Serum Antibodies to *Porphyromonas gingivalis* Chaperone HtpG Predict Health in Periodontitis Susceptible Patients

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Abstract

**Background:** Chaperones are ubiquitous conserved proteins critical in stabilization of new proteins, repair/removal of defective proteins and immunodominant antigens in innate and adaptive immunity. Periodontal disease is a chronic inflammatory infection associated with infection by *Porphyromonas gingivalis* that culminates in the destruction of the supporting structures of the teeth. We previously reported studies of serum antibodies reactive with the human chaperone Hsp90 in gingivitis, a reversible form of gingival disease confined to the oral soft tissues. In those studies, antibodies were at their highest levels in subjects with the best oral health. We hypothesized that antibodies to the HSP90 homologue of *P. gingivalis* (HtpG) might be associated with protection/resistance against destructive periodontitis.

**Methodology/Principal Findings:** ELISA assays using cloned HtpG and peptide antigens confirmed gingivitis subjects colonized with *P. gingivalis* had higher serum levels of anti-HtpG and, concomitantly, lower levels of attachment loss. Additionally, serum antibody levels to *P. gingivalis* HtpG protein were higher in healthy subjects compared to patients with either chronic or aggressive periodontitis. We found a negative association between tooth attachment loss and anti-*P. gingivalis* HtpG (p = 0.043) but not anti-*Fusobacterium nucleatum* (an oral opportunistic commensal) HtpG levels. Furthermore, response to periodontal therapy was more successful in subjects having higher levels of anti-*P. gingivalis* HtpG before treatment (p = 0.018). There was no similar relationship to anti-*F. nucleatum* HtpG levels. Similar results were obtained when these experiments were repeated with a synthetic peptide of a region of *P. gingivalis* HtpG.

**Conclusions/Significance:** Our results suggest: 1) anti-*P. gingivalis* HtpG antibodies are protective and therefore predict health periodontitis-susceptible patients; 2) may augment the host defence to periodontitis and 3) a unique peptide of *P. gingivalis* HtpG offers significant potential as an effective diagnostic target and vaccine candidate. These results are compatible with a novel immune control mechanism unrelated to direct binding of bacteria.

Introduction

*Porphyromonas gingivalis* is a gram negative obligate anaerobe that has a major etiological role in human periodontitis. The bacterium is found with high frequency in persons with periodontitis where it participates in the initiation and establishment of chronic, infectious biofilms [1,2]. These biofilms facilitate the long term survival of *P. gingivalis* and induces an inflammatory reaction that is responsible for the destruction of the hard and soft tissue supporting structures of the teeth. In addition *P. gingivalis* can invade and persist in the cells of the gingival tissue[3]. It also can escape the oral cavity and has been found in atherosclerotic plaque where it may have a role in the pathophysiology of cardiovascular disease [4].

*P. gingivalis* produces a number of chaperones as essential tools in normal cellular processes and in response to environmental stresses [5]. In addition, the role of chaperones, like the *P. gingivalis* HSP90 homologue HtpG, in immune response dynamics has become an area of intense investigation because immunomodulation by chaperones has been demonstrated [6,7]. HtpG, like most chaperones tested [8], induces a strong humoral response that...
may have consequences in the pathogenesis of periodontitis [9]. These functions are important in the establishment and perpetuation of chronic inflammatory diseases.

Recent studies have shown that antibodies to periodontal disease associated pathogens may have protective effects although the exact mechanism is still unclear. Rams et al [10] demonstrated that serum levels of IgG antibodies against A. actinomycetemcomitans or P. gingivalis in periodontitis-stable patients were higher than those in patients with active periodontitis suggesting that these antibodies have a protective effect against periodontal infections. Yamasaki et al have shown that antibodies to Hsp60 homologues increases with successful treatment [11]. We have recently described experiments that indicate that antibodies to HtpG may mitigate some of the induction of inflammatory chemokines through TLR4 and CD91 [12], a receptor expressed in human atherosclerotic lesions [13]. Taken together, these findings suggest a role for antibodies to P. gingivalis' chaperones in both periodontal and cardiovascular disease.

The possible protective role of antibodies to chaperones in periodontitis is controversial. It has been suggested that these antibodies simply reflected the high level of homology between human and bacterial proteins, a hallmark of these evolutionarily conserved molecules [14]. Other results suggest that the conserved nature of the chaperones might lead to autoimmune phenomenon due to “immune mimicry”. Earlier results from this laboratory suggested that high levels of anti-Hsp90 antibodies could have protective qualities [9]. However, that study utilized a group of individuals with minimal periodontal disease. Here we describe findings from a study of periodontitis subjects with more extensive disease that are similar to those reported earlier and support the notion of a protective role for anti-HtpG antibodies in untreated subjects. In addition, these studies suggest that the levels of these antibodies also may predict the response to treatment. These results may also reflect an immune control mechanism in chronic infections unrelated to direct binding of bacteria.

Results

Colonization of plaque by P. gingivalis and F. nucleatum

Initially we reported [9] that in gingivitis subjects probing depth and the levels of P. gingivalis were both associated with serum anti-Hsp90 antibody levels. Subjects from the gingivitis group were tested for the presence of P. gingivalis by slot immunoblot; 82 % of the subjects were positive for P. gingivalis [15,16]. Genomic DNA of pooled plaque samples from all teeth for each subject in the CP and AP groups were tested; 71% of the healthy subjects and 86% of individuals with periodontitis were positive for P. gingivalis 16S rRNA genes by PCR. The low end sensitivity of the assay was between 10 and 100 bacterial cells per sample. The mean percentage of P. gingivalis was 0.11 and 0.90 (p<0.001, t-test) in the controls and periodontitis subjects respectively. Both CP groups were 96% positive for F. nucleatum; the mean percentage of F. nucleatum was 2.77 and 3.70 (p = 0.07; t-test) in the controls and periodontitis subjects respectively (Table 1). Plaque samples from the normal controls for the AP group were not available for F. nucleatum testing and as reported previously 17% of the AP subjects were positive for P. gingivalis 16S rRNA [17].

Antibodies to P. gingivalis and F. nucleatum total cell lysates

The level of antibodies to total cell lysates of P. gingivalis and F. nucleatum was determined as a measure of the long term colonization experience that each subject had with these bacteria. These two bacteria have different roles in periodontitis, P. gingivalis as a pathogen while F. nucleatum is an opportunistic commensal in the subgingival biofilm. Anti-P. gingivalis antibodies were detected in 30 of 43 samples obtained from gingivitis subjects. All CP and AP subjects had levels of anti-P. gingivalis and anti-F. nucleatum more than 3 standard deviations above the background of the assay. In the CP group there was a statistically significant elevated levels of antibodies to P. gingivalis (p = 0.01, t-test) and F. nucleatum (p = 0.02, t-test) in the diseased subjects compared to the control group; there was no difference between levels of antibody to total P. gingivalis in the AP group (Table 2). Similar results were found in CP subjects that were colonized by P. gingivalis (Table 3) and CP subjects not colonized by P. gingivalis (Table 4) at the time the serum samples were obtained. These results suggest that most subjects in the study were likely to have been colonized with P. gingivalis prior to their participation in this study.

Total IgG Antibodies to P. gingivalis HtpG and F. nucleatum HtpG

Levels of IgG-class antibodies that specifically reacted with recombinant HtpG proteins of P. gingivalis and F. nucleatum were measured. Anti-HtpG antibody levels were measured in gingivitis subjects; subjects with more CAL (more tissue loss) had lower levels of anti-P. gingivalis HtpG.

In the CP subjects we hypothesized that the pattern of anti-HtpG would be different for the two bacteria. However, there was a trend, both in CP individuals colonized (Table 3) or uncolonized (Table 4) by P. gingivalis, to higher antibody levels to HtpG from both species in healthy subjects than in subjects with disease even though this trend did not reach statistical significance.

There were substantial and significant differences between the healthy and diseased AP subjects in all the variables tested. The three clinical measurements were all significantly higher in the

Table 1. Research Subject Demographics

| Group (n) | Gingivitis (50) | CP-Healthy (49) | CP-Diseased (50) | AP-Healthy (21) | AP-Diseased (24) |
|-----------|----------------|-----------------|-----------------|-----------------|-----------------|
| % Female  | 58             | 54              | 58              | 62              | 68              |
| Age Range in years (mean) | 18-66 (31.6) | 20-78 (43.0) | 30-77 (51.3) | 18-35 (34.4) | 22-40 (32.0) |
| Attachment Loss (mm, mean±SD) | 1.56±0.70 | 0.68±0.52 | 2.24±1.07 | 0.72±0.52 | 3.25±0.63 |
| Probing Depth (mm, mean±SD) | 2.64±0.54 | 1.60±0.21 | 2.60±0.71 | 1.50±0.22 | 2.83±1.32 |
| Bleeding on Probing (%, mean±SD) | ND | 0.24±0.12 | 0.57±0.17 | 0.13±0.06 | 0.33±0.16 |
| Gingival Index (%, mean±SD) | 0.2±0.17 | 0.18±0.19 | 0.52±0.23 | ND | ND |

ND—not determined

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Antibodies to P. gingivalis 
diseased groups (Table 5) while the anti-HtpG levels were 
subjects compared to periodontitis subjects of both groups and that 
levels of antibody to the peptide would be higher in control 
was investigated in the CP and AP subjects. We expected that the 
protective effect found in the serum of healthy subjects. Therefore 
the HtpG molecule was responsible for eliciting the apparent 
Cluster Analysis by Disease Subgroups

There were trends supporting our hypothesis when the results of 
the assays were compared between groups as they were recruited 
into the studies, basically health or disease status. However, 
examination of those groupings showed that there was overlap 
between the groups based on clinical measures, especially in the 
CP subjects, the most complete study. Therefore the subjects were 
regrouped in line with their clinical measures by K-means 
clustering, irrespective of recruitment groups. The clusters were 
used as a basis for ANOVA analysis of differences in the clinical 
measures and antibody levels.

Gingivitis subjects were regrouped into three clusters and 
differences in four clinical measures (PD, CAL, GI, PL) and two 
antibody levels (anti-P. gingivalis HtpG, anti-P. gingivalis whole cell 
lysate) sought. Cluster 1 had the greatest PD and CAL; there were 
significant differences between the clusters for PD and CAL. 
Antibody levels against P. gingivalis HtpG were lowest in the 
subjects with the highest disease measures, similar to earlier 
findings [9] for human Hsp90, but did not reach statistical 
significance (Table 6).

CP Subjects were regrouped into 4 clusters and differences 
sought in four clinical measures (PD, CAL, BOP, GI) and three 
antibody levels (anti-P. gingivalis HtpG, anti-Fn HtpG and anti-Pg 
p18). There were significant differences between the groups in all 
clinical measures (Table 7). Cluster 2 had the highest levels of all 
clinical measures and the lowest levels of anti-P. gingivalis HtpG 
and anti-P. gingivalis HtpG p18, the latter being significant 
(p = 0.00056, ANOVA).

AP subjects were regrouped into 3 clusters and differences 
sought in three clinical measures (PD, CAL, BOP) and three 
antibody levels (anti-Pg whole bacteria, anti-P. gingivalis HtpG and 
anti-Pg p18). There were significant differences in all the clinical 
measures between groups and in anti-P. gingivalis HtpG and anti-P. 
gingivalis HtpG p18, but not anti-Pg whole bacteria (Table 8). 
Cluster 2 had the highest levels of clinical measures and lowest 
levels of anti-P. gingivalis HtpG and anti-P. gingivalis HtpG p18. Both clusters 1 and 2 had 
substantially lower levels of anti-P. gingivalis HtpG compared to 
cluster 3.

diseased groups (Table 5) while the anti-HtpG levels were 
significantly lower in the diseased subjects (p<0.002, ANOVA).

Antibodies to P. gingivalis HtpG peptide p18

Previous studies [18] suggested that a peptide antigen, p18, of 
the HtpG molecule was responsible for eliciting the apparent 
protective effect found in the serum of healthy subjects. Therefore 
the serum response to a synthetic peptide antigen of this region 
was investigated in the CP and AP subjects. We expected that the 
levels of antibody to the peptide would be higher in control 
subjects compared to periodontitis subjects of both groups and that 

Table 2. Antibody responses to P. gingivalis and F. nucleatum in subjects.

| Group (n) | Gingivitis (50) | CP-Healthy (49) | CP-Diseased (50) | AP-Healthy (21) | AP-Diseased (24) |
|-----------|----------------|----------------|-----------------|----------------|-----------------|
| % positive for P. gingivalis | 82 | 71 | 86 | ND | 17 |
| % positive for F. nucleatum | ND | 96 | 96 | ND | ND |
| Antibody to total P. gingivalis (Log mean ELISA Units±S.D.) | 4.39±0.17 | 3.68±0.23 | 3.92±0.26 | 4.47±0.07 | 4.39±0.15 |
| Antibody to total F. nucleatum (Log mean ELISA Units±S.D.) | ND | 3.95±0.62 | 4.86±0.81 | ND | ND |
| ND—not determined |

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Table 3. Serum antibody (IgG) levels to bacterial antigens in CP subjects colonized by P. gingivalis.

| ELISA Units | p-value | Valid N (Health/Disease) |
|-------------|---------|--------------------------|
| Health (mean) | Disease (mean) | p-value (t-test) |
| Anti-P.gingivalis HtpG | 10,200 | 9,882 | NS | 35/41 |
| Anti-F.nucleatum HtpG | 19,959 | 19,817 | NS | 35/38 |
| Anti-P.gingivalis HtpG p18 | 7,138 | 6,254 | 0.047 | 35/40 |
| Anti-P.gingivalis W83 | 5,654 | 10,076 | 0.002 | 35/40 |
| Anti-F.nucleatum 22586 | 27,735 | 13,701 | 0.009 | 35/41 |
| NS—not significant |

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Table 4. Serum antibody (IgG) levels to bacterial antigens in CP subjects not colonized by P. gingivalis.

| ELISA Units | p-value | Valid N (Health/Disease) |
|-------------|---------|--------------------------|
| Health (mean) | Disease (mean) | p-value (t-test) |
| Anti-P.gingivalis HtpG | 9,224 | 8,594 | NS | 13/6 |
| Anti-F.nucleatum HtpG | 19,818 | 15876 | NS | 13/7 |
| Anti-P.gingivalis HtpG p18 | 6,812 | 6,477 | NS | 13/7 |
| Anti-P.gingivalis W83 | 5,347 | 11,008 | 0.002 | 13/7 |
| Anti-F.nucleatum 22586 | 17,917 | 8,473 | 0.009 | 13/7 |
| NS—not significant |

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elimination of the majority of the epitopes in the HtpG molecules, 
most of which are highly conserved between bacterial species, 
would clarify the trend observed using the whole HtpG proteins as 
ELISA target antigens. As expected, subjects in both the CP 
(Table 3 & 4) and AP (Table 5) groups diagnosed with 
periodontitis had lower levels of anti-p18 than their respective 
control groups. Although the same trend was observed in both 
colonized and un-colonized individuals using the HtpG molecule 
only the differences in p18 antibody levels in CP subjects colonized 
by P. gingivalis reached statistical significance (Table 3 & 4).
The supposition that antibodies to P. gingivalis HtpG may be protective is based on the hypothesis that high levels of antibodies should be found in subjects exhibiting healthier clinical signs and lower levels in subjects exhibiting more periodontitis related damage. Clinical measurements from each site in all the subjects were obtained and the average value determined for each subject. Relationships between those measurements and the levels of P. gingivalis and F. nucleatum anti-HtpG were sought using Pearson’s R to compare baseline antibody levels in both AP and CP groups with the clinical signs obtained at the baseline were compared. When anti-P. gingivalis HtpG levels from CP subjects were analyzed with four clinical measurements in each of the same patients there was a trend for pocket depth and attachment loss measurements to inversely correlate with antibody levels, but the correlations were small and not statistically significant. Similar results were found with anti-F. nucleatum HtpG values. However, in the AP subjects there were substantial negative associations three

### Table 5. Serum antibody (IgG/γ) levels to bacterial antigens in AP subjects.

|                | Health (mean±S.D) | Disease (mean±S.D) | p-value (ANOVA) | Valid N |
|----------------|-------------------|--------------------|-----------------|---------|
| PD (mm)        | 1.51±0.22         | 3.31±0.67          | <0.002          | 45      |
| AL (mm)        | 0.719±0.52        | 2.85±1.45          | <0.002          | 45      |
| BOP (%)        | 0.125±0.07        | 0.29±0.17          | <0.002          | 45      |
| Log anti-p18   | 4.11±0.27         | 3.97±0.16          | 0.041           | 45      |
| Log anti-HtpG  | 4.25±0.04         | 3.96±0.13          | <0.002          | 45      |
| Log anti-W83 cells | 4.47±0.07      | 4.39±0.15          | 0.032           | 45      |

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### Table 6. Analysis of clustered subjects in the Gingivitis group.

|                | Cluster 1 | Cluster 2 | Cluster 3 | p-value (ANOVA) |
|----------------|-----------|-----------|-----------|------------------|
| PD             | 3.1±0.2   | 2.8±0.4   | 2.05±0.2  | <0.001           |
| AL             | 2.2±0.6   | 1.0±0.0   | 1.11±0.3  | <0.001           |
| GI             | 27.3±5.5  | 30.0±4.8  | 5.5±2.7   | NS               |
| PI             | 45.5±5.9  | 8.0±6.3   | 27.8±4.6  | NS               |
| Anti-P. gingivalis HtpG | 9825±4720 | 14973±10178 | 12676±6928 | NS               |
| Anti-P. gingivalis W83 | 3100±6898 | 1301±177  | 7488±11008 | NS               |

NS—not significant

Subjects were clustered by K-means clustering and differences sought in means (±S.D.) of variables by ANOVA. PD and AL measured in mm; GI is the % sites with gingival redness; PI values are % sites with accumulated plaque. Antibody values are expressed in ELISA units.

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### Table 7. Cluster analysis of CP subjects.

|                | Cluster 1 | Cluster 2 | Cluster 3 | Cluster 4 | p-value (ANOVA) |
|----------------|-----------|-----------|-----------|-----------|------------------|
| PD             | 2.4±0.4   | 3.6±0.8   | 1.7±0.2   | 1.6±0.2   | <0.001           |
| AL             | 1.9±0.1   | 4.0±0.9   | 1.2±0.3   | 0.3±0.2   | <0.001           |
| BOP            | 56.8±16.2 | 68.4±16.6 | 27.7±12.7 | 24.6±14.6 | <0.001           |
| GI             | 54.3±21.1 | 60.6±24.6 | 20.2±17.9 | 21.4±21.0 | <0.001           |
| Anti-P. gingivalis HtpG | 10531±4791 | 7877±3906 | 9311±3833 | 10358±4443 | NS               |
| Anti-F. nucleatum HtpG | 20053±806 | 18530±9346 | 18231±5752 | 20748±6540 | NS               |
| Anti-P. gingivalis HtpG p18 | 6485±1920 | 4793±2120 | 7171±1782 | 6957±1068 | 0.006            |

NS—not significant

Subjects were clustered by K-means clustering and differences sought in means (±S.D.) of variables by ANOVA. PD and AL measured in mm; GI is the % sites with gingival redness. Antibody values are expressed in ELISA units.

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Correlation of Anti-P. gingivalis HtpG antibodies to clinical measures in Chronic and Aggressive Periodontitis

The supposition that antibodies to P. gingivalis HtpG may be protective is based on the hypothesis that high levels of antibodies should be found in subjects exhibiting healthier clinical signs and lower levels in subjects exhibiting more periodontitis related damage. Clinical measurements from each site in all the subjects were obtained and the average value determined for each subject. Relationships between those measurements and the levels of P. gingivalis and F. nucleatum anti-HtpG were sought using Pearson’s R to compare baseline antibody levels in both AP and CP groups with the clinical signs obtained at the baseline were compared. When anti-P. gingivalis HtpG levels from CP subjects were analyzed with four clinical measurements in each of the same patients there was a trend for pocket depth and attachment loss measurements to inversely correlate with antibody levels, but the correlations were small and not statistically significant. Similar results were found with anti-F. nucleatum HtpG values. However, in the AP subjects there were substantial negative associations three
clinical measurements and anti-HtpG levels, all of which were statistically significant (Table 9). Similar experiments using HtpG cloned from *F. nucleatum* gave no significant correlations to the clinical signs (data not shown).

**Correlation of Anti- *P. gingivalis* HtpG p18 antibodies to clinical measures in Chronic and Aggressive Periodontitis**

We then sought relationships between the same measurements and the levels of *P. gingivalis* anti-HtpG p18 by comparing antibody levels in both AP and CP groups with the clinical measurement using Pearson’s R analysis. Sera from the CP group and controls showed that levels of anti-p18 were significantly (p = 0.047) inversely correlated to CAL but not correlated to the other indices. In the AP group antibodies to p18 were significantly inversely correlated to PD (p = 0.008), CAL (p = 0.018) and BOP (p = 0.046), Table 10. Lastly, associations were sought between changes in clinical measurements 6 months after SRP treatment and the anti-p18 levels 6 months before treatment to determine if they might predict treatment success or failure. There was a significant positive correlation (p = 0.05) between the pre-treatment levels of anti-HtpG p18 and reduction in CAL.

**Discussion**

Chaperones are simultaneously highly conserved and immuno-dominant antigens which may have important roles in the pathogenesis of numerous human diseases. Although a subject of intense investigation, the role of the immune response to chaperones in human disease is currently not well understood. Periodontal diseases present a unique opportunity to examine the

### Table 8. Cluster analysis of AP subjects.

| Cluster 1 | Cluster 2 | Cluster 3 | p-value (ANOVA) |
|-----------|-----------|-----------|-----------------|
| **Localized AP n = 11** | **Generalized AP n = 12** | **Healthy n = 21** |
| PD | 3.0 ± 0.6 | 3.6 ± 0.7 | 1.5 ± 0.2 | <0.001 |
| AL | 2.2 ± 1.2 | 3.4 ± 1.5 | 0.7 ± 0.5 | <0.001 |
| BOP | 21.4 ± 11.2 | 37.9 ± 18.1 | 12.5 ± 5.7 | <0.001 |
| Anti- *P. gingivalis* HtpG p18 | 10182 ± 2437 | 8371 ± 2114 | 12390 ± 5318 | 0.0283 |
| Anti- *P. gingivalis* HtpG | 8853 ± 2493 | 10165 ± 3479 | 17742 ± 1349 | <0.001 |
| Anti- *P. gingivalis* W83 | 26899 ± 9509 | 26040 ± 12191 | 30041 ± 4977 | NS |

NS–not significant
Subjects were clustered by K-means clustering and differences sought in means (± S.D.) of variables by ANOVA. PD and AL measured in mm; BOP is the % sites bleeding on probing. Antibody values are expressed in ELISA units. NS–not significant.
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### Table 9. Correlation of Anti- *P. gingivalis* HtpG antibody levels with clinical measurements.

| A. Pretreatment | B. Post-treatment |
|-----------------|------------------|
| **Anti- *P. gingivalis* HtpG** | **Anti- *F. nucleatum* HtpG** | **Anti- *P. gingivalis* HtpG** | **Anti- *F. nucleatum* HtpG** |
| **Pearson’s R** | **p-value** | **Pearson’s R** | **p-value** | **Pearson’s R** | **p-value** | **Pearson’s R** | **p-value** |
| PD | −0.111 | 0.440 | | | | | |
| AL | −0.341 | 0.012 | | | | | |
| GI | −0.101 | 0.483 | | | | | |
| PI | −0.130 | 0.367 | | | | | |
| **CP** | | | | | | | |
| PD | −0.050 | NS | −0.019 | NS | 0.361 | 0.002 | 0.076 | NS |
| AL | −0.108 | NS | −0.113 | NS | 0.273 | 0.020 | 0.144 | NS |
| BOP | 0.144 | NS | 0.057 | NS | 0.381 | 0.001 | 0.113 | NS |
| GI | 0.093 | NS | 0.001 | NS | 0.263 | 0.026 | 0.140 | NS |
| **AP** | | | | | | | |
| PD | −0.787 | <0.001 | ND | | | | |
| AL | −0.628 | <0.001 | ND | | | | |
| BOP | −0.388 | <0.01 | ND | | | | |

NS–not significant
ND–not determined
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immune response to chaperones in a distinctive and readily accessible human environment. A few reports of antibody levels to P. gingivalis chaperones (particularly GroEL) have been published but this is the first comprehensive analysis of anti-P. gingivalis HtpG in multiple periodontal disease states. Results reported here and earlier [9,12] suggest that anti-P. gingivalis HtpG antibodies predict health in patients susceptible to periodontal disease and are protective in the untreated periodontal disease patient. These results may also reflect a previously uncharacterized immune control mechanism unrelated to direct binding of bacteria. Further, these antibodies may augment periodontitis treatment and P. gingivalis HtpG might be an attractive vaccine candidate. These results appear to be unique to P. gingivalis as they are directed at a segment of HtpG unique to P. gingivalis; parallel experiments with the HtpG homologue of F. nucleatum did not manifest these same qualities. In addition, there is reason to believe that these results may be extended to other chronic infectious diseases.

Earlier studies of a population of subjects with minimal periodontal disease suggested that antibodies to human Hsp90 related chaperones might have a protective effect. This report expands on that notion by examining the response of periodontitis subjects to the Hsp90 homologue in P. gingivalis, HtpG. In gingivitis subjects as a whole it was found that there was a distinctive, but not statistically significant, trend in support of the hypothesis. Interestingly, when anti-P. gingivalis HtpG levels and clinical measures are compared on an individual basis there is a discernable correlation with CAL. At the opposite end of the periodontitis disease spectrum, the AP subjects, there is considerable support for the hypothesis. At the group level, there are substantial and significant differences between both anti-P. gingivalis HtpG and anti-P. gingivalis HtpG p18 between clusters based on clinical measures, but no differences in the response to the whole bacterium. On an individual basis, both anti-P. gingivalis HtpG and anti-P. gingivalis HtpG p18 are inversely and significantly correlated to the clinical measures.

Support of the hypothesis was somewhat tenuous in the initial examination of the CP subjects. Those recruited as either healthy or with CP and colonized by P. gingivalis possessed a humoral response to HtpG that was higher in subject groups with less periodontal destruction and inversely related to the level of attachment loss (p<0.05). A similar relationship was not found in un-colonized individuals. This was the opposite of antibody levels to the whole bacterium which were significantly higher in subjects with disease than controls (p<0.01), a finding reported by many other laboratories. Similar trends were found when we examined the humoral response to HtpG from F. nucleatum but there was no relationship to disease status. In addition, correlations between the same antibodies and clinical measures when considered in the context of individual subjects were not found, except for a correlation between anti-P. gingivalis HtpG p18 and CAL (R = 0.205, p<0.05). This dilemma was resolved when the subjects were clustered by clinical measures. In a cluster of 10 subjects with the most severe disease there was a substantial and significantly lower level of anti-P. gingivalis HtpG and anti-P. gingivalis HtpG p18 compared to the other clusters in the CP subjects. As might be expected, the clinical measures of these subjects resemble those of the AP subjects. In summary, there is a trend for both anti-P. gingivalis HtpG and anti-P. gingivalis HtpG p18 to be lower in subjects with the more serious disease in each of the 3 periodontal disease groups. Neither trend reaches statistical significance in the gingivitis group. In the CP group anti-P. gingivalis HtpG p18 is significantly lower in the most diseased cluster; anti-P. gingivalis HtpG trends in the same direction but does not reach statistical significance. In the AP group both anti-P. gingivalis HtpG and anti-P. gingivalis HtpG p18 are significantly lower in the diseased clusters of subjects. Our initial observations (16) were probably due to the response of the most diseased individuals in that group of subjects. However, we believe that the current results are not fortuitous because 1) the relationship between the antibody levels and disease is evident not only in the original group but in groups with more serious disease in an almost dose dependent manner; 2) there is a relationship between these antibody levels and response to periodontal treatment; 3) there is a link through these antibodies to cellular receptors involved in antigen recognition and inflammation; 4) similar results have been noted in other chronic bacterial infections. In addition, there is no evidence that the lower levels of anti-P. gingivalis HtpG are due to adsorption by high circulating levels of either human Hsp90. There was no significant correlation between individual serum levels of human Hsp90 protein and anti-P. gingivalis HtpG, anti-F. nucleatum HtpG or anti-P. gingivalis HtpG p18 (Table S1). In fact, there was a trend to higher levels of anti-P. gingivalis HtpG in subjects with higher levels of human Hsp90, the opposite of what we would expect if such absorption was taking place. There was also no significant difference between levels of human Hsp90 in healthy subjects with high levels of anti-P. gingivalis HtpG antibodies and severe periodontitis subjects with low levels of anti-P. gingivalis HtpG (Table S2). Lastly, while it is possible that a subset of anti-P. gingivalis HtpG antibodies might bind other HSP90 homologues, including human Hsp90, the P. gingivalis HtpG p18 epitope is unique and antibodies to that peptide could not be adsorbed by other HSP90 family proteins (see discussion below).

Subjects with CP who were given periodontal treatment responded more effectively to that treatment the higher their original pretreatment levels of anti-P. gingivalis HtpG. There was no similar effect with antibodies to F. nucleatum HtpG. Notably, the serum samples, which are time-averaged values, correlate best
with clinical indices related to tissue destruction and less with indices related to inflammation. This suggests that these antibodies are found in subjects predisposed to a better treatment outcome or they may somehow facilitate healing after treatment. Interestingly, drugs that inhibit Hsp90 have been shown to improve outcomes or they may somehow facilitate healing after treatment.

Injuries in murine sepsis [19]. Interestingly, drugs that inhibit Hsp90 have been shown to improve outcomes or they may somehow facilitate healing after treatment.

**Table 11. Alignment of oral bacterial species HtpG amino acid sequences.**

| Species                        | HtpG Sequence                                                                 |
|-------------------------------|-------------------------------------------------------------------------------|
| A. actinomycetemcom. HtpG     | KTAVSVTNNQMTQMKLPLAAGG-QFVFKVTTLELHNLKVKYLRAED-588                          |
| F. nucleatum HtpG             | SASSLARKG-LSLEMTLSEMTNNNDPFAKEVLAIFNHVFLLNRLKSSVN 569                      |
| P. gingivalis HtpG            | AIIITQAEFRRMQDAQQFOMSFYELPS-DNVLHNLDDLIDNLSLGSGLLGLAEALAEFIRRSQRLL-684     |
| A. actinomycetemcom. HtpG     | IAD-----EQAQDNELIGQLIDLALLGSGLLTGEALAEFIRRSQRLL-614                      |
| F. nucleatum HtpG             | SVN-----TEDFNKLVDLNLQALLESENIENF-595                                       |
| P. gingivalis HtpG            | GEKESVYETLKLRAIQLRAKAKLKDDEEGRQFEI1PVATKEKLENAE-632                      |
| A. actinomycetemcom. HtpG     | -AFAFRIKILLLGSNLGLGSAEGIRRSQRLL-684                                      |
| F. nucleatum HtpG             | -AFAFRIKILLLGSNLGLGSAEGIRRSQRLL-684                                      |
| P. gingivalis HtpG            | QAAKTEGSINDQLQKTYAQHNEGQGQGLALLGLLSLLTGEALAEFIRRSQRLL-684               |

Alignment of oral bacterial species HtpG amino acid sequences in the region of the 65 amino acid “Bacteroidacae insert” (Bold, underlined characters). The insert has not been described outside the Cytophaga, Flavobacteria and Bacteroides (CFB) group. Italic, bold–p18.

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**Table 12. Alignment of Bacteroidacae HtpG C-terminal inserts**

| Species          | HtpG Sequence                                                                 |
|------------------|-------------------------------------------------------------------------------|
| B. thetaiotaomicron | LKDSQRDKEEDEPTEAEKDEHDMEKWDLLKDKKGE1IF3A-643                                     |
| B. fragilis       | LKKQEGKEDDPDEAEKDEHDMEKWDLLKDKKGE1IF3A-643                                     |
| P. intermedia     | LQEGQEDKDKEEDEPTEAEKDEHDMEKWDLLKDKKGE1IF3A-643                                 |
| T. forsythia      | LREAQKEDDEEDEPTEAEKDEHDMEKWDLLKDKKGE1IF3A-643                                 |
| P. gingivalis     | LLDEEKGDEEDEPTEAEKDEHDMEKWDLLKDKKGE1IF3A-643                                  |
| Consensus         | * . : ** :*: * . : . . . . . . . . **:                                                   |
| B. thetaiotaomicron | YASHNVRQVQLLDDLALLQNMLKGAEHNPVRSIEL-681                                      |
| B. fragilis       | YAGKNVRQVQLLDDLALLQNMLKGAEHNPVRSIEL-681                                      |
| P. intermedia     | YAGKNNAHQLDLALLQNMLKGKADKFIKRSVDDLK-685                                      |
| T. forsythia      | YAGKNPQVQQLLDDLALLQNMLKGAEHNPVRSVDDL-686                                     |
| P. gingivalis     | YAGKNELQVQQLLDDLALLQNMLKGAEHNPVRSVDDL-684                                     |
| Consensus         | ** : : : : : : : : : : : : : : : : : : : **:                                                   |

Alignment of 65 amino acid insert found in most CFB group bacteria, but not other bacterial groups. Bacteroidacae HtpG C-terminal inserts shows p18 is much less conserved than p19. Underline/bold–p18 sequence. Dashed underline–p19 sequence. Amino acid identities across all species.**

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Anti-HtpG in Periodontitis
The results reported here are similar to those of other chronic infections including *Helicobacter pylori*. World wide as much as 75% of the population has evidence of *H. pylori* infection but only subsets of these individuals manifest the peptic ulcers and stomach cancers associated with these chronic infections. The *H. pylori* chaperone hsp60 induces CXCL8 production in monocytes [22] and humoral immune response to a peptide epitope (pH9) of hsp60 is unique and seems to be associated with protection against *H. pylori* infection [23]. Intriguingly, when subjects with mucosa-associated lymphoid tissue lymphoma were examined for antibodies to the *H. pylori* hsp60 chaperone it was found that pre-treatment titers in patients whose tumors regressed after treatment were significantly higher than in patients whose tumors did not regress [24]. Low levels of antibodies to chaperones in disease subjects compared to controls has also been reported in inflammatory bowel disease [25], fungal infections [26] and other chronic bacterial infections [27].

The unique restriction of the *P. gingivalis* HtpG p18 peptide to the most important pathogen in periodontitis may have applications in vaccine and diagnostic arenas. The use of chaperones as vaccine candidates has been suggested by a number of investigators. Antibody to the Hsp90 homologue of *C. albicans* (Mycograb®; Novartis) has been shown to be effective in the treatment of disseminated fungal infections [28] in combination with amphotericin B. Other chaperones have been suggested as vaccine targets for diverse diseases in humans, cattle [29] and fish [30]. *P. gingivalis* GroEL has been used as a vaccine in a rat model of periodontitis that resulted in prevention of attachment loss [31], similar to that described here in humans. However, the authors cautioned that extensive homology between human and microbial chaperones may require use of peptides that do not induce cross-reactive antibodies with human hsp60 molecules. We believe this is the case with p18, which appears unique to *P. gingivalis* even to the exclusion of other Bacteroides species. The data presented here also suggests that serum antibodies to p18 may be useful in diagnosing periodontitis patients with extensive treatment requirements, identification of which would have substantial economic and epidemiological impact on the practice of periodontontology.

**Materials and Methods**

**Subjects**

All work with human subjects was approved by the University of Michigan Institutional Review Board or the Queen’s University, Belfast Ethics Committee, respectively. Each subject gave written individual informed consent and was advised that withdrawal from the study was available at their discretion at any time. The clinical condition of each subject was determined by examination of five clinical measurements: Probing pocket depth (PD) was determined to the nearest mm at six sites around each tooth and then averaged for all sites in each subject [32]. Bleeding on probing (BOP), was reported as a dichotomous measure and recorded as a percentage of the sites [33]. Clinical attachment loss (CAL) was determined at the same sites by measuring the distance between the cemento-enamel junction and the bottom of each pocket to the nearest mm and averaged [34]. The gingival index (GI) value represents the percentage of sites in each patient exhibiting redness associated with inflammation[35]. The plaque index value (PI) represents the percentage of sites found to have plaque biofilm accumulated at the gingival margin.

**Gingivitis Subjects**

This group was examined from archived samples described in a previous report and was included here to address the potential differences between response to *P. gingivalis* HtpG and human Hsp90. This retrospective study focused on a population of individuals living in southwestern Michigan [15,16] with gingivitis, a form of periodontal disease with no or minimal tissue destruction that is reversible with active oral hygiene. Subjects were recruited into the original study based on their membership in the rural community, not their oral health status, and all essentially presented with gingivitis or mild periodontitis. The clinical data and archived serum samples collected from 50 subjects were used. The average age was 31.6 years (range: 18–66) and 58% were female. Other characteristics of these subjects are found in Table 1.

**Chronic Periodontitis Subjects**

Subjects were recruited at the Michigan Center for Oral Health Research. Subject inclusion was based on: possession of at least 20 teeth, no periodontal treatment or antibiotic-related therapy for medical or dental reasons for 3 months before study inclusion, no history of long-term treatment with medications known to affect periodontal status, and no history of metabolic bone diseases. Healthy control subjects (n = 49) were recruited who had less than 5 mm of CAL, no pocket depth (PD) greater than 4 mm, no radiographic bone loss, and less than 20 sites with bleeding on probing. Chronic periodontitis (CP) subjects (n = 50) exhibited at least 4 sites with evidence of radiographic bone loss, mean CAL >3mm, PD >4 mm and bleeding on probing (Table 1).

**Aggressive Periodontitis Subjects**

Studies were done with serum samples from an ongoing study of Aggressive Periodontitis (AP) subjects (n = 24) and age matched control subjects (n = 21) living in Northern Ireland. AP subjects included in this investigation were 30.3±4.0 years of age at the time of clinical and radiographic examination, diagnosed with severe periodontitis, and had a minimum of 4 sites with a probing depth of at least 5 mm and CAL loss of at least 2 mm. Age matched controls without periodontitis were recruited from regular attendees at the Queen’s University School of Dentistry. Colonization of AP by *P. gingivalis* was determined by PCR using primers specific for the *P. gingivalis* 16S rRNA gene [17].

**Bacterial strains and culture conditions**

*Porphyromonas gingivalis* (ATCC 33277) and *Fusobacterium nucleatum* (ATCC 25586) were obtained from the American Type Culture Collection. *Porphyromonas gingivalis* strain W83 was a gift from Dr. Donald Clewell, University of Michigan School of Dentistry. The bacteria were maintained by weekly transfer in an anaerobe chamber (Coy Manufacturing, Grass Lake, MI) at 37°C on PRAS Brucella agar plates (Anaerobe Systems, Morgan Hill, CA) in a 5% hydrogen, 10% carbon dioxide, 85% nitrogen atmosphere. Broth cultures were grown in a mixture of 50% Brain Heart Infusion Broth, 50% Trypticase Soy Broth and 5 gram/L Yeast Extract supplemented with 0.01 gm/L Sodium Bisulfite, 5 mg/L hemin and 5ug/L Vitamin K.

**Antigen preparation**

**Cloning and purification of *P. gingivalis* and *F. nucleatum* rHtpG.** The full length sequence of *P. gingivalis* HtpG (GenBank accession number AF176245) and *F. nucleatum* HtpG (GenBank accession number EDK381176) were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). PCR primers were designed to produce full-length products that were subsequently inserted into pCR®T7 TOPO® cloning vector following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Clones of One Shot® Chemically Competent *E. coli*
Chromatography (Ni-NTA™ Agarose, Qiagen, Valencia, CA). Determined by SDS-PAGE electrophoresis, by Ni-agarose the cloned proteins. The protein was purified to 6 consecutive histidine (6xhis) residues preceding the N-terminal of thiogalactopyranoside for 4 hours to produce a fusion protein with tracers. Antigens were coated onto the plates in 25 μl sodium carbonate/bicarbonate buffer (0.05M, pH 9.5) and bovine serum albumin (PBS-BSA) added to block unoccupied protein binding sites (100 μl/well). After an additional hour of incubation at room temperature plates were washed with PBS with 0.125% NP40 three times and human serum (or control rabbit serum) washed by centrifugation at 10,000 g for 2 minutes on ice three times and centrifuged at 10,000 g for 6 minutes. Serum IgG binding was determined using the same second antibodies as described above.

ELISA assays

All assays were carried out in 384-well microtiter plates (NUNC™ black MaxiSorp, Rochester, NY) using 4-methylumbelliferone phosphate as a substrate for alkaline phosphatase-labeled tracers. Antigens were coated onto the plates in 25 μl/well volumes in sodium carbonate/bicarbonate buffer (0.05M, pH 9.5) and incubated overnight at 4°C. Plates were washed 3 times with 0.02 M phosphate buffered saline (PBS, pH 7.4) and PBS with 1% bovine serum albumin (PBS-BSA) added to block unoccupied protein binding sites (100 μL/well). After an additional hour of incubation at room temperature plates were washed with PBS with 0.125% NP40 three times and human serum (or control rabbit serum) diluted 1:100 in PBS-BSA (25 μl/well) added to the plates in triplicate and incubated overnight at 4°C. Plates were then washed 3 times with PBS with 0.125% NP40 and detection reagents added as described below. Blocked wells not coated with antigen were used as negative controls for each individual serum sample. Data is expressed as net relative florescent units (RFU) by subtracting the average of 3 control wells from the average of 3 antigen coated wells and were repeated at least 3 times each.

Antibodies to P. gingivalis Whole Cell Lysate

P. gingivalis cells (strain W83) were grown to mid-log phase, washed by centrifugation at 10,000 xg 3 times with sterile PBS and resuspended at an OD600 of 1.0 in water. The cells were sonicated for 2 minutes on ice three times and centrifuged at 10,000 xg for 20 minutes. Total protein was determined and the clarified lysate diluted to 10 μg/mL for coating. Serum IgG binding was determined as described above using alkaline phosphatase-labeled anti-human IgG(γ) antibodies and 4-methylumbelliferyl phosphate (1 μg/mL in 0.2 M TRIS, pH 9.5) (Sigma, St. Louis).

Antibodies to P. gingivalis HtpG and F. nucleatum HtpG

Recombinant HtpG proteins were dissolved in carbonate buffer to 1 μg/mL for plate coating. Total IgG(γ) binding was determined using the same second antibodies as described for the whole cell lysate assay above.

Antibodies to P. gingivalis HtpG peptide 18

The peptide was dissolved in carbonate buffer to 10 μg/mL for plate coating. Total serum IgG(γ) binding was determined using the same second antibodies as above.

Detection of P. gingivalis and F. nucleatum colonization in plaque samples

The detection of P. gingivalis in pooled plaque samples from the gingivitis subjects was done using a slot immunoblotting method as described previously [36]. Colonization of plaque samples collected simultaneously with the serum in the CP groups were evaluated by real-time PCR as described [37] using primers specific for P. gingivalis (forward: 5'-CATAGATATCAAG-AGGAATCTCGGATG-3'; reverse 5'-AAACTGTTAGCGAATCCGATGTTG-3') and F. nucleatum (forward: 5'-AAATATGTTGAGAATCTGGGAAGAAGT-3'; reverse: 5'-GCTTGACGGGCGG-TGT-3'). The presence of P. gingivalis in plaque from the AP subjects was determined by PCR as described earlier [17] using the same primers.

Statistical Analysis of anti-HtpG levels

Statistical computations were done using STATISTICA™ v. 6.0 (StatSoft, Omaha, NE). Comparison of two means was performed using t-tests. The relationships between specific indices of periodontal disease, antibody levels, and colonization were assessed by ANOVA. Fisher’s method for multiple comparisons was used for group comparisons. Log transformation of data was performed where appropriate. Results with p-values of ≤ 0.05 were considered significant.

Supporting Information

Table S2 Antibody to P. gingivalis HtpG and human Hsp90 serum levels in CP subjects.

Table S1 Correlation of human Hsp90 levels to Anti-HtpG levels in 95 CP patients and healthy controls.

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Author Contributions

Conceived and designed the experiments: DL CS. Performed the experiments: PS CS. Analyzed the data: WG CS. Contributed reagents/materials/analysis tools: WG JD JK JC BM DL CS. Wrote the paper: CS.

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