

Host and Microbiological Factors Related to Dental Caries Development

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Key Words

Atraumatic restorative treatment · Caries, general health · Caries experience, children

Abstract

Studies on dental caries suggest that in severe cases it may induce a systemic immune response. This occurs particularly when caries progresses into pulpal inflammation and results in abscess or fistula formation (AFF). We hypothesized that severe dental caries will affect the general health of children. The acute phase proteins alpha-1-acid glycoprotein (AGP), C-reactive protein (CRP) and the cytokine neopterin were chosen as parameters to monitor general health. Also, a polymorphism in the bacterial ligand CD14 (-260) was studied to investigate the relationship between genotype sensitivity for bacterial infections and AFF. In Suriname, children aged 6 years were recruited and enrolled into a dental care scheme, randomly assigned to 4 groups with different treatment strategies and monitored longitudinally. 348 children were included in the present study. Blood and saliva samples were taken at baseline and 1 year, and concentrations of serum AGP, CRP, neopterin, salivary *Streptococcus mutans* and CD14-260 C>T polymorphism were determined. There was no significant association between different treatment strategies and the serum parameters. Binary logistic regression analyses revealed a significant association between AFF as

the outcome variable and the CD14 genotype and the concentrations of CRP and of neopterin as factors ($p < 0.05$). A significant negative association was found between the CD14-260 TT and AFF ($p = 0.035$, OR = 3.3) for the whole population. For children who had 4 or more carious lesions at baseline, the significance increased ($p = 0.005$, OR = 4.8), suggesting that the CD14-260 TT genotype was protective for AFF as a consequence of dental caries.

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Dental caries is a multifactorial disease in which bacteria play an essential role [Beighton, 2005]. Among the large number of bacterial species present in dental plaque, *Actinomyces* spp., *Streptococcus mutans* and lactobacilli have been positively associated with dental caries [Corby et al., 2005].

Recently, the question whether severe dental decay has consequences for general health has been raised. It has been suggested that tooth decay in the primary dentition may lead to growth retardation, which may be explained by reduced food intake due to rampant caries [Ayhan et al., 1996; Acs and Ng, 2002; Thomas and Primosch, 2002]. An association between the acute phase protein alpha-1-acid glycoprotein (AGP) and the number of decayed, missing and filled teeth (dmft) in young Indonesian children has been reported [de Soet et al., 2003]. This obser-

vation suggests that severe dental caries may induce a systemic immune response that may especially occur when caries progresses into pulpal inflammation and results in abscess or fistula formation (AFF) [Skogedal and Tronsstad, 1977; Duggal et al., 2002]. This finding raises the question whether other acute-phase proteins or infection-related serum factors, such as C-reactive protein (CRP) and neopterin, can be found in increased concentrations in subjects with severe dental caries. The rationale to include neopterin is that this cytokine is an indicator for cell-mediated immune activation. It is released by macrophages and monocytes and is found at an increased concentration in viral infections and systemic inflammatory diseases [Shaw, 1991; Andert and Muller, 1995; Buchwald et al., 1997; Cesur, 2005]. In this context, neopterin can be used as a marker to monitor general health. The contribution of the host itself in caries development should also be taken into account.

Dental caries progression and severity have been linked in twin studies to genetic traits [Bretz et al., 2005]. In such studies, a genetic component in sugar preference has been proposed to play a role in caries susceptibility [Bretz et al., 2006]. In experiments with mice, it has been shown that chromosomal loci, possibly correlated with salivary composition and immunity, are associated with caries susceptibility [Nariyama et al., 2004; Culp et al., 2005].

It has been reported that the response to *Streptococcus mutans* is a Th1 response, resulting in elevated concentrations of interferon- γ , interleukin-10 and activation of CD8(+) T cells [Hahn et al., 2004]. In pulpal lesions, a mixed Th1/Th2 response has been found [Kim and Lim, 2002]. Gene-expression studies have shown that a range of inflammatory factors are up-regulated during infection of the pulp due to caries [Hahn and Liewehr, 2007c]. These cytokines may be responsible for the pulpal reaction to the infection, which may lead to AFF. Since in severe caries, a Th2 response is eminent, it is to be expected that mediators of the Th2 response determine the immune reaction [Zehnder et al., 2003; Adachi et al., 2007; Hahn and Liewehr, 2007a, b]. The molecule CD14 is an immune factor that is responsible for modulating Th1/Th2 responses [Baldini et al., 2002; Kedda et al., 2005]. These immune factors play an essential role in the etiology of chronic multifactorial diseases such as Crohn disease and periodontitis [Tetley, 2005; Pietruska et al., 2006; Balfour, 2007; de Sa et al., 2007]. CD14 is a coreceptor for Toll-like receptors and binds bacterial cell wall components, such as lipopolysaccharide from Gram-negative bacteria, and lipoteichoic acid (LTA) and pep-

tidoglycan (PGN) from Gram-positive bacteria [Cuzola et al., 2000; Flo et al., 2000]. The CD14:LTA and CD14:PGN complexes can bind to Toll-like receptor 2 on host cells, which leads to nuclear factor- $\kappa\beta$ pathway activation resulting in tumor necrosis factor- α and interleukin-1 production.

Different gene polymorphisms have been reported for CD14, including a polymorphism at -260 in the promoter region of the gene. This polymorphism involves replacement of a cytosine by a thymidine, resulting in transcription up-regulation of the gene. It has been found that CD14-260:T heterozygotes (CT) and homozygotes (TT) produce more cell-bound and soluble CD14 [Amar et al., 2004; Shimada et al., 2004; LeVan et al., 2006]. The TT genotype has been found more frequently in severe periodontitis [Laine et al., 2005] and in *Helicobacter pylori*-related gastritis [Zhao et al., 2007].

Given the above-mentioned systemic and genetic involvements in chronic diseases, such as dental caries, the hypothesis of this study was that caries treatment improves general health, which will result in reduced levels of the acute-phase proteins CRP and AGP, which in turn influences levels of serum neopterin. Furthermore, it was hypothesized that individuals with the T-allele of the CD14-260 gene are more sensitive to AFF as a result of severe caries.

Patients and Methods

Experimental Design

The present study was carried out in the interior of Suriname between 2002 and 2005. The study population consisted initially of 490 primary-school children aged 6 years, who were randomly assigned into 4 treatment groups. A power analysis showed that 69 children per group were needed to determine a significant clinical effect [Van Gemert-Schriks et al., 2008]. Based on an earlier study on AGP in Indonesian children, this group size is large enough to detect differences in acute-phase proteins, when applicable [de Soet et al., 2003]. The participating schools were selected from the database of the Medical Mission. Children participated in the study when they (1) were 6 years of age at the start of the study and (2) did not show a medical history of heart diseases, diabetes, hepatitis or other serious systemic chronic diseases, according to the database of the Medical Mission. Ethical clearance was obtained from the director of the Suriname Ministry of Health. Parents had to approve participation of their child by a signed letter of consent.

Oral examination was carried out by one investigator, calibrated with a gold standard (κ value 0.89) [Van Gemert-Schriks et al., 2007]. Caries was recorded according to the criteria from the World Health Organization [1987]. The prescribed decayed, missing and filled teeth (DMFT) index for caries prevalence was used for the primary and secondary dentitions. A tooth was con-

Table 1. Primers and probes used for specific TaqMan PCR for *S. mutans*

Gene	DNA oligo	Sequence
GTF	forward	5'-GCCTACAGVTCAGAGATGCTATTCT-3'
	reverse	5'-GCCATACACCACTCATGAATTGA-3'
	probe	5'-TGGAAATGACGGTCGCCGTTATGAA-3'
LDH	forward	5'-GCGACGCTCTTGATCTTAGTCA-3'
	reverse	5'-GATGGCAAGATTTTTACCAACGA-3'
	probe	5'-TGATAACAACAAGGTCAGCATCCGCACAG-3'

sidered sound if it showed no clinical evidence of treated or untreated dentine caries and decayed if any lesion in a pit or fissure or on a smooth tooth surface, had a detectable softened floor, undermined enamel or softened wall. A tooth was considered present in the mouth when any part of it was visible or could be touched with the tip of the dental probe without unduly displacing soft tissue. If a secondary and a primary tooth occupied the same tooth space, the status of the permanent tooth was recorded. AFF was recorded as the number of abscesses or fistulae in the whole mouth due to caries activity.

During the first visit, children received different treatments, as described by van Gemert-Schriks et al. [2008]. The children were evaluated after 6 months, 1, 2 and 3 years. At each visit, oral parameters (caries experience and signs of dentogenic infection) were recorded and dental treatment was performed upon indication. Furthermore, at baseline and at the evaluation visits, each child's body length and weight were determined and the body mass index was calculated (weight/length²).

Treatment

The children were allocated into 5 treatment groups [van Gemert-Schriks et al., 2008]. Briefly, a control group (group 1) consisted of all children that were clinically caries free at the start of the study (2002). Children in group 2 received full dental treatment of their primary dentition, including restoration of small carious lesions according to the atraumatic restorative treatment (ART) method [Frencken et al., 1996]. Extraction was performed for teeth with deep carious lesions in which pulp exposure was likely or where visible signs of dentogenic infection (complaints of pain, or abscess or fistula formation) were present. In group 3, the carious teeth with pulpal exposure were extracted. Children in this group did not receive any restorative care. Children in group 4 received ART of cavities that did not show pulpal involvement, while deep carious lesions were left untreated. Children in group 5 received neither restorative treatment nor extraction of carious primary teeth. In all groups, cavities in permanent molars were restored according to the ART approach or extracted when caries had progressed into the dental pulp. When a child reported dental pain, the tooth concerned was treated by extraction, irrespective of the treatment group. At the end of the study, all decayed teeth were treated.

Isolation of Serum

Blood was obtained at baseline and 1 year after treatment by finger puncture. Blood was collected in a capillary tube, coated with heparin and cells were separated from serum by gravitation for 2 h. The tubes were kept frozen (-20°C) and transported on

dry ice to Amsterdam, The Netherlands. The concentration of AGP was determined as described previously [de Soet et al., 2003]. The concentration of CRP was determined by an immunometric assay using the Immulite system (Diagnostic Products Corp., Holliston, Mass., USA). The concentration of neopterin was determined using a commercial kit (IBL, Mediphos, Renkum, The Netherlands), according to the manufacturer's instructions.

CD14-260 C>T Genotyping

To determine the -260 C>T genotype of the *CD14* gene (rs 2569190), a few drops of blood were collected onto a Whatman FTA card (Fisher Emergo, Landsmeer, The Netherlands). In Amsterdam, the DNA was eluted from the cards and the *CD14*-260 genotype was determined using a specific restriction fragment length polymorphism PCR [Laine et al., 2005; Ouburg et al., 2005]. Briefly, specific primers (5'-CCTGCAGAATCCTTCCTGTT-3' and 5'-TCACCTCCCCACCTCTCTT-3') for the -260 promoter region of the *CD14* gene were added to a PCR mix. The PCR was performed using a PE9700 PCR cycler (Applied Biosystems, Foster City, Calif., USA) with the following conditions: 5 min at 95°C, directly followed by 35 cycles of 30 s at 95°C, 30 s at 59°C and 1 min at 72°C, and finally 7 min at 72°C. The amplicons were enzymically digested by *Hae*III, resulting in an 83-bp amplicon for *CD14*-260:C and a 106-bp amplicon for *CD14*-260:T. The CC, CT and TT genotypes were made visible by standard 2% agarose gel-electrophoresis.

Detection of Salivary *S. mutans*

Saliva samples were collected at baseline by Sarstedt-Salivette (Sarstedt, The Netherlands) through active chewing on polyester rolls for 1 min. Rolls were transported at -20°C to Paramaribo, Suriname's capital, and on dry ice to Amsterdam where the saliva was isolated by centrifugation. DNA was extracted with a MagnaPure (Roche Molecular Diagnostics, Almere, The Netherlands) using a standard protocol with DNA Isolation Kit III [Boutaga et al., 2006]. The concentration of salivary *S. mutans* cells was determined by species-specific real-time PCR for glucosyl transferase and for lactate dehydrogenase, using the PCR primers and probes listed in table 1. The probes were labeled with FAM as reporter and TAMRA as quencher.

The PCR was performed as described earlier [Boutaga et al., 2005]. Briefly, real time PCR amplification was performed in a total reaction mixture volume of 25 µl. The reaction mixtures contained 12.5 µl of 2× TaqMan universal PCR master mix [PCR buffer, dNTPs, AmpliTaq Gold, reference signal (6-carboxy-X-rhodamine), uracil *N*-glycosylase, MgCl₂; Applied Biosystems], 300–900 nM of the specific primer, 50–100 nM of the

Table 2. Patient characteristics for the 5 different treatment groups

	Time, years	Control	Full treatment	Extraction only	ART filling only	No treatment
Number		53	75	66	77	77
Female, %		53	45	42	49	56
<i>S. mutans</i> , log ₁₀ CFU/ml	0	4.94 ± 0.81 ^a	5.14 ± 0.83 ^a	5.12 ± 0.82 ^a	5.18 ± 0.75 ^a	5.20 ± 0.73 ^a
dmft	0	0 ^a	6.56 ± 3.90 ^b	6.78 ± 3.15 ^b	5.78 ± 3.19 ^b	7.12 ± 3.49 ^b
DMFT	3	2.87 ± 2.94 ^{a,*}	7.46 ± 3.34 ^b	7.00 ^a ± 3.03 ^b	7.26 ± 3.28 ^{b,*}	6.58 ^a ± 3.51 ^b
AFF, cumulative n	0–3	1 ^a	19 ^b	23 ^c	37 ^c	40 ^c

Data are means ± SDs, unless otherwise stated.

Within rows, means with the same superscript letter are not significantly different (Kruskal-Wallis).

* $p < 0.05$ between baseline and 3 years (Wilcoxon).

Table 3. Mean ± SD of blood parameters at baseline and 1 year in the 5 different treatment groups

	Year	Control	Full treatment	Extraction only	ART filling only	No treatment	Total
AGP, mg/l	0	0.76 ± 0.26	0.79 ± 0.24	0.74 ± 0.25	0.80 ± 0.26	0.74 ± 0.20	0.77 ± 0.24
	1	0.70 ± 0.19	0.76 ± 0.23	0.68 ± 0.21	0.68 ± 0.21	0.72 ± 0.20	0.71 ± 0.21
CRP, nM	0	0.19 ± 0.36	0.22 ± 0.34	0.13 ± 0.23	0.56 ± 0.53	0.14 ± 0.34	0.21 ± 0.39
	1	0.24 ± 0.35	0.18 ± 0.30	0.32 ± 0.80	0.12 ± 0.21	0.17 ± 0.21	0.19 ± 0.39
Neopterin, nM	0	3.03 ± 2.07	2.72 ± 1.75	3.88 ± 2.11	2.37 ± 1.81	2.01 ± 1.29	2.68 ± 1.85
	1	2.19 ± 1.44	1.59 ± 1.18	3.12 ± 1.87	2.01 ± 1.33	1.38 ± 0.83	1.95 ± 1.42

specific probe and 5 µl of purified DNA from plaque samples. The samples were subjected to an initial amplification cycle of 50°C for 2 min and 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min, using an ABI 7000 TaqMan PCR system and its Sequence Detection System software (Applied Biosystems).

For quantification, the results from salivary samples were related to a standard curve constructed with pure-culture dilutions of *S. mutans*.

Statistical Analyses

Bacterial counts (colony-forming units/ml saliva) were converted to log₁₀. The presence of AFF was dichotomized for the presence or absence of AFF during the 3 years of the study.

Differences between the groups and between the different treatment periods were analyzed using an ANOVA with a Bonferroni correction. For data that were not normally distributed, as shown by a Kolmogorov-Smirnov test, a Kruskal-Wallis test or Wilcoxon signed-rank test for pairs was used.

For logistic regression analysis, the continuous data sets for serum proteins were dichotomized, based on the median: higher than the median was noted as 1 and lower than the median was 0. Caries experience was calculated as dmft + DMFT at baseline and at the end of the study (3 years) and dichotomized with a cut-off of 4.

The *S. mutans* counts were dichotomized with a cut-off of log₁₀ colony-forming units/ml saliva of 5.5. These data were used for further analysis.

For associations between the different serum proteins, *S. mutans* counts, *CD14* genotype and clinical data, logistic regression analyses were used by SPSS v15. To explore the associations between AFF and the *CD14* genotype serum proteins, a Fisher exact test was used. Results for Fisher exact tests are presented as p value, odds ratio (OR) and 95% confidence interval (CI). The correlation between caries experience and AFF was tested using the Pearson correlation coefficient.

In all tests, the level of significance was set at $p < 0.05$.

Results

The patient population in which all parameters were tested was 348. 142 children were excluded because they could not attend 1 or more visits of the dental team after the baseline measurement due to illness, moving from the village or any other absence from school.

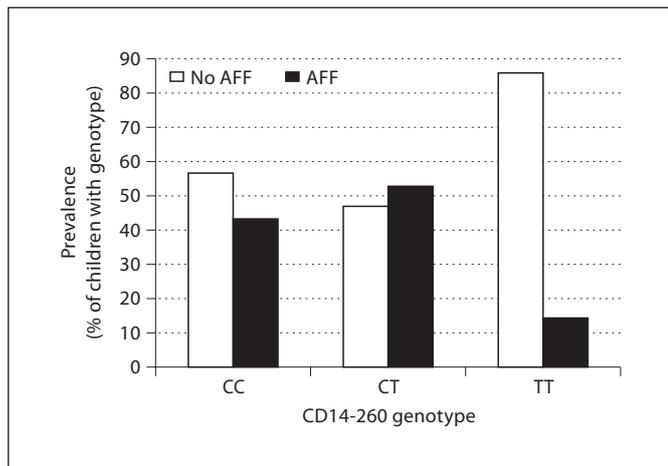


Fig. 1. The proportion of children who developed AFF during the 3-year study period, as a percentage of the *CD14-260* C>T genotype in the group of children who had a dmft + DMFT >3 at baseline.

The salivary concentration of *S. mutans* varied between 10^4 and 10^6 cells/ml saliva, with no significant differences between the groups (table 2). Caries experience was significantly lower in the control group than in the other groups ($p < 0.001$), both at baseline and after 3 years (table 2). In the ART group, the total caries experience increased significantly during the 3 years ($p < 0.001$; Wilcoxon). The association between type of treatment and clinical parameters is published elsewhere [Van Gemert-Schriks et al., 2008]. In 120 patients, AFF was observed as a result of severe caries including pulpal involvement. Caries experience at the different evaluations was significantly correlated with AFF, i.e. children with high total caries experience scores showed AFF more often (Pearson $r = 0.39$; $p < 0.006$). Only the caries-free controls and the full-treatment group showed significantly lower AFF scores than the nontreatment group ($p < 0.002$; OR 27.5, CI 6.3–121 and OR 3.2, CI 1.6–6.3, respectively; table 2).

No statistical differences in blood concentrations of AGP, CRP and neopterin between the treatment groups were observed, either at baseline or 1 year after baseline (table 3). None of these variables was correlated with caries experience at baseline, 1 and 3 years, or with BMI, gender, age or AFF.

Analyses of the frequency in *CD14-260* C>T genotypes over the whole population of 348 children revealed that the CC genotype was found most frequently (54.3%) while the TT genotype was the lowest in prevalence

(7.8%). The CT genotype was found in 37.9%. The allele frequency for this population was 73.3% for the C allele and 26.7% for the T allele. The *CD14-260* genotype distribution of the study population was in Hardy-Weinberg equilibrium.

The serum values of AGP, CRP and neopterin were dichotomized as follows: a high value was AGP >0.71 mg/l, CRP >0.0 nM and neopterin >1.61 nM.

A binary logistic regression analysis using AFF during the study period as the dependent variable revealed no significant association with gender, BMI, *S. mutans* counts at baseline or caries experience. CRP ($p = 0.002$; OR 2.2, CI 1.3–3.7), neopterin ($p = 0.003$; OR 2.7, CI 1.4–5.2) and *CD14* ($p = 0.039$, OR 3.2, CI 1.1–9.6) were significantly associated with AFF.

In figure 1, the prevalence of AFF in relation to the *CD14-260* genotype is shown. The prevalence of AFF was significantly lower in the TT genotype of *CD14-260* ($p = 0.033$; OR 3.25). This association was stronger in a subgroup of children who had caries experience >3 at baseline ($p = 0.005$; OR 4.8, CI 1.4–15.8).

Discussion

High salivary counts of *S. mutans* have been reported as indicative of an increased caries risk [Beighton et al., 2005; Colby et al., 2005]. This could not be confirmed in the present study. Even the initially caries-free controls had large numbers of salivary *S. mutans*. This is possibly due to the high sugar content and low pH of the diet consumed in these areas (there is abundant availability of sugar cane and fresh fruits). These data are not in contrast to what has been found in similar studies in Suriname, where almost all children tested were positive for cultured *S. mutans* and the colonization level was not associated with caries experience. Differences between this study and our previous study in 2002 are due to the tested population (inland rural vs. urban), age and caries incidence [de Soet et al., 2002].

The blood concentrations of AGP, CRP and neopterin were not significantly different between the 5 treatment groups or between baseline and 1 year.

The reason for measuring AGP and CRP was the previous observation where we found a relationship between the acute-phase protein AGP and caries in caries-active children in Indonesia [de Soet et al., 2003]. This was found in a population with a high proportion of dental caries (mean dmft at 6 years 8.8 ± 1.9). In the present population, the mean total caries experience at 6 years

(5.5 ± 4.0) was significantly lower ($p < 0.001$; ANOVA), which may explain the lack of association in the present study. We used neopterin as a marker to monitor the health of the children after dental treatment. All values are relatively low compared to other infectious diseases [Ip et al., 2007]. We could not find significant differences in the measured serum factors between the treatment groups, or between baseline and 1 year after initial treatment. We thus conclude that, using the serum factors CRP, AGP and neopterin, differences in general health due to dental treatment could not be established in this Suriname population. It is unlikely that these low levels have been caused by the transportation of the samples since they were transported frozen (at or below -20°C) and the factors measured were selected on the basis of their stability in epidemiological studies [Hartweg et al., 2007]. Based on the large variation in total caries experience at baseline (range 0–18), we suggest that different subpopulations may exist. In the present study, however, we could not define these subpopulations on the bases of any of the tested parameters. We should also keep in mind that the study population has not been well studied at the genetic level, which may account for differences with previous studies.

In the present study, we reported a *CD14* C-allele frequency of 73.3%. This is higher than those found for two Caucasian populations (54.0% by Eilertsen et al. [2003] and 57.3% by Laine et al. [2005]) or that found for a mixed population (45.3% by de Sa et al., [2007]). However, we must note that the current study involves a Creole population that moved 2–3 centuries ago from Africa to Suriname. The C-allele frequency in African populations is between 72 and 85%, which is similar to the level we found [Zambelli-Weiner et al., 2005; Barber et al., 2007].

Taking AFF as a sign of severe exacerbation of caries outcome, we found CRP, neopterin, total caries experience and *CD14*-260 C>T to be involved with AFF conditions. These observations show systemic involvement as a result of severe caries in a group of individuals. It was found that the caries experience was associated with AFF, which is to be expected since AFF is not likely to develop without caries lesion formation [Duggal et al., 2002].

An interesting finding was the correlation between the presence of the *CD14*-260 TT genotype and AFF. This correlation was stronger in a subgroup of children who had a relatively high number of carious lesions at baseline (>3), which is to be expected since children with a low caries activity will develop fewer abscesses or fistulae.

It has been observed for multifactorial chronic inflammatory diseases, such as Crohn's disease and periodontitis, that abscesses were significantly associated with a genetically based up-regulation of the CD14 receptor [Laine et al., 2005; Pietruska et al., 2006; Balfour, 2007].

The results of the present study suggest that the presence of *CD14*-260:TT is protective for AFF. CD14 is a molecule that is associated with complex immune modulating systems. Immune modulation may be the result of either an up-regulation or a down-regulation of CD14. Infectious diseases correlated with an up-regulation of CD14 are often due to an overproduction of cytokines that are typically associated with this genotype [Pietruska et al., 2006]. In the present study, we observed protection in relation to AFF with this genotype.

Gram-positive cell-wall products such as LTA and PGN bind less strongly to CD14, and the role of this complex has been studied than its equivalent in Gram-negative bacteria [Temple et al., 2003; Sutherland et al., 2005].

Because dental caries is associated with Gram-positive *S. mutans*, we suggest that inflammation of the dental pulp with the Gram-positive is inhibited by an up-regulation of immune factors, such as CD14, resulting in a faster clearance of the bacterial products.

In conclusion, based on the serum factors studied in this paper, we conclude that general health is not significantly affected by dental caries treatment in Creole children. Children who have the genotype *CD14*-260:TT are genetically protected in relation to the formation of abscesses or fistulae as a consequence of severe dental caries.

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