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SIRTUIN-1 IN MEDICATION-RELATED OSTEONECROSIS OF THE JAW

PhD thesis

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List of Abbreviations

ONJ	Osteonecrosis of the Jaw
MRONJ	Medication-Related Osteonecrosis of the Jaw
QoL	Quality of Life
AAOMS	American Association of Oral and Maxillofacial Surgeons
BP(s)	Bisphosphonate(s)
RANKL	Receptor Activator of NF-κB Ligand
DB	Denosumab
SRE	Skeletal-Related Events
PPi	Inorganic Pyrophosphate
FPP	Farnesyl Pyrophosphate
BRONJ	Bisphosphonate-Related Osteonecrosis of the Jaw
SNP	Single-Nucleotide Polymorphism
mTOR	mammalian Target Of Rapamycin
CYP2C8	Cytochrome P4502C8
ROS	Reactive Oxygen Species
IL-1	Interleukin-1
IL-6	Interleukin-6
OPG	Osteoprotegerin
IGF-1	Insulin-like Growth Factor 1
BMD	Bone Mineral Density
RANK	Receptor Activator of NF-κB
MMP2	Matrix Metallopeptidase 2
VEGF	Vascular Endothelial Growth Factor
PPARG	Peroxisome Proliferator-Activated Receptor Gamma
ABP1	Amiloride Binding Protein 1
CHST11	Carbohydrate Sulfotransferase 11
CROT	Carnitine O-octanoyltransferase
HLA	Human Leukocyte Antigen
NAD	Nicotinamide Adenine Dinucleotide
STAC(s)	Sirtuin-Activating Compound(s)

KO	Knockout
SOST	Sclerostin
ALFA	Allele Frequency Aggregator
PCoA	Principal Coordinate Analysis
PCA	Principal Component Analysis
WES	Whole Exome Sequencing
MM	Multiple Myeloma
eQTL	expression Quantitative Trait Loci
MSC	Mesenchymal Stem Cell
MK-4	Menaquinone-4
CGF	Concentrated Growth Factors
GWAS	Genome-wide Association Study
PRS	Polygenic Risk Score

1 Introduction

1.1 Osteonecrosis

Osteonecrosis or avascular necrosis is a broad term for conditions defined by the death of bone cells, which can be caused by several varied factors and may manifest in different localizations. Underlying causes involve traumatic (e.g., fracture, dislocation, surgery) and non-traumatic mechanisms, such as hematologic disorders (e.g., hemoglobinopathies, sickle cell disease), connective tissue disorders (e.g., systemic lupus erythematosus, rheumatoid arthritis), radiation exposure, or glucocorticoid use and antiresorptive drug administration (1). Most commonly affected sites involve the femoral head, humeral head, jaw, the vertebrae, and the small bones of the hand and foot (1).

1.2 Osteonecrosis of the Jaw (ONJ)

The mandible and maxilla are commonly affected by osteonecrosis, identified as osteonecrosis of the jaw (ONJ). Depending on the underlying mechanism, it is possible to distinguish distinct forms of the disease. Non-site-specific causes include radiation exposure (osteoradionecrosis), metastatic disease to either the maxilla or the mandible, traumatic injury, or other disorders connected to osteonecrosis. On the other hand, Medication-Related Osteonecrosis of the Jaw (MRONJ) is a distinct type of osteonecrosis, which is site-specific to the jawbones and is primarily associated with the administration of antiresorptive drugs (2).

1.3 Definition of Medication-Related Osteonecrosis of the Jaw (MRONJ)

MRONJ is a relatively rare but severe adverse effect mainly associated with antiresorptive drugs, which significantly impairs the quality of life (QoL) of the affected patients (3). The first ever recorded cases of MRONJ were reported back in 2003 by Marx et al. (4). The authors described avascular necrosis of the jawbones following treatment with pamidronate and zoledronate. The American Association of Oral and Maxillofacial Surgeons (AAOMS) 2014 position paper, which remained unchanged in the society's most recent position paper, defined MRONJ with the following three criteria:

1. Current or previous treatment with antiresorptive therapy alone or in combination with immune modulators or antiangiogenic medications.

2. Exposed bone or bone that can be probed through an intraoral or extraoral fistula(e) in the maxillofacial region that has persisted for more than 8 weeks.
3. No history of radiation therapy to the jaws or metastatic disease to the jaws (2, 5).

Antiresorptive medications, such as bisphosphonates (BPs) and receptor activator of nuclear factor kappa-B ligand (RANKL) inhibitor denosumab (DB), are effective in treating osteoporosis and skeletal-related events (SREs) (e.g., pathologic fractures, spinal cord compression) in cancer patients, as well as hypercalcemia of malignancy (6-9). BP therapy is also indicated in metabolic bone diseases, such as Paget's disease or osteogenesis imperfecta (10). BPs were first used more than half a century ago and are still utilized nowadays to prevent bone resorption by selectively targeting osteoclast cells. All BPs are inorganic pyrophosphate (PPi) analogues; however, there are two distinct groups of BPs differing in their side chains' structure and mechanism of action (11). BPs with short and simple side chains, without nitrogen content (simple BPs, e.g., clodronate, medronate, etidronate), are turned into cytotoxic metabolites in osteoclast cells, which induce apoptosis, resulting in lowered bone resorption (11). Interestingly, newer BPs, which have nitrogen-containing side chains (nitrogen-containing BPs, N-BPs, e.g., pamidronate, alendronate, ibandronate, risedronate, zoledronate), similarly to statins, act through the mevalonate pathway, resulting in a better antiresorptive capacity compared to simple BPs (11). N-BPs mainly inhibit the farnesyl-diphosphate synthase (FPP synthase, FPPS) in the mevalonate pathway, which inhibits the prenylation of proteins in the osteoclast (11). Prenylation is a crucial post-translational protein modification in several enzymes (e.g., small GTPases); thus, the inhibition of this process results in significantly lowered osteoclast function and osteoclast apoptosis (11).

DB is a fully human monoclonal antibody that inhibits RANKL, a molecule essential for osteoclast function (12). RANKL is a membrane-associated cytokine expressed by osteoblasts, a crucial part of the RANK-RANKL complex, which activates osteoclasts (13).

Other than antiresorptive drugs have also been linked with the development of MRONJ, thus MRONJ can be further divided into groups by the administered agent. These groups are bisphosphonate-related osteonecrosis of the jaw (BRONJ) and non-bisphosphonate-related osteonecrosis of the jaw (non-BRONJ). Non-BRONJ instances are caused by numerous drugs such as the anti-RANKL antibody denosumab, an inhibitor of

mammalian target of rapamycin (mTOR), everolimus, tyrosine kinase inhibitors, or antiangiogenic drugs (e.g., bevacizumab) (14).

1.4 Incidence of MRONJ

The primary disease forming the indication for antiresorptive therapy significantly influences the risk of developing MRONJ (2). In cancer patients, the incidence of the disease is at least a magnitude higher than in osteoporotic patients (2). For cancer patients treated with zoledronate, the incidence is usually found to be between 0.5 and 4 percent, depending on the duration of follow-up (15, 16). In cancer patients exposed to denosumab, the risk is comparable to that observed with BPs (17, 18).

In osteoporotic patients, the risk of MRONJ development clusters around 0.02 percent to 0.05 percent (19, 20). Interestingly, in a study with a ten-year follow-up period, the risk of MRONJ in osteoporotic patients treated with denosumab was found to be 0.3 percent, a magnitude higher than with BPs (21).

1.5 Local and systematic risk factors of MRONJ

Tooth extraction and other dentoalveolar operations are well-supported local risk factors of MRONJ (2). A Swedish four-year prospective study reported that 70 percent of MRONJ patients had a tooth extraction procedure before the development of the disease (22). Furthermore, dental, periodontal, and periapical inflammation and infection might be an even more important local initiating factor (23). Anatomic factors such as denture use and ill-fitting prosthesis are also reported to increase the risk of MRONJ development (2, 23).

Severe systemic conditions, mainly malignant diseases (e.g., multiple myeloma, breast cancer, prostate cancer), significantly increase the risk of MRONJ (23). Another greatly important risk factor is the duration and the cumulative dose of antiresorptive treatment. The risk of MRONJ increases as the duration of treatment and cumulative dose of BPs increase (24). Interestingly, MRONJ is more likely to develop in the female population; however, this phenomenon is most likely caused by the primary diseases, which require antiresorptive therapy (e.g., osteoporosis, breast cancer), being more common in females. Other previously reported systematic risk factors include corticosteroid treatment, diabetes mellitus, and smoking (2).

1.6 Diagnosis and staging of MRONJ

The diagnosis and staging of MRONJ can pose a considerable challenge, as the disease may present with widespread clinical signs and symptoms (23). Furthermore, most of the symptoms are not disease-specific and might overlap with other common pathologies, such as periodontitis or periapical disease (23). The most common clinical symptoms include odontalgia, sinus pain, pain in the jaws, and neurosensory dysfunction (e.g., numbness of the lips) (2). Findings in a clinical examination most notably involve intra- and extraoral swelling, sudden dental mobility, intraoral fistulas, non-healing extraction sockets, and purulent discharge (2, 23). Varying imaging modalities, such as panoramic radiographs, dental x-rays, and CT-based imaging techniques, are also utilized to gain a definitive diagnosis (23). The most common radiographic findings are alveolar bone sclerosis, thickening of the periodontal ligament, osteolytic changes, and sequestrum formation (2, 23).

Both the AAOMS and the Italian position paper recommend using a staging system in order to determine disease severity (2, 23). The two recommendations slightly differ in their classification system; however, both distinguish three separate stages (Stage 1, 2, and 3), with increasing severity of MRONJ (2, 23). The guideline published by AAOMS also differentiates Stage 0, in which there is no apparent sign of bone necrosis present, which is considered to be the preceding stage of MRONJ (2).

1.7 Prevention and treatment of MRONJ

Prevention strategies are important and still the most effective way to lower the burden of disease in MRONJ. Prevention strategies include patient education, emphasizing the potential risks, importance of regular dental check-ups, drug adherence, and clinical symptoms of MRONJ (23). It is also crucial to restore oral health prior to the initiation of antiresorptive therapy, as well as performing invasive dental procedures, such as tooth extractions (2, 23). During antiresorptive treatment, it is also essential to prioritize less invasive dental techniques (e.g., root restoration) and avoid dental implantation (2).

Modern treatment of MRONJ may involve a combination of conservative and surgical therapeutic interventions (2, 23). Conservative therapeutic measures include local wound treatment, antimicrobial rinsing, pain relief, or systemic antibiotic treatment (e.g., penicillin and metronidazole) (2, 23). If conservative therapy is insufficient, surgical

removal of the necrotic tissue (marginal or segmental resection) and bone reconstruction is necessary (2, 23).

1.8 Pathophysiology of MRONJ

Since the first case reported by Marx. et al, back in 2003, the exact pathomechanism of the disease is still not entirely explained. There is plenty of data in the literature about multiple factors contributing to the development of MRONJ; however, none of them is sufficient to explain it alone. Figure 1 displays the most important factors contributing to the development of MRONJ.

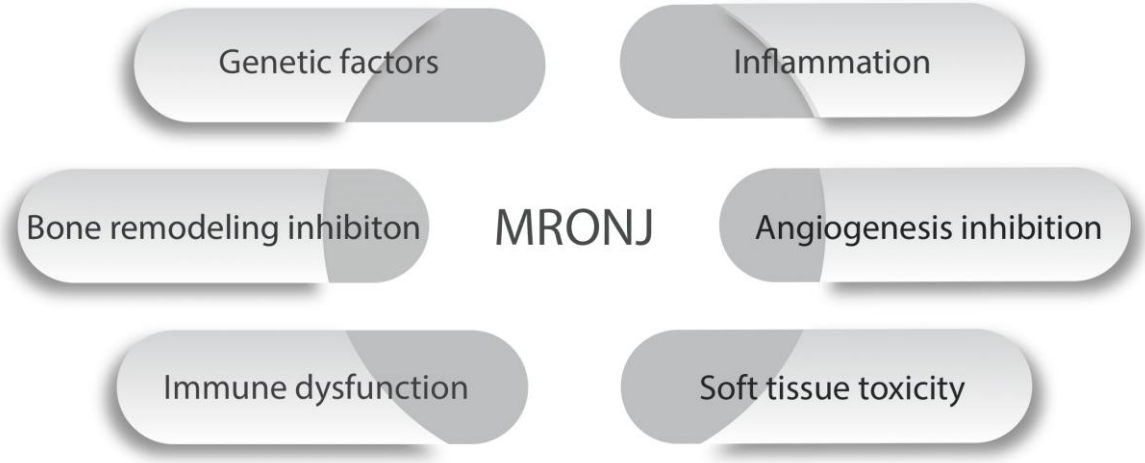

Figure 1. Main pathophysiological factors of MRONJ (25).

1.8.1 Bone remodeling inhibition

Bone remodeling inhibition is recognized as one of the key hypotheses of MRONJ pathophysiology (2). Both BPs and DB hinder bone remodeling by inhibiting osteoclast function, which is supported by the histological assessment of ONJ animal models (26). Zoledronate-treated animals displayed atypical osteoclast cells, detached from the bone surface at the osteonecrosis site (26). The parathyroid hormone (PTH) also rescued necrotic lesions and facilitated wound healing in a rat MRONJ model (27). As PTH promotes bone remodeling, this further validates bone remodeling inhibition as an important factor in the development of the disease (28).

1.8.2 Soft tissue toxicity

BPs, mainly zoledronate, have a direct cytotoxic effect on human gingival fibroblasts (29). Zoledronate might hinder wound healing by inhibiting gingival fibroblast cell proliferation and migration (29, 30). These deficits in fibroblast function can attenuate

MRONJ development. Despite having no cytotoxic effect on fibroblasts, DB still results in an incidence similar to that of BPs (31). Thus, direct soft tissue toxicity might be less important in the pathogenesis of the condition.

1.8.3 Inflammation

Inflammation is also thought to be a key factor in the development of the disease (32). An animal model of the disease demonstrated that MRONJ susceptibility was increased in periradicular disease after tooth extraction (26). In another study, the extraction of healthy teeth did not increase the chance of developing the condition, while, on the other hand, preexisting periapical disease increased the osteonecrotic susceptibility (33). Furthermore, Nakamura et al. detected that MRONJ was more likely to develop from teeth with local infections (34).

1.8.4 Angiogenesis inhibition

BPs have been demonstrated to inhibit angiogenetic pathways in multiple studies (35, 36). Moreover, locally delivered vascular endothelial growth factor (VEGF) into healing extraction sockets lowered the MRONJ incidence through a pro-angiogenetic mechanism (37). At the margins of osteonecrotic lesions, microvascular changes such as hypo-vascularized edematous areas have been detected (38). These results suggest that angiogenesis inhibition might also be a contributing factor to MRONJ development.

1.8.5 Immune dysfunction

Local immune dysfunction has also been reported as a principal factor in the development of MRONJ (39). It is well-known that patients with immune disorders (e.g., diabetes, autoimmune disorders) have higher chances of MRONJ (2). BPs mainly affect the innate immune system, such as the dendritic cells, macrophages, and neutrophil granulocytes (39). Soft tissue toxicity leads to easier infection of the gingival mucosa, while dysfunction of the innate immune system prolongs the infection and inflammation (39).

1.8.6 Genetic factors

1.8.6.1 CYP2C8

One of the most researched genes in the context of MRONJ is the *CYP2C8* gene, which encodes the cytochrome P4502C8 (CYP2C8) protein, a key member of the CYP2C family (40). The cytochrome P450 proteins function as important enzymes in the oxidative metabolism of several drugs (e.g., paclitaxel, rosiglitazone, amodiaquine) (40). Even though BPs are not directly metabolized by CYP2C8, this enzyme might have a

functional role in periodontal fibroblast cells, both in drug and reactive oxygen species (ROS) metabolism (41). Yamoune et al. found that zoledronic acid increased the ROS activity for certain genetic variants of the CYP2C8 protein (41). Increased oxidative stress has been found to be a risk factor for developing MRONJ, due to its ability to suppress bone turnover (42). Moreover, CYP2C8 also alters the metabolic pathway involved in cholesterol production, which is crucial for osteoblast and osteoclast function (43, 44).

In 2008, Sarasquete et al. conducted a genome-wide single-nucleotide polymorphism (SNP) analysis in multiple myeloma (MM) patients (45). In this study, twenty-two BRONJ cases and sixty-five matched BRONJ-free cases were compared. Four intronic SNPs, all in the CYP2C8 gene, were found to be associated with BRONJ, distinctly rs1934951, which is located at intron 8 in the CYP2C8 gene (45). Some studies, involving MM and prostate cancer patients, were unable to detect an association between the genotype of rs1934951 and BRONJ (46-48). Interestingly, Balla et al. found a significant correlation between rs1934951 and the anatomic localization of BRONJ (49). In this study, AG carriers had a significantly higher chance of developing ONJ in mandibular localization (47). Interestingly, Kastritis et al. found that the high-risk allele of the rs1934951 SNP was associated with earlier development of BRONJ (50). Thus, carriers of the high-risk allele of rs1934951 were more likely to develop BRONJ earlier compared to non-high-risk allele carrier patients (50).

Due to the contradictory results regarding the relevance of CYP2C8 rs1934951 in BRONJ, Zhong et al. performed a meta-analysis to determine the association between the polymorphism and BRONJ susceptibility (51). In the pooled analysis, there were 126 cases and 453 controls. This meta-analysis did not detect a significant correlation between the genotype of rs1934951 and BRONJ. However, a subgroup analysis, only considering MM patients, showed a significant correlation between the disease and the SNP (51).

Overall, there is still not enough evidence to conclude the significance of the *CYP2C8* gene in MRONJ. Studies with higher case numbers and matched control groups are needed to evaluate the role of *CYP2C8* in the disease.

1.8.6.2 ESR1 and CYP19A1

Estrogen receptor and aromatase enzyme coding genes have also been researched in MRONJ, as estrogens are recognized as key regulators of bone remodeling in both sexes (52). The effect of estrogens on bone cells is mediated by two nuclear receptors: ERα and

ERß, encoded by the *ESR1* and *ESR2* genes, respectively (52). Estrogens play a crucial role in maintaining bone mineral density (BMD) and mass via several pathways. These include repressing pro-osteoclastic cytokines (e.g., interleukin-1 (IL-1), interleukin-6 (IL-6)) (53, 54), anti-apoptotic effects in osteoblasts (55), and increasing the transcription of osteoprotegerin (OPG) (56). Furthermore, both *ESR1* and *ESR2* polymorphisms have been shown to affect bone mass in humans (52). Interestingly, *ESR1* also regulates insulin-like growth factor-1 (IGF-1) activation, which is involved in re-epithelialization and wound healing processes (57, 58). Based on these findings, *ESR1* polymorphisms can affect MRONJ susceptibility both by bone-specific and wound-healing mechanisms.

A study from 2023, involving 125 bisphosphonate-taking postmenopausal women, found two SNPs (rs4870056 and rs78177662) in the *ESR1* gene significantly associated with MRONJ occurrence (59). Both variants increased the risk of the condition by approximately 2.5-fold (59). Rs4870056 is located in an intronic region, and it is in a strong link with another *ESR1* SNP, rs2234693 (Pvull) (59). *ESR1* Pvull (rs2234693) is one of the most researched polymorphisms in the *ESR1* gene, associated with numerous diseases (e.g., cardiovascular diseases, breast cancer) (60, 61) and might also have an effect on BMD (62).

Aromatase, encoded by the *CYP19A1* gene, is part of the cytochrome P450 enzyme family, responsible for transforming androgen precursors to estrogenic compounds (63). The aromatase enzyme is expressed in several extraglandular sites, such as adipose tissue or bone (64). Moreover, aromatase activity is necessary for longitudinal bone growth and may significantly affect bone loss (63).

Aromatase polymorphism g.132810C>T was found to be significantly associated with BRONJ development in a study (65). Patients with a TT homozygous genotype had a twofold higher risk for the development of BRONJ (65). Interestingly, this genotype is associated with higher levels of local estrogens (66). According to the author's theory, locally higher estrogen levels and BPs inhibit bone remodeling more than BPs alone, leading to increased risk for BRONJ (65).

To conclude, genes associated with estrogen pathways present a promising possibility for understanding and predicting MRONJ better in the future. However, there is only limited data available on these genes, so further studies are very much needed.

1.8.6.3 Genes associated with osteoclast function and bone remodeling (COLIA1, RANK, OPG, MMP2, OPN)

In 2011, Katz et al. conducted a cohort study on seventy-eight MM patients taking intravenous BP therapy (47). This paper reported that, over the one year study period, twelve patients developed BRONJ. The authors compared 10 SNPs in seven genes (*CYP2C8*, *COL1A1*, *RANK*, *OPN*, *MMP2*, *OPG*, and *TNF*) in a candidate-gene study style. In this study, a combined genotype score of five SNPs (*COL1A1* rs1800012, *RANK* rs12458117, *MMP2* rs243865, *OPN* rs11730582, and *OPG* rs2073618) was able to significantly predict an 11-fold increase in MRONJ risk with a cutoff score of 5 (47).

COL1A1 encodes an important part of the type 1 collagen, and its mutations are associated with osteogenesis imperfecta. *COL1A1* rs1800012 also might be associated with ligament and tendon injuries (67). RANK is a transmembrane protein expressed on osteoclasts and several other cells. It functions as the receptor of RANKL, and its activation is very important in osteoclast differentiation, activation, and survival (68). Another very important molecule involved in osteoclastogenesis is osteoprotegerin, which functions as a decay receptor of RANKL (69). *RANK* and *OPG* genetic polymorphisms have been linked to BMD previously (70). *MMP2* encodes an important matrix metalloproteinase (matrix metallopeptidase 2, MMP2), which is involved in the cleavage of several extracellular and non-extracellular matrix molecules (71). *MMP2* has been previously suggested as a candidate gene in MRONJ, as BPs are associated with atrial fibrillation, and *MMP2* is associated with both bone and cardiovascular abnormalities (72).

These results support the role of bone remodeling abnormalities as a key factor in the pathogenesis of MRONJ and suggest that genetic testing might be an effective tool for risk screening in the future. However, the small sample size poses a major limitation when interpreting these findings.

1.8.6.4 VEGFA

As abnormal and inhibited angiogenesis is a leading hypothesis in the possible pathophysiological mechanisms, it is logical that multiple papers have reported on SNPs in the *VEGFA* gene in MRONJ patients. *VEGFA* encodes the VEGF protein, a key regulator of physiological (e.g., embryonic development) and pathological (e.g., solid tumors or intraocular neovascular syndromes) angiogenesis (73). Moreover, VEGF is also an important molecule in inflammatory disorders and wound healing (73), and it also plays a crucial role in bone angiogenesis (74). *VEGFA* polymorphisms have been linked

to several diseases, such as diabetic retinopathy, age-related macular degeneration, and different solid tumors (75-78). VEGF also plays an important role in skeletal development (79). Furthermore, postnatally osteoblast-derived VEGF regulates osteoblastogenesis and adipogenesis in bone marrow by stimulating RUNX2 and repressing PPARG2 (79). It is hypothesized that polymorphisms associated with lower VEGF expression might have a pathophysiological role in the development of the condition.

In 2011, Arduino et al. were the first to analyze *VEGFA* polymorphisms in Italian female breast cancer patients with MRONJ (80). The authors analyzed three SNPs (rs3025039, +936 C>T; rs699947, -2578 C>A; rs2010963, -634 G>C) in the *VEGFA* gene. The combined haplotype of CAC (+936 /- 2578 / -634) was associated with MRONJ susceptibility (80). All of these 3 polymorphisms were previously reported to correlate with *VEGFA* expression (81-83), with two of them being associated with a lower expression level of VEGF (-2578/-634), and one of them with a higher expression level (+936). Analyzing only the two SNPs (-2578/-634) resulted in lower expression levels that remained significantly associated with a higher incidence of MRONJ (80). Another study conducted in the Korean population also evaluated these SNPs (rs3025039, rs699947, and rs2010963), and further affirmed the association between *VEGFA* and the disease, finding that the CC genotype of both rs3025039 and rs2010963 is associated with a higher chance of developing the condition (84). These results are partially consistent with a meta-analysis in both polymorphisms, allele C being associated with MRONJ susceptibility, but not entirely identical, as there are some differences in the allele frequencies and the significant polymorphisms between the two studies, likely due to the different populations (Italian vs. Korean) studied. However, a more recent case-control study, involving osteoporosis patients exclusively, found no significant associations between rs2010963, rs3025039, and rs699947 and disease susceptibility (85). On the other hand, two SNPs (rs881858 and rs10434) were found to be associated with MRONJ. The authors argue that rs10434 might also affect VEGF expression based on different alleles being associated with different diseases; however, there is no direct proof of this in the literature (85). A meta-analysis from 2020 involving 105 MRONJ cases found that rs3025039 was significantly associated with the risk for the condition (86).

Overall, there are some contradictions in the results considering *VEGFA* in MRONJ, which can be caused by the different populations and different patients (e.g., osteoporosis

vs. cancer patients) screened in the different studies. However, polymorphisms in *VEGFA,* which are associated with a lower level of VEGF expression, might pose a promising target for future investigation. Table 1 summarizes the studies investigating SNPs in the *CYP2C8*, *SIRT1*, and *VEGFA* genes, which are among the most studied genes in MRONJ.

1.8.6.5 Other Genes Researched in MRONJ

In a 2022 study, twenty-four potentially pathogenic variants were identified using whole-exome sequencing, with allele frequencies significantly different between the MRONJ-affected and control groups (87). Nine of the polymorphisms clustered in only two genes. These genes are *KRT18* and *PABPC3*. The *KRT18* gene encodes a type 1 intermediate filament protein called keratin eighteen (KRT18) (87). It is interesting to note that the role of genes encoding other keratin proteins has been suggested in oral mucosal diseases (88). Furthermore, KRT18 plays a key role in cytoskeleton organization and in various estrogen signaling pathways (87). Malfunction of the KRT18 protein can lead to cytoskeletal dysfunction of the oral mucosal cells, which may predispose one to oral mucosal diseases and MRONJ. The *PABPC3* gene encodes a poly-A binding protein, whose functional role in the pathogenesis of the disease needs to be elucidated by further experimental and clinical studies.

A genome-wide association study identified a polymorphism (rs17024608) in the

The *RBMS3* gene, which was significantly associated with BRONJ incidence (89). Variations of this gene have previously been linked to bone mass and osteoporotic fracture risk (89).

In another study, the *PPARG* (peroxisome proliferator-activated receptor gamma) SNP rs1152003 was also found to be associated with MRONJ in MM patients as well as SNPs in *ABP1* (amiloride binding protein 1), *CHST11* (carbohydrate sulfotransferase 11), and *CROT* (carnitine O-octanoyltransferase) genes (90). *PPARG* polymorphisms have also been associated with bone remodeling and bone mineral density (90). Interestingly, a study from 2017 found that the high-risk allele of *PPARG* rs1152003, together with *CYP2C8* rs1934951, was associated with early-onset of the disease (50). Thus, these SNPs might be useful to assess the risk of early MRONJ development. Moreover, Poznak et al. found that *PPARG* rs1152003 had a significant association with MRONJ risk in an

univariate analysis; however, after covariate adjustment, this SNP did not remain significantly associated (91).

A case–control study identified *FPPS* rs2297480 allele A to be associated with MRONJ susceptibility (92). FPPS functions as an important enzyme of the mevalonate pathway, and it is targeted by several amino-bisphosphonates (93, 94).

Interestingly, the *FDPS* rs2297480 allele A was also detected to be associated with an increased response to long-term amino-bisphosphonate therapy (94).

SNPs in genes encoding interleukins 1A and 1B have also been suggested to play a potential role in the pathogenesis of the disease (95).

Infection and immune system dysfunction are regularly mentioned as major morbidity factors in MRONJ. In a case–control study of 204 patients, several variants of Human Leukocyte Antigen Class II (HLA Class II) (DRB1*15, DQB1*06:02, DRB1*01, and DQB1*05:01) were significantly more prevalent in MRONJ patients (96) [104]. These HLA antigens are involved in the differentiation of immune cells and the presentation of antigens (96).

Table 1. Summary of studies investigating SNPs of the CYP2C8, SIRT1, and VEGF genes in MRONJ

SNP	Study	Location	Study design	Sample size (cases/controls)	Study population	Drug administered
CYP2C8 rs1934951 rs1934980 rs1341162 rs17110453	Sarasquete et al. (45)	Spain	GWAS	22/65	MM patients	zoledronic acid, pamidronate
CYP2C8 rs1934951	English et al. (46)	USA	candidate gene study	17/83	advanced prostate cancer patients	zoledronic acid, alendronate, pamidronate
CYP2C8 rs1934951	Such et al. (48)	Spain, Greece	candidate gene study	42/37+45[1]	MM patients	zoledronic acid
CYP2C8 rs1934951	Katz et al. (47)	USA	candidate gene study	12/66	MM patients	pamidronate, zoledronic acid
CYP2C8 rs1934951	Balla et al. (49)	Hungary	candidate gene study	46/224	Osteoporosis, breast and other cancers	alendronate, pamidronate, zoledronate,

						ibandronate, rizedronate, clodronate
CYP2C8 rs1934951	Kastritis et al. (50)	Greece	candidate gene study	36/104	MM patients	zoledronic acid
CYP2C8 rs1934951	Poznak et al. (91)	USA	candidate gene study	76/126	MM, breast, prostate, and other cancers	zoledronic acid, pamidronate
SIRT1 rs7896005	Yang et al. (97)	USA, Hungary, Italy	WES	22/22	MM patients	zoledronate, pamidronate
SIRT1 rs7896005 rs375839 rs932658 rs2394443	Yang et al. (98)	USA, Hungary, Italy	candidate gene study	46/58	MM, breast and other cancers	-
SIRT1 rs7894483 rs7896005 rs3758391 rs932658	Bojtor et al. (99)	Hungary	candidate gene study	63/0	MM, osteoporosis, cancers	BPs, denosumab
VEGFA rs699947 rs2010963 rs302503	Arduino et al. (80)	Italy	candidate gene study	30/30	MM, breast cancer	zoledronic acid
VEGFA rs699947 rs2010963 rs302503	Choi et al. (84)	Korea	candidate gene study	26/19	osteoporosis,	alendronate, ibandronate, rizedronate, zoledronic acid
VEGFA rs202125661	Lee et al. (100)	Korea	WES	38/90	osteoporosis, cancers	BPs
VEGFA rs2010963 rs699947 rs10434 rs25648	Kim et al. (85)	Korea	candidate gene study	58/67	osteoporosis	BPs

rs3024987 rs3025022 rs3025035 rs3025039 rs998584 rs6905288 rs881858						
VEGFA rs833061 rs699947 rs2010963 VEGFC rs7664413 rs2333496 rs6838834 rs3775203	Poznak et al. (91)	USA	candidate gene study	76/126	MM, breast, prostate, and other cancers	zoledronic acid, pamidronate

[1] Number of controls, plus number of healthy individuals

1.9 Sirtuins

Sirtuins are highly conserved protein-modifying enzymes, mainly acting as histone-deacetylases (101). They are critical molecules in virtually all living organisms, including humans, as sirtuins regulate key biological pathways, such as apoptosis, cell cycle regulation, DNA repair, inflammation, and cellular metabolism (101). Sirtuins (Sirt 1-7) catalyze the deacetylation of lysine side chains in histones (mainly H3 and H4), which is dependent on free nicotinamide adenine dinucleotide (NAD^+); thus, NAD^+ metabolism and sirtuin activity are closely linked (101).

Sirtuins have attracted considerable scientific attention in recent decades due to their potential key role in aging and aging-related diseases (102). It is hypothesized that the activation of sirtuins may lead to an extended lifespan through similar molecular pathways as calorie restriction (102). There is some evidence in rodents that the overexpression of sirtuins (e.g., SIRT1, SIRT6) leads to multiple, broad beneficial effects (e.g., protection against metabolic stress and diabetes), as well as an extended lifespan in some cases (103-105). There are also studies on sirtuin activators (STACs) (e.g.,

resveratrol, SRT2104) in human subjects, which have some promising results in conditions including metabolic diseases, psoriasis, and Alzheimer's disease (106-108).

1.9.1 SIRT1 in bone biology

Sirtuins, with regard to SIRT1 in particular, play a very important role in human skeletal development and bone metabolism (109). SIRT1 knockout (KO) mice exhibited abnormal craniofacial development, as well as delayed mineralization of bone tissue in multiple locations (110-112). Furthermore, heterozygous KO of SIRT1 in adult mice resulted in lowered trabecular and cortical bone mass (113). Moreover, SIRT1 overexpression reduced the susceptibility of aging mice to reduced bone mineral density compared to wild-type controls (114). It is also evidenced that SIRT1 exerts its positive effect on bone metabolism both in direct (e.g., in osteoblast cells) and indirect pathways (e.g., sex hormone signaling pathways) (109).

These in vivo data all support the possible important role of SIRT1 in the regulation of bone remodeling; moreover, in vitro study data are also in line with the bone-forming effect of SIRT1. SIRT1 promotes mesenchymal stem cell differentiation and osteoblast activation through the Wnt signaling pathway by the deacetylation of beta-catenin (115). Furthermore, SIRT1 might also lower sclerostin (SOST) expression by the deacetylation of the *Sost* promoter (116, 117). Moreover, SIRT1 overexpression also seems to protect against osteoblast apoptosis through multiple molecular pathways (118, 119).

These in vivo and in vitro studies suggest that STAC might have a beneficial effect in osteoporosis, due to its bone-forming ability. There is evidence from more than a decade ago that resveratrol, a first-generation STAC, is able to increase trabecular and cortical bone mass in both aging and ovariectomy-induced osteoporosis (120, 121). Newer generations of STACs, such as SRT1720, SRT2104, and SRT3025, offering a more specific sirtuin activation, also showed promising results in osteoporotic animal models (122, 123).

There is limited data available with STACs in human subjects with osteoporosis; however, some clinical studies resulted in positive results. Both in middle-aged obese men with metabolic syndrome, as well as in postmenopausal women, resveratrol increased bone mineral density (124, 125).

2 Objectives

In the presented studies, our objectives were to examine four SNPs in or in proximity to the SIRT1 gene (rs932658, rs7896005, rs7894483, and rs3758391), which might have a causal role in the development of MRONJ. Figure 2 displays the schematic structure of the SIRT-1 gene with the SNPs examined. Relevant clinical data were also collected and evaluated. Furthermore, our study aimed to further understand the possible association between MRONJ and the aforementioned SNPs in the SIRT1 gene in a single-center retrospective study.

Specifically, we examined the following questions:

- To investigate the association between SNPs in the SIRT1 gene and the risk of developing MRONJ.
- To compare the frequency of SIRT1 variants in MRONJ patients and matched controls within a Central European population.
- To evaluate potential associations between SIRT1 polymorphisms and clinical variables in MRONJ patients.
- To explore possible associations between clinical factors in patients with MRONJ.
- To evaluate and determine possible MRONJ patient subgroups using multivariate analysis.

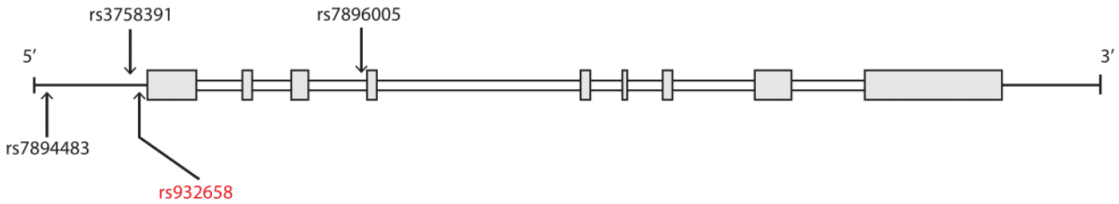

Figure 2. Schematic diagram showing the *SIRT1* gene with the SNPs evaluated. One SNP is located in the intronic region (rs7896005). One SNP is located in the 5′ upstream region (rs7894483). Two SNPs are located in the promoter region of the gene (rs932658, rs3758391). The distribution of alleles in the SNP highlighted in red is significantly different in the study population compared to the average population (99).

3 Methods

3.1 Patients

Sixty-three consecutive patients of Hungarian origin suffering from MRONJ were enrolled in this study. All MRONJ patients were treated at the Department of Oro-Maxillofacial Surgery and Stomatology, Semmelweis University. After detailed medical history taking, physical and laboratory examination, peripheral blood samples were taken from the patients. For three of them, the collection of previous medical data was unsuccessful; therefore, these patients were excluded from multivariate data analysis. All patients were informed and asked for consent before enrolling them in this study. The 1964 Declaration of Helsinki and its subsequent amendments were followed in this study. The study had the approval of the institutional ethics committee of Semmelweis University of Budapest (Semmelweis University Regional and Institutional Committee of Science and Research Ethics, protocol code: 10862-1/2016/EKU; IV/4613-4/2020/EKU). Access to human data was restricted to members of the research team. A password-protected medium was used to store the data. The authors confirm that all the methods were conducted in accordance with the applicable guidelines and regulations.

3.2 Genotyping

Four SNPs in the SIRT1 gene were genotyped in the sixty-three subjects. Peripheral blood samples were collected, and genomic DNA was extracted from each subject using the High Pure PCR Template Purification kit (Roche Diagnostics, GmbH, Mannheim, Germany). The PCR mixture (20 µL) contained 1 µL genomic DNA (50 ng/µL), 2 × 0.50 µL primer, 10 µL of JumpStart Taq Readymix (SIGMA-ALDRICH, Co., 3050 Spruce Street, St. Louis, MO 63103 USA), and 8 µL ultrapure PCR water. Cycling conditions comprised an initial cycle at 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and a final step at 72 °C for 1 min. Sanger dideoxy sequencing was performed to determine the genotype of the SNPs (Eurofins Genomics Europe GmbH, Ebersberg, Germany).

3.3 Statistical Analysis

The distribution of SIRT1 SNP genotypes and alleles in the NCBI Allele Frequency Aggregator (ALFA) reference database (https://www.ncbi.nlm.nih.gov/bioproject /PRJNA507278, accessed on 1 January 2024) and MRONJ populations was analyzed by Chi-square tests. Statistics with a p-value < 0.05 were considered significant. Chi-square tests were performed using IBM SPSS 28.0 software (SPSS Inc., Chicago, IL, USA).

For further analysis, the data were summarized in a matrix with sixty rows (patients) and twenty-six columns (variables). Conventional multivariate techniques cannot be applied to this dataset for two reasons. The variables are not homogeneous regarding the measurement scale. A few are ratio-scale variables (age, duration of treatment, and number of recurrences) for which arithmetic differences between values are meaningful. For the ordinal variables (stage, stage improvement), only the sequence of possible states can be interpreted. Most variables are nominal; that is, the only information that can be obtained by comparing two values is their equality or non-equality. Examples are sex (male or female), primary disease (lung cancer, osteoporosis, etc.), or smoking (yes or no). Also, the SNP genotypes are nominal, each value corresponding to a combination of alleles. For example, for SIRT1 rs932658, 1 means CC, 2 means AC, and 3 means AA in the position. Furthermore, the data are incomplete; 138 data points (8.5%) are unknown due to various factors (lack of measurement, etc.). In such cases, to reveal the data structure with patients or variables as units of comparison, special methods are required. Objects may be compared using the Gower dissimilarity index (126). In contrast, correlations among variables of the mixed type may be calculated by the d-correlation formula recently suggested by Podani et al. (127). Reducing dimensionality, thus revealing underlying data structure, is then made available through principal coordinates analysis (PCoA) of Gower dissimilarities and principal components analysis (PCA) from the correlation matrix. The results of both ordination procedures are displayed by scatter diagrams with axes as dimensions, most commonly selecting the first two. The relative importance of these dimensions is expressed by the percentage contributions of eigenvalues to the total variance. The larger the sum of the eigenvalues for the axes shown, the more faithful the two-dimensional representation with respect to the entire data space. Calculations were performed by the DCORR application (127) and the SYN-TAX package (128).

4 Results

4.1 Study Population

The mean age of patients included in the study was 69.3 years (range 45–91), 73% were female patients (n = 46), and 88.3% (n = 53) had a malignant disorder as primary disease, while 11.7% of patients (n = 7) suffered from osteoporosis as a reason for treatment. The majority of MRONJ cases (73.3%) were diagnosed as stage 2 disease (n = 44), 25% were deemed stage 3 (n = 15), and only one patient had stage 1 MRONJ. Descriptive statistics of the study population are shown in Table 2.

Table 2. Summary of the clinical characteristics of the study population.

Characteristics	**MRONJ Patients n = 63**
Age (years) (mean ± SD) *	69.33 ± 11.06
Sex	
Female	46
Male	17
Duration of treatment (months) (mean ± SD) *	46.42 ± 39.77
Disorder *	
Breast cancer	26
Prostate cancer	11
Myeloma multiplex	11
Osteoporosis	7
Lung cancer	2
Melanoma malignum	1
Colon cancer	1
Cervical cancer	1
Chemotherapy *	
Yes	38
No	22
Hormone deprivation therapy	
Yes	32
No	28
Antiresorptive agent *, **	
Alendronate (p.o.)	1
Ibandronate (p.o.)	4
Risedronate (p.o.)	1
Zoledronate (iv./inj.)	29
Denosumab (inj.)	29
MRONJ localization *, ***	
Maxilla	19
Mandibula	42
MRONJ stage *, ****	
1	1
2	44
3	15

* There were no available clinical data for three patients. ** Four patients had both bisphosphonate and denosumab. *** One patient had both maxillary and mandibular involvement. **** Based on the AAOMS Position Paper on Medication-Related Osteonecrosis of the Jaws—2022 update.

4.2 SNP Genotyping

There was a significant difference detected in the allele distribution of the rs932658 SNP by the Chi-square test between the MRONJ group and the healthy European population measured in the NCBI ALFA database ($p = 4.5 \times 10^{-5}$) (Table 3).

Table 3. Summary of the genotypes and allele frequencies. NCBI ALFA: National Center for Biotechnology Information Allele Frequency Aggregator for the European population. The *p*-value is for the statistical difference between the study and European populations.

SNP	Genotype 1	Genotype 2	Genotype 3	Allele Frequency	ALFA	*p*-Value
rs932658	CC: 65.1% (n = 41)	CA: 25.4% (n = 16)	AA: 9.5% (n = 6)	C:A 77.8:22.2	C:A 59.9:40.1	$p = 4.5 \times 10^{-5}$
rs7896005 *	GG: 47.4% (n = 27)	GA: 40.3% (n = 23)	AA: 12.3% (n = 7)	G:A 67.5:32.5	G:A 65.5:34.5	ns. ($p = 0.64$)
rs7894483 **	AA: 9.9% (n = 6)	AT: 39.3% (n = 24)	TT: 50.8% (n = 31)	A:T 29.5:70.5	A:T 32.9:67.1	ns. ($p = 0.43$)
rs3758391 **	CC: 50.8% (n = 31)	CT: 37.7% (n = 23)	TT: 11.5% (n = 7)	C:T 69.7:30.3	C:T 67.7:32.3	ns. ($p = 0.64$)

* Genotyping of six samples was unsuccessful. ** Genotyping of two samples was unsuccessful. ns.= not significant.

No further significant differences were seen in the other SNPs. Minor allele frequencies are displayed in Figure 3.

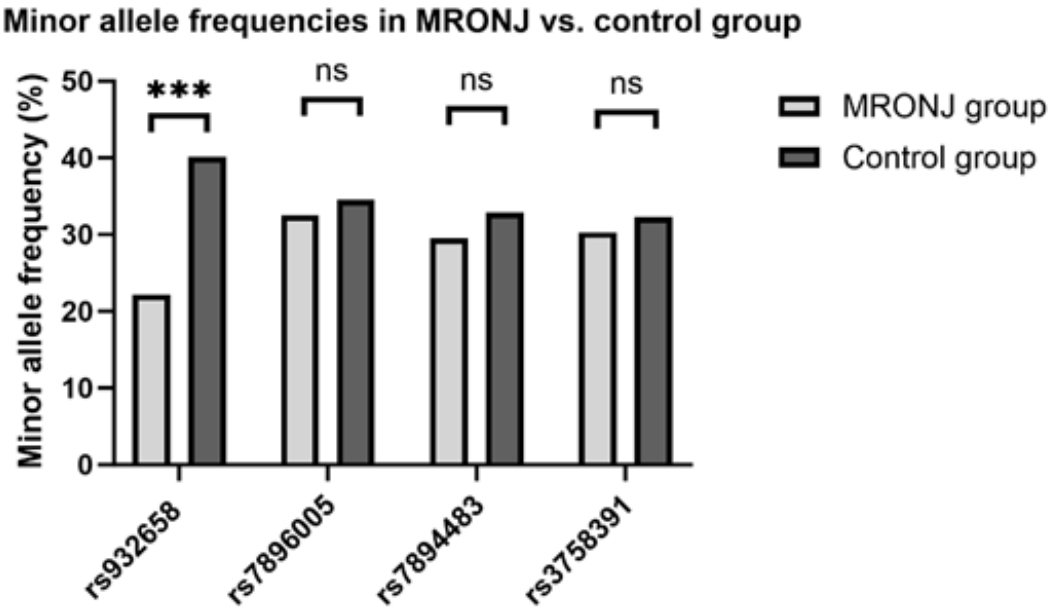

Figure 3. Bar chart showing the minor allele frequencies of the SNPs evaluated. The lighter color represents the medication-related osteonecrosis of the jaw (MRONJ) patients studied; the darker color represents the average population's value based on the NCBI Allele Frequency Aggregator (ALFA) database (accessed on 1 January 2024). A significant difference between MRONJ and the control group was detected in rs932658. *** $p = 4.5 \times 10^{-5}$. ns=not significant (99).

4.3 Multivariate Statistical Analysis

By multivariate statistical analysis of clinical and genetic variables, several strong correlations were found, which indicate the reliability of this novel statistical method. These include a high correlation (0.7063) between the type of bisphosphonate used and the method of bisphosphonate administration (e.g., oral, intravenous) or a relatively high positive correlation (0.2789) between the presence of chemotherapy and the type of bisphosphonate used. These relationships are expressed on the vertical principal component (PCA) axis (Figure 4). As a new finding, a relatively high positive correlation between the genotype of rs932658 and the number of stages improved after the appropriate non-surgical and surgical treatment was detected (0.275). This suggests that genetic factors, specifically the rs932658 SNP in the SIRT1 gene, might play a role not only in the development of MRONJ but also in the clinical course of the disease. Patients with a more favorable genotype might be less likely to have MRONJ and have a higher rate of improvement once MRONJ has developed compared with a less favorable genotype. As the diagram of Figure 4 shows, the other three genotypes are clustered; they are very highly correlated with one another (0.895 to 0.965). These appear uncorrelated

with the other variables, thus explaining the contrast along the first PCA axis. A relatively high positive correlation (0.2417) was also detected between sex and healing. Previous dentoalveolar operation, corticosteroid therapy, and diabetes mellitus did not show a correlation with other genetic or clinical variables. Smoking showed a positive correlation with the age of the patient.

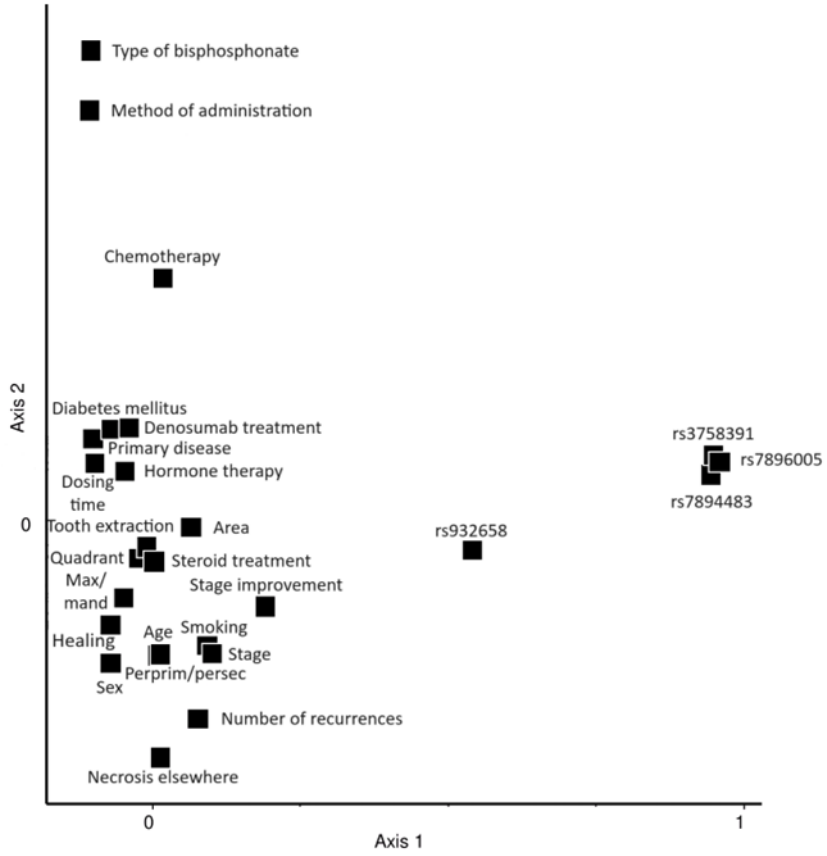

Figure 4. Principal components ordination of twenty-six variables used in the present study. Eigenvalues associated with axes 1 and 2 account for 12.8% and 8.5% of total variance, respectively. Necrosis elsewhere = osteonecrosis at a different site compared with the anatomical location of the primarily diagnosed MRONJ; Perprim/persec = per primam or per secundam wound healing after surgical treatment of MRONJ; Healing = complete healing after treatment (yes or no); Max/mand = maxillary or mandibular localization of MRONJ; Area = frontal, premolar or molar localization of MRONJ; Tooth extraction = prior tooth extraction in medical history (yes or no); Hormone therapy = prior hormone therapy in medical history (yes or no); Chemotherapy = prior chemotherapy in medical history (yes or no) (99).

The PCoA of sixty patients is displayed in Figure 5 for axes 1 and 2. Apparently, the patients form two groups with a relatively large empty area separating them. To find the original variables best explaining this grouping, correlations between variables and the ordination scores for PCoA axes 1 and 2 were calculated. Then, variables with the highest correlations were selected. These were the three SNPs, rs7894483, rs7896005, and

rs3758391, for axis 1 (r = 0.58, 0.61, and 0.59, respectively) and hormone therapy (r = 0.36) as well as the method of administration (r = −0.21) for axis 2. For visualization, the correlations were rescaled to best fit the ordination of patients, as usual in biplots, and arrows were drawn to point to their positions in Figure 5. As seen, the principal factors affecting the grouping of patients are the three genotypes, whereas none of the other clinical or genetic variables is influential.

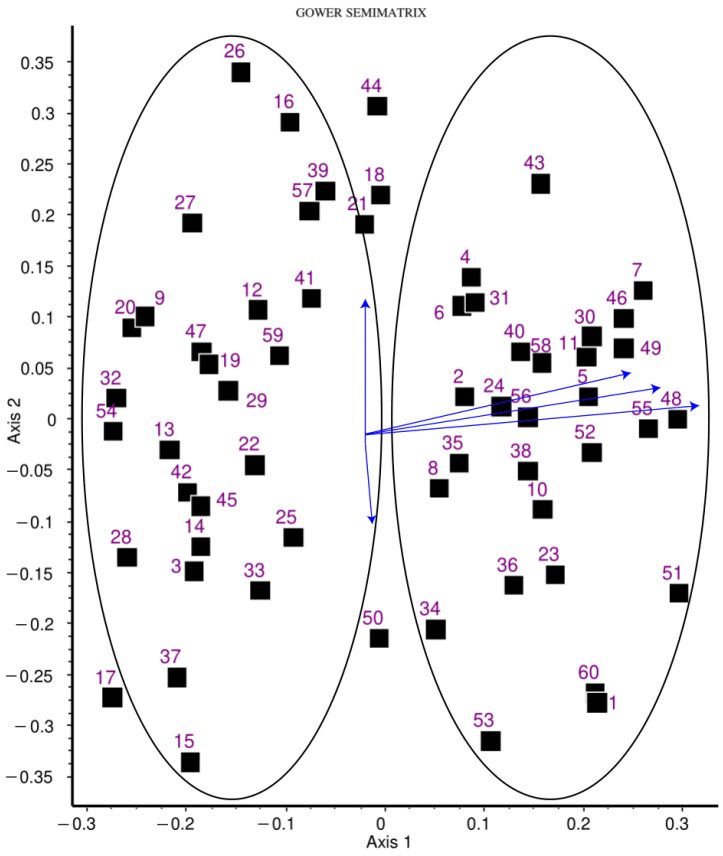

Figure 5. Principal coordinates ordination of sixty patients (full symbols). Eigenvalues associated with axes 1 and 2 account for 15% and 11% of total variance, respectively. Blue arrows and labels refer to variables with the highest correlations with either axis. Note that variable–axis correlation values were scaled down for best fit to the ordination, so that their relative positions and relative lengths matter only (99).

5 Discussion

The studies presented investigated the role of the SIRT1 gene and protein in the development of MRONJ in a relatively large Hungarian single-center patient cohort. The findings validated the potential role of the *SIRT1* rs932658 SNP in the pathomechanism of MRONJ, as a significant difference was found between the allele distribution in MRONJ patients and the healthy European population. Furthermore, a positive correlation was found between the genotype of the same SNP and MRONJ stage improvement, which further validates the role of certain genetic factors in the development of this adverse drug reaction. These results provide a novel insight into the pathomechanism of MRONJ, as well as support the role of SIRT1 in bone metabolism. Furthermore, besides understanding the pathophysiology of MRONJ better, the identification of contributing genetic factors might aid in the development of a personalized MRONJ risk assessment system.

5.1 Comparison with previous studies

SIRT1 and its SNPs have been previously linked with the risk of MRONJ development. In 2018, a whole-exome sequencing (WES) study conducted in MM patients with or without BRONJ revealed a significant association between MRONJ susceptibility and two SNPs in chromosome 10, one in the *SIRT1* gene (rs7896005) (97). In this study, *SIRT1* rs7896005, an intronic SNP, was associated with lower odds of developing the condition. Furthermore, in silico analysis revealed that this SNP was an expression quantitative trait locus (eQTL) of the SIRT1 gene in whole blood, with the minor A allele increasing the gene expression, resulting in a lower risk for MRONJ (97). In a follow-up study, Yang et al. aimed to identify causal SNPs explaining the association between rs7896005 and MRONJ susceptibility (98). They found that rs932658, an SNP in the promoter region of the SIRT1 gene, was causally related to susceptibility to the disease (98). In this study, a higher expression of the SIRT1 gene was detected with the minor A allele of the rs932658 SNP, which might act as a protective factor in MRONJ (98). Our results, that the frequency of allele A in rs932658 is significantly lower in MRONJ patients and that MRONJ patients with allele A tend to heal better if the disease has already developed, both suggest that the rs932658 allele A might act as a protective factor in the development of MRONJ. This SNP, like other polymorphisms evaluated in this study (e.g., rs3758391), is located in the promoter region of the gene, which is universally

understood to be crucial in the regulation of gene expression. One possible explanation of the functional importance of rs932658 might be its relative distance from the 5′-end of the SIRT1 gene. However, further studies are called for to understand the mechanism of the effect of the rs932658 SNP on SIRT1 gene expression.

5.2 Biological interpretation

Sirtuin proteins play a significant role in bone biology. SIRT1 promotes bone formation by several molecular mechanisms. These include activating osteoblasts via stimulating the Wnt signaling pathway and decreasing the expression of sclerostin, an inhibitor of bone formation (109). These findings established the start of several clinical trials investigating sirtuin-activating compounds for osteoporosis (109). Despite considerable progress over the past few years, many research questions have yet to be answered about sirtuin activators as potential therapeutic targets.

SIRT1-dependent signaling pathways have also been studied in the context of ONJ. An in vivo study conducted on mice found that the overexpression of SIRT1 and resveratrol treatment resulted in increased alveolar bone mass, via promoting mesenchymal stem cell (MSC) proliferation and osteogenic differentiation, as well as inhibiting MSC senescence (129). Another in vitro study found that LPS-induced inflammation aggravated oncologic-dose zoledronate-induced oxidative stress and mitochondrial dysfunction via an SIRT1-dependent pathway in human oral keratocyte cell cultures (130). These results might suggest that inflammation and zoledronate treatment concurrently can lead to mucosal healing deficiencies in a SIRT1-dependent manner (130). Furthermore, menaquinone-4 (MK-4), a special vitamin K2 isoform, was found to alleviate zoledronate-induced ONJ through inhibiting osteoblast apoptosis, via a SIRT1-dependent pathway (131). Borsani et al. found that resveratrol cotreatment with concentrated growth factors (CGF) increased osteoblast proliferation, as well as protected against bisphosphonate-induced osteoblast damage (132).

Consistent with the data in the literature [29], our study suggests that the prevalence of MRONJ is higher in women than in men. This is likely due to the higher prevalence of the primary diseases treated with antiresorptive agents in the female population (e.g., breast cancer, osteoporosis). However, the relatively high positive correlation between the sex of the patient and healing suggests that the patient’s sex might not be irrelevant in the clinical course of MRONJ. In our study group, female patients also had a higher

tendency to heal from MRONJ than male patients. Between the sex of the patient and the genotype of rs932658, there was no positive correlation detected, so there might be factors other than genetic factors determining the correlation between the sex of the patient and healing. Other previously described risk factors, like corticosteroid therapy or diabetes mellitus, did not show a correlation with other clinical or genetic variables in our study. Principal coordinates analysis revealed distinct patient groupings, primarily influenced by the genotypes of the three SNPs, i.e., rs7894483, rs7896005, and rs3758391, respectively ($r = 0.58, 0.61, 0.59$). The patients in the distinct groups might act differently in some clinical respects, so further studies may be needed to identify the relevance of this apparent patient grouping.

5.3 Advantages and limitations

In discussing the outcomes of our investigation, it is imperative to acknowledge the advantages that underpin the significance of the findings presented in this article. This study was conducted on a relatively large and independent cohort of Central European MRONJ patients. This enabled us to detect genetic associations with greater reliability. Furthermore, a novel multivariate statistical analysis was deployed to assess the possible correlations between clinical and genetic variables. This innovative statistical method enabled us to compare a wide variety of variables, including genetic and heterogenous clinical factors. These analyses allowed us to detect possible associations between SNPs, which are categorical variables, and other categorical and quantitative variables concurrently. Furthermore, the incorporation of PCA to analyze the outcomes of our research aided us in reducing the dimensionality of our study, while also minimizing information loss. Moreover, the visualization of results enables us to detect the underlying data structures in our cohort. The results of the multivariate statistical analysis further validated the findings made by genotyping. This innovative methodological approach not only adds knowledge to current discourse but also might set a precedent for further investigations in this field.

It is also crucial to acknowledge the limitations of this study. The sample size of sixty-three patients, while providing valuable insights, may not fully capture the diversity of genetic variations that could contribute to MRONJ. Furthermore, the relatively low sample size might reduce our ability to generalize the outcomes of our study. Since all patients have a relatively homogenous Hungarian origin, there may also be some genetic

population bias, which might also alter the interpretation of our results. Differences in ancestry, allele frequencies, and linkage disequilibrium patterns across populations could have influenced our association results and limited their transferability to broader or more diverse cohorts. As a result, the applicability of our findings to populations with different genetic backgrounds needs further study. Additionally, the study was based on a slightly incomplete dataset, and further research with larger and more diverse cohorts is required to validate and extend these findings.

5.4 Clinical implications

The findings presented here might be useful in the future due to multiple reasons. Firstly, the possible role of SIRT1 in MRONJ and, more broadly, in bone biology was further validated by our findings. The association of MRONJ with the identified SNP implicates the significant role of sirtuins in the pathomechanism of this adverse drug reaction, resulting in a severe decrease in the quality of life of affected patients. These results suggest that the modulation of sirtuins by pharmacological agents (e.g., STACs) might present a promising new field of therapeutics in MRONJ. Moreover, with the improvement of knowledge on the genetic background of MRONJ, new risk stratification tools based on the genetic predisposition of the patient can be developed, further aiding personalized medicine. Furthermore, these SNPs can be determined relatively easily and cost-effectively and might help patients avoid MRONJ, which could ease the load on caregivers and help patients and doctors concentrate on the management of the often malignant primary disease. Genetic testing for MRONJ susceptibility could potentially identify high-risk individuals who carry polymorphisms associated with the development of the disease. These high-risk individuals might benefit from lower intensity antiresorptive treatment, such as administering lower doses or for shorter durations. Moreover, in these individuals, a more rigorous, targeted dental monitoring could also be beneficial to detect MRONJ as soon as possible. Some patients also might need invasive dental procedures during antiresorptive treatment. Genetic risk stratification could also guide clinical decision-making in these scenarios on whether a patient needs a drug holiday, prophylactic antibiotic treatment, or even a modified surgical intervention.

There is still a definite need for further research regarding the wide genetic landscape of MRONJ and, specifically, the role of the *SIRT1* gene in the disease. Since genetic factors may vary slightly in the investigated population, further validation of the association

between rs932658 and the risk of MRONJ should be conducted in populations with different genetic backgrounds. Known risk factors, combined with newly identified genetic predisposing factors, could be used to develop a personalized algorithm including genetic diagnostics to screen patients at elevated risk for MRONJ. Greater therapeutic attention to them (e.g., performing necessary dental procedures before antiresorptive therapy) could reduce the incidence of osteonecrotic side effects when administering antiresorptive agents. This could improve the quality of life of patients as well as improve the success of the treatment of the often malignant underlying disease. This will require further large case–control studies in the future to confirm the role of known genetic factors and identify new genetic risk factors. The introduction of novel scientific approaches can help further improve the knowledge of MRONJ genetics. For instance, the founding of a biobank of MRONJ samples and related genetic data could facilitate large-scale studies on multi-center study groups. Longitudinal genetic studies would also be beneficial to better understand the development of this disease. Furthermore, analyzing epigenomic data could help understand the effects of environmental factors on gene expression and genetic susceptibility. Finally, the implementation of polygenic risk scores (PRS) based on genome-wide association study (GWAS) data can provide an overview of the genetic effects in MRONJ by aggregating the combined effect of several lower-impact polymorphisms. By calculating the PRSs, high-risk individuals can be identified, which could lower the MRONJ incidence in patients at risk.

In summary, this research provides insights into the genetic and clinical factors underlying MRONJ, thus promoting a better understanding of its development and progression. The introduction of novel multivariate statistical analysis might also contribute to increasing our knowledge of MRONJ. There are some MRONJ risk assessment questionnaires published in the literature (133, 134), whereas no prevalent, internationally accepted, and validated a priori risk estimation algorithm for MRONJ has been available. In addition, there is absolutely no procedure that takes any genetic data into account in the assessment. The potential integration of genetic data into clinical management offers the possibility of personalized treatment in the future. A future goal would be to screen out high-risk patients for whom antiresorptive therapy should or should not be considered. Contribution to that goal with the results of this study is intended.

6 Conclusions

1. We have successfully identified an SNP (rs932658) in the *SIRT1* gene promoter region, whose allele distribution was significantly different in our MRONJ patient cohort compared to the average European population.
2. We were able to identify that the rs932658 allele A is significantly less frequent in MRONJ patients compared to the average European population.
3. We were able to identify a positive correlation between the genotype of rs932658 and the number of stages improved in MRONJ patients.
4. We have successfully identified a positive correlation between the female sex of the patient and a higher tendency to heal from MRONJ.
5. Principal coordinates analysis revealed distinct patient groupings, primarily influenced by the genotypes of the three SNPs, i.e., rs7894483, rs7896005, and rs3758391.
6. After validation in patient groups with different genetic backgrounds, our results might contribute to a better understanding of MRONJ pathophysiology and to early genetic risk assessment of patients treated with antiresorptive drugs.

7 Summary

Medication-Related Osteonecrosis of the Jaw (MRONJ) is a relatively scarce, but substantial adverse drug reaction, primarily associated with antiresorptive drugs, such as bisphosphonates and denosumab. Its exact pathophysiology is still not entirely understood; however, there is increasing evidence that certain genetic factors, particularly the *SIRT1* gene, are involved in the development of the disease.

In our studies, we have successfully identified SNP rs932658 in the promoter region of the *SIRT1* gene to be associated with the risk of MRONJ development. Furthermore, rs932658 is also associated with the stage improvement of patients treated with adequate therapy. Our results suggest that the rs932658 allele A is a protective factor in MRONJ, as patients with this favorable genetic variant have less chance of developing MRONJ, as well as heal better once the disease has already occurred.

These findings further confirm SIRT1 as a potentially important regulator in MRONJ, as well as more broadly, in bone biology. Elucidating the exact pathomechanism of MRONJ holds great significance as it may contribute to enhanced effectiveness in the prevention and treatment of this adverse drug reaction. Furthermore, the identification of SIRT1 and SIRT1-dependent molecular pathways as potentially important biological targets in MRONJ might aid the development of innovative MRONJ therapy.

After further validation in populations with heterogeneous genetic backgrounds, our results can also aid the implementation of personalized medical care in the field of antiresorptive treatment. Early risk stratification systems supplemented by genetic testing might be useful to detect patients with an elevated chance of MRONJ development; thus, we might be able to manage and prevent MRONJ better, enabling physicians to treat the often malignant primary disease more effectively.

In conclusion, our findings strengthen the evidence that genetic factors, particularly variants of the *SIRT1* gene, play a meaningful role in the susceptibility and clinical course of MRONJ. The identification of the rs932658 allele A as a protective allele not only provides novel insight into the disease pathogenesis but also raises the possibility of incorporating genetic information into routine patient management. Ultimately, these advances could contribute to safer antiresorptive therapies, improved patient outcomes, and the development of more effective, targeted interventions.

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9 Bibliography of the candidate's publications

9.1 Publications directly related to the thesis

- **B. Bojtor**, M. Vaszilko, R. Armos, B. Tobias, J. Podani, S. Szentpeteri, B. Balla, B. Lengyel, H. Piko, A. Illes, A. Kiss, Z. Putz, I. Takacs, J. P. Kosa, and P. Lakatos, "Analysis of SIRT1 Gene SNPs and Clinical Characteristics in Medication-Related Osteonecrosis of the Jaw," *INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES*, vol. 25, no. 7, 2024.
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- **B. Bojtor**, B. Balla, M. Vaszilko, S. Szentpeteri, Z. Putz, J. P. Kosa, and P. Lakatos, "Genetic Background of Medication-Related Osteonecrosis of the Jaw: Current Evidence and Future Perspectives," INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES, vol. 25, no. 19, 2024.
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  IF: 0

9.2 Publications directly not related to the thesis

- R. Armos, **B. Bojtor**, M. Papp, I. Illyes, B. Lengyel, A. Kiss, B. Szili, B. Tobias, B. Balla, H. Piko, A. Illes, Z. Putz, A. Kiss, E. Toth, I. Takacs, J. P. Kosa, and P. Lakatos, "MicroRNA Profiling in Papillary Thyroid Cancer," INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES, vol. 25, no. 17, 2024.
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  IF: 4,9

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