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SHORT COMMUNICATION

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Hemophore-like proteins produced by periodontopathogens are recognized by the host immune system and react differentially with IgG antibodies

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ABSTRACT

Aims: Hemophore-like proteins sequester heme from host hemoproteins. We aimed to determine whether the host immune system can recognize not only *Porphyromonas gingivalis* HmuY but also its homologs expressed by other periodontopathogens, and how periodontitis influences the production of respective antibodies.

Methods: The reactivity of total bacterial antigens and purified proteins with serum IgG antibodies of 18 individuals with periodontitis and 17 individuals without periodontitis was examined by enzyme-linked immunosorbent assay (ELISA). To compare IgG reactivity between groups with and without periodontitis and within the various dilutions of sera, statistical analysis was performed using the Mann-Whitney U-test and two-way ANOVA test with the post-hoc Bonferroni test.

Results: Individuals with periodontitis produced IgG antibodies reacting more strongly not only with total *P. gingivalis* antigens ($P = 0.0002$; 1:400) and *P. gingivalis* HmuY ($P = 0.0016$; 1:100) but also with *Prevotella intermedia* PinA ($P = 0.0059$; 1:100), and with low efficiency with P. intermedia PinO ($P = 0.0021$; 1:100). No increase in the reactivity of IgG antibodies with *Tannerella forsythia* Tfo and *P. gingivalis* HusA was found in individuals with periodontitis.

Conclusions: Although hemophore-like proteins are structurally related, they are differentially recognized by the host immune system. Our findings point to specific antigens, mainly *P. gingivalis* HmuY and *P. intermedia* PinA, whose immunoreactivity could be further investigated to develop markers of periodontitis.

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Introduction

The healthy oral cavity is inhabited by a microbiome that consists of more than 500 species, with the species of *Streptococcus* occupying a broad range of oral habitats [1–3]. The development of periodontal diseases, described by the dysbiosis and keystone pathogen model, is linked to an environmental shift in the oral microbiome, leading to the dominance of anaerobic Gram-negative pathogenic bacteria over aerobic Gram-positive commensal, early colonizers [1–6]. The most frequent bacterial species isolated from subgingival sites, which are associated with the clinical features of periodontitis, are *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, keystone bacteria related to periodontal dysbiosis, previously considered to constitute the 'red complex' [1,3]. *P. gingivalis* and *T. forsythia* have been described as late colonizers prevalent in microbial consortia within subgingival pockets associated with periodontitis. Other bacteria, such as *Prevotella intermedia*, formerly considered to belong to the 'orange complex', serve as early colonizers of dental biofilm and act as bridging species with late colonizers. Periodontal diseases comprise inflammatory conditions and disorders of tooth supporting tissues [7]. In contrast to mild, reversible gingivitis, periodontitis is an advanced, irreversible disease, resulting in the destruction of gingiva and alveolar bone loss. From a clinical point of view, periodontitis is characterized by deep periodontal pockets, resulting from the loss of alveolar bone and connective tissue attachment to the tooth, leading eventually to tooth loss. The severity of bleeding upon probing depends on the intensity of the gingival inflammation, at least in part caused by an exaggerated inflammatory response of the host cells against bacteria and agents produced by them [7].

Epidemiological studies have revealed that the key pathogen considered responsible for the initiation and progression of periodontitis is *P. gingivalis* [7]. The presence of whole *P. gingivalis* bacteria and their outer membrane vesicles or DNA can also be detected in non-

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oral sites, suggesting that the bacterium or its components can be involved not only in local but also in systemic inflammatory diseases, including diabetes, atherosclerosis, cardiovascular diseases, rheumatoid arthritis, and Alzheimer's disease [8,9]. The bacterium can also enter gingival epithelial and immune cells, remaining viable within host cells, and can spread systemically to other tissues, which allows for its survival and engagement in comorbidities [10–12].

As part of the infective process, host-associated oral members of the Bacteroidetes phylum (represented mainly by *P. gingivalis*, *T. forsythia* and *P. intermedia*) must acquire heme, which is indispensable for life and enables them to survive and multiply at the infection site. *P. gingivalis* uses a newly discovered unique Hmu heme uptake system, in which a leading role is played by the HmuY protein [13–16]. HmuY, the first member of a novel family of hemophore-like proteins, is responsible for acquiring heme and increasing virulence of this periodontopathogen. Other hemophore-like proteins, which belong to the HmuY family, are *T. forsythia* Tfo [15] and *P. intermedia* PinO (the protein encoded on the megaplasmid) and *P. intermedia* PinA (the protein encoded on the large chromosome) [17]. Also, *P. gingivalis* HusA exhibits hemophore-like properties, albeit distinct from HmuY protein [18,19].

Although the homologs of *P. gingivalis* HmuY have been identified in other periodontopathogens, we found that the unique HmuY protein may serve as a specific marker of *P. gingivalis* [15,17,20,21]. Indeed, antibodies raised against purified *P. gingivalis* HmuY protein or against selected epitopes of the HmuY molecule were highly specific for the purified and cell-associated protein and did not recognize the closest homologous proteins produced by *P. intermedia* and *T. forsythia* [21]. Moreover, we found higher levels of anti-HmuY antibodies in sera of individuals with periodontitis, as compared to individuals without periodontitis [22], suggesting high abundance and/or high immunoreactivity of this protein.

The aim of this study was to determine whether the host immune system can recognize not only *P. gingivalis* HmuY but also hemophore-like proteins expressed by other periodontopathogens, and how periodontitis influences production of respective antibodies.

Materials and methods

Examination of participants and sample collection

This study was approved by the Ethics Committee of Feira de Santana State University (Feira de Santana, Bahia, Brazil) (CAAE number: 32535914.4.0000.0053) and conducted in accordance with the Helsinki Declaration as revised in 2013. Participants involved in this study were informed and signed the consent forms. A structured questionnaire was applied to obtain information about the general characteristics of the participants. Then, periodontal clinical examination and collection of peripheral blood were performed. Periodontal condition was classified according to the periodontal criteria reported by Gomes-Filho et al. [23]. The examined group consisted of 35 systemically healthy Brazilian individuals, including 18 individuals with moderate and severe periodontitis (periodontitis group) and 17 control individuals without periodontitis (non-periodontitis group), who attended the Dental Clinic of Feira de Santana State University from February to July 2015 (Table 1). Participants were selected considering exclusion criteria: age less than 18 years, number of teeth less than 10, history of systemic diseases, current pregnancy, current periodontal treatment or until 1 year before the selection, current or previous cigarette smoking, use of antibiotics and anti-inflammatories at six and two months before data collection, respectively. The periodontal examination was made at six sites per tooth, and all teeth were evaluated, excluding third molars. The clinical definition of periodontitis was the presence of at least four teeth with one site presenting probing depth ≥4 mm, clinical attachment level ≥3 mm and bleeding on probing at the same site. Peripheral venous blood was drawn from the cubital fossa of each individual. Blood samples (5 ml) were collected into tubes and centrifuged after clot formation. Collected serum samples were stored at −20°C until used.

Bacterial stains, growth conditions, and preparation of bacterial cell lysates

P. gingivalis A7436 [16], *P. intermedia* 17 [17], and *T. forsythia* ATCC 43,037 [15] were grown anaerobically

Table 1. Demographic and clinical characteristics of participants. Periodontal diagnosis was performed according to the criteria reported by Gomes-Filho et al. [23].

Group	Participants (Male/Female)	Age (years)	Number of teeth[#]	Bleeding on Probing (%)	Probing Depth ≥4 (%)	Clinical Attachment Level ≥5 (%)	Clinical Attachment Level ≥3 (%)
Non-periodontitis	17 (3/14)	30.17 ± 7.29	24.7 ± 3.8	9.2 (4.9–17.9)	0 (0–0.7)	0 (0–0)	1.2 (0–10.4)
Periodontitis	18 (5/13)	36.05 ± 9.33	23.4 ± 5.9	47.7 (32.7–59.4)	15.3 (9.4–27.3)	14.7 (4.8–29.1)	32.8 (14.3–45.5)

[#]Number of teeth excluding the 3rd molars.
Data are shown as mean±standard deviation (mean±SD) or as median and interquartile range (25–75%).

(Whitley A35 anaerobic workstation; Bingley, UK) at 37°C for 5 days on blood agar plates composed of Schaedler broth containing hemin and L-cysteine, and supplemented with 5% sheep blood and menadione (Argenta, Poznań, Poland and Biomaxima, Lublin, Poland). Bacterial colonies were used to inoculate the liquid basal medium (BM) composed of 3% trypticase soy broth (Becton Dickinson, Sparks, MD, USA), 0.5% yeast extract (Biomaxima), supplemented with 0.5 mg/l menadione (Fluka, Munich, Germany), 0.05% L-cysteine (Sigma-Aldrich, St. Louis, MO, USA), and 7.7 μM hemin chloride (Fluka). To grow *T. forsythia*, liquid and solid media were additionally supplemented with *N*-acetylmuramic acid (Sigma-Aldrich) to the final concentration of 10 μg/ml.

To prepare cell lysates, bacteria were grown for three passages in liquid cultures, centrifuged (4000×*g*, 20 min, 4°C) and washed twice with 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl (phosphate-buffered saline; PBS). Cell pellets obtained from 10-ml cultures were resuspended in 5 ml of PBS, supplemented with Protease Inhibitor Cocktail (Bimake, Houston, TX, USA) and stored at −80°C until used. Samples were thawed on ice and sonicated (Sonopuls HD 4100, Bandelin, Berlin, Germany). Protein concentration in the obtained bacterial cell lysates was determined using ROTI Nanoquant (Carl Roth, Karlsruhe, Germany).

Overexpression and purification of proteins

HmuY, Tfo, PinO, PinA and HusA proteins in their native, soluble forms (lacking respective signal peptides) were overexpressed in *Escherichia coli* BL21-CodonPlus-RIL cells (Agilent Technologies, Santa Clara, CA, USA) using previously constructed expression plasmids [15,17,24]. To construct expression plasmids, nucleotide sequences encoding examined proteins were PCR amplified using genomic DNA isolated from the above-mentioned strains. Recombinant proteins possessing N-terminal His-MBP fusion tag were purified from soluble fractions obtained from *E. coli* cell lysates and the fusion tag was removed according to the manufacturer's protocol (New England Biolabs). Purified proteins were concentrated using Amicon Ultra-15 10-kDa cut-off filter units (Millipore, Billerica, MA, USA). Purity of proteins was examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie Brilliant Blue G-250 and Western blotting as described previously [15,17,24]. Concentration of the purified proteins was determined spectrophotometrically using the empirical molar absorption coefficients ($mM^{-1}cm^{-1}$): HmuY = 36.68, Tfo = 26.32, PinA = 45.91, PinO = 23.87, and HusA = 33.81 [14,15,17,24].

Enzyme-linked immunosorbent assay (ELISA)

Reactivity of antibodies present in sera with respective antigens was examined using ELISA. For this purpose, 96-well polystyrene plates (Sarstedt, Waltham, MA, USA) were coated for 1 h at 37°C with respective purified proteins or HmuY-derived synthesized peptides (GKKKDEPNQPSTPE, SKGEVVNVTDYKNDL, and EMGPDGHQMEYEEQ) (GenScript Inc., Piscataway, NJ, USA) [21] prepared in PBS (100 ng/100 μl/well) or bacterial cell lysates containing 5 μg of protein per ml of the sample (500 ng/100 μl/well). The plates were washed five times with 200 μl of PBS prior to overnight blocking at 4°C with 200 μl/well of 2% fetal bovine serum (FBS; Cytogen, Zgierz, Poland) diluted in PBS, containing 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), and then washed five times with 200 μl/well of PBS. Subsequently, the respective human sera diluted in 0.1% BSA in PBS (100 μl/well; 1:100–1:12800) or sera obtained from rabbits immunized with purified HmuY (GenScript Inc.), Tfo, PinO, PinA proteins or peptides (100 μl/well; 1:6400) (ProteoGenix, Schiltigheim, France) [15,17,21] were applied and incubated for 1 h at 37°C. After washing with PBS, 100 μl/well of goat anti-human or goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Sigma-Aldrich) diluted in 0.1% BSA in PBS (1:10000) were applied (100 μl/well) and incubated for 1 h at 37°C. After five final washes with PBS, a substrate solution containing 0.05% *o*-phenylenediamine (Sigma) in citrate/phosphate buffer pH 5.0 (containing 48.5 mM citric acid and 103 mM Na_2HPO_4) and 0.01% H_2O_2 was added (100 μl/well) and incubated at room temperature for color development. The reaction was stopped after 15 min by adding 25 μl of 12.5% H_2SO_4 and the absorbance was measured at 450 nm using a GloMax Discover plate reader (Promega, Madison, WI, USA).

Dot blotting

Alternatively to ELISA, reactivity of antibodies present in sera with respective antigens was examined using dot blotting. For this purpose, 100 ng (5 μl of 20 μg/ml solution) of purified HmuY, Tfo, PinO and PinA proteins or peptides prepared in PBS were applied on spots onto nitrocellulose membranes (Cytiva, Marlborough, MA, USA). After the samples had dried, the membranes were blocked with 5% skim milk in PBS with an addition of 0.2% Tween 20 (Carl Roth) (PBST) for 1 h at room temperature. Subsequently, rabbit sera containing antibodies raised against purified HmuY, Tfo, PinO and PinA proteins or against peptides (1:6400 in PBST with addition of 0.1% skim milk) [15,17,21] or pooled sera of individuals with periodontitis and control individuals without periodontitis (1:800 in PBST with addition of 0.1% skim milk)

were applied and incubated overnight at 4°C. Then, the membranes were washed four times with PBST with the addition of 0.1% skim milk and incubated for 1 h at room temperature with respective goat anti-rabbit or goat anti-human IgG antibodies conjugated with horseradish peroxidase diluted in PBST with the addition of 0.1% skim milk (1:10000). After four final washes, the reaction was developed using chemiluminescence staining (PerkinElmer, Waltham, MA, USA) and visualized using the ChemiDoc imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

Bioinformatics analysis

Amino acid and DNA sequences were compared using the Multiple Sequence Alignment Clustal Omega tool available from the European Bioinformatics Institute (EMBL-EBI) [25] and then presented graphically using Jalview [26] in the form of sequence alignment and phylogenetic tree. Three-dimensional protein structures of HmuY (PDB ID: 6EWM), PinO (PDB ID: 6R2H), Tfo (PDB ID: 6EU8), HusA (PDB ID: 6CRL), and AlphaFold-predicted structure of PinA [27,28] (UniProt ID: A0A1P8JJ12) were visualized using Swiss-PdbViewer [29].

Statistical analysis

Clinical and demographic characteristics of participants are shown as mean ± standard deviation (mean ± SD; e.g. age and number of teeth) or as the median and interquartile range (25%–75%; other parameters, which do not exhibit normal distribution). To compare the reactivity of serum IgG between groups with and without periodontitis, the Mann-Whitney U-test was used. To compare the reactivity of serum IgG between groups with and without periodontitis and within the various dilutions of sera tested against each antigen employed in this study, a two-way ANOVA test with the post-hoc Bonferroni test was employed. Values of $P < 0.05$ were considered statistically significant. IgG responses in tested individuals against antigens were further compared by generating a receiver operating characteristic (ROC) curve and calculation of the area under the curve (AUC). The GraphPad software (GraphPad Prism 8.0 Inc., San Diego, CA, USA) was used for all statistical analyses.

Results

Influence of periodontitis on reactivity of IgG antibodies with hemophore-like proteins

We hypothesize that the HmuY protein may be a unique *P. gingivalis* marker because low amino acid identity has been found between HmuY and its closest homologs belonging to the HmuY family and identified in other periodontopathogens (Figures 1a-c) [15,17,20,21]. Although proteins from the HmuY family are structurally related (Figure 1d) [14,15,17], they significantly differ in amino acid sequences (Figure 1c) [20,21]. The highest structural similarity is found mainly in the core protein structures (Figures 1e,f), whereas the regions with the lowest amino acid sequence identity are localized on the proteins' surfaces [14,15,17]. This suggests the possibility of formation of various epitopes, which could be differentially recognized by the host immune system. Another hemophore-like protein produced by *P. gingivalis*, HusA, but not belonging to the HmuY family, exhibits a different three-dimensional protein structure (Figure 1g).

In the present study, we first aimed to find whether hemophore-like proteins produced by other periodontopathogens can also be recognized in vivo by the host immune system and how periodontitis influences production of antibodies raised against respective proteins. We demonstrated that all examined hemophore-like proteins (HmuY and its homologs, as well as HusA) were recognized by the host IgG antibodies as follows: PinA>HmuY>HusA>Tfo>PinO (Figures 2a–2c). It is worth mentioning here that in contrast to antibodies directed against HmuY, which were highly specific and recognized this protein only (Supplementary Figure S1A, C,D), cross reactivity can be found in the case of antibodies directed against PinA and Tfo (Supplementary Figure S1A,D).

The main findings of our study showed that, as expected, IgG antibodies present in sera of individuals with periodontitis reacted more strongly with *P. gingivalis* HmuY, as compared to IgG antibodies present in sera of individuals without periodontitis (Figure 2a). Interestingly, significantly higher reactivity was also determined with *P. intermedia* PinA in the group comprising individuals with periodontitis (Figure 2b). In relation to *P. intermedia* PinO, only slightly higher reactivity was detected in individuals with periodontitis (Figure 2b). In contrast to those findings, no difference was found for immunoreactivity of *T. forsythia* Tfo between the two examined groups (Figure 2c). Also, *P. gingivalis* HusA reacted with similar ability with IgG antibodies present in both sera derived from individuals with periodontitis and sera derived from individuals without periodontitis (Figure 2a).

It is also worth noting that reactivity of IgG antibodies with examined antigens varied between individuals with periodontitis and did not always correlate with the clinical status (data not shown). In addition, the greatest variation of results in the reactivity of IgG antibodies was observed in the case of PinA (Figure 2b).

We also aimed to examine whether synthetic peptides derived from the HmuY amino acid sequence [21] could be used in ELISA as antigens. Although

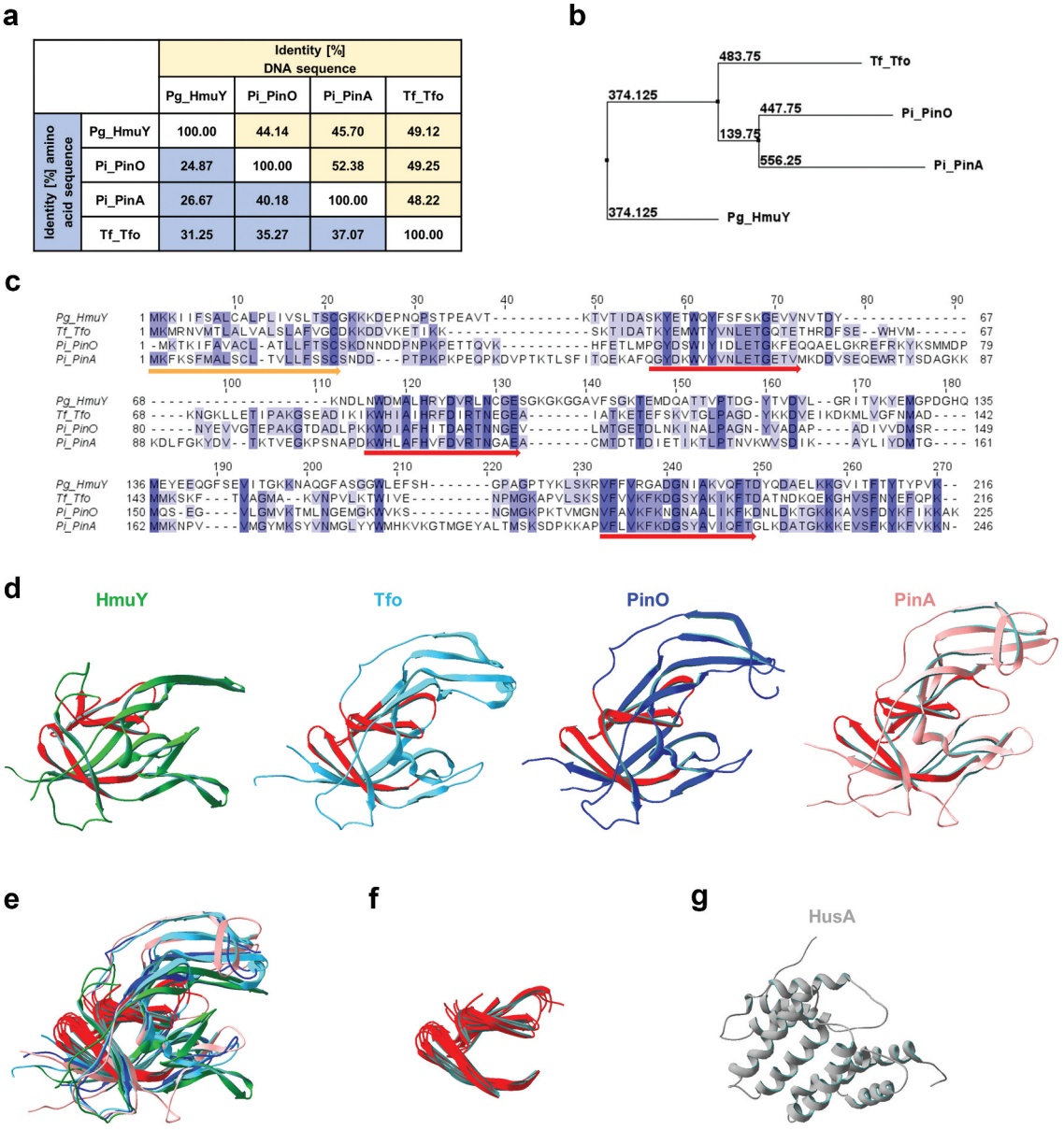

Figure 1.Comparison of amino acid and DNA sequences of the best-characterized hemophore-like proteins. Identity of amino acid and DNA sequences (a), phylogenetic tree (b) and amino acid alignment (c). Identical amino acids are shown in purple, highly homologous regions are indicated by red arrows. An orange arrow indicates signal peptides. Comparison of three-dimensional protein structures of HmuY and its homologs produced by periodontopathogens (d), their overlapped protein structures (e) or overlapped core protein structures (f), and HusA protein structure (g). Pg, *Porphyromonas gingivalis*; Tf, *Tannerella forsythia*; Pi, *Prevotella intermedia*.

the antibodies raised against peptides recognized the respective peptides specifically (Supplementary Figure S1B,C) [21], no reactivity of IgG antibodies present in pooled sera in both examined groups with peptides was found (Supplementary Figure S2).

Influence of periodontitis on reactivity of IgG antibodies with total periodontopathogens' antigens

Then, we aimed to examine how total antigens produced not only by *P. gingivalis* but also by other periodontopathogens are recognized in vivo by the host immune system and how periodontitis influences production of antibodies. The reactivity of IgG antibodies with antigens present in cell lysates was as follows: *P. intermedia*>*P. gingivalis*>*T. forsythia* (Figure 2a-c). We found that only *P. gingivalis* antigens elicited statistically significantly higher reactivity of IgG antibodies in sera of individuals with periodontitis as compared to sera of individuals without periodontitis (Figure 2a). We observed general low IgG reactivity in the case of total antigens present in cell lysates of *T. forsythia* (Figure 2c). In contrast to *P. gingivalis* and *T. forsythia* cell lysates, we demonstrated higher reactivity of *P. intermedia* antigens with IgG antibodies present in sera derived from non-periodontitis individuals, as compared to sera derived from individuals with periodontitis (Figure 2b).

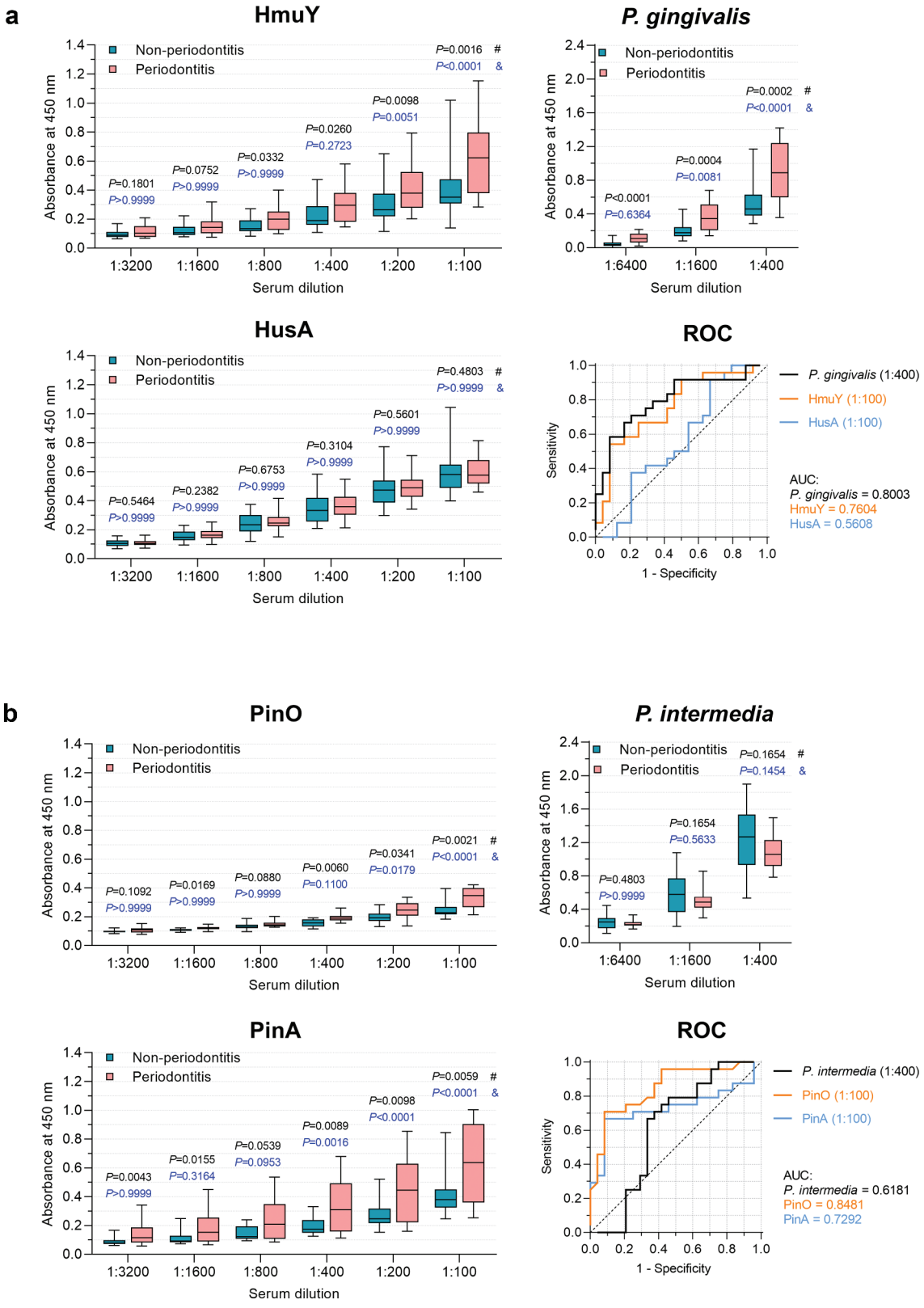

Figure 2. Reactivity of periodontopathogens' antigens with serum IgG antibodies. Immunoreactivity of examined antigens with IgG antibodies present in sera derived from individuals with periodontitis (periodontitis group) and sera from individuals without periodontitis (non-periodontitis group) was examined using enzyme-linked immunosorbent assay (ELISA). The following antigens were analyzed: *P. gingivalis* total antigens and purified HmuY protein or HusA protein (a), *P. intermedia* total antigens or purified PinO protein or PinA protein (b), and *T. forsythia* total antigens or purified Tfo protein (c). As total antigens, cell lysates were used. P values relate to the comparison between periodontitis group and non-periodontitis group at each dilution of sera. Values of $P<0.05$ were considered statistically significant. IgG responses in tested individuals against antigens were compared by generating a receiver operating characteristic (ROC) curve and calculation of the area under the curves (AUC). #P values calculated using Mann-Whitney U test; &P values calculated using two-way ANOVA test with post-hoc Bonferroni test.

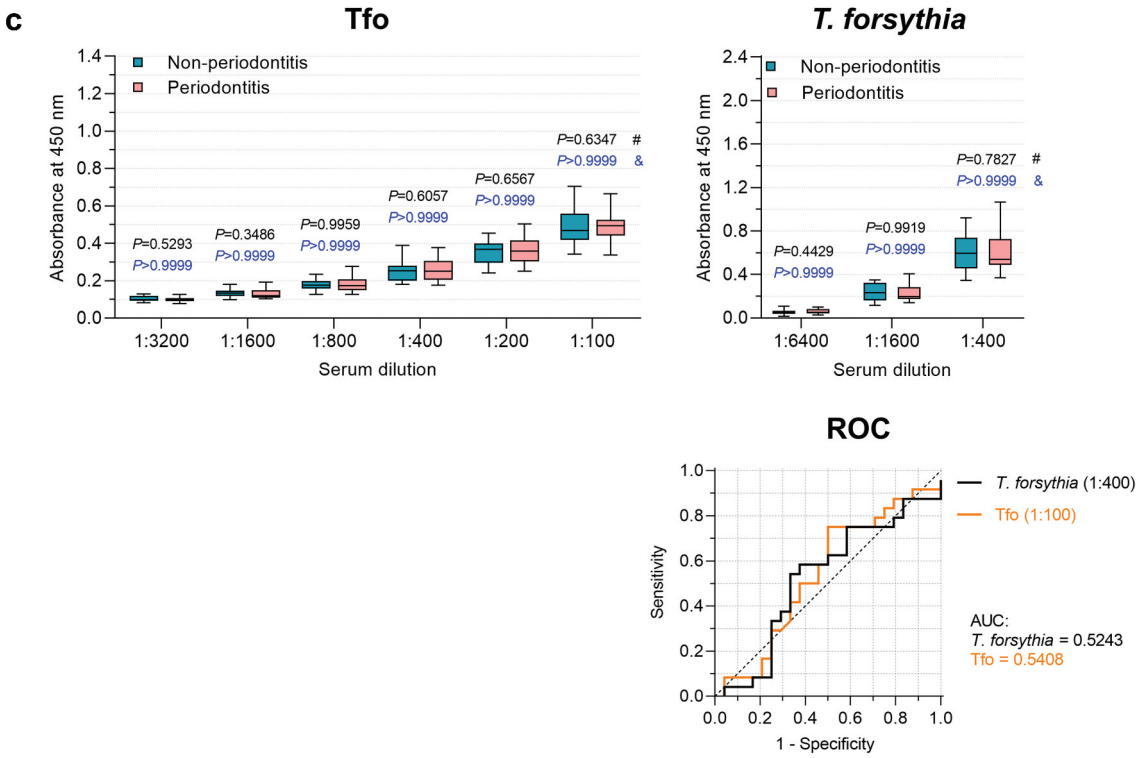

Figure 2.(Continued).

Similar to analysis carried out using purified proteins, reactivity of IgG antibodies with antigens present in *P. gingivalis* and *P. intermedia* cell lysates varied between individuals with periodontitis and did not always correlate with the clinical status (data not shown).

Choosing better antigen – purified proteins versus bacterial cell lysates

Based on our results it seems that HmuY could serve as a specific *P. gingivalis* antigen. First, antibodies raised against homologous proteins did not recognize HmuY protein (Supplementary Figure S1). Second, as shown in Figure 2a, analysis of an area under the ROC curve obtained for reactivity with HmuY protein demonstrated similar value as compared to the value obtained for *P. gingivalis* cell lysate (AUC 0.7604 versus 0.8003). In the case of PinA (AUC 0.7292) (Figure 2b), because of cross reactivity of antibodies directed against PinA and Tfo (Supplementary Figure S1), further analysis is required.

Discussion

In understanding the importance of our findings, it is necessary to remember that a variety of periodontopathogens' antigens are differentially recognized by the adaptive immune system, which results in the production of antibodies [30,31]. One such antigen is *P. gingivalis* HmuY protein, which is constitutively expressed, but significantly higher levels in both mRNA and protein forms are measured when bacteria grow in vitro in low-iron/heme conditions or as a biofilm constituent on abiotic surfaces, environments typical for the healthy oral cavity or early stages of periodontitis, as well as intracellularly in host cells [12,16,32]. Significantly higher HmuY expression in *P. gingivalis* grown in the form of a biofilm together with *Candida albicans* [33] and in a plaque formed in patients with periodontitis [6,34,35] confirms the importance of this protein in in vivo conditions. Similar to HmuY, also in *T. forsythia* and *P. intermedia* higher expression of homologous proteins was observed when bacteria were cultured in vitro in low-iron/heme conditions [15,17]. It is also worth mentioning that *P. gingivalis* HmuY protein is associated with the bacterial outer membrane through a lipid anchor but can also be shed as an intact, soluble protein as a result of the limited proteolytic processing performed by *P. gingivalis* lysine-specific gingipain Kgp [14,29,36], which allows its distribution in vivo. Other hemophore-like proteins can be shed from the bacterial surface, albeit with a lower efficiency [15,17,19].

Outer membrane vesicles produced by periodontopathogens contain virulence factors, which are released into the host environment and can be recognized as antigens. Among them are *P. gingivalis* HmuY and HusA [15,18,29,36–40], *T. forsythia* Tfo [15,35,36], *P. intermedia* PinO and PinA [17,41], and *P. gingivalis* gingipains [36,42]. It is also worth mentioning here that *P. gingivalis* outer

membrane vesicles are enriched in gingipains and HmuY, which constitute their main cargo [36,42]. Such distribution of hemophore-like proteins, especially HmuY, might be advantageous for their dissemination into the different host niches and therefore increase production of antibodies [43].

One should also take into consideration the fact that proteins belonging to the HmuY family differ in their stability and susceptibility to proteolysis [14,15,17,44]. Besides *P. gingivalis* HmuY, other hemophore-like proteins are easily degraded by *P. gingivalis* proteases. This means that they might not be exposed to the host immune system as long as HmuY. However, when *P. intermedia* dominates over *P. gingivalis*, this might promote PinA distribution, allowing for higher production of antibodies raised against this protein. Lower recognition of PinO, HusA and Tfo by the host immune system might suggest lower immunogenicity of these proteins.

Our preliminary study, using pooled sera samples, demonstrated that *P. gingivalis* HmuY protein elicited higher production of IgG antibodies in patients with periodontitis, suggesting that this antigen is available for recognition by the host immune system [22]. Using individually tested serum samples, we confirmed higher titers of IgG antibodies (mainly IgG1 subclass) raised against HmuY in sera of patients with periodontitis, as compared to healthy individuals [22]. Results obtained in the present study confirmed that HmuY may be a unique *P. gingivalis* marker. We also identified another antigen, namely PinA protein, which could serve as a marker of periodontitis. We assume that analysis carried out using a combination of these two potential markers could be more useful in the assessment of periodontitis progression and monitoring of the disease therapy. However, potential application of PinA requires further investigation, since cross reactivity was found in vitro between antibodies raised against PinA and Tfo.

It is worth noting that the reactivity of IgG with *P. gingivalis* antigens may vary between individuals with periodontitis, which may result, for example, from different numbers of particular bacterial species residing at diseased sites and genetic susceptibility of the host [45–47]. We observed such a discrepancy in this study (data not shown) and found it in our previous studies [46,47], where a dichotomy in the humoral response against *P. gingivalis* antigens within the group with periodontitis was observed. In the present study, lower reactivity of IgG antibodies with HmuY in sera derived from some individuals with periodontitis (data not shown) might suggest lower colonization by *P. gingivalis*. One may assume that other periodontopathogens, such as *P. intermedia*, are present in those individuals in higher numbers. This was true, but not for all individuals, and may be explained, at least in part, by higher immunoreactivity of serum IgG antibodies with *P. intermedia* PinA in those individuals.

Several reports have documented elevated levels of antibodies produced in individuals with periodontitis in response to total antigens present in periodontopathogens [45,46]. In contrast to *P. gingivalis* and *T. forsythia* cell lysates, we demonstrated higher reactivity of IgG antibodies present in sera derived from non-periodontitis individuals with total *P. intermedia* antigens, as compared to sera derived from individuals with periodontitis, which is partly consistent [48] and at the same time different [49] from other studies. This discrepancy could be explained, at least in part, by differences in the general characteristics of the participants in our study versus those in previous studies, such as the demographics. The age range of participants in our study was 20–43 in the non-periodontitis group and 24–56 in the periodontitis group, while the other studies presented results obtained from the examination of the elderly (age above 60) [48,49]. Another explanation for such an effect could be a difference in antigenic determinants in strains infecting patients [50]. *P. gingivalis* has the ability to alter the expression of antigens that are recognized by the host and may be important in resulting protection or susceptibility to periodontal diseases [51–56]. Differences in *P. gingivalis* phenotypes and pathogenicity are caused mainly by variation in capsular polysaccharides, the major and minor fimbriae, adhesion domains of gingipains and hemagglutinin A, and A-LPS structures [54,57–62]. On the contrary, *hmuY* gene and its product belong to highly conserved components [20,58]. Therefore, we assume that HmuY may be a marker that would be more useful because it is both specific for *P. gingivalis* but is likely to be universally recognized and conserved across all strains.

One might speculate that *P. intermedia* could suppress the host immune response, as it has been suggested by others [63], which results in lower reactivity of IgG antibodies with total antigens during the course of periodontitis, but, surprisingly, not with *P. intermedia* PinA protein. We also found general low IgG reactivity in the case of total antigens present in cell lysates of *T. forsythia*, suggesting that this bacterium produces antigens, which are poorly recognized by the host immune system, which is in accordance with other published reports [48,64].

We also demonstrated some reactivity with all antigens examined in this study in the group comprising individuals without periodontitis. However, this is not surprising since the opportunistic periodontopathogens can colonize a healthy oral cavity in low numbers or some cross-reactivity might occur [19,65,66]. Although we found cross reactivity between PinA and Tfo in vitro, analysis of their reactivity with serum IgG antibodies in individuals did not reflect such an effect.

Final remarks

Based on our results we suggest that determination of immunoreactivity against particular bacterial antigens, especially HmuY, could be a better marker of periodontitis as compared to the immunoreactivity against total bacterial antigens.

We are aware that our study has some limitations. First, the analysis has been performed on small groups, and in this type of studies, the sample size is an important limitation. Therefore, no detailed analysis on the correlation between clinical parameters and biological results has been performed and the present study should be treated as a preliminary report. Second, no microbiological analysis of oral samples has been performed. Therefore, no correlation between immunoreactivity of selected antigens and number of particular species has been carried out.

In conclusion, the findings of this initial study point to antigens, which could be further examined in relation to the host response and IgG production to develop potential markers of periodontitis. We assume that determination of reactivity of antibodies directed against *P. gingivalis* HmuY, and possibly against *P. intermedia* PinA, could be used in the future not only to examine progression of periodontitis but also to monitor the efficiency of therapy.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contribution

All authors have made substantial contributions to the conception and design of the study. EKNS, ISGF, and SCT have been involved in examination of participants and samples collection. All authors have been involved in data collection, data analysis and data interpretation. TO and SCT have been involved in drafting the manuscript. All authors have been involved in revising of the manuscript and given final approval of the version to be published.

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Data availability statement

All data generated in this study are included in this published article and are available from the corresponding author upon request.

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