Does Smoking Impair Bone Regeneration in the Dental Alveolar Socket?

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Received: 24 June 2019 / Accepted: 27 August 2019 / Published online: 18 September 2019
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Abstract

Smoking is a major risk factor for dental implant failure. In addition to higher marginal bone loss around implants, the cellular and molecular responses to injury and implant physicochemical properties are also differentially affected in smokers. The purpose of this work is to determine if smoking impairs bone microstructure and extracellular matrix composition within the dental alveolar socket after tooth extraction. Alveolar bone biopsies obtained from *Smokers* (> 10 cigarettes per day for at least 10 years) and *Ctrl* (never-smokers), 7–146 months after tooth extraction, were investigated using X-ray micro-computed tomography, backscattered electron scanning electron microscopy, and Raman spectroscopy. Both *Smokers* and *Ctrl* exhibited high inter- and intra-individual heterogeneity in bone microstructure, which varied between dense cortical and porous trabecular architecture. Regions of disorganised/woven bone were more prevalent during early healing. Remodelled lamellar bone was predominant at longer healing periods. Bone mineral density, bone surface-to-volume ratio, mineral crystallinity, the carbonate-to-phosphate ratio, the mineral-to-matrix ratio, the collagen crosslink ratio, and the amounts of amino acids phenylalanine and proline/hydroxyproline were also comparable between *Smokers* and *Ctrl*. Bone microstructure and composition within the healing dental alveolar socket are not significantly affected by moderate-to-heavy smoking.

Keywords Bone · Smoking habit · Alveolar process · Extracellular matrix · Bone regeneration · Raman spectroscopy

Introduction

Smoking is a global health problem, with increased healthcare and societal costs owing to diminished healing capacities and risk of with premature mortality [1, 2]. In 2012 alone, the global cost of smoking-attributable diseases (excluding second-hand smoking) was equivalent to 5.7% of the global health expenditure [3]. In addition to being a risk factor for poor bone mineral density [4] and increased cortical porosity [5], the effects of smoking on periodontal health are well-documented [6, 7]. Although dental rehabilitation using osseointegrated implants is a reliable treatment

modality with high survival rates [8, 9], smoking can modulate the expression of osteogenic and inflammatory factors in alveolar bone [10], implant-adherent cells [11], and in the peri-implant crevicular fluid [12, 13]. Consequently, dental implant failures are more prevalent in smokers [14–16], particularly prior to functional loading [17]. Smoking is implicated in an increased number of periodontally diseased sites [18], greater vertical periodontal bone loss [19], and peri-implant/marginal bone loss [20]. Exposure of peri-implant tissues to tobacco smoke [21] and compromised blood supply during early healing [22] have also been proposed as the mechanisms underlying implant failure in smokers. It is hardly surprising that smoking also changes the peri-implant microbiome [23]. Experiments conducted in rats indicate diminished bone-implant contact and bone area due to inhalation of cigarette smoke [24–26]. Smoking has also been implicated in decreased width and radiographic density of the dental alveolar socket, 6 months after tooth extraction [27]. However, it is not known whether smoking also impairs bone regeneration within the dental alveolar socket and/or induces alterations in extracellular matrix composition, e.g. defective mineral phase, changes in the secondary

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structure of collagen, and atypical variations in mineral content. For this purpose, bone biopsies obtained between 7 and 146 months after tooth extraction were investigated using Raman spectroscopy in addition to X-ray micro-computed tomography (micro-CT) and backscattered electron scanning electron microscopy (BSE-SEM). It is demonstrated, for the first time, that the extracellular matrix composition of the healed site is not affected significantly by smoking, and therefore poor bone quality at the implant recipient site may be disregarded as a potential explanation for implant failures in moderate-to-heavy smokers.

Materials and Methods

Bone Specimens and Inclusion Criteria

The study subjects were selected from patients referred to the Department of Periodontology, Institute for Postgraduate Dental Education, Jönköping, Sweden, between January 2016 and January 2017, in accordance with the following inclusion criteria: *Smokers* were individuals who had smoked an average of > 10 cigarettes per day for at least 10 years, and *Ctrl* were never-smokers by history. Additional requirements were: (i) adequate volume of alveolar bone for implant placement, according to the Lekholm and Zarb classification [28], (ii) absence of risk factors that may affect levels of bone-related gene expression, including osteoporosis, chronic use of anti-inflammatory agents, use of bisphosphonates, or severe metabolic diseases such as diabetes, and (iii) a minimum of 6 months between tooth extraction and implant placement. Partially or completely edentulous individuals were *not* excluded. From 32 systemically healthy individuals (Table 1), cylindrical, 2 × 3 mm, alveolar bone biopsies were obtained under local anaesthesia from premolar regions, during implant site preparation, using a trephine drill and were immediately immersed in 10% neutral buffered formalin, followed by dehydration in a graded ethanol series (50–100%) and resin embedding (LR White, London Resin Co. Ltd., UK). The study protocol was approved by the Institutional Review Board at the University of Linköping (Dnr 2016/319-31). Informed consent was obtained from all participants. The study was conducted in accordance with the guidelines of Good Clinical Practice for Trials on Medicinal Products in the European Community, the International Conference on Harmonisation (ICH) guideline for Good Clinical Practice, the Declaration of Helsinki, and the CONSORT guidelines for clinical studies. Post-extraction healing times (mean values ± standard deviation) of *Smokers* ($n = 16$; 38 ± 21 months) and *Ctrl* ($n = 16$; 49 ± 31 months) were comparable ($p = 0.209$).

Table 1 Patient demographics and sample details

Group	Sample code	Healing time (months)	Age (years)	Gender	Jaw
<i>Smokers</i>	H375	7	47	M	Maxilla
	H376	7	45	M	Mandible
	H431	7	66	M	Mandible
	H429	10	44	M	Maxilla
	H428	16	67	M	Maxilla
	H361	37	40	M	Mandible
	H393	46	59	M	Maxilla
	H389	48	62	M	Maxilla
	H390	48	60	M	Maxilla
	H430	48	46	M	Maxilla
	H398	48	62	M	Mandible
	H409	48	52	F	Mandible
	H410	48	36	F	Mandible
	H422	48	49	F	Mandible
	H423	60	50	F	Mandible
	H432	74	65	M	Mandible
<i>Ctrl</i>	H399	9	71	F	Maxilla
	H404	14	56	M	Maxilla
	H425	15	55	F	Mandible
	H374	32	68	F	Maxilla
	H387	48	54	M	Maxilla
	H400	48	35	F	Maxilla
	H405	48	52	F	Maxilla
	H377	48	68	F	Mandible
	H388	48	50	M	Mandible
	H392	48	52	F	Mandible
	H394	48	70	M	Mandible
	H395	48	72	M	Mandible
	H424	60	56	M	Maxilla
	H426	60	69	M	Maxilla
	H427	60	56	M	Mandible
	H397	146	71	M	Maxilla

Bone Microstructure

Bone mineral density (BMD) and bone surface-to-volume ratio (BS/BV) were evaluated using X-ray micro-computed tomography (micro-CT). In a Skyscan 1172 (Bruker micro-CT, Kontich, Belgium) operating at 49 kV energy with an Al filter (0.5 mm), all samples were scanned over a 180° rotation at a step size of 0.5° with an average of four frames and an image pixel size of 7.92 µm. BMD calibration phantoms (0.25 g/cm³ and 0.75 g/cm³ stoichiometric hydroxyapatite in epoxy resin, 2 mm diameter) were scanned using the same settings. Reconstruction, analysis,

and visualisation were performed using associated Sky-scan software (NRecon, Image viewer, CTVOx, CTAn).

Resin embedded blocks were wet polished using 400–4000 grit SiC paper for backscattered electron scanning electron microscopy (BSE-SEM) in a Quanta 200 environmental SEM operated at low vacuum, 20 kV accelerating voltage, 10 ± 0.1 mm working distance, 40 μm objective aperture, $\times 100$ magnification, and 2048×1768 pixels (pixel size = $1.26 \mu\text{m}$). The extraction voltage and emission current were closely monitored. Brightness and contrast levels were kept constant throughout. Low Z- (atomic number) contrast features arising from the embedding medium were removed by applying a mean filter (radius = 1 pixel) followed by image segmentation using the Otsu algorithm in ImageJ (<https://imagej.nih.gov/ij/>). From the greyscale (8-bit) histogram of each sample, the degree of mineralisation (dMn) was estimated as a dimensionless parameter, by integrating the product of each greyscale intensity, i , and the corresponding pixel count, for all values of i (where $0 < i \leq 255$), divided by the total number of pixels [29]. Mineralisation profiles were obtained from greyscale histograms. The data were binned in 16 greyscale intensity steps (i.e. 0–15, 16–31, 32–47, etc.).

Raman Spectroscopy

Raman spectroscopy was performed using a confocal Raman microscope (WITec alpha300 R, Ulm, Germany), equipped

with a 532 nm laser. The composition of the extracellular matrix was analysed at $20 \times 20 \mu\text{m}$ regions of interest. For each sample, 8–10 randomly selected locations were measured. On the surface of polished resin embedded blocks, the laser was focused down on to the sample using a $\times 100$ objective having a numerical aperture of 0.9. Spectra were collected in the $300\text{--}1800 \text{ cm}^{-1}$ spectral range behind a 600 mm^{-1} grating, at a spectral resolution of $\sim 6 \text{ cm}^{-1}$, an integration time of 3 s per pixel, and an isotropic pixel size of 5 μm . Background fluorescence subtraction was performed in WITEC Control FIVE software. Curve fitting and quantification of integral areas were done using MagicPlot (www.magicplot.com). The Raman metrics investigated included mineral crystallinity, taken as the reciprocal of the full-width at half-maximum ($1/\text{FWHM}$) of the $\nu_1 \text{PO}_4^{3-}$ peak, the carbonate-to-phosphate ratio ($\nu_1 \text{CO}_3^{2-}/\nu_2 \text{PO}_4^{3-}$), the mineral-to-matrix ratio ($\nu_2 \text{PO}_4^{3-}/\text{amide III}$), and the collagen crosslink ratio (CXL), taken as the percentage area of the amide I sub-component at $\sim 1662 \text{ cm}^{-1}$ [30, 31]. To identify sub-component peaks in the amide I region, second-derivative spectra were calculated, followed by deconvolution of the $1540\text{--}1740 \text{ cm}^{-1}$ envelope by Gaussian curve fitting. The amount of amino acid phenylalanine relative to the main phosphate peak at 959 cm^{-1} ($\text{Phe}/\nu_1 \text{PO}_4^{3-}$) and the amount of amino acids proline/hydroxyproline ($\text{Pro} + \text{Hyp}$) were also investigated. The integral areas were $\nu_1 \text{PO}_4^{3-}$ ($960 \pm 15 \text{ cm}^{-1}$), $\nu_2 \text{PO}_4^{3-}$ ($437 \pm 25 \text{ cm}^{-1}$), ν_1

Fig. 1 X-ray micro-computed tomography overview of sample geometry and inter-individual heterogeneity of tissue microstructure in *Smokers* and *Ctrl*

CO_3^{2-} ($1072 \pm 15 \text{ cm}^{-1}$), amide III ($1259 \pm 40 \text{ cm}^{-1}$), amide I ($1660 \pm 40 \text{ cm}^{-1}$), and Pro + Hyp ($850 \pm 30 \text{ cm}^{-1}$).

Statistical Analysis

The Kruskal–Wallis test was used for all statistical analyses (SPSS Statistics, v.25, IBM Corporation) between *Smokers* and *Ctrl*; p values < 0.05 were considered statistically significant. Mean values $\pm$ standard deviations are presented.

Results

Bone Microstructure

In both *Smokers* and *Ctrl*, micro-CT revealed considerable inter-individual heterogeneity, varying between dense cortical to porous trabecular structure (Fig. 1), but with no apparent association with healing time or jaw. The BMD of *Smokers* ($1.20 \pm 0.06 \text{ g/mm}^3$) and *Ctrl* ($1.21 \pm 0.08 \text{ g/mm}^3$) was similar ($p = 0.821$). The BS/BV of *Smokers* ($16.96 \pm 5.42 \text{ mm}^{-1}$) and *Ctrl* ($16.76 \pm 6.38 \text{ mm}^{-1}$) was also similar ($p = 0.821$) (Table 2).

Using BSE-SEM, high intra-individual and inter-individual heterogeneity in tissue microstructure were observed in both *Smokers* and *Ctrl*, including variation in mineral distribution and dimensions of non-lacunar porosity (i.e. blood vessels and marrow spaces). Regions of woven bone characterised by the presence of disorganised clusters of osteocytes were, typically, more prevalent during early healing (e.g. up to 16 months), while remodelled lamellar bone was predominant at longer healing periods (Fig. 2). The degree of mineralisation (dMn) in *Smokers* (112.97 ± 10.38) and *Ctrl* (112.78 ± 15.75) was similar ($p = 0.940$). The mineralisation profiles were also similar ($p > 0.15$ for all bins) in *Smokers* and *Ctrl* (Fig. 3).

Extracellular Matrix Composition

Typical Raman spectral features of mineralised bone were seen for *Smokers* and *Ctrl*. The major peak/band assignments were $\nu_1 \text{ PO}_4^{3-}$ at 961 cm^{-1} , $\nu_2 \text{ PO}_4^{3-}$ at 432 cm^{-1} , $\nu_4 \text{ PO}_4^{3-}$ at 579 cm^{-1} and $\nu_1 \text{ CO}_3^{2-}$ at 1072 cm^{-1} , amide III ($1219\text{--}1299 \text{ cm}^{-1}$), amide I ($1620\text{--}1700 \pm 40 \text{ cm}^{-1}$), Pro + Hyp ($820\text{--}880 \text{ cm}^{-1}$), and Phe (1004 cm^{-1}). The mineral crystallinity, the carbonate-to-phosphate ratio, the mineral-to-matrix ratio, the collagen crosslink ratio, Phe, and Pro + Hyp in *Smokers* were similar ($p > 0.05$ for all) to *Ctrl* (Fig. 4, Table 3). No qualitative differences were noted between the maxilla and the mandible (Fig. 5).

Table 2 Bone mineral density (BMD) and bone surface-to-volume ratio (BS/BV) determined using X-ray micro-computed tomography

Group	Sample code	BMD g/mm^3	BS/BV mm^{-1}
<i>Smokers</i>	H375	1.171	28.210
	H389	1.197	15.427
	H390	1.319	10.715
	H393	1.224	20.866
	H428	1.101	14.261
	H429	1.070	14.322
	H430	1.191	12.544
	H361	1.236	18.748
	H376	1.218	19.905
	H398	1.248	21.147
	H409	1.233	9.916
	H410	1.213	13.658
	H422	1.237	13.938
	H423	1.240	11.708
	H431	1.146	26.477
	H432	1.227	19.540
<i>Ctrl</i>	H374	1.236	11.417
	H387	1.228	10.593
	H397	1.386	17.016
	H399	1.169	22.988
	H400	1.077	29.369
	H404	1.097	20.489
	H405	1.223	23.741
	H424	1.210	15.887
	H426	1.176	23.125
	H377	1.319	10.295
	H388	1.279	11.296
	H392	1.239	12.157
	H394	1.236	11.603
	H395	1.252	8.250
	H425	1.159	16.140
	H427	1.139	23.779

Discussion

Inter-individual variation in the time needed for dental alveolar socket healing to complete is well-documented [32, 33]. Histological examination of alveolar bone biopsies from post-extraction sites in smokers and non-smokers reveals similar proportions of lamellar bone, woven bone, osteoid, bone marrow, and fibrous tissue [34]. Here, alveolar bone microstructure and the mineralised component in particular were probed using BSE-SEM, which in agreement with BMD values obtained using micro-CT, indicated similar mineralisation profiles and the overall degree of mineralisation (dMn) in *Smokers* and *Ctrl*. In contrast to histology, and with the exception of cells surrounded by bone mineral (e.g.

Fig. 2 Inter- and intra-individual heterogeneities in microstructure in *Smokers* and *Ctrl*, evaluated using backscattered electron scanning electron microscopy (BSE-SEM). For visual comparison, a 16-level

lookup table has been applied. Post-extraction healing time is indicated in months (mo). Low Z: $i=0$. High Z: $i=255$

Fig. 3 Mineralisation profiles in *Smokers* and *Ctrl*. **a** Low Z- (atomic number) contrast areas arising from the embedding medium were filtered using the Otsu algorithm (BSE-SEM; 16-level lookup table).

b Comparison of greyscale (8-bit) histograms of the same sample, H430, with (*Otsu+*) and without (*Otsu-*) Otsu filtering. **c** Mineralisation profiles binned in 16 greyscale intensity steps

Fig. 4 **a** Average Raman spectra of *Smokers* ($n=16$) and *Ctrl* ($n=16$). Spectra are normalised to the intensity of the 960 cm^{-1} peak. Inset: Detail of the $1040\text{--}1140\text{ cm}^{-1}$ spectral region showing the $\nu_1\text{ CO}_3^{2-}$ bands. **b** Detail of the $1525\text{--}1765\text{ cm}^{-1}$ spectral region showing the amide I band and the absolute difference Raman spectrum (black line). Inset: Second-derivative spectrum (left) for identification of sub-component peaks in the amide I region and sub-component deconvolution of the $1540\text{--}1740\text{ cm}^{-1}$ envelope by fitting Gaussian

curves centred at $\sim 1555\text{ cm}^{-1}$, $\sim 1572\text{ cm}^{-1}$, $\sim 1589\text{ cm}^{-1}$, $\sim 1604\text{ cm}^{-1}$, $\sim 1616\text{ cm}^{-1}$, $\sim 1621\text{ cm}^{-1}$, $\sim 1638\text{ cm}^{-1}$, $\sim 1662\text{ cm}^{-1}$, $\sim 1669\text{ cm}^{-1}$, $\sim 1691\text{ cm}^{-1}$, $\sim 1708\text{ cm}^{-1}$, $\sim 1720\text{ cm}^{-1}$, and $\sim 1736\text{ cm}^{-1}$. The percentage area of the amide I sub-component at $\sim 1662\text{ cm}^{-1}$ represents the collagen crosslink ratio. **c** Detail of the $825\text{--}900\text{ cm}^{-1}$ spectral region showing the Pro+Hyp bands and the absolute difference Raman spectrum (black line). **d** Raman metrics for extracellular matrix composition

osteocytes and osteoblastic osteocytes), cellular components and intracellular contents cannot be observed directly using BSE imaging, and for this purpose, different contrast staining methods have been proposed [35].

On the extracellular matrix level, the individual constituents contribute greatly to bone strength and mechanical competence [36]. The strength of bone is governed not only by the mineral content, but is also influenced by the degree of mineral crystallinity [37, 38]. The $\nu_2\text{ PO}_4^{3-}$ /amide III ratio is the most reliable determinant of the mineral-to-matrix ratio [39], while mineral crystallinity can be estimated from both the position and the width of the $\nu_1\text{ PO}_4^{3-}$ peak [40]. In synthetic carbonated apatites, an inverse relationship exists between the carbonate content and mineral crystallinity (i.e. the FWHM $\nu_1\text{ PO}_4^{3-}$ increases) [41]. The crystallographic c -axis, (002) reflection measured using X-ray diffraction, shrinks from $\sim 86\text{ nm}$ (FWHM $\nu_1\text{ PO}_4^{3-} = 7.3\text{ cm}^{-1}$) at $0.3\text{ wt\% CO}_3^{2-}$ to $\sim 21\text{ nm}$ (FWHM $\nu_1\text{ PO}_4^{3-} = 18.2\text{ cm}^{-1}$) at $10.3\text{ wt\% CO}_3^{2-}$ [42]. In the $1000\text{--}1100\text{ cm}^{-1}$ region, the $\nu_1\text{ CO}_3^{2-}$ peak overlaps the $\nu_3\text{ PO}_4^{3-}$ band. The latter resolves into sub-components mainly at 1029 cm^{-1} ,

1040 cm^{-1} , 1047 cm^{-1} , 1053 cm^{-1} , with weaker contributions at 1062 cm^{-1} and 1076 cm^{-1} that encroach upon the $\sim 1070\text{--}1072\text{ cm}^{-1}$ $\nu_1\text{ CO}_3^{2-}$ peak. In B-type CO_3^{2-} (i.e. substituting for PO_4^{3-}) apatites, the $\nu_3\text{ PO}_4^{3-}$ band is most obvious up to $\sim 3\text{ wt\% CO}_3^{2-}$, but is almost completely enveloped by the $\nu_1\text{ CO}_3^{2-}$ peak in bone [43], where the CO_3^{2-} content is much higher, and typically around $7\text{--}9\text{ wt\%}$ [44].

Intermolecular crosslinking governs the arrangement of collagen molecules within a fibril and ultimately imparts certain mechanical properties to the fibril [30]. The secondary structure of collagen can be interpreted from the amide I band [45, 46]. Alterations in collagen crosslinking, even when limited to microanatomical locations, can significantly influence the mechanical competence on the whole bone level, independent of changes in the mineral phase [47]. The collagen crosslink ratio, taken as the % area of the 1662 cm^{-1} sub-component, corresponds to biochemically determined pyridinoline content and represents pyridinoline trivalent crosslinks—one of the several naturally occurring crosslinks in collagen [30]. Here, the collagen crosslink

Table 3 Raman metrics for extracellular matrix composition

Group	Sample code	FWHM ν_1 PO ₄ ³⁻	ν_1 CO ₃ ²⁻ / ν_2 PO ₄ ³⁻	ν_2 PO ₄ ³⁻ / amide III	CXLR	Phe/ ν_1 PO ₄ ³⁻	Pro + Hyp
		†	††	††	#	‡	‡‡
<i>Smokers</i>	H375	18.352	0.888	0.745	16.74	0.163	4.162
	H389	17.505	1.036	0.711	23.87	0.120	2.815
	H390	17.483	0.972	0.769	8.07	0.213	2.714
	H393	17.067	0.893	0.901	5.69	0.305	2.468
	H428	18.566	1.042	0.694	31.89	0.127	3.713
	H429	17.877	0.880	0.834	18.55	0.292	3.157
	H430	17.254	1.027	0.712	24.61	0.200	2.461
	H361	17.638	0.920	0.868	5.97	0.317	3.460
	H376	17.621	0.891	0.815	12.99	0.265	3.296
	H398	17.544	0.951	0.820	16.94	0.241	2.835
	H409	17.068	1.019	0.685	25.48	0.122	2.898
	H410	17.762	0.868	0.893	17.01	0.290	3.256
	H422	17.406	0.932	0.676	29.19	0.155	3.205
	H423	17.525	1.006	0.711	28.57	0.150	2.820
	H431	17.646	0.947	0.818	29.37	0.238	2.976
	H432	17.391	0.954	0.852	29.01	0.221	2.525
<i>Ctrl</i>	H374	17.309	0.956	0.942	23.61	0.208	2.626
	H387	17.060	1.087	0.714	29.78	0.085	2.075
	H397	17.486	0.965	0.964	5.00	0.278	2.609
	H399	17.475	0.848	0.689	21.05	0.302	3.098
	H400	17.806	0.860	0.579	14.00	0.224	4.372
	H404	17.718	0.930	0.750	31.20	0.302	4.239
	H405	17.706	0.943	0.816	15.58	0.250	3.119
	H424	17.379	1.062	0.697	26.17	0.126	2.315
	H426	17.588	0.996	0.837	29.02	0.118	2.019
	H377	17.052	0.969	0.791	20.48	0.126	2.295
	H388	16.709	0.907	0.833	6.68	0.221	2.050
	H392	16.735	0.968	0.840	9.16	0.167	1.831
	H394	16.629	0.984	0.825	5.76	0.184	1.722
	H395	17.166	1.054	0.716	4.05	0.128	2.622
	H425	17.755	1.025	0.625	8.32	0.143	3.362
	H427	17.839	0.901	0.806	21.32	0.327	3.497

†cm⁻¹

††Relative integral area

#% 1662 cm⁻¹ area

‡Relative intensity

‡‡Integral area

ratio did not differ significantly between *Smokers* and *Ctrl*. Phe levels decline with increasing tissue maturity [48] and are typically recorded at around 9–12% with respect to the intensity of the phosphate peak at 960 cm⁻¹ [49, 50] in peri-implant bone. Here, Phe levels for *Smokers* (12–32%) and *Ctrl* (8–33%) were relatively higher, which reflects the contribution of rapidly formed woven bone. Pro + Hyp content also declines with tissue age [29], but did not differ between the two groups.

In composite materials, the volume fractions (V) of the constituents, i.e. carbonated apatite and type-I collagen in bone, directly influence the physical properties. The Young's modulus (E) of the extracellular matrix can thus be predicted using the *rule of mixtures* equation [$E_c = E_f V_f + E_m (1 - V_f)$], where subscripts *c*, *f*, and *m* represent the composite, the filler phase, and the matrix phase, respectively. Atomistic simulations indicate that a mineralised collagen fibril reaches maximum elastic modulus at a mineral density

Fig. 5 Average Raman spectra and absolute difference Raman spectra (black lines) of alveolar bone biopsies from the maxilla and the mandible in **a** *Smokers* (maxilla, $n=7$; mandible, $n=9$), and **b** *Ctrl* (maxilla, $n=9$; mandible, $n=7$)

of $\sim 30\%$ [51]. Similar mineral fraction values of ~ 0.438 (*Smokers*) and ~ 0.435 (*Ctrl*), estimated from the mineral-to-matrix ratios, in addition to similar collagen crosslink ratios, mineral crystallinity, and carbonate-to-phosphate ratios for *Smokers* and *Ctrl*, imply that both groups exhibit comparable mechanical competence. A recent study also reports no difference in the ultimate load and the energy-to-failure in vertebrae of mice exposed to cigarette smoke [52].

Smoking adversely affects various aspects of bone metabolism, directly and indirectly [53]. Through a dose-dependent inhibitory effect of nicotine on vascular endothelial growth factor (VEGF) production [54], impaired vascular perfusion of bone is particularly noteworthy [55]. Extracellular matrix composition, however, is not representative of biological events occurring at the bone surface or in the bone marrow. Bone specimens typically present substantial amounts of bone surface (characterised by the cellular components including osteoblasts and osteoclasts) and marrow spaces, where most of the osteogenic and inflammatory activity takes place. Therefore, there is minimal overlap between biomarkers of osteogenesis/inflammation (e.g. detected using gene expression analysis) and extracellular matrix composition. The present patient cohort is categorised as moderate intensity smokers with a 5.4% population prevalence in the USA [56]. It is not known whether these subjects are, in fact, exposed to a sub-critical dose of cigarette smoke and if a higher dose can be expected to induce detectable alterations in bone quality. It is, therefore, remarkable that in addition to bone microstructure, extracellular matrix compositional parameters, all, in *Smokers* remain comparable to *Ctrl*. It appears likely that in contrast to the effects reported on biomarkers of osteogenesis and inflammation [10, 11, 57], the extracellular matrix is less sensitive to the direct and indirect influences of cigarette smoke. Small patient cohorts in the present work preclude statistically reliable comparisons between maxillary and

mandibular sites. Since bone turnover rates differ between maxillary and mandibular alveolar processes [58], disparate expression of osteogenic and/or inflammatory biomarkers between the two anatomical sites in smokers cannot be excluded. Further, the present observations do not exclude that cellular and molecular activities (i.e. tissue response) to trauma and the modulatory effects of different implant properties on these activities may differ between *Smokers* and *Ctrl*. In fact, the implant surface properties tend to play an important role in the expression of markers of inflammation and bone formation as well as marginal bone loss in smokers [11–13, 15]. Nevertheless, we demonstrate that bone microstructure and extracellular matrix composition are not affected significantly by smoking. Poor bone quality is, therefore, not a plausible cause for implant failures in moderate-to-heavy smokers.

A potential pitfall of this work is chemical fixation using formalin, which is deemed sub-optimal for spectroscopic analysis of bone. Compared to unprocessed and/or cryo-sectioned tissues, and fixation with 70–100% ethanol, glycerol, etc., formalin may affect various compositional parameters [59]. However, both *Smokers* and *Ctrl* samples were processed in an identical manner, limiting experimental error(s) attributable to formalin fixation.

Conclusions

Alveolar bone regeneration following tooth extraction follows similar patterns in *Smokers* and *Ctrl*, with a predominance of disorganised woven bone during the early stages that is later replaced by organised lamellar bone. This study demonstrates, for the first time, that bone microstructure and the relative contents and specific characteristics of the inorganic and the organic phases are not significantly altered by moderate-to-heavy smoking. In contrast to the various cells

found in bone that tend to respond by producing a number of osteogenic and inflammatory biomarkers, the mineralised extracellular matrix, which itself is a product of cellular and physicochemical/biophysical processes, appears less sensitive to the direct and indirect effects of smoking.

Acknowledgements Open access funding provided by University of Gothenburg. The authors wish to thank Lena Emanuelsson and Märta-Sofie Geijer for technical assistance. Financial support is acknowledged from the Swedish Research Council (2018-02891), Svenska Sällskapet för Medicinsk Forskning (SSMF) postdoctoral scholarship, the Swedish state under the agreement between the Swedish government and the county councils, the ALF agreement (ALFGBG-725641), the Adlerbertska Foundation, the IngaBritt and Arne Lundberg Foundation, the Wilhelm and Martina Lundgren Foundation, Stiftelsen Konrad och Helfrid Johanssons fond, the Dr. Felix Neubergh Foundation, Promobilia, the Hjalmar Svensson Foundation, Futurum—Academy for Health and Care, Jönköping County Council, Jönköping, the Osteology Foundation (YRG 17-047), and the Materials Science Area of Advance at Chalmers and the Department of Biomaterials, University of Gothenburg within the Strategic Research Area initiative launched by the Swedish Government.

Compliance with Ethical Standards

Conflict of interest Furqan A. Shah, Shariel Sayardoust, Omar Omar, Peter Thomsen, and Anders Palmquist declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent The study protocol was approved by the Institutional Review Board at the University of Linköping (Dnr 2016/319-31). Informed consent was obtained from all participants. The study was conducted in accordance with the guidelines of Good Clinical Practice for Trials on Medicinal Products in the European Community, the International Conference on Harmonisation (ICH) guideline for Good Clinical Practice, the Declaration of Helsinki, and the CONSORT guidelines for clinical studies.

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