Received: 8 February 2023 | Revised: 9 March 2023 | Accepted: 30 March 2023

DOI: 10.1111/clr.14067

CLINICAL ORAL IMPLANTS RESEARCH WILEY

ORIGINAL ARTICLE

# Anti-Apoptotic and Pro-Apoptotic Bcl-2 Family Proteins in Peri-Implant Diseases

Dogukan Yilmaz[1,2] | Mervi Gürsoy[2] | Ulvi Kahraman Gürsoy[2]

[1]Department of Periodontology, Faculty of Dentistry, Sakarya University, Sakarya, Turkey

[2]Department of Periodontology, Institute of Dentistry, University of Turku, Turku, Finland

**Correspondence**
Dogukan Yilmaz, Department of Periodontology, Institute of Dentistry, University of Turku, Lemminkäisenkatu 2, 20520, Turku, Finland.
Email: dogukan.yilmaz@utu.fi

**Funding information**
Suomen Hammaslääkäriseura Apollonia; Türkiye Bilimsel ve Teknolojik Araştırma Kurumu

## Abstract

**Objectives:** Intrinsic apoptosis, which is regulated by Bcl-2 family proteins, has an important role in chronic inflammatory diseases. The aim of the study was to identify the tissue levels and ratios of anti- and pro-apoptotic Bcl-2 family proteins in peri-implant diseases.

**Materials and Methods:** Twenty-three individuals with peri-implant mucositis, 25 individuals with peri-implantitis, and 24 controls were included. The following clinical parameters were recorded: keratinized mucosa width, modified bleeding index, probing depth, modified plaque index, modified gingival index, and keratinized tissue thickness. Marginal alveolar bone assessments were performed by a software program. Granulation tissues were collected during treatments of peri-implant diseases. The control tissue samples were collected during the second stage of implant surgery. The tissue levels of Bcl-2 family pro-apoptotic (Bak, Bax, active caspase-3) and anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1) proteins were determined by multiplex immunoassay method.

**Results:** The pro-apoptotic proteins; Bak, Bax and anti-apoptotic proteins Bcl-2, Bcl-xL, Mcl-1 were detected significantly higher in controls compared with patients with peri-implant mucositis and peri-implantitis ($p < .001$), respectively. The higher active caspase-3 levels were also detected in controls in comparison with peri-implant mucositis ($p = .018$) and peri-implantitis ($p = .005$). Anti-apoptotic: pro-apoptotic protein ratios (Bcl-2:Bax, $p < .001$; Bcl-2:Bak, $p = .01$; Bcl-xL: Bax, $p = .006$, Bcl-xL:Bak, $p = .011$; Mcl-1:Bak, $p < .001$) were significantly increased in diseased groups. A positive correlation was demonstrated between clinical variables and anti-apoptotic: pro-apoptotic ratios.

**Conclusion:** Our findings indicate dysregulation of the Bcl-2 family proteins in peri-implant diseases. This unregulated response may disturb the homeostasis of peri-implant tissue.

KEYWORDS
apoptosis, apoptosis regulatory proteins, peri-implantitis

## 1 | INTRODUCTION

Dental implants are indispensable treatment options for the rehabilitation of partially or fully edentulous individuals; however, a great number of them develop biological complications (Derks et al., 2022). Peri-implant diseases are chronic inflammatory disorders related to bacterial dysbiosis (Schwarz et al., 2018). The prevalence of peri-implant diseases, encompassing peri-implant mucositis (soft tissue inflammation alone) and peri-implantitis (inflammation with hard and soft tissue loss), is relatively high. It has been reported that approximately one out of three patients and one out of five dental implants experienced peri-implantitis (Kordbacheh Changi et al., 2019). Peri-implant mucositis is assumed to be the precursor of peri-implantitis, but the etiological and pathophysiological processes from peri-implant mucositis to peri-implantitis are still obscure (Ravidà et al., 2020). Several treatment options exist for peri-implant diseases (Schwarz et al., 2021). Nonetheless, the treatments are challenging, and the outcomes may be unsatisfactory. Additionally, current evidence does not support a gold-standard treatment protocol (Ramanauskaite et al., 2021). The underlying mechanism of the peri-implant diseases keeps its enigmatical paradigm and evolving the peri-implant diseases as a major burden for dental community (Kumar et al., 2018).

Apoptosis is a programmed cell death mechanism, characterized by coordinated disintegration of dying cells into smaller fragments that ensure sequestration of intracellular component (Green, 2019). This specific type of cell death mechanism is triggered by multi-signal pathways and regulated by intrinsic and extrinsic pathways (Ketelut-Carneiro & Fitzgerald, 2022). The intrinsic pathway initiates through signals that promote disruption of the mitochondrial integrity. When the mitochondrial integrity disrupts, cytochrome C release into cytosol and activates the executioner caspase 3 (Picca et al., 2021). Bcl-2 family proteins can directly control the mitochondrial membrane permeability (Lalier et al., 2022). These proteins can be classified into different groups based on their structural and functional properties. According to their capability to inhibit or to stimulate cytochrome C release, they defined as anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1) or pro-apoptotic proteins (Bax, Bak) (Youle & Strasser, 2008). Bcl-2 family proteins can form homo- and heterodimers with one another and overall, the ratio of pro to anti-apoptotic regulators may determine whether a cell is condemned to die or survive (Galluzzi et al., 2012).

Dysregulation of intrinsic apoptotic response, due to increase of anti-apoptotic: pro-apoptotic protein ratios, has been implicating in various chronic inflammatory diseases including, Crohn's disease, rheumatoid arthritis, and periodontitis (Figueredo et al., 2019; Itoh et al., 2001; Williams et al., 2018). According to these studies, increased anti-apoptotic: pro-apoptotic protein ratios and decreased intrinsic apoptosis response cause persistent chronic inflammatory environment. Uncontrolled inflammatory response eventually leads to hard and/or soft tissue loss. The alterations in intrinsic apoptotic response can also contribute to the pathogenesis of peri-implant diseases, which have a chronic inflammatory nature (Schwarz et al., 2018). Studies regarding on the apoptosis in peri-implant diseases are scarce (Bullon et al., 2004; Ganesan et al., 2022). To the best of our knowledge, Bcl-2 family protein levels and their ratios in peri-implant diseases have not been studied before. Based on this information, our hypothesis was that the severity of peri-implant disease relates to the unregulated intrinsic apoptotic response. The aim of the study was to detect the Bcl-2 family protein levels in healthy peri-implant mucosa and in inflamed peri-implant granulation tissues. Additionally, we investigated the anti-apoptotic: pro-apoptotic Bcl-2 family protein ratios in relation to the severity of peri-implant disease.

## 2 | MATERIALS AND METHODS

### 2.1 | Participant selection and clinical evaluation

The ethical permit of the study was approved by the Clinical Research Committee of the Sakarya University Faculty of Medicine (Approval number: E-16214662-050.01.04-85,162-217) in accordance to the ethical guidelines of Helsinki Declaration. The study protocol was explained to all participants, and their written informed consents were obtained.

All patients, who applied to clinics of the Department of Periodontology, Sakarya University, between March 2021 and June 2022, were invited to the study. Patients were either treated by dental implants and in the maintenance phase or were still under dental implant treatment or applied with a complaint of their dental implants, which was performed by another center. For the selection of the study participants, the following inclusion criteria were applied: (1) partially/totally edentulous patients rehabilitated with a single/multiple implant-supported prosthesis, (2) dental implant supported restorations, which were in function for at least 1 year, (3) the presence of at least one screw-type (one- or two-part) titanium implant diagnosed with peri-implant mucositis or peri-implantitis and indicated for treatment (for diseased study groups), (4) being non-smoker (who were never smoked) and former smoker (quit smoking for more than 5 years before the initiation of the study) (Levin et al., 2008). The exclusion criteria were as follows: (1) having general contraindications for dental and surgical treatments, (2) being diagnosed with ongoing periodontal disease, (3) being pregnant or lactating, (4) having autoimmune or/and inflammatory diseases, (5) having uncontrolled diabetes (HbA1c > 7), (6) having corticosteroid therapy, and (7) being smoker. Overall, 72 participants and 72 dental implants (systemically healthy 23 individuals with peri-implant mucositis, 25 individuals with peri-implantitis, and 24 healthy controls) were recruited to the study.

The demographic variables and the medical and dental backgrounds of participants were obtained by interviews. After full-mouth periodontal examination, the following clinical parameters were recorded at each implant site using by manual periodontal probe (Hu-Friedy, Chicago, IL, USA): (1) keratinized mucosa width (KTW), (2) modified bleeding index (mBI) (Mombelli et al., 1987), (3)

probing depth (PD), (4) modified PI (mPI) (Mombelli et al., 1987), (5) modified gingival index (mGI) (Mombelli et al., 1987), and (6) keratinized tissue thickness (Kan et al., 2003), from six sites of dental implants by single calibrated periodontist (Kappa: 0.92). Marginal alveolar bone assessments and bone loss percentages were accomplished through radiological evaluation using a software program (Image J, Wisconsin, USA). The health status of peri-implant tissues was diagnosed according to 2017 classification of Periodontal and Peri-Implant diseases (Berglundh et al., 2018). Peri-implant tissues that did not have any sign of inflammation and the absence of radiological bone loss were considered as healthy. Peri-implant tissues with presence of bleeding and/or suppuration on probing and absence of bone loss beyond crestal bone level changes resulting from initial bone remodeling (when follow-up radiographs were compared with initial radiograph) were defined as peri-implant mucositis. While diagnosis of peri-implantitis required the presence of bleeding and/or suppuration on probing, probing depths of ≥6 mm and radiological bone levels ≥3 mm apical of the most coronal portion of the intraosseous part of dental implants were required. Additionally, the severity of peri-implantitis was classified according to the defect length from the implant neck and the ratio of marginal bone loss relative to the total implant length (bone loss %) (slight <25% of the implant length ($n=11$), moderate ≥25%–50% of the implant length ($n=9$), advanced >50% of the implant length ($n=5$)) (Monje et al., 2019).

## 2.2 | Tissue sampling

Following baseline clinical evaluation, full-mouth prophylaxis or a periodontal maintenance was performed without subgingival debridement. Patients, who were diagnosed with peri-implant mucositis or peri-implantitis, were treated by single periodontist (DY). The granulation tissue samples were harvested during the treatments of peri-implant mucositis (non-surgical) and peri-implantitis (resective, reconstructive, or combined treatment) (Menezes et al., 2016; Schwarz et al., 2022). Briefly, in nonsurgical treatment of peri-implant mucositis, inflamed granulation tissues were obtained by titanium curettes (Hu-Friedy, Titanium implant scaler mini-five, Chicago, IL, USA) during the mechanical debridement under local anesthesia. During the surgical treatment of peri-implantitis, following local anesthesia (articaine, 1:200,000), mucoperiostal flaps were elevated at both vestibular and oral aspects to expose the peri-implant defect. All granulation tissue was carefully harvested from the respective bony defect area by using titanium curettes (Hu-Friedy, Titanium implant scaler mini-five, Chicago, IL, USA). When peri-implant disease was diagnosed in more than one area, the granulation tissue was obtained from the peri-implant diseased tissue with the deepest probing depth. All patients with peri-implant diseases received maintenance therapy after the completion of their treatments. The healthy peri-implant mucosa tissue samples were collected during the second stage of surgery phase in regular dental implant treatments with an incisional biopsy including the mucosa, which was in contact with the implant surface. All granulation and mucosal tissue samples were placed in Eppendorf tubes and stored at −80°C until being transferred to Institute of Dentistry, University of Turku, in dry ice for the biochemical analyses.

## 2.3 | Quantification of the intrinsic apoptotic proteins

The defrosted tissue samples were weighed on a microbalance and then cut in small pieces by scalpels (Disposable scalpel #11, #15, Feather Safety Razor Co., Ltd, Osaka, Japan). Afterwards, samples were transferred to 2 mL tubes containing four ultrapure zirconium microspheres of 3 mm (beads) and 400 μL of lyzer buffer. The samples were homogenized with a high-speed tissue homogenizer (The TissueLyzer LT, Qiagen, Hilden, Germany) at 2000 rpm with four cycles of 60 s each, with an interval of 20 s on ice. After homogenization, samples were centrifuged at 10000 rpm for 4 min, and the beads were removed. Then, the samples were ultrasonicated (UP50H, Hielscher, Teltow, Germany) at 0.5 cycle, 100% amplitude with three cycles of 5 s each, with an interval of 20 s on ice; thereafter, they were centrifuged at 10,000 rpm for 4 min. The supernatants were transferred to another tube. Total protein levels of each sample were determined by Bradford assay (Hammond & Kruger, 1988).

Pro-apoptotic proteins (Bak, Bax, active caspase-3) and anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1) levels were determined by a bead-based immunoassay technique (Luminex xMAP, Luminex Corporation, Austin, TX, USA) with commercial kits (Bio-Plex Pro, RBM Apoptosis Assays, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction. The detection range of assays was as follows: Bak (0.98–592 ng/mL), Bax (0.69–280 ng/mL), active caspase-3 (0.041–90 ng/mL), Bcl-2 (0.78–510 ng/mL), Bcl-xL (0.11–47 ng/mL), Mcl-1 (0.24–190 ng/mL). The pro- and anti-apoptotic protein levels were normalized to the total protein levels of each sample. The tissue levels of related proteins were presented as ng/μg protein.

## 2.4 | Statistical analyses

The statistical analyses were performed using by SPPS V.28.0.1.1 (SPPS, Chicago, IL, USA). As no study existed in the literature that investigated the tissue levels of Bcl-2 family proteins in peri-implant tissue samples, the effect size 0.668 was defined based on a previous research reporting significantly different gingival tissue levels of caspase-3 between systemically and periodontally healthy participants (Johnson & Wikle, 2014). With a power of 95% and $\alpha=0.05$, a minimum number of 21 participants was required for the comparison. Power analyses were performed by the G*Power 3.1 (Universitat Kiel, Kiel, Germany). The data were presented as means (standard deviations) and medians. In normally distributed samples, one-way ANOVA with Bonferroni post-hoc analyses was applied to compare study groups. Non-parametric Kruskal–Wallis (for multiple comparisons) and Dunn–Bonferroni post-hoc methods were used

when comparing nonnormally distributed parameters. The Chi-square test was used to compare the categorical variables between the study groups. Spearman correlation test was applied to determine the possible correlation between related proteins with clinic variables. A *p*-value of <.05 was considered statistically significant. The report of this study follows the STROBE guidelines.

## 3 | RESULTS

Demographic and periodontal clinic variables of participants are presented in Table 1. The mean age of the participants was 49.6 ± 9.7 years. No difference was observed in the age and gender distributions of the study groups. The mean functional time of dental implants at which participants were included in the study was 4.5 ± 1.7 years. As expected, highest PD, mPI, mGI, and mBI values were detected in peri-implantitis group followed by peri-implant mucositis group. Contrary, KTW of control and peri-implant mucositis groups was higher than that of peri-implantitis group (control vs peri-implantitis, $p = .021$; peri-implant mucositis vs peri-implantitis, $p = .027$). Among the subclassification of peri-implantitis, clinical parameters except PD ($p < .001$) and bone loss percentage ($p < .001$) did not show any difference.

The levels of pro-apoptotic proteins; Bak, Bax and anti-apoptotic proteins Bcl-2, Bcl-xL, Mcl-1 were significantly higher in controls compared with patients with peri-implant mucositis and peri-implantitis ($p < .001$). The higher active caspase-3 levels were also detected in controls in comparison with peri-implant mucositis ($p = .018$) and peri-implantitis ($p = .005$), which are illustrated in Figure 1. There was not any difference between the peri-implant mucositis and peri-implantitis patients regarding their tissue pro- and anti-apoptotic protein levels. Interestingly, our results demonstrated that anti-apoptotic: pro-apoptotic protein ratios (Bcl-2:Bax; peri-implant mucositis vs. control, $p < .001$, peri-implantitis vs. control, $p < .001$; Bcl-2:Bak; peri-implant mucositis vs. control, $p = .01$, peri-implantitis vs. control, $p < .001$; Bcl-xL: Bax; peri-implant mucositis vs. control, $p = .006$, Bcl-xL:Bak; peri-implant mucositis vs. control, $p = .011$; Mcl-1:Bak; peri-implant mucositis vs. control, $p < .001$, peri-implantitis vs. control, $p < .001$) were significantly increased in diseased groups compared with controls (Figure 2). Regarding the subclassification of peri-implantitis, neither Bcl-2 family protein levels nor anti-apoptotic: pro-apoptotic ratios demonstrated any significant differences among groups ($p > .05$).

Correlations regarding clinical parameters, intrinsic apoptotic proteins, and anti-apoptotic: pro-apoptotic protein ratios are demonstrated in Table 2. Briefly, a positive correlation was demonstrated between clinical variables and anti-apoptotic: pro-apoptotic ratios (Bcl-2:Bax, Bcl-2:Bak, Bcl-xL:Bak, Mcl-1:Bak), while a negative correlation existed between clinical variables and Bcl-2 family proteins, also between pro-apoptotic and anti-apoptotic protein levels: Bax with Bcl-2 ($r$:−.465, $p < .001$), Bcl-xL ($r$:−841, $p < .001$), and Mcl-1 ($r$:−783, $p < .001$); Bak with Bcl-2 ($r$:−.536, $p < .001$), Bcl-xL ($r$: −839, $p < .001$), and Mcl-1 ($r$: −868, $p < .001$); Active caspase 3 with Mcl-1 ($r$:−.323, $p = .013$).

## 4 | DISCUSSION

In the present study, we have demonstrated that Bcl-2 family protein-dependent increased anti-apoptotic: pro-apoptotic protein ratios and significant decreased levels of executive active caspase-3 in peri-implant mucositis and peri-implantitis in comparison with controls. To the best of our knowledge, peri-implant tissue levels of Bcl-2 family proteins and their ratios in patients with peri-implant diseases were investigated for the first time.

Apoptosis is a fundamental physiological cellular activity that plays an important role in homeostasis (Green, 2019). Levels and types of Bcl-2 family proteins contribute to death or survival of the host cells by regulating intrinsic apoptosis (Ketelut-Carneiro & Fitzgerald, 2022). These proteins of intrinsic apoptosis can form

**TABLE 1** Demographic and clinical data of study participants.

| | Control $N = 24$ | Peri-implant mucositis $N = 23$ | Peri-implantitis $N = 25$ | $p$ |
|---|---|---|---|---|
| Age (years) | 47.5 ± 8.2 | 49.8 ± 10.3 | 51.4 ± 10.5 | .379 |
| Female (%) | 66.6 | 52.17 | 60 | .099 |
| PD (mm) | - | 3.58 ± 0.57 | 7.29 ± 1.15 | **<.001** |
| mPI | - | 1.03 ± 0.8 | 2.25 ± 0.42 | **<.001** |
| mGI | - | 1.33 ± 0.55 | 2.27 ± 0.33 | **<.001** |
| mBI | - | 1.76 ± 0.32 | 2.35 ± 0.44 | **<.001** |
| KTW (mm) | 2.5 ± 0.83 | 2.47 ± 1.23 | 1.54 ± 1.45 | **.009** |
| Thick Biotype (%) | 50.8 | 43.47 | 36 | .078 |
| Bone loss percentage (%) | - | - | 40 ± 18.8 | **<.001** |

*Note*: All data presented in mean ± standard deviation values. One-way ANOVA and Bonferroni corrections were used to analyze clinical variables. Chi-square test was used to compare categorical variables between study groups. Statistically significant *p*-values are in bolded.

Abbreviations: mBI, Modified bleeding index; mGI, Modified Gingival Index; mPI, Modified Plaque Index; PD, Probing depth; KTW, Keratinized tissue width.

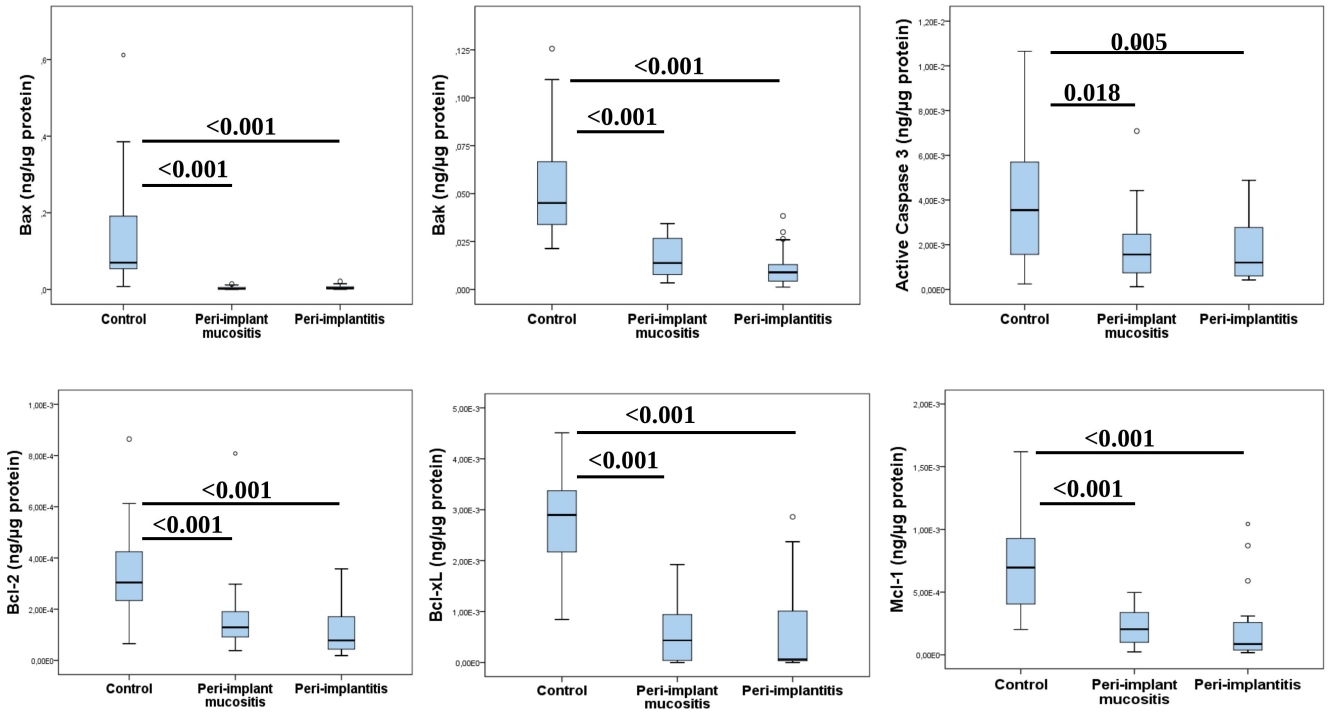


**FIGURE 1** Granulation and the healthy peri-implant mucosal tissue levels of pro-apoptotic proteins (Bax, Bak, active caspase 3) and anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1) levels in relation to participants' peri-implant health status. Statistically significant *p*-values are in presented.

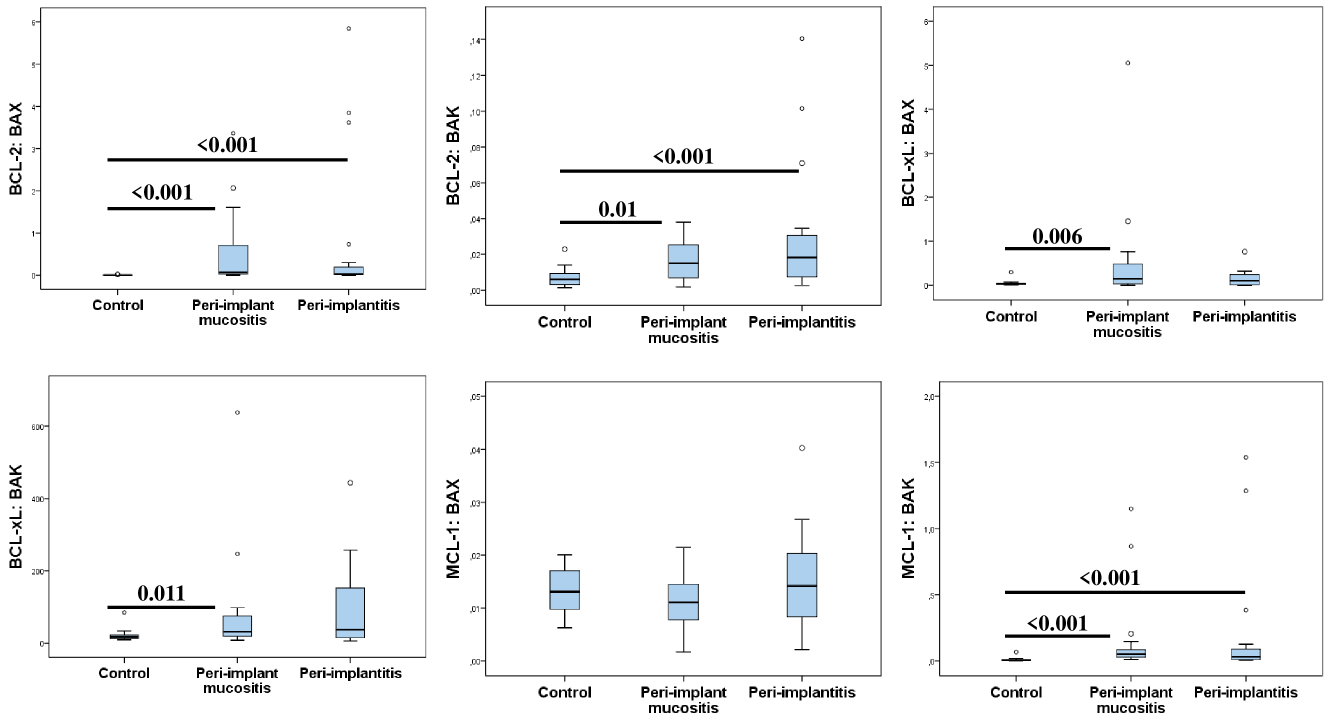


**FIGURE 2** Anti-apoptotic: pro-apoptotic protein ratios in relation to participants peri-implant health status. Statistically significant *p*-values are in presented.

homo- and heterodimers with one another, so what actually dictates the final faith of the cell is regulated by the ratios of Bcl-2 family proteins (Galluzzi et al., 2012). Therefore, we evaluated three distinct parameters including absolute expression levels, ratio of anti-apoptotic: pro-apoptotic protein levels, and the correlation between these proteins and clinical variables. It is known that systemic and host-related determinants can modify the apoptotic response. For instance, senescence, gender, and smoking status arise

**TABLE 2** Correlations between clinical variables, Bcl-2 family proteins, and anti-apoptotic: pro-apoptotic protein ratios.

| | Age | PD | mPI | mGI | mBI | KTW | Bone loss % |
|---|---|---|---|---|---|---|---|
| Bcl-2:Bax | **.244, 0.050** | **.497, <0.001** | **.467, <0.001** | **.571, <0.001** | **.489, <0.001** | −.235, 0.06 | .228, 0.68 |
| Bcl-2:Bak | .148, 0.241 | **.435, <0.001** | **.345, 0.005** | **.405, 0.001** | **.445, <0.001** | −.123, 0.327 | **.368, 0.003** |
| Bcl-xL: Bax | −.012, 0.920 | .148, 0.233 | .112, 0.365 | .040, 0.745 | .167, 0.289 | −.126, 0.31 | .003, 0.982 |
| Bcl-xL:Bak | **.323, 0.011** | **.298, 0.02** | **.255, 0.047** | **.380, 0.003** | **.367, 0.008** | −.051, 0.698 | .144, 0.268 |
| Mcl-1:Bax | −.135, 0.308 | .005, 0.971 | −.042, 0.752 | −.030, 0.839 | −038, 0.786 | −.191, 0.147 | .127, 0.337 |
| Mcl-1:Bak | .216, 0.100 | **.553, <0.001** | **.567, <0.001** | **.571, <0.001** | **.589, <0.001** | −.205, 0.119 | **.270, 0.039** |
| Bax | **−.286, 0.015** | **−.616, <0.001** | **−.594, <0.001** | **−.699, <0.001** | **−.645, <0.001** | .196, 0.098 | **−.320, 0.006** |
| Bak | −.191, 0.108 | **−.683, <0.001** | **−.618, <0.001** | **−.742, <0.001** | **−.675, <0.001** | .200, 0.093 | **−.498, <0.001** |
| Active caspase 3 | −.017, 0.890 | **−.318, 0.007** | **−.274, 0.020** | **−.239, 0.043** | **−.268, 0.017** | .117, 0.326 | −.215, 0.070 |
| Bcl-2 | −.064, 0.610 | **−.587, <0.001** | **−.587, <0.001** | **−.634, <0.001** | **−.615, <0.001** | .112, 0.375 | **−.428, <0.001** |
| Bcl-xL | −.281, 0.021 | **−.651, <0.001** | **−.625, <0.001** | **−.752, <0.001** | **−.649, <0.001** | .111, 0.370 | **−.392, 0.001** |
| Mcl-1 | −.147, 0.266 | **−.625, <0.001** | **−.641, <0.001** | **−.641, <0.001** | **−.633, <0.001** | .235, 0.073 | **−.433, 0.001** |

*Note*: Data presented as Spearman correlation coefficient, *P*-value. Statistically significant *p*-values are in bolded.

Abbreviations: mBI, Modified bleeding index; mGI, Modified gingival Index; mPI, Modified plaque Index; PD, Probing depth; KTW, Keratinized tissue width.

differences in apoptotic response in gingival tissues (Das et al., 2009; Johnson & Wikle, 2014; Karatas et al., 2020). The age and gender-matched study groups and the strict exclusion criteria of common risk factors such as smoking in peri-implant diseases were the main strength of the present study. In addition, including the peri-implant mucositis and peri-implantitis patients, together with the subclassification of peri-implantitis, allowed us to demonstrate the variations in Bcl-2 family protein levels. However, the present study's cross-sectional design prevents us from monitoring possible fluctuations of related proteins in relation to active or inactive inflammatory status, which is a limitation. In our study, tissue samples from peri-implant disease patients were harvested from the deepest probing depth. Granulation tissue was selected as a sample material, as it can be considered as the most active and appropriate site reflecting an ongoing disease (Özdemir et al., 2020). As there was no ethical justification and clinical indication to excise healthy peri-implant mucosal tissues from participants, healthy control tissues were obtained during second stage of surgery or implant replacement. Therefore, the anatomical characteristics of healthy peri-implant mucosa and granulation tissues were different, and it can be considered as a second limitation.

According to the present findings, both pro and anti-apoptotic Bcl-2 family proteins were decreased in peri-implant mucositis and peri-implantitis compared with healthy controls. Similar observations were previously presented in peri-implant diseases. For instance, peri-implant crevicular fluid levels of sirtuin 1 (SIRT1), which is a molecule that can regulate apoptosis, decrease in peri-implant mucositis and peri-implantitis compared with healthy controls (Wang, 2022). As well as negative correlation of SIRT1 with pro-inflammatory cytokines indicated decreased apoptotic response in peri-implant diseases, which was in accordance with present findings. Under physiological circumstances, increasing or decreasing levels of pro-apoptotic proteins of Bcl-2 family should be accompanied by the anti-apoptotic counterpart (Youle & Strasser, 2008). Strong negative correlation of pro-apoptotic proteins with anti-apoptotic proteins in present study confirmed this expectation. Our results demonstrated higher anti-apoptotic: pro-apoptotic protein ratios in tissue samples of peri-implant disease compared with controls. Dysregulation of intrinsic apoptotic response, due to increase of anti-apoptotic: pro-apoptotic ratios, has been demonstrated in other chronic inflammatory diseases, including Crohn's disease, rheumatoid arthritis, and periodontitis (Figueredo et al., 2019; Itoh et al., 2001; Williams et al., 2018). Higher expressions of pro-apoptotic protein levels and apoptosis-related genes in gingival tissues and peripheral bloods of periodontitis patients compared with healthy controls were demonstrated (Liu et al., 2016; Lundmark et al., 2015). On the contrary, Figueredo et al. (2019) presented anti-apoptotic traits in gingival tissues of chronic periodontitis. Peri-implantitis differs from periodontitis with respect to anatomical features, cellular composition of the lesion, and the disease progression rate (Salvi et al., 2017). Due to different characteristics of the periodontal and peri-implant diseases, it is expected that peri-implant diseases are associated with different apoptotic profile than the one that is observed in periodontitis. Finally, based on our findings, Mcl-1: Bax ratios do not differ among the study groups. This disparity can be explained by the feature of Mcl-1. It has been demonstrated that Mcl-1 may not be as potent a protector against apoptosis as Bcl-2, and it does appear to be an important anti-apoptotic protein in some cell types including neutrophils (Denis et al., 2020).

Apoptosis has shown to be directly involved in the integrity of mucosal surface and the regulation of chronic inflammatory and immune responses. Simultaneous activation of intrinsic apoptosis may be considered pathologic at the individual cell level (Green, 2019). Polymorphonuclear leukocytes represent the host's first line of defense against different microorganisms, but their hyperactivity can cause tissue damage and may prolong the extent and severity of chronic inflammatory diseases including peri-implant mucositis and peri-implantitis (Noronha Oliveira et al., 2018). Delayed apoptosis of

neutrophils has been shown in peripheral blood and gingival tissues from patients with periodontitis (Gamonal et al., 2003; Lakschevitz et al., 2013). As chronic inflammatory infiltrate in peri-implantitis granulation tissue is dominated by neutrophils and plasma cells (Rakic et al., 2022), it can be speculated that increased anti-apoptotic trait and positive correlations between clinical variables and anti-apoptotic: pro-apoptotic protein ratios originated from neutrophil and plasma cells. However, peri-implant tissue consists of various kinds of resident and immune-cell types including fibroblasts, epithelial cells, and macrophages, which differ in terms of their apoptotic traits in inflammation and infection (Cheng et al., 2015; Fujita et al., 2012). Our analysis defines intrinsic apoptotic changes at the tissue level, without taking the cellular type into account. Further studies are warranted to determine the cell types that are responsible for the anti-apoptotic trait of peri-implant diseases.

One possible mechanism of the increased anti-apoptotic: pro-apoptotic protein ratios and decreased intrinsic apoptotic trait in peri-implant diseases could be the dysbiotic character of the peri-implant disease. It is known that apoptosis can be hijacked by bacteria and/or fungus to exit host cells, acquire nutrients, and evade host defenses (Ashida et al., 2011). It has been indicated that *P. gingivalis* and *C. albicans* can block the intrinsic apoptosis to maintain their intracellular lifestyle in gingival and oral epithelial cells, respectively, and eventually decrease the intrinsic apoptotic trait (Castro et al., 2017; Villar et al., 2012). Indeed, White et al. (2020) showed that *Streptococcus gordonii* and *Streptococcus sanguinis*, which are known as oral commensal bacteria, can induce apoptotic response in oral epithelial cells to maintain bacterial colonization and tissue homeostasis in health. These findings are in accordance with the present data, which indicate increased intrinsic anti-apoptotic trait in peri-implant diseases. Future studies are warranted to investigate the potential effect of peri-implant subgingival microbiome and host–bacteria interactions regarding intrinsic apoptotic response.

Apoptosis can also be mediated by extrinsic pathway. Extrinsic pathway of apoptosis is known as caspase-dependent and induced by extracellular stimulation (Green, 2019). Briefly, the external stimulus initiate the death-inducing signaling complex (DISC) and the adequate DISC can directly activate caspase-8 to trigger the stimulation of caspase-3 to initiate apoptosis (Singh et al., 2016). According to literature, intrinsic and extrinsic apoptotic pathways may not be coupled (Bock & Tait, 2020). Further studies would be beneficial to clarify the possible effect of extrinsic pathway constituents on anti-apoptotic traits in peri-implant diseases. Programmed cell death mechanisms, including apoptosis, autophagy, pyroptosis and necroptosis, are actively communicated and coordinate with each other to maintain tissue homeostasis (Ketelut-Carneiro & Fitzgerald, 2022). It has been demonstrated that pyroptosis can be triggered by inflammation in peri-implantitis (Chen et al., 2021), and autophagy played an essential role in osteoblast cell differentiation on titanium base surfaces (Zhang et al., 2021). However, literature also illustrated that increased autophagy, pyroptosis, and necroptotic traits in different cells and tissues of periodontitis and peri-implantitis patients may inhibit cell apoptosis (Li et al., 2021; Yang et al., 2021). It can be hypothesized that the other cell death mechanisms may overcome the intrinsic apoptosis, and this may explain the increased intrinsic anti-apoptotic response in peri-implant disease. The exact switch between these cell death mechanisms is still unclear and needs further investigation (Nikoletopoulou et al., 2013). It was shown that complex mechanisms can regulate the intrinsic apoptotic response in host cells (Picca et al., 2021). By understanding the mechanisms that inhibit the intrinsic apoptosis and provoke higher clinical parameters that seen in peri-implant diseases, diagnostic and therapeutic strategies may be developed by future studies.

## 5 | CONCLUSION

Within the limitation of present study, intrinsic anti-apoptotic trait is increased in peri-implant diseases. Dysregulation in the expression profiles of Bcl-2 family proteins in peri-implant tissues may take part in disruption of healthy tissue homeostasis, which may lead to the chronic inflammatory disease. Future studies are needed to describe the potential mechanisms.

### AUTHOR CONTRIBUTIONS

D.Y contributed to conception, design, data acquisition, analysis and interpretation, and preparation of the draft of original manuscript; M.G. contributed to the analysis and critically revised the manuscript; U.K.G contributed to conception, design, data acquisition, analysis and interpretation, and critically revised the manuscript.

### ACKNOWLEDGMENTS

Authors thank Katja Sampalahti from University of Turku for her technical assistance during laboratory analyses. This study was supported by TUBITAK, Turkey (D.Y, Grant no: BIDEB-1059B192100836) and Finnish Dental Society Apollonia, Finland. Authors confirm that there is no conflict of interest.

### FUNDING INFORMATION

This study was supported by TUBITAK, Turkey (Grant no: BIDEB-1059B192100836) and Finnish Dental Society Apollonia, Finland.

### CONFLICT OF INTEREST STATEMENT

The authors report no conflict of interest related to this study.

### DATA AVAILABILITY STATEMENT

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

### ETHICS STATEMENT

The ethical permit of the study was approved by the Clinical Research Committee of the Sakarya University Faculty of Medicine (Approval number: E-16214662-050.01.04-85,162-217) within the accordance to the ethical guidelines of Helsinki Declaration. The study protocol was explained to all participants, and their written informed consents were obtained.

## ORCID

*Dogukan Yilmaz* https://orcid.org/0000-0003-2576-0885
*Mervi Gürsoy* https://orcid.org/0000-0001-8545-6821
*Ulvi Kahraman Gürsoy* https://orcid.org/0000-0002-1225-5751


## REFERENCES


Ashida, H., Mimuro, H., Ogawa, M., Kobayashi, T., Sanada, T., Kim, M., & Sasakawa, C. (2011). Cell death and infection: A double-edged sword for host and pathogen survival. *The Journal of Cell Biology*, *195*(6), 931–942. https://doi.org/10.1083/jcb.201108081

Berglundh, T., Armitage, G., Araujo, M. G., Avila-Ortiz, G., Blanco, J., Camargo, P. M., Chen, S., Cochran, D., Derks, J., Figuero, E., Hämmerle, C. H. F., Heitz-Mayfield, L. J. A., Huynh-Ba, G., Iacono, V., Koo, K. T., Lambert, F., McCauley, L., Quirynen, M., Renvert, S., … Zitzmann, N. (2018). Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 world workshop on the classification of periodontal and Peri-implant diseases and conditions. *Journal of Clinical Periodontology*, *45*(Suppl 20), S286–S291. https://doi.org/10.1111/jcpe.12957

Bock, F. J., & Tait, S. W. G. (2020). Mitochondria as multifaceted regulators of cell death. *Nature Reviews. Molecular Cell Biology*, *21*(2), 85–100. https://doi.org/10.1038/s41580-019-0173-8

Bullon, P., Fioroni, M., Goteri, G., Rubini, C., & Battino, M. (2004). Immunohistochemical analysis of soft tissues in implants with healthy and peri-implantitis condition, and aggressive periodontitis. *Clinical Oral Implants Research*, *15*(5), 553–559. https://doi.org/10.1111/j.1600-0501.2004.01072.x

Castro, S. A., Collighan, R., Lambert, P. A., Dias, I. H., Chauhan, P., Bland, C. E., Milic, I., Milward, M. R., Cooper, P. R., & Devitt, A. (2017). *Porphyromonas gingivalis* gingipains cause defective macrophage migration towards apoptotic cells and inhibit phagocytosis of primary apoptotic neutrophils. *Cell Death & Disease*, *8*(3), e2644. https://doi.org/10.1038/cddis.2016.481

Chen, C., Jiang, Z., Jiang, Q., Dai, W., Shao, Q., Chen, Q., Wang, Y., & Yang, G. (2021). Caspase-3 and gasdermin E detection in peri-implantitis. *Biochimica et Biophysica Acta. Molecular Basis of Disease*, *1867*(11), 166217. https://doi.org/10.1016/j.bbadis.2021.166217

Cheng, R., Choudhury, D., Liu, C., Billet, S., Hu, T., & Bhowmick, N. A. (2015). Gingival fibroblasts resist apoptosis in response to oxidative stress in a model of periodontal diseases. *Cell Death Discovery*, *1*, 15046. https://doi.org/10.1038/cddiscovery.2015.46

Das, P., Chopra, M., Sun, Y., Kerns, D. G., Vastardis, S., & Sharma, A. C. (2009). Age-dependent differential expression of apoptosis markers in the gingival tissue. *Archives of Oral Biology*, *54*(4), 329–336. https://doi.org/10.1016/j.archoralbio.2009.01.008

Denis, C., Sopková-de Oliveira Santos, J., Bureau, R., & Voisin-Chiret, A. S. (2020). Hot-spots of Mcl-1 protein. *Journal of Medicinal Chemistry*, *63*(3), 928–943. https://doi.org/10.1021/acs.jmedchem.9b00983

Derks, J., Ichioka, Y., Dionigi, C., Trullenque-Eriksson, A., Berglundh, J., Tomasi, C., & Graziani, F. (2022). Prevention and management of peri-implant mucositis and peri-implantitis: A systematic review of outcome measures used in clinical studies in the last 10 years. *Clinical Oral Implants Research*, 1–13. https://doi.org/10.1111/clr.13925

Figueredo, C. M., Alves, J. C., de Souza Breves Beiler, T. F. C., & Fischer, R. G. (2019). Anti-apoptotic traits in gingival tissue from patients with severe generalized chronic periodontitis. *Journal of Investigative and Clinical Dentistry*, *10*(3), e12422. https://doi.org/10.1111/jicd.12422

Fujita, T., Alotaibi, M., Kitase, Y., Kota, Y., Ouhara, K., Kurihara, H., & Shuler, C. F. (2012). Smad2 is involved in the apoptosis of murine gingival junctional epithelium associated with inhibition of Bcl-2. *Archives of Oral Biology*, *57*(11), 1567–1573. https://doi.org/10.1016/j.archoralbio.2012.08.011

Galluzzi, L., Kepp, O., & Kroemer, G. (2012). Mitochondria: Master regulators of danger signalling. *Nature Reviews. Molecular Cell Biology*, *13*(12), 780–788. https://doi.org/10.1038/nrm3479

Gamonal, J., Sanz, M., O'Connor, A., Acevedo, A., Suarez, I., Sanz, A., Martínez, B., & Silva, A. (2003). Delayed neutrophil apoptosis in chronic periodontitis patients. *Journal of Clinical Periodontology*, *30*(7), 616–623. https://doi.org/10.1034/j.1600-051x.2003.00350.x

Ganesan, S. M., Dabdoub, S. M., Nagaraja, H. N., Mariotti, A. J., Ludden, C. W., & Kumar, P. S. (2022). Biome-microbiome interactions in peri-implantitis: A pilot investigation. *Journal of Periodontology*, *93*(6), 814–823. https://doi.org/10.1002/JPER.21-0423

Green, D. R. (2019). The coming decade of cell death research: Five riddles. *Cell*, *177*(5), 1094–1107. https://doi.org/10.1016/j.cell.2019.04.024

Hammond, J. B., & Kruger, N. J. (1988). The Bradford method for protein quantitation. *Methods in Molecular Biology*, *3*, 25–32. https://doi.org/10.1385/0-89603-126-8:25

Itoh, J., de La Motte, C., Strong, S. A., Levine, A. D., & Fiocchi, C. (2001). Decreased Bax expression by mucosal T cells favours resistance to apoptosis in Crohn's disease. *Gut*, *49*(1), 35–41. https://doi.org/10.1136/gut.49.1.35

Johnson, R. B., & Wikle, J. C. (2014). Sex differences in inflammatory and apoptotic signaling molecules in normal and diseased human gingiva. *Journal of Periodontology*, *85*(11), 1612–1619. https://doi.org/10.1902/jop.2014.130718

Kan, J. Y., Rungcharassaeng, K., Umezu, K., & Kois, J. C. (2003). Dimensions of peri-implant mucosa: An evaluation of maxillary anterior single implants in humans. *Journal of Periodontology*, *74*(4), 557–562. https://doi.org/10.1902/jop.2003.74.4.557

Karatas, O., Balci Yuce, H., Tulu, F., Taskan, M. M., Gevrek, F., & Toker, H. (2020). Evaluation of apoptosis and hypoxia-related factors in gingival tissues of smoker and non-smoker periodontitis patients. *Journal of Periodontal Research*, *55*(3), 392–399. https://doi.org/10.1111/jre.12723

Ketelut-Carneiro, N., & Fitzgerald, K. A. (2022). Apoptosis, Pyroptosis, and necroptosis-oh my! The many ways a cell can die. *Journal of Molecular Biology*, *434*(4), 167378. https://doi.org/10.1016/j.jmb.2021.167378

Kordbacheh Changi, K., Finkelstein, J., & Papapanou, P. N. (2019). Peri-implantitis prevalence, incidence rate, and risk factors: A study of electronic health records at a U.S. dental school. *Clinical Oral Implants Research*, *30*(4), 306–314. https://doi.org/10.1111/clr.13416

Kumar, P. S., Dabdoub, S. M., Hegde, R., Ranganathan, N., & Mariotti, A. (2018). Site-level risk predictors of peri-implantitis: A retrospective analysis. *Journal of Clinical Periodontology*, *45*(5), 597–604. https://doi.org/10.1111/jcpe.12892

Lakschevitz, F. S., Aboodi, G. M., & Glogauer, M. (2013). Oral neutrophil transcriptome changes result in a pro-survival phenotype in periodontal diseases. *PLoS One*, *8*(7), e68983. https://doi.org/10.1371/journal.pone.0068983

Lalier, L., Vallette, F., & Manon, S. (2022). Bcl-2 family members and the mitochondrial import machineries: The roads to death. *Biomolecules*, *12*(2), 162. https://doi.org/10.3390/biom12020162

Levin, L., Hertzberg, R., Har-Nes, S., & Schwartz-Arad, D. (2008). Long-term marginal bone loss around single dental implants affected by current and past smoking habits. *Implant Dentistry*, *17*(4), 422–429. https://doi.org/10.1097/ID.0b013e31818c4a24

Li, Y., Ling, J., & Jiang, Q. (2021). Inflammasomes in Alveolar Bone Loss. *Frontiers in Immunology*, *12*, 691013. https://doi.org/10.3389/fimmu.2021.691013

Liu, Y. Z., Maney, P., Puri, J., Zhou, Y., Baddoo, M., Strong, M., Wang, Y. P., Flemington, E., & Deng, H. W. (2016). RNA-sequencing study of

peripheral blood monocytes in chronic periodontitis. *Gene*, *581*(2), 152–160. https://doi.org/10.1016/j.gene.2016.01.036

Lundmark, A., Davanian, H., Båge, T., Johannsen, G., Koro, C., Lundeberg, J., & Yucel-Lindberg, T. (2015). Transcriptome analysis reveals mucin 4 to be highly associated with periodontitis and identifies pleckstrin as a link to systemic diseases. *Scientific Reports*, *5*, 18475. https://doi.org/10.1038/srep18475

Menezes, K. M., Fernandes-Costa, A. N., Silva-Neto, R. D., Calderon, P. S., & Gurgel, B. C. (2016). Efficacy of 0.12% chlorhexidine gluconate for non-surgical treatment of Peri-implant mucositis. *Journal of Periodontology*, *87*(11), 1305–1313. https://doi.org/10.1902/jop.2016.160144

Mombelli, A., van Oosten, M. A., Schurch, E., Jr., & Land, N. P. (1987). The microbiota associated with successful or failing osseointegrated titanium implants. *Oral Microbiology and Immunology*, *2*(4), 145–151. https://doi.org/10.1111/j.1399-302x.1987.tb00298.x

Monje, A., Pons, R., Insua, A., Nart, J., Wang, H. L., & Schwarz, F. (2019). Morphology and severity of peri-implantitis bone defects. *Clinical Implant Dentistry and Related Research*, *21*(4), 635–643. https://doi.org/10.1111/cid.12791

Nikoletopoulou, V., Markaki, M., Palikaras, K., & Tavernarakis, N. (2013). Crosstalk between apoptosis, necrosis and autophagy. *Biochimica et Biophysica Acta*, *1833*(12), 3448–3459. https://doi.org/10.1016/j.bbamcr.2013.06.001

Noronha Oliveira, M., Schunemann, W. V. H., Mathew, M. T., Henriques, B., Magini, R. S., Teughels, W., & Souza, J. C. M. (2018). Can degradation products released from dental implants affect peri-implant tissues? *Journal of Periodontal Research*, *53*(1), 1–11. https://doi.org/10.1111/jre.12479

Özdemir, M., Caglayan, F., Bikker, F. J., Pussinen, P., Könönen, E., Yamalik, N., Gürsoy, M., Fteita, D., Nazmi, K., Güncü, G. N., Pietiäinen, M., Tolvanen, M., & Gürsoy, U. K. (2020). Gingival tissue human beta-defensin levels in relation to infection and inflammation. *Journal of Clinical Periodontology*, *47*(3), 309–318. https://doi.org/10.1111/jcpe.13227

Picca, A., Calvani, R., Coelho-Junior, H. J., & Marzetti, E. (2021). Cell death and inflammation: The role of mitochondria in health and disease. *Cell*, *10*(3), 537. https://doi.org/10.3390/cells10030537

Rakic, M., Radunovic, M., Petkovic-Curcin, A., Tatic, Z., Basta-Jovanovic, G., & Sanz, M. (2022). Study on the immunopathological effect of titanium particles in peri-implantitis granulation tissue: A case-control study. *Clinical Oral Implants Research*, *33*(6), 656–666. https://doi.org/10.1111/clr.13928

Ramanauskaite, A., Fretwurst, T., & Schwarz, F. (2021). Efficacy of alternative or adjunctive measures to conventional non-surgical and surgical treatment of peri-implant mucositis and peri-implantitis: A systematic review and meta-analysis. *International Journal of Implant Dentistry*, *7*(1), 112. https://doi.org/10.1186/s40729-021-00388-x

Ravidà, A., Galli, M., Siqueira, R., Saleh, M. H. A., Galindo-Moreno, P., & Wang, H. L. (2020). Diagnosis of peri-implant status after peri-implantitis surgical treatment: Proposal of a new classification. *Journal of Periodontology*, *91*(12), 1553–1561. https://doi.org/10.1002/JPER.20-0124

Salvi, G. E., Cosgarea, R., & Sculean, A. (2017). Prevalence and mechanisms of Peri-implant diseases. *Journal of Dental Research*, *96*(1), 31–37. https://doi.org/10.1177/0022034516667484

Schwarz, F., Alcoforado, G., Guerrero, A., Jönsson, D., Klinge, B., Lang, N., Mattheos, N., Mertens, B., Pitta, J., Ramanauskaite, A., Sayardoust, S., Sanz-Martin, I., Stavropoulos, A., & Heitz-Mayfield, L. (2021). Peri-implantitis: Summary and consensus statements of group 3. The 6th EAO consensus conference 2021. *Clinical Oral Implants Research*, *32*(Suppl 21), 245–253. https://doi.org/10.1111/clr.13827

Schwarz, F., Derks, J., Monje, A., & Wang, H. L. (2018). Peri-implantitis. *Journal of Clinical Periodontology*, *45*(Suppl 20), S246–S266. https://doi.org/10.1111/jcpe.12954

Schwarz, F., Jepsen, S., Obreja, K., Galarraga-Vinueza, M. E., & Ramanauskaite, A. (2022). Surgical therapy of peri-implantitis. *Periodontology 2000*, *88*(1), 145–181. https://doi.org/10.1111/prd.12417

Singh, N., Hassan, A., & Bose, K. (2016). Molecular basis of death effector domain chain assembly and its role in caspase-8 activation. *FASEB Journal*, *30*(1), 186–200. https://doi.org/10.1096/fj.15-272997

Villar, C. C., Chukwuedum Aniemeke, J., Zhao, X. R., & Huynh-Ba, G. (2012). Induction of apoptosis in oral epithelial cells by Candida albicans. *Molecular Oral Microbiology*, *27*(6), 436–448. https://doi.org/10.1111/j.2041-1014.2012.00648.x

Wang, Z. (2022). Peri-implant crevicular fluid SIRT1 levels decrease in patients with peri-implant inflammatory: A prospective observational study. *Transplant Immunology*, *74*, 101659. https://doi.org/10.1016/j.trim.2022.101659

White, T., Alimova, Y., Alves, V. T. E., Emecen-Huja, P., Al-Sabbagh, M., Villasante, A., Ebersole, J. L., & Gonzalez, O. A. (2020). Oral commensal bacteria differentially modulate epithelial cell death. *Archives of Oral Biology*, *120*, 104926. https://doi.org/10.1016/j.archoralbio.2020.104926

Williams, B., Dharmapatni, A., & Crotti, T. (2018). Intracellular apoptotic pathways: A potential target for reducing joint damage in rheumatoid arthritis. *Inflammation Research*, *67*(3), 219–231. https://doi.org/10.1007/s00011-017-1116-5

Yang, Y., Huang, Y., & Li, W. (2021). Autophagy and its significance in periodontal disease. *Journal of Periodontal Research*, *56*(1), 18–26. https://doi.org/10.1111/jre.12810

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: Opposing activities that mediate cell death. *Nature Reviews. Molecular Cell Biology*, *9*(1), 47–59. https://doi.org/10.1038/nrm2308

Zhang, T., Jiang, M., Yin, X., Yao, P., & Sun, H. (2021). The role of autophagy in the process of osseointegration around titanium implants with micro-nano topography promoted by osteoimmunity. *Scientific Reports*, *11*(1), 18418. https://doi.org/10.1038/s41598-021-98007-7

**How to cite this article:** Yilmaz, D., Gürsoy, M., & Gürsoy, U. K. (2023). Anti-Apoptotic and Pro-Apoptotic Bcl-2 Family Proteins in Peri-Implant Diseases. *Clinical Oral Implants Research*, *00*, 1–9. https://doi.org/10.1111/clr.14067