters and expression of GO. In this context we hypothesise that increased GO is linked to reduced residual NFAT activity indicating a more pronounced CsA effect in an individual subject. Therefore we evaluated whether incidence and expression of CsA-induced GO was correlated with the transcription level of NFAT-regulated genes. In addition pharmacokinetic parameters and periodontal variables were determined as secondary outcome variables.

Material and Methods

Experimental design and patients selection

All renal transplant patients on CsA-based immunosuppressive maintenance from the transplant outpatient clinic of the Department of Nephrology were screened for presence of GO by oral inspection from May 2006 to August 2008. Every subject fulfilling the inclusion criteria was provided with detailed information about the study and invited to participate. Inclusion criteria were (i) single or multiple renal transplant recipient, (ii) CsA intake for at least 6 months, and (iii) age $\geqslant$ 18 years. A total of 144 patients could be screened, of whom 36 subjects showed local or generalised enlargement of the gingiva (25%, Fig. 1). Thirty-nine patients did not match the inclusion criteria (27.1%; 25 subjects had a CsA intake of $<$6 months or the duration of drug administration was not clearly evident from the records and 14 patients went off CsA in the meantime). Forty-seven patients refused to participate in the study (32.6%).

Finally, 18 subjects with GO and 18 pair-matched controls without GO were enrolled in this study. Included patients were matched for age ($\pm$ 5 years) and gender. After informed consent was obtained the next follow-up appointment in the Department of Nephrology was coordinated with the periodontal examination in the Section of Periodontology to allow all parameters to be assessed on the same day. For the analysis patients were classified as responders and non-responders according to the extend of GO assessed on models. Eventually, 18 patients had been included with signs of GO based on the oral screening. However, one of these patients had a GO score (GOS) of $<$10% and was finally assigned to the non-responder group (Fig. 1).

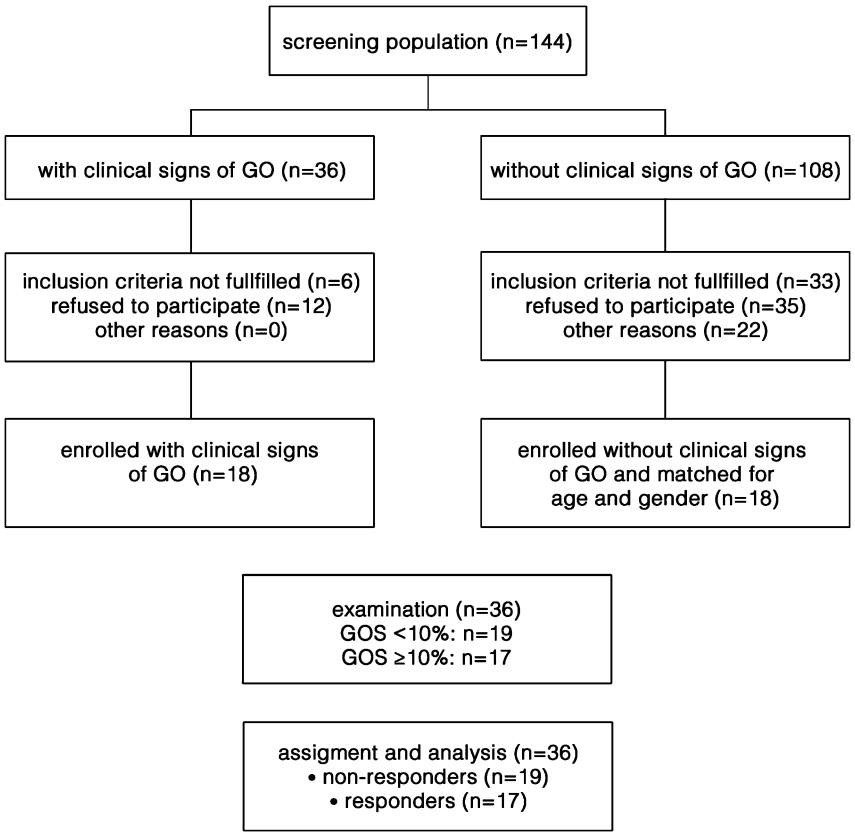

Fig. 1. Study flow chart: a total of 144 patients could be screened. Thirty-nine patients did not match the inclusion criteria, 47 patients refused to participate in the study. Subsequently 18 subjects with clinical signs of gingival overgrowth (GO) and 18 pair-matched (match to age and gender) controls without GO were enrolled in this study. One of the patients with GO had a GO score (GOS) of $<$10% and was finally assigned to the non-responder group.

This cross-sectional trial was approved by the local ethics committee of the Medical Faculty of the Heidelberg University (Application S-004/2007).

Assessment of GO

GO was assessed on plaster models by one experienced examiner (B. D.). Models were scored using a system described before (Seymour et al. 1985). This was expanded to allow the recording of all inter-dental sites. A GOS between 0 and 5 was assigned to each inter-dental papilla depending on the amount of both horizontal and vertical enlargement. The papilla distal to the last-standing molar and areas with an adjacent edentulous space were not measured. Basically, a total of 60 such papillae could be examined giving a potential maximum of 300; GOS was expressed as a percentage. Patients with a GOS $\geqslant$ 10% were classified as responders and the other subjects as non-responders.

To evaluate consistency of repeated measurements intra-examiner reproducibility was determined. Therefore, GOS was measured on two occasions three weeks apart in 10 subjects not involved in the study, each showing various degrees of GO. The calculated intra-class correlation coefficient of 0.988 (95% confidence interval 0.951–0.997, $p<0.001$) demonstrated a very good correlation of both measurements. Hence, only a single determination of GOS was performed in this study.

Periodontal variables

The following parameters were measured by one experienced examiner (E. K.): (i) number of teeth, (ii) tooth mobility (increased mobility was classified

as follows: Grade I slightly more than normal; Grade II moderately more than normal; Grade III severe mobility facial-lingually and/or mesio-distally combined with apical displacement) (Carranza & Takei 2006), (iii) full-mouth plaque score (FMPS) (O'Leary et al. 1972), (iv) full-mouth bleeding score (FMBS) (Ainamo & Bay 1975), (v) probing pocket depths (PPD) to the nearest 1 mm at six sites per tooth using a standardised periodontal probe (PCP-UNC-15, Hu-Friedy, Chicago, IL, USA), and (vi) bleeding on probing (BOP).

Quantitative analysis of NFAT-regulated gene expression

The NFAT-regulated gene expression was measured in peripheral blood lymphocytes stimulated with phorbol-12-myristate-13-acetate (PMA)/ionomycin (Sigma-Aldrich, Steinheim, Germany). For further details, we refer to recent reports (Giese et al. 2004a, b). The strongest inhibition was seen for interleukin-2 (IL-2), interferon-γ (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Giese et al. 2004b). To avoid individual differences in the expression of a particular single gene, NFAT-regulated gene expression was evaluated by measuring three different genes.

Briefly, gene expression was quantified using real-time PCR with the LightCycler. Target sequences were amplified using commercially available LightCycler Primer Sets (Search-LC, Heidelberg, Germany) with the LightCycler FastStart DNA Sybr Green I Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The transcript concentration for the measured genes was calculated from a virtual standard curve, obtained by plotting a known input concentration of a plasmid to the PCR cycle number at which the detected fluorescence intensity reached a fixed value. mRNA input was normalised by a constant expression value of two housekeeping genes (β-actin and cyclophilin B). The data of the two independent analyses for each sample and each parameter were averaged and presented as the ratio between the expression level before (C_0) and 2 h (C_2) after CsA intake (residual NFAT activity = $C_2/C_0 \times 100$). For all three genes, the residual gene expression was averaged and presented as mean residual gene expression.

Demographic data, concomitant medication, parameters of kidney function, and measurement of CsA blood level

Data regarding age, gender, weight, number of transplantations, post-transplantation time, daily CsA dosage, and concomitant medication were obtained from the patients' records. Creatinine was assessed as serum marker of renal function and the glomerular filtration rate (GFR) was estimated using the MDRD (modification of diet in renal disease) equation (Levey et al. 1999).

Patients were requested to fast from the collection of the CsA trough level (C_0) in the morning until after the 2-h sample had been obtained. CsA blood levels were measured by quantitative multiplied immunoassay technique (EMIT™; Dade-Behring, Marburg, Germany).

Determination of salivary CsA concentration

CsA concentration was determined in stimulated as well as unstimulated saliva. Before use, all plasticware was treated with AquaSil™ Siliconizing Fluid (Pierce, Rockford, IL, USA) (Mendonza et al. 2004). Samples were taken 2 h after CsA intake under standardised conditions (Söderling 1989), subsequently frozen and collectively referred to the Institute of Pharmacology, Hannover Medical School, Germany, for analysis, applying liquid chromatography-tandem mass spectrometry technique (LC-MS/MS) as described before (Koal et al. 2004, Mendonza et al. 2004).

Statistical analysis

Statistical analysis was performed using a commercially available software package (SPSS, Version 16.1 for Mac OS X, SPSS Inc., Stanford, CA, USA). To compare means between both groups *t*-test for independent samples was applied for continuous, normally distributed data. For nonparametric parameters Mann–Whitney *U*-test was used. Nominal or ordinal data were analysed using χ^2 statistics.

To elucidate the impact of various CsA-specific and periodontal variables (independent variables) on expression of GO (dependent variable) data were evaluated applying univariate and multiple regression analysis. Missing values of salivary CsA concentration were replaced by the calculated median. Transformation was performed to linearise the association between dependent and independent variables.

Results

Experimental population and kidney function

A total of 36 subjects were enrolled in this study (Fig. 1). The mean age was 45 years; 25% of the studied patients were female (Table 1). Since patients were matched, both groups showed a similar distribution for gender and age. Post-transplantation time was on average 91 months. This time span was considerably longer in the responder group (106 ± 73 months) than in the non-responder group (78 ± 68 months); however, this difference was statistically not significant (Table 1).

Non-responders revealed a slightly better kidney function than responders demonstrated by a lower creatinine level and a higher GFR, but the difference between both groups failed to reach statistical significance.

CsA dosage, duration of drug intake, CsA concentration in blood and saliva

The daily dosage for CsA was 172.36 ± 48.9 mg (2.47 ± 0.86 mg/kg body weight, accordingly) for all studied patients. Non-responders showed a higher dosage (183.42 ± 51.52 mg) than responders (160.0 ± 44.02 mg), but this difference failed to reach statistical significance (Table 1). Within responders mean duration for CsA intake was longer (106.47 ± 72.77 months) compared with non-responders (71.63 ± 67.4 months), but the observed difference was statistically not significant. Trough and 2-h CsA blood level were comparable in both groups (Table 1).

In one patient collection of unstimulated saliva yielded no sufficient volume for analysis; another patient showed a CsA concentration below the detection limit (1 ng/ml) in both samples. In one patient CsA was administered as suspension and the measured concentration in both samples exceeded the average of the other patients by the factor 100. These subjects were not included for descriptive evaluation of the data. Overall mean CsA concentration in stimulated saliva was less than in unstimulated samples. Responders showed a higher CsA concentration in both samples than non-responders; however, the difference was statistically not significant (Table 1).

Table 1. Demographic and CsA-relevant parameters for responders showing a gingival overgrowth score (GOS) ⩾ 10% and non-responders (GOS < 10%)

	Responders ($n = 17$)	Non-responders ($n = 19$)	p
Age in years (mean ± SD; range)	44 ± 14; 24–67	46 ± 16; 19–69	NS
Female sex (no./total no.; %)	4/17; 23.5%	5/19; 26.3%	NS
Weight in kg (mean ± SD; range)	71 ± 15; 48–95	72 ± 11; 53–90	NS
Post-transplantation time in months (mean ± SD; range)	106 ± 73; 14–246	78 ± 68; 11–224	NS
Creatinine in mg/dl (mean ± SD; range)	1.55 ± 0.46; 1.01–2.69	1.38 ± 0.5; 0.51–2.67	NS
MDRD in ml/min/1.73 m^2 (mean ± SD; range)	54.35 ± 19.68; 28.89–94.3	64.97 ± 30.28; 18.52–144.65	NS
Duration of CsA therapy in months (mean ± SD; range)	106.47 ± 72.77; 14–246	71.63 ± 67.4; 12–225	NS
CsA dosage/day in mg (mean ± SD; range)	160.0 ± 44.02; 85–235	183.42 ± 51.51; 100–260	NS
CsA dosage/kg BW (mean ± SD; range)	2.31 ± 0.81; 1.32–4.9	2.6 ± 0.9; 1.22–4.91	NS
CsA-C_0 blood level in ng/ml (mean ± SD; range)	102.24 ± 29.43; 39–157	110.58 ± 32.8; 30–161	NS
Dose-adjusted CsA-C_0 blood level in ng/ml per mg/kg BW (mean ± SD; range)	46.97 ± 15.35; 19.66–70.68	47 ± 17.32; 8.7–68.08	NS
CsA-C_2 blood level in ng/ml (mean ± SD; range)	628.29 ± 238.92; 216–1182	647.79 ± 214.27; 346–1178	NS
Dose-adjusted CsA-C_2 blood level in ng/ml per mg/kg BW (mean ± SD; range)	285.61 ± 108.25 (108.86–496.44)	274.61 ± 105.25 (70.53–465)	NS
Residual NFAT activity in % (mean ± SD; range)	7.88 ± 4.92; 2–17	18.11 ± 11.78; 3–46	< 0.01
CsA level in unstimulated saliva in ng/ml (median; IQR; no.)	5.9; 3.78–9.37; $n = 16$	3.9; 2.9–8.5; $n = 17$	NS
CsA level in stimulated saliva in ng/ml (median; IQR; no.)	4.5; 2.8–6.6; $n = 17$	3.2; 2.35–4.35; $n = 17$	NS

BW, body weight; C_0, before CsA administration; C_2, 2 h after intake; CsA, cyclosporine A; eGFR, estimated glomerular filtration rate; IQR, inter-quartile range; MDRD, modification of diet in renal disease; NFAT, nuclear factor of activated T cells; NS, not statistically significant; SD, standard deviation.

Residual expression of NFAT-regulated genes (residual NFAT activity)

Regarding the residual NFAT activity we could detect a statistically significant difference between both groups (Table 1 and Fig. 2). Non-responders had a more than twofold higher residual NFAT activity than responders (18.1% and 7.9%, respectively; $p < 0.01$).

To evaluate the correlation between residual NFAT activity as dependent variable to and C_2 blood level, data were displayed as scatter diagrams (not shown). Correlation of residual NFAT activity and CsA-C_2 blood concentration was inverse (residual NFAT activity $= 24.619 + (-0.0018 \times$ CsA-C_2 blood level), $p = 0.023$, $R^2 = 14.4\%$).

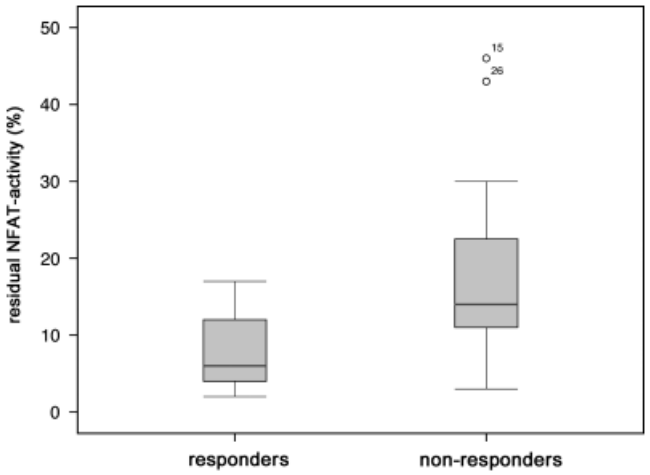

Fig. 2. The boxplot diagram illustrates the residual activity of the transcription factor NFAT (nuclear factor of activated T-cells; residual NFAT activity) 2 h after cyclosporin A (CsA) administration (C_2) displayed as percent. For responders showing a gingival overgrowth score (GOS) ⩾ 10% mean residual NFAT activity was significantly lower ($p < 0.01$) than for non-responders (GOS < 10%). The box encloses the middle half of the values from the lower to the upper quartile and the whiskers represent the highest/lowest values that are no outliers or extreme values. Mild outliers (between 1.5 and three times the inter-quartile range) are denoted by a dot (patient no. 15 and 26). The red line within the box shows the median.

Concomitant medication

Patients were also treated with several other supplemental drugs: 35 subjects (97.2%) received an antihypertensive medication, in 32 patients (91%) two or more antihypertensive agents were combined. More than half of the studied patients (58.3%) took calcium channel blockers (CCB) additionally to CsA (Table 2). The proportion was significantly higher in the responder group (76.5%) than in the non-responder group (42.1%; $p < 0.05$). The following CCB were applied: dihydropyridine (amlodipine, felodipine or lercanidipine; overall 57.2%), benzothiazepine (diltiazem; overall 33.3%), and in two patients compounds of both groups (Table 2). Median GOS in patients medicated with calcium channel blockers was 21.3% compared with 14.6% in subjects without this additional medication. However, the difference between both groups was statistically not significant.

Periodontal variables and GO score (GOS)

Overall patients had a mean of 24.9 teeth; the number of teeth was similar in both groups (Table 3). Only 5.3% of teeth had an increased mobility, the majority (79.2%) of grade I. Responders showed significantly more teeth with increased tooth mobility of grade II or III (39.1%) than non-responders (4%).

The mean value for all assessed PPD was about 0.5 mm deeper in responders than in non-responders ($p < 0.01$). In responders the most pronounced GO was observed in anterior teeth (GOS 22%). Most notably, lower anterior teeth showed severe GO (GOS 18%), whereas

Table 2. Intake of calcium channel blockers (CCB) for responders showing a gingival overgrowth score (GOS) ⩾ 10% and non-responders (GOS < 10%)

	Responders ($n = 17$)	Non-responders ($n = 19$)	p
CCB (no./total no.; %)	13/17; 76.5%	8/19; 42.1%	< 0.05
Benzothiazepine (no./total no.; %)	5/13; 38.5%	2/8; 25.0%	NS
Dihydropyridine (no./total no.; %)	7/13; 53.8%	5/8; 62.5%	NS
Benzothiazepine + dihydropyridine (no./total no.; %)	1/13; 7.7%	1/8; 12.5%	NS

CCB, calcium channel blocker; NS, not statistically significant.

Table 3. Periodontal variables for responders showing a gingival overgrowth score (GOS) ⩾ 10% and non-responders (GOS < 10%)

	Responders ($n = 17$)	Non-responders ($n = 19$)	p
Number of teeth (mean ± SD; range)	24.18 ± 7.58; 3–31	25.63 ± 4.5; 14–32	NS
Number of mobile teeth (no./total no.; %)	23/411; 5.6%	25/487; 5.1%	NS
FMBS in % (median; IQR)	14.7; 7.6–23.3	15.2; 12.5–20.5	NS
FMPS in % (mean ± SD; range)	25.37 ± 15.96; 8.3–62.5	24.88 ± 12.47; 8.3–53.9	NS
BOP in % (median; IQR)	12.5; 8.5–19.3	11.4; 7.3–14.5	NS
PPD in mm (mean ± SD; range)	2.81 ± 1.13; 1–7	2.35 ± 0.7; 1–8	< 0.01
GOS in % (median; IQR)	14.62; 10.21–18.65	2.94: 0.56–4.62	< 0.01
GOS maxilla in % (median; IQR)	15.0; 10.0–41.15	4.0; 0–6.67	< 0.001
GOS mandible in % (median; IQR)	10.77; 5.96–22.02	1.33; 0–4.55	< 0.01
GOS anterior teeth in % (median; IQR)	22.0; 10.5–31.12	3.33; 0–6.25	< 0.01
GOS posterior teeth in % (median; IQR)	11.88; 10–15.5	1.88; 0–4.29	< 0.01
GOS anterior teeth maxilla in % (median; IQR)	10.0; 10–40	4.0; 0–10	NS
GOS anterior teeth mandible in % (median; IQR)	18.0; 7–33	2.0; 0–4	< 0.01
GOS posterior teeth maxilla in % (median; IQR)	14.29; 8.38–33.75	3.75; 0–7.5	< 0.01
GOS posterior teeth mandible in % (median; IQR)	8.75; 4.73–19.38	0.0; 0–2	< 0.01
GOS labial sites in % (median; IQR)	17.69; 12.69–26.21	5.38; 0–7.06	< 0.01
GOS oral sites in % (median; IQR)	11.25; 7.91–17.08	0.0; 0–2.31	< 0.01

BOP, bleeding on probing; FMBS, full-mouth bleeding score; FMPS, full-mouth plaque score; GOS, gingival overgrowth score; IQR, inter-quartile range; NS, not statistically significant; PPD, probing pocket depth; SD, standard deviation.

Table 4. Univariate regression analysis investigating the relationship between gingival overgrowth score (GOS) and cyclosporin A (CsA)-specific parameters as well as periodontal variables

Independent variables	Transformation	Equation	p-value	SE	95%	R^2 (%)
Dose-adjusted CsA-C_2 blood level (ng/ml per mg/kg BW)		x0.063–5.364	0.026	0.027	0.008–0.118	13.8
Residual NFAT activity (%)	SQRT(NFAT)	$x(-5.525)+31.124$	0.013	−0.41	−9.807–(−1.243)	16.8
CsA level in unstimulated saliva samples (ng/ml)		x1.786–0.452	< 0.0001	0.804	1.325–2.246	64.6
CsA level in stimulated saliva samples (ng/ml)		x3.535–3.998	< 0.0001	0.832	2.688–4.383	69.3
FMBS (%)	FMBS^2	x0.006+9.339	0.002	0.502	0.002–0.009	25.2

BW, body weight; CI, confidence interval; C_2, 2 h after intake; CsA, cyclosporine A; FMBS, full-mouth bleeding score, nuclear factor of activated T cells; SE, standard error; SQRT, square root.

no statistically significant difference could be observed between both groups in the area of the upper anterior teeth (responders: 10%, non-responders: 4%). GO was more pronounced buccally than at oral sites, and more severely in the maxillary gingiva than in the mandibular gingiva (Table 3).

Correlation and extend of variability of GOS to CsA-specific and periodontal parameters

Firstly an univariate regression analysis with GOS as dependent variable was performed to evaluate an association between GOS and CsA-specific parameters and periodontal variables. As indicator of the overall fit of the model, F-statistic was assessed, revealing that models with acceptable suitability could only be calculated for dose-adjusted CsA-C_2 blood level, residual NFAT activity, CsA concentration in unstimulated and stimulated saliva and FMBS (Table 4). Dose-adjusted CsA-C_2 blood level ($p = 0.026$, $R^2 = 13.8\%$), square root (SQRT)-transformed values of residual NFAT activity ($p = 0.013$, $R^2 = 16.8\%$), quadratic-transformed values of FMBS ($p = 0.002$, $R^2 = 25.2\%$), and salivary CsA concentration in unstimulated ($p < 0.0001$, $R^2 = 64.6\%$) and stimulated saliva samples ($p < 0.0001$, $R^2 = 69.3\%$) demonstrated a statistically significant correlation to the expression of CsA-induced GO (Table 4).

Subsequently, multiple regression analysis was applied to evaluate which of the factors considered in this study have an impact on the expression of CsA-induced GO. All variables, which had demonstrated a significant association on GOS in univariate analysis, were included at the start. Variables without regression coefficient differing significantly from zero were gradually excluded from the model (dose-adjusted

Table 5. Multivariate regression analysis investigating the relationship between gingival overgrowth score (GOS) and residual NFAT (nuclear factor of activated T cells) activity, full-mouth bleeding score (FMBS) and cyclosporin A (CsA) level in unstimulated saliva sample

	Coefficient	SE	95% CI	*p*-value
Constant	10.925	4.776	1.196–20.654	0.029
SQRT(NFAT)	− 3.766	1.165	− 6.138–(− 1.394)	0.003
$FMBS^2$	− 0.004	0.002	− 0.007–0.000	0.027
CsA unstimulated saliva	2.253	0.311	1.619–2.887	<0.001

CI, confidence interval; CsA, cyclosporine A; FMBS, full-mouth bleeding score; NFAT, nuclear factor of activated T-cells; SE, standard error; SQRT, square root.

CsA-C_2 blood level and CsA concentration in stimulated saliva). According to the results depicted in Table 5, expression of GOS can be explained by the following equation: $GOS = 10.925 - 3.766 \times SQRT(NFAT) - 0.004 \times FMBS^2 + 2.253 \times$ CsA unstimulated saliva.

All coefficients were significantly different from zero with a significance level of 5%. FMBS, residual NFAT activity, and CsA concentration in unstimulated saliva could predict the variability of GOS up to 77% ($R^2 = 0.768$ and $R = 0.876$).

Discussion

The aetiology of CsA-induced GO is multi-factorial in nature and numerous risk factors have been identified previously (Seymour et al. 2000, Seymour 2006). Since pharmacokinetic parameters do not seem to be reliable prognostic factors for GO, the present study aims to evaluate the correlation of GO and expression of NFAT-regulated genes, as a specific pharmacodynamic parameter of CsA therapy. Moreover, periodontal variables and pharmacokinetic parameters were studied accordingly.

Analysis of the data revealed no statistically significant difference between both groups for post-transplantation time, duration of CsA intake, daily CsA dosage, daily CsA dosage related to the body weight, and CsA blood level at C_0 and C_2. However, these results can be related to the low threshold level of $\geqslant 10\%$, which may be too low to identify significant differences between both groups.

Subsequently, association between measured variables and expression of GO was examined applying simple regression analysis in addition to the comparison of groups. This analysis demonstrated a marginal statistically significant positive correlation to the severity of GO only for the dose-adjusted CsA-C_2 blood level. In recent years numerous studies have focused on the relationship between pharmacokinetic parameters and the incidence and severity of GO showing conflicting results. The majority could not demonstrate a significant association between CsA concentration in blood/serum and GO (Wysocki et al. 1983, Daley et al. 1986, Friskopp & Klintmalm 1986, McGaw et al. 1987, Ross et al. 1989, Seymour & Smith 1991, King et al. 1993, Cebeci et al. 1996a, b, Margiotta et al. 1996, Afonso et al. 2003, Thomason et al. 2005, Cota et al. 2010). In contrast, other authors detected an association between CsA daily dosage, CsA blood level and GO (Hefti et al. 1994, Somacarrera et al. 1994, Thomas et al. 2000, Radwan-Oczko et al. 2003, de Oliveira Costa et al. 2006). Regarding the discussed results, pharmacokinetic data such as CsA daily dosage and blood level do not seem eligible as prognostic indicators for patients with increased risk for GO.

Most investigations evaluated the expression of GO either to the trough level or it was not clearly described at which time after CsA intake blood concentration was determined. For the new galenic formulation of CsA (Sandimmun® Neoral, Novartis Pharma, Nüremberg, Germany), the 2-h level provides a better calculation of the CsA exposure compared with the previously favoured pre-dose level (Keown et al. 1996).

So far, only few studies have evaluated the association of salivary CsA concentration and occurrence and expression of GO (McGaw et al. 1987, King et al. 1993, Hefti et al. 1994, Margiotta et al. 1996). As yet only one group shows that GO is significantly correlated with the salivary CsA level (McGaw et al. 1987). Additionally, authors reported that CsA concentration in saliva exceeded the corresponding serum level by as much as factor 12 (McGaw et al. 1987). Patients in the study were medicated with a CsA suspension and oral mucosal exposure to CsA by administration of a mixture is approximately 130 times higher than in capsule form (Modeer et al. 1992). We could not show any significant difference between both groups regarding salivary CsA concentration, yet in contrast to the investigated literature, we observed a statistically significant positive correlation between the salivary CsA level and the severity of GO. The mean CsA concentration in stimulated salivary samples was significantly lower than in unstimulated saliva. However, increased oral fluid production changes the pH and as a result salivary concentration of the drug (Drummer 2006).

In contrast to the pharmacokinetic data, we could detect a statistically significant difference between responders and non-responders regarding the residual NFAT activity as well as a significant inverse correlation between expression of NFAT-regulated genes and severity of GO. Patients with GO demonstrated less residual NFAT activity as consequence of CsA-induced calcineurin inhibition while CsA blood concentration differed not significantly between both groups. The association of pharmacodynamic parameters and GO has not been evaluated so far. Nevertheless a specific association between the residual NFAT activity and CsA related adverse events such as recurrent infections or malign transformations have been proven (Sommerer et al. 2006, 2008b, Konstandin et al. 2007, Billing et al. 2010). These events are associated with the immunosuppressive effect of CsA. Though it is questionable whether the immunomodulative effect of CsA is relevant for the aetiology of GO, these surrogate parameters allow a more precise estimation of the individual bioavailability of CsA compared with the conventionally applied therapeutic drug monitoring (Sommerer et al. 2008a, 2009). Further intervention studies have to elucidate if GO reduces when CsA dosage is adjusted according to the residual NFAT activity.

Numerous studies have described that a concomitant medication with CCB does not consistently increase the incidence but significantly aggravates the expression of GO (Pernu et al. 1993, Thomason et al. 1993, 1996, Khoori et al. 2003, Ellis et al. 2004, Vescovi et al. 2005, Cota et al. 2010). In the present study 76.5% of responders but only 42.1% of non-responders were

additionally medicated with CCB. The median GOS was increased for patients taking CCB, but the difference failed to reach statistical significance. The class of CCB includes different substances. Clinical trials show that various agents have a different effect on the occurrence and severity of GO (Cebeci et al. 1996a, b, James et al. 2000, Radwan-Oczko et al. 2003). However, the number of patients enrolled in the present study is too small and intake too heterogeneous to allow evaluation of the impact of concomitant medication with CCB on GO.

The mean FMPS calculated in the present study was only 25.4% and no association could be detected for FMPS and severity of GOS. In many cases GO is so extensive, that much of the tooth crown is covered. Evaluation of plaque scores on the basis of application of a disclosing solution may therefore underestimate the presence of plaque. Bacterial plaque and subsequently gingival inflammation still have to be considered as risk factors for GO, even if a direct association may be masked by the above mentioned problems. Although the incidence and expression of GO might not only be related to the amount but also to the qualitative composition of the microbiota (Romito et al. 2004, Cota et al. 2010) challenging the mere quantitative assessment of plaque as appropriate tool for the estimation of the association between plaque and GO (Romito et al. 2004).

Plaque-induced gingivitis was identified as an important risk factor for GO in the majority of previous clinical studies corroborating our results (Tyldesley & Rotter 1984, McGaw et al. 1987, Ross et al. 1989, King et al. 1993, Pernu et al. 1993, Thomason et al. 1993, 2005, Hefti et al. 1994, Somacarrera et al. 1994, Cebeci et al. 1996a, b, Margiotta et al. 1996, Afonso et al. 2003, Ellis et al. 2004, de Oliveira Costa et al. 2006, Lima et al. 2008, Cota et al. 2010). As in our trial, most of the previous investigations are cross-sectional trials, which cannot differentiate whether the gingival inflammation was present before onset of GO or was subsequently induced by aggravated plaque accumulation as consequence of GO.

Assessment of the clinical attachment in patients with GO often shows a considerable measurement error due to the difficulty to identify the cemento-enamel junction. Therefore we measured PPD but not the attachment level. This however is surely a shortcoming of our study design, as we could not determine the loss of periodontal attachment and the potential influence on expression of GO.

Conclusion

In the present study we could demonstrate that gingival inflammation, salivary CsA level, and residual NFAT activity significantly influence the severity of GO; 77% of GO's variability could be attributed to these three factors in a multi-variate analysis. These factors are therefore important indicators for the incidence of GO.

Acknowledgements

The kind assistance of the clinical staff of the Section of Periodontology and Department of Nephrology, University Clinic Heidelberg is gratefully acknowledged. The authors also wish to thank Burkhard Glass for his valuable support in the statistical analysis of the data.

We wish to thank Karlheinz Weber and Dr. Almut von Schwedler, MBBS (Tas), Matcham, NSW, Australia for her valuable and competent support in the preparation of this manuscript.

References

Afonso, M., Bello Vde, O., Shibli, J. A. & Sposto, M. R. (2003) Cyclosporin A-induced gingival overgrowth in renal transplant patients. *Journal of Periodontology* **74**, 51–56.

Ainamo, J. & Bay, I. (1975) Problems and proposals for recording gingivitis and plaque. *International Dental Journal* **25**, 229–235.

Billing, H., Giese, T., Sommerer, C., Zeier, M., Feneberg, R., Meuer, S. & Tonshoff, B. (2010) Pharmacodynamic monitoring of cyclosporine A by NFAT-regulated gene expression and the relationship with infectious complications in pediatric renal transplant recipients. *Pediatric Transplantation* **14**, 844–851.

Carranza, F. A. & Takei, H. H. (2006) Clinical diagnosis. In: Newman, M. G., Takei, H. H., Klokkevold, P. R. & Carranza, F. A. (eds). *Clinical Periodontology*, 10th edition, pp. 540–560. St. Louis: Saunders Elsevier.

Cebeci, I., Kantarci, A., Firatli, E., Aygun, S., Tanyeri, H., Aydin, A. E., Carin, M., Guc, U. & Tuncer, O. (1996a) Evaluation of the frequency of HLA determinants in patients with gingival overgrowth induced by cyclosporine-A. *Journal of Clinical Periodontology* **23**, 737–742.

Cebeci, I., Kantarci, A., Firatli, E., Carin, M. & Tuncer, O. (1996b) The effect of verapamil on the prevalence and severity of cyclosporine-induced gingival overgrowth in renal allograft recipients. *Journal of Periodontology* **67**, 1201–1205.

Cota, L. O., Aquino, D. R., Franco, G. C., Cortelli, J. R., Cortelli, S. C. & Costa, F. O. (2010) Gingival overgrowth in subjects under immunosuppressive regimes based on cyclosporine, tacrolimus, or sirolimus. *Journal of Clinical Periodontology* **37**, 894–902.

Daley, T. D., Wysocki, G. P. & Day, C. (1986) Clinical and pharmacologic correlations in cyclosporine-induced gingival hyperplasia. *Oral Surgery, Oral Medicine, Oral Pathology* **62**, 417–421.

de Oliveira Costa, F., Diniz Ferreira, S., de Miranda Cota, L. O., da Costa, J. E. & Aguiar, M. A. (2006) Prevalence, severity, and risk variables associated with gingival overgrowth in renal transplant subjects treated under tacrolimus or cyclosporin regimens. *Journal of Periodontology* **77**, 969–975.

Drummer, O. H. (2006) Drug testing in oral fluid. *Clinical Biochemist Review* **27**, 147–159.

Ellis, J. S., Morgan, C. L., Kirby, J. A., Taylor, J. J. & Thomason, J. M. (2004) Plasma TGF-beta1 as a risk factor for gingival overgrowth. *Journal of Clinical Periodontology* **31**, 863–868.

Friskopp, J. & Klintmalm, G. (1986) Gingival enlargement. A comparison between cyclosporine and azathioprine treated renal allograft recipients. *Swedish Dental Journal* **10**, 85–92.

Giese, T., Zeier, M. & Meuer, S. (2004a) Analysis of NFAT-regulated gene expression in vivo: a novel perspective for optimal individualized doses of calcineurin inhibitors. *Nephrology, Dialysis, Transplantation* **19** (Suppl. 4), iv55–iv60.

Giese, T., Zeier, M., Schemmer, P., Uhl, W., Schoels, M., Dengler, T., Buechler, M. & Meuer, S. (2004b) Monitoring of NFAT-regulated gene expression in the peripheral blood of allograft recipients: a novel perspective toward individually optimized drug doses of cyclosporine A. *Transplantation* **77**, 339–344.

Hartel, C., Fricke, L., Schumacher, N., Kirchner, H. & Muller-Steinhardt, M. (2002) Delayed cytokine mRNA expression kinetics after T-lymphocyte costimulation: a quantitative measure of the efficacy of cyclosporin A-based immunosuppression. *Clinical Chemistry* **48**, 2225–2231.

Hefti, A. F., Eshenaur, A. E., Hassell, T. M. & Stone, C. (1994) Gingival overgrowth in cyclosporine A treated multiple sclerosis patients. *Journal of Periodontology* **65**, 744–749.

James, J. A., Marley, J. J., Jamal, S., Campbell, B. A., Short, C. D., Johnson, R. W., Hull, P. S., Spratt, H., Irwin, C. R., Boomer, S., Maxwell, A. P. & Linden, G. J. (2000) The calcium channel blocker used with cyclosporin has an effect on gingival overgrowth. *Journal of Clinical Periodontology* **27**, 109–115.

Keown, P., Landsberg, D., Halloran, P., Shoker, A., Rush, D., Jeffery, J., Russell, D., Stiller, C., Muirhead, N., Cole, E., Paul, L., Zaltzman, J., Loertscher, R., Daloze, P., Dandavino, R., Boucher, A., Handa, P., Lawen, J., Belitsky, P. & Parfrey, P. (1996) A randomized, prospective multicenter pharmacoepidemiologic study of cyclosporine microemulsion in stable renal graft recipients. Report of the Canadian Neoral Renal Transplantation Study Group. *Transplantation* **62**, 1744–1752.

Khoori, A. H., Einollahi, B., Ansari, G. & Moozeh, M. B. (2003) The effect of cyclosporine with and without nifedipine on gingival overgrowth in renal transplant patients. *Journal of the Canadian Dental Association* **69**, 236–241.

King, G. N., Fullinfaw, R., Higgins, T. J., Walker, R. G., Francis, D. M. & Wiesenfeld, D. (1993) Gingival hyperplasia in renal allograft recipients receiving cyclosporin-A and calcium antagonists. *Journal of Clinical Periodontology* **20**, 286–293.

Koal, T., Deters, M., Casetta, B. & Kaever, V. (2004) Simultaneous determination of four immunosuppressants by means of high speed and robust online solid phase extraction-high performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences* **805**, 215–222.

Konstandin, M. H., Sommerer, C., Doesch, A., Zeier, M., Meuer, S. C., Katus, H. A., Dengler, T. J. & Giese, T. (2007) Pharmacodynamic cyclosporine A-monitoring: relation of gene expression in lymphocytes to cyclosporine blood levels in cardiac allograft recipients. *Transplant International* **20**, 1036–1043.

Lima, R. B., Benini, V. & Sens, Y. A. (2008) Gingival overgrowth in renal transplant recipients: a study concerning prevalence, severity, periodontal, and predisposing factors. *Transplantation Proceedings* **40**, 1425–1428.

Levey, A. S., Bosch, J. P., Lewis, J. B., Greene, T., Rogers, N. & Roth, D. (1999) A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of diet in renal disease study group. *Annals of Internal Medicine* **130**, 461–470.

Margiotta, V., Pizzo, I., Pizzo, G. & Barbaro, A. (1996) Cyclosporin- and nifedipine-induced gingival overgrowth in renal transplant patients: correlations with periodontal and pharmacological parameters, and HLA-antigens. *Journal of Oral Pathology and Medicine* **25**, 128–134.

McGaw, T., Lam, S. & Coates, J. (1987) Cyclosporin-induced gingival overgrowth: correlation with dental plaque scores, gingivitis scores, and cyclosporin levels in serum and saliva. *Oral Surgery, Oral Medicine, Oral Pathology* **64**, 293–297.

Mendonza, A., Gohh, R. & Akhlaghi, F. (2004) Determination of cyclosporine in saliva using liquid chromatography-tandem mass spectrometry. *Therapeutic Drug Monitoring* **26**, 569–575.

Modeer, T., Wondimu, B., Larsson, E. & Jonzon, B. (1992) Levels of cyclosporin-A (CsA) in saliva in children after oral administration of the drug in mixture or in capsule form. *Scandinavian Journal of Dental Research* **100**, 366–370.

O'Leary, T. J., Drake, R. B. & Naylor, J. E. (1972) The plaque control record. *Journal of Periodontology* **43**, 38.

Pernu, H. E., Pernu, L. M., Knuuttila, M. L. & Huttunen, K. R. (1993) Gingival overgrowth among renal transplant recipients and uraemic patients. *Nephrology Dialysis Transplantation* **8**, 1254–1258.

Radwan-Oczko, M., Boratynska, M., Klinger, M. & Zietek, M. (2003) Risk factors of gingival overgrowth in kidney transplant recipients treated with cyclosporine A. *Annals of Transplantation* **8**, 57–62.

Rao, A., Luo, C. & Hogan, P. G. (1997) Transcription factors of the NFAT family: regulation and function. *Annual Review of Immunology* **15**, 707–747.

Rateitschak-Plüss, E. M., Hefti, A., Lortscher, R. & Thiel, G. (1983) Initial observation that cyclosporin-A induces gingival enlargement in man. *Journal of Clinical Periodontology* **10**, 237–246.

Romito, A. G., Pustiglioni, F. E., Saraiva, L., Pustiglioni, A. N., Lotufo, R. F. M. & Stolf, N. A. G. (2004) Relationship of subgingival and salivary microbiota to gingival overgrowth in heart transplant patients following cyclosporin A therapy. *Journal of Periodontology* **75**, 918–924.

Ross, P. J., Nazif, M. M., Zullo, T., Zitelli, B. & Guevara, P. (1989) Effects of cyclosporin A on gingival status following liver transplantation. *SDC Journal of Dentistry for Children* **56**, 56–59.

Seymour, R. A. (2006) Effects of medications on the periodontal tissues in health and disease. *Periodontology* **2000**, 40##120–129.

Seymour, R. A., Ellis, J. S. & Thomason, J. M. (2000) Risk factors for drug-induced gingival overgrowth. *Journal of Clinical Periodontology* **27**, 217–223.

Seymour, R. A. & Smith, D. G. (1991) The effect of a plaque control programme on the incidence and severity of cyclosporin-induced gingival changes. *Journal of Clinical Periodontology* **18**, 107–110.

Seymour, R. A., Smith, D. G. & Turnbull, D. N. (1985) The effects of phenytoin and sodium valproate on the periodontal health of adult epileptic patients. *Journal of Clinical Periodontology* **12**, 413–419.

Söderling, E. (1989) Practical aspects of salivary analyses. In: Tenovuo, J. O. (ed). *Human Saliva: Clinical Chemistry and Microbiology*, 1st edition, pp. 1–24. Boca Raton: CRC Press Inc..

Somacarrera, M. L., Hernandez, G., Acero, J. & Moskow, B. S. (1994) Factors related to the incidence and severity of cyclosporin-induced gingival overgrowth in transplant patients. A longitudinal study. *Journal of Periodontology* **65**, 671–675.

Sommerer, C., Giese, T., Meuer, S. & Zeier, M. (2009) Pharmacodynamic monitoring of calcineurin inhibitor therapy: is there a clinical benefit? *Nephrology, Dialysis, Transplantation* **24**, 21–27.

Sommerer, C., Giese, T., Schmidt, J., Meuer, S. & Zeier, M. (2008a) Ciclosporin A tapering monitored by NFAT-regulated gene expression: a new concept of individual immunosuppression. *Transplantation* **85**, 15–21.

Sommerer, C., Hartschuh, W., Enk, A., Meuer, S., Zeier, M. & Giese, T. (2008b) Pharmacodynamic immune monitoring of NFAT-regulated genes predicts skin cancer in elderly long-term renal transplant recipients. *Clinical Transplantation* **22**, 549–554.

Sommerer, C., Konstandin, M., Dengler, T., Schmidt, J., Meuer, S., Zeier, M. & Giese, T. (2006) Pharmacodynamic monitoring of cyclosporine a in renal allograft recipients shows a quantitative relationship between immunosuppression and the occurrence of recurrent infections and malignancies. *Transplantation* **82**, 1280–1285.

Thomas, D. W., Newcombe, R. G. & Osborne, G. R. (2000) Risk factors in the development of cyclosporine-induced gingival overgrowth. *Transplantation* **69**, 522–526.

Thomason, J. M., Seymour, R. A. & Ellis, J. S. (2005) Risk factors for gingival overgrowth in patients medicated with ciclosporin in the absence of calcium channel blockers. *Journal of Clinical Periodontology* **32**, 273–279.

Thomason, J. M., Seymour, R. A., Ellis, J. S., Kelly, P. J., Parry, G., Dark, J., Wilkinson, R. & Ilde, J. R. (1996) Determinants of gingival overgrowth severity in organ transplant patients. An examination of the role of HLA phenotype. *Journal of Clinical Periodontology* **23**, 628–634.

Thomason, J. M., Seymour, R. A. & Rice, N. (1993) The prevalence and severity of cyclosporin and nifedipine-induced gingival overgrowth. *Journal of Clinical Periodontology* **20**, 37–40.

Tyldesley, W. R. & Rotter, E. (1984) Gingival hyperplasia induced by cyclosporin-A. *British Dental Journal* **157**, 305–309.

Vescovi, P., Meleti, M., Manfredi, M., Merigo, E. & Pedrazzi, G. (2005) Cyclosporin-induced gingival overgrowth: a clinical-epidemiological evaluation of 121 Italian renal transplant recipients. *Journal of Periodontology* **76**, 1259–1264.

Wysocki, G. P., Gretzinger, H. A., Laupacis, A., Ulan, R. A. & Stiller, C. R. (1983) Fibrous hyperplasia of the gingiva: a side effect of cyclosporin A therapy. *Oral Surgery, Oral Medicine, Oral Pathology* **55**, 274–278.

Address:
Priv.-Doz. Dr. med. dent. Bettina Dannewitz
Department of Conservative Dentistry
Section of Periodontology
Clinic for Oral, Dental and Maxillofacial Diseases
University Hospital Heidelberg
Im Neuenheimer Feld 400, 69120 Heidelberg
Germany
E-mail: bettina_dannewitz@med.uni-heidelberg.de

Clinical Relevance

Scientific rationale of the study: Pharmacokinetic parameters are not reliable prognostic indicators for CsA-induced GO due to the highly variable bioavailability of CsA. Nuclear factor of activated T cells (NFAT)-regulated gene expression as a specific pharmacodynamic parameter displays directly the biologic effect of CsA and allows estimation of the individual immunosuppressive responsiveness to the drug. Association of GO and residual NFAT activity has not been evaluated so far.
Principal findings: Extend of GO correlates highly significantly with residual NFAT activity. Residual NFAT activity, gingival inflammation and salivary CsA concentration explain up to 77% of the variability of GO expression.
Practical implications: Residual NFAT activity, inflammatory status of the gingival and salivary CsA-level could be used by clinicians to identify patients on CsA with increased risk for GO.