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Effects of bisphosphonate treatment on DNA methylation in osteonecrosis of the jaw

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Abstract

Bisphosphonates are used in the treatment of hypocalcaemia, mainly in cancer and osteoporosis. Some patients experience adverse events, such as BP-related osteonecrosis of the jaw (BRONJ). DNA methylation plays a key role in gene regulation in many tissues, but its involvement in bone homeostasis is not well characterized, and no information is available regarding altered methylation in BRONJ. Using the Illumina Infinium HumanMethylation27 BeadChip assay, we performed an epigenome-wide association study in peripheral blood samples from 68 patients treated with nitrogenous BP, including 35 with BRONJ. Analysis of the estimated cumulative BP exposure distribution indicated that the exposure of the case group to BP was slightly higher than that of the control group; more severely affected cases (i.e., with BRONJ in both mandible and maxilla) were significantly more exposed to BP than were those with BRONJ only in the mandible or maxilla (one-sided Wilcoxon rank sum test, $p = 0.002$). Logistic regression analysis confirmed the positive association between cumulative bisphosphonates exposure and risk of BRONJ (OR 1.015 per mg of cumulative exposure, 95%CI 1.004–1.032, $p = 0.036$). Although no statistically significant differences were observed between case and control groups, methylation levels of probes mapping on three genes, ERCC8, LEPREL1 and SDC2, were strongly associated with cumulative BP exposure levels ($p < 1.31E-007$). Enrichment analysis, combining differentially methylated genes with genes involved in the mevalonate pathway, showed that BP treatment can affect the methylation pattern of genes involved in extracellular matrix organization and inflammatory responses, leading to more frequent adverse effects such as BRONJ. Differences in DNA methylation induced by BP treatment could be involved in the pathogenesis of the bone lesion.

Keywords: Illumina Infinium HumanMethylation27, BeadChip, Bisphosphonate treatment, Alendronate, Zoledronate, Bisphosphonate-related osteonecrosis of the jaws

1. Introduction

Bisphosphonates (BP) are widely used for treatment of hypocalcaemia associated with metastatic cancer and multiple myeloma, Paget disease, osteogenesis imperfecta, and osteoporosis [1–3]. BP have high bone mineral affinity, preferentially binding to the bone surface at sites of active remodeling [4]. The amount of BP taken up by the skeleton depends on several factors, especially renal function and rate of bone turnover [5]. BP drugs can be broadly classified into two main groups with different mechanisms of action: non-N-containing and N-containing. Non-N-containing BP (e.g., clodronate, tiludronate, and etidronate) act by incorporation into ATP. The newer, more effective N-containing BPs (N-BP, e.g., Pamidronate, Alendronate, Ibandronate, Risedronate, and Zoledronate), which are also most associated with adverse reactions, act by inhibiting farnesyl pyrophosphate synthase, a key enzyme of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase pathway (mevalonate pathway) [6]. Although usually well tolerated, BP treatment can cause adverse events, such as atrial fibrillation, acute phase responses, or BP-related osteonecrosis of the jaw (BRONJ) [7–11]. The pathogenesis of BRONJ is not completely understood, but several risk factors have been identified, such as types of BP (especially the N-BP), methods of administration (mainly intravenous), duration of therapy, concurrent use of chemotherapeutic drugs or glucocorticoids, advanced age, and presence of co-morbid conditions, for example, obesity and the effects of tobacco and alcohol abuse [12]. Of all environmental factors, dental trauma is considered to be the most relevant risk factor for the development of BRONJ [13]. BRONJ appears to be caused by N-BP related inhibition of osteoclasts and, hence, of bone remodeling, as well as decreased capillary formation and inhibition of endothelial growth factors [10,14]. BP adverse effects are presumed to be more relevant in the jaws, because BP are more concentrated there than in

other bones – due to the greater blood supply and a faster bone turnover related to the presence of teeth [10]. Bone turnover is essential to maintain bone viability. If osteoclast activity decreases severely, osteocytes are not replaced and the bone capillary network is not maintained, allowing the development of avascular bone necrosis [15]. Studies to elucidate the molecular mechanisms that lead to osteoclast inhibition and BRONJ have focused mainly on gene expression changes. Koch and co-workers demonstrated the effect of BP on alpha V beta 3 integrin (ITGAV gene ID 3685 and ITGB3 gene ID 3690) and tenascin C (TNC gene ID 3371) expression levels affecting human osteoblast adhesion and migration, while Scheller and co-workers showed that in vitro treatment of oral keratinocytes with Zoledronate inhibited p63 [16,17]. A study on rat osteoblasts demonstrated that the BP Risedronate selectively upregulated several genes associated with cell differentiation and apoptosis, including genes coding for proteins of the bone morphogenetic protein (BMP) family – known for its ability to promote growth of bone and cartilage [18]. A member of this family is encoded by bone morphogenetic protein 6 gene (BMP6 Gene ID 654). Cytosine methylation in CpG-rich regions of the genome influences gene transcription in many tissues, but its role in bone homeostasis is not clear (either under physiological conditions or during BP treatment), and no information is available on its role in BRONJ [19]. Nevertheless, it has recently been reported that DNA methylation is involved in the differentiation of cells into the osteoblastic lineage and the osteoblast–osteocyte transition; moreover, a genome-wide methylation profiling of bone samples revealed differentially methylated regions in osteoporosis and osteoarthritis [20,21]. Therefore, we hypothesized that differences in DNA methylation might be involved in the pathogenesis of bone mass changes. In order to test this hypothesis, we performed a genome-wide methylation profiling in leukocytes from subjects treated with N-BP (Alendronate and Zoledronate) who eventually developed BRONJ. Methylation levels of 27,578 CpG sites (14,475 consensus coding sequences and 110 miRNA promoters) were assessed in blood samples from 68 patients, 35 of whom had developed BRONJ by the time of enrolment. The subjects were treated with BP as part of treatment for cancers (54subjects) or for osteoporosis (12subjects).

2. Materials and methods

2.1. Study design

Unrelated patients of European ancestry with BRONJ, and controls, participated in the study, which was approved by the Ethics Committee of the University of Turin (No. 2 CEI-396). Informed consent was obtained. Participants with BRONJ were recruited from subjects referred to the Oral Medicine and Oral Surgery Sections, Lingotto Dental School, University of Turin, Italy. The case definition of BRONJ patients used was as follows: presence of non-healing exposed bone in the maxilla and/or mandible that has persisted for more than 8 weeks in a patient who has received bisphosphonates, but not local radiation therapy [12]. Controls were unrelated patients with a history of BP use but no evidence of osteonecrosis after oral and dental examination. All patients were clinically evaluated by a group of oral health care providers. Information collected during the visit includes gender, age, presence of systemic diseases, use of other medications, and a detailed history of BP therapy (e.g., dose, frequency and duration of treatment). Each participant was also asked to provide a 20 ml peripheral venous blood sample. The clinical appearance, size and site of oral involvement, as well as signs of secondary infection and the presumed initiating event were also recorded for participants diagnosed with BRONJ, which were further referred to undertake a radiological examination that included dental panoramic radiographs and computed axial tomography scans. To exclude possible bone involvements without clinical signs of BRONJ, all other participants underwent a dental panoramic radiograph only [22]. Peripheral venous blood samples were obtained from 68 individuals, 35 patients with BRONJ and 33 patients with history of bisphosphonates treatment but no sign of BRONJ at the time of the recruitment (Table 1).

2.2. Cumulative BP exposure estimation

Because of the different potencies of the two BP used in cancer and osteoporosis treatment regimes, we were unable to establish a single measure of BP exposure. To overcome this problem, we assumed comparability between the osteoporosis treatment using Alendronate (described above) and yearly intravenous administration of a 5 mg dose of Zoledronate; this allowed us to estimate BP exposures of 48 mg/year and 5 mg/year for the treatment of cancer and osteoporosis, respectively [23]. Individual cumulative BP exposure (CBPE) could then be readily estimated by multiplying these values by the treatment durations.

2.3. DNA methylation analysis

Whole genome DNA methylation analysis has been conducted using the Illumina Infinium HumanMethylation27 BeadChip (27K beadchip). Samples were randomly distributed within six beadchips. DNA was extracted from blood cell fractions with the QIAasympphony DNA Midi Kit (Qiagen Cat. No. 931255). DNA (500 ng) was bisulfite-converted with the EZ-96 DNA Methylation-Gold Kit, according to the manufacturer's protocol (Zymo Research Cat. No. D5008). Bisulfite-converted DNA was used for hybridization on the 27K beadchip, following the Illumina Infinium Methylation protocol (Illumina Ca. No. WG-314-1002). Briefly, a whole genome amplification step was followed by enzymatic end-point fragmentation, and hybridization to the 27K beadchip at 48 °C for 17 h, followed by single nucleotide extension. The incorporated nucleotides were labeled with biotin (ddCTP and ddGTP) and 2,4-dinitrophenol (DNP) (ddATP and ddTTP). After the extension step and staining, the BeadChip was washed and scanned using the Illumina HiScan SQ scanner. The intensities of the images were extracted using the GenomeStudio (v2011.1) Methylation module (v1.9.0). Background subtraction based on negative control probes was subsequently performed, and the β value was computed as the ratio of the methylated channel (B) over the total intensity (A + B). Finally, probes that contained missing values in more than 20% of the samples were excluded, leaving a total of 21,851 probes (79% of the original set of probes) for further analyses.

2.4. Statistical analyses

All statistical analyses were carried out using R (version 2.15.2) [24]. The significance level was set at $\alpha = 0.05$. To assess the impact of the comparability assumption made in the estimation of cumulative BP exposures, we repeated all analyses twice, once including all participants, and a second time excluding participants receiving BP as treatment for osteoporosis. Case/control analyses were performed by means of logistic regression. Due to the nature of the data, DNA methylation levels (expressed as β values) were analyzed using per-probe beta regression models as implemented in the betareg package (version 3.0-2) [25]. In particular, to assess the effect of CBPE on DNA methylation, we estimated separate beta regression models for each probe, adjusting for the beadchip on which each sample was processed, as well as for gender, age, case/control status, treatment reason, and use of corticosteroids of each participant. We then extracted the p-values associated with the regression coefficients for the estimated CBPE and applied the multiple-testing correction method of Benjamini and Hochberg [26].

2.5. Gene Set Enrichment Analysis

Genes for which at least one probe was found to be significantly associated with cumulative BP exposure (hereby reported as differentially methylated – DM genes) were further subjected to Gene Set Enrichment Analysis (GSEA) [27]. The lists of significant genes were tested for over-representation, using the Web-based Gene Set Analysis Toolkit V2 (<http://bioinfo.vanderbilt.edu/webgestalt/>) in various biological contexts, such as the GO (gene ontology) gene set, and, for canonical pathway databases, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. We used as reference set the genes listed in the Illumina 27K beadchip annotation file (v1.2); the significance of each gene set enrichment was assessed by means of a p-value computed from a hypergeometric distribution [28–30]. Additionally, we looked for interactions between proteins codified by DM genes and proteins especially involved in N-BP mechanism of action, i.e., part of the HMG-CoA reductase pathway. We searched, for the combination of the two lists of proteins, STRING 9.0 (Search Tool for Recurring Instances of Neighbouring Genes), a database dedicated to protein–protein interactions, including both physical and functional interactions [31]. Additionally, from the initial list of DM genes, we extracted the relevant information stored in Pubmed and Medline (through December 2012) using a series of key words, as described in Fig. 4. We did this research automatically using a software named ProteinQuest (<https://www.proteinquest.com> BioDigitalValley), a platform for biomedical literature retrieval and analysis which integrates data from the scientific literature, data repositories and biological images.

3. Results

3.1. Patient characteristics

Participants with a history of N-BP therapy (68 total) were enrolled in the study: 54 as part of their cancer treatment (monthly 4 mg doses of Zoledronate, administered intravenously over 15 min), and the remaining 14 patients under treatment for osteoporosis (weekly 70 mg oral doses of Alendronate). Among the cancer patients, 31 were diagnosed with breast cancer, 11 with prostate cancer, and 12 with multiple myeloma. Among the 68 patients, 33 subjects had no sign of BRONJ at the time of recruitment and were considered as controls; 35 patients were diagnosed with BRONJ, with lesions only in the mandible (19), only in the maxilla (9), and in both mandible and maxilla (7); (Fig. 1). The characteristics of the study population are summarized in Table 1.

3.2. Cumulative BP exposure and BRONJ

Preliminary investigations of the CBPE distribution indicated that the cases were slightly more exposed than the controls. As shown in Fig. 2, the median CBPE in cases diagnosed with BRONJ in both mandible and maxilla is significantly higher than in controls (one-sided Wilcoxon rank sum test, $p = 0.002$). The overall distribution is also shifted toward the higher end of the range of exposure compared to cases diagnosed with BRONJ in either mandible or maxilla. Case/control analysis by means of logistic regression (Table S1) confirmed the positive association between cumulative BP exposure and risk of BRONJ (OR 1.015, 95% CI 1.004–1.032, $p = 0.036$).

3.3. DNA methylation profiles in the case–control study

We tested the methylation levels at 21,851 CpG sites for their association with BP exposure. This number represents approximately 80% of the probes in the Illumina Infinium HumanMethylation27 BeadChip and corresponds to the subset of probes that were retained after quality controls. Logistic regression analysis was used to estimate the magnitude of the association between methylation levels at each site and the presence of BRONJ, controlling for potential confounders (gender, age at diagnosis, treatment reason, use of corticosteroids and cumulative BP exposure). No significant differences were found for methylation levels between cases and controls.

3.4. DNA methylation levels and cumulative BP exposure

The analysis of models including all participants identified 34 probes that were significantly associated with CBPE (Table 2). Repeating the same analyses on the subset of participants who received BP as part of cancer treatment identified 38 probes (Table 3), of which 29 were in common with the first subset. To investigate effect sizes, we used models including all participants to predict the relationship between CBPE and DNA methylation (β values). Because of the heterogeneity of the population, we analyzed all possible combinations of gender and treatment motivation, averaged over age, case/control status, and use of corticosteroids. Fig. 3 shows this relationship for the four most significantly associated probes (that map on three distinct genes), with each line corresponding to a different combination of gender and treatment motivation. Inspection of these plots shows that the change in percentage of DNA methylation across the range of exposure is always greater than 5%, and exceeds 10% for the two most significantly associated probes. This variation is considerable, and strengthens the plausibility of the associations.

3.5. Gene Set Enrichment Analysis (GSEA)

The analyses have been conducted on the outcomes provided by the model including all participants since, for analyses conducted excluding the osteoporosis patients, the results were remarkably similar. The list of significant 34 probes included in Table 2 mapped 33 genes (differentially methylated – DM genes), see Table S2 for further details. For the ERCC8 gene, two probes resulted significant and differentially methylated in the same direction. The analyses have been conducted in three steps: first we looked for connection within the DM genes; next, between the DM genes and genes involved in BP pathway; and

finally, between BP genes and a shortened DM genes list including only those more related to BP side effects.

3.5.1. GSEA analyses for DM

GAL and BMP6 are the genes responsible in the hormone metabolic process (GO: 0032350) for the GO biological process regulation enrichment in our gene list (adjP = 0.0417). In contrast, no KEGG pathway is significantly enriched for the provided list.

3.5.2. GSEA analyses for DM genes combined with genes involved in BP pathway

To examine whether our list of DM genes had any relevance to the genes involved in BP pathway (as described in Gong and colleagues), we searched the STRING database to identify known and predicted protein associations between the two sets of genes [32]. From the initial network of associations, we have investigated each connection for its relevance to our study. The most relevant interactions are those involving COL1A1 and COL1A2, from the BP pathway and IGFBP3, BMP6 and SDC2 from the list of DM genes (Table 4). Since the two lists are interrelated, we performed a GSEA analysis combining all genes. Both GAL and BMP6 were responsible for the GO enrichment for the steroid metabolic process (GO: 0008202, adjP = 6.10E-05) and regulation of the hormone metabolic process (GO: 0032350, adjP = 0.0083). Moreover, BMP6 also resulted in the enrichment for the steroid biosynthetic process (GO: 0006694, adjP = 1.74E-05) and the lipid biosynthetic process (GO: 0008610, adjP = 0.0037). Regarding KEGG pathways, the extracellular matrix (ECM)- receptor pathway was enriched (adjP = 0.0500) due to the SDC2 gene, which belongs to the list of the significant genes, together with COL1A1 gene, which is part of the BP pathway.

3.5.3. GSEA analyses for genes involved in BP pathway combined with DM genes filtered for relevance to BRONJ and BP treatment

A further investigation was done, filtering out from the DM gene list those genes that should be less involved with BRONJ and BP treatment, according to a literature search. We entered in ProteinQuest all DM genes, retrieving 18,527 papers from MedLine and Pubmed, from which we focused only on those related to Homo Sapiens (11,295 papers). The paper list was subsequently restricted with consecutive selections, specific to (i) the GO biological processes (8518), (ii) the dental/bone/necrosis diseases (964), (iii) the body part (318), and (iv) tissues (182) relevant to our topic. The studies included in the final list take into consideration seven genes out of our initial query of 33 (Fig. 4). We combined the ProteinQuest gene list with the previously described list of genes involved in the BP pathway and ran the GSEA analysis again [32]. The roles of GAL and BMP6, or BMP6 alone, in the significant enrichment for the steroid metabolic process (GO: 0008202, adjP = 7.78E-08), the cellular lipid metabolic process (GO: 0044255, adjP = 4.84E-05), the steroid biosynthetic process (GO: 0006694, adjP = 7.78E-08) and the lipid biosynthetic process (GO: 0008610, adjP = 1.42E-05) were again confirmed. Moreover, two additional GO biological processes were significantly enriched: the ECM structural constituent (GO: 0005201, adjP = 0.0347), due to TFPI2 among the PQ genes, and growth factor binding (GO: 0019838, adjP = 0.0461), involving the PQ IGFBP3 gene. The results of the GSEA analyses are reported in detail in Table S3.

4. Discussion

The present study aimed to identify methylation changes in relation to bisphosphonate (BP) therapy in peripheral blood leukocytes of patients who eventually developed BRONJ. To the best of our knowledge, this represents the first investigation on this topic using a genome-wide approach. Methylation levels of 23,367 CpG sites (corresponding to 13,463 genes) have been assessed.

4.1. Methylation levels in cases and controls

A total of 35 patients developed BRONJ by the time of enrolment, while control subjects were 33 patients with a history of BP treatment but with no sign of BRONJ. We found small differences in methylation levels between cases and controls, but none of them reached statistical significance at genome-wide level. This is possibly due to two main reasons: the small size of the study population (especially for osteoporosis patients) and the fact that the samples were collected over 2 years; during this time, the guidelines for the management

of the dental and periodontal diseases in BP-treated patients were produced and gradually adopted. For this reason, patients who underwent dental extractions at different times are not totally comparable; the small number of patients did not allow us to match cases and controls by time of the dental surgery. Moreover, not all of the controls underwent dental extraction, so the analyses could not be adjusted for this important covariate. Our study was conducted on 68 subjects treated with N-BP, 54 as adjuvant therapy for cancer (11 prostate cancers, 12 multiple myelomas, and 31 breast cancers) and 14 for osteoporosis. BP treatment is a well-known risk factor for BRONJ, and alterations of expression patterns of a number of genes have been reported in blood and tissue samples [18]. Interestingly, in our group of patients, the levels of BP exposure (evaluated as mg/year) were correlated with the risk of developing of BRONJ in a more severe form (both mandible and maxilla involved). Thus, we evaluated the effect of the BP exposure on methylation levels in our subjects regardless of BRONJ occurrence.

4.2. Differentially methylated genes associated with BP exposure

We identified a large number of CpG sites associated with BP exposure and, after multiple-testing correction, 34 remained statistically significant – with three of them, which map on ERCC8, LEPREL1, and SDC2 genes – showing a high level of significance ($2.07E-026$, $4.74E-009$, and $1.31E-007$, respectively). ERCC8 (excision repair cross-complementing rodent repair deficiency, complementation group 8) codes for a substrate recognition component of the Cockayne syndromeA(CSA) complex involved in transcription-coupled nucleotide excision repair. An involvement of DNA repair in response to BP treatment is plausible: Zoledronate treatment, for instance, induces a DNA damage response in oral keratinocytes, resulting in cell cycle arrest and repressive effects on cell viability, proliferation, and epithelial turnover in a dose-dependent manner [33]. N-BP treatment also induces oxidative stress, the response to which may involve the CSA complex as well [34,35]. In particular, an increase in lipid peroxidation and glutathione peroxidase levels was observed in patients treated with risedronate, while oxidative stress in rabbit liver has been induced by Zoledronate [36,37]. LEPREL1 (leprecan-like 1/Myxoid liposarcoma-associated protein 4) codes for an enzyme that catalyze the post-translational formation of 3-hydroxyproline in collagen, which plays a key role for collagen stability [38]. Ravosa and colleagues demonstrated that treatment of oral fibroblasts with Zoledronate inhibits the expression of both the COL1A1 and COL1A2 chains of type-I collagen. These pieces of evidence support a model wherein Zoledronate treatment blocks the growth and migratory capacity of oral fibroblasts as well as downregulates the transcription of type-I collagen. This protein is required for deposition of the granulation tissue needed for reepithelialization, and its absence may thus delay wound healing of the oral mucosal barrier and contribute to BRONJ pathogenesis [39]. It is possible that a similar effect may be exerted through the modification of LEPREL1 promoter methylation and, possibly its expression level. The third of the identified differentially methylated genes is SDC2 (syndecan 2), which codes for a cell surface proteoglycan that participates in cell proliferation, cell migration, cell–matrix interactions and is involved in wound healing. At the site of a tissue injury, the syndecan-1 ectodomains shed from cell surfaces into wound fluids and inhibit fibroblast growth factor 2 (FGF-2) mitogenic activity. Degradation of these regions by platelet heparanase activates FGF-2 mitogenicity [40]. Alteration of SDC2 promoter methylation could alter this balance, resulting in less effective healing.

4.3. Predicted variation of β values is influenced by the tumor site

We used the beta regression models including all participants to predict the relationship between estimated CBPE and β values for the above-described genes. As expected, considering that the analysis has been performed on blood samples, the predicted variation of values is small, between 6 and 10%. Interestingly, identified relationships seem to be influenced by treatment reason. This might be partly explained by the fact that treatment is gender-specific for all diseases but multiple myeloma; the design of the present study, however, does not allow us to entirely rule out a potential modifying effect of tumor type. Further studies are needed to test and characterize these differences.

4.4. Differentially methylated genes and genes that code for proteins of the HMG-CoA reductase pathway are connected

Additional analyses were conducted for potential biological significance of the whole list of differentially methylated genes, revealing functionally relevant enrichment for biological processes and pathways previously shown to be involved in BP treatment. This observation is plausible, since a single CpG site alteration cannot explain alone a massive increase of BP exposure-related risk, as opposed to a combination of several minor alterations in methylation patterns of genes in related pathways. In this context, the use of GSEA and STRING approach helped us to reveal two main connections between DM genes and genes that code for proteins of the HMG-CoA reductase pathway (a biosynthetic pathway responsible for the production of cholesterol, other sterols and isoprenoid lipids), and that are involved in the N-BP mechanism of action [41]. The first connection is with the extra-cellular matrix (ECM) and in particular with collagen proteins. An altered methylation pattern in this context may affect the ECM function and possibly have a role in reduction of wound healing, since BPs seem to inhibit human osteoblast adhesion and migration – affecting integrin gene expression [16]. Three differentially methylated genes, IGFBP3, BMP6 and SDC2, code for proteins that have been predicted by STRING search to interact at various levels with collagen proteins COL1A1 and COL1A2 [42,43]. The insulin-like growth factor binding protein-3 (IGFBP3) possesses both growth-inhibitory and potentiating effects on cells, and has been demonstrated by Liu and colleagues to bind type I alpha collagen. The physiological effects of these interactions may involve modulation of cell adhesion [42]. The bone morphogenetic protein 6 (BMP6) is part of a family of secreted signaling molecules that were initially identified by their ability to promote the growth of bone and cartilage *in vivo*, but little is known about their regulation in skeletal cells [44]. Nevertheless, Simic and colleagues demonstrated that BMP-6 *in vivo* increased bone formation [43]. SDC2, the third protein found associated to collagen by STRING, shares with COL1A2 the same KEGG pathway –i.e., the ECM receptor interaction pathway. The ECM consists of a complex combination of structural and functional macromolecules and has an crucial role in the maintenance of cell and tissue structure and function. The relationship between SDC2 and COL1A2 has also been confirmed by enrichment analysis, when we ran the analysis of the DM list of genes together with the genes involved in BP pathway. The second interaction highlighted by GSEA is between two DM genes (GAL and again BMP6, either alone or together) and genes involved in the steroid metabolic and biosynthetic process at different levels. Both GAL and BMP6 seem to be involved in inflammatory processes; thus, a possible explanation could be that modification of methylation pattern of these genes, due to BP treatment, alters the inflammatory response subsequent to a dental trauma, slowing down the wound healing. Galanin is a neuropeptide, encoded by the GAL gene, implicated in many different biological functions, among others acting as a trophic factor [45]. Galanin is present in tooth pulp and is upregulated upon inflammation. When overexpressed in transgenic mice, it showed a significant decrease in plasma extravasation upon activation of neurogenic inflammation, while Gal KO mice demonstrated abnormal neurogenic inflammatory responses in murine skin as compared to strain-matched wild-type mice [46–49]. BMP6 improves osteoblast function, but BMP family members have also been considered to influence inflammatory processes in adults, due to their chemotactic activity on fibroblasts, myocytes, and inflammatory cells [50]. Since the majority of the test subjects in our study had advanced stage tumors, it is likely that the tumor itself, together with the antitumoral therapies, massively influenced the methylation pattern of the samples. To confirm our results, we tried to sift out from the DM significant signals those which, according to the currently published literature, were less related with BP treatment and its side effects. The selection resulted in a list of seven genes, five of which (IGFBP3, BMP6, SDC2, GAL, and ERCC8) were also highlighted from the GSEA analysis on the whole DM genes list. The analysis on this reduced list together with the genes involved in BP pathway confirmed the previous results and added another significant signal for the TFPI2 gene that resulted enriched in the GO class “ECM structural constituent” (GO: 0005201), again confirming a possible role of the alteration of the methylation pattern of the ECM constituent as a side effect of the BP treatment. The tissue factor pathway inhibitor 2 gene (TFPI2) encodes for matrix associated Kunitz-type serine proteinase inhibitor. The protein can inhibit a variety of serine proteases and has been reported to effectively regulate the activity of metalloproteinases involved in ECM remodeling [51]. Some methodological limitations of this preliminary study should be considered, in particular the small number of samples, the limited information about the subjects, and the analysis of methylation profiles in blood samples. Regarding the last point, in many studies it has been reported that methylation assessed in lymphocyte/blood-derived DNA is a good surrogate biomarker for various tumors, opening the way to the discovery of DNA methylation-based markers in peripheral blood cells’ DNA [52–58]. Additionally, blood-based assays can be informative of the target tissue, which is often not easily available. For instance, IGF2 and ER- α methylation patterns in leukocytes resemble that of colon tissue, and candidate loci methylation analysis profiles in blood and buccal cells showed a good correlation [59,60].

Some questions have been raised concerning the reliability of DNA methylation measurement in blood cells, due to their high turnover and dissimilarities in their proportion among subjects [61–64]. Different white blood cell types have different patterns of DNA methylation, and it is therefore possible that our observations are the result of a selection in blood cell populations due to BP exposure. Thus, DNA methylation at the loci we identified should be highly specific for a subset of white blood cells. However, to our knowledge, none of the loci we discovered have been described in the literature as differentially methylated in any subset of white blood cells. In our study, where we found altered methylation levels as the results of BP exposure, we can hypothesize that this can be due either to an alteration of gene methylation or to a selection of particular peripheral blood cells. In any case, since blood is the vehicle for the systemic distribution of BP, and jaw bones are highly vascularized, both scenarios can be involved in BRONJ susceptibility and deserve to be further investigated.

5. Conclusions

This study represents a first attempt to evaluate the impact of BP treatment on DNA methylation of genes, by using a genome-wide approach. Even though a significant correlation between BRONJ onset and specific differentially methylated genes was not found, what emerged, as an interesting and novel finding, is that BP treatment can affect a patient's methylation pattern. ECM organization/remodeling and the inflammatory response seem to be the cellular processes most involved. This can be especially relevant when, after a tooth extraction, the extraction site heals through a series of precise phases. Disturbing the healing at any stage, particularly the formation of the provisional matrix, could compromise the entire process and lead to a more frequent occurrence of side effects such as BRONJ [65]. Additional studies are warranted to test this hypothesis and to assess whether differences in methylation levels are associated with differential gene expression.

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Table 1
Study population characteristics.

	Cases							Controls			
	Gender		Age	CBPE	Location			Gender		Age	CBPE
	M	F			MN	MX	Both	M	F		
Multiple myeloma	3	5	61.50 (49-79)	71.50 (32.00-200.00)	5	1	2	2	2	59.50 (56-62)	85.00 (64.00-116.00)
Breast cancer		15	67.73 (46-81)	132.00 (20.00-368.00)	6	5	4		16	64.69 (44-79)	51.00 (24.00-120.00)
Prostate cancer	5		73.00 (66-85)	87.20 (20.00-180.00)	3	2	0	6		65.33 (47-73)	54.67 (48.00-68.00)
Osteoporosis		7	71.14 (64-83)	5.30 (2.30-11.50)	5	1	1		7	72.14 (60-79)	5.97 (1.92-12.65)
Overall population	8	27	67.74 (46-85)	86.43 (2.30-368.00)	19	9	7	8	25	65.76 (44-79)	46.24 (1.92-120.00)

Age and cumulative BP exposure reported as mean (range). MN, mandible; MX, maxilla.

* Significant difference ($p < 0.05$) between cases and controls (two-sided t-test).

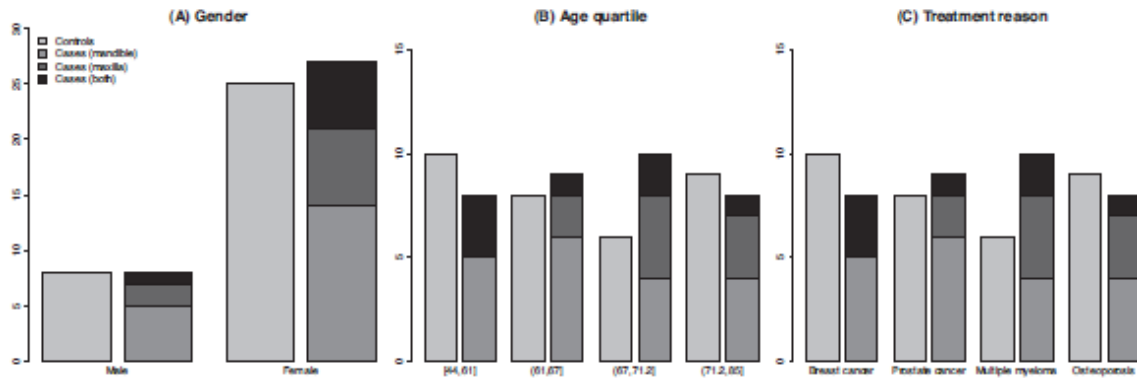


Fig. 1. Study participants stratified by (A) gender, (B) age at diagnosis and (C) BP treatment reason. Cases are categorized for BRONJ localization.

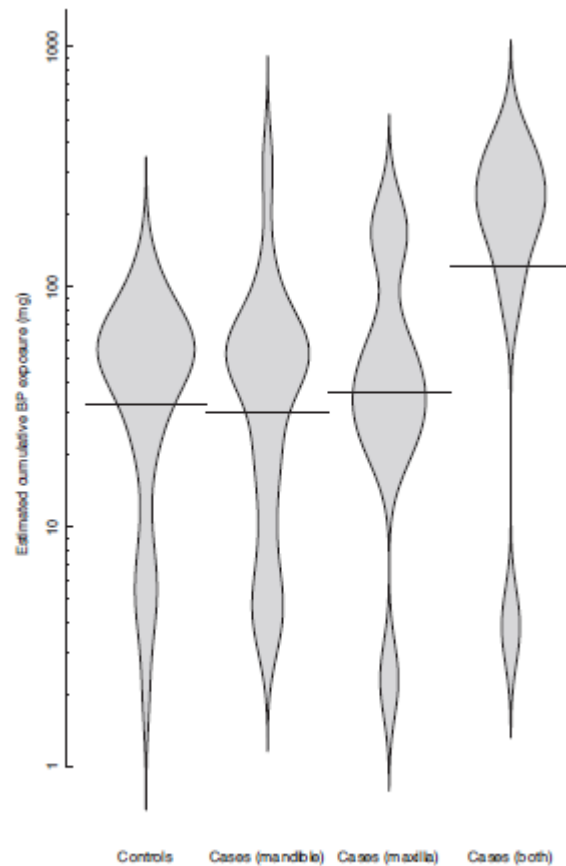


Fig. 2. Beanplots depicting the difference in CBPE between cases and controls. Cases are stratified by location of osteonecrosis (BRONJ) diagnosed only in mandible, only in maxilla, or in both bones. Horizontal lines represent median of CBPE levels.

Table 2

Genomic information, direction of associations, p-values for probes significantly associated with CBPE distribution (model including all participants) ranked by statistical significance. p-Values are derived from a beta regression model and adjusted for the relevant confounders. adjP-values are corrected applying the multiple-testing correction method of Benjamini and Hochberg.

Illumina Probe ID	Gene name	Chr.	Location	Association	p-Value	adjP-value
cg16928795	ERCC8	5	60276574	+	2.07E-026	4.53E-022
cg18626709	LEPBL1	3	191321387	+	4.74E-009	5.18E-005
cg25070637	SDC2	8	97575044	-	1.31E-007	9.53E-004
cg12045002	DPYSL5	2	26923479	-	2.07E-006	8.13E-003
cg07479491	BMP6	6	7671107	+	2.15E-006	8.13E-003
cg11649654	ERCC8	5	60276709	+	2.33E-006	8.13E-003
cg03931808	RLN3	19	14000099	-	2.68E-006	8.13E-003
cg08785534	GAL	11	68208403	+	2.98E-006	8.13E-003
cg13562542	GPR27	3	71886029	+	5.85E-006	1.42E-002
cg11768886	STK32B	4	5103696	+	7.41E-006	1.62E-002
cg07922606	HIST1H3E	6	26333368	+	9.96E-006	1.98E-002
cg10935550	BTG2	1	201541283	+	1.20E-005	2.19E-002
cg16716983	DRP2	X	100360903	-	1.63E-005	2.57E-002
cg22807551	MTUS1	8	17703658	+	1.76E-005	2.57E-002
cg10526232	ZNF688	16	30490543	-	1.96E-005	2.57E-002
cg04970117	SLCGA20	3	45813041	+	2.01E-005	2.57E-002
cg23180489	LYNX1	8	143856788	-	2.21E-005	2.57E-002
cg09558850	TFPI2	7	93358381	+	2.29E-005	2.57E-002
cg05607127	KCNG3	2	42575085	+	2.30E-005	2.57E-002
cg16604218	EIF2B3	1	45225192	+	2.41E-005	2.57E-002
cg08831744	IGFBP3	7	45927871	+	2.47E-005	2.57E-002
cg04059714	TCTN2	12	122721547	+	2.62E-005	2.60E-002
cg08578641	DNAI1	9	34447440	-	2.87E-005	2.72E-002
cg13994177	SNRPN	15	22847557	-	3.17E-005	2.89E-002
cg01783386	RFT1	3	53139429	-	3.54E-005	2.91E-002
cg13240332	ARPC2	2	218789924	+	3.57E-005	2.91E-002
cg00725777	NGFRAP1	X	102517067	-	3.60E-005	2.91E-002
cg20225681	CLP	15	63290500	+	4.39E-005	3.43E-002
cg15522719	GLS	2	191453493	+	4.84E-005	3.64E-002
cg17460386	FAM3	1	205162291	-	5.31E-005	3.87E-002
cg27504117	ANKMY1	2	241146336	-	5.84E-005	4.07E-002
cg25651984	MAGEC1	X	140819258	-	5.97E-005	4.07E-002
cg03658471	SGMS1	10	52053444	-	6.97E-005	4.53E-002
cg20959523	FLCN	2	164300723	-	7.04E-005	4.53E-002

Table 3

Genomic information, direction of association, p-values for probes significantly associated with CBPE distribution (model excluding participants treated for osteoporosis) ranked by statistical significance. adjP-values are corrected applying the multiple-testing correction method of Benjamini and Hochberg. Probes above the dotted line are in common between the two models (including all participants and excluding osteoporosis patients).

Illumina Probe ID	Gene Name	Chr.	Location	Association	p-Value	adjP-value
cg16928795	ERCC8	5	60276574	+	1.50E-028	3.25E-024
cg07479491	BMP6	6	7671107	+	6.05E-017	6.56E-013
cg18626709	LEPBL1	3	191321387	+	3.71E-009	2.68E-005
cg11649654	ERCC8	5	60276709	+	3.15E-008	1.71E-004
cg25070637	SDC2	8	97575044	-	3.82E-007	1.66E-003
cg12045002	DPYSL5	2	26923479	-	7.65E-007	2.76E-003
cg13240332	ARPC2	2	218789924	+	2.19E-006	6.16E-003
cg20225681	CLP	15	63290500	+	2.27E-006	6.16E-003
cg13562542	GPR27	3	71886029	+	4.15E-006	6.92E-003
cg10935550	BTG2	1	201541283	+	4.04E-006	6.92E-003
cg05607127	KCNG3	2	42575085	+	4.07E-006	6.92E-003
cg17460386	FAM3	1	205162291	-	4.12E-006	6.92E-003
cg22807551	MTUS1	8	17703658	+	6.70E-006	1.04E-002
cg11768886	STK32B	4	5103696	+	1.16E-005	1.48E-002
cg10526232	ZNF688	16	30490543	-	1.60E-005	1.92E-002
cg25651984	MAGEC1	X	140819258	-	2.07E-005	2.24E-002
cg04970117	SLCGA20	3	45813041	+	2.33E-005	2.31E-002
cg16604218	EIF2B3	1	45225192	+	2.35E-005	2.31E-002
cg08785534	GAL	11	68208403	+	2.66E-005	2.50E-002
cg03931808	RLN3	19	14000099	-	3.03E-005	2.60E-002
cg00725777	NGFRAP1	X	102517067	-	2.92E-005	2.60E-002
cg16716983	DRP2	X	100360903	-	3.35E-005	2.69E-002
cg15522719	GLS	2	191453493	+	3.60E-005	2.78E-002
cg23180489	LYNX1	8	143856788	-	3.98E-005	2.97E-002
cg07922606	HIST1H3E	6	26333368	+	4.54E-005	3.28E-002
cg09558850	TFPI2	7	93358381	+	5.89E-005	3.76E-002
cg04059714	CT2orf38	12	122721547	+	5.82E-005	3.76E-002
cg03658471	TMEM23	10	52053444	-	5.82E-005	3.76E-002
cg08578641	DNAI1	9	34447440	-	8.12E-005	4.74E-002
cg22637941	WISP1	8	134273023	+	3.99E-006	6.92E-003
cg19388557	GPR174	X	78313533	-	1.00E-005	1.45E-002
cg27519424	HLC5	21	37284290	+	1.07E-005	1.45E-002
cg27613076	INPP5F	10	121474934	+	2.01E-005	2.24E-002
cg01914242	ATP2A3	17	3814817	+	3.12E-005	2.60E-002
cg20583073	EDG6	19	3129759	-	5.76E-005	3.76E-002
cg08935301	LMCD1	3	8518732	+	7.09E-005	4.39E-002
cg17849956	ZNF304	19	62554292	+	7.57E-005	4.56E-002
cg15384392	ARHGAP1	11	46678497	+	8.31E-005	4.74E-002

Table 4
Most relevant interactions between DM genes and genes involved in BP pathway, as predicted by STRING.

STRING evidence	Evidence for specific actions	Evidence suggesting a functional link
BMP6 – COL1A1	Activation (score 0.745) BMP-6 increased <i>in vivo</i> the bone expression of collagen type I	Co-Mentioned in 18 PubMed Abstracts (score 0.821)
BMP6 – COL1A2	Activation (score 0.745) BMP-6 increased <i>in vivo</i> the bone expression of collagen type I	Co-Mentioned in 17 PubMed Abstracts (score 0.761)
IGFBP3 – COL1A1	Binding (score 0.620) Type I alpha collagen binds IGFBP-3	Experimental/Biochemical Data (score 0.620) Protein-protein interaction detected by co-purification assay Co-Mentioned in 47 PubMed Abstracts (score 0.333)
SDC2 – COL1A1	-	Association in Curated Databases (score 0.800) ECM-receptor interaction annotated pathway (KEGG) Co-Mentioned in 50 PubMed Abstracts (score 0.761)
SDC2 – COL1A2	-	Association in Curated Databases (score 0.800) ECM-receptor interaction annotated pathway (KEGG) Co-Mentioned in 50 PubMed Abstracts (score 0.618)

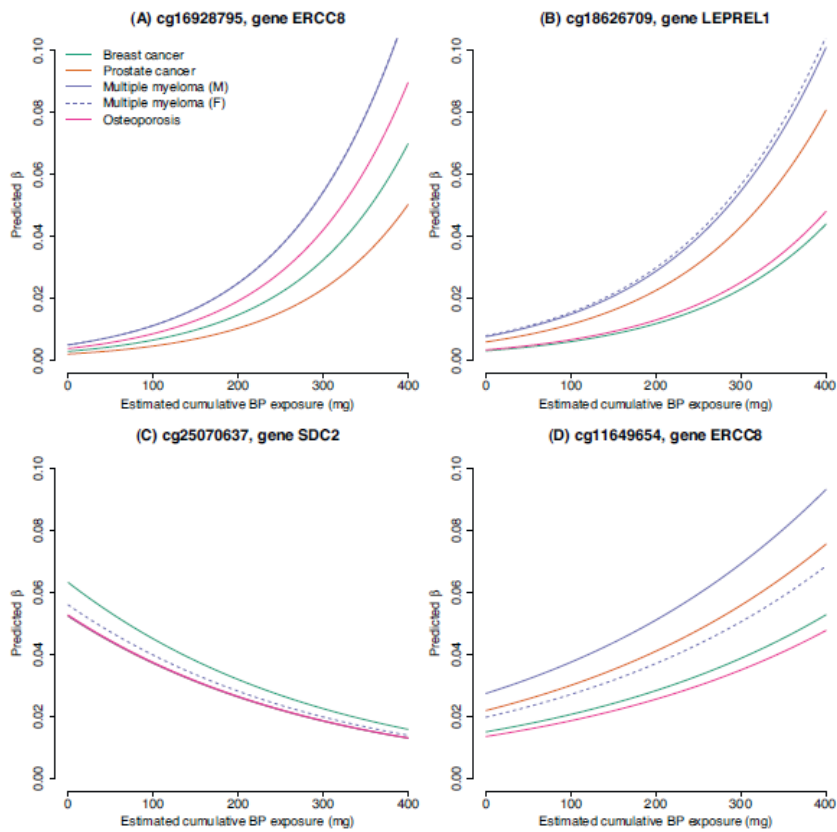


Fig. 3. Relationship between CBPE and β values for ERCC8, GAL and SDC2 as predicted by the associated regression models.

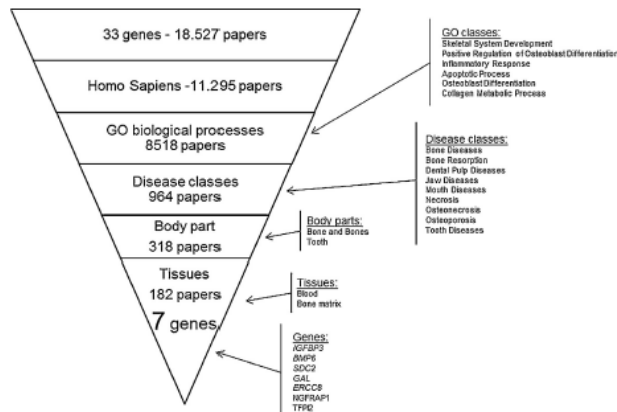


Fig. 4. ProteinQuest Pubmed query details.