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Integrated Proteomic Profiling of Gingival Crevicular Fluid and Tooth Surface-Collected Material Reveals Potential Inflammatory Signatures in Periodontitis Post-Therapy: An Exploratory Study

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Abstract

Background: Characterizing the molecular microenvironment of periodontitis (PD) is essential for identifying non-invasive biomarkers. This study compared the total protein content and qualitative protein profiles of gingival crevicular fluid (GCF) and tooth surface-collected material (TSCM) from healthy individuals and patients with PD before and after non-surgical periodontal therapy (NSPT). **Methods**: GCF and TSCM samples were collected at baseline (T0) and 3 months after NSPT (T1) from 20 systemically healthy subjects: 10 healthy controls and 10 patients with stage III–IV PD. The control and PD groups were not age- and gender-matched. In PD patients, GCF was sampled from healthy and pathological sites. Total protein concentration was quantitatively assessed by the Bradford assay, while proteins were qualitatively characterized in differential SDS-PAGE fractions by LC-MS/MS analysis of pooled samples. **Results**: NSPT significantly improved clinical periodontal parameters ($p < 0.05$). Total protein concentration was higher in PD patients than in controls ($p < 0.05$). Baseline analysis revealed differential protein patterns, with identification of acute-phase and immune-related proteins in pathological GCF and TSCM samples. These alterations were markedly reduced after NSPT, accompanied by the identification of proteins associated with tissue protection and homeostasis. Healthy sites in PD patients also displayed distinct protein profiles, suggesting the presence of a subclinical inflammatory state. **Conclusions**: Combined proteomic analysis of GCF and TSCM provided exploratory evidence of molecular changes associated with PD and response to therapy. Given the small, non-matched groups and pooled-sample design, further validation is required to assess their potential applicability for the future management of PD.

Keywords: periodontitis; gingival crevicular fluid; tooth surface-collected material; proteomics; mass spectrometry; non-surgical periodontal therapy

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1. Introduction

Periodontitis (PD) is a chronic multifactorial inflammatory disease characterized by the progressive destruction of the tooth-supporting tissues, ultimately leading to tooth

loss if left untreated [1]. Current evidence recognizes PD as a biofilm-associated, host-mediated disease in which dysbiosis of the oral microbiota triggers a dysregulated immune-inflammatory response, leading to oxidative stress, extracellular matrix remodeling, and progressive degradation of periodontal soft and hard tissues [2–5]. Beyond its local effects, PD has been consistently associated with several systemic disorders, particularly cardiovascular diseases and diabetes mellitus, further supporting the concept of a bidirectional relationship between oral and systemic health [6–9].

Although periodontal diagnosis and staging currently rely on well-established clinical parameters, including probing pocket depth (PPD), clinical attachment level (CAL), and bleeding on probing; these measurements primarily reflect the cumulative consequences of tissue destruction and provide limited information regarding the ongoing biological activity of the disease and the molecular changes associated with disease progression and response to therapy. Consequently, increasing attention has been devoted to the identification of molecular biomarkers capable of complementing conventional clinical assessment by capturing the biological processes underlying PD and its response to treatment [10–13].

Among the biological substrates available for periodontal biomarker discovery, gingival crevicular fluid (GCF) and tooth surface-collected material (TSCM) provide complementary yet distinct sources of biological information. GCF is a serum-derived inflammatory exudate that reflects the local host response and contains cytokines, enzymes, antimicrobial proteins, and immune mediators, including components of the IL-23/IL-17 signaling axis [14–16]. In contrast, TSCM consists of heterogeneous biological material mechanically collected from the tooth surface and gingival margin, including biofilm-associated microorganisms, salivary proteins, desquamated epithelial cells, inflammatory exudates, extracellular matrix components, and cellular debris. Therefore, TSCM may represent a source of human proteins associated with the local periodontal microenvironment, providing information complementary to GCF [17].

Non-surgical periodontal therapy (NSPT), based on scaling and root planing, remains the standard non-surgical treatment for PD by disrupting the subgingival biofilm and reducing the inflammatory burden, thereby promoting periodontal healing [18].

Recent advances in mass spectrometry-based proteomics have considerably expanded our understanding of periodontal biology by enabling comprehensive characterization of the molecular composition of different oral substrates [10–13,19]. However, direct comparisons between GCF and TSCM in the same study remain limited. Consequently, the extent to which these substrates share common molecular signatures or reflect complementary biological processes within the periodontal microenvironment has not yet been fully established. Likewise, it is still unclear whether these protein profiles change following NSPT and whether alterations observed in clinically diseased sites are also detectable at clinically healthy sites within patients affected by PD [19].

Therefore, the aim of the present study was to compare the protein profiles of GCF and TSCM collected from periodontally healthy subjects and from PD patients, before and after NSPT. Specifically, in patients with PD, GCF samples were obtained from both clinically healthy sites (HS) and pathological sites (PS). The primary hypothesis was that GCF and TSCM would display distinct but partially complementary molecular profiles associated with periodontal health, disease, and changes following NSPT. TSCM was evaluated primarily as a potential source of human protein markers associated with the periodontal microenvironment. GCF and TSCM protein content was quantitatively measured and qualitatively characterized, respectively, by the Bradford assay and through SDS-PAGE coupled to LC-MS/MS analysis.

2. Materials and Methods

2.1. Chemicals and Reagents

All reagents used were of analytical or mass spectrometry grade. Dithiothreitol (DTT), 2x Laemmli sample buffer, ampholytes, Coomassie Brilliant Blue G-250, Precision Plus Protein™ Dual Color Standard, and the protein assay dye reagent were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Urea, thiourea, iodoacetamide (IA), Tris, bovine serum albumin (BSA), trifluoroacetic acid (TFA), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and 2-mercaptoethanol were obtained from Merck KGaA (Darmstadt, Germany). Bolt™ 4–12% Bis-Tris Plus precast gels and MES SDS running buffer were procured from Life Technologies (Milan, Italy). Trypsin Gold (MS grade) was supplied by Promega Corporation (Madison, WI, USA). Sterile Versylene water was obtained from Fresenius Kabi (Bad Homburg, Germany). Acetone, glacial acetic acid, and acetonitrile (ACN) were purchased from Carlo Erba Reagents (Milan, Italy).

2.2. Study Population and Ethical Approval

The study was carried out at the University Hospital of Modena, within the Unit of Periodontology, Department of Dentistry and Oro-Maxillofacial Surgery. Ethical approval was granted by the Emilia-Romagna Regional Health Service Ethics Committee (protocol no. 3968/2017, registration no. 315/17; amendment AOU 0026796/22). All procedures complied with the ethical principles of the Declaration of Helsinki, and written informed consent was obtained from each participant prior to enrollment [20]. Subjects were consecutively recruited between January 2023 and February 2024 from patients attending routine periodontal examinations at the University Hospital of Modena.

Eligibility criteria included an age between 18 and 70 years, non-smoking status, absence of systemic diseases, and no current pregnancy or breastfeeding. Individuals were excluded if they had metabolic, autoimmune, or bone disorders, diabetes mellitus, unstable medical conditions, active antibiotic therapy within the previous 3 months, or indications for antibiotic prophylaxis.

2.3. Clinical Assessment and Patient Grouping

Each participant underwent a comprehensive periodontal assessment, including medical history collection, a full-mouth clinical examination, and radiographic evaluation to support the periodontal diagnosis. All clinical periodontal examinations and parameters were recorded by the same trained examiner using a PCP-UNC 15 periodontal probe. Spearman correlation analysis for the calibration session demonstrated an intrarater agreement ranging from 0.89 to 1.000 ($p < 0.001$). The following parameters were evaluated: probing pocket depth (PPD), mean PPD, number of sites with PPD $\geq$ 5 mm, clinical attachment level (CAL), full-mouth plaque score (FMPS), full-mouth bleeding score (FMBS), and gingival recession. Based on these clinical findings, participants were stratified into two distinct groups: Group H ($n = 10$), comprising periodontally healthy individuals without signs of gingivitis or PD, and Group P ($n = 10$), including patients diagnosed with Stage III or IV, Grade C PD at baseline according to the current international classification criteria [13,21,22]. Given the exploratory and pilot nature of the present study, no a priori sample size calculation was performed. The resulting sample was considered appropriate for generating preliminary data on the protein profiles of GCF and TSCM and for informing the design of future studies with larger cohorts.

2.4. Experimental Design and Therapeutic Intervention

The experimental workflow consisted of a baseline evaluation (T0), a three-month NSPT period, and a post-treatment follow-up (T1). GCF and TSCM samples were col-

lected from all participants at T0. Patients in Group P then received NSPT according to the European Federation of Periodontology (EFP), Clinical Practice Guidelines [23]. Treatment was delivered within this evidence-based therapeutic framework and tailored to individual patient needs, as well as to clinical and behavioral response. At T1, clinical evaluation and sample collection were repeated in Group P to assess therapy-related changes in protein profiles [24,25].

All periodontal treatment was performed by the same experienced periodontist (CB). The initial phase was directed toward behavioral change and control of periodontal risk factors, including patient motivation and instruction in effective supragingival biofilm control. Dental and periodontal conditions were assessed and treated according to individual needs. Periodontal charting and diagnostic-quality periodontal radiographs, when indicated, were obtained at baseline (T0) in both periodontally healthy participants (Group H) and patients with PD (Group P); periodontal charting was repeated in Group P at the 3-month reassessment (T1).

In Group P, supra- and subgingival NSPT was performed using both manual and powered instrumentation. A full-mouth disinfection protocol was not used. Patients attended weekly treatment sessions until all affected sextants had been completely treated, with the active treatment phase completed in all cases within approximately 6 weeks. At each weekly appointment, adherence to individualized home oral-hygiene instructions was verified and reinforced. After completion of active NSPT, patients were monitored every 2 weeks until the T1 reassessment. Additional instrumentation could be performed when clinically indicated; however, no retreatment was required in this cohort before T1.

Quality control of biofilm/deposit removal and oral-hygiene performance was supported by a plaque-disclosing agent (GC Tri Plaque ID Gel, GC Corporation, Tokyo, Japan). Subgingival debridement was additionally clinically verified by instrumentation to ensure adequate removal of subgingival deposits. No systemic or local antibiotic therapy was administered. Chlorhexidine was used only in association with more invasive treatment procedures in sextants presenting pockets ≥5 mm and for short periods, as a 0.12% mouthrinse and a 0.05% toothpaste.

2.5. Sample Collection and Total Protein Extraction

GCF and TSCM samples were collected according to the study design. GCF sampling was always performed in the morning and before periodontal probing. Before sampling, all participants followed the same standardized pre-collection instructions. They were required to abstain from food, sugary drinks, fruit juices, carbonated beverages, coffee, and alcohol for at least 8 h. Water was permitted when needed, although excessive intake was discouraged. Participants were also instructed to refrain from toothbrushing, and mouthwash use for at least 8 h before sampling.

GCF collection was performed by the same trained periodontist (CB). The standardized collection sequence consisted of: (a) a 30 s rinse with tap water; (b) a 60 s waiting period after rinsing; (c) intraoral positioning of an OptraGate latex-free lip and cheek retractor (Ivoclar, Bendererstrasse 2, FL-9494 Schaan, Liechtenstein), followed by isolation of the sampling sites and gentle air-drying to minimize salivary contamination [26]; (d) insertion of sterile filter paper strips (Whatman, 67 g/m^2; TISCH Scientific, Cleves, OH, USA) into the gingival sulcus or periodontal pocket for approximately 30 s [26]; (e) transfer of the samples into sterile storage tubes. Samples suspected of blood or saliva contamination were promptly discarded. Immediately before transfer into the sterile storage tubes, the portion of each paper strip not saturated with GCF was removed using sterile scissors and discarded, thereby further limiting potential contamination from the oral environment or tooth surface.

GCF sampling differed according to periodontal status and study time point. In periodontally healthy participants (Group H), GCF was collected only at baseline (T0). Four sites were sampled at each of the six Ramfjord teeth—buccal, mesiobuccal, distopalatal/distolingual, and palatal/lingual—resulting in 24 clinically healthy sampling sites per participant. All selected sites had PPD ≤ 3 mm and no clinical signs of inflammation.

In patients with PD (Group P), GCF was collected both at baseline (T0) and three months after NSPT (T1). Clinically healthy sites (HS; PPD ≤ 3 mm and no bleeding on probing) were sampled according to the same four-site scheme used in Group H, preferentially at the six Ramfjord teeth. When a Ramfjord tooth did not fulfill the criteria for healthy-site sampling because of the presence of periodontal pockets, an adjacent tooth fulfilling the HS criteria was selected. PS were not restricted to a predetermined number: all sites presenting PPD ≥ 6 mm at T0 were sampled. PPD ≥ 6 mm was selected as the threshold for PS sampling because pockets of this depth are considered deep periodontal pockets and represent a recognized clinical feature of Stage III PD, consistent with the severe periodontal phenotype investigated in this study [21]. The tooth and site location of each pathological sample were recorded at T0, and the same sites were resampled at T1 regardless of their clinical response to NSPT, thereby allowing direct intra-individual longitudinal comparison of GCF composition at corresponding sites before and after treatment.

TSCM samples were collected by sterile curettage from the supragingival tooth surface and gingival margin of the selected HS in periodontally healthy subjects at T0 (TSCM-HT0). In patients with PD, TSCM samples included in the analysis were collected in correspondence with PS (PPD ≥ 6 mm at T0) at baseline (TSCM-PT0), and the same sites were resampled at T1 (TSCM-PT1). Samples were transferred to sterile tubes and immediately stored at −80 °C until further processing. Care was taken to avoid collecting subgingival material or gingival tissue; if bleeding occurred during sampling, collection at that site was immediately discontinued to prevent blood contamination.

For GCF protein extraction, individual collection strips were incubated in 200 µL of phosphate-buffered saline at 4 °C for 2 h under agitation. After centrifugation, proteins were precipitated with cold acetone (1:12, v/v) overnight at −20 °C. Protein pellets were collected by centrifugation and resuspended in rehydration buffer (RB), composed of 6 M urea, 2 M thiourea, 4% CHAPS, 25 mM DTT, and 0.2% ampholytes. At this stage, GCF samples from individual subjects were kept separate to allow subsequent protein quantification on each individual extract.

For TSCM protein extraction, based on our previous experience with these samples, which consistently yielded limited amounts of protein, three independent pools for each experimental condition were generated prior to any chemical processing by physically combining multiple raw TSCM specimens together. These pooled TSCM samples were then incubated overnight in lysis buffer (7 M urea, 2 M thiourea, 3% CHAPS, 40 mM Tris-HCl pH 8.3, 1% ampholytes pH 3–10, and Roche Complete EDTA-free protease inhibitor), after which proteins were precipitated with acetone and resuspended in RB. The pellet fraction was further extracted and combined with the supernatant to maximize protein recovery.

2.6. Proteomic Analytical Workflow and Protein Quantification

To achieve a comprehensive characterization of the proteomic profiles associated with periodontal health and disease, the analytical workflow was structured into sequential phases. First, a quantitative assessment of total protein content was performed. Total protein concentration was determined by the Bradford assay, employing BSA as standard in the concentration range of 0.2 µg–6 µg/µL and the protein assay dye reagent. Absorbance was measured at 595 nm using a Multiskan™ FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). All measurements were performed in duplicate.

Total protein quantification was utilized to normalize sample loading for subsequent electrophoretic analysis.

For GCF, final experimental pools were generated only after this quantification step by combining equal protein amounts from the individual extracts, resulting in three independent pools for each experimental condition (HS and PS). For TSCM, the quantification was performed directly on the three previously prepared independent protein pool for each experimental condition to determine the final concentration needed for downstream analysis.

2.7. SDS-PAGE and Densitometric Analysis

A qualitative and comparative evaluation was subsequently carried out via SDS-PAGE followed by densitometric analysis, enabling the identification of protein bands showing differential relative intensity among the experimental groups and across treatment time points.

For each pooled sample, 20 µg of protein were mixed with Laemmli sample buffer containing 2-mercaptoethanol and heated at 95 °C using a Thermomixer Comfort (Eppendorf, Milan, Italy) to ensure complete denaturation. Samples were then loaded onto precast 4–12% Bis-Tris gels and electrophoresed using MES running buffer at a constant voltage of 200 V. Following electrophoresis, gels were stained with Coomassie Brilliant Blue G-250 and destained with 5% acetic acid. Gel images were acquired using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA), and densitometric analysis was performed using Quantity One 1D software (version 4.6.7, Bio-Rad Laboratories), enabling the identification of differentially expressed protein bands among experimental groups. After automatic band detection, gel images were background-corrected by selecting a 5 × 5 pixel filter size to reduce background noise, and only clearly resolved bands were included in the comparative analysis. The quantity of each band was then measured by its intensity and expressed as percentage of the total intensity of all the bands in the lane (relative band intensity).

2.8. Mass Spectrometry Analysis

Protein bands of interest were manually excised from the gels and subjected to an in-gel digestion protocol, as previously described [27]. Briefly, gel pieces were destained with ACN, followed by protein reduction with 10 mM DTT at 56 °C and alkylation with 55 mM IA. Proteins were then enzymatically digested using sequencing-grade trypsin. The resulting peptide mixtures were extracted with a solution containing 1% TFA/50% ACN and then analyzed by nano-UHPLC-MS/MS, using an UltiMate 3000 system coupled to an Exploris 480 Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Reinach, Switzerland). For protein identification, MS data were analysed by the MASCOT search engine (v. 2.4, Matrix Science, Boston, MA, USA) by matching peptide mass fingerprints and MS/MS fragmentation spectra against the human Swiss Prot database for peptide sequences, and cRAP database for contaminants, respectively. The analyses were managed by Xcalibur ™ software (v. 29 build 2926), and the raw data from one or more peptides were converted to Mascot Generic Format by MsConvert (v. 3.0.10730, ProteoWizard tools, Palo Alto, CA, USA). The ESI source worked in positive ionization mode with the operating parameters set to 3.5 kV for the capillary voltage, and 320 °C for the capillary temperature. The five most intense multi-charged ions were selected for MS^2 nitrogen-promoted collision-induced dissociation (normalized collision energy: 28%). A precursor active exclusion of 20 s was set, and peptide-like isotope pattern ions were preferred. Moreover, to ensure mass accuracy, the instrument was calibrated with a standard mix before each sample batch examination.

Database searches were performed assuming trypsin specificity with up to one missed cleavage. Carbamidomethylation of cysteine residues was set as a fixed modification, whereas methionine oxidation and asparagine/glutamine deamidation were considered variable modifications. Mass tolerances were set to 10 ppm for precursor ions and 0.05 Da for fragment ions, with a false discovery rate (FDR) below 1%. Protein identifications were accepted based on standard scoring criteria and peptide sequence coverage. To ensure reproducibility, protein identification was repeated at least twice by analyzing bands cut from replicated gel lanes.

2.9. Statistical Analysis

Statistical analyses were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Data normality was assessed to ensure the appropriateness of parametric testing. Continuous variables, including clinical parameters and relative band intensity values, were expressed as mean ± standard deviation (SD).

To evaluate differences in baseline clinical features (FMPS, FMBS, PPD and CAL) before and after NSPT, and total protein content between the two independent experimental groups (Group H and Group P), the unpaired Student's *t*-test was applied. For densitometric analysis, the relative intensity values of each electrophoretic band (% of total lane intensity) were compared between the three independent protein pools of healthy and pathological samples using the unpaired two-tailed Student's *t*-test. The protein pool was considered the statistical unit ($n = 3$ per group). To assess the longitudinal effects of NSPT within the PD cohort, clinical and changes in protein profiles between baseline (T0) and post-treatment (T1) time points were analyzed using the paired Student's *t*-test.

The T0 and T1 pools were generated from the same subjects, allowing paired longitudinal comparisons. For all analyses, a p-value < 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the Study Population

The study population consisted of 20 subjects divided into two groups: Group H and Group P. Group H included exclusively female subjects with a mean age of 24.9 ± 4.4 years (mean ± SD) (range: 18–32 years). Clinical periodontal examination confirmed periodontal health, as indicated by a low FMPS (8.1%), a low FMBS (3.6%), a mean PPD of 1.2 mm, and a mean CAL of 1.2 mm (Table 1).

Table 1. Demographic and clinical periodontal parameters of the study population.

	HT0	PT0	PT1
N (Female)	10 (10)	10 (5)	
Age (years)	24.9 ± 4.4	52.9 ± 8.5 §	
FMPS (%)	8.12 ± 6.03	41.10 ± 22.20 *	20.72 ± 9.68 #
FMBS (%)	3.62 ± 3.12	33.80 ± 15.34 *	16.90 ± 10.80 #
PPD (mm)	1.24 ± 0.15	2.63 ± 0.65	1.94 ± 0.42 #
CAL (mm)	1.22 ± 0.14	3.56 ± 0.75	2.95 ± 0.67 #

HT0: periodontally healthy subjects, baseline; PT0: PD patients, baseline; PT1: PD patients, post-NSPT; FMPS: Full-Mouth Plaque Score (percentage); FMBS: Full-Mouth Bleeding Score (percentage); PPD = Probing Pocket Depth, full-mouth mean (mm); CAL = clinical attachment level, full-mouth mean (mm). Data were expressed as mean ± SD. Significantly different demographic data between healthy and PD subjects: § $p = 1.6 \times 10^{-8}$. Clinical parameters significantly different between groups: * HT0 vs. PT0; # PT1 vs. PT0.

Group P consisted of 5 females and 5 males with a mean age of 52.9 ± 8.5 years (range: 43–66 years). At baseline (T0), these patients exhibited clinical findings consistent with severe PD, including an increased FMPS (41.10%), an increased FMBS (33.80%),

a full-mouth mean PPD of 2.63 mm, and a full-mouth mean CAL of 3.56 mm. Following NSPT (T1), a statistically significant reduction in periodontal clinical parameters was observed at the 3-month reassessment (T1), with FMPS decreasing to 20.72%, FMBS to 16.90%, full-mouth mean PPD to 1.94 mm, and full-mouth mean CAL to 2.95 mm (Table 1 and Figure 1).

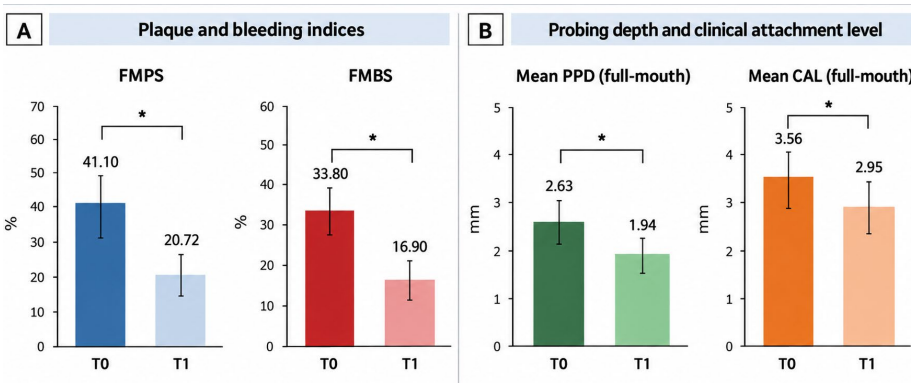

Figure 1. Changes in clinical periodontal parameters following NSPT in patients with PD (Group P). (**A**) Full-Mouth Plaque Score (FMPS) and Full-Mouth Bleeding Score (FMBS); (**B**) full-mouth mean probing pocket depth (PPD) and mean clinical attachment level (CAL), at baseline (T0) and 3 months after NSPT (T1). Data are expressed as mean ± SD. Statistical comparisons between T0 and T1 were performed using a paired Student's *t*-test. Statistically significant differences are indicated as follows: * $p < 0.05$, T1 vs. T0.

3.2. Total Protein Content

Quantitative analysis of GCF pooled samples revealed a significantly higher total protein content in patients with PD compared with healthy subjects at both experimental time points (Figure 2; Supplementary Table S1). Specifically, statistically significant differences were observed between baseline GCF pools from Group H and all corresponding GCF pooled samples collected from Group P, including both clinically HS and PS, at T0 and T1. Interestingly, no significant differences in total protein content were detected between intra-individual HS and PS pools, either before (T0) or after (T1) therapy.

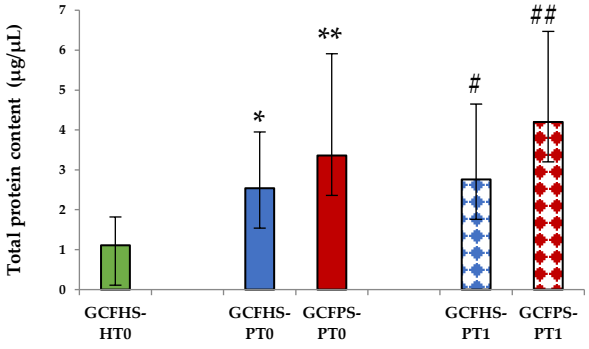

Figure 2. Total protein concentration in GCF pooled samples. GCFHS-HT0: healthy controls at baseline; GCFHS-PT0: HS of PD patients at baseline; GCFPS-PT0: PS of PD patients at baseline; GCFHS-PT1: HS of PD patients after NSPT (T1); GCFPS-PT1: PS of PD patients after NSPT (T1). Data are expressed as mean ± SD of three independent pools for each experimental condition. Baseline comparisons were performed using an unpaired two-tailed Student's *t*-test, whereas T0–T1 comparisons within the PD group were performed using a paired Student's *t*-test. Statistically significant differences are indicated as follows: * $p < 0.05$ and ** $p < 0.01$ vs. GCFHS-HT0; # $p < 0.05$ and ## $p < 0.01$ vs. the corresponding baseline time point (T0).

Quantitative analysis of TSCM pooled samples revealed significantly higher total protein content in PD patients than in healthy controls at both time points. Specifically, total protein concentration was higher at baseline (T0) and significantly decreased after therapy (T1), while remaining higher than in healthy controls (Figure 3; Supplementary Table S1). Following NSPT, total protein concentration decreased significantly, although it remained higher than in healthy controls.

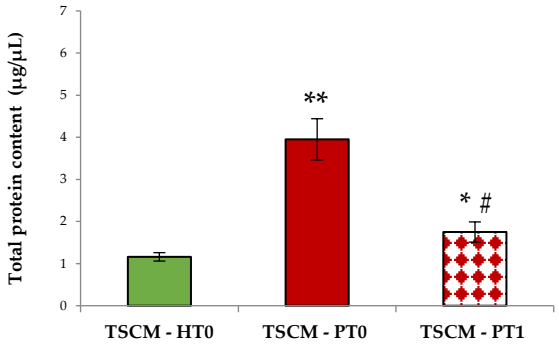

Figure 3. Total protein concentration in TSCM pooled samples. TSCM-HT0: healthy controls at baseline; TSCM-PT0: PD patients at baseline; TSCM-PT1: PD patients after NSPT (T1). Data are expressed as mean ± SD of three independent pools for each experimental condition. Baseline comparisons were performed using an unpaired two-tailed Student's *t*-test, whereas T0–T1 comparisons within the PD group were performed using a paired Student's *t*-test. Statistically significant differences are indicated as follows: * $p < 0.05$ and ** $p < 0.01$ vs. TSCM-HT0; # $p < 0.05$ vs. the corresponding baseline time point (TSCM-PT0).

3.3. Electrophoretic Protein Profiles and Densitometric Analysis

SDS-PAGE yielded clear and reproducible protein profiles across all clinical groups and time points. Quantity One densitometric evaluation identified ten significantly altered bands in GCF group comparisons (Figure 4) and three in TSCM profiles (Figure 5), respectively. The original, uncropped, gel images for both GCF and TSCM samples are reported in Supplementary Figures S1 and S2.

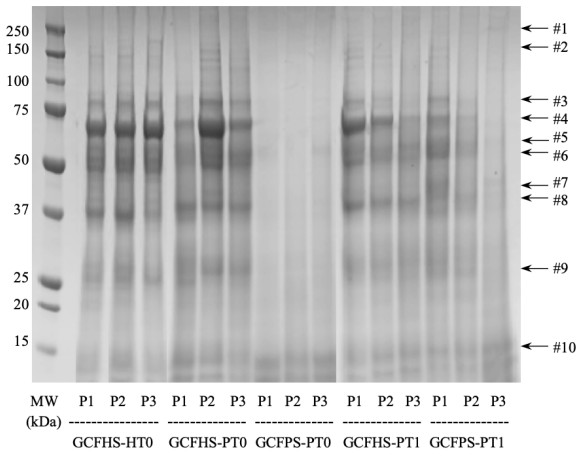

Figure 4. Representative SDS-PAGE profile of GCF pooled samples. The left lane displays the molecular weight (MW) marker expressed in kilodalton (kDa). The ten differentially expressed protein bands, labeled from #1 to #10 on the right, correspond to those detailed in Table 2 and Supplementary Table S2. Their respective estimated MW are: #1 (250 kDa), #2 (180–200 kDa), #3 (85–90 kDa), #4 (70–72 kDa), #5 (55–60 kDa), #6 (48–50 kDa), #7 (40–42 kDa), #8 (35–37 kDa), #9 (25 kDa), and #10 (14–15 kDa). Individual biological replicates are presented within each pool (P1–P3). GCFHS-HT0, healthy controls baseline; GCFHS-PT0, PD patients' healthy sites baseline;

GCFPS-PT0, PD patients' pathological sites baseline; GCFHS-PT1, PD patients' healthy sites post-NSPT (T1); GCFPS-PT1, PD patients' pathological sites post-NSPT (T1). Samples are resolved on Coomassie Blue-stained 12% acrylamide gel.

Table 2. Differential expression of GCF protein bands.

Time Point	Comparison	Statistically Decreased Band (*p*-Value)	Statistically Increased Band (*p*-Value)
T0	GCFHS-PT0 vs. GCFHS-HT0	Band #4 ($p = 0.003$)	Band #7 ($p = 0.002$)
	GCFPS-PT0 vs. GCFHS-HT0	Band #3 ($p = 0.003$) Band #4 ($p = 0.001$) Band #5 ($p = 0.001$) Band #6 ($p = 0.0002$) Band #7 ($p = 0.01$)	Band #1 ($p = 0.01$) Band #2 ($p = 0.01$) Band #10 ($p = 0.0001$)
	GCFHS-PT0 vs. GCFPS-PT0	Band #10 ($p = 0.005$)	Band #5 ($p = 0.004$) Band #6 ($p = 0.01$) Band #7 ($p = 4.7 \times 10^{-11}$) Band #8 ($p = 0.002$)
T1	GCFHS-PT1 vs. GCFHS-HT0	Band #4 ($p = 0.001$) Band #9 ($p = 0.003$) Band #10 ($p = 0.005$)	Band #7 ($p = 0.004$)
	GCFPS-PT1 vs. GCFHS-HT0	Band #4 ($p = 0.001$) Band #6 ($p = 0.01$)	Band #9 ($p = 0.02$)
	GCFHS-PT1 vs. GCFPS-PT1	None	Band #6 ($p = 0.02$) Band #8 ($p = 0.04$)

Decreased and increased bands were determined by comparing GCF samples from healthy subjects and PD patients at baseline (T0) and after NSPT (T1). Reported *p*-values indicate statistically significant differences in band expression between the compared groups. Statistical analysis was performed using unpaired Student's *t*-test for comparisons between healthy subjects and PD patients, and paired Student's *t*-test for pre- and post-NSPT comparisons within the PD group, with significance set at $p < 0.05$. Statistical comparisons refer to densitometric analysis.

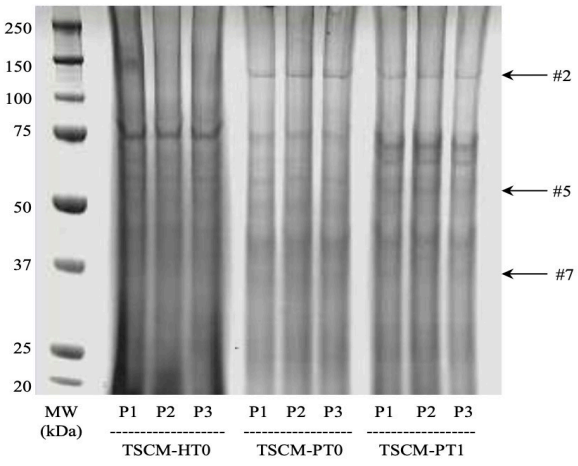

Figure 5. Representative SDS-PAGE profile of TSCM pooled samples. Arrows on the right indicate the specific protein bands identified and analyzed via LC-MS/MS (detailed in Supplementary Table S2) that match the differential expression profile of GCF: #2 (180–200 kDa), #5 (55–60 kDa), and #7 (42–45 kDa). Individual biological replicates (P1–P3) are presented within each pool: TSCM-HT0, healthy controls baseline; TSCM-PT0, PD patients' baseline; TSCM-PT1, PD patients' post-NSPT (T1).

In GCF, the primary comparison at baseline between PS in PD patients and HS in controls (GCFPS-PT0 vs. GCFHS-HT0) involved eight target bands, three of which were

up-regulated (#1, #2, #10) and five resulted down-regulated (#3, #4, #5, #6, #7), indicating substantial disease-associated proteome remodeling (Figure 4).

Beyond this primary axis, the statistical framework was extended to the remaining planned experimental cross-comparisons, which successfully mapped both broad patient-level discrepancies between healthy controls and PD patients, as well as localized, intra-individual site-level variations between HS and PS, as systematically detailed in Table 2. The extremely low p-value observed for Band #7 in the comparison between GCFHS-PT0 and GCFPS-PT0 ($p = 4.7 \times 10^{-11}$) was associated with very low within-group variability in the relative band intensity values.

Regarding TSCM, baseline analysis (TSCM-PT0 vs. TSCM-HT0) revealed only 3 significantly overexpressed bands (#2, #5, and #7) (Figure 5) which remarkably overlapped with those previously identified in GCF, suggesting a convergence between fluid- and surface-associated proteomic alterations.

Furthermore, the analysis demonstrated substantial proteomic changes following NSPT (T1). In the TSCM profiles, after therapy a notable down-regulation was detected for band #5 and band #7 (TSCM-PT1 vs. TSCM-PT0, $p = 1.1 \times 10^{-7}$), indicating a partial normalization toward the proteomic profile observed in healthy controls.

All the complete expression trends for TSCM are summarized in Table 3.

Table 3. Differential expression of TSCM protein bands.

Time Point	Comparison	Statistically Decreased Band (p-Value)	Statistically Increased Band (p-Value)
T0	TSCM-PT0 vs. TSCM-HT0	None	Band #2 (0.007) Band #5 (0.002) Band #7 (0.001)
T1	TSCM-PT1 vs. TSCM-HT0	None	Band #5 (0.016) Band #7 (0.002)
	TSCM-PT1 vs. TSCM-PT0	Band #5 (1.1×10^{-7}) Band #7 (1.1×10^{-7})	None

Decreased and increased bands were determined by comparing TSCM samples collected from healthy subjects and PD patients at baseline (T0) and after NSPT (T1). Statistical analysis was performed using unpaired Student's t-test for comparisons between healthy subjects and PD patients, and paired Student's t-test for pre- and post-NSPT comparisons within the PD group, with significance set at $p < 0.05$. Statistical comparisons refer to densitometric analysis.

3.4. Protein Identification by Mass Spectrometry

The differentially expressed electrophoretic bands were subjected to nano-UHPLC-MS/MS analysis to identify the proteins associated with each fraction, in both GCF and TSCM samples. Overall, the identified proteins were predominantly involved in innate and adaptive immune responses, oxidative stress regulation, antimicrobial defense, metabolic adaptation, proteostasis, and cytoskeletal organization.

Representative proteins for each band are shown in Table 4, along with their main biological function, proposed significance, and the expression trends observed across the clinical groups (the complete protein dataset is reported in Supplementary Table S2).

The identified proteins belonging to distinct functional categories distributed across the periodontal microenvironment.

Band #1 was characterized by the presence of complement and acute-phase proteins, including Complement C3, α-2-Macroglobulin, and α-2-Macroglobulin-like protein 1.

Table 4. Functional annotations of proteins identified by LC-MS/MS in the differentially expressed bands from GCF and TSCM samples.

Band	Proteins Identified	Predominant Biological Function	Proposed Biological Significance	Observed Expression Pattern
# 1	α2-Macroglobulin, Complement C3, α2-macroglobulin-like protein 1	Complement activation, proteolysis	Host inflammatory response	Increased in PS at T0
#2 *	Ceruloplasmin, Ubiquitin-like modifier-activating enzyme 1	Oxidative stress, ubiquitin pathway, cytoskeletal regulation	Stress-related host response associated with periodontal inflammation	Increased in GCF and TSCM from PD patients
#3	Lactoperoxidase, Lactotransferrin, Transglutaminase 3	Antimicrobial defense	Host-derived antimicrobial activity	Reduced in PS
#4	HSP70 family proteins, Endoplasmic reticulum chaperone BiP, Ezrin, Plastin-2, Plastin-3	Stress response, protein folding, cytoskeletal organization	Protective and stress-response mechanisms	Reduced in HS and PS of PD patients
#5 *	α-Amylase 2B, Immunoglobulins, Hemopexin, BPI fold-containing family B member1/2	Immune response, plasma-derived proteins	Host response at the biofilm interface	Increased at T0 and partially reduced after NSPT
#6	α-Enolase, Phosphoglycerate kinase 1, Serpins, Cathepsin D, Isocitrate dehydrogenase	Glycolysis, proteolysis, inflammatory regulation	Metabolic and proteolytic dysregulation	Reduced in PS
#7 *	Actins, Aldolase A, Serpins, Mitogen-activated protein kinase 1	Cytoskeletal remodeling, inflammation, tissue adaptation	Structural and inflammatory remodeling associated with periodontal disease	Increased in HS, PS, and TSCM
#8	Glyceraldehyde-3-phosphate dehydrogenase, Annexins A1/A2, L-lactate dehydrogenase A chain, Transaldolase	Metabolism, inflammation resolution, redox balance	Metabolic adaptation during inflammation and repair	Increased after NSPT
#9	Triosephosphate isomerase, Peroxiredoxin-6, Phosphoglycerate mutase 1, 14-3-3 proteins	Redox regulation, metabolic recovery	Reactivation of metabolic and antioxidant pathways	Increased after NSPT
#10	Glutathione S-transferase P, Peroxiredoxin-1, Interleukin-18, Ras-related protein, Superoxide dismutase [Mn], mitochondrial	Oxidative stress, intracellular trafficking, inflammatory signaling	Persistent inflammatory and oxidative microenvironment	Increased in PS and persistent after NSPT

(*) Bands differentially expressed simultaneously in both GCF and TSCM samples.

Band #2 contained Ceruloplasmin, Ubiquitin-like modifier-activating enzyme 1 (UBA1), and Vinculin, representing proteins involved in oxidative stress response, ubiquitin-mediated pathways, and cytoskeletal organization.

Band #3 was enriched in host-derived antimicrobial proteins, including Lactoperoxidase, Lactotransferrin, and Protein-glutamine gamma-glutamyltransferase E (Transglutaminase 3).

Band #4 contained proteins involved in cellular stress responses and cytoskeletal organization, including Heat shock protein 70 kDa family members, Endoplasmic reticulum chaperone BiP (GRP78/HSPA5), Ezrin, Plastin-2, Plastin-3, and Serum albumin.

Band #5 was characterized by plasma-derived and immune-related proteins, including Immunoglobulin A2 heavy chain, Immunoglobulin G1 heavy chain, Hemopexin, and members of the Bactericidal/permeability-increasing fold-containing (BPIF) family.

Band #6 contained proteins involved in cellular metabolism, protease regulation, and cytoskeletal remodeling, including α-Enolase (ENO1), Phosphoglycerate Kinase 1 (PGK1), Cytoplasmic isocitrate dehydrogenase (IDH1), Actin-related protein 3 (ACTR3) Cathepsin D, α-1-antitrypsin, and Serpins B3, B4, and B13.

Band #7 was enriched in structural and regulatory proteins, including actin isoforms, Leukocyte elastase inhibitor (Serpin B1), Serpins B6 and B8, Fructose-bisphosphate aldolase A (ALDOA), and Mitogen-activated protein kinase 1 (MAPK1).

Band #8 contained proteins involved in glycolytic metabolism and redox regulation, including Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), L-lactate dehydrogenase A chain (LDHA), Transaldolase (TALDO1), Annexin A1, Annexin A2, and Malate dehydrogenase.

Band #9 was characterized by the presence of Triosephosphate isomerase (TPI1), Phosphoglycerate mutase 1 (PGAM1), Peroxiredoxin-6 (PRDX6), 14-3-3 proteins, members of the Bactericidal/permeability-increasing fold-containing (BPIF) family, and Flavin reductase.

Band #10 contained proteins associated with antioxidant defense, intracellular signaling, and cellular stress responses, including Glutathione S-transferase P (GSTP1), Peroxiredoxin-1 (PRDX1), Superoxide dismutase [Mn], mitochondrial (SOD2), Interleukin-18 (IL-18), Ras-related proteins, Heat shock protein beta-1 (HSPB1), and Proteasome subunits.

Collectively, the proteins identified across the ten differentially expressed bands were assigned to distinct functional categories, including innate and adaptive immunity, antimicrobial defense, oxidative stress response, cellular stress and proteostasis, cytoskeletal organization, intracellular signaling, and energy metabolism.

4. Discussion

PD is increasingly recognized as a complex biofilm-associated, host-mediated inflammatory disease characterized by dynamic interactions between the subgingival microbiota and the host immune response rather than by isolated microbial infection alone [2–5]. In the present study, the combined proteomic analysis of GCF and TSCM provides an exploratory overview of protein patterns associated with these interactions, including proteins related to innate immunity, oxidative stress, proteostasis, metabolic processes, cytoskeletal remodeling, and tissue repair. Rather than representing independent biological events, these protein groups appear to coexist within the periodontal microenvironment, where host-derived inflammatory mediators and biofilm-associated components contribute to shaping the local molecular landscape. Collectively, our findings suggest that PD is associated with a complex protein composition involving multiple interconnected biological processes across different periodontal substrates.

4.1. Functional Characterization of Proteins Identified in Differential Electrophoretic Bands

Beyond the identification of individual proteins, LC-MS/MS analysis revealed that the proteins identified in differentially intense electrophoretic bands could be grouped into interconnected functional categories rather than isolated protein clusters. These categories included proteins associated with innate immunity, cellular stress adaptation, host–biofilm interactions, metabolic processes, tissue remodeling, and antioxidant defense [28–30].

The first functional module, corresponding predominantly to Bands #1-#2-#3 and #4, included proteins associated with acute inflammatory responses, complement activation, antimicrobial defense, and cellular adaptation to stress. The identification of Complement

C3 together with α2-Macroglobulin and α2-Macroglobulin-like protein 1 is consistent with activation of innate immune mechanisms and acute-phase responses that have been reported in GCF proteomic studies of PD [31,32]. Likewise, Ceruloplasmin is involved in iron homeostasis and antioxidant defense and has been associated with protection against oxidative damage, a recognized feature of periodontal inflammation [33–35].

The concomitant identification of Ubiquitin-like modifier-activating enzyme 1 may be compatible with the involvement of protein quality-control mechanisms that contribute to cellular homeostasis under inflammatory conditions. Host antimicrobial defense was represented by Lactoperoxidase and Lactotransferrin, two components of the oral innate immune system that contribute to limiting bacterial growth and maintaining mucosal homeostasis [26,29,36]. In addition, the identification of multiple heat shock proteins, the Endoplasmic reticulum chaperone BiP, Ezrin, and Plastin family members is consistent with the presence of proteins involved in stress adaptation, protein homeostasis, cytoskeletal organization, and preservation of epithelial integrity during chronic inflammation [29,30,37].

The second functional module, mainly represented by Bands #5-#6-#7-#8 and #9, was associated with host–biofilm interactions, extracellular matrix remodeling, proteolytic regulation, and metabolic adaptation. Band #5 contained Immunoglobulin heavy chains, Hemopexin, and members of the bactericidal/permeability-increasing (BPI) fold-containing family, indicating the presence of proteins involved in immune and antimicrobial responses at the host–biofilm interface. Immunoglobulins participate in bacterial recognition and neutralization, whereas Hemopexin and BPI family proteins contribute to antimicrobial defense and modulation of local inflammatory responses [32,36,38]. Together, these findings further support the complementary biological role of GCF and TSCM in reflecting the dynamic interactions between the host and the subgingival biofilm.

Bands #6 and #7 were enriched in proteins involved in cytoskeletal organization and extracellular matrix turnover. The coexistence of Cathepsin D and several Serpin family members is compatible with the presence of proteins involved in the regulation of proteolytic activity during periodontal inflammation. Similarly, the identification of Actins, Mitogen-activated protein kinase 1 (MAPK1), and other structural proteins is consistent with biological processes related to cytoskeletal organization, inflammatory cell migration, epithelial adaptation, and connective tissue remodeling, in agreement with previous proteomic studies of periodontal tissues and GCF [10,11,14].

A prominent feature of Bands #6-#7-#8 and #9 was the identification of enzymes involved in glycolysis and cellular energy metabolism, including ENO1, PGK1 ALDOA, GAPDH, LDHA, TPI1, PGAM1, TALDO1, and IDH1. Although metabolic changes have been associated with chronic periodontal inflammation [39,40], the presence of these metabolic enzymes in differentially intense protein bands should be interpreted cautiously, as it does not demonstrate metabolic reprogramming or pathway activation.

The third functional module, corresponding mainly to Bands #9 and #10, was enriched in proteins involved in antioxidant defense and redox homeostasis. Peroxiredoxins 1 and 6 (PRDX1 and PRDX6), GSTP1, and Mitochondrial Superoxide Dismutase (SOD2) are components of endogenous antioxidant system that protects tissues from reactive oxygen species. Their identification is consistent with the recognized role of oxidative stress in periodontal disease pathogenesis [33,34]. The persistence of these proteins likely reflects sustained activation of antioxidant defense mechanisms aimed at counteracting chronic oxidative stress rather than complete restoration of tissue homeostasis. The detection of these proteins after NSPT may reflect the continued presence of proteins associated with antioxidant responses; however, their detection alone does not demonstrate sustained pathway activation or incomplete restoration of tissue homeostasis.

Overall, the LC-MS/MS findings indicate that the identified proteins could be grouped into functional categories reflecting biological processes within the periodontal microenvironment, including innate immunity, antimicrobial defense, cellular stress adaptation, tissue remodeling, metabolic processes, and antioxidant protection. These functional associations should be considered exploratory, as the LC-MS/MS analysis was qualitative and performed on pooled samples.

4.2. Evidence of an Inflammatory Priming in Clinically HS

One relevant finding of the present study was that clinically HS from patients with PD exhibited proteomic profiles that differed from those of periodontally healthy subjects. Several proteins identified in HS were also detected in PS, although their presence and relative distribution varied according to the biological substrate. These findings suggest that clinically healthy site within patients with PD may display protein profiles that differ from those observed in periodontally healthy subjects and that partially overlap with those of pathological sites.

This finding may support the concept that clinical health does not necessarily correspond to complete molecular similarity with periodontally HS. Conventional periodontal diagnosis relies on clinical parameters, including PPD, CAL, and bleeding on probing, which primarily reflect established tissue damage and may not detect early biological alterations preceding clinical manifestations [21,22]. In contrast, proteomic analysis enables identification of molecular changes that remain undetectable during routine clinical examination.

HS samples exhibited proteins involved in oxidative stress regulation, cytoskeletal remodeling, inflammatory signaling, and cellular adaptation. Ceruloplasmin, Ubiquitin-like modifier-activating enzyme 1, Actins, MAPK1, and Serpin family members, identified within Bands #2 and #7, were also detected in PS, although with different expression patterns depending on the biological substrate. Rather than indicating active tissue destruction, these proteins likely reflect adaptive responses aimed at maintaining tissue homeostasis under persistent inflammatory challenge but alone cannot establish active inflammation or tissue damage and may reflect different local biological processes.

The coexistence of these molecular signatures in HS and PS supports the concept of a subclinical inflammatory field, whereby the biological effects of PD extend beyond clinically detectable lesions. This interpretation agrees with recent proteomic studies reporting inflammatory and stress-response proteins in clinically HS of patients with PD, suggesting a generalized host response rather than inflammation confined to individual periodontal pockets [14,30,41,42]. Importantly, these findings do not imply that all clinically HS will inevitably progress to disease. Instead, they suggest that molecular differences associated with PD may extend beyond sites showing overt clinical signs of disease. The identification of proteins associated with oxidative stress, inflammatory signaling, and cellular adaptation may provide a biological context for these differences, although the present data do not allow us to determine whether such alterations represent active inflammation, adaptive responses, or other local biological processes [33–35].

The present findings suggest that PD may involve molecular alterations that are not completely captured by conventional clinical assessment alone. Consequently, proteomic profiling may provide complementary information on periodontal site biology, although its clinical relevance requires validation in larger cohorts, using individual samples and quantitative targeted approaches.

4.3. Quantitative Proteomic Changes Across Biological Substrates

In addition to the qualitative differences revealed by LC-MS/MS, the present study demonstrated significant quantitative variations in total protein content among the different biological substrates and clinical conditions. These findings indicate that PD affects not only protein composition but also the overall protein abundance within the periodontal microenvironment.

PS showed the highest protein concentrations, which may be related to increased vascular permeability and inflammatory exudation associated with periodontal tissue inflammation. The accumulation of plasma-derived proteins, inflammatory mediators, antimicrobial molecules, and extracellular matrix components is consistent with previous GCF proteomic studies [14,26,38].

TSCM also showed substantial protein content despite representing a biologically distinct substrate. Because it integrates host-derived proteins with biofilm-associated material, its relatively high protein yield further supports its potential utility as a complementary substrate for exploratory periodontal proteomic investigations.

Interestingly, clinically HS from patients with PD exhibited higher protein concentrations than healthy controls, although lower than PS. This quantitative observation is consistent with the qualitative proteomic findings discussed above and indicates that clinically HS within patients with PD may differ in their overall protein content from sites in periodontally healthy subjects.

Following NSPT, total protein content decreased markedly in both GCF and TSCM, consistent with the reduction in local inflammation burden following periodontal treatment [23,24]. However, protein concentrations did not completely return to those observed in periodontally healthy individuals. This difference may reflect persisting differences in the local protein composition of the sampled substrates; however, total protein concentration alone does not allow us to determine whether these differences represent persistent inflammation, tissue remodeling, or other biological processes.

These quantitative findings complement the qualitative proteomic analyses by demonstrating differences in both protein composition and total protein concentration within the periodontal microenvironment.

4.4. Complementary Protein Profiles of GCF and TSCM

One of the main findings of the present study was the identification of three protein bands shared between GCF and TSCM. Although these substrates originate from distinct biological compartments, they shared several identified proteins and functional categories, suggesting that they provide complementary information related to the same periodontal microenvironment rather than representing completely independent molecular compartments.

A particularly relevant finding was the identification of Bands #2, #5, and #7 as common molecular signatures shared by both substrates. Although these bands showed slight differences in electrophoretic migration, LC-MS/MS analysis demonstrated a substantial overlap in the major proteins identified within the corresponding bands, including proteins involved in oxidative stress regulation, immune responses, host–biofilm interactions, and tissue remodeling. Together, these findings suggest that the biological information is shared between the soluble and surface-associated compartments of the periodontal environment.

This biological convergence is consistent with the continuous exchange of biological material within the periodontal pocket. GCF continuously bathes the tooth surface, allowing plasma proteins, inflammatory mediators, antimicrobial molecules, and cellular products to adsorb onto the acquired pellicle and biofilm matrix. Conversely, epithelial cells, extracellular vesicles, bacterial products, and extracellular matrix components be-

come incorporated into TSCM, creating a heterogeneous biological material that integrates molecular information from both the microbial community and the host response.

Minor differences in electrophoretic migration were observed between corresponding GCF and TSCM bands. At first inspection, these shifts could suggest differences in protein composition between the two substrates. However, LC-MS/MS analysis showed substantial overlap in the proteins identified in corresponding bands. Therefore, the observed differences in apparent molecular weight may reflect electrophoretic migration variability rather than differences in the major proteins detected. Such variability may arise from differences in protein conformation, post-translational modifications, detergent binding, sample composition, or matrix-dependent effects [43–45]. The combined electrophoretic and mass spectrometry findings therefore support the presence of partially shared protein profiles between GCF and TSCM, while also indicating that the two substrates are not identical.

From a translational perspective, these findings are of potential interest for future biomarker research. GCF is widely recognized as one of the most informative substrates for periodontal biomarker discovery because it closely reflects the local inflammatory response [26,36]. However, its collection is technique-sensitive and often yields limited sample volumes. TSCM contains a heterogeneous mixture of host- and biofilm-associated material, while simultaneously providing both host- and biofilm-derived molecular information. Although further validation is required, these findings suggest that TSCM may represent a practical complementary substrate for future proteomic studies and biomarker discovery. Its clinical utility, however, cannot be established from the present study alone and will require assessment of sampling reproducibility, sample standardization, and biomarker performance in larger cohorts.

Importantly, the present findings do not indicate that GCF and TSCM are interchangeable. Rather, GCF primarily reflects the soluble inflammatory response, whereas TSCM contains biological material derived from both the host and the tooth-associated environment. Their combined analysis therefore provides a more comprehensive characterization of the periodontal microenvironment than either substrate alone, and supports the application of multi-substrate proteomic approaches in periodontal research.

4.5. Longitudinal Proteomic Changes Following NSPT

The longitudinal design of the present study enabled evaluation of change in protein patterns following NSPT. Changes in relative band intensity and in proteins identified in selected bands were observed in both GCF and TSCM.

Several proteins associated with inflammation, host–biofilm interactions, and metabolic adaptation decreased after therapy, whereas proteins involved in metabolic recovery and cellular homeostasis became more prominent.

In particular, Bands #8 and #9 showed changes involving GAPDH, Annexins, TALDO1, TPI1, PGAM1, and PRDX6. These observations are compatible with changes in local protein composition following NSPT; however, the qualitative LC-MS/MS approach and pooled sample analysis do not allow determination of whether these changes reflect metabolic recovery, pathway activity, or other biological processes. Previous studies have demonstrated molecular changes following periodontal therapy alongside clinical improvement [14,18]. Despite the changes observed after treatment, several proteins associated with oxidative stress, inflammatory regulation, and cellular stress remained detectable. These findings indicate that post-treatment protein profiles were not identical to those observed at baseline or in periodontally healthy subjects. However, the persistence of individual proteins should not be interpreted as direct evidence of persistent inflammation or incomplete clinical healing. Current concepts of periodontal healing recognize that resolution of inflammation and

tissue remodeling may involve processes extending beyond the immediate clinical response to therapy [23,24].

Overall, the longitudinal findings indicate that NSPT was associated with changes in the protein composition of GCF and TSCM. These results support the potential value of proteomic approaches for investigating molecular changes following periodontal therapy, while longer follow-up and individual-sample quantitative analyses are required to determine the biological and clinical significance of these changes.

4.6. Study Strengths, Limitations and Future Perspectives

The present study has several limitations that should be considered when interpreting the findings. First, it was designed as an exploratory pilot investigation and included a small sample of 20 systemically healthy subjects. Given the exploratory and pilot nature of the study, no a priori sample size calculation was performed. The resulting sample was used to generate preliminary data and identify protein patterns that may warrant further investigation. Second, the Group H and Group P were not age- and gender-matched. This was related to the small sample size and the limited number of eligible participants available during the enrollment period who provided informed consent. Therefore, differences in age and gender distribution should be considered when interpreting the present findings, as these factors may potentially influence periodontal clinical parameters and protein profiles. In addition, proteomic analyses were performed on pooled samples, a strategy required to obtain sufficient protein yield from GCF and TSCM but preventing assessment of inter-individual variability. For GCF, individual extracts were quantified before pooling, whereas TSCM specimens were pooled before chemical processing because of the limited protein yield observed in our previous experience with these samples.

The discovery-based proteomic approach was qualitative at the protein-identification level and therefore did not provide quantitative validation of individual proteins. Moreover, the three-month follow-up allowed assessment of short-term molecular changes following NSPT, but was insufficient to establish long-term molecular restoration, periodontal stability, or recurrence risk. Finally, the absence of correction for multiple comparisons should be considered when interpreting the statistical findings of this exploratory study.

Despite these limitations, the present study provides preliminary data from a direct comparison of GCF and TSCM protein profiles in periodontal health, disease, and following NSPT. The combined SDS-PAGE and LC-MS/MS approach and the longitudinal design allowed the identification of proteins associated with different clinical conditions, as well as the evaluation of therapy-associated changes. The direct comparison of two biological substrates and the evaluation of both pathological and clinically healthy sites represent additional strengths of the study.

Future studies should include larger, appropriately age- and gender-matched cohorts, individual samples, targeted quantitative approaches, and longer follow-up periods. Particular attention should be given to validating the proteins identified in Bands #2, #5, and #7, as well as to assessing their reproducibility and their relationship with clinical parameters and response to periodontal therapy. Such approaches will be necessary to determine whether the molecular differences observed in the present exploratory study have reproducible biological and clinical relevance.

5. Conclusions

The present study provides preliminary evidence that GCF and TSCM display distinct but partially overlapping protein profiles associated with periodontal health, PD, and changes following NSPT. Clinically HS in patients with PD showed protein profiles that

differed from those of periodontally healthy subjects, suggesting that molecular differences associated with PD may extend beyond sites showing overt clinical signs of disease.

The qualitative LC-MS/MS analysis of pooled samples indicates that these findings should be considered exploratory and does not allow assessment of individual protein abundance or pathway activation. The partially overlapping profiles of GCF and TSCM support their potential complementary value in periodontal proteomic investigations.

Larger studies using individual samples, quantitative validation, and longer follow-up are needed to confirm the reproducibility and clinical relevance of these findings.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/sci8110339/s1, Figure S1: Original SDS-PAGE gel image of GCF protein profile; Figure S2: Original SDS-PAGE gel image of TSCM protein profile; Table S1: Total protein concentration in GCF and TSCM samples; Table S2: LC-MS/MS identification of proteins detected in differentially expressed bands from GCF and TSCM samples.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All relevant data and the original contributions presented in this study are included in the article and in the Supplementary Materials. Further inquiries can be directed to the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CAL	Clinical attachment level
EFP	European Federation of Periodontology
FMBS	Full-Mouth Bleeding Score
FMPS	Full-Mouth Plaque Score
GCF	Gingival Crevicular Fluid
LC-MS/MS	Liquid Chromatography–Mass Tandem Mass Spectrometry
PD	Periodontitis
PPD	Probing Pocket Depth
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
NSPT	Non-Surgical Periodontal Therapy
TP0	Time Point 0
TP1	Time Point after NSPT
GCFSS-ST0	GCF from healthy site of healthy subjects at T0 (pre-NSPT)
GCFSS-PT0	GCF from healthy site of PD patients at T0 (pre-NSPT)
GCFSP-PT0	GCF from diseased site of PD patients at T0 (pre-NSPT)

GCFSS-PT1	GCF from healthy site of PD patients at T1 (post-NSPT)
GCFSP-PT1	GCF from diseased site of PD patients at T1 (post-NSPT)
TSCM	Tooth surface collected material
TSCM-ST0	Tooth surface collected material from healthy subjects at T0
TSCM-PT0	Tooth surface collected material from PD patients at T0
TSCM-PT1	Tooth surface collected material from PD patients at T1

References

1. Sanz, M.; Quirynen, M.; European Workshop in Periodontology Group A. Advances in the aetiology of periodontitis. Group A consensus report of the 5th European Workshop in Periodontology. *J. Clin. Periodontol.* **2005**, *32*, 54–56. [CrossRef]
2. Lamont, R.J.; Koo, H.; Hajishengallis, G. The oral microbiota: Dynamic communities and host interactions. *Nat. Rev. Microbiol.* **2018**, *16*, 745–759. [CrossRef]
3. Hajishengallis, G. Periodontitis: From microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* **2015**, *15*, 30–44. [CrossRef]
4. Curtis, M.A.; Diaz, P.I.; Van Dyke, T.E. The role of the microbiota in periodontal disease. *Periodontol. 2000* **2020**, *83*, 14–25. [CrossRef]
5. Salvatori, R.; Generali, L.; Bellei, E.; Bergamini, S.; Bertoldi, C. The Inflammation-Initiating and Resolving Mechanisms and Oxidation: Could Periodontal Therapy and Nutritional Strategy Improve the Systemic Health? A Narrative Review. *Food Sci. Nutr.* **2025**, *13*, e70096. [CrossRef]
6. Bertoldi, C.; Salvatori, R.; Pinti, M.; Mattioli, A.V. Could the periodontal therapy improve the cardiologic patient health? A narrative review. *Curr. Probl. Cardiol.* **2024**, *49*, 102699. [CrossRef]
7. Tonetti, M.S.; Van Dyke, T.E.; Working Group 1 of the Joint EFP/AAP Workshop. Periodontitis and atherosclerotic cardiovascular disease: Consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. *J. Periodontol.* **2013**, *84*, S24–S29. [CrossRef]
8. Borgnakke, W.S.; Ylöstalo, P.V.; Taylor, G.W.; Genco, R.J. Effect of periodontal disease on diabetes: Systematic review of epidemiologic observational evidence. *J. Clin. Periodontol.* **2013**, *40*, S135–S152. [CrossRef]
9. Chapple, I.L.; Genco, R.; Working Group 2 of the Joint EFP/AAP Workshop. Diabetes and periodontal diseases: Consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. *J. Periodontol.* **2013**, *84*, S106–S112. [CrossRef]
10. Bertoldi, C.; Bellei, E.; Pellacani, C.; Ferrari, D.; Lucchi, A.; Cuoghi, A.; Bergamini, S.; Cortellini, P.; Tomasi, A.; Zaffe, D.; et al. Non-bacterial protein expression in periodontal pockets by proteome analysis. *J. Clin. Periodontol.* **2013**, *40*, 573–582. [CrossRef]
11. Monari, E.; Cuoghi, A.; Bellei, E.; Bergamini, S.; Lucchi, A.; Tomasi, A.; Cortellini, P.; Zaffe, D.; Bertoldi, C. Analysis of protein expression in periodontal pocket tissue: A preliminary study. *Proteome Sci.* **2015**, *13*, 33. [CrossRef]
12. Gürsoy, U.K.; Kabalak, M.O.; Gürsoy, M. Advances in periodontal biomarkers. *Adv. Clin. Chem.* **2024**, *120*, 145–168. [CrossRef]
13. Bertoldi, C.; Nasi, M.; Salvatori, R.; Pinti, M.; Montagna, S.; Tonetti, M.; Generali, L.; Bellei, E.; Zaffe, D.; Selleri, V.; et al. Salivary Biomarker Analysis to Distinguish Between Health and Periodontitis Status: A PreliminaryStudy. *Dent. J.* **2025**, *13*, 436. [CrossRef]
14. Bellei, E.; Bertoldi, C.; Monari, E.; Bergamini, S. Proteomics Disclose the Potential of Gingival Crevicular Fluid (GCF) as a Source of Biomarkers for Severe Periodontitis. *Materials* **2022**, *15*, 2161. [CrossRef]
15. Alarcón-Sánchez, M.A.; Guerrero-Velázquez, C.; Becerra-Ruiz, J.S.; Rodríguez-Montaño, R.; Avetisyan, A.; Heboyan, A. IL-23/IL-17 axis levels in gingival crevicular fluid of subjects with periodontal disease: A systematic review. *BMC Oral Health* **2024**, *24*, 302. [CrossRef]
16. Bellei, E.; Monari, E.; Bertoldi, C.; Bergamini, S. Proteomic Comparison between Periodontal Pocket Tissue and Other Oral Samples in Severe Periodontitis: The Meeting of Prospective Biomarkers. *Sci* **2024**, *6*, 57. [CrossRef]
17. Bergamini, S.; Bellei, E.; Generali, L.; Tomasi, A.; Bertoldi, C. A Proteomic Analysis of Discolored Tooth Surfaces after the Use of 0.12% Chlorhexidine (CHX) Mouthwash and CHX Provided with an Anti-Discoloration System (ADS). *Materials* **2021**, *14*, 4338. [CrossRef]
18. Bergamini, S.; Bellei, E.; Selleri, V.; Salvatori, R.; Micheloni, G.; Nasi, M.; Pinti, M.; Bertoldi, C. The Impact of Nonsurgical Periodontal Therapy on the Salivary Proteome: A Pilot Study. *Int. J. Dent.* **2025**, *2025*, 6655743. [CrossRef]
19. Bertoldi, C.; Bergamini, S.; Ferrari, M.; Lalla, M.; Bellei, E.; Spinato, S.; Tomasi, A.; Monari, E. Comparative proteomic analysis between the gingival crevicular fluid and the corresponding periodontal pocket: A preliminary study. *J. Biol. Regul. Homeost. Agents* **2019**, *33*, 983–986.
20. World Medical Association. World Medical Association Declaration of Helsinki: Ethical principles for medical research involving human subjects. *JAMA* **2013**, *310*, 2191–2194. [CrossRef]

21. Tonetti, M.S.; Greenwell, H.; Kornman, K.S. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. *J. Periodontol.* **2018**, *89*, S159–S172. [CrossRef]
22. Trombelli, L.; Farina, R.; Silva, C.O.; Tatakis, D.N. Plaque-induced gingivitis: Case definition and diagnostic considerations. *J. Clin. Periodontol.* **2018**, *45*, S44–S67. [CrossRef]
23. Sanz, M.; Herrera, D.; Kebschull, M.; Chapple, I.; Jepsen, S.; Beglundh, T.; Sculean, A.; Tonetti, M.S.; EFP Workshop Participants and Methodological Consultants. Treatment of stage I–III periodontitis-The EFP S3 level clinical practice guideline. *J. Clin. Periodontol.* **2020**, *47*, 4–60. [CrossRef]
24. Krajewski, A.; Perussolo, J.; Ercal, P.; Gkranias, N.; Donos, N. The Effect of Non-Surgical Periodontal Therapy on Subgingival Microbiota: A Systematic Review and Meta-Analysis. *J. Periodontal Res.* **2025**, *60*, 963–993. [CrossRef]
25. Corbella, S.; Calciolari, E.; Alberti, A.; Donos, N.; Francetti, L. Systematic review and meta-analysis on the adjunctive use of host immune modulators in non-surgical periodontal treatment in healthy and systemically compromised patients. *Sci. Rep.* **2021**, *11*, 12125. [CrossRef]
26. Khurshid, Z.; Mali, M.; Naseem, M.; Najeeb, S.; Zafar, M.S. Human Gingival Crevicular Fluids (GCF) Proteomics: An Overview. *Dent. J.* **2017**, *5*, 12. [CrossRef]
27. Bellei, E.; Cuoghi, A.; Monari, E.; Bergamini, S.; Fantoni, L.I.; Zappaterra, M.; Guerzoni, S.; Bazzocchi, A.; Tomasi, A.; Pini, L.A. Proteomic analysis of urine in medication-overuse headache patients: Possible relation with renal damages. *J. Headache Pain* **2012**, *13*, 45–52. [CrossRef]
28. Bostanci, N.; Bao, K. Contribution of proteomics to our understanding of periodontal inflammation. *Proteomics* **2017**, *17*, 1500518. [CrossRef]
29. de Paula Sumback Sivila Souza, L.; Reis Dias, D.; Portolese Sversutti Cesar, G.; de Almeida Bianco, D.; Araújo, M.G.; Matarazzo, F. MS-Based Crevicular Fluid Proteomics for the Study of Periodontal and Peri-Implant Conditions: A Systematic Review. *J. Proteome Res.* **2026**, *25*, 3217–3233. [CrossRef]
30. Ahmad, P.; Escalante-Herrera, A.; Marin, L.M.; Siqueira, W.L. Progression from healthy periodontium to gingivitis and periodontitis: Insights from bioinformatics-driven proteomics—A systematic review with meta-analysis. *J. Periodontal Res.* **2025**, *60*, 8–29. [CrossRef]
31. Bostanci, N.; Bao, K.; Li, X.; Maekawa, T.; Grossmann, J.; Panse, C.; Briones, R.A.; Resuello, R.R.G.; Tuplano, J.V.; Garcia, C.A.G.; et al. Gingival Exudatome Dynamics Implicate Inhibition of the Alternative Complement Pathway in the Protective Action of the C3 Inhibitor Cp40 in Nonhuman Primate Periodontitis. *J. Proteome Res.* **2018**, *17*, 3153–3175. [CrossRef]
32. Blanco-Pintos, T.; Regueira-Iglesias, A.; Suárez-Rodríguez, B.; Seijas-Otero, N.; Relvas, M.; Bravo, S.B.; Balsa-Castro, C.; Tomás, I. Characterisation of the periodontal proteome in gingival crevicular fluid and saliva using SWATH-MS. *Front. Cell. Infect. Microbiol.* **2025**, *15*, 1576906. [CrossRef]
33. Chapple, I.L.C.; Matthews, J.B. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol. 2000* **2007**, *43*, 160–232. [CrossRef]
34. Sá Carneiro Sczepanik, F.; Lima Grossi, M.; Casati, M.; Goldberg, M.; Glogauer, M.; Fine, N.; Tenenbaum, H.C. Periodontitisis an inflammatory disease of oxidative stress: We should treat it that way. *Periodontol. 2000* **2020**, *84*, 45–68. [CrossRef]
35. Jiang, W.; Wang, Y.; Cao, Z.; Chen, Y.; Si, C.; Sun, X.; Huang, S. The role of mitochondrial dysfunction in periodontitis: From mechanisms to therapeutic strategy. *J. Periodontal Res.* **2023**, *58*, 853–863. [CrossRef]
36. Bostanci, N.; Belibasakis, G.N. Gingival crevicular fluid and its immune mediators in the proteomic era. *Periodontol. 2000* **2018**, *76*, 68–84. [CrossRef]
37. Hetz, C.; Zhang, K.; Kaufman, R.J. Mechanisms, regulation and functions of the unfolded protein response. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 421–438. [CrossRef]
38. Bibi, T.; Khurshid, Z.; Rehman, A.; Imran, E.; Srivastava, K.C.; Shrivastava, D. Gingival Crevicular Fluid (GCF): A Diagnostic Tool for the Detection of Periodontal Health and Diseases. *Molecules* **2021**, *26*, 1208. [CrossRef]
39. Ming, H.; Li, Y.; Chen, T.; Wu, X.; Xie, X. Glycolysis Plays a Critical and Dual Role in Periodontitis. *J. Cell. Physiol.* **2025**, *240*, e70098. [CrossRef]
40. Bellei, E.; Bergamini, S.; Salvatori, R.; Bertoldi, C. Evaluation of Salivary GAPDH as a Predictor Biomarker for Periodontitis. *Int. J. Mol. Sci.* **2025**, *26*, 10441. [CrossRef]
41. Bostanci, N.; Grant, M.M.; Kebschull, M. Biological definition of periodontal diseases: A historical review of host-response diagnostics and their implications for disease classification. *Periodontol. 2000* **2025**, *00*, 1–13. [CrossRef]
42. Miranda, T.S.; Figueiredo, N.F.; Figueiredo, L.C.; Silva, H.D.P.; Rocha, F.R.G.; Duarte, P.M. Cytokine profiles of healthy and diseased sites in individuals with periodontitis. *Arch. Oral Biol.* **2020**, *120*, 104957. [CrossRef]
43. Rath, A.; Glibowicka, M.; Nadeau, V.G.; Chen, G.; Deber, C.M. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 1760–1765. [CrossRef]

44. Shi, Y.; Mowery, R.A.; Ashley, J.; Hentz, M.; Ramirez, A.J.; Bilgicer, B.; Slunt-Brown, H.; Borchelt, D.R.; Shaw, B.F. Abnormal SDS-PAGE migration of cytosolic proteins can identify domains and mechanisms that control surfactant binding. *Protein Sci.* **2012**, *21*, 1197–1209. [CrossRef]
45. Choi, I.; Yang, Y.I.; Don Song, H.; Lee, J.S.; Kang, T.; Sung, J.J.; Jongheop, Y.I. Lipid molecules induce the cytotoxic aggregation of Cu/Zn superoxide dismutase with structurally disordered regions. *Biochim Biophys. Acta* **2011**, *1812*, 41–48. [CrossRef]

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