Serum proteins associated with periodontitis relapse post-surgery: A pilot study

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Abstract

**Background:** The knowledge of which genes and proteins that are connected to the susceptibility to gingivitis with subsequent local tissue degradation seen in periodontitis is insufficient. Changes of serum proteins associated with recurrence of bleeding on probing (BOP) and increased periodontal pocket depths (PPD) after surgical treatment of periodontitis could reveal molecules that could be early signals of tissue destruction and/or of importance for systemic effects in other tissues or organs.

**Methods:** We performed a longitudinal pilot study and followed 96 inflammation-related proteins over time in serum from patients who underwent surgical treatment of periodontitis (n= 21). The samples were taken before (time 0), and then at 3, 6, and 12 months after surgery. Changes in protein levels were analysed in relation to the clinical outcome measures, that is, proportion of surfaces affected by BOP and PPD.

**Results:** Changes in treatment outcomes with early signs of relapse in periodontitis after surgical treatment, for example, increased BOP and PPDs, were during 12-months follow up associated with increased serum levels of high-sensitivity C-reactive protein (hs-CRP) and programmed death-ligand 1 (PD-L1), and reduced serum levels of cystatin D protein.

**Conclusion:** This study shows that clinical signs of recurrence of periodontitis after surgery are reflected in serum, but larger studies are needed for verification. Our novel findings of an association between increased PD-L1- and decreased cystatin D-levels and recurrence in periodontitis are interesting because PD-L1 has been shown to facilitate bacterial infections and chronic inflammation and cystatin D to inhibit tissue destruction. Our results justify mechanistic studies regarding the role of these molecules in periodontitis.

**KEYWORDS**
bone resorption, infection control, inflammation, periodontal diseases, periodontal pocket
1 | INTRODUCTION

Tooth-associated commensal bacteria can initiate inflammatory and immune responses, which lead to gingivitis, a reversible process that affects almost all individuals at some point.\(^1\) In individuals susceptible to periodontitis, the inflammatory process destroys the periodontal connective tissues, which activates bone-resorbing osteoclast-cells, and the tooth-supporting jawbone is irreversibly reduced.\(^2\) Periodontitis is the result of a complex interplay between pathobiotic bacteria in the dental biofilm and the host. The role of specific microorganisms and their products in periodontitis initiation and propagation remain unclear.\(^3\) However, host genetic, inflammatory, immunological, and environmental factors, especially smoking, have been shown to contribute to periodontitis susceptibility.\(^4\) The aetiological process of periodontitis is not yet fully understood.

Currently, the diagnostic tools used in dental care can only discover periodontitis after the loss of tooth-supporting tissues has occurred. Although the population’s oral hygiene efforts have increased in most countries and that many societies spend enormous resources on professional cleaning of teeth, \(\approx 10\%\) of the populations’ worldwide is affected by advanced periodontal disease.\(^5\) Current periodontitis treatment practices involve anti-infectious agents for resolving inflammation and preserving tooth-supporting tissues. In severe cases, periodontal surgery is required to access root surfaces. In most cases, both non-surgical and surgical treatments are effective for treating periodontitis; however, in a recent study \(\approx 14\%\) of the treated patients were considered to respond poorly to treatment; thus, despite treatment, they experience a disease relapse with tissue degradation.\(^6\) However, it remains unknown which individuals, despite good oral hygiene, supportive periodontal treatment, and no smoking, will experience periodontitis relapses after treatment.

Clinical and epidemiological data have indicated that periodontitis is associated with other chronic inflammatory diseases, such as cardiovascular diseases, diabetes, and rheumatoid arthritis.\(^7\) Nevertheless, the potential mechanisms underlying this association are poorly understood. Many hypotheses exist to explain how periodontitis might be linked to other chronic inflammatory diseases.\(^7\) One hypothesis is that oral inflammatory molecules, autoantibodies, oral bacteria, or bacterial products are spread systemically and trigger inflammatory reactions in other parts of the body, distant from the oral cavity.\(^8-10\) Another suggestion is that inflammatory molecules released from unresolved periodontal inflammation might contribute to chronic inflammatory states at different parts of the body.\(^11,12\) A third option is that the link between periodontitis and other systemic inflammatory diseases could be explained by a genetic background, with a more pronounced proinflammatory constitution among individuals that are exposed to this type of diseases.\(^12\) Most interestingly, poor response to periodontal treatment has been suggested to predict future cardiovascular disease.\(^9\) These diseases may have a common denominator linked to the inability to resolve inflammation.

In this pilot prospective study, we aimed to evaluate inflammatory-associated serum proteins related to changes in clinical treatment outcome after surgical therapy associated with early signs of relapse of periodontitis.

2 | MATERIALS AND METHODS

This study was approved by the Regional Ethics Committee, Uppsala, Sweden (Dnr 2009/029). Signed informed consent was obtained from all patients. The trial is registered at ClinicalTrials.gov (NCT04663165) and the study conform to STROBE Guidelines\(^13\) and conducted in accordance with the Helsinki Declaration.

2.1 | Study design

In this pilot prospective clinical intervention study, we followed periodontal healing capacity after periodontal surgery and the protein profile in serum. Twenty-one consecutive patients referred to the department of Periodontology (Public Dental Health County Council of Gävleborg, Gävle, Sweden) in need of periodontal surgery were recruited to the study. All participants received a thorough case presentation, instruction in oral hygiene and non-surgical subgingival treatment consisting of scaling of the root surfaces using ultrasonic and hand instruments before they were included in the study. The criteria for periodontal surgery in at least two quadrants were: a minimum of at least two sites that showed periodontal probing depths (PPDs) \(\geq 6\) mm, combined with bleeding on probing (BOP) and/or pus; and radiographs that displayed a marginal bone loss \(\geq 4\) mm from the cemento-enamel junction. Periodontal healing was followed with clinical measurements (BOP and PPD) and blood collections at 3-, 6-, and 12-months after surgical therapy. None of the participants had received any periodontal treatment or taken antibiotics within three months prior to surgery, and none had taken an anti-inflammatory medication within two weeks prior to surgery.
2.2 | Clinical registration, serum collection, sample preparation, and storage

Blood samples were drawn and handled by the chemical laboratory at Gävle hospital, at the time of therapy and at follow-up visits. For each patient, five 1 mL serum aliquots were labeled and stored at -80°C. At each time point, blood samples were collected before the clinical six sites/tooth measurements using dental probes and the deepest 4-sites/tooth were registered (BOP and PPD).

2.3 | Treatment

A periodontal specialist (AH) performed all surgeries. Briefly, after an intra-crevicular incision, a flap was raised, and inflamed tissue, dental calculus, and bacterial biofilm were removed with an ultrasonic cleaner fitted with a PE39 tip and curettes. The area was rinsed with saline solution, and the flap was closed with nonresorbable suture. Patients were advised to rinse twice daily with 10 mL of a 2 mg/mL chlorhexidine solution for 12 weeks and not to chew or brush on the treated side for 2 weeks. Sutures were removed after 2 weeks. Supportive care was provided at 6 weeks and every third month, for 12 months after surgery.

2.4 | Protein analysis

Protein arrays were performed on serum samples collected pre-surgery (time 0) and at 3-, 6-, and 12-months after surgical treatment. The concentrations of 92 proteins were assessed with a proximity extension assay and the inflammation panel provided (Table S1.docx). The assay comprised oligonucleotide-labeled antibody pairs, which allowed pair-wise binding to target proteins, and proteins were quantified with high-throughput real-time PCR. Data were presented as normalized protein expression levels, measured in terms of Olink Proteomics’ arbitrary unit, and plotted on a log2 scale. The V-PLEX Vascular Injury Panel 2 Human Kit was used to assess concentrations of four other proteins: high-sensitivity C-reactive (hs-CRP), intercellular adhesion molecule 1 (ICAM-1), serum amyloid A (SAA), and vascular cell adhesion molecule 1 (VCAM-1).

2.5 | Statistics

Descriptive statistics were performed with SPSS including the mean and 95% confidence interval (95% CI) estimates. Spearman’s correlation method was performed to evaluate correlations between variables. Jonckheere–Terpstra test was used for evaluating trends. All tests were two-sided and corrected for multiple comparisons with the Benjamini–Hochberg false discovery rate (FDR < 0.05) when appropriate. Generalized estimated equation (GEE) was used for repeated measurements, that is, 3-, 6-, and 12-months samples, as it allows the use of non-normal distributed data and includes all available data for each subject. GEE approach was used to evaluate if gender, smoking, age, other diseases, or number of teeth associated with BOP or PPD over 3 to 12 months post-surgery. Generalized linear regression model (GLM) was performed to evaluate variables at individual time points. Multivariate partial least square regression (PLS) model was performed to generate an overview of the independent inflammatory markers and the dependent BOP variable. PLS regression was chosen because it allows co-variation among the independent x-variables. GLM was used for time point specific, and GEE models for repeated measurements, to evaluate associations between serum proteins and BOP or PPD, after adjusting for gender, age, smoking, number of teeth, and other diseases.

3 | RESULTS

3.1 | Pre- and post-surgery comparison

At time point 0, the mean (95% CI) patient age was 57.5 years (53.2-61.7). Among the 21 patients, 52.4% were female and 57.1% were smokers. Patients had an average (95% CI) of 25.7 (24.0-27.4) teeth, BOP was observed in 39.5% (31.7-47.3) of tooth surfaces, and the PPD was > 4 mm in 41.2% (34.4-48.0) and > 6 mm in 10.5% (7.7-13.4) of tooth surfaces (Table S2.docx). Five patients displayed potential confounding medical symptoms, including: diabetes (n = 2), high blood pressure (n = 2) and cardiovascular disease (n = 1), which were adjusted for in all models. One patient did not complete the 12 months follow-up examination.

Before surgery (time 0), we found no associations between gender, smoking, other diseases, number of teeth, or plaque index (PLI), with BOP or PPD (P > 0.05). In contrast, at time 0, age was negatively correlated with the number of teeth (r = -0.578, P = 0.006) and PLI (r = -0.453, P < 0.05). There were no significant correlations between BOP or PPD and other disease variables (Table S3.docx).
FIGURE 1  Correlations between clinical outcomes and indicated BOP and PPD relapse after surgery. Scatterplots show correlations between participants' age, number of teeth, plaque index (PLI), the percentage of sites with bleeding on probing (BOP), and the percentage of sites with peridontal probing depths (PPDs) larger than 4 or 6 mm, measured at (A) time point 0 and (B) for time point 3, 6, and 12 months post-surgery. Trend lines show Spearman correlations, with the 95% CI. The correlation coefficient (r), and P-value are shown and significant correlations are color indicated based on strength. Box plots show the percent sites (%-sites) with (C) BOP and (D) PPD > 4 mm measured at time points, 3-, 6-, and 12-months post-surgery.

Correlations were also observed between BOP and PPD > 4 mm (r = 0.777, P < 0.001) and BOP and PPD > 6 mm (r = 0.669, P = 0.001). In addition, correlations were observed between PLI and BOP (r = 0.604, P = 0.004), PLI and PPD > 4 mm (r = 0.549, P = 0.010), and PLI and PPD > 6 mm (r = 0.442, P = 0.045) (Figure 1A). Similar trends were observed post-surgery throughout the 12-month follow-up (3 to 12 months; Figure 1B) and at the individual time points of 3-, 6-, and 12-months (Figure S1.docx).

Surgical treatment reduced the frequencies of BOP (P < 0.0001) and PPD (P < 0.0001) (data not shown). Post-surgery, change in BOP was not associated with gender, smoking, age, the number of teeth, or other diseases (P > 0.05), however, change in PPD after surgery was associated with other diseases (P = 0.044), but not with gender, smoking, age, or the number of teeth (all P > 0.05; based on GEE of data from 3 to 12 months). Notably, both BOP and PPD > 4 mm increased over-time post-surgically, indicating early signs of relapse (GEE, 3 to 12 months samples, adjusted for gender, smoking, age, and other diseases) (Figure 1C and 1D).

3.2  Multivariate analysis of associations between serum inflammatory markers and BOP

We performed a PLS regression to generate an overview of the 96 serum proteins and their potential associations with changes in clinical treatment outcome at different time points after surgery. Due to the highly correlated links between BOP, PPD, and PLI, we primarily used BOP as the dependent variable. The model results (R^2 = 0.89, Q^2 = 0.41) for samples collected at time point 0 indicated that hs-CRP and cytokines IL8, IL20RA, ENRAGE, IL12B, and CCL4 were positively associated with BOP (Figure 2A). At 12 months after surgery, model results (R^2 = 0.86, Q^2 = 0.14) showed that CXCL5, MMP10, IL8, OPG, CCL11, hs-CRP, and MCP4 were positively associated with BOP (Figure 2B). For samples taken at 3, 6, and 12 months after surgery, model results (R^2 = 0.77, Q^2 = 0.46) showed that BOP associated with increased serum levels of CXCL5, TNFβ, DNER, MMP10, CXCL6, CD6, OPG, IL10, IL8, and hs-CRP, and reduced level of cystatin-D (Figure 2C).

3.3  Inflammatory markers associated with BOP and PPD

Next, we used GLM and GEE models to evaluate associations between serum proteins and BOP, after adjusting for potential confounders (i.e., gender, smoking, age, and other diseases). Before surgery, hs-CRP, CXCL5, IL8, and HGF were positively associated with BOP (Table 1). At 12 months after surgery, hs-CRP and CXCL5 were positively associated with BOP, and cystatin-D was negatively associated with BOP (Table 1). A relapse of BOP between 3...
FIGURE 2 Multivariate partial least square (PLS) regression analysis show associations between serum proteins and BOP. PLS models included BOP as the dependent variable ($y$) and levels of 96 serum proteins as the independent $x$-variables. Samples were collected at (A) time point 0 (pre-surgery), (B) at 12 months post-surgery, or (C) at 3, 6, and 12 months post-surgery (combined). (Left) Score plots summarize the relationship between the serum proteins and the dependent variable, BOP. (Right) Bar plot shows the variable importance for the projection (VIP), which summarizes the importance of the variable in explaining the correlation between $X$ and $Y$. Serum proteins were considered significant, when VIP > 1.0 and 95% CI was above zero. Significant factors are indicated in red. $w^*$ describes the PLS weights from the combination of the original variables in the X-swarm; $c[1]$ and $c[2]$ are components 1 and 2, respectively.

and 12 months after surgery was associated with elevated levels of hs-CRP, PD-L1, CXCL5, CD6, and TNFβ, and reduced levels of cystatin-D (Table 1).

Due to concern about the confounding effects of missing teeth, we constructed models that were adjusted for gender, smoking, age, other diseases, and the number of teeth. Those results suggested that elevations in hs-CRP and PD-L1, and a reduction in cystatin-D, were associated with a relapse of BOP after surgical treatment (Figure 3A-C). Serum proteins that were associated with BOP were then analyzed for relationships to PPD. Consistent with the highly correlated structure between BOP and PPD, we found that elevated levels of hs-CRP were associated with both PPD > 4 mm ($P < 0.0005$) and PPD > 6 mm ($P < 0.0005$); we also found that reduced levels of cystatin-D were associated with elevations in PPD > 4 mm ($P = 0.041$) and PPD > 6 mm ($P = 0.001$). Elevated levels of PD-L1 were associated with an elevation in PPD > 4 mm ($P = 0.026$), but not PPD > 6 mm ($P = 0.188$; Figure 3D-I).
TABLE 1 Association of inflammatory markers with bleeding on probing

<table>
<thead>
<tr>
<th>Factor</th>
<th>Pre-surgery</th>
<th>Post-surgery 12 months</th>
<th>P Value</th>
<th>Post-surgery 3, 6, and 12 months</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-CRP</td>
<td>33427 (11394–55460)</td>
<td>191641 (105699–277582)</td>
<td>&lt;0.0005</td>
<td>171484 (100081–242888)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Cystatin–D</td>
<td>−0.005 (−0.015–0.005)</td>
<td>−0.023 (−0.035–0.011)</td>
<td>&lt;0.0005</td>
<td>−0.032 (−0.046–0.019)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>PD–L1</td>
<td>0.006 (−0.002–0.013)</td>
<td>0.027 (0.001–0.052)</td>
<td>0.038</td>
<td>0.030 (0.015–0.044)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>CXCL5</td>
<td>0.018 (0.006–0.029)</td>
<td>0.002 (0.030–0.085)</td>
<td>&lt;0.0005</td>
<td>0.044 (0.020–0.067)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>TNFβ</td>
<td>0.004 (−0.003–0.012)</td>
<td>0.013 (−0.011–0.037)</td>
<td>0.30</td>
<td>0.020 (0.008–0.033)</td>
<td>0.002</td>
</tr>
<tr>
<td>CD6</td>
<td>0.004 (−0.008–0.015)</td>
<td>0.013 (−0.006–0.033)</td>
<td>0.18</td>
<td>0.025 (0.008–0.041)</td>
<td>0.003</td>
</tr>
<tr>
<td>IL8</td>
<td>0.017 (0.007–0.028)</td>
<td>0.001 (0.027–0.011–0.061)</td>
<td>0.13</td>
<td>0.012 (−0.010–0.034)</td>
<td>0.29</td>
</tr>
<tr>
<td>HGF</td>
<td>0.009 (0.003–0.014)</td>
<td>0.001 (0.012–0.011–0.036)</td>
<td>0.31</td>
<td>0.009 (−0.006–0.024)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

To evaluate associations between the 96 serum proteins and bleeding on probing, we constructed a general linear model to examine measurements at each individual visit, and a generalized estimated equation to evaluate the 3- to 12-month repeated measurements; β: Beta values; 95% CI: 95% confidence interval; all models were adjusted for age, sex, smoking, and other diseases. Bold font indicates proteins that showed significant associations after adjusting for multiple comparisons with the Benjamini-Hochberg equation, at a false discovery rate of 5%.

4 | DISCUSSION

This pilot prospective study focused on the early signs of periodontitis relapses over 12 months after surgical intervention. The levels of 96 serum proteins were evaluated to determine associations with BOP and PPD. Our main finding revealed that increases in BOP and PPD after surgery were associated with increased serum levels of hs-CRP and PD-L1, and reduced levels of cystatin-D.

CRP is a highly conserved, type I acute-phase protein, and its plasma concentration can increase up to 1000-fold in response to a stimulus, like an infection or tissue damage; thus, it is an important clinical diagnostic tool. Minor elevations in serum CRP levels can be detected with hs-CRP assays. Studies have shown that hs-CRP is a reliable marker of systemic low-grade inflammation associated with a variety of conditions and several genetic polymorphisms. In this study relapse of periodontitis after surgery were associated with increased levels of hs-CRP and PD-L1, and reduced levels of cystatin-D.

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In this study we followed treatment outcome at different timepoints for 12 months. Signs of relapse of periodontitis regarding the clinical parameters PPD and BOP were associated to changes of some serum proteins of interest for the healing process. That clinical signs of relaps of periodontitis could be monitored in serum further strengthens the possibility that periodontitis could contribute to the systemic inflammatory burden. Together, these findings suggested that serum proteins could be early indicators, of increased risk for inflammatory associated diseases, present in individuals that do not respond well to periodontal treatment or experience relapses of the disease.

The balance between bone resorption and bone formation is central in bone metabolism-related diseases and conditions, such as periodontitis and autoimmune arthritis. Here, we showed that reduced serum cystatin-D levels were associated with post-surgery periodontitis relapses. In this context, it is interesting that up-regulation of cystatin-D is shown to inhibit the activation of osteoclasts and bone resorption. Cystatin-D is a type-2 cysteine protease inhibitor, predominantly found in saliva. Thus, cystatin-D inhibits proteases, such as cathepsin S that degrade extracellular matrix proteins. Cathepsin S is produced by for example macrophages, lymphocytes, dendritic cells, and periodontal ligament cells. Inflammatory and microbial stimuli can affect the levels of cathepsin S, which suggests that this protease might play a role in oral inflammatory diseases. Gene expression analysis of gingival biopsies from periodontitis sites identified cathepsin S as a central hub protein, which suggested that it was involved in periodontitis. Cathepsin S was also shown to affect bone remodeling. When cathepsin S expression was disrupted in knockout mice, the balance between adipocyte and osteoblast differentiation was altered, bone turnover increased, and bone microarchitecture changed. Cathepsin S has also been implicated in
Figure 3 Generalized estimated equation results show hs-CRP, cystatin-D, and PD-L1 associations with bleeding on probing (BOP) and deep periodontal probing depths (PPDs). Generalized estimated equation models, including measurements from time points 3, 6, and 12 months post-surgery, were used to evaluate associations between the indicated proteins and percent surfaces with (A–C) BOP, (D–F) PPD > 4 mm, or (G–I) PPD > 6 mm. All models were adjusted for gender, age, smoking, other diseases, and the number of teeth. Each graph includes trend lines with 95% CIs.

Osteoimmunological diseases, such as rheumatoid arthritis, and in vascular and metabolic complications related to obesity, like diabetes mellitus, diseases shown to associate with periodontal diseases. Studies have also shown that cathepsin S knockout mice had a reduced disease burden in experimental models of arthritis. However, a potential direct or indirect role of cystatin-D in periodontal diseases needs to be further evaluated.

In addition, we found that PD-L1 was associated with BOP and PPD. PD-L1 is present on macrophages and antigen-presenting cells. PD-L1 can also be induced in other cell types, including endothelial, epithelial, and B cells, during inflammation. When PD-L1 interacts with the programmed death protein, it can adjust the immune response by down-regulating active T-cells, which protects the host from uncontrolled immune responses and inflammation-induced tissue damage. However, elevated PD-L1 expression might lead to a PD-L1-dependent attenuation of the immune system during inflammation or even during acute inflammation, which
Periodontitis-associated bacteria (e.g., Porphyromonas gingivalis) can induce elevations in PD-L1 expression. This activity down-regulates the immune response, attenuates bacterial clearance, and thus, facilitates a chronic bacterial infection. Indeed, persistently elevated PD-L1 levels can drive a chronic inflammatory disease, like periodontitis. This study had some strengths and limitations that should be recognized and considered when evaluating the results. The study strengths included the prospective study design with frequent follow-ups and the focus on the recurring frequency and progression of the disease for 12 months after surgery. Furthermore, serum was collected at each time point and screened against a wide panel of inflammatory markers. A major limitation was the modest clinical sample size. Still, this small sample size facilitated relatively frequent recalls. The selected study design did not include a healthy control group. Therefore, we might potentially have overlooked inflammatory markers that were elevated in a population affected by periodontitis. However, that was not the focus of this study as it is changes in protein levels after treatment that we are investigating. In future studies, the set of serum proteins that we found were associated with periodontitis should be validated in an independent sample, which preferably includes a large biobank of material and reliable periodontitis scores. Further studies are also needed to evaluate if serum levels of these biomarkers could be mirrored in the local environment such as gingival crevicular fluid or saliva, which are more easily accessible.

5 | CONCLUSION

This pilot study showed that clinical signs of periodontal disease relapse after surgery were associated with changes in several inflammatory associated serum proteins. In particular, relapses were associated with elevated PD-L1 and hs-CRP concentrations and reduced cystatin-D concentrations. Increased PD-L1 expression can lead to T-cell inactivation, local immune suppression, and attenuated bacterial clearance, which facilitate chronic bacterial infections and can potentially drive chronic inflammation. The increase in hs-CRP might indicate the involvement of a pathway in common with other diseases or a response to unresolved PD-L1-driven inflammation. The reduced cystatin-D levels could lead to bone resorption by osteoclasts and/or increased cathepsin activity, which leads to tissue destruction and can induce auto-immune processes.

ACKNOWLEDGMENTS

This work received financial support from the the County Council of Västerbotten, Sweden, Spjutspetsmedel, grant number RV 396172146, and the County Council of Västerbotten, Sweden, Internal Research Foundation, grant number RV 396172134 and the Public Dental Health County Council of Gävleborg, Gävle, Sweden, and from the Centre for Research and Development, Uppsala University/Region Gävleborg, Gävle, Sweden. None of the funding bodies had any influence on the study design, the data collection, analysis, or interpretation, or the writing of the manuscript. We also want to acknowledge SciLifeLab and the Clinical Biomarkers facility, Uppsala, Sweden.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Anders Esberg: data analysis and interpretation; drafting and critical revision of the manuscript
Catrine Isehed: study conception and design; data analysis and interpretation; drafting and critical revision of the manuscript.
Anders Holmlund: study conception and design; data interpretation; and critical revision the manuscript.
Susanne Lindquist: data analysis and interpretation; drafting and critical revision of the manuscript.
Pernilla Lundberg: data acquisition, analysis, and interpretation; drafting, and critical revision of the manuscript. All authors gave final approval of the manuscript and agree to be accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES


**SUPPORTING INFORMATION**

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[https://doi.org/10.1002/JPER.21-0089](https://doi.org/10.1002/JPER.21-0089)