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Salivary proteotypes of gingivitis tolerance and resilience

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Abstract

Aim: This study aimed to characterize the salivary proteome during the induction and resolution of gingival inflammation in the course of human experimental gingivitis (EG), and to cluster the proteomic profiles based on the clinically defined "slow" and "fast" response patterns.

Materials and Methods: A total of 50 unstimulated whole saliva were obtained from the EG model which was induced over 21 days (days 0, 7, 14 and 21), followed by a two-week resolution phase (day 35). Label-free quantitative proteomics using liquid chromatography–tandem mass spectrometry was applied. Regulated proteins were subject to Gene Ontology enrichment analysis.

Results: A total of 804 human proteins were quantified by ≥ 2 peptides. Principal component analysis depicted significant differences between "fast" and "slow" responders. Despite gingival and plaque scores being similar at baseline among the two groups, "fast" responders presented with 48 proteins that were at > 4-fold higher levels than "slow" responders. These up-regulated proteins showed enrichment in "antigen presentation" and "proteolysis."

Conclusions: Together, these findings highlight the utility of integrative systems-level quantitative proteomic approaches to unravel the molecular basis of "salivary proteotypes" associated with gingivitis dubbed as "fast" and "slow" responders. Hence, these differential responses may help prognosticate individual susceptibility to gingival inflammation.

KEYWORDS
biomarkers, experimental gingivitis, proteomics, saliva, salivary proteotypes

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1 | INTRODUCTION

Periodontitis progression is infrequent and episodic, with varying susceptibility across individuals (Hajishengallis & Korostoff, 2017). Susceptibility to periodontitis appears to be largely determined by the nature of the inflammatory host response and other causes like tobacco smoking. To identify what proximal make an individual susceptible to the disease, it is important to have a deep understanding of molecular basis for disease initiation, progression and resolution. Gingivitis can advance to periodontitis, but some individuals seem to be more tolerant to oral biofilm exposure than others (Shaw et al., 2016). However, the connection between characteristics of gingivitis and periodontitis pathogenesis remains controversial; a link between them has been postulated but not proved.

It is suggested that an individual host's resilience to resolve gingivitis may render them less susceptible to the progression to periodontitis (Freire & Van Dyke, 2013; Marsh & Zaura, 2017). Resilience is the ability of an ecosystem to revert to its original state following exposure to a potentially harmful stimulus. The resilience of the oral microbiome has been studied in response to external environmental factors, such as antibiotics and smoking (Joshi et al., 2014; Zaura et al., 2015). Resilience can also allude to the equilibrium of interactions between proteins, the disruption of which may fundamentally affect the outcome of any biological processes (Zitnik, Sosič, Feldman, & Leskovec, 2019). Inter-individual (and intra-individual) variability in the development of gingival inflammation has long been observed in experimental gingivitis (Loe, Theilade, & Jensen, 1965; Schincaglia et al., 2017). More recently, two different patterns of gingivitis development, dubbed "fast" and "slow" responders to plaque accumulation, have been identified and documented (Nascimento, Danielsen, Danielsen, Baelum, & Lopez, 2019). The key difference between "fast" and "slow" responders is related to the time required to develop clinical signs of gingival inflammation given similar amount of plaque is accumulated. While most host response variations during onset of experimentally induced gingivitis (gingival phenotypes) have been attributed to the gene–environment interactions (Jönsson et al., 2011; Yu et al., 2015), mechanistic insights into the host protein network disturbance or proteome resilience remain elusive. Although the assessment of gingival crevicular fluid (GCF) proteome in experimental gingivitis models gave very important insights on host and microbial protein dynamics in the periodontium, these examinations were limited to only few sites in a given individual and did not attempt to cluster into different clinical phenotypes (Bostanci et al., 2013; Grant et al., 2010). Accordingly, it remains unclear whether and to what extent differences in gingival inflammatory profiles could be attributed to identifiable "protein signatures/proteotypes" among individuals with different response patterns of gingival inflammation development from oral biofilm accumulation. Furthermore, saliva, compared to gingival tissue or GCF, allows for a simple, non-invasive and repetitive collection and subsequently for longitudinal analyses, and it provides a reflection of the overall inflammatory profile in the oral cavity rather than a site-specific view (Öztürk, Belibasakis, Emingil, & Bostanci, 2016; Taylor & Preshaw, 2016; Willi, Belibasakis, & Bostanci, 2014; Zhang et al., 2016). Hence, finding proteins in saliva by application of contemporary proteomics technologies that provide biological evidence of gingivitis susceptibility may help diagnose early disease and identify susceptible populations. Here, we provide novel evidence by use of contemporary high-throughput quantitative proteomics supported with protein network tools to unravel the molecular basis of salivary host response patterns in response to oral biofilm accumulation (induction of gingivitis) and removal (resolution of gingivitis).

Clinical relevance

Scientific rationale for the study: Understanding the inter-individual variability in the degree of gingival inflammation in response to abstinence of oral hygiene practices requires a deep knowledge of the molecular basis for disease initiation, progression and resolution. Two clinically defined host types ("slow" and "fast" responders) with distinct susceptibility to gingivitis are known.

Principal findings: The characterization of the salivary proteome during the experimental induction and resolution of gingival inflammation revealed significant differences between "fast" and "slow" responders.

Practical implications: Inflammation control was not a predictable end point in the present study. These differential responses may help prognosticate the individual susceptibility to gingival inflammation and may help diagnose early disease.

2 | MATERIALS AND METHODS

2.1 | Study population and design

The study was approved by the Ethics Committee of Central Denmark (number 1-10-72-402-14) (Nascimento, Baelum, et al., 2019; Nascimento, Danielsen, et al., 2019; Silbereisen, Hallak, et al., 2019) and conforms to the STROBE guidelines for observational studies. Details regarding the selection of study participants and saliva collection are depicted in Supporting File 1 (Nascimento, Baelum, et al., 2019; Nascimento, Danielsen, et al., 2019). Based on the development of gingival inflammation, and using factor analysis, participants were classified into "fast" and "slow" responders (Nascimento, Danielsen, et al., 2019).

2.2 | Label-free quantitative proteomic analysis

Label-free quantification (LFQ) was performed by the Progenesis QI for Proteomics software (version 4.1, Nonlinear Dynamics, Newcastle upon Tyne, UK) as previously described (Bostanci et al., 2010, 2013,

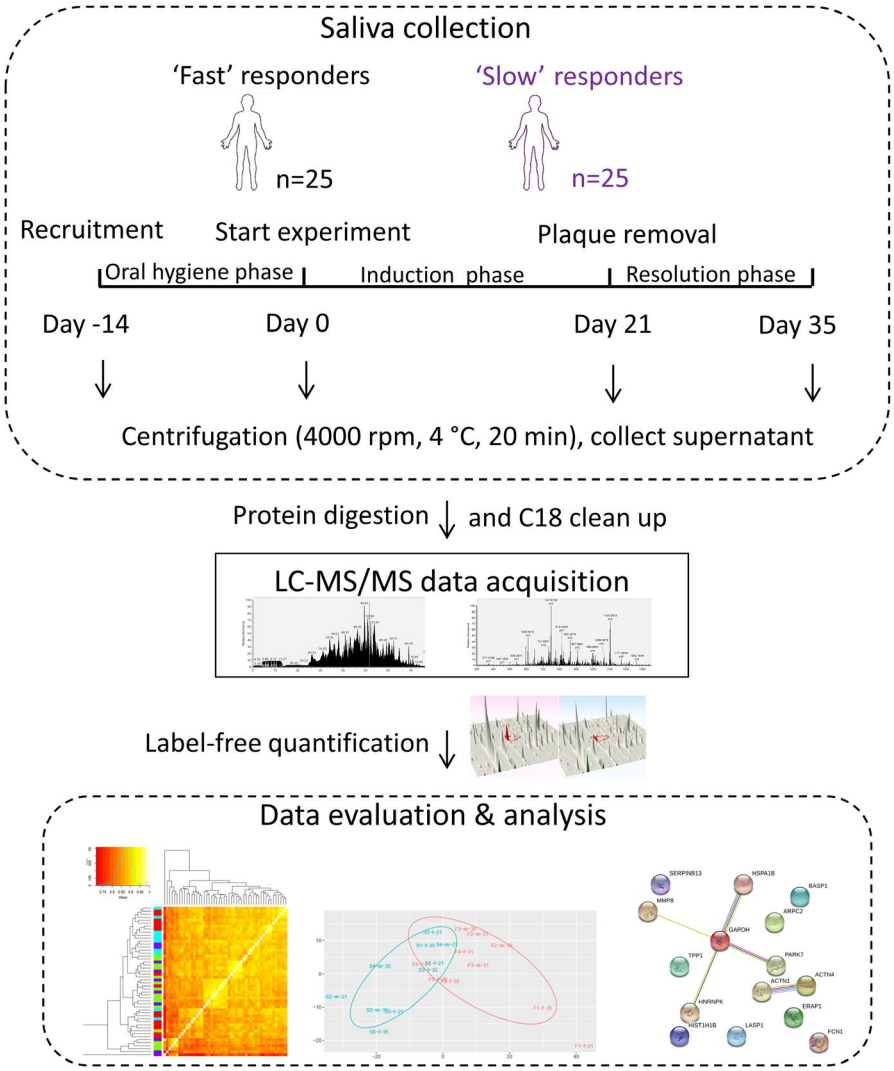

FIGURE 1 Schematic representation of the study design from saliva collection to protein discovery using label-free quantitative (LFQ) proteomics

2018). Details regarding the LFQ proteomics protocol and liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis are depicted in Supporting File 1. The entire workflow, from saliva collection until data evaluation and analysis, is illustrated in Figure 1. Supporting MS proteomics data are accessible via the PRIDE (Perez-Riverol et al., 2019) partner repository (dataset identifier: PXD015220).

2.3 | Statistical and bioinformatic analysis

To analyse the protein expressions, significant differences between pairwise comparisons were conducted using Progenesis QI based on the hyperbolic arcsine-transformed normalized abundances of quantified proteins. Proteins with peptides ≥ 2 and p-value < .05 were considered as statistically differentially regulated in different conditions. Proteins in "fast" versus "slow" responder comparisons were considered significantly regulated with peptides ≥ 2, p-value < .05 and fold changes ≥ 4. The significantly regulated proteins between the different groups and experimental phases of the model were subject to analyses for process network and GO process enrichment, and protein–protein interactions. Details are depicted in Supporting File 1.

3 | RESULTS

3.1 | Clinical findings

The current study analysed 50 saliva samples and included "fast" responders (mean age: 22.0 years; F:M: 3:2), "slow" responders (mean age: 24.0 years; F:M: 3:2) and total sample ("fast" and "slow" responders combined, mean age: 23.1 years; F:M: 6:4). The mean gingival inflammation (MGI) and plaque (TQHPI) scores for "fast" and "slow" responders during the induction and resolution phases

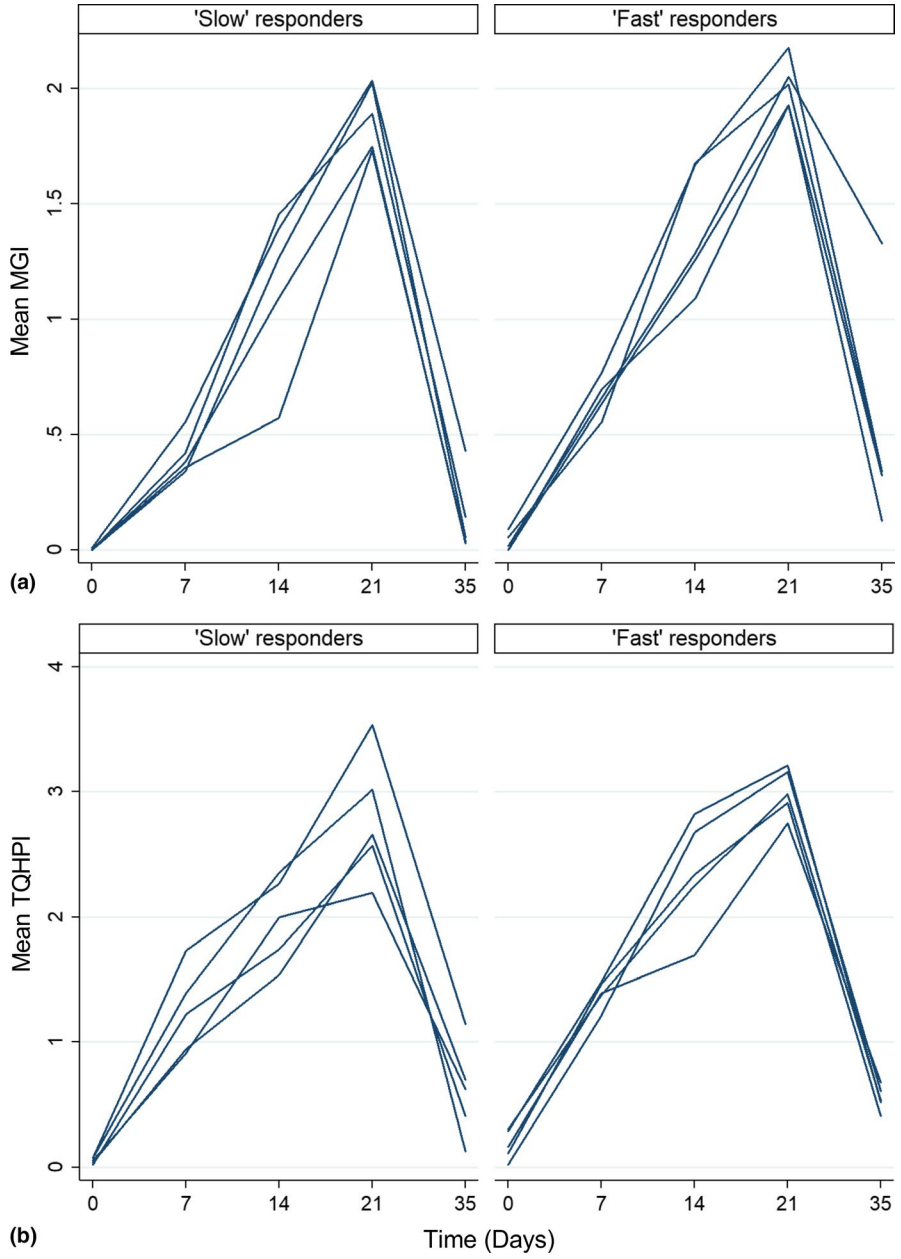

FIGURE 2 Gingival inflammation (MGI, a) and plaque (TQHPI, b) scores (Mean ± *SD*) for the two trajectories, "fast" responders (n = 5) and "slow" responders (n = 5), during the induction (days 0, 7, 14 and 21) and resolution (days 21 and 35) phases of experimentally induced gingivitis (Original cohort [n = 42]: Nascimento, Baelum, et al., 2019; Nascimento, Danielsen, et al., 2019)

of gingival inflammation are summarized in Figure 2 and Supporting File 2 (Silbereisen, Alassiri, et al., 2019).

3.2 | Protein quantification

LFQ overall identified 897 proteins (13,418 peptides) among all (total sample n = 50) saliva samples. Eighty-six human proteins were quantified by one peptide, while 804 human proteins were quantified by ≥ 2 peptides (proFDR = 0.75%) (Supporting File 3), after exclusion of reverse sequences and contaminants (rev = 6, contaminants = 15). The latter were used for further comparative analysis.

3.3 | Principal component analysis (PCA) and heat maps

Protein abundances of the 804 quantified human proteins are presented in the heat maps of Figure 3, with proteins either depicted in a correlation matrix (Figure 3a) or clustered in rows and samples in columns (Figure 3b). The scale of Figure 3a represents the

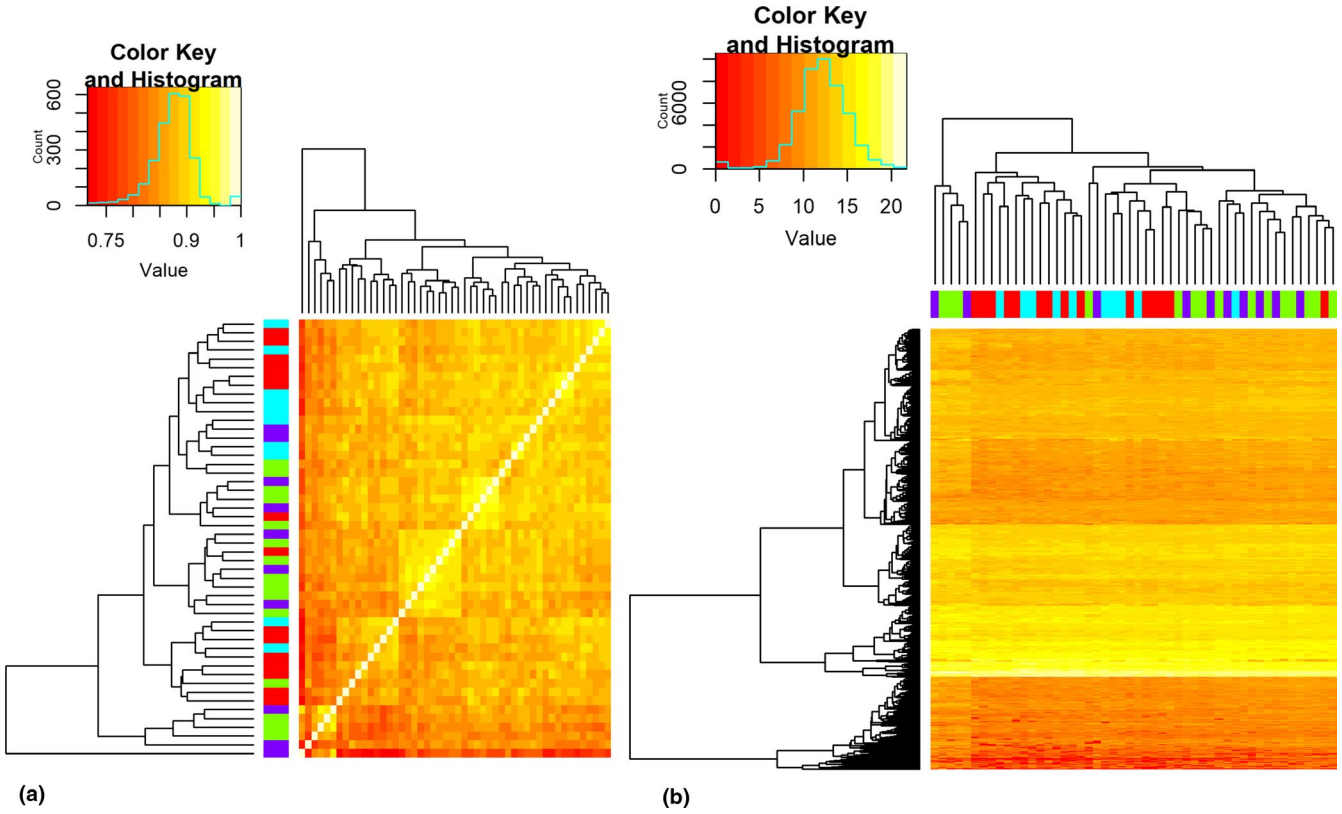

FIGURE 3 Heat maps presenting arcsinh-normalized protein abundances of the 804 quantified proteins in a correlation matrix (a) and normalized protein abundances of the 804 quantified proteins clustered in rows and samples in columns (b). Abundance range: low (red) to high (yellow). "Fast" responders (n = 5): induction phase (red; days 0, 7, 14 and 21) and resolution phase (blue; days 21 and 35). "Slow" responders (n = 5): induction phase (green; days 0, 7, 14 and 21) and resolution phase (purple; days 21 and 35)

correlation of two samples based on their arcsinh-normalized protein abundance levels, while for Figure 3b, the scale represents the normalized protein abundances, both ranging from low (red) to high (yellow) protein abundances.

To determine the capability of the saliva proteome to predict the two trajectories of gingival inflammation development ("fast" and "slow" responders), the 804 quantified human proteins were further analysed using principal component analysis (PCA)-based clustering (Figure 4). "Fast" and "slow" responders separated from each other, during both the induction and resolution phases.

3.4 | Protein dynamics and protein network analysis in "slow" and "fast" responders during induction and resolution of gingivitis

3.4.1 | Induction and resolution phases

Out of 804 quantified proteins, in "fast" responders, 15 (induction) and 22 (resolution) proteins, and in "slow" responders, 35 (induction) and 39 (resolution) proteins were regulated (p < .05) (Table 1, Supporting File 4). "Slow" responders shared two regulated common proteins between the induction and resolution phase, whereas "fast" responders shared none (Supporting File 5). Comparisons between "fast" responders and "slow" responders revealed one shared protein during the induction phase and three shared proteins during the resolution phase. All three groups (total sample, "fast" responders and "slow" responders) shared one common protein during the induction phase (ficolin-1) and three proteins during the resolution phase (transthyretin, zymogen granule protein 16 homolog B and pigment epithelium-derived factor). Additional results for the total sample are provided in Supporting File 2.

The analysis of process networks identified "actin filaments," "protein folding nucleus" and "synaptic contact" (induction), and "blood coagulation", "innate inflammatory response" and "IL-6 signaling" (resolution) as the most significantly regulated categories (top three) in "fast" responders (Table 2, Supporting File 6). The specific proteins involved in these process networks were LIM and SH3 domain protein 1 (LASP1), alpha-actinin-1, alpha-actinin-4, actin-related protein 2/3 complex subunit 2 (ARPC2), HPA1B and DJ-1 (induction), and annexin V, fibrinogen-alpha, fibrinogen-beta, fibrinogen-gamma, BPI fold-containing family A member 1 (PLUNC), monocyte differentiation antigen CD14 and sCD14 (resolution) (Supporting File 6). In "slow" responders, "IL-6 signaling," "blood coagulation" and"antigen presentation" (induction), and "protein folding in normal condition", "response

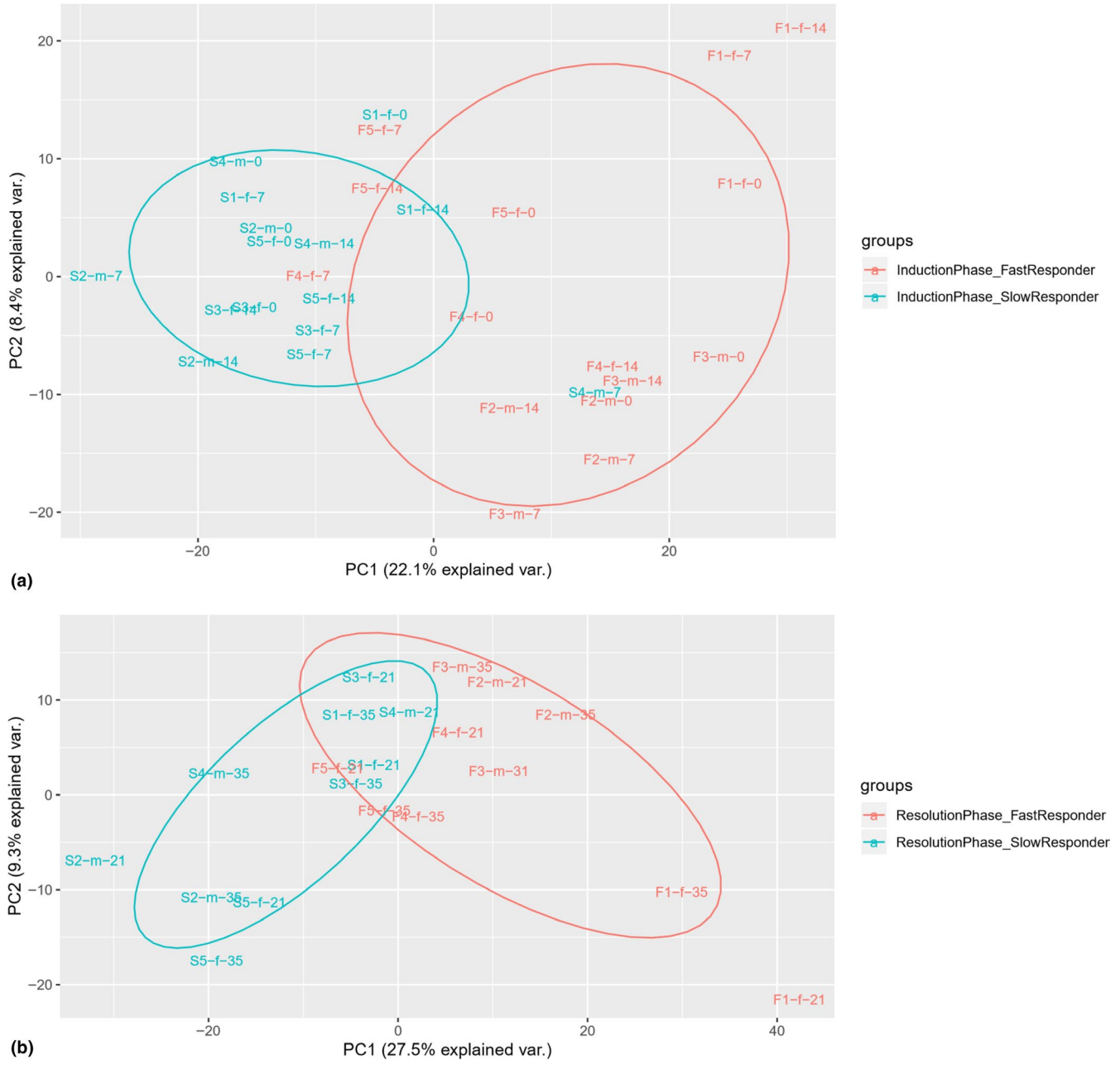

FIGURE 4 Clustered principal component analysis (PCA) showing sample grouping based on protein abundances of the 804 quantified proteins during the induction phase (a) and the resolution phase (b). "Fast" responders (red) and "slow" responders (blue)

to unfolded proteins" and "translation in mitochondria" were the top three significantly regulated process networks. The specific proteins involved were hemopexin, alpha-1-antitrypsin, fibrinogen-alpha, fibrinogen-beta, thrombospondin 1, haptoglobin (HP), CCAAT/enhancer-binding protein beta (C/EBP-beta), proteasome subunit alpha type-1 (PSMA1), proteasome subunit alpha type-2 (PSMA2), protein disulphide-isomerase A3 (PDIA3) and cathepsin L (induction), and endoplasmin, FKBP12, heat-shock 27 kDa protein (HSP27), heat-shock protein 90-alpha (HSP90-alpha) and GlyRS (resolution).

3.4.2 | "Induced" and "resolved" gingival inflammation versus no inflammation

In addition, protein regulation ($p < .05$) and process networks were investigated between individual days compared to day 0 ("non-inflamed" state). In "fast" responders, 39 (19 up, 20 down) (day 21 vs. day 0) and 51 (40 up, 9 down) (day 35 vs. day 0) proteins were identified to be regulated (Table 1, Supporting File 4). "Slow" responders demonstrated 52 (40 up, 12 down) (day 21 vs. day 0) and 57 (42 up, 15 down) (day 35 vs. day 0) regulated proteins. In

TABLE 1 List of regulated proteins

Regulated proteins (up/down)*	Total sample (*n* = 50)	"Fast" responders (*n* = 25)	"Slow" responders (*n* = 25)
Induction phase (Days 0, 7, 14 and 21)	65	15	35
Resolution phase (Days 21 and 35)	34 (30/4)	22 (20/2)	39 (13/26)
"Induced gingival inflammation" vs. "non-inflamed"			
Day 7 vs. Day 0	51 (8/43)	64 (45/10)	52 (23/29)
Day 14 vs. Day 0	29 (8/21)	21 (8/13)	42 (25/17)
Day 21 vs. Day 0	70 (41/29)	39 (19/20)	52 (40/12)
"Resolved gingival inflammation" vs. "non-inflamed"			
Day 35 vs. Day 0	92 (68/24)	51 (40/9)	57 (42/15)
Regulated proteins (up/down)**	"Fast" vs. "Slow" responders		
Induction phase (Day 0, 7, 14, 21)	25		
Resolution phase (Day 21, 35)	35 (35/0)		
Day 0	50 (48/2)		
Day 7	6 (4/2)		
Day 14	35 (32/3)		
Day 21	10 (10/0)		
Day 35	29 (29/0)		

*$p < .05$.

**$p < .05$, fold change ≥ 4.

"fast" responders "protein folding ER and cytoplasm," "response to unfolded proteins" and "ESR1-nuclear pathway" (day 21 vs. day 0), and "DNA damage BER-NER repair," "regulation of cytoskeleton rearrangement" and "BMP TGF-β signalling" (day 35 vs. day 0) were the top three significantly regulated process networks (Table 2, Supporting File 6). In "slow" responders, the top three regulated process networks were "MIF signalling," "regulation of angiogenesis" and "mRNA processing" (day 21 vs. day 0), and "response to hypoxia and oxidative stress," "cadherins" and "mRNA processing" (day 35 vs. day 0). The following proteins were involved in these processes: Heat-shock cognate 71 kDa protein (HSC70), protein disulphide-isomerase (P4HB), endoplasmic reticulum chaperone BiP (GRP78), heat-shock 27 kDa protein (HSP27) and lactoferrin ("fast," day 21 vs. day 0); ubiquitin receptor RAD23B, DNA-(apurinic or apyrimidinic site) lyase (APEX), transforming protein RhoA, radixin, ADP-ribosylation factor 3 (ARF3), actin, alpha cardiac muscle 1 (ACTC), thymosin beta-10, 14–3–3 protein eta and homeodomain-only protein (LAGY) ("fast," day 35 vs. day 0); mitogen-activated protein kinase 1 (ERK2), acidic leucine-rich nuclear phosphoprotein 32 family member A (PHAP1), protein S100-A7, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), hnRNP A2 and thrombospondin 1 ("slow," day 21 vs. day 0); and heat-shock protein HSP90-beta, catalase, superoxide dismutase [Cu-Zn] (SOD1), extracellular superoxide dismutase [Cu-Zn] (SOD3), glutathione S-transferase omega-1 (GSTO1), desmocollin 3, desmoglein 1, alpha-actinin 4, hnRNP A2 and polypyrimidine tract-binding protein 1 (PTBP1) ("slow," day 35 vs. day 0) (Supporting File 6).

3.4.3 | "Slow" versus "fast" responders

Comparing the two response patterns, "fast" and "slow" responders, 25 and 35 proteins were differentially regulated ($p < .05$, fold changes ≥ 4) during the induction and resolution phases, respectively (Table 1, Supporting File 4). The top three regulated process networks between "fast" and "slow" responders were "interferon signaling," "hemopoiesis, erythropoietin pathway" and "cell cycle G2-M" in the induction phase, and "complement system" "translation in mitochondria" and "mRNA processing" in the resolution phase (Table 2, Supporting File 6). Individual day comparisons for days 0, 7, 14, 21 and 35 revealed 50 (48 up, 2 down), 6 (4 up, 2 down), 35 (32 up, 3 down), 10 (10 up, 0 down) and 29 (29 up, 0 down) regulated proteins ($p < .05$, fold changes ≥ 4), respectively, between "fast" and "slow" responders (Table 1, Supporting File 4). At day 0, the top three regulated process networks between "fast" and "slow" responders were "phagosome in antigen presentation", "antigen presentation" and "ubiquitin-proteasomal proteolysis" involving proteasome subunit alpha type-1 (PSMA1), PSMA4, PSMA5, PSMA6, proteasome subunit beta type-1 (PSMB1), PSMB3, PSMB6, endoplasmin, heat-shock protein 90-beta (HSP90-beta), alpha-actinin 4 and protein disulphide-isomerase A4 (ERp72) (Table 2, Supporting File 6).

3.5 | Protein–protein interactions

Protein–protein interactions of the differentially expressed proteins in "fast" and "slow" responders and experimental phases of

TABLE 2 List of top three regulated process networks

Process networks	Total sample (*n* = 50 from 10 individuals)	"Fast" responder sample (*n* = 25 from 5 individuals)	"Slow" responder sample (*n* = 25 from 5 individuals)
Induction phase (Day 0, 7, 14, 21)	IL-6 signalling, Th17-derived cytokines, DNA damage BER-NER repair	Actin filaments, protein folding nucleus, synaptic contact	IL–6 signalling, blood coagulation, antigen presentation
Resolution phase (Day 21, 35)	Blood coagulation, IL-6 signalling, anti-apoptosis mediated by external signals via PI3K/AKT	Blood coagulation, innate inflammatory response, IL-6 signalling	Protein folding in normal condition, response to unfolded proteins, translation in mitochondria
"Induced gingival inflammation" vs. "non-inflamed"			
Day 7 vs. Day 0	Cell–matrix interactions, Jak-STAT pathway, insulin signalling	Phagocytosis, integrin-mediated cell–matrix adhesion, leucocyte chemotaxis	Innate inflammatory response, chemotaxis, iron transport
Day 14 vs. Day 0	Cell–matrix interactions, manganese transport, protein folding nucleus	Protein folding nucleus, apoptotic nucleus, transcription by RNA polymerase II	Manganese transport, BMP and GDF signalling, cell–matrix interactions
Day 21 vs. Day 0	ESR1-nuclear pathway, Th17-derived cytokines, proteolysis in cell cycle and apoptosis	Protein folding ER and cytoplasm, response to unfolded proteins, ESR1-nuclear pathway	MIF signalling, regulation of angiogenesis, mRNA processing
"Resolved gingival inflammation" vs. "non-inflamed"			
Day 35 vs. Day 0	Platelet aggregation, blood vessel morphogenesis, ubiquitin-proteasomal proteolysis	DNA damage BER-NER repair, regulation of cytoskeleton rearrangement, BMP TGF-β signalling	Response to hypoxia and oxidative stress, cadherins, mRNA processing
Process networks	"Fast" vs. "Slow" responders		
Induction phase (Day 0, 7, 14, 21)	Interferon signalling, hemopoiesis, erythropoietin pathway, cell cycle G2-M		
Resolution phase (Day 21, 35)	Complement system, translation in mitochondria, mRNA processing		
Day 0	Phagosome in antigen presentation, antigen presentation, ubiquitin-proteasomal proteolysis		
Day 7	IFN-γ signalling, cell cycle G1-S, innate inflammatory response		
Day 14	Interferon signalling, cell cycle S phase, inflammasome		
Day 21	mRNA processing, oxytocin signalling, apoptotic mitochondria		
Day 35	IgE signalling, translation in mitochondria, cell cycle G2-M		

the model were investigated using the STRING database (Szklarczyk et al., 2017). Figure 5 summarizes the protein interactions for "fast" and "slow" responders during both the induction and resolution phases. In "fast" responders, 5 (among 7 proteins) and 19 (among 24 proteins) pairs of protein interactions were identified in the induction and resolution phases, respectively, while in "slow" responders, 37 (13 proteins) and 50 (27 proteins) pairs of interaction were discovered (Supporting File 7). The analysis revealed that some proteins interacted with much more other regulated proteins than the others and served as "centre nodes" among the protein interaction networks. In "fast" responders, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was one of these centre nodes that strongly over-connected with many proteins regulated in the induction phase, including important immune regulators such as matrix metalloproteinase-8 (MMP8) and heat-shock protein family A member 1B (HSPA1B). During the resolution phase, fibrinogen-alpha chain (FGA) and fibrinogen-gamma chain (FGG) were the centre nodes, and both interacted with 6 other proteins. In "slow" responders,

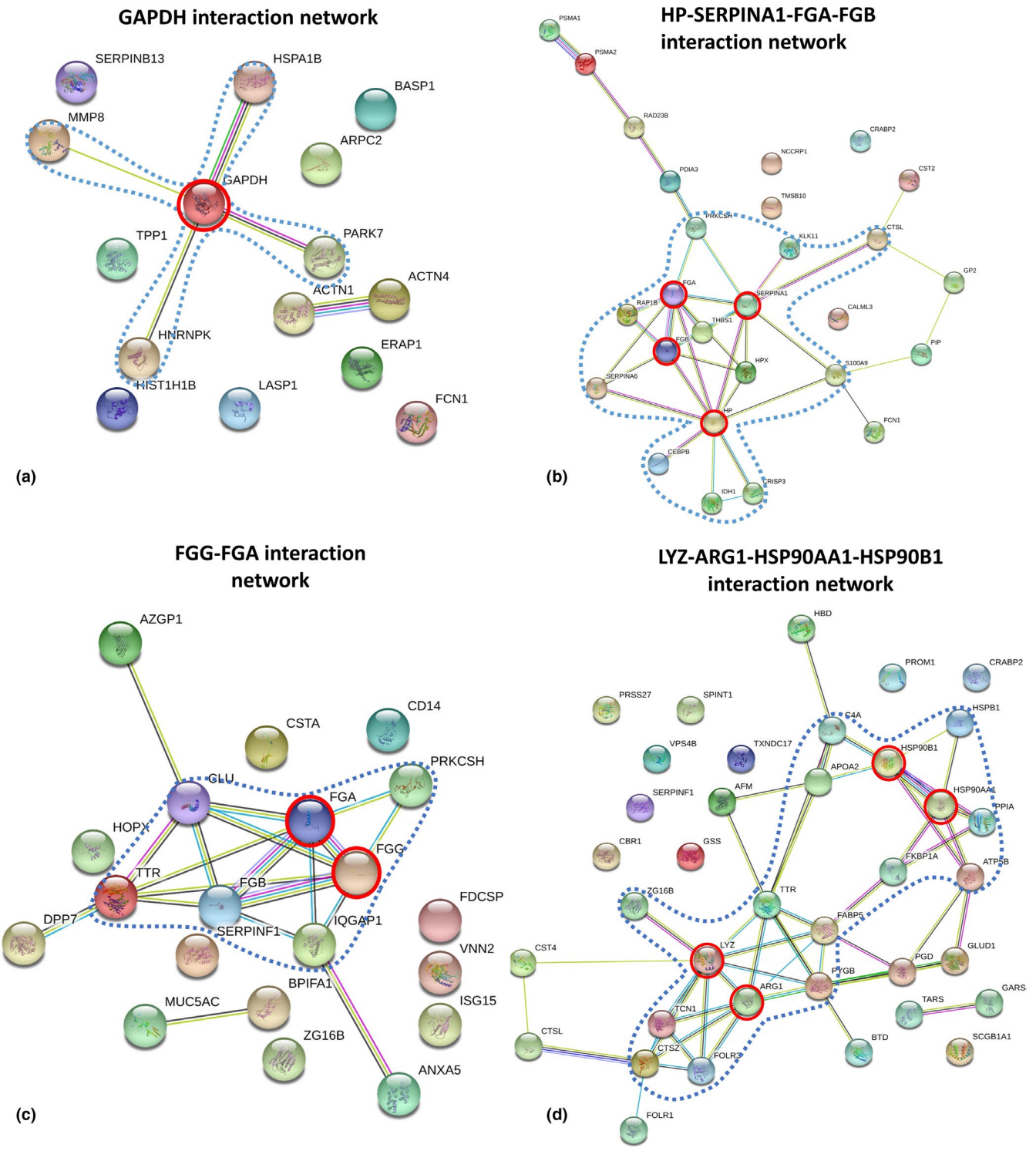

FIGURE 5 STRING visualization of the regulated proteins in "fast" responders (A + C) and "slow" responders (b + d) during the induction (a + b) and resolution (c + d) phases. The nodes represent the proteins and the connecting lines the STRING interactions. Network established using STRING 10.5 (Supporting File 6) based on the medium confident (0.4) of regulated tissue proteins with stringent cutoff. Lines indicate different types of protein–protein interactions. Blue and purple lines indicate known interaction determined from the curated database and experimental results, respectively. Green, red and dark blue lines indicate predicated interaction determined from gene neighbourhood, gene fusions and gene co-occurrence, respectively. Yellow, black and light blue lines indicate interactions from text mining, co-expression and protein homology, respectively. Centre nodes were highlighted in red circles, while their interacted proteins were circled within blue dash lines

the top centre nodes for the induction phase were haptoglobin (HP), alpha-1-antitrypsin (SERPINA1), fibrinogen-alpha chain (FGA) and fibrinogen-beta chain (FGB), which interacted with 13, 9, 8 and 7 other regulated proteins, respectively. During the resolution phase, the top regulated proteins were lysozyme C (LYZ), arginase-1 (ARG1), endoplasmin (HSP90B1) and heat-shock protein HSP 90-alpha (HSP90AA1), which interacted with 9, 7, 6 and 5 other regulated proteins, respectively.

4 | DISCUSSION

Inter-individual variability in the degree of gingival inflammation has long been noticed in response to abstinence of oral hygiene practices and traditionally been attributed to an "individual host response" (Lang, Oberling, Giron, & Mayer, 1977). These variations during the onset of experimentally induced gingivitis ("gingival phenotypes") have been attributed to the gene–environment interactions (Joshi et al., 2014). Two host types ("slow" responders vs. "fast" responders) have been identified, with distinct sensitivity/susceptibility to gingivitis. "Fast" responders feature averagely over two times more acute disease development than "slow" responders, whereas the heterogeneity of plaque microbiota among hosts may in part explain the interhost phenotypic variations of gingivitis sensitivity and possibly susceptibility to disease reoccurrence (Huang et al., 2014).

Mechanistic insights into the involved protein networks and processes that confer susceptibility or resilience to gingival inflammation in some individuals remain elusive. Here, we provide novel evidence by use of contemporary high-throughput quantitative proteomics, supported with process network and GO process enrichment tools, to unravel the molecular basis of the long-observed inter-individual susceptibility to gingival inflammation, previously dubbed as "fast" and "slow" responders. The release of host proteins into saliva appears to conform the "fast" and "slow" response patterns during both the induction and resolution phases (Bikker et al., 2019; Nascimento, Baelum, et al., 2019; Silbereisen, Hallak, et al., 2019). The "slow" responders can be considered as individuals less sensitive to the development of inflammation in response to microbial challenge. The most marked salivary proteome differences between the two response groups were observed at baseline, as early as one week after abstinence of oral hygiene (i.e. biofilm accumulation on tooth surface), and persisted after the reinstatement of oral hygiene and the reestablishment of "gingival health" ("resolved gingival inflammation"). Whether the salivary proteome at baseline might predict susceptibility to future gingivitis remains an intriguing possibility for future follow-up studies.

Overall, the process network and GO process enrichment analyses supported a starkly deregulated neutrophil-mediated immunity in the "fast" responders. The "slow" responders displayed more "regulated" proteins in their saliva, compared to "fast" responders (35 vs. 15 proteins), which may seem at odds with that the former group tends to demonstrate less clinical changes over the same period of time (van der Veen, Volgenant, Keijser, Ten Cate, & Crielaard, 2016). More interestingly, at the peak of inflammation (day 21) when gingival inflammation is established, the "slow" responders displayed more up-regulated proteins as compared to baseline (day 0) in their saliva, while during the same period, the "fast" responders presented with more than 50% of the detected proteins being down-regulated. Protein down-regulation could either be an active cellular process or simply the result of enzymatic degradation of proteins by proteolytic bacteria following secretion by the host cells (Bostanci et al., 2015; Silbereisen, Hallak, et al., 2019). Remarkably, specific microbiological patterns alone in saliva may not adequately explain the distinguishment of the inflammatory response patterns in experimental gingivitis (Holm-Pedersen, Agerbaek, & Theilade, 1975; Johnson, Reinhardt, Payne, Dyer, & Patil, 1997).

We further studied whether different response patterns share common or distinct biological processes. As the first week is decisive in differentiating clinical response patterns, we identified which salivary protein networks are highly regulated during this period. Despite MGI and TQHPI scores (Nascimento, Danielsen, et al., 2019) being similar at day 0 among the two clinically defined response groups, "fast" responders presented with 48 proteins that were at > 4-fold higher levels than "slow" responders. The top three regulated process networks associated with these proteins were "phagosome in antigen presentation", "antigen presentation" and "proteolysis." Higher proteolytic activity in saliva may be related to specific salivary microbial clusters, representing different oral ecotypes (Zaura et al., 2017). A proteolytic and proinflammatory saliva profile may commensurate an early dysbiosis.

Interestingly, during the induction phase, the GO processes of proteins regulated in the "fast" responders were mainly represented by "positive regulation of interleukin (IL)-8 production." These findings further delineate the role of specific processes or protein networks (such as HSPA1B, HSPA1A, Ficolin-1, DJ-1, HP70) in excessive gingival inflammation. Interleukin-8 is a well-characterized chemokine produced mainly by gingival epithelial cells in response to oral biofilm exposure (Belibasakis, Thurnheer, & Bostanci, 2013) and presents a concentration gradient within the inflamed periodontal tissues that facilitate neutrophil recruitment in the gingival crevice (Darveau, 2010). In "fast" responders, all metabolic enzymes mapping to the glycolysis/gluconeogenesis pathway were also up-regulated, potentially securing high amounts of energy for polymorphonuclear leucocyte (PMN) migration in this population. In contrast, "slow" responders may have a more homeostatic glycolic local environment in their gingival tissues during inflammation. Indeed, the regulation of glucose/insulin responses in chronic inflammation associated with periodontitis is not as pronounced as in acute inflammation (Yu et al., 2015). In a recent experimental gingivitis study, one-third of the subjects displayed severe gingival inflammation with an exaggerated influx of PMNs, whereas the remaining two-thirds experienced a lower degree of inflammation and minimal PMN influx (Wellappuli et al., 2018), corroborating the results of the present study. The "slow" responders displayed absence of certain key proteins such as C/EBP-beta, C5a, ceruloplasmin and lipocalin 2,

which are involved in the regulation of genes involved in inflammatory responses (Bassoy, Towne, & Gabay, 2018; Chinery, Brockman, Dransfield, & Coffey, 1997; Kinoshita, Akira, & Kishimoto, 1992; Pless et al., 2008; Roy et al., 2002). Further, high concentrations of IL-36 may amplify the expression of antimicrobial proteins by gingival epithelial cells, thereby prohibiting bacterial growth (Heath, Scholz, Veith, & Reynolds, 2019). The combination of increased proinflammatory cytokines and reduced acute phase response proteins could shift the homeostatic equilibrium to render the tissue towards a more resilient biofilm challenge over longer periods without pronounced inflammatory damage (Reddi & Belibasakis, 2012; Westerlund et al., 1996).

Differences in the salivary proteomes of "fast" and "slow" responders were also observed, once the oral hygiene practices were restored (the resolution phase). At day 35 (two weeks after oral hygiene instilment), clinical inflammation was resolved, yet approximately 50 proteins remained regulated in both groups, compared to baseline. In particular, among "slow" responders, 2/3 of regulated proteins were down-regulated at day 35 compared to day 21. In the "fast" responder group, proteins were mainly up-regulated at day 35 compared to day 21, whereas the only two proteins whose levels were "decreased" were ubiquitin-like protein interferon-stimulated gene 15 (ISG15) and dipeptidyl peptidase 2, a serine peptidase. ISG15 is secreted by granulocytes and lymphocytes, and promotes secretion of Interferon (IFN)-γ by natural killer (NK) cells, thus exerting an anti-mycobacterial activity (Swaim, Scott, Canadeo, & Huibregtse, 2017). Reduction of ISG15 during the course of periodontal inflammation may denote enhanced susceptibility of the host to endogenous microbiota of the accumulated biofilm. A plausible explanation for the proteome differences observed during the resolution phase is that the inherent capacity of "slow" responders to reduce the expression of the biofilm-induced inflammatory mediators is more efficient compared to that of "fast" responders, which remain at high levels even after the biofilm has been removed.

While it is established that GCF is the more appropriate proximal fluid than saliva in assessing local gingival inflammation, there are considerable impracticalities regarding its collection for full-mouth assessment of the oral inflammatory status. GCF is washed out from the gingival pocket into saliva and can thus be mirrored in the salivary proteome. Despite considerable effort and the application of the state-of-the art MS methods, we may still not have full mapping of the salivary proteome of gingival inflammation (Bostanci et al., 2018). This is mainly mandated by matrix complexity and a large dynamic range of protein expression in saliva. Extensive prefractionations at the expense of sample size may be required for deeper coverage of salivary proteome (Amado, Ferreira, & Vitorino, 2013; Grassl et al., 2016).

In conclusion, this study successfully identified distinct "salivary proteotypes" associated with gingivitis that correlates with clinical phenotypes. Furthermore, the proteomic profile of experimental gingivitis during the induction and resolution phases of inflammation, corresponding to biofilm accumulation and removal, highlights the utility of integrative systems-level quantitative proteomic approaches to unravel the molecular basis of "salivary proteotypes" dubbed as "fast" and "slow" responders. It also provides an accessible resource to the research community that moves towards a broad and comprehensive understanding of specific pathways and processes that relate to gingivitis.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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SUPPORTING INFORMATION

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