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Correlation of Mutans Streptococci Bacteriocin Genes to Children's Caries Status

Author
Markova-Mitev, Iveta

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Correlation of Mutans Streptococci Bacteriocin Genes to Children's Caries Status

by

Iveta Sashkova Markova-Mitev, DDS

THESIS

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ABSTRACT

Purpose: to assess whether single or combinations of three novel antimicrobial peptide genes (*NN2, PNS1, TKS1*) of mutans Streptococci (MS) were associated with dental caries in 2-5 year old children.

Methods: Eighty-two caries-free (CF) and 37 severe early-childhood caries (S-ECC) 2-5 year-old children were recruited in a pilot and current studies. Caries status was recorded utilizing the International Caries Detection and Assessment System. Oral MS levels were enumerated from oral swab by selective culture. In the pilot study, MS were isolated from each subject with the genes identified by high-throughput illumina sequencing. In the current study, the genes were identified by SYBR green qPCR assays. The prevalence of the single gene or gene combinations were compared by Chi Square test/Fisher's exact test between CF and S-ECC group and Odds Ratios were calculated. LogMS levels and decayed surfaces in subjects with or without the single gene or combination of the genes were compared by Student t and non-parametric tests.

Results: Thirty-nine CF and 36 S-ECC subjects had MS infection. The prevalence of any of the three genes was 21% in CF and 58% in S-ECC. In the individual gene analysis, *PNS1* was the most commonly detected gene (38%) and was significantly more prevalent in S-ECC than in the CF group while *NN2* and *TKS1* were less prevalent (16% and 19% respectively) and showed no statistically significant difference in prevalence between S-ECC and CF groups. In analysis of combinations of any two or all three genes, the prevalence of all gene combinations with *PNS1* were significantly higher in the S-ECC group combination ($P<0.05$) with the highest Odds ratio in *PNS1 – TKS1* (9.17). MS levels between subjects with or without any single gene or combinations of genes were not significantly different ($P>0.05$). However, mean decayed surfaces were significantly higher in the presence of the genes or gene combinations except for *NN2+TKS1*.

Conclusion: *PNS1* and combination of *PNS1* with *NN2* and/or *TKS1* may contribute to caries development and risk. Future studies are needed to study the mechanism of these genes' function to develop tools for caries prediction and prevention.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Dental caries is the most prevalent chronic disease in children in the U.S. (1). Although the general trend is for the prevalence of untreated carious primary teeth to be on the decline in the U.S., children aged 2 to 5 years who are from low socioeconomic status still have a high percentage of untreated severe early childhood caries (S-ECC) (2,3). Distribution of dental caries in children is skewed (4) in that 7% of children account for 80% of the decay load in primary teeth (5).

Dental Caries is an infection disease that results from interactions of acidogenic/aciduric bacteria heavily colonizing the tooth surface and the oral environment. The bacterial group of Mutans Streptococci (MS) is one of the major etiological agents of dental decay (6,7). The MS group includes the species *S.mutans* and *S. Sobrinus*, each of which appear often in human dental plaque biofilms. The critical time for MS colonization in children is from birth to 3 years although it can last lifelong (7,8). Infants can be infected with MS before tooth eruption but MS colonization increases with increasing age (7). By 3 years of age, up to 84% of children could experience MS colonization (7,8,9). MS are readily acquired through vertical transmission from mother to child especially at that early stage (10), however, horizontal transmission from siblings and playmates may also occur among children (11,12). Children with early MS colonization are at higher risk for caries than those who had later MS colonization or no colonization at 4 years of age (12). Therefore, screening of MS has been suggested as a part of caries risk assessment in children (13).

However, MS levels alone are not the accurate predictor of future caries lesions because it yields highly variable specificity and sensitivity (ranging between 27-93%). A significant portion of caries-active children (up to 40%) could have low oral MS levels and about 30% caries-free (CF)

children could have high MS levels (14,15,16). Therefore, in addition to MS levels, MS virulence may also be evaluated to improve the accuracy of caries risk assessment in children. Further, the virulence of MS is also directly related to any mechanism that enables MS to become the dominant species within the supragingival plaque that leads to caries initiation (17,18).

Microbiology studies on MS virulence have shown that, certain virulence factors such as biofilm formation, acidogenicity, acid tolerance, and bacteriocin activity, may play a role in determining whether the microbiota will be cariogenic or not (19,20). Bacteriocins are protein or peptides produced by bacteria that kill or inhibit the growth of similar or closely related bacterial strains and may play a significant role in *S. mutans* virulence. Bacteriocins produced by MS are named 'mutacins' (21, 22). Two types of mutacins have been characterized: the lantibiotics and the non-lantibiotics. The wide spectrum lantibiotics may consist of a single peptide, such as mutacins I, II, III, 1140, B- NY266, and K8 (encoded by MutR gene) or two peptides such as mutacin Smb (exact genes unknown). The narrow spectrum non-lantibiotics mutacins are much more prevalent among strains of *S. mutans* which play a significant role in both community-level and population-level interactions in the dental biofilm. Four of them have been characterized mutacins IV, V, VI, and mutacin N and the genes that encode for them are ComE, HdrR, BrsR (23). Mutacins are regulated complex system involving multiple genetic and environmental factors that frequently overlap (23). Further, several studies have identified some evidence of mutacin genes that are related to caries activities or bacterial transmission (22,29). Isolates that had a broad inhibitory spectrum against multiple species also produced larger inhibition zones than isolates that inhibited fewer strains (22). Moreover, MS isolates that produced mutacins against several closely related strains and yielded bigger inhibition zones were more easily

transmitted to their children than were isolates producing mutacins against fewer strains with smaller inhibition zones (29). However, there are limited published studies that account for all virulence factors due to the inter-strain diversity and complexity of MS genotype (19). Therefore, sequencing of the whole genome of MS may help to systemically screen genes coding specific virulence factors of MS (24).

Preliminary Study:

Dr. DeRisi's laboratory at UCSF has recently developed a barcoded high-throughput Illumina Genomic Sequencing system which reduces the genome sequencing price significantly from approximately $10,000 per sample to approximately $50 per sample. This advancement has enabled Ling Zhan and co-workers to conduct full-genome sequencing of 85 MS isolates from 43 children with or without caries, funded by U54 DE 019285 (CANDO center grant) from NIDCR. This approach allowed for systematic evaluation of MS genes that are critical for virulence of MS to induce dental caries. The results indicated that complex biosynthetic gene clusters (BGCs), assumed to be mutacin genes, constitute the largest variable genetic component among the MS isolated from children with or without caries (accounts for 50-70% of variation). In these clusters of the potential antimicrobial genes, 14 genes (12 of them were novel) were detected in *S. mutans* and 4 genes (3 of them were novel) were detected in *S. sobrinus*. Five genes in MS (*NN2*, *PNS1*, Mut III, *TKS1* and Lan SMB genes) were associated with the child's caries status, MS bacteriocin activity and MS cariogenicity. *PNS1* and Mut III genes (a known mutacin) were only present in MS isolates from S-ECC children while the presence of *NN2*, LanSMB and *TKS1* genes were more likely to be found in MS from CF children. In addition, the presence of the *PNS1* and Mut III genes was associated with stronger mutacin activity against *S. sanguinis*, a commensal bacterial species in dental plaque that is associated with good oral health

and suppresses the over population of MS on the tooth surface.

Therefore, they hypothesized that some of these small molecules may play an important role in modulating MS interaction with other health oral commensal bacteria to determine the stability and over population of MS in dental plaque to induce caries formation. To prove their hypothesis, they have generated a *PNS1* knock-out strain and studied its ability in producing dental caries in an in vitro caries model. They found that *PNS1* knock-out resulted in a decrease of MS in the biofilm and also resulted in decreased demineralization (caries formation) on bovine enamel when co-cultured with *S. sanguinis* (Figure 1A and 1B). However, knock-out of *PNS1* did not affect the cariogenicity of MS in mono-culture (Figure 1B). These results indicate that *PNS1* regulates MS cariogenic virulence by modulating its competition to *S. sanguinis*. Although the data is very preliminary it indicated the potential of using *PNS1* gene to guide the need for continuation or initially starting an antimicrobial regimen. Until now, they have generated knockout strains for *NN2* gene and a serious knockout of a strain with both *PNS1* and *TKS1*. Similarly, the *in vitro* caries model indicated that the knockout of these genes alone does not alter the cariogenicity of this *S. mutans* strain.

Dr. Zhan et al. has identified a specific primer pair (MSB2-1 and MSB2-2) for qPCR that can effectively identify MS from saliva samples and yield high correlation with conventional selective culture method (Pearson correlation $r=0.74$, $p< 0.001$, funded by U54 DE 019285 pilot grant from NIDCR). These primer pairs can be used together with the virulence genes for fast identification of total MS levels in saliva.

The above data indicates that the presence of antimicrobial genes have shown potential correlation with caries formation and may play a critical role in determining the dominance of MS in dental plaque in competition with other oral commensal bacteria. However, the initial

research results are very underpowered and cannot conclusively verify the results. A larger sample size is needed to determine the validity of the preliminary study results.

AIMS AND HYPOTHESIS

We hypothesize that the presence of single or combination of the three novel genes (*NN2*, *PNS1*, and *TKS1*) of MS in saliva will be related to caries status of children aged 2 to 5 years old.
The aim of the current study is to assess the feasibility and proof of concept of screening of three of the antimicrobial peptide genes of MS in oral swab samples of children with S-ECC or CF by qPCR and to determine whether the presence or the levels of individual or combinations of the antimicrobial peptide genes are related to the caries status in 2 to 5 year old children.

METHODS

This was a cross sectional cohort study. The research protocol was approved by the UCSF Committee on Human Research (Approval number 12-10366). Informed consent was obtained from the guardians of all study subjects. Seventy nine subjects (62 caries free children and 17 children with caries) were recruited from the UCSF Pediatric Dental clinic from March to November 2016. In addition, forty subjects (20 caries free and 20 children with caries) who were recruited previously by Dr. Zhan in the preliminary study were added to the current study to increase sample size.
The inclusion criteria of both groups included 2-5 year old healthy children who registered as a patient at the UCSF Pediatric Dentistry Clinic. For S-ECC group, children were required to meet the definition of S-ECC by the American Association of Pediatric Dentistry. The CF children group, no clinically detectable white spot or cavitated caries lesions and no restorations within the past 2 years were required.

Exclusion criteria for both groups were children with chronic systemic diseases, periodontal disease, or history of use of antibiotics or other medications that might affect oral flora or salivary flow in the past 3 months.

The following information was collected by a questionnaire: age, gender, race/ethnicity, and demographic data, together with a standard caries risk assessment form. Dental caries scores were collected using the International Caries Detection and Evaluation System (ICDAS) scoring system for each child by the principle investigators. A cotton swab saliva sample was collected at the beginning of the appointment before any treatment was initiated. The saliva sample was transported on ice for microbiological assays within 24 hours. Selective culture on Mitis-Salivarius-Bacitracin agar (MSSB) was used to confirm MS presence, followed by DNA extraction and the qPCR assay, which screened for the presence of *NN2*, *PNS1*, and *TKS1* genes.

Sample Size Calculation:

In the preliminary study conducted by Zhan et al., 40 children aged 4-5 year old were recruited (20 each with ECC or CF); however, only one-third of the CF children had detectable MS. Therefore, it was planned to use a 1:3 sample size ratio for children with ECC and CF in the current study to provide adequate power. Given the presence of the *PNS1* gene as 31% in the ECC group and 0.6% in the CF group at a two sided alpha=0.0125 and power=80%, a sample size of 18 children with severe ECC and 56 CF children will be needed to detect a significant difference in the proportion of children with the gene between ECC and CF children based on Fisher's exact test. It was proposed to have 20% extra-subjects to ensure the power of the study. Therefore, the total number of subjects will be 22 children with severe ECC and 67 CF children.

Saliva Collection, Microbiology Assays and Sample Storage:

Saliva samples were collected using a cotton swab prior to any dental treatment (25). The swab

was placed into a tube with 2.5 ml of PBS. Samples were stored on ice or at 4ºC and processed for culture within 24 hours. The swab sample was vortexed and 0.3 ml of the aliquot was use for enumeration of MS on Mitis Salivarius sucrose bacitracin (MSSB), Rogosa tomato juice plates for LB enumeration and Brain Heart Infusion blood plates for total viable bacterial enumeration (14). The plates were incubated anaerobically at 37°C for 72 hours before enumeration under a dissecting microscope (24).

The rest of the swab saliva sample was stored as follows: 1 ml of the swab saliva sample was saved in a labeled tube and 1 ml was placed in 40% Glycerol solution. Both samples were frozen in a -80˚ freezer for future qPCR assay screening. All MS colonies grown on MSSB medium at the lowest dilution were collected by cotton swab, dispersed in 1ml PBS, centrifuged to collect the cell pellet and frozen in a -80˚ freezer for future qPCR study.

DNA Extraction and qPCR Assays:

The DNA of the cell pellet from MS cells grown on MSSB agar of each sample was extracted using ZR Fungal/bacterial DNA MiniPrep™ kit and stored in -80˚ freezer until qPCR assays. The concentration of the DNA samples was measured by the Nano drop spectrometer. Specific primer sets for *PNS1*, *NN2*, and *TKS1* genes were used. The specificity and efficacy of the primer sets were verified using the MS genotypes with known full genome sequence. The levels of each gene in the ECC or CF children were evaluated using qPCR via the ABI SYBR green master kit. Each sample was run in duplicate against a standard curve generated by DNA samples from a known MS genotype with the test gene and negative control from DNA of known MS genotype in which the tested gene is absent. Each assay was run in 20µl volume with a template concentration of 1ng. The condition of the assay was at 50ºC for 2 mins, 95ºC for 10 mins; 40 cycles of 10s at 95 ºC, 1min at 55 ºC, 1min at 74ºC; and 1 cycle of dissociation phase:

15S at 95ºC, 15S at 60ºC with a ramp time of 19:59min to 15S at 95ºC in an ABI 7500 thermocycler.

Statistical Analysis:

The primary analysis compared the proportion of children who had single or any combination of the three genes between two groups with Chi square test/Fisher's exact test with Bonferroni correction and odds ratios were calculated for caries prediction based on the presence of the genes. Secondary analyses included testing bacterial levels and severity of decayed surfaces in subjects with or without the gene or gene combinations, analyzed by Student t test and ANOVA.

RESULTS

Subject Demographics, Bacterial Levels and Caries Status:

Figure 2. illustrates the study population and study design. The current study recruited seventy-nine subjects (CF n= 62 and S-ECC n=17). One subject did not meet the inclusion criteria and was excluded from the CF group because of 4 existing white spot lesions. Due to the time limit of the project only the proposed sample size was reached. To ensure power of the study, the pilot study subjects (n=40 total, n=20 of each group) were added. Therefore a total of one hundred and eighteen subjects were included with total CF n=82 and S-ECC n=36. Among them, 39 CF and 36 S-ECC subjects had MS infection and were included in the analysis for the MS gene study (Figure 2).

Summaries of subject demographics, bacterial levels and caries data of the two groups are presented in Table 2. The mean age of the study population was similar in both groups with an average of 3.4 years old for CF group and 3.6 years old for S-ECC. Although there were some differences in the gender and ethnicity distributions between CF and S-ECC groups, they are not statistically significantly different. Diverse race/ethnicity was observed among subjects residing

in the San Francisco Bay area in the CF group with a higher percentage of Asian (45%) and Hispanic (33%) populations in the S-ECC group. Log of MS and LB levels were higher in the S-ECC group in comparison to CF ($p<0.05$). The S-ECC group presented with a mean± SD of 12.8 (± 3.9) carious teeth and 34.2(±11.8) carious surfaces. None of the S-ECC subjects had any operative dental treatment that resulted in missing (due to caries) or filled surfaces. Therefore, we only used ds or dt in the data analysis.

Distribution of *NN2*, *PNS1* and *TKS1* in the Study Population:

Distributions of *NN2*, *PNS1* and *TKS1* in subjects are presented in Table 2. *PNS1* was the most commonly detected gene (in 13% CF and 41% of S-EEC groups respectively) that can present alone or co-exist with *NN2* and *TKS1*. *NN2* was less commonly found in the subjects (8% for CF and 20% for S-ECC) while *TKS1* gene always presented in conjunction with *PNS1* (10% for CF and 19% for S-ECC). Overall, the three genes were less frequently detected in the CF group (21%) in comparison to the S-ECC group (58%) in single existence or co-existence.

Prevalence of the Single Gene or Co-existence of Genes in CF and S-ECC Groups:

We first compared the presence of any single gene in CF and S-ECC (Figure 3). Since *TKS1* always presents with *PNS1*, it is illustrated as *PNS1+TKS1*. Although genes were generally more prevalent in the S-ECC group, only *PNS1* gene was significantly more prevalent in the S-ECC group than CF group (odds ratio = 4.32, Chi square test $p<0.05$).

Prevalence of the Combinations of Genes in CF and S-ECC Groups:

We also analyzed the prevalence of combinations of any two or all three genes in CF and S-ECC group (Figures 4-8). In this case, presence of any single gene or co-existence of two or three genes was all considered as positive for the analysis. When *PNS1* was combined with *NN2*, only one subject in S-ECC group had both genes. Either *NN2* or *PNS1* were present in 31% CF and

69% S-ECC (Figure 4) and the difference was statistically significant between the two groups (OR=4.33, p<0.05). Prevalences in the S-ECC group were also significantly higher for *PNS1* and *TKS1* (OR=4.33, p<0.05, Figure 5) and all three gene combinations (OR 4.31, P<0.05, Figure. 8). However, although the trend of prevalence of *NN2* and/or *TKS1* combination was higher in the S-ECC group than CF group, it was not statistically significant (OR=2.58, p =0.08, Figure 6). We also explored the *PNS1-TKS1* combination because *TKS1* gene showed no benefits for MS competition with commensal bacteria and its co-existence with *PNS1* gene also showed negative impact on *PNS1* function in our *in vitro* preliminary study. Therefore, *PNS1* gene present without *TKS1* gene may be more virulent and *PNS1-TKS1* combination will allow us to investigate the function of *PNS1* alone (Fig. 7). The prevalence of *PNS1* only in the S-ECC group was significantly higher than the CF group with the highest odds ratio in predicting S-ECC (OR 9. 17, p<0.05, 88% for S-ECC and 12% for CF).

MS Levels in Subjects with or without Any Single Gene or Gene Combinations:

We also studied the MS levels in the presence or absence of a single gene or gene combinations to explore whether certain gene or gene combinations are related to the over population of MS. Figures 9-10 depict logMS levels in absence or presence of any single gene or gene combinations. Even though we observed higher MS levels with the presence of *PNS1* and *PNS1+TKS1* genes, and lower MS levels in the presence of *NN2* gene, no statistical significance was found (p>0.05, Figure 9). MS levels in presence of the gene combinations were slightly higher but were not statistically significantly different (p>0.05, Figure 10).

Decayed Surfaces in Subject with or without Certain Single Gene or Gene Combinations:

Figures 11-12 demonstrate mean ds scores in subjects with presence or absence of a single or gene combinations, which represent caries severity. Subjects with *PSN1* gene (29.2±19.7,

p<0.001) and *PNS1+TKS1* (27.3±23.0, p<0.02) had significantly more ds than those without the genes (12.3±17.0 for *PSN1* and 14.0±18.1for *PNS1+TKS1*). However, no statistically significant differences on ds were found in subjects with or without *NN2* (18.7±16.5 vs 16.2±19.4 respectively, p>0.05, Figure 11). Subjects with presence of all gene combinations except for *NN2+TKS1* had consistently significantly higher ds than those who did not (p<0.05, Figure 12). The highest caries severity was observed in the presence of *PNS1* alone (*PNS1-TKS1* combination, 31.74±5.4), followed by *TKS1+PNS1* (29.2±19.7), *NN2+TKS1+PNS1* (25.6±19.4), *NN2+PNS1* (25.5±19.8), and *NN2+TKS1* (23.4±20.3).

DISCUSSION

In our study, we focused on examining the association of three novel antimicrobial peptide genes (*NN2*, *PNS1*, *TKS1*) of MS with dental caries in 2-5 year old children. The most striking result in our study was that the prevalence of the *PNS1* gene in S-ECC subjects in comparison to CF subjects was highly significantly different. This result supports the finding from the pilot study that MS with *PNS1* gene present suppressed the growth of the commensal organism *S. Sanguinis* and resulted in more demineralization in the *in vitro* artificial caries model (unpublished results). In addition, the preliminary data also indicated that MS with *NN2* gene present also suppressed *S. mutans* competition with *S. Sanguinis* and resulted in more demineralization compared to its knockout strains. However, the prevalence of *NN2* gene was low in MS strains. Hence, we did not find a significant difference in *NN2* between CF and S-ECC children although there was a trend.

Interestingly, the *TKS1* gene was never present alone and was always found in combination with the *PNS1* gene. Our preliminary *in vitro* study indicated that *TKS1* did not assist MS competition with *S. sanguinis* when co-existed with *PNS1*. To further evaluate the function of *PNS1* alone

and *TKS1*, we run the analysis for *PNS1-TKS1* combination, and we found the highest odds ratio of *PNS1* alone gene association with S-ECC compared to CF group. Therefore, the study results confirmed the preliminary study hypothesis of association of *PNS1* with caries as well as indicated that *TKS1* may not *be* related to dental caries. Hence, the sample size in the current study was calculated based on the presence of *PNS1* gene from the pilot study. Therefore, a larger sample size is required to confirm the results for *NN2* and *TKS1* genes.

Looking at gene combinations, we found that all subjects with the *TKS1* gene also have *PNS1* gene present, with significant difference in S-ECC group. Further, we compared the prevalence of *PNS1* by itself and the combination of *PNS1+TKS1*. Higher prevalence of *PNS1* alone was observed in S-ECC group in comparison when combined with *TKS1*.This finding leads to the conclusion that MS with *PNS1* gene alone is more likely to cause cavities in children than when it is combined with *TKS1*. In the pilot study, it was found that *TKS1* was present in the CF group, therefore *TKS1* may be associated with less virulent MS. Lastly, only one subject from S-ECC group had both *PNS1* and *NN2* genes together, which may lead to the conclusion that if this combination is present, MS might be very virulent. Further study is needed.

In addition to gene prevalence among S-ECC and CF groups, we also explored whether presence of the gene or gene combinations are related to MS levels. We did not find any significant difference when we compared the MS levels to the presence or absence of a single gene or gene combinations. That indicates that the *PNS1* gene may not affect MS colonization level but may influence the commensal bacterial level as we have shown in the in vitro caries model when co-cultured with *S. sanguinis*. The result supports the conclusion that bacteria levels alone are not sufficient enough when determining caries risk in children (16, 26). Since MS resides in a diverse, multispecies biofilm, there is constant competition among species for ecological niches.

(27). Therefore, understanding the specific function of the studied genes is important to establish the mechanism of virulence.

In our study, we looked at mean ds in single gene or gene combinations to establish the relationship between caries severity and the presence or absence of gene or gene combinations. There are statistically significant differences in caries severity (ds) and *PNS1*, *PNS1+TKS1*, and *PNS1-TKS1* and *PNS1+NN2* gene patterns. *PNS1* gene alone has shown the highest severity in caries, followed by *PNS1+TKS1* and *PNS1+NN2*, respectively. This result is supported by the proposed function of MS genes established by Zhan et al. in *in vitro* carious model. They found that *NN2* and *PNS1* are promoting MS competition with *S. sanguinis* and induce caries formation. In contrast, the *TKS1* gene did not promote MS competition with *S. sanguinis* and did not increase caries formation. In our study, *TKS1* always coexisted with *PNS1* and we see reduction in caries severity in comparison to the *PNS1* alone (*PNS1-TKS1*). No significant difference was found between *NN2* gene alone or with *NN2+TKS1* combination, which may have resulted from the low prevalence of these two genes.

The potential limitation to the study is the small sample size. The sample size was calculated based on *PNS1* gene, which was the most prevalent gene among all three genes. We were able to achieve significant results for *PNS1*, however, a larger sample size study is needed to further evaluate *TKS1* and *NN2* genes.

Since we know that non-lantibiotics mutacins are more prevalent in *S.mutans* (23), it is important to establish if the mutacins produced from the three novel genes belong to that group. Moreover, the relationship between the known genes ComE, HdrR, BrsR that encode the known mutacins IV, V, VI, and mutacin N (23) and the three novel genes need to be studied. Lastly, the mutacins encoded by the three novel genes and their functions need to be confirmed and inhibitory

spectrum of those genes need to be established to evaluate the ease of transmission to children and the effect on caries formation.

CONCLUSIONS

PNS1 and combination of *PNS1* with *NN2* and/or *TKS1* may contribute to caries development and risk. These genes may be used as a screening tool for caries risk assessment, especially *PNS1*. Further study with larger sample sizes will be needed to verify the presence and importance of these three novel genes. Moreover, the mechanism of these genes' function needs to be established in order to develop tools for caries prediction and prevention.

Table 1. Demographics for CF and S-ECC participants

	CF with MS n=39	S-ECC with MS n= 36	p Values
Age (years), mean±(SD)	**3.44 ±1.05**	**3.6 ±1.06**	**0.27**
Sex, n (female/male)	**26/13**	**17/19**	**0.07**
Race/Ethnicity, n (%)			**0.06**
Asian	**7(18%)**	**16(45%)**	
African American	**5(13%)**	**1(3%)**	
Latino/Hispanic	**10(26%)**	**12(33%)**	
Caucasian	**9(23%)**	**3(8%)**	
Other/No Answer	**8(20%)**	**4(11%)**	
Bacteria counts (log), mean ± (SD)			
Mutans Streptococci	**3.87±1.55**	**6.11±1.49**	**0.00**
Lactobacillus	**0.24±0.70**	**3.08±2.10**	**0.00**
Total Viable Bacteria	**7.46±0.50**	**7.94±1.53**	**0.04**
Caries, mean (SD)			
Decayed teeth (dt)*	**0**	**12.8 (3.9)**	**0.00**
Decayed surfaces (ds)= ds*	**0**	**34.2 (11.8)**	**0.00**

* No subjects had any restored or extracted teeth or surfaces in the S-ECC group. Therefore, only dt and ds were reported.

Table 2. Distribution of single gene and co-existence of genes in the study population

Gene or Gene combinations	CF n=39	S-ECC n=36	Total n=75
NN2 only	3 (8%)	5(17%)	8 (11%)
PNS1 only	1 (3%)	7(19%)	8 (11%)
NN2+PNS1	0 (0%)	1 (3%)	1 (1%)
PNS1+TKS1	4 (10%)	7(19%)	11 (14%)
Total	8 (21%)	20(58%)	28 (37%)

Figure 1. Biofilm composition and demineralization of bovine enamel in the artificial caries model

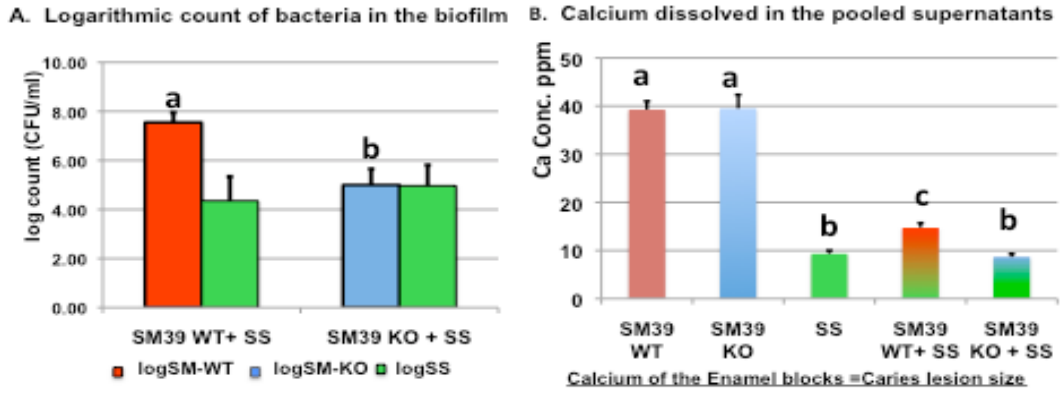


Figure 1 Biofilm composition and demineralization of bovine enamel in the artificial caries model

A. In co-culture of SM39 WT or PNS1 KO with *S. sanguinis* (SS), level of SM39 PNS1 KO significantly decreased creased compared to SM39WT.

B. The demineralization was comparable between the monoculture of MS3 WT or PNS1 KO. However, knock-out of PNS1 resulted in significant reduction in demineralization when co-cultured with *S. sanguinis* compared to MS3 WT.

(Groups with different letters of a, b, c are statistically significantly different from each other, $p<0.05$)

Figure 2. Study population and study design

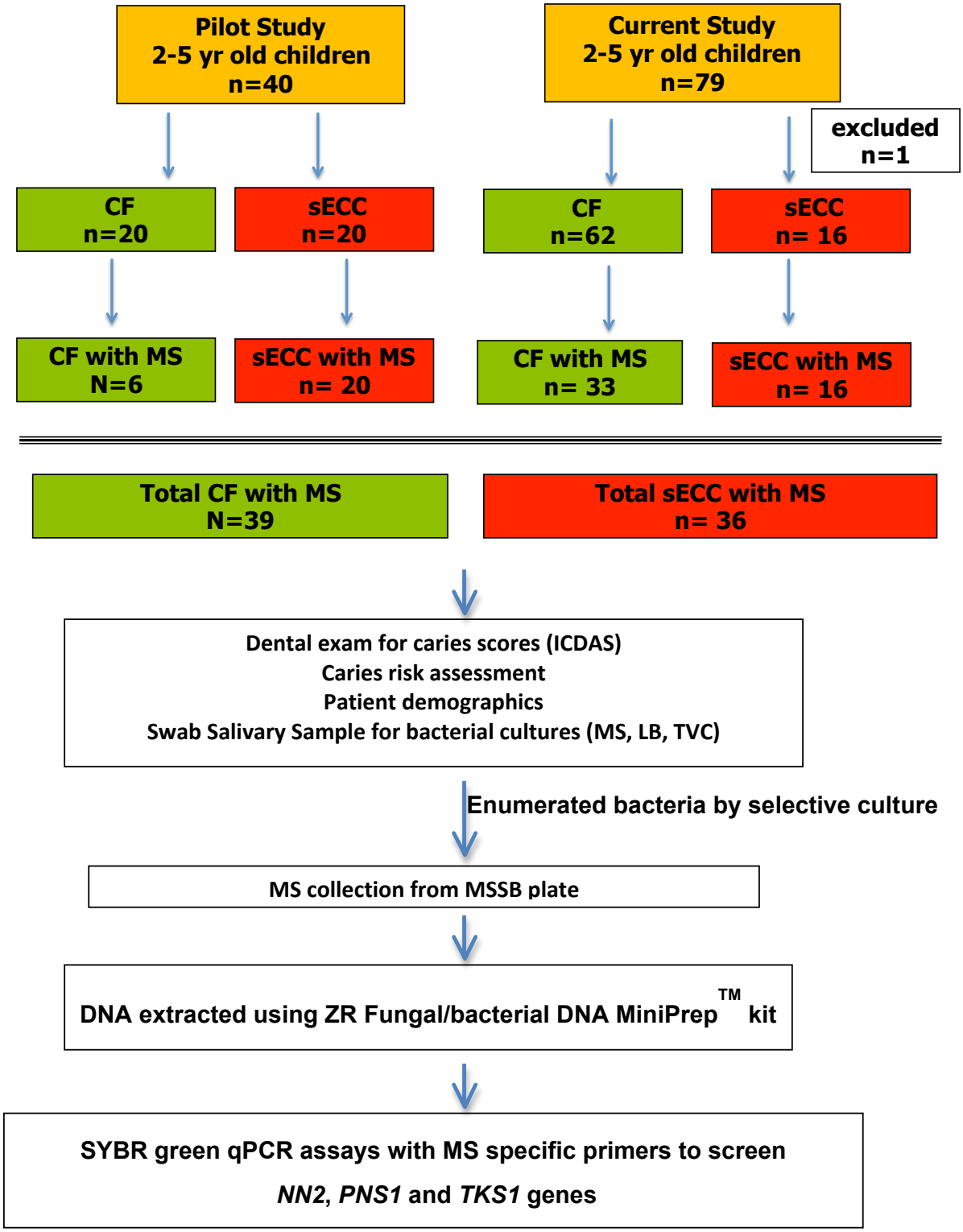
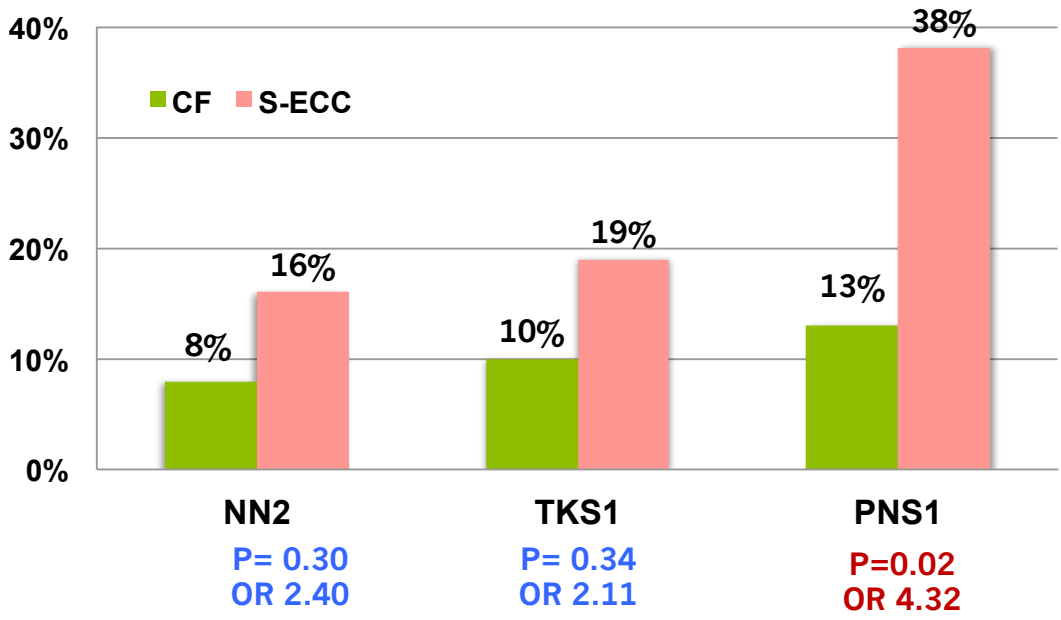


Figure 3. Prevalence of single gene (*NN2, TKS1* or *PNS1)* presence in CF and S-ECC groups



* Significant high percentage of *PNS1* gene was found in S-ECC group ($p<0.05$)

Figure 4. Prevalence of *NN2* and *PNS1* combinations in CF and S-ECC groups

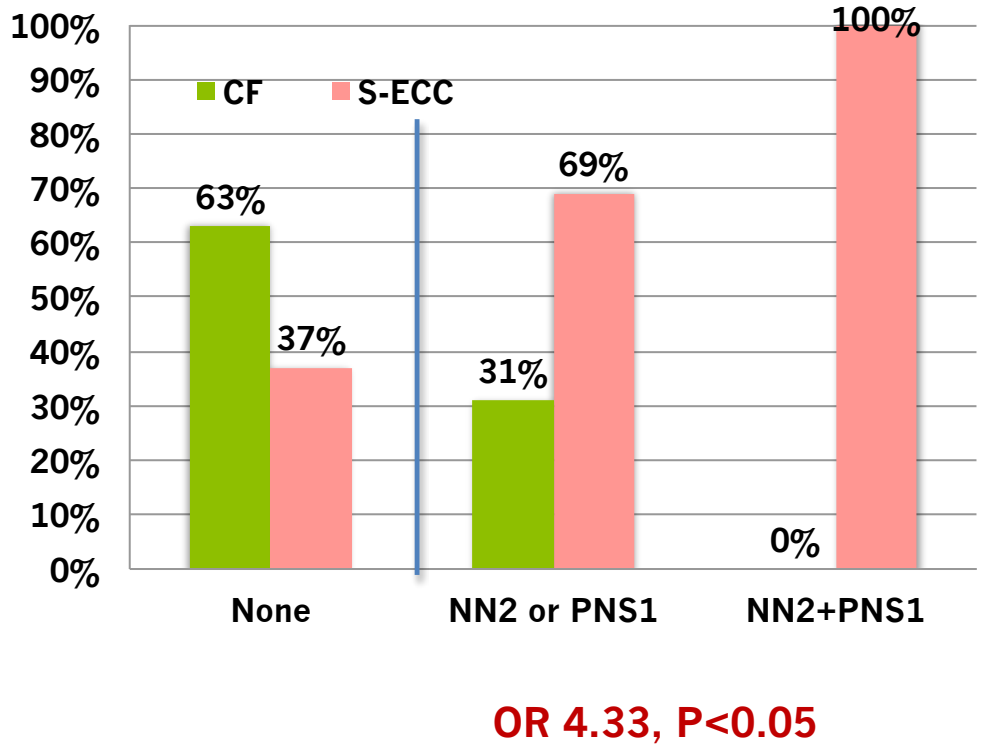


Figure 5. Prevalence of *PNS1* and *TKS1* combinations in CF and S-ECC groups

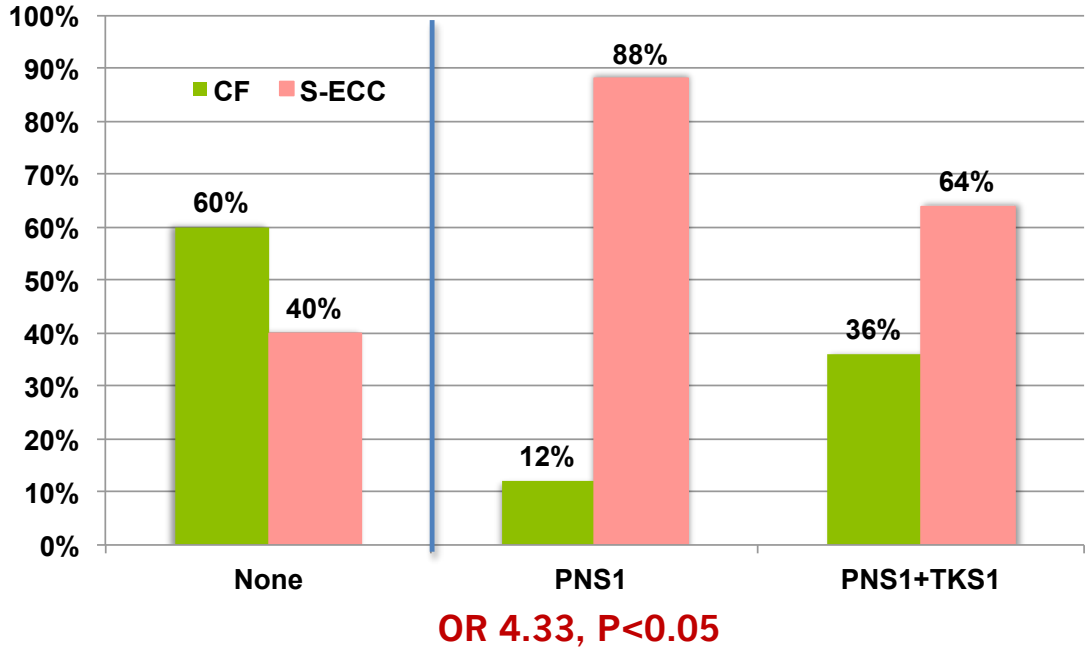
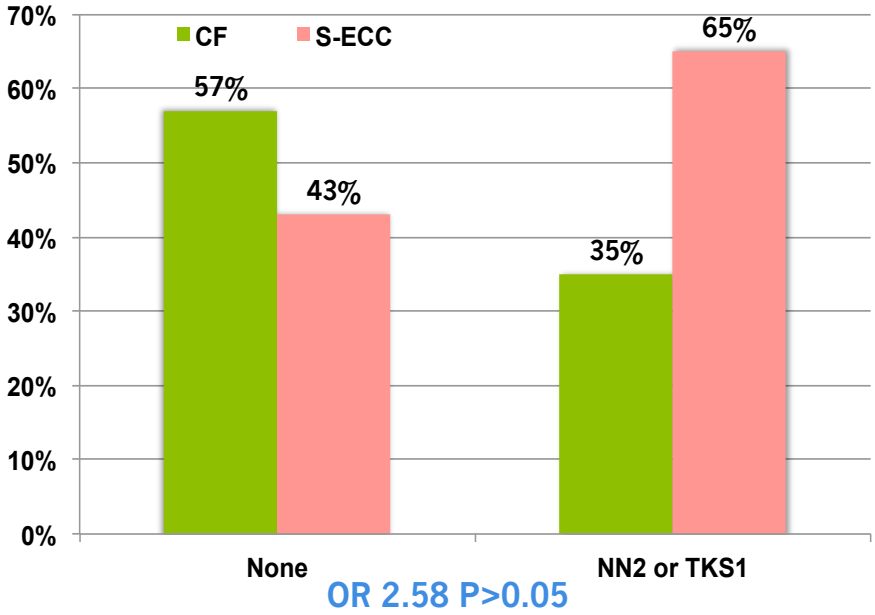
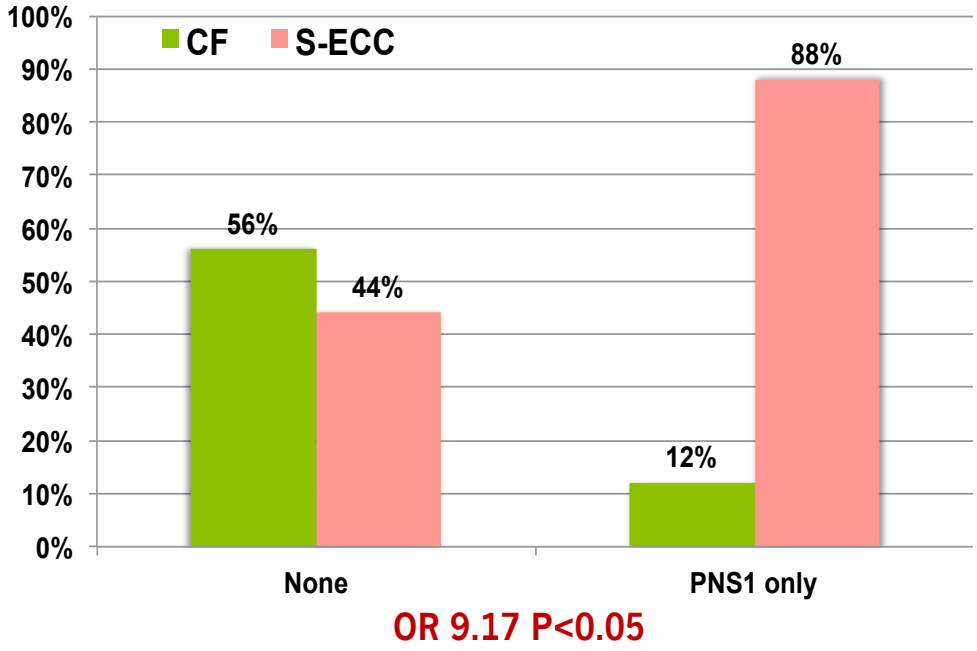


Figure 6. Prevalence of *NN2* or *TKS1* combinations in CF and S-ECC groups



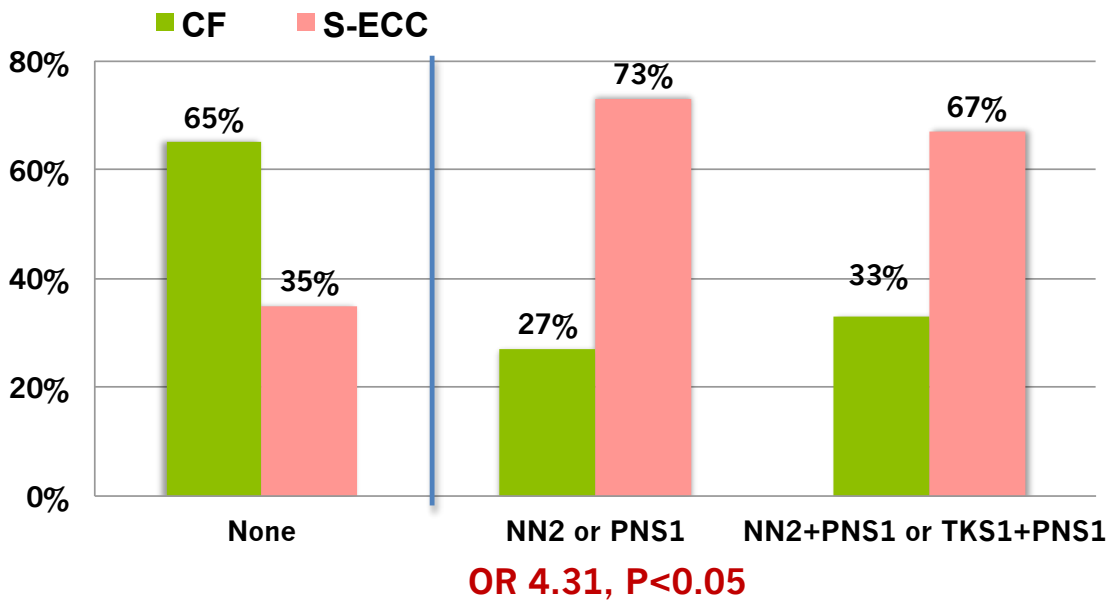
* *NN2 +TKS1* confirms the absence of both genes present together among subjects in both groups.

Figure 7. Prevalence of *PNS1-TKS1* (*PNS1* only) status in CF and S-ECC groups



* *TKS1* - *PNS1* gene combination confirms absence of *TKS1* gene present alone in both groups. (Student t test $p < 0.05$).

Figure 8. Prevalence of *NN2* and/or *PNS1* and/or *TKS1* combinations in CF and S-ECC groups



*All three genes (*NN2* and *PNS1* and *TKS1*) combination confirms the absence of all three genes present together among subjects in both groups.

Figure 9. Log MS levels in subjects with or without the gene (*NN2*, *PNS1* or *TKS1+PNS1*)

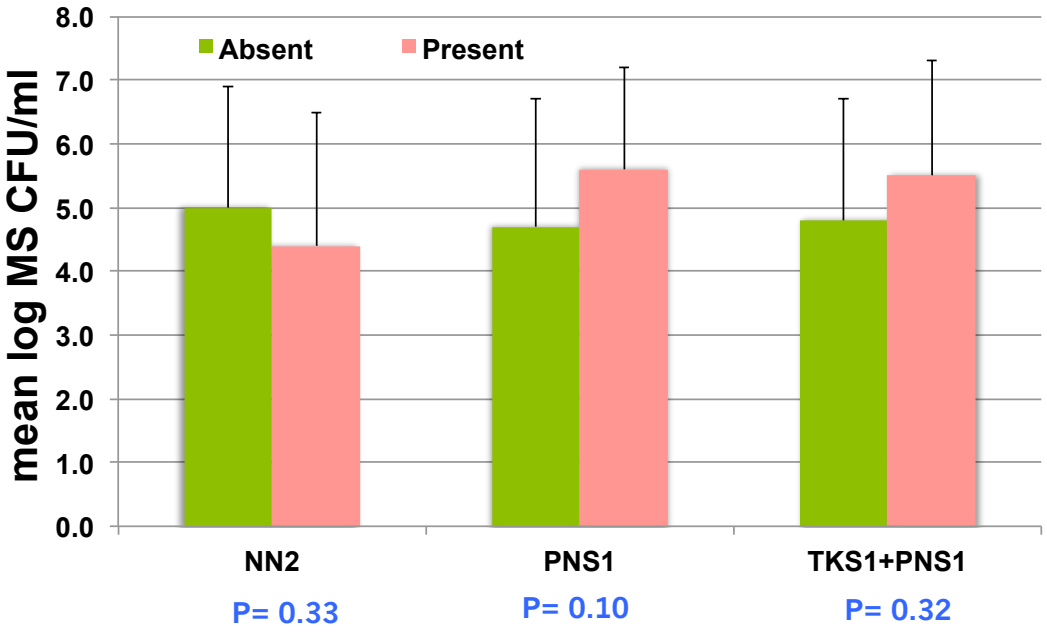


Figure 10. Log MS levels in subjects with or without certain gene combinations

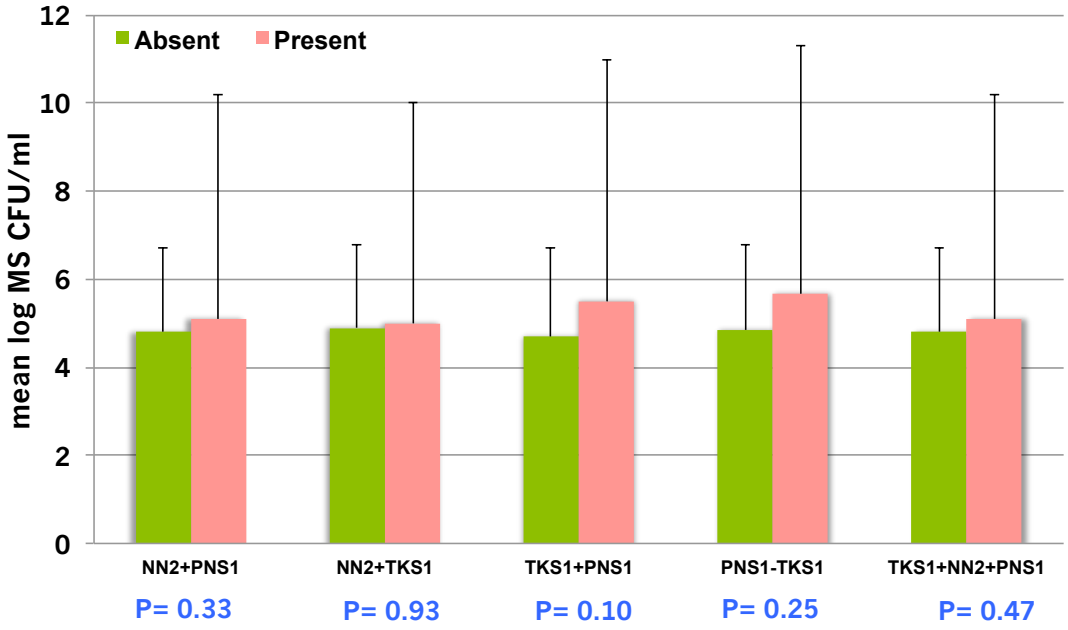


Figure 11. Mean ds levels in subjects with or without a single gene (*NN2*, *PNS1*, *TKS1*)

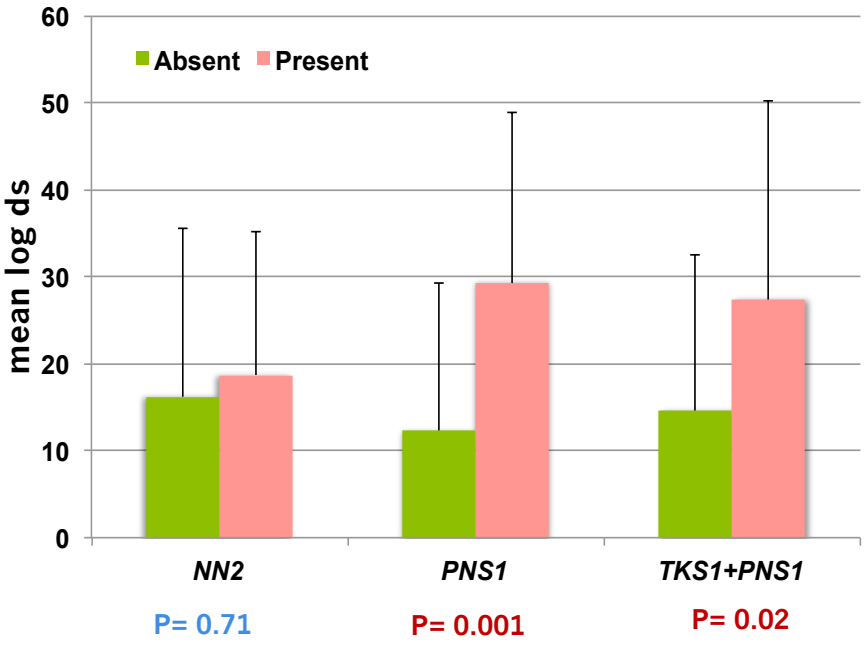
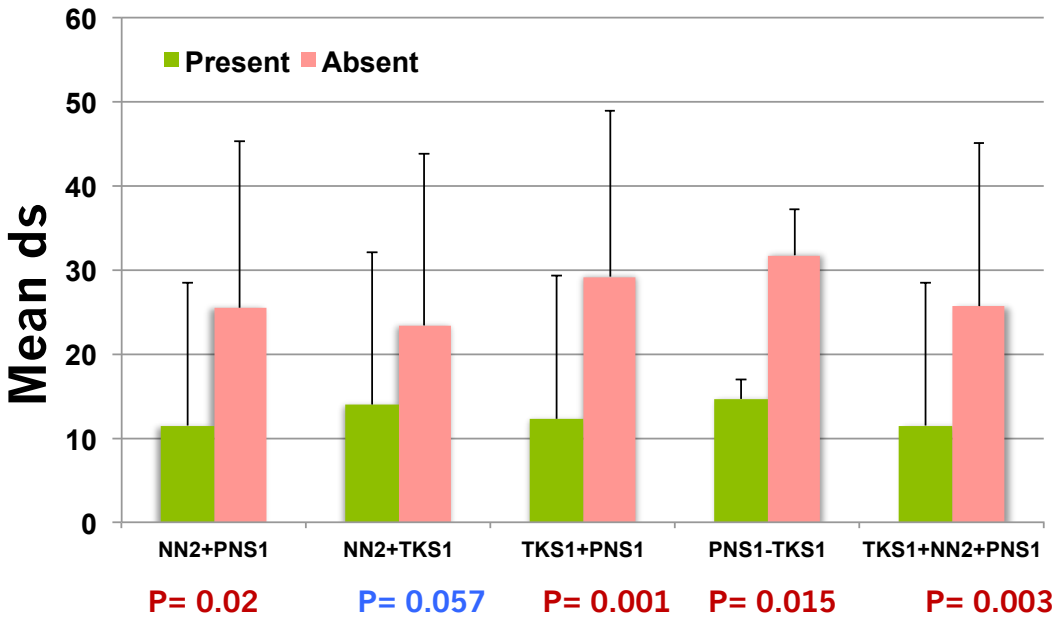


Figure 12. Mean of ds in subjects with or without certain gene combinations



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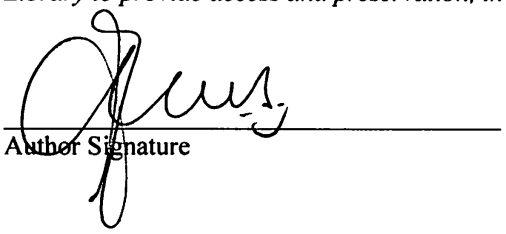

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