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Cigarette Smoke as a Risk Factor for Delayed Fracture Healing: Role of Oxidative Stress Impairs Primary Cilia on Osteogenic Differentiation

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Abbreviations

1,25(OH)2D3	1,25 dihydroxy vitamin D3
25(OH)D3	25 hydroxy vitamin D3
ACAN	aggrecan
ALK	activin receptor-like kinases
AP	alkaline phosphatase
ARE	antioxidant response element
BAP	bone specific alkaline phosphatase
bFGF	basic fibroblasts growth factor
BMD	bone mineral density
BMP-2	bone morphogenetic protein 2
BMPs	bone morphogenic proteins
BSA	bovine serum albumin
CAGA	promotor region of TGF-β target genes
CAT	catalase
CCL2	C-C motif chemokine 2
CH	chloral hydrate
CICP	type I C-terminal collagen pro-peptide
COLXA1	collagen X
COL2A1	collagen II
CS	cigarette smoke
CSE	cigarette smoke extract
CTX-1	C-terminal telopeptide of type I collagen
DCFH-DA	2´, 7´ dichlorfluorescein-diacetate
DGE	Deutsche Gesellschaft für Ernährung (German nutrition society)
DHE	dihydroethidium
ECM	extracellular matrix
ELISA	Enzyme Linked Immunosorbent Assay
ERK1/2	extracellular signal-regulated kinases

FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
Gli2	zinc finger protein
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
H_2O_2	hydrogen peroxide
Hh	Hedgehog signalling
HOCl	hypochlorite
IFN-γ	interferon gamma
IFT	intraflagellar transport system
IGF	insulin-like growth factor
IL	interleukin
JNK	c-Jun-N terminal kinase
Keap-1	Kelch-like ECH associating protein 1
MAPKs	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MMPs	matrix metalloproteinases
MSC	mesenchymal stem cell
NAC	N-acetylcysteine
NO	nitrogen monoxide
NOX	nicotinamide adenine dinucleotide phosphate oxidase
Nrf2	nuclear factor erythroid-2-related factor-2
NRS	nutritional risk screening 2002 questionnaire
NUP	nucleoporin protein
O_2^-	superoxide anion
OH^-	hydroxyl radical
OPG	osteoprotegerin
OPN	osteopontin
PC2	polycystin 2

PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
Ptch1	receptor Patched
PY	pack-years
RANKL	receptor activator of nuclear factor kappa-B ligand
RLU	relative fluorescent unit
ROO •	peroxyl radicals
ROS	reactive oxygen species
RT	room temperature
RUNX2	runt-related transcription factor 2
SARA	Smad anchor for receptor activation
SCP-1	single-cell-picked clone 1 (single-cell-derived stem cell line)
Smo	Smoothened
SOD	superoxide dismutase
sRANKL	soluble receptor activator of nuclear factor kappa-B ligand
SRB	sulforhodamine B
TBS-T	tris-buffered Saline-Tween
TGF-β	transforming growth factor beta
THS	Tobacco Heating System
TIMP-1 and TIMP-2	tissue inhibitor of metalloproteinases 1 and 2
TJA	total joint arthroplasty
TNF-α	tumor necrosis factor alpha
TRAP5b	tartrate-resistant acid phosphatase

Figures

1. Introduction

1.1 Bone Structure and Function

The **skeletal system** is responsible for **providing shape, mechanical structure, physical support, and protection to the organs** of the body (Boskey, 2013, Cech and Martin, 2012). Together with cartilage, ligaments, tendons, bones are the principal component of the skeletal system. The skeletal system play a **crucial role in maintaining endocrine energy metabolism balance** by controlling the equilibrium of calcium and other essential ions (Marolt *et al*., 2010, Bronner, 2001, Christov and Jüppner, 2018). Bone composition may be affected by several factors like ageing, diseases, and unhealthy habits (*e.g.* cigarette smoking). **Changes in bone composition may directly disturb bone properties and strength** due to altered cell function *via* dysregulated protein concentrations and activities (Boskey and Coleman, 2010, Boskey and Robey, 2013, Bailey *et al*., 1999).

1.2 Bone Cells

Osteoblasts are a type of **mononuclear bone cells desendent from mesenchymal stem cells** (MSCs); they are **in charge for the creation of "new" bone**. This cell type comprises 4–6% of all cells found in bone (Figure 1) (Johannesdottir and Bouxsein, 2018). MSCs can also give race into other cell types, like chondrocytes, adipocytes, myocytes, and endothelial cells (Aubin and Triffitt, 2002).

Bone morphogenic proteins (BMPs) are principally liable for controlling the osteogenic differentiation of MSCs. BMPs belongs to the transforming growth factor-β (TGF-β) superfamily of proteins. They act as morphogens by influencing fundamental mechanisms, for instance, bone formation during embryonic development and bone cells functions during postnatal bone remodeling (Bassi *et al*., 2011). Osteoprogenitor cells under osteogenic differentiation secrete collagen I to form osteoids. Mature osteoblasts precipitate calcium salts and phosphate from the blood, which binds to osteoids (forming hydroxyapatite crystals) in order to form the mineralized bone tissue (Orimo, 2010). Osteoblasts also synthesize alkaline phosphatase (AP), which hydrolyzes pyrophosphate and provides inorganic phosphate to promote bone

mineralization (Orimo, 2010). Moreover, increased AP expression is related with improved differentiation of pre-osteoblasts and is frequently used to identify early osteoblastogenic differentiation (Huang *et al*., 2007).

Osteoblasts that are entirely surrounded by mineralized bone matrix are called osteocytes, the most mature cells in bone (Figure 1) (Dallas and Bonewald, 2010). These cells extend appendages (called filopodia) into the canaliculi (approximately 0.03–0.2 μm canals between the lacunae of the ossified bone) to maintain communication and direct contact between them (Palumbo *et al.*, 1990). **Osteocytes** are the **furthermost cell type in bone**, about 90–95% of the total cell count (Johannesdottir and Bouxsein, 2018). These cells can **regulate the bone remodeling process** *via* the production of factors that impact the homeostasis of neighboring bone due to regulating osteoblast/osteoclast function (Nakashima *et al.*, 2011, Poole *et al.*, 2005, Standal e*t al*., 2014). Additionally, active osteoblasts that become quiescent are found lining the surface of bone (Figure 1). It has been shown that **bone lining cells** favor osteoclast attachment to the bone surface and remove excess collagen from resorption sites (Howship's lacunae) to **facilitate bone remodeling** (Everts *et al*., 2002). If necessary, these cells have the capacity to be activated as functional osteoblasts. Furthermore, they communicate with osteocytes through gap junctions and promote the differentiation of hematopoietic stem cells (Matic *et al*., 2016, Reitsma *et al.*, 2017, Brown *et al.*, 2013).

Osteoclasts, multinucleated cells derived from the fusion of hematopoietic stem cells, have the **ability to resorb bone** (Figure 1) (Teitelbaum, 2007). Osteoclasts represent about 1–2% of bone cells (Johannesdottir and Bouxsein, 2018). The osteoclast membrane is associated with the surface of bone; they use a ruffled border to bind and secrete enzymes that dissolve the matrix, an action that produces resorption pits (Howship's lacunae) (Filgueira, 2010, Parvizi and Kim, 2010, Feher, 2017).

It is known that cigarette smoke directly affects the appropriate balance between bone-forming and bone-resorbing cells (Al-Bashaireh *et al.*, 2018). Interestingly, in the oral cavity, where cigarette smoke directly contacts bone tissue, there is a reported increase in bone resorption under enhancing osteoclast activity (Behfarnia *et al*., 2016, Buduneli *et al*., 2008, Buduneli *et al*., 2009, Ozcaka *et al.*, 2010). In long bones where the compounds present in cigarette smoke should arrive through the bloodstream,

there is an apparent decrease in osteoblast activity (Al-Bashaireh *et al.*, 2018, Holzer *et al*., 2012).

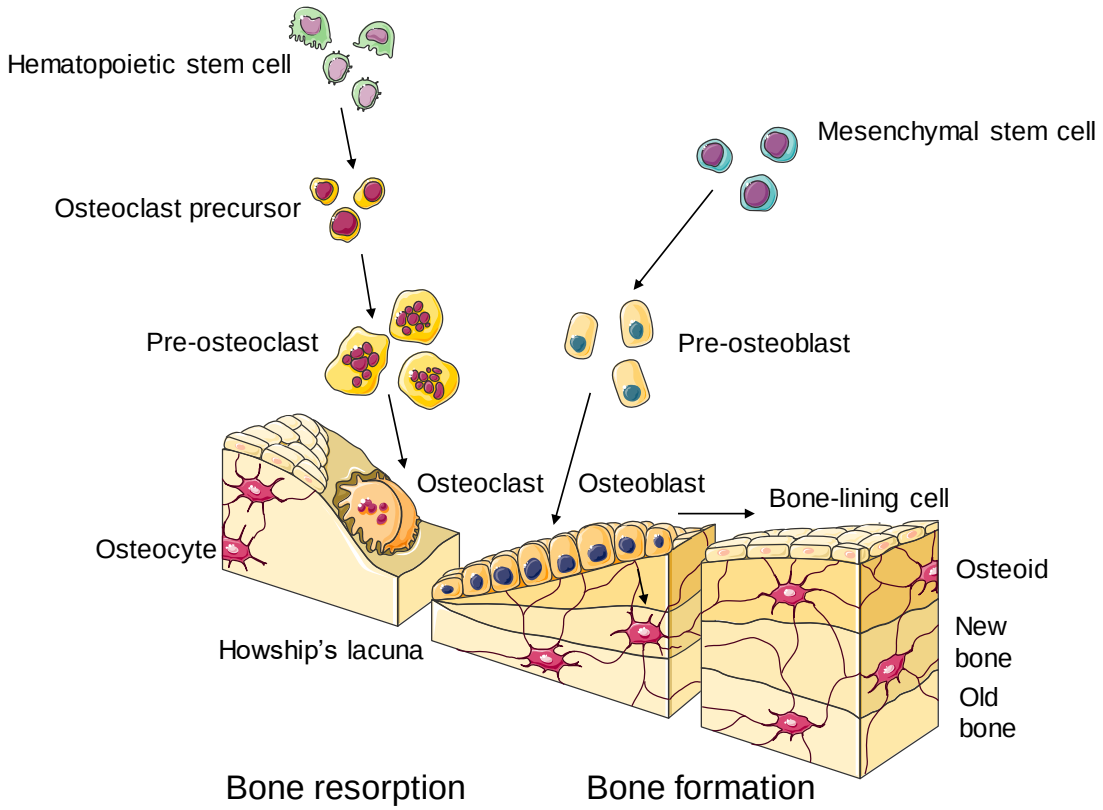

Figure 1: Bone cell types and origin. Mesenchymal stem cells may differentiate into pre-osteoblasts, which further differentiate into bone-forming cells (osteoblasts) on the surface of the bone. Osteocytes are integrated osteoblasts encased in the bone matrix; they coordinate bone remodeling. Bone lining cells are inactive osteoblasts on the surface that facilitate bone remodeling. Osteoclasts are bone-resorbing cells; they originate from precursors of the hematopoietic lineage that become pre-osteoclasts and merge together to give generate mature multinuclear osteoclasts. This figure was produced using graphic components obtained from http://www.servier.com.

1.3 Fracture Healing

Human bone is a highly metabolically active and perfused organ that continuously changes. Osteoblasts and osteoclasts are the central cells involved in this process, defined as remodeling, which maintains the constant equilibrium of bone tissue (Hadjidakis and Androulakis, 2006, Tanaka *et al*., 2005b). A fracture occurs when the continuity of the bone is disrupted due to high force impact, stress, or other medical conditions (*e.g.* osteoporosis, cancer, or osteogenesis imperfecta). This condition is the most common reason for orthopedic procedures.

Fracture healing comprises the recapitulation of several ontological events that occur during embryogenic skeletal development (Einhorn and Gerstenfeld, 2015). Therefore, fracture healing is considered a **regenerative process that restores the**

disrupted organ to the pre-injury condition (cellular regeneration and the return of structure and mechanical properties) without the formation of a fibrous scar (Marsell and Einhorn, 2011). Bone healing is determined by the gap between the bone fragments and the stability of the fracture. It may be divided into two models: primary (direct, cortical) and secondary (indirect, spontaneous) healing (Marsell and Einhorn, 2011). **Direct bone healing** is the faster process; it includes intramembranous bone formation and direct cortical bone remodeling without endochondral ossification (Marsell and Einhorn, 2011, Isaksson *et al.*, 2007). This type of healing can only occur when the stability of fracture is anatomically ensured with a low inter-fragmentary strain (Epari *et al*., 2010, Tsiridis *et al.*, 2007). In contrast, **indirect bone healing** (fracture gap > 0.5 mm) is a slower, spontaneous process that includes endochondral ossification with callus formation as well as intramembranous ossification (LaStayo *et al.*, 2003). This type of bone healing is the most common form and is inhibited by rigid fixation and improved by mobilization (Dimitriou *et al.*, 2005).

1.3.1 Phases of Fracture Healing

After a fracture occurs, there are three phases of the bone healing process: the early inflammatory phase, the callus formation reparative phase, and the remodeling phase (Marsell and Einhorn, 2011). However, it should be noted that these three phases overlap and thus generate a continuous healing process. During the remodeling phase, there are two states: degradation of the initial soft cartilage callus, followed by the replacement of mineralized tissue (hard callus). However, during normal bone development, remodeling refers to the degradation of mineralized tissue by osteoclasts and replacement of new mineralized tissue by osteoblasts (Figure 2) (Oryan *et al.*, 2015).

Inflammatory phase: Bone structure disruption leads to the destruction of several blood vessels from the medullary canal and the periosteum, with subsequent bleeding that causes a hematoma in the damaged area. A **fracture hematoma** avoids hemorrhage; it provides a fibrin network for appropriated cellular migration (Grundnes and Reikeras, 1993b, Grundnes and Reikeras, 1993a) and plays a vital role in initiating several cellular signaling events that are necessary for fracture healing to proceed (Bolander, 1992). Increased levels of proinflammatory cytokines, such as interleukin 1 (IL-1), IL-6, IL-11, IL-18, and tumor necrosis factor α (TNF-α), are elevated after

trauma; their chemotactic properties attract inflammatory cells (Mountziaris and Mikos, 2008). A hematoma represents a hypoxic and acidic environment, and osteocytes become necrotic due to a lack of nutrients (Geris *et al.*, 2008). The hypoxic–acidic conditions stimulate the activity of macrophages that phagocytize the necrotic area (Hollinger and Wong, 1996) and initiate the regeneration stage by releasing growth factors (*e.g.* BMP-2, BMP-5, BMP-7, basic fibroblasts growth factor [bFGF], TGF-β, platelet-derived growth factor [PDGF], and insulin-like growth factor [IGF]). These proteins are responsible for the migration, recruitment, and proliferation of MSCs. Those cells then differentiate into fibroblasts, angioblasts, chondrocytes, and osteoblasts that promote vascularization and establish granulation tissue on the fracture gap (Goldhahn *et al.*, 2012, Wang *et al.*, 2013). A reparative granuloma (soft callus) that decreases the mobility at the fracture site develops during the inflammatory phase. This phase of fracture healing is fast and usually lasts about 1–2 weeks under normal conditions (Figure 2A) (Loi *et al.*, 2016, Oryan *et al.*, 2015).

Reparative phase: While the necrotic tissue is reabsorbed by osteoclasts (derived from the circulating monocytes in the blood) and monoblastic precursor cells (from the bone marrow) (Haverstock and Mandracchia, 1998), MSCs from the damaged tissue are recruited through the bloodstream and differentiate into fibroblasts, chondrocytes, and osteoblasts (Goldhahn *et al.*, 2012, Wang *et al.*, 2013). During soft callus formation, there is vascular ingrowth, collagen synthesis, and secretion of osteoid (Pilitsis *et al.*, 2002), which will be replaced with mineralized tissue *via* intramembranous or endochondral ossification (Goldhahn *et al.*, 2012). **Intramembranous ossification** occurs by the formation of woven bone tissue; this process is mediated by osteoblasts situated in opposition to the cortex and distal from the fracture site (Einhorn, 1998, LaStayo *et al.*, 2003). Bone formation in this area occurs due to direct osteoblast differentiation from precursor cells, without an intermediate cartilage formation step. The area where this type of bone formation occurs is termed the hard callus. This process happens in the external callus without the formation of cartilage (Bolander, 1992, Sfeir *et al.*, 2005). **Endochondral ossification** transpires while intramembranous ossification is taking place. MSCs from the periosteum and adjacent external soft tissues differentiate to chondrocytes (cartilage-forming cells) in the central, hypoxic fracture area (McKibbin, 1978, Einhorn, 1998, Sfeir *et al.*, 2005). Under the stimulation of several growth factors like TGF-α2, PDGF, IGF-1, TGF-β2, TGF-β3, and BMPs (BMP-2, BMP-4, BMP-5, and BMP-6),

chondrocytes begin to synthesize cartilaginous tissue (soft callus) (Mountziaris and Mikos, 2008, Geris *et al.*, 2008). The cartilage callus is replaced by mineralized tissue *via* hypertrophic chondrocytes and osteoblasts that secrete vesicles with calcium phosphate and neutral proteases (*e.g.* proteoglycanases) and AP. As the reparative phase progresses, the acidic conditions begin to decrease, favoring the activity of AP – exoenzyme produced by hypertrophic chondrocytes and osteoblasts. These cells have a main function in the inorganic ions' precipitation onto the collagen soft callus. Due to the essential role of proteoglycanases, that digest the proteoglycan-rich matrix, and AP, which supply phosphate ions for the mineralization of the soft callus *via* the hydrolyzes of high-energy phosphate esters (Einhorn *et al*., 1989, Brighton and Hunt, 1986, Sfeir *et al.*, 2005). Consequently, the callus becomes more rigid due to the mineralization, and the fracture or osteotomy site is considered to be immobilized (Haverstock and Mandracchia, 1998). As a result, an irregular woven bone callus connects the fracture gap (Bolander, 1992). The reparative phase of the fracture healing occurs before the inflammatory phase recedes and take place between 3 and 6 weeks (Figure 2B).

Remodeling phase: This is the final step in fracture healing and involves the conversion of irregular woven bone callus into lamellar bone through mineralization (Puzas *et al*., 2003, Schindeler *et al*., 2008, Hollinger and Wong, 1996). Woven bone is resorbed by osteoclasts, which attach to the bone surface through podosomes, form a ruffle border, and secrete acid and proteinases (*e.g.* tartrate-resistant acid phosphatase [TRAP] and cathepsin K) that create resorption pits called 'Howship's lacunae'. Osteoblasts are deposited in the resorbed area and initiate mineralization by deposition of hydroxyapatite (a mineral rich in calcium and phosphate) (Schindeler *et al.*, 2008). The result of this phase is the restoration of the bone's mechanical strength and optimal stability: the bone architecture is similar to before the trauma (Puzas *et al*., 2003). This phase of fracture healing usually lasts about 8 weeks to 2 years (Figure 2C). Bone remodeling is also a continuous process in the healthy skeleton that maintains the appropriate level of bone homeostasis; it has major similarities with the fracture healing process. The remodeling phase is regulated by an orchestra of cytokines and hormones (Mountziaris and Mikos, 2008).

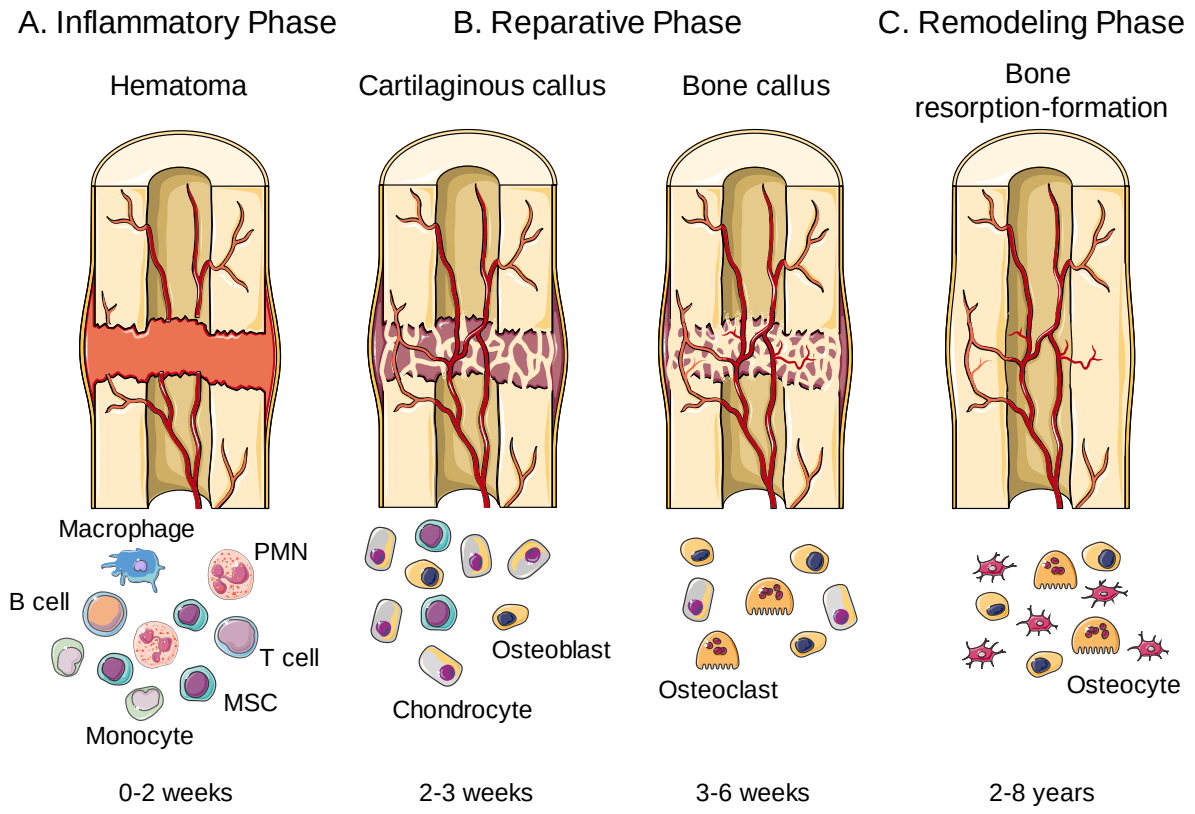

Figure 2: The states of fracture healing. (A) Inflammatory phase: following injury, the blood supply is disturbed, leading to hematoma formation. (B) Reparative phase: Ingrowth from vessels provides blood supply to the damaged area. During this phase, the soft callus (cartilage) is assembled by endochondral ossification, while the external callus is produced by intramembranous ossification. Osteoblast and hypertrophic activity mineralize the soft callus. (C) Remodeling phase: the irregular woven bone callus is replaced with lamellar bone by osteoclasts (resorption) and osteoblasts (mineralization). The figure was produced using graphic components obtained from http://www.servier.com and based on a previous study (Einhorn and Gerstenfeld, 2015)

1.4 Cigarette Consumption – Incidence

Cigarette smoking (CS) is worldwide the most popular and common way of consuming tobacco. Additionally, a cigarette is the most prevalent legal drug; it has a high addiction potential and is a leading worldwide cause of premature and preventable death (Pötschke-Langer *et al*., 2015, Aspera-Werz *et al.*, 2018). According to the World Health Organization, CS will be the reason for 8 million deaths per year globally in 2030. In 2015, **nearly 1 billion people smoked worldwide**, of which around 16.3 million took place in Germany alone (Reitsma *et al*., 2017). Moreover, CS causes more than 100,000 premature dead per year in Germany (Pötschke-Langer *et al*., 2015). CS represents a significant health hazard that adversely impact the entire human body and is linked to several chronic, non-communicable health disorders (*e.g.* coronary heart disease, chronic obstructive pulmonary disease, cerebrovascular disease, and

cancer) (Burns, 2003, Sasco *et al.*, 2004, Domagala-Kulawik, 2008, Benowitz, 2003, Aspera-Werz *et al.*, 2018).

Smoking consists of combusting tobacco at around 800°C and then smoking it and inhaling the more than 6,500 molecular compounds (of which more than 150 have been identified as toxic) (Rothem *et al.*, 2009, Pappas, 2011, Cooke, 2010, 2012, Rodgman and Perfetti, 2016, Aspera-Werz *et al*., 2020). The most harmful substances to the human body are hydrocarbons, oxygen compounds, nitrogen compounds, and metals that can easily pass into the bloodstream through the lung alveoli and then get distributed to the whole body, a phenomenon that affects the appropriate function of several organs (Reitsma *et al.*, 2017, Cooke, 2010).

Nicotine, a major CS additive, is very pharmacologically active and directly and indirectly negatively impact cellular metabolism. Approximately 70–80% of nicotine is metabolized to cotinine in the liver (Hukkanen *et al.,* 2005, Benowitz *et al.*, 2009, Aspera-Werz *et al.*, 2018). Nicotine and cotinine concentrations in the musculoskeletal system reach the same levels as arterial blood (Benowitz *et al.*, 2009). After smoking one cigarette, the nicotine concentration in arterial blood is 20–60 ng/ml. However, cotinine reaches higher blood levels than nicotine, with concentrations of 250–300 ng/ml, as a result of the longer half-life (16 h for cotinine *vs.* 2 h for nicotine) (Benowitz *et al*., 2009, Hukkanen *et al*., 2005, Lunell *et al.*, 2000, Rose *et al*., 1999, Gourlay and Benowitz, 1997, Aspera-Werz *et al*., 2018).

1.5 Cigarette Smoking – Bone

It has been known since 1976 that there is a positive correlation between the number of cigarettes consumed and reduced bone mineral density (Daniell, 1976, Aspera-Werz *et al*., 2018). Several studies have confirmed this association (Cusano, 2015, Wong *et al*., 2007) and have demonstrated that CS may lead to a **disproportion in bone remodeling mechanisms**, resulting in secondary osteoporosis, osteoarthritis, and fracture (Ward and Klesges, 2001, Kanis *et al*., 2005, Amin *et al*., 2007, Abrahamsen *et al*., 2014). Moreover, CS enhances the risk of delayed fracture healing (Adams *et al*., 2001, Aspera-Werz *et al.*, 2020), non-union (Scolaro *et al*., 2014), and complications (Mills *et al*., 2011), and leads to longer hospital stays (Scolaro *et al*., 2014, Sloan *et al*., 2010, Abate *et al*., 2013, Singh *et al*., 2015, Ehnert *et al.*, 2019). Based on clinical observations, the risk of non-union after ankle arthrodesis increased

3.75 fold in smokers (Cobb *et al.*, 1994). In patients with pseudoarthrosis, there was a reported 40% non-union rate for smokers compared with 4% for non-smokers (Brown *et al.*, 1986). Additionally, smokers undergoing orthopedic surgeries experienced a higher risk of postoperative complications (*e.g.* infections, implant revisions) compared with non-smokers (Singh *et al*., 2015, Høidrup *et al*., 2000).

Several factors are responsible for the detrimental effects of CS on fracture healing (Ward and Klesges, 2001). Nicotine induces catecholamine production by the adrenal gland, causing vasoconstriction and reduced tissue perfusion (Reus *et al*., 1984). Additionally, nicotine downregulates the level of prostacyclin, a prostaglandin that inhibits platelet activation and causes vasodilatation (Nadler *et al*., 1983), as well prostaglandin I_2, which affects endothelial cell development (Effeney, 1987). Moreover, smokers present higher levels of carboxyhemoglobin in comparison to non-smokers (1–20% *vs.* 0.5–1%, respectively (McVeigh *et al.*, 1996)) due to inhalation of carbon monoxide present in CS; this gas reduces the oxygen-carrying capacity of the blood and leads to hypoxia (Birnstingl *et al*., 1971). Therefore, the combination of these factors decreases the blood flow and oxygen supply to the fracture site due to vasoconstriction, contributing to the delay in bone healing or non-union.

Delays in fracture healing, non-union, an increased complication rate, and extended hospital stay lead to an increase in health system costs. In Germany, the direct and indirect health system costs caused by cigarette are estimated at around 80 billion euros per year (Effertz and Viarisio, 2015). Therefore, the development of treatment strategies that improve bone healing or protect the bone structure from the deleterious effects of CS are strongly needed.

Cell therapy is a promising and continuously growing alternative to treat musculoskeletal diseases and improve bone healing (Pountos *et al*., 2006, Lin *et al*., 2017). MSCs have been one of the primary researches focuses for cell therapy because

e they can contribute to the regeneration of mesenchymal tissues (*e.g.* bone, cartilage, vasculature, fat, tendon, and muscle) (Jiang *et al*., 2002, Pittenger *et al.*, 1999), as well as activate tissue regeneration through paracrine stimulation (Wang *et al*., 2014a). In the bloodstream, MSCs come into direct contact with the inhaled cigarette smoke components and can be damaged by them (Tura-Ceide *et al*., 2017).

Several studies have shown, directly and indirectly, the negative effects of CS on osteoblasts and MSCs *in vitro* (Ehnert *et al.*, 2012a, Ehnert *et al*., 2012b, Holzer *et al*., 2012, Braun *et al.*, 2011, Greenberg *et al.*, 2017, Cyprus *et al*., 2018, Liu *et al.*, 2001). In the same line, additional studies have revealed the detrimental impact of nicotine on MSC proliferation and differentiation (Tanaka *et al.*, 2005a, Marinucci *et al.*, 2018). However, other studies have shown that lower nicotine concentrations have stimulatory effects on MSCs and bone-forming cells (Daffner *et al.*, 2012, Kim *et al*., 2012). Furthermore, benzo[a]pyrene, a polycyclic aromatic hydrocarbon present in cigarette smoke unfavorably influences osteoblast function and MSC differentiation. (Monnouchi *et al.*, 2016, Zhou *et al.*, 2017). Thus, understanding the molecular mechanisms and associated harmful compounds by which CS exerts its effects on MSCs and bone cells is crucial for designing therapeutic treatments that abate CS-mediated damage to bone tissue.

1.6 TGF-β: A Mediator of Bone Healing

As discussed in section 1.3, fracture repair is a complex biological process that commits several phases: inflammation, migration and recruitment of MSCs, generation of cartilaginous soft callus, vascularization, resorption of soft callus and subsequent mineralization, and remodeling. The systemic migration and recruitment of MSCs to the fracture site is most important for the healing outcome; it is mainly mediated by TGF-β. This **multifactorial regulatory protein** belongs to TGF-β superfamily, which is a large and expanded group of over 30 structurally related cell regulatory peptides like BMPs, activins, inhibins, growth and differentiation factors, glial-derived neurotrophic factors, Müllerian inhibiting substance, left-right determination factor, and nodal growth differentiation factor (Javelaud and Mauviel, 2004).

The TGF-β family contains **three related mammalian isoforms** (TGF-β1, TGF-β2, and TGF-β3) that are derived from a common ancestor. All three isoforms can be found in human bone (Cho *et al*., 2002): there are high TGF-β1 and TGF-β3 levels in soft callus, while TGF-β2 is mainly expressed in cartilage during endochondral ossification. TGF-β1 and TGF-β2 are detected in mineralized areas and TGF-β3 is extensively distributed during intramembranous ossification (Horner *et al.*, 1998). However, **TGF-β1 is the most abundant isoform present in bone matrix** (200 µg/kg (Bonewald and Dallas, 1994, Janssens *et al.*, 2005)) and one of the main factors in

skeletal tissue because it maintains the homeostasis of cartilage and bone metabolism as a result of recapitulation of embryonic endochondral ossification (Vortkamp *et al.*, 1998, Ferguson *et al.*, 1999).

TGF-β1 is secreted by bone cells and stored in the extracellular matrix (ECM) in its inactive form by non-covalent associations with the latency-associated protein (which avoids the interaction of the epitope(s) with TGF-β receptor). **Functional osteoclasts** secrete proteases, generate an acidic microenvironment, and **release active TGF-β1** (Oreffo *et al.*, 1989, Oursler, 1994). Therefore, **TGF-β** significantly **influences bone cell function** and plays a main role in **maintenance of bone homeostasis** (Erlebacher *et al.*, 1998, Poniatowski *et al.*, 2015, Robey *et al*., 1987, Bonewald and Mundy, 1990). This molecule **activates the expression** of **ECM compounds**, such as collagen type I, fibronectin, osteopontin (OPN), osteonectin, thrombospondin, and proteoglycans, all of which **support to fracture repair** (Poniatowski *et al.,* 2015, Moghaddam *et al.*, 2010, Harris *et al.*, 1994). Blumenfeld *et al.* demonstrated that lower TGF-β levels in rats weaken bone strength and microarchitecture. However, facture healing may be improved in this model by local application of TGF-β and IGF-1 (Blumenfeld *et al.*, 2002).

1.7 TGF-β1 Signaling

Once active, TGF-β1 binds to type II transmembrane serine/threonine kinase receptor (five types in human), followed by recruitment of type I activin receptor-like kinases (ALKs; seven types in human, ALK5 is the only one involved in bone cells) into a hetero-tetrameric receptor complex with two type I receptors (signal propagators) and two type II receptors (activators) (Massagué, 2012). This activity induces the transphosphorylation and activation of ALK by a type II receptor, resulting in the intracellular transmission of the signal (Figure 3) (Massagué, 2012, Massague, 1998).

Receptor-regulated Smad2 and Smad3 transcription factors can bind to the activated receptor complex, with the assistance of adaptor proteins (*e.g*. Smad anchor for receptor activation [SARA]) (Tsukazaki *et al.*, 1998, Wu *et al.*, 2000). ALK phosphorylates Smad2/3, an action that induces a conformational change that dissociates phospho-Smad2/3 from the receptor complex. Phospho-Smad2/3 interacts with a common-partner Smad (Smad4) and assembles the active complex phospho-Smad2/3/4, which translocates to the nucleus (Wu *et al*., 2001, Chacko *et al*., 2004).

Once in the nucleus, the active phospho-Smad2/3/4 complex may directly bind to Smad-responsive motifs, such as Smad-binding element or CAGA (Itoh *et al.*, 2019) in DNA, or recruit other cofactors to promote the expression of TGF-β target genes (Figure 3) (Shi and Massague, 2003, Poniatowski *et al*., 2015). The nuclear pore importin complex may translocate the Smad3/4 active complex (mediated by importin-β1) (Kurisaki *et al*., 2001, Xiao *et al*., 2000) or through nuclear pore proteins (NUP214/NUP153) (Xu *et al*., 2003). In contrast, nuclear translocation of active Smad2/4 only involves nuclear pore proteins (Xu *et al*., 2002). Therefore, Smad3/4 is a tentative target to consider due to its multiple nuclear translocation mechanisms.

Interesting, Smad4 is essential to maintain appropriate bone homeostasis (Tan *et al.*, 2007). *In vitro* studies have revealed an enhanced hypertrophic phenotype in conditional Smad4 knockout chondrocytes (Zhang *et al.*, 2005). Moreover, *in vitro* studies have demonstrated that MSC osteogenesis could be improved *via* Smad4 interaction with transcriptional factors like Runx2 and AP-1 (c-Fos/JunD) (Lai and Cheng, 2002). Additionally, Smad2-deficient mice present early embryonic lethality; however, Smad3 knockout mice are viable and fertile. Hence, Smad2 function may rescue Smad3 deficiency, but Smad3 cannot compensate for the lack of Smad2 (Brown *et al.*, 2007).

Smad-independent pathway could also be triggered by TGF-β. Non-canonical signaling comprises numerous branches of mitogen-activated protein kinase (MAPK) pathways, Rho-like guanosine triphosphate hydrolase enzyme signaling pathways, or phosphatidylinositol-3-kinase/protein kinase B signaling. This action leads to extracellular-signal-regulated kinases (ERK1/2), c-Jun-N terminal kinase (JNK), and p38 activation (Johnson and Lapadat, 2002, Mulder, 2000, Zhang, 2008 Aspera-Werz *et al.*, 2019). MSCs and osteoblasts also express type III receptors (betaglycan and endoglin), while osteoclasts only express endoglin. These receptors are directly involved in signaling through the modulation of TGF-β1, and additional ligands specify binding (Walsh *et al.*, 2003, Kim *et al*., 2019, Janssens *et al.*, 2005).

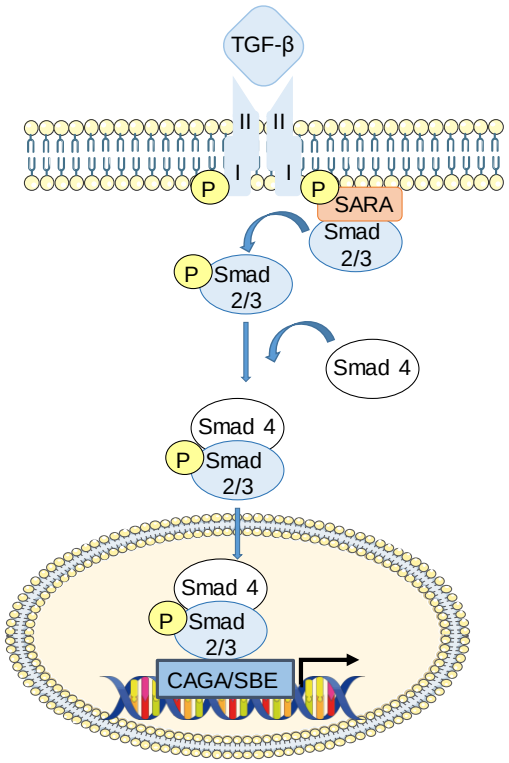

Figure 3: The canonical transforming growth factor β (TGF-β) signaling pathway. TGF-β binds to the type II transmembrane serine/threonine kinase receptor, which recruits a type I activin receptor-like kinase dimer, forming a hetero-tetrameric complex. The activated complex phosphorylates receptor-regulated Smad2/3 with the assistance of the adaptor protein Smad anchor for receptor activation (SARA). Activated Smad2/3 loses the affinity for the receptor complex and interacts with the common partner Smad4. The active Smad2/3/4 complex is translocated to the nucleus and modulates the expression of target genes. This figure is based on previous studies (Massagué, 2012, Massague, 1998).

1.8 TGF-β1 in Smokers

A **dual role of TGF-β1** has been described during bone healing: TGF-β1 is **involved in bone resorption and bone formation**. On the one hand, functional osteoclasts may activate TGF-β1, which *via* negative feedback impairs osteoclastogenesis (suppresses hematopoietic precursor cell proliferation) and downregulates bone resorption. On the other hand, TGF-β1, through its chemoattractant properties, may recruit MSCs to the fracture site and promote their proliferation (Lucas, 1989, Pfeilschifter *et al*., 1990, Hughes *et al*., 1992). Additionally, this cytokine regulates osteoblast function (induces early and inhibits late differentiation (Alliston *et al*., 2001, Maeda *et al*., 2004)), appropriate chondrogenic differentiation (Xia *et al*., 2017, Tuli *et al*., 2003, James *et al*., 2009), and, consequently, influences endochondral ossification. Additionally, TGF-β1 shows anti-apoptotic properties on osteoblast, supporting their transition to osteocytes (Chua *et al.*, 2002, Karsdal *et al*., 2002, Bodine *et al*., 2005, Dufour *et al*., 2008, Jilka *et al*., 1998). Besides the direct effects on bone cells, TGF-β

can enhance the impact of other factors that positively regulate later osteogenic differentiation and mineralization and, thus, improve bone healing (*e.g.* BMPs and IGFs) (Zimmermann *et al*., 2005, Li *et al*., 2012, Li *et al*., 2015).

It should be highlighted that **active TGF-β1 levels increase after a fracture** (during the inflammatory phase), and high levels of active TGF-β1 positively correlate with differentiated and functional osteoblasts and chondrocytes during endochondral ossification (Si *et al*., 1997). Nevertheless, it was demonstrated that **cigarette smoking** is associated with **reduce blood serum TGF-β levels** (Poniatowski *et al*., 2015, Moghaddam *et al.*, 2010). Therefore, there is a clear **association between delayed fracture repair and a decrease in systemic TGF-β1 levels in smokers** (Zimmermann *et al.*, 2005). Several *in vivo* and *in vitro* studies have proved that recombinant human TGF-β1 improves fracture healing (Poniatowski *et al*., 2015, Robey *et al.*, 1987, Noda and Camilliere, 1989, Tang *et al*., 2009). Hence, it may be possible to utilise recombinant human TGF-β1, as well as therapies that boost or restore the impair TGF-β signaling pathway, as a treatment approach to enhance the delay fracture repair that smokers undergo (Aspera-Werz *et al.*, 2019).

1.9 Oxidative Stress – Bone

The **intracellular redox state** of cells is regulated by the appropriate **balance the levels of pro-oxidants, oxidizing agents, and antioxidants** (Kurutas, 2016, Valko *et al.*, 2007). Reactive oxygen species (ROS) are highly reactive radical and non-radical oxygen molecules, such as superoxide anion (O_2^-), hydroxyl radical (OH), hypochlorite (HOCl), peroxyl radicals (ROO), nitrogen monoxide (NO), and hydrogen peroxide (H_2O_2) (Jaimes *et al.*, 2001). ROS are derived from normal cellular metabolic processes (*e.g.* immune cell signaling *via* nicotinamide adenine dinucleotide phosphate oxidase [NOX], mitochondrial oxidases, cytochrome P450, endoplasmic reticulum, peroxisomes, and lysosomes) as well as in response to exogenous exposure, such as pollutants, heavy metals, tobacco smoke, ultraviolet light, ionizing radiation, xenobiotics, or drugs (de Mochel *et al*., 2010, Milkovic *et al.*, 2019). **Physiological ROS levels** play an essential role as **second messengers in several cell signaling** and other physiological cellular events (Bae *et al*., 1997, Sundaresan *et al*., 1995, Forman *et al*., 2004), regulating processes such as proliferation, differentiation, inflammation, and apoptosis (Ray *et al*., 2012, Ji *et al*., 2010, Rhee,

2006, Migliario *et al*., 2014). However, **excessive ROS negatively affects cell homeostasis** and, consequently, damages several tissues.

Changes in the redox state or the cellular antioxidant systems are also associated with the bone remodeling process by regulating the synchronized action of osteoclasts, osteoblasts, and osteocytes (Wauquier *et al*., 2009). In fact, non-physiological levels of oxidative stress alter the bone remodeling process through activation of osteoclast differentiation and generate an imbalance between bone forming and bone resorbing cells, an eventuality that may lead to metabolic bone disorders such as osteoporosis (Lean *et al*., 2005, Baek *et al*., 2010).

Human marrow mononuclear cell exposure to H_2O_2 show increased levels of TRAP as well as a high number of active osteoclasts compared with control conditions (Baek *et al.*, 2010). Several *in vitro* studies have demonstrated that the upregulation of osteoclast activity is *via* activation of receptor activator of nuclear factor kappa-B ligand (RANKL) signaling through ROS as an intracellular signaling mediator for JNK and p38 activation (Figure 4) (Lee *et al*., 2005, Ha *et al.*, 2004, Boyle *et al*., 2003, Ikeda *et al*., 2004). Additionally, non-physiological ROS levels induce osteoblast and osteocyte apoptosis through activation of MAPKs, such as ERK1/2, JNK, and p38 kinase; this phenomenon enhances the imbalance between bone forming and bone resorbing cells (Fontani *et al*., 2015, Plotkin *et al*., 2005). Interestingly, apoptotic osteocytes induce osteoclast recruitment and differentiation (Plotkin, 2014, Bellido *et al*., 2018, Kennedy *et al*., 2014, Al-Dujaili *et al*., 2011). Moreover, oxidative stress downregulates the production of OPG by osteoblasts, causing an increase in the RANKL/OPG ratio in favor of bone resorption (Figure 4) (Brzóska and Rogalska, 2013, Tolba *et al*., 2017). Therefore, targeting excessive ROS production *via* treatment with ROS scavengers, inductors of antioxidative cell system, or an inhibitor/activator of osteoclast/osteoblast function may be an exciting approach to prevent bone damage in bone metabolic diseases related to increased bone resorption (Song *et al*., 2018, Yamaguchi *et al*., 2018).

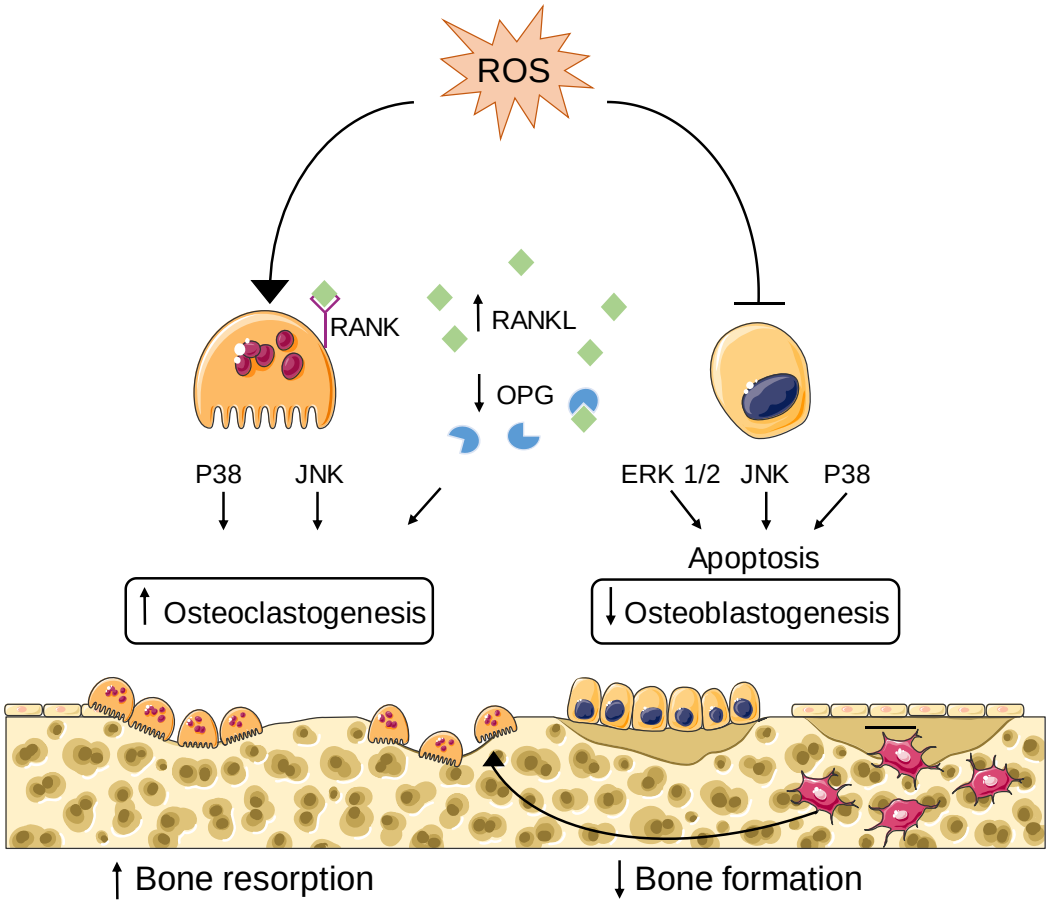

Figure 4: The effect of oxidative stress on bone homeostasis. Changes in oxidative stress levels are associated with bone remodeling by regulating the function and genesis of osteoclasts, osteoblasts, and osteocytes. Non-physiological levels of reactive oxygen species (ROS) trigger activation of c-Jun-N terminal kinase (JNK) and p38, resulting in the induction of the receptor activator of nuclear factor-kappa-B ligand (RANKL) signaling, which favors osteoclast activity. Additionally, oxidative stress decreases osteoprotegerin (OPG) levels, causing an increase in the RANKL/OPG ratio, which induce osteoclast function. Moreover, ROS lead the activation of mitogen-activated protein kinase (MAPKs), such as extracellular signal-regulated kinases (ERK1/2) and JNK and p38 kinase, an eventuality that induces osteoblast and osteocyte apoptosis. Apoptotic osteocytes positively influence osteoclast recruitment and differentiation. Thus, osteoclast bone reabsorption is increased and osteoblast bone formation is reduced by ROS. This figure was produced using graphic components obtained from http://www.servier.com and based on a previous study (Wauquier *et al*., 2009).

1.10 ROS – Cigarette Smoke

After burning a cigarette, the smoke that directly goes into the environment between puffing is defined as sidestream smoke. Mainstream smoke refers to the aerosol drawn into the mouth and inhaled by smokers (National Research Council Committee on Passive, 1986). **Mainstream smoke** from regular cigarettes **contains between 120–150 nmol H_2O_2** (Zhao and Hopke, 2012). Moreover, **1×10^{15} free radical constituents are inhaled with each puff** and distributed throughout the body *via* the bloodstream (Rahman *et al*., 1996). Additionally, cigarette smoke contains several pro-oxidative compounds, which also induce and **activate endogenous ROS production by cells**

(Figure 5) (Csiszar *et al.*, 2009). Unfortunately, cigarette filters are not an option to reduce the levels of ROS and harmful compounds inhaled in mainstream smoke (Huang *et al.*, 2005). Consequently, oxidative stress causes cellular damage due to lipid peroxidation, structural damage of the membranes, oxidation of proteins, nucleic acid mutations, and altered gene expression (Byon *et al*., 2008, Duan *et al*., 2005). These dysregulations affect various organs and systems, including the skeletal system by **impairing bone formation and accelerating bone resorption** (Abate *et al.*, 2013, Barreiro *et al*., 2010).

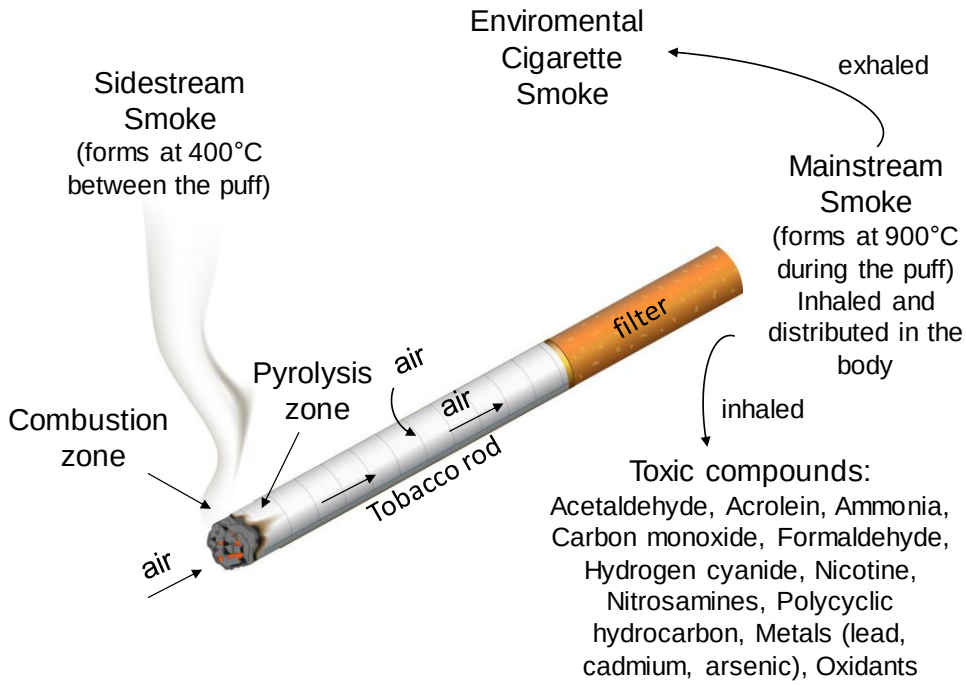

Figure 5: Cigarette combustion and associated compounds. Tobacco combustion at 800°C in a cigarette form generates sidestream and mainstream smoke. Mainstream smoke is inhaled, and the associated toxic compounds are distributed throughout the body *via* the bloodstream. Sidestream smoke and exhaled mainstream smoke contribute to environmental cigarette smoke. This figure is based on previous studies (Guerin, 1987, Centers for Disease *et al*., 2010).

1.11 Antioxidant Defense System in Bone Cells

With the aim of preventing oxidative stress damage, bone cells activate a major antioxidant defense system mechanism: **nuclear factor erythroid-2-related factor-2 (Nrf2) signaling** (Ma, 2013). As a consequence of the activation of Nrf2 signaling due to oxidative stress, several antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), and others associated in the glutathione (GSH) homeostasis (*e.g.*

glutathione peroxidase [GPx]) transcription was upregulated (Osburn and Kensler, 2008, Nguyen, 2009, Venugopal and Jaiswal, 1996, Aspera-Werz *et al*., 2019). In normal conditions, cytoplasmic Nrf2 is bound to Kelch-like ECH associating protein 1 (Keap-1) (Guo *et al*., 2017), which inhibits the transcriptional activity of Nrf2 *via* ubiquitination and proteasomal degradation. Under **oxidative stress conditions**, Nrf2 is dissociated from Keap-1 through changes in its structure (stabilization of its thiol groups), which obstruct the association to Nrf2. Once free, cytoplasmic **Nrf2 is phosphorylated** and transported into the nucleus. Once in the nucleus, **phosphorylated Nrf2 binds to the DNA antioxidant response element** (ARE) to **promote the transcription of antioxidative enzymes** and genes involved in the glutathione system (Figure 6) (Massague and Weis-Garcia, 1996, Aspera-Werz *et al*., 2019).

Several studies demonstrated that Nrf2 plays an essential protective role against the harmful effects of cigarette smoke induced oxidative stress on cell metabolism. Disruption of Nrf2 signaling enhances the adverse outcome of mice exposured to cigarette smoke (Rangasamy *et al.*, 2004). Furthermore, Keap-1 knockdown augments the antioxidative defense system and reduces lung injury caused by smoking (Blake *et al*., 2010). Nevertheless, the role of the Nrf2 signaling pathway on bone cells is controversial. On the one hand, Nrf2 knock out upregulated osteoclast activity and decreased osteoblast activity in bone tissue, and consequently, reduces bone mineral density (BMD) (Sun *et al*., 2015b, Sun *et al*., 2015a). On the other hand, Nrf2 activation in MC3T3 cells treated with H_2O_2 impairs osteogenic differentiation (Lee *et al*., 2015). Therefore, it is essential to elucidate whether there is a protective role of the Nrf2 signaling pathway on bone cells oxidative stress adverse consequences caused by CS.

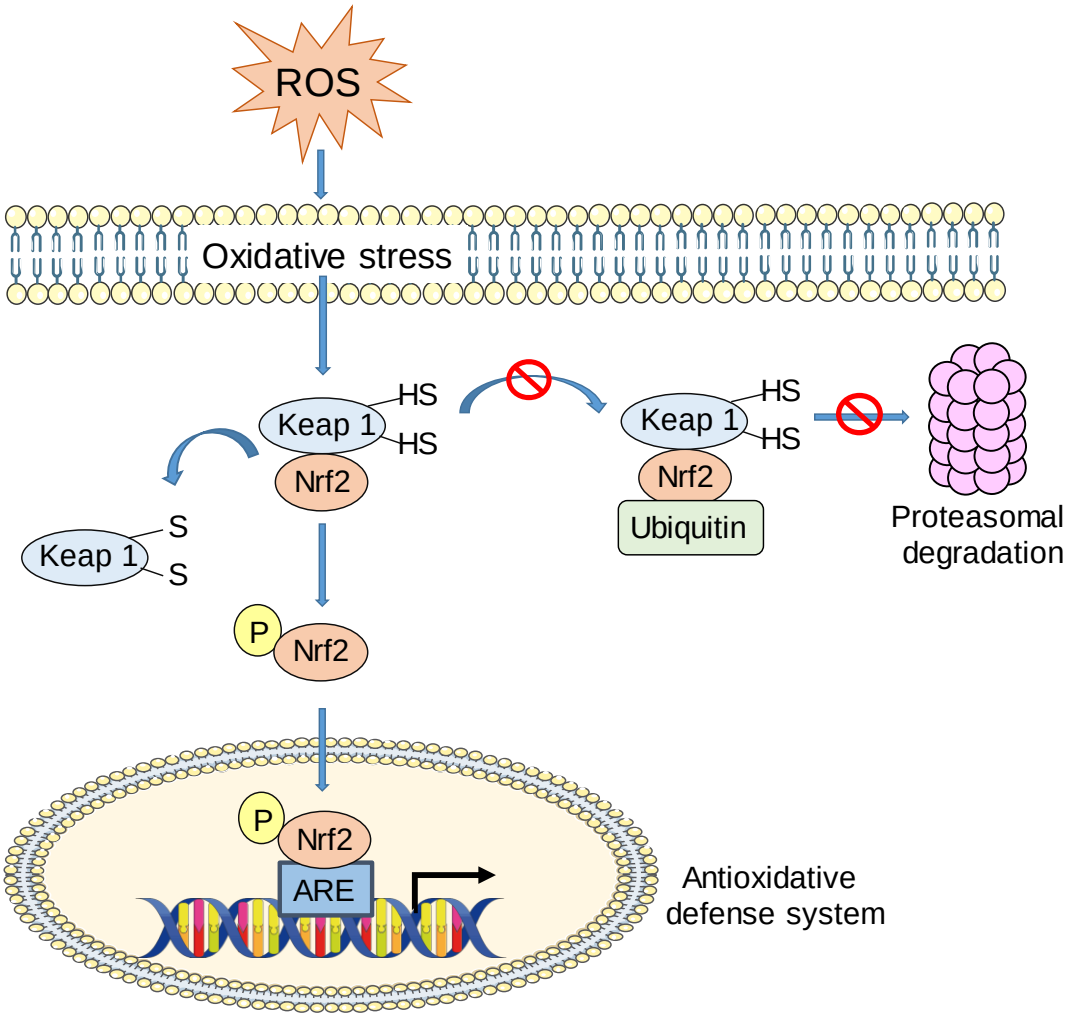

Figure 6: The nuclear factor erythroid-2-related factor-2 (Nrf2) signaling pathway, which is the central antioxidant defense system in cells. Normally, Nrf2 interact with Kelch-like ECH associating protein 1 (Keap-1), leading to the ubiquitination of Nrf2 and its proteasomal degradation. Reactive oxygen species can oxidize Keap-1, leading to increased Nrf2 stability. Upon release and activation, phospho-Nrf2 can translocate to the nucleus and bind to and activate the antioxidant response element (ARE) and regulate the expression of target genes. This figure is based on a previous study (Aspera-Werz *et al.*, 2018).

1.12 Antioxidant – Treatments

In physiological conditions, the cellular antioxidant system may scavenge free radicals and maintain the balance between oxidation and antioxidation. However, exposure to cigarette smoke induces the production of non-physiological ROS levels, and the cells' antioxidative system cannot compensate with this change. Therefore, the intake of exogenous antioxidants should reduce the harm caused by oxidative stress through their radical scavenger properties as well as regulation of the cell antioxidative system The **natural antioxidants** found in cells are **thiol compounds** – the most important and abundant of which in humans is GSH – non-thiol compounds such as **polyphenols** – predominantly contained in various plants (*e.g.* resveratrol) – **vitamins** such as

ascorbic acid, as well as various **antioxidant enzymes** capable of eliminating ROS, such as SOD, CAT, and enzymes that regulate GSH homeostasis (*e.g.* glutathione reductase [GR], GPX, etc.) (Domazetovic *et al*., 2017). Figure 7 shows a schematic view of the cell defense system with the respective reactions and enzymes involved.

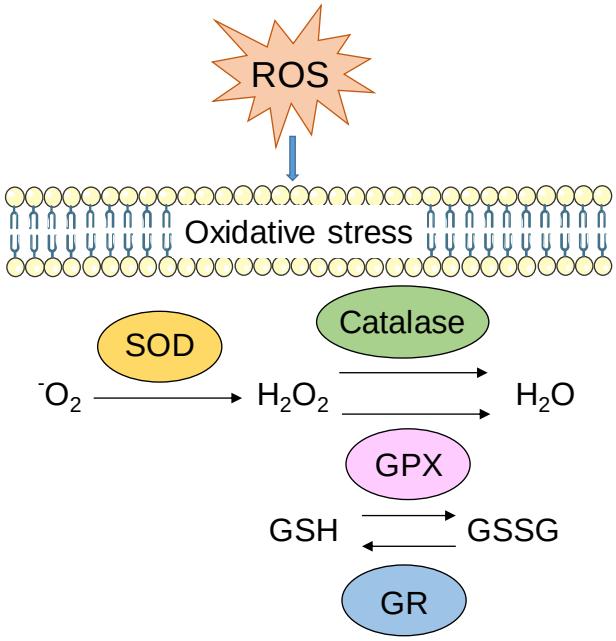

Figure 7: The cellular antioxidant cell defense system. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radical ($O_2^{\cdot-}$) into oxygen and hydrogen peroxide (H_2O_2). H_2O_2 is reduced by catalase to water and O_2, completing the detoxification process initiated by SOD. Additionally, glutathione peroxidase (GPx) can catalyze the degradation of H_2O_2 to H_2O *via* oxidation of reduced glutathione (GSH) into its oxidized, disulfide form (GSSG). Glutathione reductase catalyzes the reduction of GSSG to the sulfhydryl form, carrying out GSH recycling. This figure is based on a previous study (Aspera-Werz *et al*., 2018).

1.12.1 N- Acetyl Cysteine (NAC): A Precursor for the Master Antioxidants in Human Cells

GSH is the most important and abundant antioxidant in human cells; the concentration is between 1 and 10 mM in the cytoplasm for most cells, and 98% is found in the active reduced-thiol form (Forman *et al*., 2009). The oxidation to glutathione disulfide (GSSG) and downregulation of the **GSH/GSSG ratio positively correlates with oxidative stress** (Owen and Butterfield, 2010). Therefore, GSH biosynthesis, reduction, and exogenous intake are essential to balance the cellular redox status. **NAC** is an exogenous thiol derivative of the amino acid L-cysteine and a **precursor for GSH**. NAC intake increases GSH/GSSG (Aruoma *et al.*, 1989). The molecular structure of NAC is represented in Figure 8.

Additionally, NAC compensates for the harmful effect of H_2O_2 exposure on MC3T3-E1 cells during osteogenic differentiation (Lee *et al*., 2015). Furthermore, an *in vitro* and *in vivo* study demonstrated that NAC enhances AP activity, matrix formation in bone marrow MSCs, and accelerates bone formation in rats (Yamada *et al*., 2013). NAC inhibits adipogenesis of bone marrow stem cells and induces osteogenesis *via* activation of Wnt signaling (Ji *et al.*, 2011). Moreover, NAC favors osteogenic differentiation and enhances bone formation by inhibiting apoptosis and senescence caused by oxidative stress (Jun *et al*., 2008, Watanabe *et al*., 2018). Accordingly, NAC is a potential therapy to protect bone from the deleterious effects of cigarette smoke, enhance bone formation in osteoporosis, or ameliorate delayed fracture healing under oxidative stress conditions.

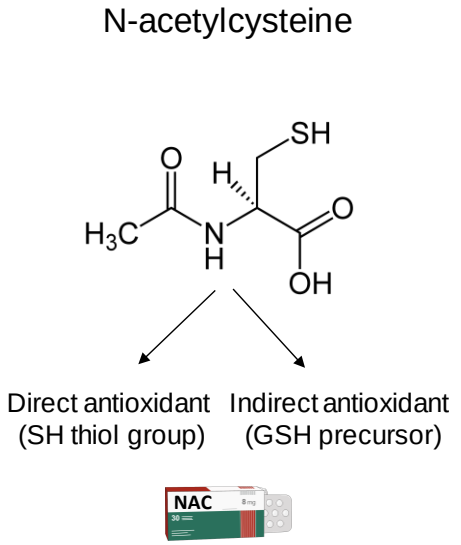

Figure 8: The molecular structure of N-acetyl cysteine (NAC). This figure was produced using graphic components obtained from http://www.wikipedia.de.

1.12.2 L-ascorbate: A Free Radical Scavenger with Low Levels in Smokers

L-ascorbate, also known as vitamin C, is **present in many fruits and vegetables** (*e.g.* broccoli, brussels sprouts, cauliflower, green and red peppers, spinach, cabbage, other leafy greens, sweet and white potatoes, tomatoes, citrus, and berries). L-ascorbate is absorbed in the intestine and distributed through the bloodstream to the whole body, reaching concentrations of 35 mg/kg in the musculoskeletal system (Figueroa-Méndez and Rivas-Arancibia, 2015). **Humans**, unlike most animals and plants**, are unable to synthesize L-ascorbate** endogenously due to the lack of gulonolactone oxidase (an enzyme that catalyzes the final step in the biosynthesis), so

L-ascorbate has to be supplemented *via* nutrition (Naidu, 2003, Linster and Van Schaftingen, 2007, Gabbay *et al*., 2010). The German Nutrition Society (DGE) recommends an intake of 110 mg/day for healthy non-smokers (German Nutrition Society, 2015). Interestingly, **smokers serum L-ascorbate levels are reduced** compared with non-smokers due to higher metabolic requirement and defective L-ascorbate recycling (Kelly, 2003, Smith and Hodges, 1987, Aspera-Werz *et al.*, 2018). Therefore, the DGE recommends a daily intake of 155 mg for smokers to reach similar L-ascorbate blood levels as non-smokers (Schectman, 1993, Smith and Hodges, 1987). Besides, its **antioxidants properties**, as a free radical scavenger, L-ascorbate **stimulates osteoblast proliferation** (Alcain and Buron, 1994, Takamizawa *et al*., 2004) and **supports bone and cartilage formation** *via* contribution to collagen production and maturation (Ganta *et al*., 1997). Additionally, L-ascorbate is a precursor for the synthesis of bone glycosaminoglycans (Kao *et al.*, 1990). Therefore, low L-ascorbate intake slows bone growth and bone remodeling, causing delays in fracture healing and osteoporosis (Malmir *et al.*, 2018). Interestingly, several prospective studies have shown a positive correlation between L-ascorbate intake and increased BMD in post-menopausal women as well as reduced bone mineral loss in older men (Morton *et al.*, 2001, Sahni *et al.*, 2008). Furthermore, lower L-ascorbate levels in the blood are associated with an increased risk of osteoporosis and fractures (Martinez-Ramirez *et al*., 2007). Melhus *et al.* reported that insufficient L-ascorbate intake increases the risk of hip fracture in smokers. (Melhus *et al.*, 1999). *In vitro* experiments have shown that L-ascorbate upregulates the expression of osteogenic genes such as *AP*, osteocalcin, *OPN*, and the master transcriptional factor for osteogenesis runt-related transcription factor 2 (*RUNX2*); it also increases matrix formation (Franceschi *et al.*, 1994, Franceschi and Iyer, 1992, Xiao *et al*., 1997). Furthermore, a study with ovariectomised rats (lack of estrogen, a model of osteoporosis (Kharode *et al.*, 2008)) treatment with L-ascorbate prevents bone loss, increases BMD, and upregulates gene expression of osteoblast markers (*RUNX2*, osterix, bone sialoprotein, and *BMP-2*) (Zhu *et al.*, 2012). A murine model deficient in L-ascorbate biosynthesis also displays high levels of RANKL that may be regulated with L-ascorbate supplementation (Park *et al*., 2012). Therefore, **L-ascorbate seems to have a dual role in bone homeostasis**. On the one hand, due to its free radical scavenger properties, it **reduces the number of functional osteoclasts**. On the other hand, L-ascorbate **stimulates MSC proliferation and osteogenic differentiation** (Choi *et al.*, 2019).

Therefore, under smoking conditions where there is increased oxidative stress that disrupts normal bone homeostasis and favors bone resorption, L-ascorbate may represent a promising therapy to improve bone formation and support fracture healing. The molecular structure of L-ascorbate is shown in Figure 9.

L- ascorbate

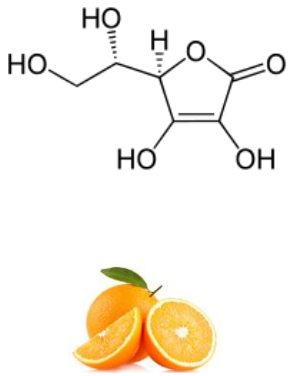

Figure 9: The molecular structure of L-ascorbate. This figure was produced using graphic components obtained from http://www.wikipedia.de.

1.12.3 Resveratrol: An Antioxidant with Beneficial Effects on Bone Homeostasis

Resveratrol is a natural **polyphenol and stilbenoid** that has potent **antioxidant and anti-inflammatory properties** (Alarcon De La Lastra and Villegas, 2005, Gülçin, 2010). More than 70 species of plants synthesize it in response to stressful conditions (*e.g.* pathogen infection, damage, or injury). Resveratrol may be **obtained by the ingestion of several fruits**, grapes, peanuts, nuts, pistachios, cocoa, berries, giant knotweed, cassia seeds, passion fruit, white tea, and red wine. In addition to its scavenger properties, several studies have demonstrated the beneficial effects of resveratrol on bone homeostasis (Casarin *et al.*, 2014, Tou, 2015). An *in vivo* and *in vitro* study revealed that in a murine osteoporosis model induced with excess iron, resveratrol intake upregulates the expression of osteogenic genes such as *Runx2*, *osteocalcin*, and *type I collage*n and as a consequence prevents bone loss. Additionally, resveratrol decreases the RANKL/OPG ratio by inhibiting osteoclast function in MC3T3-E1 cells and an osteoporotic mouse model (Zhao *et al.*, 2015).

Resveratrol also promotes osteogenic differentiation of MSCs *via* induction of SIRT1/FoxO1 signaling pathway and upregulation of various *BMPs*, *RUNX2*, and *OPN*

(Shakibaei *et al.*, 2012, Casarin *et al.*, 2014). Furthermore, a randomized, placebo-controlled clinical study with overweight patient showed a positive correlation between increased AP activity, BMD, and resveratrol concentration after 4 months of treatment (Ornstrup *et al.*, 2014). Regarding smoking conditions, several studies in animal models have revealed that resveratrol inhibits periodontitis bone loss (Ribeiro *et al.*, 2017), enhances bone formation after mechanical bone damage, and promotes titan implant healing (Franck *et al*., 2018, Ribeiro *et al.*, 2019) exposure to smoking conditions. These results suggest that **resveratrol influences bone homeostasis by stimulation of osteoblast and inhibition of osteoclast function**. Given these actions, resveratrol may be a potential treatment strategy to impair bone remodelling in smokers (Jiang *et al*., 2020). Figure 10 presents the molecular structure of resveratrol.

Resveratrol

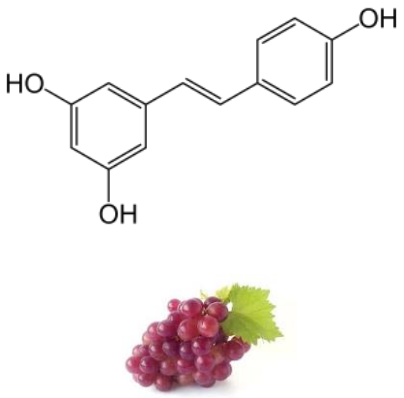

Figure 10: The molecular structure of resveratrol. This figure was produced using graphic components obtained from http://www.wikipedia.de.

2. Aim

This thesis aimed to evaluate the role of cigarette smoke–induced oxidative stress on MSCs under osteogenic differentiation, specifically with regard to primary cilia–associated signaling pathways and its relation with delayed fracture healing and impaired bone homeostasis. This aim was achieved by addressing the following points:

- Identify the correlation between age, gender, body mass index, complications, and duration of hospital stay with the number of cigarettes consumed in patients who underwent orthopedic surgery;

- Investigate the underlying mechanism for delayed fracture healing in orthopedic patients who smoke, through screening blood serum levels of bone remodeling, osteoblast/osteoclast function, oxidative stress markers, and cytokines in smoker and non-smoker patients receiving an orthopedic surgery;

- Evaluate the effect of cigarette smoke, nicotine, and cotinine on MSCs during osteogenic differentiation with regard to the primary cilia structure;

- Determine which type of ROS are induced by cigarette smoke, nicotine, or cotinine, and their implications on the antioxidative system of MSCs during osteogenic differentiation;

- Assess the effect of antioxidants on impaired osteogenic differentiation of MSCs caused by cigarette smoke and the outcome in the restoration of primary cilia structure and signaling efficiency;

- Determine at which step TGF-β signaling is disrupted by cigarette smoke and its implications on migration, proliferation, and appropriate chondrogenic differentiation of MSCs.

These aims were addressed in this thesis in the following publications: Ehnert *et al*., 2019, Sreekumar *et al*., 2018, Aspera-Werz *et al.*, 2018 and Aspera-Werz *et al.*, 2019.

3. Results

3.1 Smoking Dependent Alterations in Bone Formation and Inflammation Represent Major Risk Factors for Complications Following Total Joint Arthroplasty

Ehnert, S.; **Aspera-Werz, R.**; Ihle, C.; Trost, M.; Zirn, B.; Flesch, I.; Schroter, S.; Relja, B.; Nussler, A. *J Clin Med*, 2019.

3.1.1 Summary and Major Findings

Since 1976, several studies have reported a direct association between conventional cigarette consumption and reduced amount of bone tissue in the body (Daniell, 1976, Benson and Shulman, 2005, Rudang *et al.*, 2012). Moreover, smokers present a raised hazard of suffering delayed fracture healing (Adams *et al*., 2001, Sloan *et al.*, 2010), non-union (Scolaro *et al*., 2014, Pearson *et al.*, 2016), or complications (Mills *et al.*, 2011, Singh *et al*., 2015, Hess *et al.*, 2018) after orthopedic trauma. Cigarette smoke may affect bone homeostasis either directly through harmful effects on bone-forming and bone-resorbing cells or indirectly by altering hormone levels, disrupting angiogenesis, perturbing immune responses, or inducing hypoxia (Barreiro *et al*., 2010, Qiu *et al*., 2017, Church and Pryor, 1985, Patel *et al*., 2013, Staempfli and Anderson, 2009, Ejaz and Lim, 2005).

The following manuscript aimed to corroborate that cigarette consumption is a significant hazard for adverse outcomes in orthopedic trauma patients. From 817 patients that received a total joint replacement, we observed that patients who received a revision surgery consumed more cigarettes than patients who did not need the surgery. Interestingly, there was a 2-fold increase in complications for patients who were heavy smokers (>20 packs-year). However, there were no differences between non-smokers and moderate smokers (1–20 packs-year) regarding complications. Additionally, heavy smokers stayed 0.6 or 3.1 days longer in the hospital compared with moderate smokers or non-smokers, respectively. Although heavy and moderate smokers were on average 5.4 years youngers compared with non-smokers, the number of comorbidities was similar in all groups (Ehnert *et al*., 2019).

In order to investigate the cellular mechanisms responsible for complications and, consequently, delayed fracture healing in heavy smokers, we analyzed markers for bone remodeling, oxidative stress, and inflammation in serum samples before and after surgery. There was a positive correlation between an increasing number of pack-years and increased cytokine levels, specifically leptin (inhibits appetite), monocyte chemoattractant protein 1 (MCP-1) (oxidative stress marker), OPN (supports osteoclast anchoring), and tissue inhibitors of metalloproteinases. Nevertheless, there was a dose-dependent decrease in the levels of several cytokines – IL-6, IL-1β, interferon γ (pro-inflammatory), TGF-β1 (immune regulatory chemoattractant), and OPG (inhibitor osteoclastogenesis) – as the number of cigarettes consumed increased. Bone-specific AP was upregulated 2.08 fold in non-smokers compared with heavy smokers as well as 1.65 fold compared with moderate smokers. Additionally, the bone formation marker type I C-terminal collagen pro-peptide (an indicator of collagen I synthesis) was downregulated 35.5 fold in heavy smokers and 7.8 fold in moderate smokers compared with non-smokers (Ehnert *et al*., 2019).

Conversely, the osteoclast marker TRAP, as well as the level of the bone resorption marker C-terminal telo-peptide of type I collagen (an indicator of collagen I degradation), showed no correlation with the number of cigarettes consumed. However, OPN expression was on average 5.9 fold higher in serum samples from heavy and moderate smokers compared with non-smokers. Interestingly, OPG:receptor activator of RANKL (an important determinant of bone mass and skeletal integrity) ratio was not disturbed in smokers because both cytokines increased with the number of cigarettes consumed (Ehnert *et al.*, 2019).

These results demonstrated that cigarette consumption increased the risk of complications after orthopedic surgeries. Additionally, the rate of revision surgery for smokers was significantly higher. The serum findings suggest that an immunosuppressive status in smokers may be correlated with increased complications at an early age in comparison with non-smokers. Moreover, orthopedic patients who smoke have an osteoblast–osteoclast imbalance due to strong harmful effects on bone-forming cells and a mild increase in bone-resorbing cells function that might be correlated with increased oxidative stress (Ehnert *et al*., 2019). Therefore, *in vitro* studies that evaluate alternatives to increase the function of bone-forming cells as well as reduce oxidative stress and reverse immunosuppression are necessary to

determine therapies to enhance the outcome in smokers after trauma or orthopedic surgical procedure.

3.1.2 Personal Contribution

I assisted with performing the experiments and critically revised the manuscript draft.

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Smoking Dependent Alterations in Bone Formation and Inflammation Represent Major Risk Factors for Complications Following Total Joint Arthroplasty

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Abstract: Numerous studies have described a correlation between smoking and reduced bone mass. This not only increases fracture risk but also impedes reconstruction/fixation of bone. An increased frequency of complications following surgery is common. Here, we investigate the effect of smoking on the clinical outcome following total joint arthroplasty (TJA). 817 patients receiving primary or revision (including clinical transfers) TJA at our level-one trauma center have been randomly interviewed twice (pre- and six months post-surgery). We found that 159 patients developed complications (infections, disturbed healing, revisions, thrombosis, and/or death). Considering nutritional status, alcohol and cigarette consumption as possible risk factors, OR was highest for smoking. Notably, mean age was significantly lower in smokers (59.2 ± 1.0a) than non-smokers (64.6 ± 0.8; $p < 0.001$). However, the number of comorbidities was comparable between both groups. Compared to non-smokers (17.8 ± 1.9%), the complication rate increases with increasing cigarette consumption (1–20 pack-years (PY): 19.2 ± 2.4% and >20 PY: 30.4 ± 3.6%; $p = 0.002$). Consequently, mean hospital stay was longer in heavy smokers (18.4 ± 1.0 day) than non-smokers (15.3 ± 0.5 day; $p = 0.009$) or moderate smokers (15.9 ± 0.6 day). In line with delayed healing, bone formation markers (BAP and CICP) were significantly lower in smokers than non-smokers 2 days following TJA. Although, smoking increased serum levels of MCP-1, OPG, sRANKL, and Osteopontin as well as bone resorption markers (TRAP5b and CTX-I) were unaffected. In line with an increased infection rate, smoking reduced 25OH vitamin D3 (immune-modulatory), IL-1β, IL-6, TNF-α, and IFN-γ serum levels. Our data clearly show that smoking not only affects bone formation after TJA but also suppresses the inflammatory response in these patients. Thus, it is feasible that therapies favoring bone formation and immune responses help improve the clinical outcome in smokers following TJA.

Keywords: total joint arthroplasty (TJA); cigarettes; smoking; pack-years (PY); complications; infection; delayed wound healing; revision surgery; bone metabolism

1. Introduction

Smoking indisputably affects human health, e.g. increases the risk of cancers, cardiovascular and respiratory diseases, reproductive problems, and other medical maladies. However, one of the little known effects of smoking is that on injuries. A correlation between cigarette consumption and reduced bone mass was described for the first time in 1976 [1]. This correlation has been confirmed in several epidemiologic studies since then [2,3]. Consequently, smokers seem to be at higher risk for fragility fractures [4,5]. For example, in patients with distal radius fractures, smokers show more frequently post-surgical tenderness, wrist stiffness, non-union, and revision surgeries as compared to non-smokers [6]. Similarly, Pearson et al. reported not only a significant delay in fracture healing, but also a two-fold increase in the risk for developing a non-union after fracture, spinal fusion, osteotomy or arthrodesis in smokers [7]. In orthopedic surgeries, smokers frequently show delayed fracture healing, higher frequency of complications, and prolonged hospital stays as compared to non-smokers [8,9]. This might be partly due to alterations in bone density and structure, which in turn impede reconstruction and fixation of the bone. However, the underlying mechanisms affecting bone quality are not yet fully understood.

Bone metabolism in smokers can be affected either directly via toxic effects on the bone cells or indirectly via changes in hormone status, vasculature, immune responses, or oxygenation [8,10]. This in turn may compromise wound and fracture healing. Most research has been done on bone loss in the jaw of smokers with periodontitis. In these patients, serum levels of osteoprotegerin (OPG) are reduced while serum levels of sRANKL (soluble receptor activator of nuclear factor kappa-B ligand) are unchanged, proposing an increased bone turnover [11–17]. However, it is questionable if these observations can be translated to skeletal bone. In the oral cavity, effects on bone are not only mediated by factors distributed via the blood stream (as with most sites of skeleton) but may, to a large part, be mediated by direct toxic effects following contact with the cigarette smoke. Osteogenic differentiation of bone marrow-derived mesenchymal stem cells, for example, is inhibited when exposed to cigarette smoke extract [18,19]. Furthermore, primary human osteoblast viability is strongly affected when exposed to cigarette smoke extract [20]. Oxidative stress, which is well accepted to be increased in smokers, seems to be a key regulator for this.

Oxidative stress may cause increased synthesis of monocyte chemoattractant protein 1 (MCP-1), also known as C-C motif chemokine 2 (CCL2), an important chemotactic factor for monocytes and macrophages [21]. It has been shown that MCP-1 is instrumental in favoring the formation of osteoclasts [22–25]. There are reports, showing that MCP-1 may favor osteoclast fusion and osteoclastogenesis both in an autocrine and paracrine manner [23]. Expression of MCP-1 by osteoclasts is proposed to be regulated by sRANKL [24]. Similarly, 25 hydroxy vitamin D_3 ($25(OH)D_3$) and its metabolite 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) were identified as key regulators of MCP-1, OPG, and sRANKL in this process [26,27]. However, regulation of the individual genes is different: MCP-1 expression is reported to be reduced by $25(OH)D_3$ [28], and sRANKL expression is reported to be increased by $25(OH)D_3$ [27]. Paradoxically, supplementation with vitamin D_3 improves bone mineral density in patients via inhibition of osteoclasts [26,29]. Both insufficient uptake or synthesis of vitamin D and insufficient processing are supposed to decrease $25(OH)D_3$ and $1,25(OH)_2D_3$ serum levels in smokers [30], which in turn might favor bone resorption [31]. However, $25(OH)D_3$ is also known as a potent regulator of immune responses [32].

In the oral cavity of smokers (with and without periodontitis), inflammatory markers, e.g. IL-1β, IL-6, or TNF-α, are frequently increased [33,34]. This is in clear contrast to reports describing a general immune suppression in smokers [35–37], affecting their ability to fight infections. Indeed, epidemiologic studies suggest that smokers are at higher risk for developing infections following injuries [30]. Besides the ability to fight infections, an ongoing inflammation response in the early fracture hematoma is required to induce fracture healing [38].

Although it is well described that smoking increases the risk for fractures and complications during the following healing process, little is known about the possible underlying mechanisms and

thus specific treatment options. Despite great progress in implant and surgical technologies, handling of smokers during and after orthopedic and trauma surgeries remains a huge challenge. Therefore, we wanted to first confirm that smoking is a major risk factor for complications (infections, disturbed wound healing, required revision surgery, thrombosis, and/or death) up to six months following surgery in our study cohort comprised of 817 both in-house and transfer patients that received a primary or revision total joint arthroplasty (TJA). Then, we conducted a closer investigation of the study cohort regarding age, gender, BMI, complications, and duration of hospital stay, with respect to the amount of cigarettes consumed. In order to identify possible underlying mechanisms for delayed bone healing and infections, we measured blood serum markers for bone formation and resorption, oxidative stress, and inflammation two days post-surgery in non-, moderate, and heavy smokers. Based on the available literature, we hypothesize a shift in bone metabolism in smokers, characterized by a decrease in bone formation markers, as well as an increase in oxidative stress and osteoclast markers. Furthermore, a decreased inflammatory response following surgery is expected based on the proposed higher complication rate in smokers.

2. Experimental Section

2.1. Ethics Statement

The study, including patient material, was performed in accordance with the Declaration of Helsinki (1964) in its latest amendment. Patient survey and collection of the clinically relevant data was performed in accordance with the ethical vote 193/2014BO2. Additional blood sampling was performed during a routine blood sampling (ethical vote 538/2016BO2). All study participants have signed a written informed consent.

2.2. Patient Recruitment and Survey

Study participants were recruited between June 2014 and January 2018. A consecutive series of patients at our arthroplasty center of maximum care as well as patients that were transferred to our septic surgery department from other hospitals because of complications following TJA of the hip or knee have been included. Patients who were hospitalized for two or more nights and agreed to participate in the presented study were included. Patients suffering from dementia, patients with insufficient knowledge of the study language, and patients who could not answer the questions because of severe health conditions were excluded. All patients were screened at hospital admission during a bedside interview and in telephone interviews in the following six months. To avoid observer-dependent bias, all observers were trained for two weeks. General patient data and nutritional status as well as alcohol and cigarette consumption were detected. After discharge (in general, 12–14 days after surgery and initial mobilization with the in-house physiotherapist), complications were captured out of the hospital information system. As described in a prior publication of our working group, complications were defined as death, infections, wound healing disorders, further operations, and thrombosis [39,40]. All adverse events were weighted equally and assessed during hospitalization as well as six months post-surgery.

2.3. Blood Sampling

A 10 ml blood sample (5 ml serum and 5 ml EDTA) was obtained during a routine blood sampling. Blood samples were centrifuged at 1000 *g* for 10 min at room temperature within a time frame of 30 min after sampling. Resulting serum and plasma samples were stored in aliquots at −80 °C until further use.

2.4. Cytokine Array

To determine relative serum levels of cytokines, a RayBio® Human Cytokine Array C5 (BioCat, Heidelberg, Germany) was performed according to the manufacturer's instructions. For the signal

development, the dot blot membranes were incubated for 1 min with ECL substrate solution. Chemiluminescent signals were detected with a CCD camera (INTAS, Göttingen, Germany). Signal intensities were measured using the ImageJ software (NIH, Bethesda, MA, USA). On each array membrane, the 6 spots (positive controls) were used for normalization.

2.5. Enzyme Linked Immunosorbent Assay (ELISA)

Target proteins in serum samples were quantified with the help of ELISA kits, performed as indicated by the manufacturer. An overview is given in Table 1.

Table 1. Overview on the performed enzyme linked immunosorbent assays (ELISAs).

Target	Function	ELISA Kit		Dilution Factor
		Order No.	Company	
25(OH)D_3	25OH vitamin D_3	AC-57DF1	IDS	-
BAP	Osteoblast activity	AC-20F1	IDS	-
TRAP5b	Osteoclast activity	SB-TR201A	IDS	-
CTX-I	Bone resorption	AC-57SF1	IDS	3
CICP	Collagen synthesis	8003	TecoMedical	12.5
MCP-1	Stress marker	900-K31	Peprotech	20
sRANKL	Favors osteoclastogenesis	900-K142	Peprotech	20
TIMP-1	Inhibitor for MMPs	900-M438	Peprotech	20
IL-1β	Inflammatory marker	900-K95	Peprotech	10
IL-6	Inflammatory marker	900-K16	Peprotech	10
TNF-α	Inflammatory marker	900-K25	Peprotech	10
IFN-γ	Inflammatory marker	900-K27	Peprotech	10
TIMP-2	Inhibitor for MMPs	ELH-TIMP2	BioCat	100
OPG	Inhibitor for sRANKL	ELH-OPG	BioCat	20
OPN	Anchor for osteoclasts	ELH-OPN	BioCat	20

25(OH)D_3: 25 hydroxy vitamin D_3; BAP: bone specific alkaline phosphatase; TRAP5b: tartrate-resistant acidic phosphatase; CTX-I: C-terminal telo-peptide of type I collagen; CICP: type I C-terminal collagen pro-peptide; MCP-1: Monocyte chemoattractant protein-1; sRANKL: soluble receptor activator of nuclear factor kappa-B ligand; TIMP-1 and TIMP-2: tissue inhibitor of metalloproteinases; OPG: Osteoprotegerin; OPN: Osteopontin; IL-1β: interleukin 1 beta; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; IFN-γ: interferon gamma. Order No.: Order number of the company.

2.6. Statistics

Distributions within groups are represented as Venn diagrams, pie charts, or contingency tables. Results are represented either as box blots (Box and Whiskers–Tukey to visualize outliers) or as scatter diagrams (mean ± 95% confidence interval). The number of donors (N) and technical replicates (n) is given in the figure legends. Comparison of multiple groups was done using the Kruskal–Wallis H-test followed by Dunn's multiple comparison test. The Mann–Whitney U-test (2-sided) was used to compare two single groups with each other. Data are summarized as mean ± SEM; 95% confidence interval. Statistical analysis was performed using the GraphPad Prism Software (Version 5, El Camino Real, California, CA, USA). $p < 0.05$ at an $\alpha = 0.05$ was taken as minimum level of significance.

2.7. Data Availability

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

3. Results

3.1. Patient Recruitment and Description of The Study Cohort

In total, 817 patients (359 women and 458 men) receiving TJA (primary or revision), including patients that were transferred to our hospital because of post-surgical complications, were

randomly interviewed for this study. However, 29 patients were lost to follow up because of missing data sets.

Overall, 510 patients received primary TJA and 278 patients received revision TJA. In both groups together, 159 patients developed complications (infections, disturbed wound healing, required revision surgery, thrombosis, and/or death) within six months following surgery (Figure 1A). The complication rate was significantly lower in patients with primary TJA (15.5%; Figure 1B) than in patients with revision TJA (28.8%; OR = 2.204; $p = 0.009$; Figure 1D). As expected, patients with complications stayed significantly longer in hospital (Primary: 24.1 ± 18.9 days; 19.9–28.4 days. Revision: 25.7 ± 16.3 days; 22.1–29.4 days) than patients that did not develop adverse events (Primary: 12.6 ± 5.6 days; 12.1–13.2 days; $p < 0.001$. Revision: 16.6 ± 7.6 days; 15.5 – 17.6 days; $p < 0.001$; Figure 1C,E).

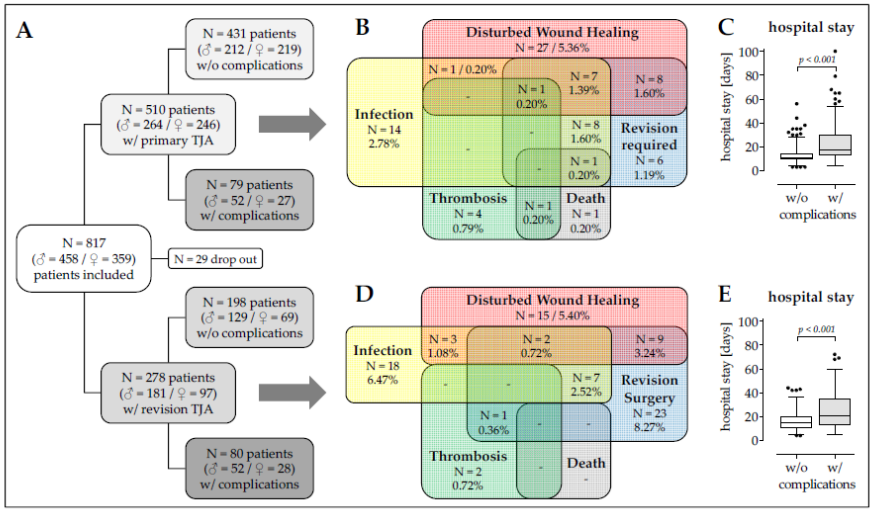

Figure 1. Overview on the study population. (**A**) Flow chart on the patient recruitment: Between June 2016 and January 2018, a total of 817 patients with total joint arthroplasties (TJA) were interviewed for our study. However, 29 patients had to be excluded from the study because of missing data sets. Of the remaining 788 patients, 510 (62.4%) received primary TJA and 284 (37.6%) had a revision TJA. Of the patients with primary TJA, 431 (84.5%) had no complications and 79 (15.5%) had complications up to six months following surgery. Of the patients with revision TJA, 189 (71.2%) had no complications and 80 (28.8%) had complications up to six months following surgery. Venn diagrams on the complications, with the number in patients affected (N) and the relative occurrence (in %) with regard to (**B**) the patients with primary TJA and (**D**) the patients with revision TJA. Duration of hospital stay (in days) of the study participants with (**C**) primary TJA and (**E**) revision TJA.

Differentiating between primary and revision TJA, the mean age was comparable between the group that developed complications (59.9 ± 14.7a) and the group that did not develop complications (Primary: Δ_{mean} 2.3a; $p = 0.106$. Revision: Δ_{mean} 0.4a; $p = 0.437$). When considering primary TJA, there were more male patients in the group with complications (65.8%) than in the group without complications (49.2%; $p = 0.022$). Interestingly, this imbalance in gender distribution was not present in patients receiving revision TJA. For both primary and revision TJA, BMI was comparable between patients developing or not complications (Primary: Δ_{mean} 0.9 kg/m^2; $p = 0.263$. Revision: Δ_{mean} 0.8 kg/m^2; $p = 0.809$). Similarly, the number of comorbidities (primary: Δ_{mean} 0.39; $p = 0.821$/revision: Δ_{mean} 0.29; $p = 0.574$) and medication (Primary: Δ_{mean} 0.69; $p = 0.844$. Revision: Δ_{mean} 0.01; $p = 0.795$) were comparable between both groups. Interestingly, while in patients receiving primary TJA, the frequency of malaise (nausea and/or vomitus) prior surgery only trends to be higher in the group that developed complications (17.7%) when compared with the group that did not develop

adverse events (9.3%; OR = 2.220; $p = 0.097$), this effect was highly significant in patients receiving revision TJA (8.6% *vs.* 31.3%; OR = 4.543; $p < 0.001$). For overview, see Table 2.

Table 2. Overview on the study population.

			w/o Complications	w/ Complications	All Patients	*p*-Value
Primary Athroplasties		Age (a)	63.1 ± 14.9 (61.7–64.5)	60.8 ± 14.3 (57.5–64.0)	62.7 ± 14.8 (61.5–64.0)	0.106
	Gender distribution	Male	49.2% (*N* = 212)	65.8% (*N* = 52)	65.4% (*N* = 264)	0.022
		Female	50.8% (*N* = 219)	34.2% (*N* = 27)	34.6% (*N* = 246)	
		BMI (kg/m^2)	28.1 ± 5.1 (27.7–28.6)	29.0 ± 5.8 (27.7–30.3)	28.3 ± 5.2 (27.8–28.7)	0.263
		Number of comorbidities	3.47 ± 2.65 (3.22–3.73)	3.86 ± 3.49 (3.08–4.64)	3.54 ± 2.80 (3.29–3.78)	0.821
		Number of drugs	3.50 ± 3.25 (3.19–3.81)	4.19 ± 4.66 (3.15–5.23)	3.61 ± 3.51 (3.30–3.92)	0.844
		Frequency of malaise (%)	9.3% (*N* = 40)	17.7% (*N* = 14)	10.6% (*N* = 54)	0.097
Revision Athroplasties		Age (a)	60.3 ± 16.7 (58.0–62.7)	59.1 ± 15.2 (55.8–62.5)	60.0 ± 16.3 (58.1–61.9)	0.437
	Gender distribution	Male	65.2% (*N* = 129)	65.0% (*N* = 52)	65.1% (*N* = 181)	1.000
		Female	34.8% (*N* = 69)	35.0% (*N* = 28)	34.9% (*N* = 97)	
		BMI (kg/m^2)	28.2 ± 5.8 (27.4–29.1)	29.0 ± 6.8 (27.5–30.5)	28.5 ± 6.1 (27.7–29.2)	0.809
		Number of comorbidities	3.34 ± 2.87 (3.94–3.74)	3.63 ± 3.12 (2.93–4.32)	3.42 ± 2.94 (3.07–3.77)	0.574
		Number of drugs	3.55 ± 3.39 (3.08–4.03)	3.56 ± 3.62 (2.75–4.37)	3.56 ± 3.45 (3.14–3.97)	0.795
		Frequency of malaise (%)	8.6% (*N* = 17)	31.3% (*N* = 25)	15.1% (*N* = 42)	<0.001

3.2. Higher Frequency of Malnourished Patients and Smokers in The Complication Group

The risk for malnutrition was determined with the help of the nutritional risk screening 2002 questionnaire (NRS) [40]. Patients who developed complications following primary TJA scored significantly higher (2.03 ± 1.04; 1.78–2.26; $p = 0.003$) than patients who did not develop complications (1.65 ± 0.93; 1.56–1.74; Figure 2A). There was a higher frequency of patients (primary TJA) at risk for malnutrition (NRS ≥ 3) in the group developing complications, resulting in an OR of 2.315 ($p = 0.026$). Interestingly, the observed difference in NRS score (and frequency of malnutrition) was not existent in patients with revision TJA (1.90 ± 0.99; 1.76–2.04 vs. 1.91 ± 0.98; 1.69–2.13), which already had a higher NRS in the group without complications (Figure 2D). In both primary and revision TJA, the rate of daily alcohol consumption and alcohol abuse is higher in the group with complications than in the group without complications, without a marked difference between patients receiving primary or revision TJA (Figure 2B,E). More pronounced was the difference in smoking behavior. There was a clear difference between the patients receiving primary and revision TJA observed. Overall, patients who received a primary TJA smoked less than patients who received a revision TJA. In this group, patients who did not develope a complication had comparable smoking behavior to patients receiving a primary TJA but developed a complication. Overall, the proportion of non-smokers was lower in the group without complications. The proportion of moderate smokers (1–20 pack-years [PY]) was comparable between all four groups investigated. The rate of heavy smokers was almost twice as high in the group with complications as in the group without complications (Figure 2C,F). In line with this, the determined odds ratios increase with increasing PY. For primary TJA: (i) >0 PY: OR = 1.601, (ii) >10 PY: OR = 1.624, and (iii) >20 PY: OR = 1.875; $p = 0.034$. For revision TJA: (i) >0 PY: OR = 1.453, (ii) >10 PY: OR = 1.527, and (iii) >20 PY: OR = 2.062; $p = 0.015$. Interestingly, there was no significant difference in the rate of active smokers to former smokers between the four groups investigated.

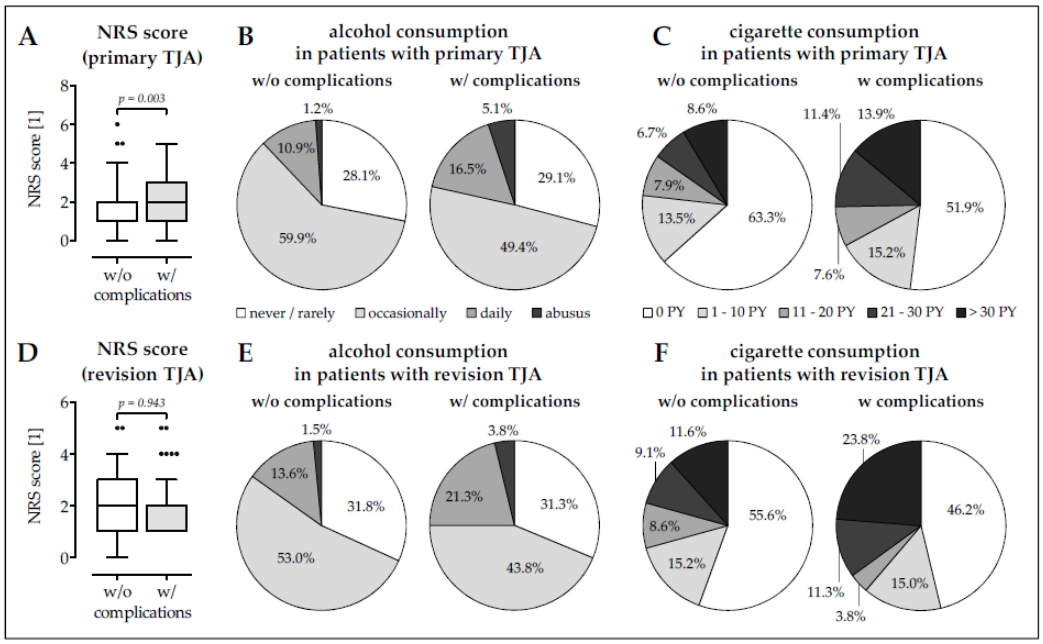

Figure 2. Nutritional status and alcohol and cigarette consumption in patients receiving (**A**–**C**) primary TJA or (**D**–**F**) revision TJA. (**A**,**D**) Nutritional status was obtained with the help of the nutrition risk screening 2002, which defines a nutritional risk for obtained scores ≥3. The data are presented as box blot Tukey to mark ouliers (*). (**B**,**E**) Pie diagrams showing the alcohol consumption in the study population. Alcohol consumption was defined as never/rarely, occasionally, daily (one glass of wine or beer), and abuse (daily more than one glass of wine, beer and/or hard liquor). (**C**,**F**) Pie diagrams showing the smoking behavior of the study population. Cigarette consumption was measured in pack-years (PY), with the number of PY being ((number of cigarettes smoked per day/20) × (number of years smoked)).

3.3. Smokers Have More Complications at a Younger Age

We investigated the effect of smoking in the clinical outcome in our patient cohort. Compared to non-smokers (17.3 ± 1.9%; 13.5–21.1%), the complication rate was not significantly increased in moderate smokers (1–20 PY: 18.2 ± 2.4%; 13.4–23.0%) but almost doubled (31.2 ± 3.7%; 23.8–38.6%; $p \leq 0.004$) in heavy smokers (≥20 PY; Figure 3A). Consequently, the mean hospital stay for heavy smokers was significantly longer (18.4 ± 1.0 days; 16.4–20.4 days) compared to non-smokers (15.3 ± 0.5 days; 14.3–16.4 days; $p = 0.009$) or moderate smokers (15.9 ± 0.6 days; 14.8–17.1 days; Figure 3B). It is noteworthy that the mean age of moderate smokers (59.1 ± 1.0a; 57.2–61.1a; $p < 0.001$) and heavy smokers (59.3 ± 0.9a; 57.2–61.2a; $p < 0.001$) was significantly lower than the mean age of non-smokers (64.6 ± 0.8a; 63.0–66.2a; Figure 3C). Overall, smoking was more prominent in male patients than female patients (69.0% male smokers vs. 43.0% male non-smokers; $p = 0.003$; Figure 3D). Interestingly, the patients' mean BMI was not affected by smoking (Figure 3E). Despite the younger age, the average number of comorbidities was comparable between smokers (3.40 ± 0.15; 3.11–3.70) and non-smokers (3.59 ± 0.14; 3.33–3.86; Figure 3F).

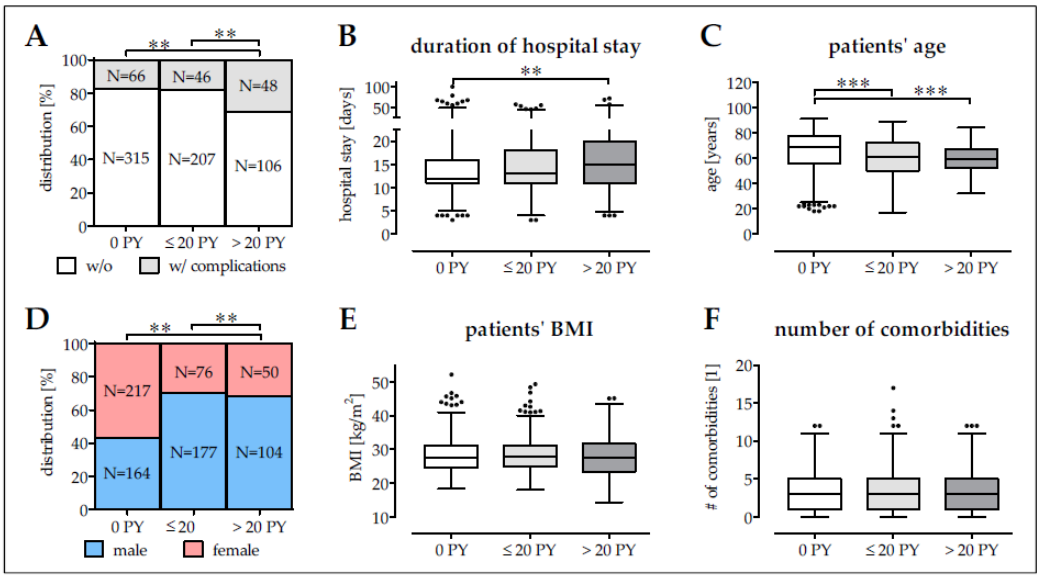

Figure 3. Description of the study population based on the smoking behavior. The analysis differentiated between non-smokers (0 PY; N = 381), moderate smokers (1–20 PY; N = 253) and heavy smokers (>20 PY; N = 154). (**A**) Complication rate is given in % and total numbers (N). (**B**) Time of hospitalization was documented in days. (**C**) Patients' age is given in years. (**D**) Gender distribution within the groups is given in % and total numbers (N). (**E**) Patients' BMI is given in kg/m^2. (**F**) Number of comorbidities. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as indicated.

3.4. Cytokine Levels are Altered in Smokers' Blood

In order get an idea on the possible regulatory mechanisms involved, we screened cytokine levels in the blood (pre-surgical) of patients receiving total joint arthroplasties (five each of non-smokers, light smokers: 1–10 PY, moderate smokers: 11–20 PY, and heavy smokers: >20 PY). Relative cytokine levels were determined with the help of the RayBio®Human Cytokine Array C5. As expected, blood serum levels of the appetite suppressant leptin and the pro-oxidative MCP-1 were increased with increasing number of pack-years. Similarly, blood serum levels of regulators of tissue integrity e.g. OPN (osteopontin favors adherence of osteoclasts) and the tissue inhibitors of metalloproteinases TIMP-1 and -2, were elevated in smokers. On the contrary, cytokine levels of pro-inflammatory IL-6, IL-1β, and IFN-γ were decreased in smokers. Blood serum levels of the immune regulatory TGF-β1 also decreased with increasing number of pack-years. Osteoprotegerin (OPG), the soluble decoy receptor of receptor activator of nuclear factor kappa-B ligand (sRANKL), was also decreased in smokers. These effects were more pronounced the more the patients smoked (Figure 4), suggesting that smokers have an imbalance in osteoblast-osteoclast function and a suppressed immune response.

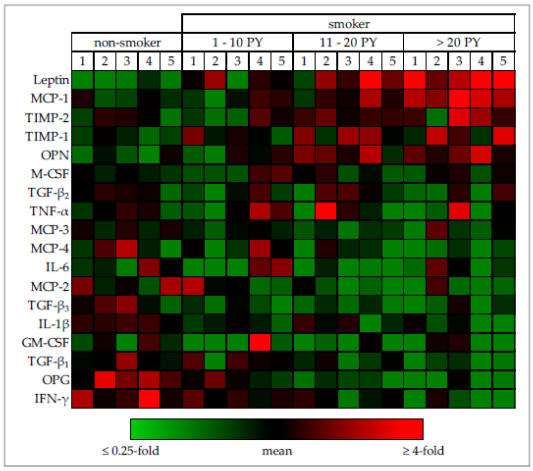

Figure 4. Effect of smoking on blood circulating factors. Relative cytokine levels in (pre-surgical) serum samples from non-smokers (0 PY), light smokers (1–10 PY), moderate smokers (11–20 PY) and heavy smokers (>20 PY) were determined with the help of the RayBio®Human Cytokine Array C5 (N = 5 per group). For the heat map, signal intensities were first normalized to the internal (positive) control followed by a second normalization to the mean signal intensity of each cytokine. Under-represented cytokines are colored green; over-represented cytokines are colored red.

3.5. Decreased Osteoblast Activity in Smokers Following Surgery

We first quantified blood serum levels of osteoblasts and osteoclast markers in our patients two days following surgery. Blood serum levels of bone-specific alkaline phosphatase (BAP) were significantly higher in non-smokers (6.10 ± 0.32 µg/L; 5.44–6.76 µg/L) than smokers, with heavy smokers showing lower BAP levels (4.04 ± 0.27 µg/L; 3.47–4.59 µg/L; $p < 0.001$) than moderate smokers (4.45 ± 0.22 µg/L; 3.99–4.90 µg/L; $p = 0.003$; Figure 5A). In line with this, blood serum levels of the bone formation marker type I C-terminal collagen pro-peptide (CICP) decreased with increasing number of pack-years (127.9 ± 6.0 ng/mL; 115.4–140.3 ng/mL vs. 120.0 ± 5.9 ng/mL; 107.8–132.1 ng/mL vs. 92.7 ± 9.7 ng/mL; 72.7–112.8 ng/mL; $p = 0.004$; Figure 5B). Interestingly, blood serum levels of the osteoclast marker tartrate-resistant acidic phosphatase (TRAP5b) and the bone resorption marker C-terminal telo-peptide of type I collagen (CTX-I) were not significantly affected by smoking (Figure 5C,D). Blood serum levels of both sRANKL and its decoy receptor OPG increased with increasing number of pack-years, such that the resulting OPG:sRANKL ratio was not affected by smoking (Figure 5E–G). This is interesting, as its regulator, 25(OH) vitamin D_3, was significantly decreased in blood of smokers (8.3 ± 0.8 nmol/L; 6.6–9.9 nmol/L; $p = 0.013$, and 8.2 ± 0.8 nmol/L; 6.5–9.9 nmol/L, $p = 0.012$) when compared to the blood of non-smokers (12.9 ± 1.2 nmol/L; 10.4–15.4; Figure 5H). It is noteworthy that blood serum levels of OPN, which favors osteoclast adherence, were significantly increased in heavy smokers (10.2 ± 1.8 ng/ml; 6.5–13.8 ng/mL) when compared to non-smokers (4.6 ± 0.7 ng/ml; 3.1–6.0 ng/mL; $p = 0.016$) or moderate smokers (4.0 ± 0.6 ng/mL; 2.8–5.1 ng/mL; $p = 0.002$, Figure 5I). As expected, blood serum levels of MCP-1, a marker increased by oxidative stress, is significantly increased in heavy smokers (3.0 ± 0.3 ng/mL; 2.4–3.6 ng/mL) when compared to non-smokers (2.4 ± 0.2 ng/mL; 2.0–2.7 ng/mL; $p = 0.045$) or moderate smokers (2.5 ± 0.2 ng/mL; 2.2–2.8 ng/mL; $p = 0.026$, Figure 5J). In line with the cytokine array, blood serum levels of TIMP-1 were significantly increased in heavy smokers (23.5 ± 1.0 ng/mL; 21.4–25.6 ng/mL) when compared to non-smokers (17.1 ± 0.8 ng/mL; 15.6–18.7 ng/mL; $p < 0.001$) or moderate smokers (18.9 ± 0.9 ng/mL; 17.0–20.8 ng/mL; $p = 0.002$; Figure 5K). In contrast, blood serum levels of TIMP-2 were significantly decreased in heavy smokers (6.8 ± 0.5 ng/mL; 5.7–7.9 ng/mL) when compared to non-smokers (9.8 ± 0.7 ng/ml; 8.3–11.3 ng/mL; $p = 0.020$) or moderate smokers (7.9 ± 0.3 ng/mL; 7.3–8.4 ng/mL; Figure 5L).

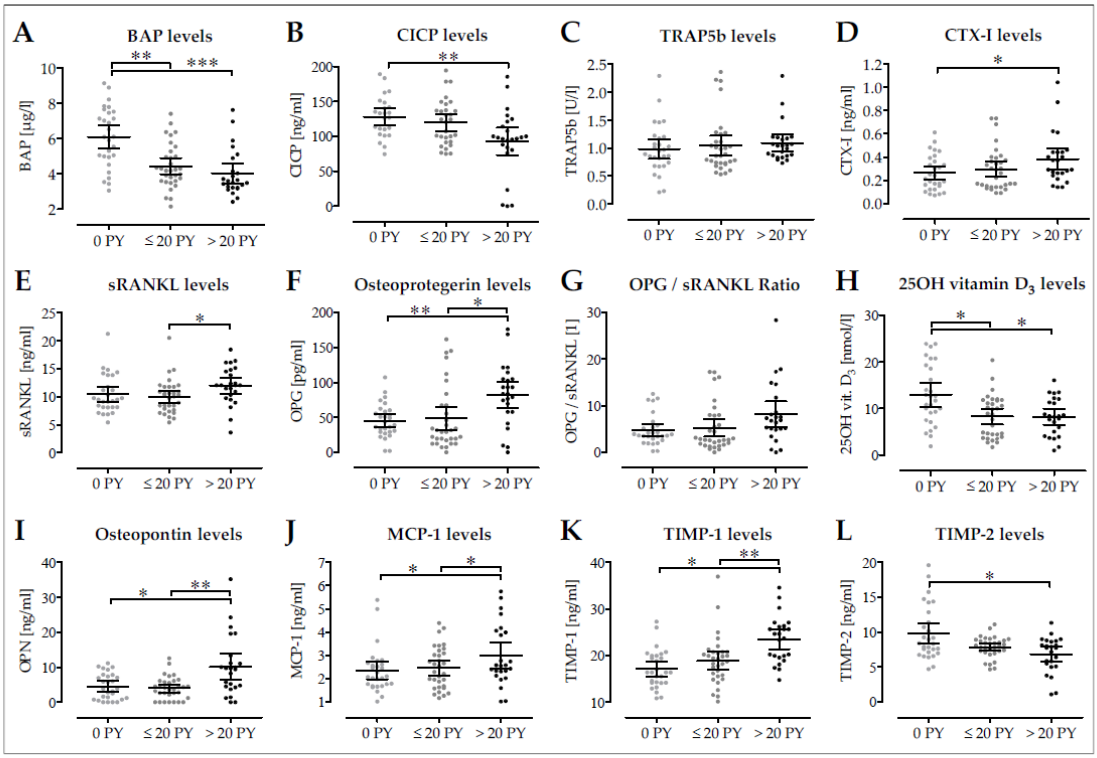

Figure 5. Effect of smoking on serum levels of markers for bone formation and resorption. The analysis differentiated between non-smokers (0 PY; $N = 14$), moderate smokers (1–20 PY; $N = 16$) and heavy smokers (>20 PY; $N = 12$). All ELISAs were performed in duplicates. As bone formation markers, serum levels of (**A**) bone specific alkaline phosphatase (BAP) and (**B**) type I C-terminal collagen pro-peptide (CICP) were determined. As bone resorption markers, serum levels of (**C**) tartrate-resistant acidic phosphatase (TRAP5b) and (**D**) C-terminal telo-peptide of type I collagen (CTX-I) were determined. As regulators for osteoclastogenesis, serum levels of (**E**) soluble receptor activator of nuclear factor kappa-B ligand (sRANKL), (**F**) Osteoprotegerin (OPG), (**G**) the resulting OPG:sRANKL ratio, and (**H**) the regulatory 25OH vitamin D3 were determined. (**I**) Osteopontin serum levels were determined as a marker for osteoclast adherence. (**J**) Monocyte chemoattractant protein-1 (MCP-1/CCL2) serum levels were determined as a stress marker. In addition, serum levels of the tissue inhibitor of metalloproteinases (**K**) TIMP-1 and (**L**) TIMP-2 were determined as an indicator for tissue turnover. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as indicated.

3.6. Pro-Inflammatory Cytokine Levels are Decreased in Smokers Following Surgery

In the next step, we quantified blood serum levels of pro-inflammatory cytokines in our patients two days following surgery. IL-1β serum levels were significantly lower in moderate smokers (2.6 ± 0.3 ng/mL; 1.9–3.3 ng/mL; $p = 0.005$) and heavy smokers (2.4 ± 0.3 ng/mL; 1.8–3.0 ng/mL; $p = 0.002$) when compared to non-smokers (9.7 ± 1.8 ng/mL; 5.9–13.4 ng/mL; Figure 6A). IL-6 blood serum levels were lower in moderate smokers (4.3 ± 0.3 ng/ml; 4.0–5.0 ng/mL; $p < 0.001$) and heavy smokers (5.2 ± 0.3 ng/mL; 4.6–5.8 ng/mL; $p = 0.047$) than in non-smokers (6.3 ± 0.3 ng/mL; 5.6–6.9 ng/mL; Figure 6B). Similarly, TNF-α blood serum levels were significantly lower in moderate smokers (5.5 ± 0.3 ng/mL; 4.9–6.2 ng/mL; $p < 0.001$) and heavy smokers (6.6 ± 0.4 ng/mL; 5.7–7.5 ng/mL; $p = 0.002$) than in non-smokers (10.5 ± 0.9 ng/mL; 8.8–12.3 ng/mL; Figure 6C). Blood serum levels of IFN-γ were significantly lower in heavy smokers (2.9 ± 0.3 ng/mL; 2.2–3.6 ng/mL; $p = 0.008$) and moderate smokers (3.1 ± 0.4 ng/ml; 2.3–3.9 ng/mL; $p = 0.021$) than in non-smokers (4.9 ± 0.6 ng/mL; 3.6–6.1 ng/mL; Figure 6D).

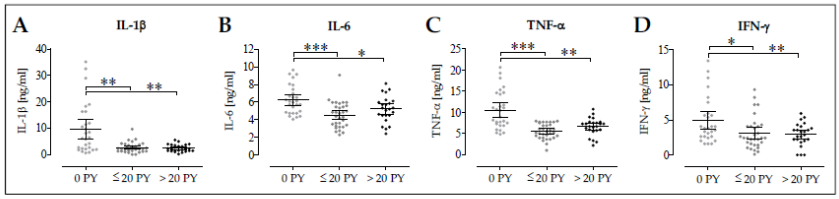

Figure 6. Effect of smoking on serum levels of inflammatory markers. The analysis differentiated between non-smokers (0 PY; $N = 14$), moderate smokers (1–20 PY; $N = 16$) and heavy smokers (>20 PY; $N = 12$). All ELISAs were performed in duplicates. Serum levels of (**A**) interleukin 1 beta (IL-1β), (**B**) interleukin 6 (IL-6), (**C**) tumor necrosis factor alpha (TNF-α), and (**D**) interferon gamma (IFN-γ) were determined. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as indicated.

4. Discussion

Despite frequent reports on smoking as a risk factor for osteoporosis, fragility fractures, and associated post-surgical complication (e.g., delayed/impaired healing, infections, or revision surgeries) [3–9], little is known about the underlying mechanisms. Consequently, handling of smokers during and after musculoskeletal surgeries remains a huge challenge, as no specific treatment strategies exist for these patients. Thus, we set out to better characterize the possible underlying mechanisms that might cause delayed or impaired healing and infections in smokers receiving a TJA in order to propose possible supportive treatment strategies.

In our study cohort, which consisted of 817 patients that received a TJA (primary or revision), smoking was confirmed as a major risk factor for complications (infections, disturbed wound healing, required revision surgery, thrombosis, and/or death). Compared to patients at risk for malnutrition (NRS ≥ 3) and patients with daily alcohol intake, the frequency of complications was higher in smokers. Furthermore, there was a positive correlation between the risk for complications and the amount of smoked cigarettes, which is in line with epidemiological reports [30]. Looking closer at the smokers in our study cohort revealed a higher proportion of men than women in this subgroup. The obtained female to male ratio of approx. 0.43 is lower than that reported for Germany by the World Health Organization (approx. 0.6) [41]. However, this difference might be due to the overall higher amount of men ($N = 445$) than women ($N = 343$) in our study cohort. Although representative for Germany, the female to male ratio among orthopedic/trauma patients might be different in other countries, as the reported smoking behavior varies strongly [41]. Interestingly, in our patients, the mean number of comorbidities seemed to not be increased in smokers. However, this might be explained by the stringing difference in the patients' age. Smokers in our study population were significantly younger than non-smokers (on average 5.4 years), suggesting that smokers are at higher risk for fragility fractures at a much younger age than non-smokers. This is in line with the five-year longitudinal study of Rudang et al. reporting impaired bone mass development and associated higher risk for fragility fractures in young adult men [3]. An extended meta-analysis by Pearson et al. showed not only significantly delayed fracture healing but also a higher frequency of non-unions after fracture, spinal fusion, osteotomy, or arthrodesis in smokers [7]. This finding is fostered by the retrospective study of Hess et al. reporting a higher frequency of post-surgical tenderness, wrist stiffness, non-unions, and revision surgeries in smokers with distal radius fractures (when compared to non-smokers) [6].

Thus, it is indisputable that smoking affects bone health, increases fracture risk, impairs bone healing, and increases the risk for complications. Despite many novel implants and great progresses in surgical techniques, there is still a high frequency of complications in smokers following trauma/orthopedic surgery. As smoking cannot be forbidden in these patients, there is a great need for specific treatment strategies in order to reduce associated complications, e.g., altered antibiosis, immune-modulators, and drugs favoring bone formation or inhibiting bone resorption. However, to establish such secondary treatment strategies, the underlying mechanisms have to be better understood.

Reports on periodontitis patients propose increased bone resorption in the jaw of smokers, as these patients frequently show decreased OPG:sRANKL ratios [11–17]. This is not supported by our finding. Although the cytokine array showed pre-surgically decreased OPG levels in our smokers, quantification of the OPG levels with ELISA two days post-surgery showed contrary results, suggesting the stimulation of OPG expression by the surgery in heavy smokers. Similar results hold for the associated sRANKL, which is strongly induced in heavy smokers two days following surgery, such that the OPG:sRANKL ratio was not significantly altered. These partly contradictory results might be due to the fact that the oral cavity is more affected by direct contact to the cigarette smoke than the skeleton where the effectors have to reach the bone via the blood stream. Considering the stable OPG:sRANKL ratios, it was not surprising that TRAP5b serum levels were comparable between smokers and non-smokers in our patients. Expression of sRANKL is known to be upregulated following orthopedic surgery as a surgery-induced oxidative stress response and a possible immune response towards the implanted material [42]. Just recently, Blaschke et al. showed that sRANKL expression was induced in mesenchymal cells by a combination of the inflammatory cytokines IL-1β, IL-6, and TNF-α [43]. Interestingly, serum levels of these pro-inflammatory markers were reduced in our smokers, which is in line with other reports stating a general immune suppression in smokers [35–37]. This might explain why smokers are more susceptible to infections [30,36]. $25(OH)D_3$ is a well described regulator of MCP-1, OPG, and sRANKL expression [26,27]. Thus, decreased $25(OH)D_3$ serum levels in our cohort of smokers smokers go well together with the increased MCP-1 serum levels in these patients. Although sRANKL expression is increased by $25(OH)D_3$ in vitro [27], $25(OH)D_3$ supplementation improves bone mineral density in patients via inhibition of osteoclasts [26,29]. Thus, it is not surprising that the bone resorption marker CTX-I was mildly induced in heavy smokers with decreased $25(OH)D_3$ serum levels. General supplementation of $25(OH)D_3$ in these patients has to be carefully considered, as $25(OH)D_3$ is also known as a potent regulator of immune responses [32] with strong immune-suppressive action [44]. Further studies are necessary to investigate whether supplementation of $25(OH)D_3$ in immune-suppressed smokers increases their risk for infections.

Serum levels of the oxidative stress marker MCP-1 were increased in our heavy smokers, which is in accordance with other reports [45,46]. Accumulation of Reactive Oxygen Species (ROS) can directly induce formation of osteoclasts from mononuclear cells [47,48] and also by upregulation of MCP-1, a strong inducer of osteoclast fusion and osteoclastogenesis [22–24]. MCP-1 can be induced not only by oxidative stress but also by sRANKL [21,24], which was also increased in these patients. Increased serum levels of OPN, a glycoprotein in bone tissue which functions as an anchor for osteoclasts [38], might further favor osteoclastogenesis in heavy smokers. Thus, it was not surprising that serum levels of CTX-I were mildly induced in heavy smokers, indicating increased bone resorption. Furthermore, we found a smoking-dependent imbalance in TIMP-1 and TIMP-2 serum levels, which may affect matrix resorption via matrix metalloproteinases (MMPs) [49]. Interestingly, TIMP-1 knock-out mice showed significantly stronger inflammatory responses after injury than wild-type mice, suggesting that TIMP-1 may restrict inflammation following injury [50]. Thus, it is feasible that increased TIMP-1 levels in smokers might contribute to the observed decrease in inflammatory response following surgery in these patients. TIMP-2, which was decreased in our smokers, is supposed to play a crucial role in protecting the extracellular matrix (ECM) from proteolysis during fracture healing [51]. Its overexpression in turn may cause pathophysiological ECM accumulation in patients with Dupuytren's disease [52].

However, smoking effects were more pronounced on osteoblastic cells. BAP serum levels were already significantly reduced in moderate smokers, indicating reduced bone formation. This finding is underlined by the reduced serum levels of the bone formation marker CICP in these patients. Experiments with bone marrow-derived mesenchymal stem cells show the strong inhibitory effects of cigarette smoke extract on the cells osteogenic differentiation [18,19]. This effect was not mediated directly by nicotine or its more stable metabolite cotinine [18] but to a big part by oxidative stress caused by reactive substances in cigarette smoke formed in the burning process. When oxidative

stress from cigarette smoke extract accumulates, it strongly affects the viability of primary human osteoblasts [20].

Thus, alternative smoking devices, e.g., e-cigarettes or tobacco heat systems, are advertised as potentially less harmful alternatives [53]. However, long term studies showing the effect of these potential alternatives to cigarettes on bone are still missing. Therefore, smokers are still encouraged to abstain from smoking by studies on patients with acute fracture surgery, which showed less complications, when offered a standardized smoking cessation program for six weeks following surgery [54]. As acute intervention has already shown promising results, further studies on pre-operative smoking cessation followed, which similarly showed reduced complication rates in patients with pre-operative smoking cessation [55,56]. Active intervention, e.g., with standardized smoking cessation programs, kept patients from smoking for longer times [57]. Offering of alternative products, e.g., e-cigarettes or tobacco heat systems, were helpful in reducing acute cravings for cigarettes [58]. However, the effects of this kind of intervention on the clinical outcome of orthopedic/trauma surgery have yet to be investigated.

5. Conclusions

Our data confirm that smoking is a major risk factor for complications following TJA, even at an early age. This holds true for primary TJA, where the overall rate of smokers is lower, as well as for revision TJA, where the overall rate of smokers was significantly higher. Thus, smokers should be encouraged to abstain from smoking to improve the outcome of orthopedic surgeries, especially while no specific treatment strategies are available for these patients.

We could identify alterations in serum levels suggesting a mild increase in bone resorption in heavy smokers. Bone formation was already strongly affected in mild smokers. Thus, bone anabolic drugs might be feasible to stimulate bone formation in smokers following orthopedic or trauma surgery. Post-surgical activation of the immune system is strongly reduced in smokers, suggesting an impaired immune response in these patients, which makes them potentially susceptible for infections. Therefore, general administration of $25(OH)D_3$, which is a strong immune suppressant, to stabilize bone metabolism should be carefully deliberated. We further identified an imbalance in TIMP-1 and TIMP-2 serum levels in smokers, which might represent novel regulators and thus therapeutic targets for both bone regeneration and immune responses in these patients.

Author Contributions: Conceptualization: S.E., C.I., R.A.W., and A.K.N.; formal analysis, S.E., C.I., and R.A.W.; investigation, R.A.W., M.T., and B.Z.; resources, C.I., I.F., and S.S.; writing—original draft preparation, S.E. and C.I.; writing—review and editing, R.A.W., M.T., B.Z., I.F., S.S., B.R., and A.K.N.; visualization, S.E.; supervision, S.E. and C.I.; project administration, A.K.N.; funding acquisition, S.E., R.A.W., and A.K.N.

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3.2 Resveratrol Protects Primary Cilia Integrity of Human Mesenchymal Stem Cells from Cigarette Smoke to Improve Osteogenic Differentiation *In Vitro*

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3.2.1 Summary and Major Findings

The previous study (section 3.1) provided evidence that smokers present an imbalance between bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). Serum levels of osteoblast activity and collagen precursors were strongly affected. As a consequence, smokers presented more complications and delayed fracture repair compared with non-smokers (Ehnert *et al*., 2019). Several studies have demonstrated MSC migration and appropriate differentiation are crucial for successful fracture healing (Ho *et al*., 2015, Devine *et al*., 2002, Einhorn and Gerstenfeld, 2015, Marsell and Einhorn, 2011).

The purpose of the following research was to explore the impact of CS exposure on the osteogenic differentiation of MSCs as well as their cellular effects. Long-term exposure to cigarette smoke extract (CSE) significantly, and in a dose-dependent manner, reduced MSC viability as well as AP activity and matrix formation as early and late osteogenic differentiation markers, respectively (Sreekumar *et al*., 2018).

Primary cilia are described as essential thought to function as cellular sensors. This organelle integrates and transduces extracellular clues into functional responses thought activation of several signaling pathways (Anderson *et al*., 2008, Malone *et al.*, 2007, Goetz *et al*., 2009, Nozawa *et al.*, 2013). Smokers present disturbed cilia in lung epithelial tissue (Leopold *et al*., 2009, Aufderheide *et al*., 2015). Thus, we explored the primary cilia morphology in CSE-exposed MSCs. CSE-treated cells showed shorter and fewer primary cilia. In order to prove that primary cilia play a crucial role during osteogenic differentiation, we analyzed whether pharmacological removal of primary cilia (with chloral hydrate [CH] treatment) positively correlated with impaired osteogenic differentiation in MSCs. Disruption of primary cilia significantly decreased AP activity. Besides, sequential time-based analysis demonstrated that delayed osteogenic differentiation in MSCs exposed to CSE may be a consequence of primary cilia disruption (Sreekumar *et al*., 2018).

Increasing evidence has suggested that elevated ROS production plays a central role in cigarette smoke–induced bone tissue injury (Church and Pryor, 1985, Kubo *et al.*, 2019, Muinos-Lopez *et al.*, 2016). Cigarette smoke has large amounts of ROS, nitric oxide, peroxynitrite, and free radicals of organic compounds (Nakayama *et al*., 1989, Pryor *et al.*, 1983, Pryor *et al.*, 1998, Zang *et al*., 1995). In addition to these highly reactive and short-lived molecules, cigarette smoke also contains prooxidant substances that have the potential to upregulate cellular ROS levels (Swain *et al*., 1969, Squadrito *et al*., 2001). Water-soluble components of cigarette smoke may reach the skeletal system through the bloodstream, and thus they can directly harm macromolecules – *e.g.* nucleic acids, proteins, and lipids – by promoting oxidative stress in bone cells (Duan *et al*., 2005, Miranda *et al.*, 2008, Barbouti *et al*., 2002). We showed that increased ROS levels from cigarette smoke or H_2O_2 negatively affected the structure of primary cilia. Furthermore, treatment with resveratrol, a phytoalexin with antioxidant properties, decreased the cigarette smoke–induced ROS level 2 fold, and attenuated the harmful effects on AP activity, matrix production, and protect primary cilia integrity on MSCs (Sreekumar *et al*., 2018).

Upon co-stimulation with resveratrol, we observed a 2-fold increase in the gene expression of *polycystin 2* (*PC2*), a primary cilia marker, *zinc finger protein* (*Gli2*), a hedgehog (Hh) signaling transcription factor, *RUNX*2, a key transcription factor associated with osteoblast differentiation, *BMP-2,* a growth factor, *OPG*, an inhibitor of osteoclastogenesis, and *RANKL*, an activator of osteoclastogenesis. Gene expression analysis was mostly in line with the previously described enhanced osteogenic differentiation after resveratrol treatment (Sreekumar *et al.*, 2018).

These results further substantiate the possible regulatory function of ROS-induced primary cilia disruption for impaired bone homeostasis in smokers. In summary, our results showed that CS-induced ROS impaired MSC osteogenesis partially *via* reducing primary cilia length and ciliated cells. This knowledge opens up new treatment options for trauma patients who smoke and have chronically elevated ROS levels, a condition that frequently delays fracture healing.

3.2.2 Personal Contribution

I was involved in the conception of the experiments. I helped perform the experiments and analyze the data. I was responsible for the data presentation. I contributed to

writing the manuscript, including its critical revision according to the reviewers' suggestions.

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ORGAN TOXICITY AND MECHANISMS

Resveratrol protects primary cilia integrity of human mesenchymal stem cells from cigarette smoke to improve osteogenic differentiation in vitro

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Abstract

Several studies have explored the negative effects of cigarette smoke on bone healing; however, the complex pathogenesis still remains unclear. One crucial and primary factor determining effective fracture repair is the recruitment and differentiation of mesenchymal stem cells (MSCs) into bone-forming cells. Recently, primary cilia, microtubule-based sensory organelles, have been shown to be critical in lineage commitment and differentiation of MSCs. Our present study indicates that exposure to cigarette smoke extract (CSE 0.1–10%) impaired osteogenic differentiation of human mesenchymal stem cell line (SCP-1) and interestingly, also affected primary cilia distribution and integrity in these cells during the differentiation. Furthermore, significant amounts of free radicals generated by CSE could be causative of primary cilia loss since treatment with 0.01% of hydrogen peroxide, a prime free radical in CSE, destroyed primary cilia in these cells. The debilitated differentiation of CSE-exposed SCP-1 cells also correlated with the significantly reduced expression of transcription factor and target genes of primary cilia-specific hedgehog signalling, a key player in osteogenic differentiation. As a treatment strategy, co-incubation of the CSE-exposed SCP-1 cells with the antioxidant resveratrol (1 µM) had a protective effect as it significantly reduced free radical production, protected the primary cilia and enhanced osteogenic differentiation. The current study shows for the first time that cigarette smoke affects primary cilia in human MSCs during osteogenic differentiation and treatment with resveratrol could reverse the effects and enhance differentiation, thus opening up potential therapeutic alternatives to treat fracture healing in smokers, in particularly, when delayed fracture healing is assumed.

Keywords Cigarette smoke · Primary cilia · SCP-1 cells · Reactive oxygen species · Hedgehog signalling · Bone regeneration

Introduction

In the past, several studies have attested the deleterious effects of the cigarette smoke (CS) on the musculoskeletal system (Abate et al. 2013). The numerous toxins and free radicals contained in cigarette smoke induce a chain of damage throughout the skeletal system by destroying bone cells, impairing new bone formation and accelerating bone loss (Abate et al. 2013; Barreiro et al. 2010). Consequently, these factors could delay or inhibit bone healing after surgery or trauma in smokers as evidenced by several clinical studies (Patel et al. 2013; Shibli et al. 2010; Wong et al. 2007). Being a complex process, bone healing is influenced by numerous biological, mechanical and systemic factors. Additionally, it involves well-orchestrated series of events, where the infiltration and differentiation of mesenchymal stem cells (MSCs) play decisive roles (Einhorn and Gerstenfeld 2015; Marsell and Einhorn 2011; Marsh and Li 1999). Several adverse effects of cigarette smoke have been reported such as immune reactivity, cytotoxicity, damage of cellular organelles such as mitochondria, and damage of nucleic acids, lipids and proteins; however, its effect on the cellular sensors has not been studied extensively (Sung et al. 2015; Wahl et al. 2016).

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Since differentiation of MSCs into different lineages depends on the local cues that are present in their environment, a crucial factor influencing this is the ability of cells to respond to various stimuli (mechanical forces, chemical stimulus, osmolarity, gravity, etc.) (Tummala et al. 2010), via immotile microtubule-based primary cilia which protrudes from the cell membrane like a cellular antenna and translates the external stimulus to molecular changes, thereby activating genes involved in proliferation and differentiation of bone cells (Huang and Ogawa 2010; Klein-Nulend et al. 2012; Tummala et al. 2010). Primary cilia play vital roles in the initiation of osteogenic differentiation of MSCs and in the phenotypic maintenance of differentiated cells (James 2013; Tummala et al. 2010) via families of signalling proteins that are specifically regulated by primary cilia. Genetic studies in mice showed that primary cilia are specialised for hedgehog signalling (Hh signalling), a highly conserved signalling process that plays a crucial role in osteoblast differentiation from mesenchymal progenitors during endochondral bone formation (Huangfu et al. 2003; Mak et al. 2008; Nozawa et al. 2013). Alterations of this signalling have been linked to skeletal deformities (Temiyasathit and Jacobs 2010; Xiao et al. 2008). The Hh signalling pathway initiates by binding of Hh to receptor Patched (Ptch1), releasing Ptch1 inhibition on membrane protein Smoothened (Smo) which then translocates to the primary cilium. Ciliary Smo activates transcription factor Gli (Goetz et al. 2009; He et al. 2016). It has been shown that Hh/Gli2 signalling promotes osteoblast differentiation by regulating the runt-related transcription factor (RUNX2) expression and function (Shimoyama et al. 2007).

During our study which sought to explore the molecular link between CS and impaired osteogenic differentiation, we found that CSE caused a significant distortion of primary cilia. The disruption of primary cilia also correlated with the downregulation of transcription factor and target genes of Hh signalling pathway which plays crucial roles in osteogenic differentiation. It is an undisputable fact that toxins and free radicals generated by the CS induce extensive cellular damage, which could contribute to primary cilia distortion in our cells. Therefore, an early treatment using antioxidant resveratrol was aimed to quench the large amounts of free radicals generated and to protect the cilia, consequently enhancing the osteogenic differentiation. In addition to being a potent antioxidant, resveratrol is a natural stilbenoid proven to possess both anti-inflammatory and bone-protective properties (Casarin et al. 2014). Resveratrol is able to modulate osteogenesis by stimulating the Sirt1 pathway, and inhibits osteoclast differentiation by suppressing RANKL (Shakibaei et al. 2012). Furthermore, it can activate bone morphogenetic proteins, the transcription factor RUNX2 and osteopontin (OPN), thereby promoting the osteogenic differentiation of MSCs (Casarin et al. 2014).

Co-incubation of MSCs with CSE and resveratrol was able to protect the primary cilia integrity and further promoted its osteogenic differentiation. Thus, our study reports a novel effect of CS on debilitated osteogenic differentiation through the distortion of cellular sensors and delivers new insights into the protective functions of resveratrol in reversing these effects and enhancing osteogenic differentiation of MSCs, thus opening up potential therapeutic alternatives to treat fracture healing in smokers.

Materials and methods

Cigarette smoke extract (CSE) preparation

Two commercial cigarettes (Marlboro, Philip Morris USA) containing 0.8 mg nicotine and 10 mg tar each, were continuously combusted with a peristaltic pump (Cyclo II, Carl Roth, Germany). The smoke was bubbled through 50 ml pre-warmed SCP-1 differentiation medium without Foetal bovine serum (FBS) in a standard gas washing bottle, at a speed of 95 bubbles/min. The concentration of CSE was determined and normalised by its optical density at 320 nm (OD 320) in a plate reader (BMG Labtech GmbH, Offenburg, Germany) (Braun et al. 2011). The solution with an optical density of 0.7 was considered 100% CSE, was prepared fresh and filtered through a 0.22-µm pore filtre. This was further diluted (0.1, 1, 5 and 10%) with SCP-1 differentiation medium to reach the concentration up to 3.2–320 ng/ml nicotine. The CSE concentrations correspond approximately to exposures associated with smoking slightly less than 0.01 pack cigarettes/day (0.1%) to 1 pack per cigarettes/day (10%) (Su et al. 1998).

Culture and osteogenic differentiation of SCP-1 cells

Human-immortalised mesenchymal stem cells [SCP-1 cells, kind gift by Dr. Matthias Schieker (Bocker et al. 2008)] were cultured in MEM alpha medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin (SCP-1 growth media) in a water-saturated atmosphere of 5% CO_2 at 37 °C (Ehnert et al. 2015). Osteogenic differentiation was induced by supplementing 200 µM *L*-ascorbate-2-phosphate, 10 mM β-glycerol-phosphate, 25 mM HEPES, 1.5 mM $CaCl_2$ and 100 nM dexamethasone to MEM alpha medium with 1% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin (SCP-1 differentiation media). For the experiments different concentrations of CSE (0–10%) were added to the media. Medium with or without CSE was changed every 3–4 days during osteogenic differentiation which was sustained for 28 days.

Resazurin conversion assay

Viability and proliferation was indirectly determined by Resazurin conversion (mitochondrial activity). SCP-1 cells were incubated with 1/10 volume Resazurin solution (0.025% in DPBS) for 30 min at 37 °C. Resulting fluorescence was measured (ex/em = 540/590 nm) and corrected to background control (Ehnert et al. 2016).

Live/dead staining with calcein AM and ethidium homodimer

Cell viability was determined by intracellular esterase activity with calcein AM and dead cells were determined by membrane passage of Ethidium homodimer. SCP-1 cells stimulated with or without CSE were washed three times with PBS and were incubated with calcein AM (2 µM), Ethidium homodimer (4 µM) and Hoechst 33,342 (1:1000 in PBS) at room temperature (RT) for 30 min. Cell images were taken (Epifluorescence: EVOS FL, life technologies, Darmstadt, Germany) following washing with PBS.

Alkaline phosphatase (AP) assay

AP activity was measured by incubating SCP-1 cells with reaction buffer (0.2% 4-nitrophenyl-phosphate, 50 mM glycine, 1 mM $MgCl_2$, 100 mM TRIS, pH 10.5) at 37 °C for 40 min. Generated 4-nitrophenol was determined photometrically ($\lambda = 405$ nm), corrected to background control and normalised to relative cell numbers determined by resazurin conversion (Ehnert et al. 2010).

Alkaline phosphatase (AP) staining

Cells were washed with DPBS and fixed with 4% (w/v) paraformaldehyde for 10 min at RT. After a brief wash, they were permeabilised with 0.2% Triton-X-100 solution for 20 min at RT, washed with DPBS and stained with AP staining solution (0.06% Fast blue BB salt, 0.01% Naphthol AS-MX phosphate disodium salt, 0.5% Dimethylformamide, $MgCl_2$ 2 mM and TRIS 0.1 M) for 30 min at 37. After an additional washing step, images were taken, where AP showed as blue stain.

Alizarin red staining for mineralised matrix

Matrix mineralisation is associated with the final phase of osteoblast differentiation and was assessed by Alizarin red staining. Cells were fixed with ethanol at − 20 °C for 60 min, washed three times with tap water and stained with 0.5% Alizarin red solution (pH 4.0) for 30 min at RT. After three additional washing steps, the resulting staining was resolved with 10% cetylpyridium chloride solution and quantified photometrically ($\lambda = 562$ nm).

Immunostaining of primary cilia

Cells were washed with DPBS and fixed with 4% (w/v) paraformaldehyde for 10 min at RT. After a brief wash, they were permeabilised with 0.2% Triton-X-100 solution for 20 min at RT, washed with DPBS and treated with 2% (w/v) paraformaldehyde for 10 min at RT. Unspecific binding sites were blocked (5% BSA in DPBS) for 1 h at RT. Then, cells were incubated with anti-acetylated α-tubulin antibody SC-23,950 (1:100 in DPBS, Santa Cruz, Heidelberg, Germany) overnight at 4 °C, washed and incubated with ALEXA488 labelled secondary antibody (1:2000 in DPBS, Life Technologies, Darmstadt, Germany) for 2 h at RT. Nuclei were stained with Hoechst 33,342 (1:1000). Images were taken with an epifluorescence microscope (EVOS FL, life technologies, Darmstadt, Germany). Pictures were processed and primary cilia length was analysed using the ImageJ software (line tool) by two independent investigators in a blinded fashion.

Free radical production analysis with DCFH-DA assay

Prior to experiments, cells were washed twice with DPBS. Subsequently, they were incubated with 10 µM 2′, 7′-dichlorfluorescein-diacetate (DCFH-DA) in serum-free culture medium for 30 min at 37 °C. After washing the cells twice with DPBS, cells were stimulated with CSE. The fluorescence intensity, representing reactive oxygen species (ROS) levels, was quantified using a plate reader (ex/em = 485/520 nm) (Braun et al. 2011).

Semi-quantitative RT-PCR

Total RNA was isolated from cells using TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's protocol and quantified using a spectrophotometer (Omega plate reader, BMG Labtech, Offenburg, Germany). cDNA was synthesised with the First Strand cDNA Synthesis Kit (Fermentas St, Leon-Rot, Germany). Semi-quantitative RT-PCR was performed using KAPA2G Fast Ready Mix (Peqlab, Erlangen, Germany). Primer sequences and PCR conditions are summarised in Table 1. GAPDH was used as an internal control for normalisation. PCR products were resolved by 1.5% agarose gel electrophoresis and visualised by ethidium bromide. Densitometric analysis was performed using the ImageJ software (NIH, Bethesda, USA).

Table 1 Summary of PCR conditions

Gene	Forward primer	Reverse primer	Amplicon [bp]	Tm [°C]	GenBank accession [NM_]
BMP2	CCCCCTACATGCTAGACCTGT	CACTCGTTTCTGGTAGTTCTTCC	150	60	1200.3
GLI2	CACCAACCAGAACAAGCAGA	ACCTCAGCCTCCTGCTTACA	195	59	5270.4
OPG	CCGGAAACAGTGAATCAACTC	AGGTTAGCATGTCCAATGTG	313	60	2546.3
PC2	CCAGCTCTCGACAACCATGT	TCGACCTGAGTGCCAAAGAC	120	60	297.3
RANKL	TCCCAAGTTCTCATACCCTGA	CATCCAGGAAATACATAACACTCC	245	56	33012.3
RUNX2	TCCTATGACCAGTCTTACCCCT	GGCTCTTCTTACTGAGAGTGGAA	190	62	1024630.3
GAPDH	GTCAGTGGTGGACCTGACCT	AGGGGTCTACATGGCAACTG	420	56	1289746.1

Statistics

Data analysis was performed using Graph Pad Prism software (El Camino Real, CA, USA) and PAST.exe (http://folk.uio.no/ohammer/past/index.html). All the data are presented as mean ± SEM ($N = 3$, $n = 2$). Data were analysed using Kruskal–Wallis H test followed by a Dunn's test for comparisons between multiple groups. $p \leq 0.05$ was considered to be minimum level of significance. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Results

CSE exposure reduced viability, AP activity and matrix mineralisation during osteogenic differentiation of SCP-1 cells

SCP-1 cells were osteogenically differentiated by culturing them in SCP-1 differentiation media containing 0, 0.1, 1, 5 and 10% CSE for 28 days. Live–dead staining showed that treatment with CSE caused a significant reduction in the viability of SCP-1 cells when compared to non-exposed controls in a concentration-dependent manner (Fig. 1a). Similarly, the mitochondrial activity (indirect indicator of viability and proliferation) of cells measured by Resazurin conversion also showed a significant reduction. SCP-1 cells treated with 5 and 10% CSE showed a significant reduction in viability at day 7 (5%, $^{*}p \leq 0.05$ and 10%, $^{***}p \leq 0.001$) and day 14 (5%, $^{***}p \leq 0.001$ and 10%, $^{***}p \leq 0.001$), respectively. Since a significant reduction in viability of SCP-1 cells were observed upon CSE treatment, it was interesting to investigate its effect on the functionality of the osteogenically differentiated SCP-1 cells. AP activity of CSE (5 and 10%)-exposed SCP-1 cells showed a significant reduction at day 14 (Fig. 2a, b). However, CSE concentration 0.1% showed a marginally increased AP activity when compared to control cells. It has been reported previously that nicotine affects the AP activity of osteoblasts in a dose-dependent manner and low concentrations of nicotine have been shown to enhance osteoblast activity (Kim et al. 2012). Consistent with the AP activity, 5 and 10% of CSE also significantly reduced the matrix mineralisation after 28 days post-exposure when compared to control non-exposed SCP-1 cells which is quite evident with the alizarin red staining of the cells (Fig. 2c, d $^{*}p \leq 0.05$, $^{***}p \leq 0.001$).

CSE exposure caused a significant reduction in primary cilia length and distribution in SCP-1 cells during osteogenic differentiation

Primary cilia are the mechanosensors of the osteoblasts and are microtubule-based organelles. The integrity of the cilia is maintained by the acetylation of the microtubules (Takemura et al. 1992). To determine the integrity and distribution of primary cilia in the SCP-1 cells cultured in the presence or absence of CSE, the cells were stained for acetylated α-tubulin (primary cilia) and Hoechst 33,342 (nucleus) (Fig. 3a–e). The length and distribution of primary cilia was determined by double blinded analysis. Exposure to CSE 1, 5 and 10% for 14 days caused a significant reduction ($^{***}p \leq 0.001$) in the length of ciliated cells (Fig. 3f). Also the number of ciliated cells after 5 and 10% CSE exposure was seen significantly reduced (Fig. 3g, $^{***}p \leq 0.001$).

Pharmacological removal of primary cilia-impaired osteogenic differentiation in SCP-1 cells

To determine the role of primary cilia in osteogenic differentiation of SCP-1 cells, the cells were differentiated in the presence of 2 mM chloral hydrate for 14 days, since chloral hydrate of concentration of 4 mM and lower has been reported to be non-toxic to cells but efficient enough to disrupt the primary cilia (Malone et al. 2007; Praetorius and Spring 2003). Chloral hydrate treatment not only caused a significant 66% reduction in primary cilia length in SCP-1 cells during osteogenic differentiation but also resulted in a significant reduction of ciliated cells (Fig. 4a, $^{***}p \leq 0.001$;

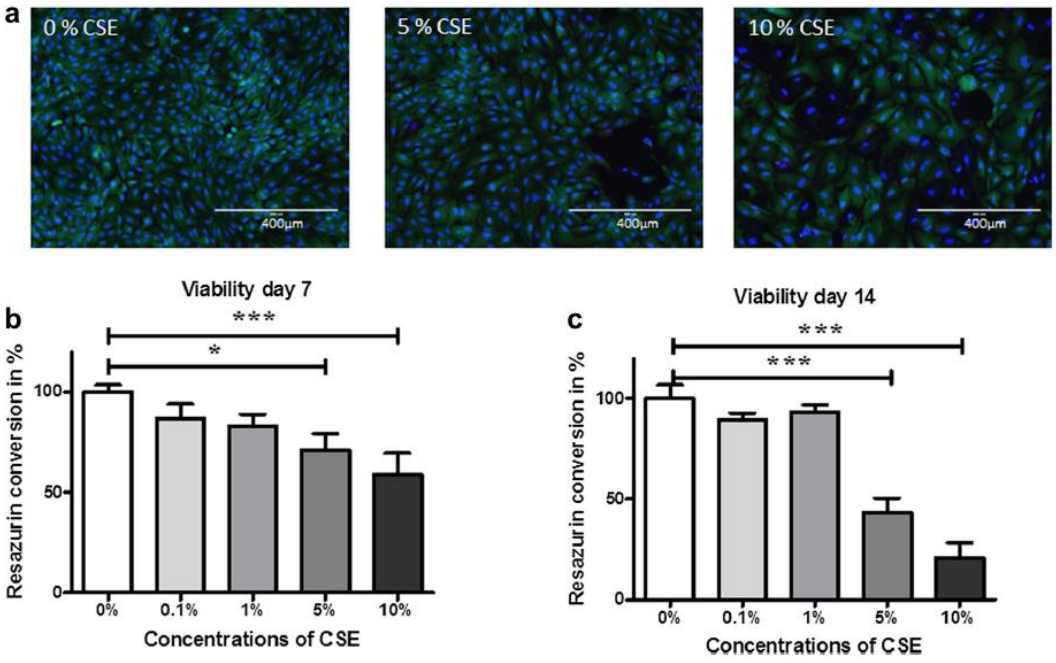

Fig. 1 CSE exposure affected viability during osteogenic differentiation of SCP-1 cells. SCP-1 cells were osteogenically differentiated by culturing them in SCP-1 differentiation media containing 0, 0.1, 1, 5 and 10% CSE. **a** Live–dead staining using calcein AM (green)/Ethidium homodimer (red) and nuclear staining using Hoechst 33,342 (blue) is shown after growing the SCP-1 cells in the differentiating media with or without CSE at day 14. **b–c** Viability indicated by the Resazurin conversion is shown for SCP-1 cells cultured in differentiating media with or without CSE at day 7 and 14, respectively. For all quantifications ($N=3$, $n=2$), statistical significance was determined by Kruskal–Wallis H test followed by Dunn's post-test. Data are represented as mean ± SEM and significance represented as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (Color figure online)

b, *$p \leq 0.05$). Interestingly, the chloral hydrate treatment also significantly reduced the AP activity in these cells compared to control cells after 14 days of osteogenic differentiation (Fig. 4c, *$p \leq 0.05$).

CSE induced loss of primary cilia in SCP-1 cells correlated with impaired AP activity during osteogenic differentiation

Since we observed loss of primary cilia and impaired AP activity/matrix mineralisation of SCP-1 cells in the presence of CSE at 14 days of osteogenic differentiation, we were interested in investigating whether the loss of primary cilia was a cause or consequence of impaired osteogenic differentiation. The presence of primary cilia was evident in SCP-1 cells only after 8 days of osteogenic differentiation since it has been reported previously that primary cilia is lost during active cell division phase (Izawa et al. 2015). Primary cilia integrity and AP activity were, therefore, co-analysed during 9, 10 and 12 days of osteogenic differentiation as a time course study. At day 9, significant reduction in primary cilia (Fig. 5; **$p \leq 0.01$) was already observed with no significant changes in the AP activity. However, both primary cilia length and AP activity on day 10 and 12 of osteogenic differentiation seemed to be progressively affected (Fig. 5a, b). Since disruption of primary cilia was observed before significant changes in AP activity, it could be concluded that impaired differentiation could be a consequence of loss of primary cilia.

Increased ROS generated by CSE countered by resveratrol treatment

It is a well-established fact that one of the crucial factors responsible for the negative effects of CS is the generation of considerable amounts of ROS that is beyond the antioxidant scavenging ability of the system (Church and Pryor 1985). The ROS generated due to the CSE concentrations used in the study also revealed significant surge in a concentration-dependent manner. CSE concentration of 1% and above generated significantly high amounts of ROS (**$p \leq 0.01$) when compared to untreated cells (Fig. 6a). However, it was interesting to test whether if the generation of ROS had an effect on primary cilia integrity. Treatment of SCP-1 cells with 0.01% hydrogen peroxide for 1 h, which represents a chief ROS present in cigarette smoke, significantly reduced

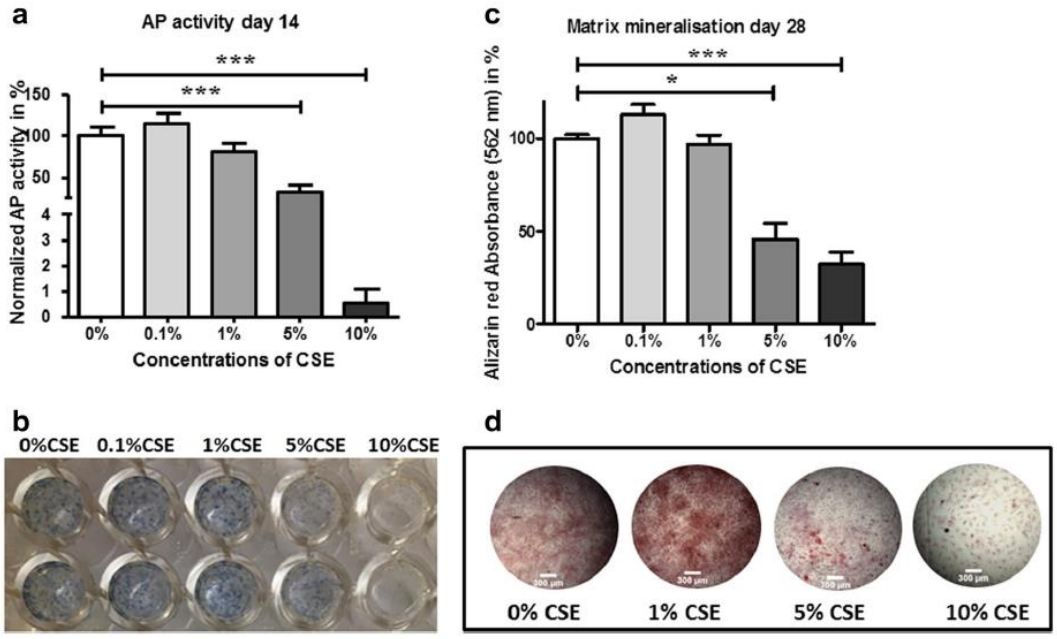

Fig. 2 CSE exposure affected the AP activity and matrix mineralisation. SCP-1 cells were osteogenically differentiated by culturing them in SCP-1 differentiation media containing 0, 0.1, 1, 5 and 10% CSE. **a** The graph here represents the AP activity of SCP-1 cells undergoing osteogenic differentiation in the presence and absence of different concentrations of CSE at day 14. **b** A representative AP staining in the cells is shown. **c** The graph here represents the formation of mineralised matrix indicated by alizarin red staining after osteogenically differentiating the SCP-1 cells for 28 days with or without CSE. **d** Representative Alizarin red staining indicating the extent of matrix mineralisation of the cells with and without treatment with CSE is shown. For all quantifications ($N=3$, $n=2$), statistical significance was determined by Kruskal–Wallis H test followed by a Dunn's post-test. Data are represented as mean ± SEM and significance represented as * $p \leq 0.05$, *** $p \leq 0.001$

primary cilia length in these cells (Fig. 6b), thus linking the high ROS production by CSE to the loss of primary cilia integrity in SCP-1 cells during its osteogenic differentiation. To quench the free radical generated, the SCP-1 cells were co-incubated with CSE and the antioxidant resveratrol for 14 days. Initially, to determine the most beneficial concentration of resveratrol, SCP-1 cells were treated with various concentrations of resveratrol (0–40 µM) for 14 days (Fig. 6c). Based on cell survival rate, the physiological concentration of 1 µM resveratrol was used for the further studies. Co-incubation of 5 and 10% CSE-exposed SCP-1 cells with 1 µM concentration of resveratrol for 14 days significantly reduced the ROS generation by at least twofold (Fig. 6d, ***$p \leq 0.001$).

Resveratrol enhanced viability, AP activity, matrix mineralisation and protected the primary cilia in CSE-exposed SCP-1 cells

Co-incubation of 5 and 10% CSE exposed SCP-1 cells with 1 µM resveratrol for 14 days significantly increased the viability when compared with 5 and 10% CSE-exposed SCP-1 cells without resveratrol treatment (Fig. 7a, ***$p \leq 0.001$). Additionally, resveratrol treatment also significantly enhanced the AP production in 5 and 10% CSE-exposed SCP-1 cells, bringing it up to nearly the AP activity in control SCP-1 cells unexposed to CSE (Fig. 7b, ***$p \leq 0.001$). Upon 28 days of co-incubation, the matrix mineralisation was analysed photometrically and via Alizarin red staining. Treatment with 1 µM resveratrol significantly improved matrix mineralisation in CSE-exposed SCP-1 cells (Fig. 7d, e, *$p \leq 0.05$).

Resveratrol treatment rescued the CSE-induced loss of primary cilia integrity and the alterations in the basal gene expression of Hh transcription factor and Hh target genes

Interestingly, in addition to improving the function and matrix mineralisation, resveratrol treatment for 14 days also significantly increased the cilia length and the number of ciliated SCP-1 cells exposed to 5 and 10% of CSE (Fig. 8c, f). Primary cilia are essential for transducing the developmental signals, especially the Hh signalling which plays an important role in osteoblast differentiation and bone remodelling (Goetz et al. 2009). Since we observed loss of primary cilia

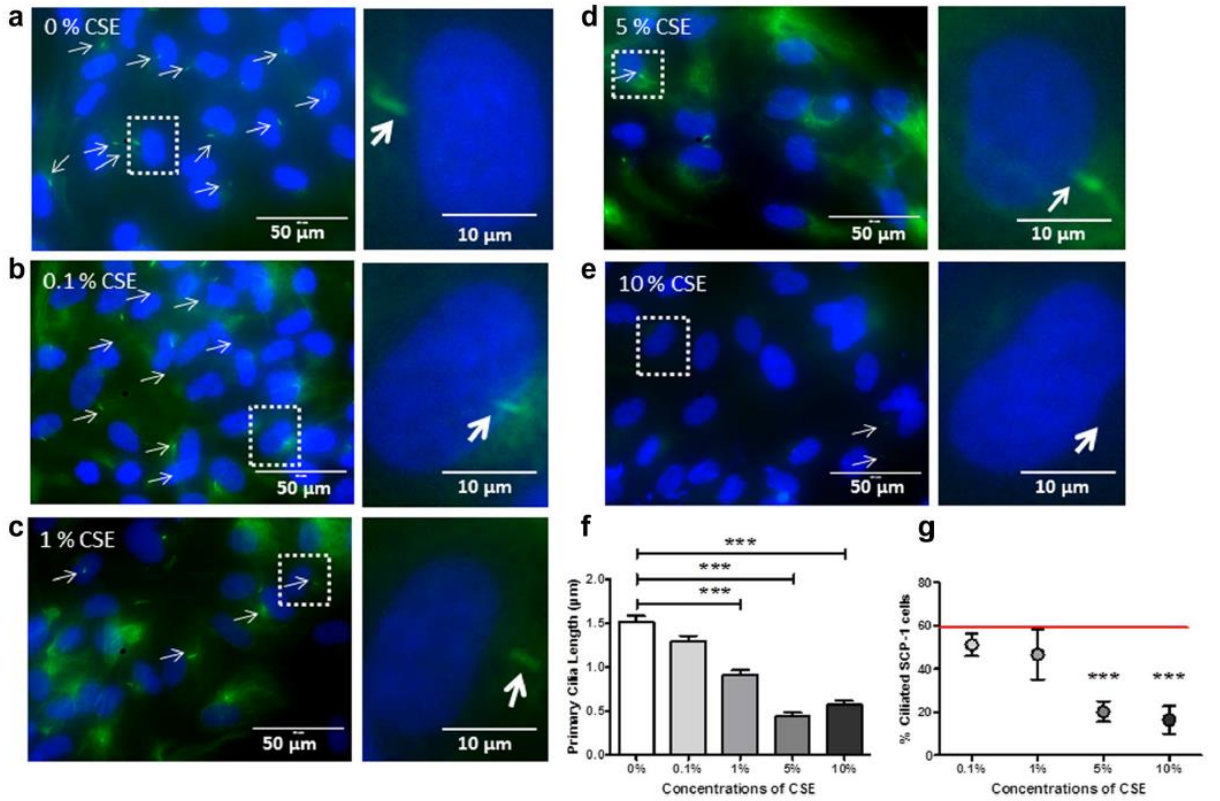

Fig. 3 CSE exposures caused a significant reduction in number of ciliated cells. Immunostained images of primary cilia in SCP-1 cells at day 14 with and without exposure to CSE are shown (**a**–**e**). Primary cilia staining by acetylated tubulin (green), and nucleus Hoechst (blue) is shown. Primary cilia are indicated by white arrows. **f** Graph here represents the primary cilia length of SCP-1 cells undergoing osteogenic differentiation for 14 days with or without CSE. **g** The percentage of ciliated SCP-1 cells is shown following osteogenic differentiation for 14 days with CSE compared to untreated cells (represented as red line). For all quantifications ($N=3$, $n=3$), statistical significance was determined by Kruskal–Wallis H-test followed by a Dunn's post-test. Data are represented as mean ± SEM and significance represented as *** $p \leq 0.001$. (Color figure online)

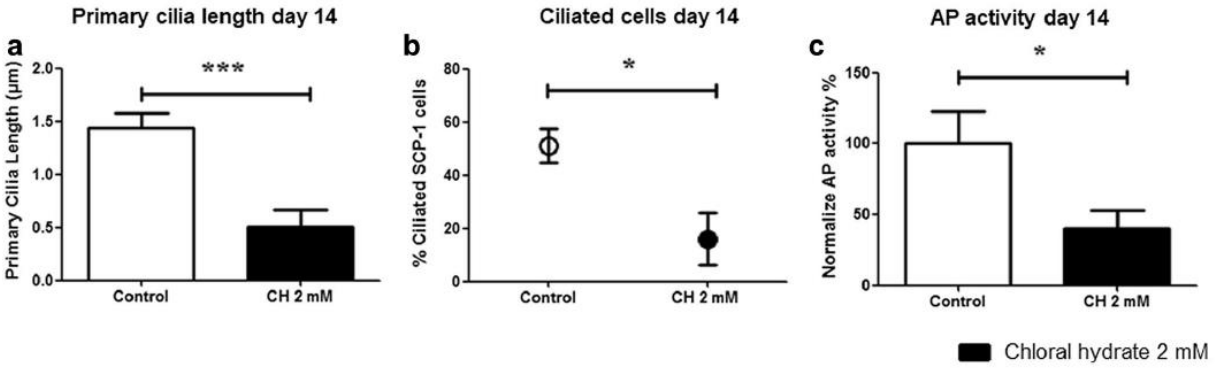

Fig. 4 Disruption of primary cilia by chloral hydrate affected SCP-1 osteogenic differentiation. SCP-1 cells were osteogenically differentiated with 2 mM chloral hydrate for 14 days. **a** Primary cilia length quantification of SCP-1 cells is shown following osteogenic differentiation for 14 days with chloral hydrate 2 mM. **b** The percentage of ciliated SCP-1 cells was shown following osteogenic differentiation for 14 days with chloral hydrate 2 mM compared to untreated cells. **c** The graph here represents the AP activity of SCP-1 cells undergoing osteogenic differentiation with chloral hydrate 2 mM at day 14 compared to control cells. For all quantifications ($N=3$, $n=2$), statistical significance was determined by Kruskal–Wallis H test followed by Dunn's post-test. Data are represented as mean ± SEM and significance represented as *$p \leq 0.05$, ***$p \leq 0.001$

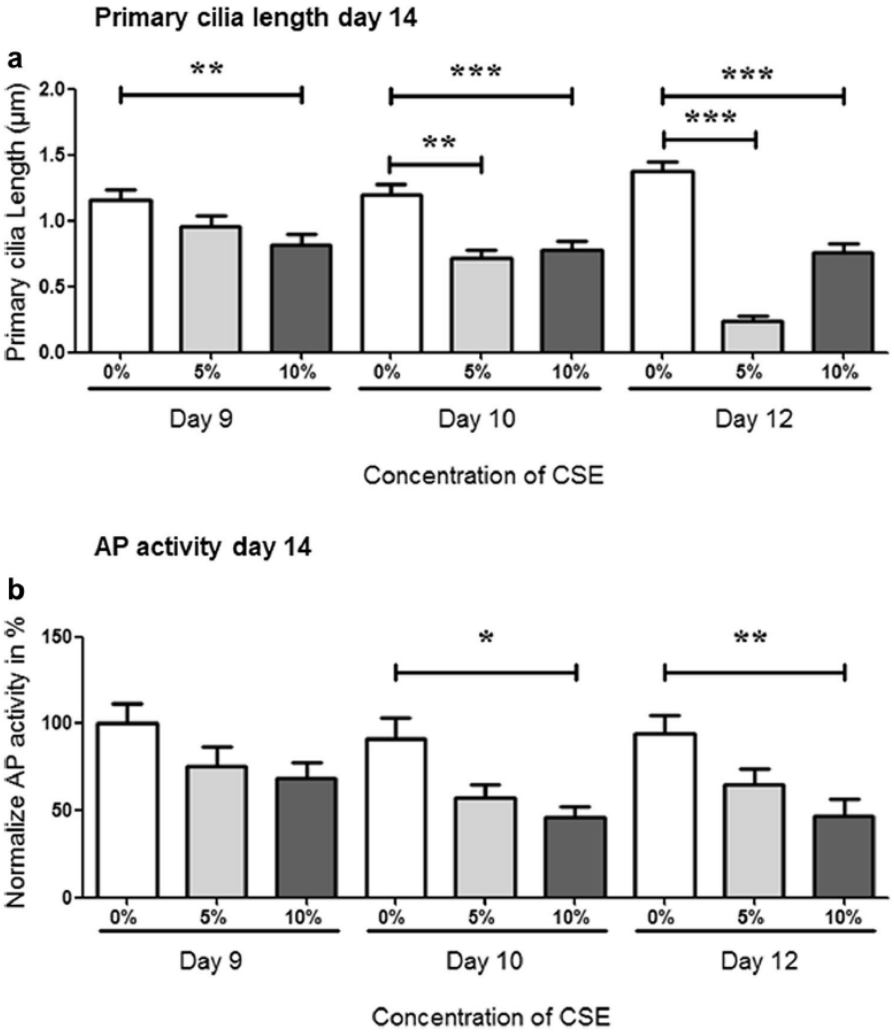

Fig. 5 Loss of primary cilia by CSE precedes the impairment in osteogenic differentiation. SCP-1 cells were osteogenically differentiated by culturing them in SCP-1 differentiation media containing 0, 5 and 10% CSE. **a** The graph represents the quantification of primary cilia length of SCP-1 cells undergoing osteogenic differentiation in the presence and absence of different concentrations of CSE at day 9, 10 and 12. **b** AP activity of SCP-1 cells undergoing osteogenic differentiation with and without CSE exposure at day 9, 10 and 12. For all quantifications ($N=3$, $n=3$), statistical significance was determined by Kruskal–Wallis H test followed by Dunn's post-test. Data are represented as mean ± SEM and significance represented as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$

integrity, it was further interesting to investigate its effect on the Hh signalling in the SCP-1 cells during its osteogenic differentiation. Semi-quantitative gene expression analysis revealed that expression of the primary cilia marker *PC2* was significantly downregulated in cells exposed to CSE ≥ 1% at day 14 when compared to non-exposed controls (Fig. 8a). Similarly, the Hh signalling transcription factor *Gli2* was also significantly downregulated in these cells (Fig. 8b). Since the transcription factor of the Hh signalling was affected, it was interesting to investigate the expression of Hh target genes. *RUNX2* induces the differentiation of multipotent mesenchymal cells into immature osteoblasts and is also the target gene of Hh signalling. We observed that the treatment of CSE ≥ 1% resulted in reduced gene expression of *RUNX2* (Fig. 8c). 5 and 10% CSE-exposed SCP-1 cells displayed reduced gene expression of another Hh target gene *BMP-2* (Fig. 8d) which taken together could have critically affected the osteogenic differentiation of these cells. Similarly, gene expression of Osteoprotegerin (*OPG*)—inhibitor of osteoclastogenesis and receptor activator NF-kB ligand (*RANKL*)—activator of osteoclastogenesis were also significantly downregulated in SCP-1 cells treated with 5 and 10% CSE (Fig. 8e, f). Treatment with 1 µM resveratrol caused nearly twofold increases in the gene expression of *PC2, Gli2, RUNX2, BMP-2, OPG* and *RANKL* (Fig. 8a–f).

Discussion

Cigarette harbours nearly 150 toxic components (toxic gases, nicotine, tar, aromatic amines, heavy metals, carcinogens, etc.), of which nicotine is the prime addiction factor that gets readily absorbed in the body (Braun et al. 2011; Pappas 2011). The concentrations of nicotine in the arterial blood raise up to 100 ng/ml shortly after smoking a cigarette which then gets distributed to various tissues including the skeletal

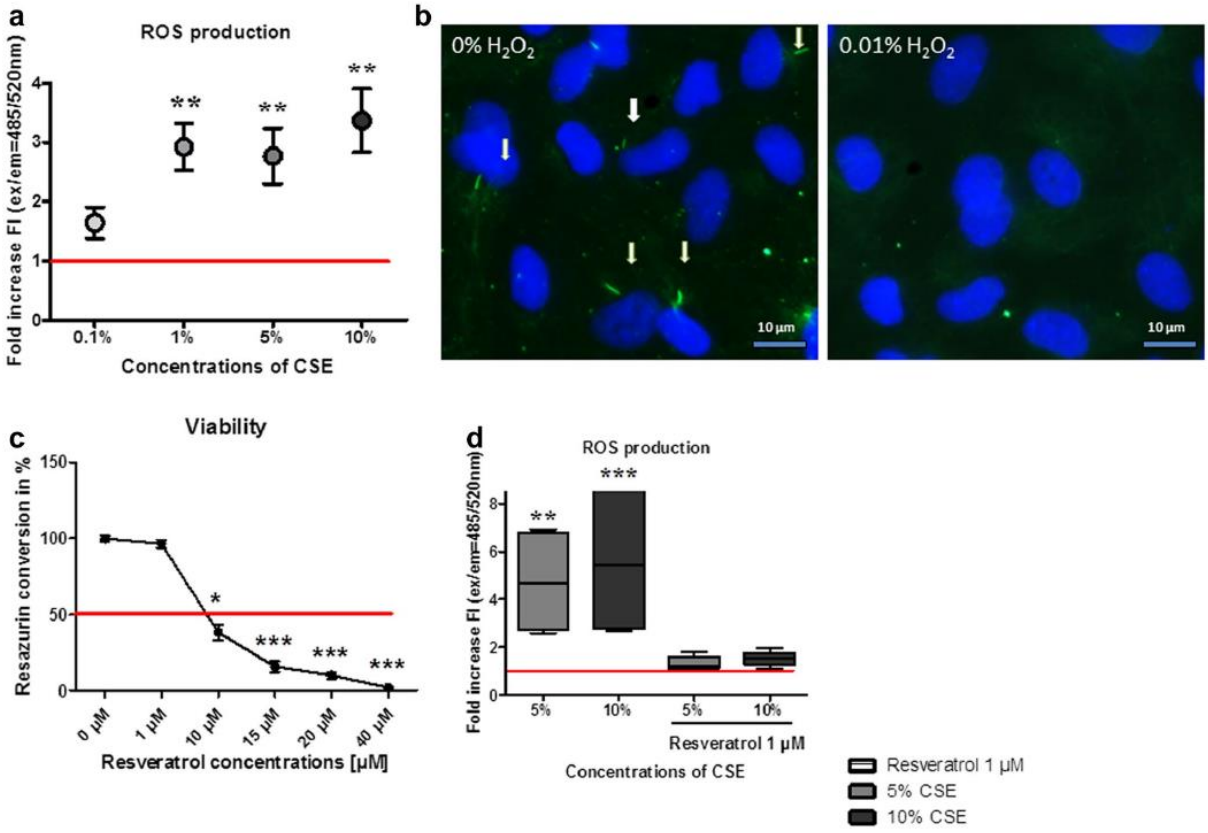

Fig. 6 Resveratrol treatment decreased ROS generated by CSE protecting the primary cilia integrity. ROS production of SCP-1 cells after treatment with different concentrations of CSE and co-incubation with resveratrol 1 µM was determined by DCFH-DA assay. **a** The graph represents fold increase in ROS production in CSE-treated cells compared to untreated cells (represented in red line). **b** Immunostained images of the SCP-1 cells in the presence and absence of 0.01% hydrogen peroxide. Primary cilia staining by acetylated α-tubulin (green) and nucleus by Hoechst (blue) is shown. Primary cilia are indicated by white arrows. **c** Graph here represents the viability indicated by the Resazurin conversion for SCP-1 cells cultured in differentiation medium and increasing concentrations of resveratrol at day 14. **d** Fold change in ROS production of SCP-1 cells treated with or without CSE and co-incubated with resveratrol 1 µM compared to untreated cells (represented in red line). For all quantifications ($N=3$, $n=2$), statistical significance was determined by Kruskal–Wallis H test followed by a Dunn's post-test. Data are represented as mean ± SEM and significance represented as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. (Color figure online)

system (Benowitz et al. 2009). There are conflicting results on the influence of nicotine on osteoblast proliferation and differentiation and is found mostly to be dose dependent (Benowitz et al. 2009; Daffner et al. 2012; Kim et al. 2012). Nevertheless, toxins contained in cigarettes have been shown to induce hypoxia, oxidative stress, inflammatory response, hormonal alterations, reduced calcium absorption, reduced blood supply, etc. which has profound roles in impairing bone regeneration (Barreiro et al. 2010; Church and Pryor 1985; Patel et al. 2013; Staempfli and Anderson 2009). These factors could be accountable for delayed fracture healing in smokers; however, the pathogenesis is not completely understood. Since osteogenic differentiation of MSCs is an early and critical event during fracture healing, we attempted to understand the long-term consequence of CSE containing physiological concentrations of cigarette smoke on the osteogenic differentiation of immortalised human mesenchymal stem cells (SCP-1).

We have previously reported that CSE has a direct toxic effect on primary osteoblasts (Braun et al. 2011). Additionally, in this study we confirm that exposure of CSE on the osteogenic precursor cells (SCP-1) for a period of 4 weeks (time taken for the SCP-1 to fully differentiate to osteoblasts), furthermore, compromised the osteogenic differentiation which was evident by the significant reduction in the proliferation, AP activity (early osteogenic marker) as well as matrix mineralisation (late osteogenic marker) in these cells (Huang et al. 2007). However, interestingly, we observed that the debilitated osteogenic differentiation of SCP-1 cells also correlated with a significant reduction

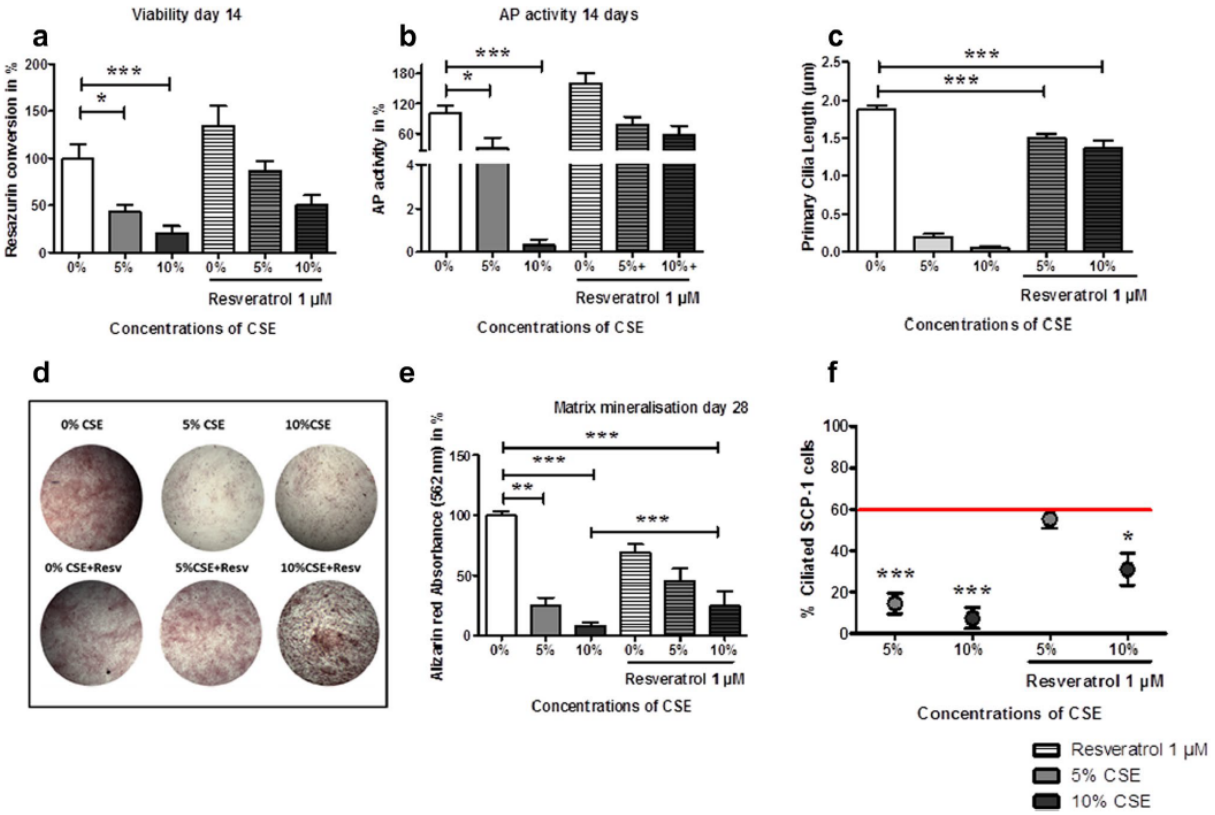

Fig. 7 Resveratrol enhanced osteogenic differentiation from SCP-1 cells exposure to CSE. SCP-1 cells were osteogenically differentiated by culturing them in SCP-1 differentiation media containing 0, 5 and 10% CSE and co-incubated with or without resveratrol 1 µM for 14 days. **a** Viability indicated by the resazurin conversion is shown for the different treatments at day 14. **b** The graph here represents the AP activity of SCP-1 cells undergoing osteogenic differentiation in the different conditions at day 14. **d** Alizarin red staining indicating the extent of matrix mineralisation of the cells after 28 days of differentiation is shown. **e** Quantification of the mineralised matrix by alizarin red staining is shown. **c** Graph here represents the quantification of primary cilia length of SCP-1 cells undergoing osteogenic differentiation with co-incubation CSE and resveratrol 1 µM for 14 days. **f** The percentage of ciliated SCP-1 cells was shown following osteogenic differentiation for 14 days with co-incubation CSE and resveratrol 1 µM compared to untreated cells (represented in red line). For all quantifications ($N=3$, $n=2$), statistical significance was determined by Kruskal–Wallis H test followed by Dunn's post-test. Data are represented as mean ± SEM and significance represented as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. (Color figure online)

of ciliated cells and the length of primary cilia. CS has been shown to shorten the epithelial airway cilia in smokers which accounts for reduced mucosal clearance (Leopold et al. 2009), however, its effects on primary cilia in osteoblasts have not been reported before. Though remained unacknowledged till 1990s, recent studies have found the central role of primary cilia in skeletal development and loss of which has been linked to skeletal deformities (Temiyasathit and Jacobs 2010; Xiao et al. 2008). Moreover, primary cilia are crucial for lineage commitment of stem cells (Tummala et al. 2010). Since we observed a significant reduction of primary cilia in SCP-1 cells during osteogenic differentiation with CSE, we pursued to investigate whether the loss of cilia integrity induced by the CSE could also contribute to impaired differentiation. As a first step, we examined how CSE could affect cilia length. It is a well-established fact that CS induces considerable oxidative stress by producing large amounts of free radicals (Church and Pryor 1985; Pappas 2011). ROS measurement in our samples exposed to CSE showed a significant increase in free radical production in a concentration-dependent manner. Furthermore, when we treated the SCP-1 with 0.01% of hydrogen peroxide which is a prime free radical present in the CSE, it caused a significant reduction in the primary cilia length in these cells. Therefore, we concluded that increased free radical production during smoking could be one of the responsible factors for the observed loss of cilia integrity and consequently could cause delayed fracture healing. Next, we also showed that loss of primary cilia can cause impaired differentiation by the pharmacological aberration of cilia by chloral hydrate. Furthermore, we performed a sequential time-based analysis of both the cilia integrity and loss of AP activity

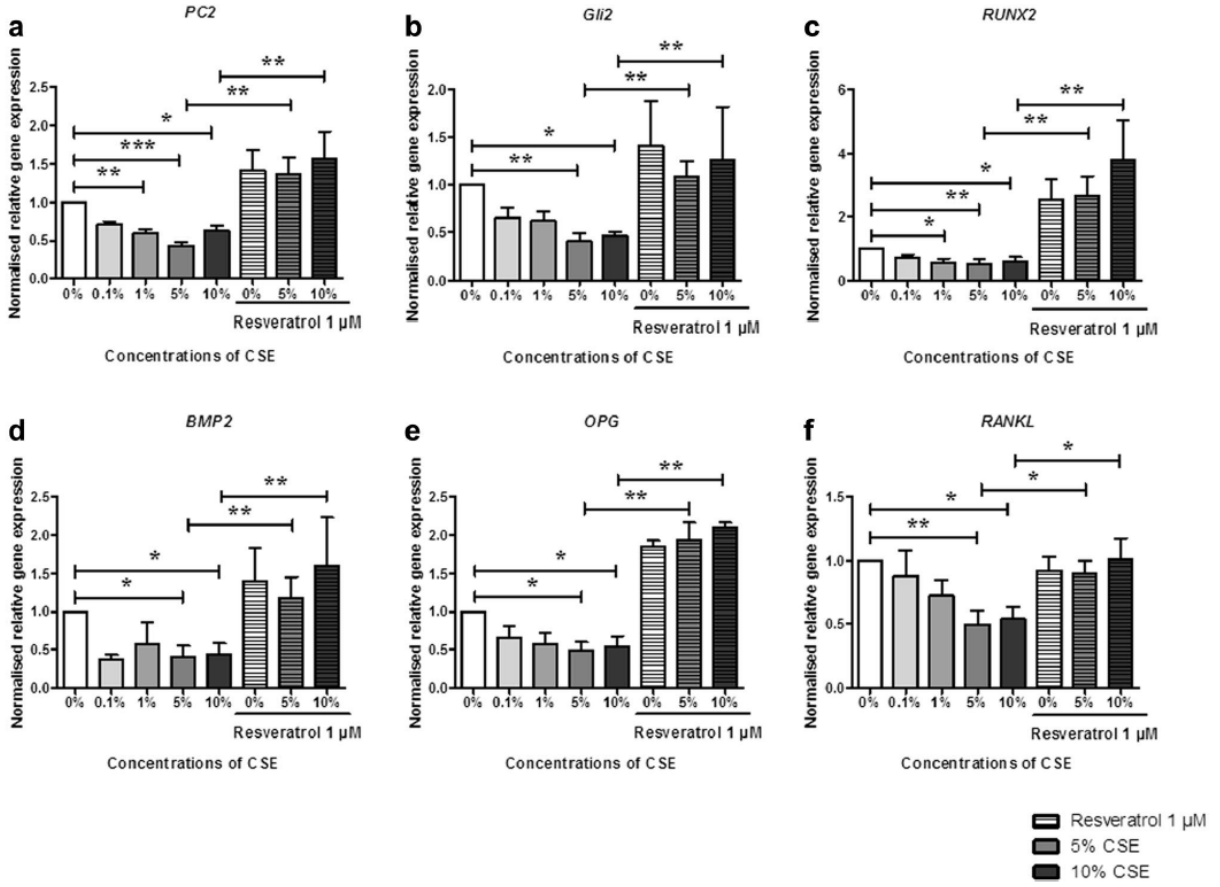

Fig. 8 Loss of primary cilia under CSE exposure affected Hedgehog signalling and target genes could be reversed by co-incubation with resveratrol. The graph represents the normalised (housekeeping *GAPDH*) relative (without CSE treatment) gene expression of **a** *PC2*, **b** *Gli2*, **c** *RUNX2*, **d** *BMP2*, **e** *OPG* and **f** *RANKL* after osteogenically differentiating SCP-1 cells for 14 days with or without CSE and resveratrol 1 µM. For all quantifications ($N=4$, $n=2$), statistical significance was determined by Kruskal–Wallis H test followed by a Dunn's post-test. Data are represented as mean ± SEM and significance represented as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$

during different days of osteogenic differentiation of SCP-1 cells in the presence of CSE. We observed significant reduction of primary cilia length but no significant loss of AP activity at day 9 of osteogenic differentiation which progressively worsened from day 10 onwards. From these results, we concluded that the impaired differentiation was the cause rather than the consequence of impaired differentiation.

Subsequently, we investigated the molecular changes caused by primary cilia loss which could directly or indirectly affect osteogenic differentiation of SCP-1 cells. Cilia-dependent Hh signalling cascade is a critical regulator of osteoblast differentiation and bone formation. Gene expression studies in our CSE-exposed cells showed a significant downregulation of primary cilia-specific *PC2* gene as well as the Hh transcription factor *Gli2*. It has been reported that activated Gli regulates bone morphogenetic protein (BMP-2) and runt-related transcription factor 2 (RUNX2) (Shimoyama et al. 2007; Zhao et al. 2006). Besides, BMP signalling acts downstream of the Hh pathway during osteoblast cell differentiation from perichondrial cells (Yang et al. 2015). Hh and BMP-2 synergistically stimulate osteoblast differentiation of mesenchymal stem cells, signifying the combined action of Hh and BMP-2 signalling in maintaining bone health (Zhao et al. 2006). Interestingly, exposure to CSE ≥ 1% resulted in a significant reduction in the expression of *BMP-2* and *RUNX2* alongside *Gli2* in our cells. Thus, the CSE-induced loss of primary cilia integrity possibly impaired the osteogenic differentiation of SCP-1 cells by downregulating the Hh signalling and its associated genes *BMP-2* and *RUNX2*. Furthermore, it has been shown that Hh regulates bone formation by controlling the *RANKL* expression (Mak et al. 2008) which was found to be also reduced in our CSE-treated cells. Likewise, CSE

treatment of SCP-1 cells also significantly reduced the *OPG* expression. Both OPG and RANKL are crucial for bone remodelling since they balance the osteoblast and osteoclast-mediated bone formation and resorption. Since treatment with CSE significantly reduced both *RANKL* and *OPG* expression in our cells, the overall bone homeostasis could be compromised. Consequently, CSE conceivably contributes to delayed fracture healing not only by impairing the early osteogenic differentiation of mesenchymal stem cells but also by disrupting the balance of mature osteoblast and osteoclast, thereby affecting the bone remodelling in fractured bones.

We here showed a novel toxic effect of cigarette smoke on the cellular sensors of osteoblasts and how it could also contribute to impaired osteogenic differentiation. As a next step, we wanted to reverse the ill effects of CSE using 1 µM resveratrol treatment. We decided to use this concentration of resveratrol treatment since it possesses potent antioxidative, bone-protective functions and moreover, the concentration used is normal physiological concentration achievable in human system (in vivo) without inducing any toxicity (Zunino and Storms 2015). Resveratrol treatment significantly reduced the free radical production and protected cilia against damage during osteogenic differentiation of the MSCs exposed to high concentrations of CSE (5 and 10%). Moreover, it also helped the SCP-1 cells survive better and for longer periods, improved its AP activity as well as improved differentiation which was evident with better matrix mineralisation an upregulation of *OPG* and *RUNX2* gene expression. This treatment also rescued the expression of Hg signalling factors, osteogenic transcription factor *RUNX2* and bone morphogenetic protein *BMP-2* which together resulted in achieving enhanced osteogenic differentiation.

Currently, fracture healing treatments involve two key phases—immobilisation or reparative phase (fixation of fracture) followed by remodelling phase (mechanical loading/physiotherapy) (Sfeir et al. 2005). Our data clearly suggest that restoration of primary cilia could ensure better differentiation and osteogenic lineage commitment of mesenchymal stem cells in the initial immobilisation phase of fracture treatment. Similarly, the success of remodelling treatment strategies such as physiotherapy, electric stimulation, and low-intensity pulsed ultrasound could also rest on the integrity of the primary cilia since they are the cellular sensors to perceive these signals. Taking cues from our study, future treatment options involving early intervention using resveratrol treatment (pill/injections) to restore primary cilia followed by additional rehabilitation measures could prove effective in accelerating fracture healing in smokers.

Conclusion

Our study addresses an additional impact of cigarette smoke on delayed fracture healing by loss of primary cilia integrity and impaired MSCs osteogenic differentiation. Maintenance and restoration of cilia integrity, and its molecular transductions could be critical in promoting the development of potential therapies to enhance fracture healing in smokers. Resveratrol treatment proved efficient in protecting and promoting the osteogenic differentiation of MSCs exposed to CSE.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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3.3 Nicotine and Cotinine Inhibit Catalase and Glutathione Reductase Activity Contributing to the Impaired Osteogenesis of SCP-1 Cells Exposed to Cigarette Smoke.

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3.3.1 Summary and major findings

The negative effect of cigarette smoke is often associated with alterations in oxidative stress levels (Barreiro *et al*., 2010, Munakata *et al*., 2018). The previous study (section 3.2) showed that chronic exposure to CSE impaired MSC osteogenic differentiation due to enhanced oxidative stress (Sreekumar *et al*., 2018). Therefore, in the present work, we evaluated the role of nicotine and its major metabolite cotinine on MSC osteogenic differentiation. We also explored which types of reactive oxygen species were promoted by CSE, nicotine, or cotinine and their effects in the antioxidative defense responses (Aspera-Werz *et al.*, 2018).

Nrf2 is the major antioxidative signaling pathway in human cells (Ma, 2013). Under a stress-free condition, Nrf2 is bound with Keap-1, an interaction that leads to ubiquitination follow proteasomal degradation in the cytoplasm (Guo *et al.*, 2017). In an oxidative stress condition, Nrf2 is phosphorylated and free in the cytoplasm due to stabilization of thiol groups in Keap-1, a change that disturbs its interaction to Nrf2 (Massague and Weis-Garcia, 1996, Aspera-Werz *et al.*, 2018). Phosphorylated and activated Nrf2 translocates into the nucleus and binds to partners such as the antioxidant response element. This action commences the expression of several antioxidative enzymes and other proteins involved in GSH homeostasis (Aspera-Werz *et al.*, 2018).

In our model, the treatment with nicotine and cotinine did not affect cell viability and osteogenic differentiation, assessed by AP activity (early marker) and calcium deposition (late marker) (Aspera-Werz *et al.*, 2018). In contrast, CSE exposure associated with smoking 10 cigarettes per day reduced (2.7-fold) cell viability and delayed MSC differentiation, denoted by reduced (2.13-fold) AP activity after 14 days and diminished (2.13-fold) matrix mineralization after 21 days (Aspera-Werz *et al.*, 2018).

Primary cilia are microtubule-based organelles that play an essential role in MSC osteogenic differentiation and the preservation of the cellular phenotype (Tummala *et al.*, 2010). Interesting, nicotine and cotinine did not affect primary cilia structure, in contrast to CSE exposure (a 0.60-fold reduction in primary cilia length). The detrimental effects of CSE on MSC osteogenesis were mainly due to increased ROS levels (2-fold), a fact that was not observed with nicotine and cotinine treatment (Aspera-Werz *et al.*, 2018).

Furthermore, we elucidated that $^{\cdot}O_2^-$ is the main ROS responsible for the oxidative stress generated by CSE exposure. Additionally, CSE decreased the main antioxidant present in the cells (GSH); these data demonstrated that CSE affects the antioxidant capacity of MSCs. Interestingly, the addition of L-ascorbate (antioxidant present in lower concentration in smokers (Ueta *et al.*, 2003)) or NAC (GSH precursor) entirely abolished the adverse effect of CSE on MSCs ROS levels, primary cilia structure and osteogenic differentiation (Aspera-Werz *et al.*, 2018).

On the molecular level, co-stimulation with NAC or L-ascorbate enhanced Nrf2 phosphorylation and SOD-1 and catalase protein synthesis. These results suggest that the antioxidant protective effect occurs through a Nrf2-dependent pathway and not only as a result of their free radical scavenging activities (Aspera-Werz *et al.*, 2018). Besides, SOD and catalase enzymatic activities were affected by the CSE stimulation. The observed changes in protein expression and activity may partly explain the accumulation of $^{\cdot}O_2^-$ and H_2O_2 in the CSE-treated cells (Aspera-Werz *et al.*, 2018). However, CSE significantly increased GPX activity (with also catalyzes the reduction of H_2O_2 to H_2O *via* oxidation of GSH) and significantly reduced GR activity, triggering an unsuitable GSH recycling under smoking conditions (Aspera-Werz *et al.*, 2018). Additionally, nicotine and cotinine significant reduced catalase and GR activity (Aspera-Werz *et al*., 2018). In summary, by inhibiting catalase and GR function, nicotine and cotinine enhance the ROS levels observed in CSE treated cells and partially contribute to the negative effects on MSCs impair osteogenic differentiation with CSE.

3.3.2 Personal Contribution

I participated in the conception of the experiments. I performed most of the experiments, analyzed the data and wrote the manuscript draft. I critically revised the manuscript according to the reviewers' suggestions.

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Research Article

Nicotine and Cotinine Inhibit Catalase and Glutathione Reductase Activity Contributing to the Impaired Osteogenesis of SCP-1 Cells Exposed to Cigarette Smoke

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Cigarette smoking has been identified as a major risk factor for osteoporosis decades ago. Several studies have shown a direct relationship between cigarette smoking, decreased bone mineral density, and impaired fracture healing. However, the mechanisms behind impaired fracture healing and cigarette smoking are yet to be elucidated. Migration and osteogenesis of mesenchymal stem/stromal cells (MSCs) into the fracture site play a vital role in the process of fracture healing. In human nicotine, the most pharmacologically active and major addictive component present in tobacco gets rapidly metabolized to the more stable cotinine. This study demonstrates that physiological concentrations of both nicotine and cotinine do not affect the osteogenic differentiation of MSCs. However, cigarette smoke exposure induces oxidative stress by increasing superoxide radicals and reducing intracellular glutathione in MSCs, negatively affecting osteogenic differentiation. Although, not actively producing reactive oxygen species (ROS) nicotine and cotinine inhibit catalase and glutathione reductase activity, contributing to an accumulation of ROS by cigarette smoke exposure. Coincubation with N-acetylcysteine or L-ascorbate improves impaired osteogenesis caused by cigarette smoke exposure by both activation of nuclear factor erythroid 2-related factor 2 (Nrf2) signaling and scavenging of ROS, which thus might represent therapeutic targets to support fracture healing in smokers.

1. Introduction

Smoking is the most common method of consuming tobacco which is the most popular substance smoked. For that, tobacco is combusted and the smoke with the active substances is inhaled. Smoking cigarettes represents a major health risk, which increases morbidity and mortality. It affects the whole human body and is linked to various health disorders. Deleterious effects of cigarette smoking on bone integrity have been shown, with a positive correlation between the number of cigarettes smoked per day and the years of exposure. Furthermore, smoking affects patients submitted to orthopedic surgery negatively by delaying the fracture healing, increasing the frequency of complications, and prolonging the hospital stay [1, 2].

Over 150 of the 6000 molecular species present in cigarette smoke have been identified as toxic compounds [3, 4]. It is still not completely elucidated which of these compounds are responsible for the negative effects of smoking on bone metabolism and fracture healing. Nicotine is the most pharmacologically active component present in tobacco which directly and indirectly affects cellular metabolism. Several studies have shown dose-dependent positive effects of nicotine on proliferation and differentiation of mesenchymal stem/stromal cells (MSCs) [5, 6]. However, the concentration of nicotine in these *in vitro* studies was much

lower than the concentration found in blood samples from smokers [5]. Other studies revealed negative effects of nicotine on MSC proliferation as well as differentiation [7, 8]. Therefore, the effect of nicotine on the osteogenic differentiation of MSCs still remains unclear.

Cotinine is the most important metabolite of nicotine. 70–80% of nicotine is converted to cotinine in the liver. This metabolite is also present in the blood from smokers, with an average of 250–300 ng/ml cotinine which reaches higher blood levels than nicotine (50–100 ng/ml), which might be due to the longer half-life of cotinine (nicotine 2 h, cotinine 16 h) [9].

Recently, we reported that oxidative stress induced by cigarette smoke extract (CSE) [10] could be one of the responsible factors for the impaired osteogenic differentiation of SCP-1 cells. Coincubation with the antioxidant resveratrol protected the SCP-1 cells from the CSE deleterious effect [11]. However, the underlying mechanisms are not completely understood.

Nuclear factor erythroid-2-related factor-2 (Nrf2) signaling is known as a major mechanism in the cellular defense against oxidative stress which is activated in response to stress conditions [12]. In an unstressed condition, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH associating protein 1 (Keap-1) [13] which favors its proteasomal degradation. Under stress conditions, Keap-1 changes its structure by stabilizing its thiol groups, which interferes with its binding to Nrf2. Free in the cytoplasm, Nrf2 is activated [14] and translocates into the nucleus, where it binds to the antioxidant response element (ARE) in the promoter region of genes, e.g., antioxidative enzymes and genes involved in glutathione (GSH) homeostasis, regulating their expression. Some studies in mice have shown that disruption of Nrf2 impairs the induction of cellular defense pathways and increases the negative effects of oxidative stress induced by cigarette smoke [15]. Moreover, upregulating Nrf2 signaling by knockdown of Keap-1 increases antioxidative defense and diminishes lung injury caused by smoking [16]. However, there are controversial findings on the roles of antioxidant signaling pathways on bone metabolism under oxidative stress. On the one hand, it was shown that MC3T3 cells exposed to H_2O_2 activation of Nrf2 signaling negatively affect osteogenic differentiation—a mechanism inhibited by N-acetylcysteine (NAC) [17]. On the other hand, deletion of Nrf2 in bone tissue leads to a poor bone mineral density not only due to increased osteoclast activity but also because of a lack of functional osteoblasts [18, 19].

Up to now, it is not known if and how nicotine and cotinine affect the osteogenic differentiation of MSCs. Therefore, the aim of the present study was to evaluate the effect of nicotine and cotinine on MSCs during osteogenic differentiation and, furthermore, to investigate which type of reactive oxygen species (ROS) is induced by CSE, nicotine, or cotinine and how these substances affect the cell response to oxidative stress.

2. Materials and Methods

Anti-acetylated-α-tubulin, anti-SOD-1, and anti-rabbit HRP-labeled secondary antibodies were obtained from Santa Cruz (SC-23950, SC-11407, and SC-2004; Heidelberg, Germany). Anti-GAPDH antibody was obtained from Sigma-Aldrich (G9545; Munich, Germany). Anti-phospho-p38 MAPKinase and anti-catalase antibodies were obtained from Cell Signaling (Frankfurt am Main, Germany). Anti-phospho-Nrf2 antibody was obtained from Abcam (ab76026; Cambridge, United Kingdom). Alexa Fluor 448-labeled secondary antibody was obtained from Invitrogen (Karlsruhe, Germany). N-Acetylcysteine (NAC) and nicotine were obtained from Carl Roth (Karlsruhe, Germany). L-Ascorbic acid was obtained from Sigma-Aldrich (Darmstadt, Germany). Cotinine was obtained from Alfa Aesar (Karlsruhe, Germany).

2.1. Generation of Cigarette Smoke Extract (CSE). CSE was freshly prepared for every experiment. In total, the smoke of two commercial cigarettes (Marlboro, Philip Morris, New York City, USA) containing 0.8 mg nicotine and 10 mg tar each was continuously bubbled through a 50 ml prewarmed SCP-1 differentiation medium (0% FCS) in a standard gas wash bottle, as described before [11]. The CSE was normalized by its optical density at 320 nm (OD_{320}), with an OD_{320} of 0.7 considered 100% CSE [20]. After sterile filtration (0.22 μm pore filter), the CSE was diluted with SCP-1 differentiation medium to receive 5% *V*/*V* CSE, which corresponds to exposures associated with smoking up to 10 cigarettes/day [21].

2.2. Culture and Osteogenic Differentiation of SCP-1 Cells. Human immortalized mesenchymal stem cells (SCP-1 cells, provided by Dr. Matthias Schieker [22]) were cultured in MEM alpha medium (10% *V*/*V* FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin) in a water-saturated atmosphere of 5% CO_2 at 37°C [23]. SCP-1 cells were osteogenically differentiated for 21 days in MEM alpha medium (1% *V*/*V* FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 200 μM L-ascorbate-2-phosphate, 10 mM β-glycerol-phosphate, 25 mM HEPES, 1.5 mM $CaCl_2$, and 100 nM dexamethasone). The medium was changed every 3–4 days.

2.3. Resazurin Conversion Assay. Cell viability (mitochondrial activity) was measured by Resazurin conversion assay. Briefly, cells were covered with 0.0025% *W*/*V* Resazurin in PBS. After 30 min incubation at 37°C, the resulting Resorufin fluorescence was measured (excitation = 544 nm/emission = 590 nm) as described [24, 25]. The incubation time was optimized based on the high metabolic activity of SCP-1 cells.

2.4. Sulforhodamine B (SRB) Staining to Assess Total Protein Content. Total protein content was determined by SRB staining of ethanol-fixed (1 h at −20°C) cells. Cells were stained with 0.4% *W*/*V* SRB (in 1% *V*/*V* acetic acid) for 20 min at ambient temperature. Cells were washed 4–5 times with 1% acetic acid to remove unbound SRB. Bound SRB was resolved with 10 mM unbuffered TRIS solution (pH ~10.5). Resulting absorption (λ = 565 nm) was determined with a plate reader [26].

2.5. Alkaline Phosphatase (AP) Activity Assay. AP activity was measured as an early osteogenic marker. Briefly, cells were incubated with AP reaction buffer (0.2% *W*/*V* 4-nitrophenyl-phosphate, 50 mM glycine, 1 mM $MgCl_2$, 100 mM TRIS, and pH 10.5) for 30 min at 37°C. Formed 4-nitrophenol was determined photometrically ($\lambda = 405$ nm) as described and normalized to relative cell numbers by SRB staining. Changes in AP activity are displayed relative to untreated cells [24, 25].

2.6. Alizarin Red Staining. Matrix mineralization was measured as a late osteogenic marker. Cells were fixed with ice-cold ethanol for 1 h. After washing with tap water, cells were incubated with 0.5% *W*/*V* Alizarin Red solution (pH 4.0) for 30 min at ambient temperature. After 3 additional washing steps, the resulting staining (red) was assessed microscopically. After resolving the stain with 10% *W*/*V* cetylpyridinium chloride, Alizarin Red staining was quantified photometrically at $\lambda = 562$ nm [24, 25].

2.7. Immunofluorescence Staining. Cells were fixed with 4% *V*/*V* paraformaldehyde solution and permeabilized with 0.2% *V*/*V* Triton-X-100 for 10 min each. Unspecific binding sites were blocked with 5% *W*/*V* BSA for 1 h. Incubation with primary antibodies (1 : 100) was performed overnight at 4°C, followed by incubation with ALEXA488 labeled secondary antibodies (1 : 2.000) for 1 h. Images were taken with a fluorescence microscope (EVOS FL AF 4301, Life Technologies, Darmstadt, Germany). The excitation and emission wavelengths were used as specified by the manufacturer. Pictures were analyzed using the ImageJ software (line tool) (National Institute of Health, Bethesda, USA) by 4 independent investigators in a blinded fashion. Based on the microscopic pictures taken, cilia length was determined by the maximum intensity projection method [27].

2.8. Determination of ROS Levels. To measure the formation of ROS, different fluorescent probes were used [28]:

(i) For the most unspecific 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) assay, cells were incubated with 10 μM DCFH-DA for 25 min at 37°C. After washing twice with PBS, cells were stimulated with CSE according to the experimental setup. As positive control, SCP-1 cells were stimulated with 0.01% *V*/*V* (882 μM) H_2O_2. After 0, 5, 10, and 15 min, the increase in fluorescence (excitation = 485 nm/emission = 520 nm) was detected by a plate reader, representing levels of $^{\cdot}O_2^-$, H_2O_2, $HO^{\cdot}$, and $ONOO^-$ [29]. To trap the ROS, cells were coincubated with either 25 μM α-tocopherol ($^{\cdot}O_{2i}^-$), 10 mM sodium-pyruvate (H_2O_{2i}), 250 mM DMSO ($HO^{\cdot}_i$), or 100 μM uric acid ($ONOO^-_i$) [28]

(ii) To determine $^{\cdot}O_2^-$ levels, cells were incubated with 10 μM dihydroethidium (DHE) for 25 min at 37°C. After washing twice with PBS, cells were stimulated with CSE according to the experimental setup. As negative control (assay specificity), SCP-1 cells were stimulated with 0.01% *V*/*V* (882 μM) H_2O_2. After 0, 5, 10, and 15 min, the increase in fluorescence (excitation = 544 nm/emission 590 nm) was detected by a plate reader. The slope of the linear part of the curve, resembling the product formation rate, was calculated. Cellular localization of the fluorescence was confirmed by fluorescence microscopy

2.9. Determination of Total Glutathione. The total GSH measurement was performed according to the Ellman assay: after stimulation, protein precipitation of cellular lysates was carried out with 3% *W*/*V* m-phosphoric acid. The protein samples were reneutralized with 5 mM EDTA in 0.1 M potassium phosphate buffer (pH = 7.4), and the total GSH was determined. For the determination, 20 μl of sample was incubated for 30 seconds with 120 μl of a mixture (1 : 1) of 1.68 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 2.5 U/ml glutathione reductase in 0.1 M potassium phosphate buffer. Then, 60 μl of NADPH 0.8 mM was added and absorbance was measured at $\lambda = 412$ nm for 15 min [30].

2.10. Western Blot Analysis. Cells were lysed in freshly prepared ice-cold RIPA buffer. 30 μg total protein was separated by SDS page and transferred to nitrocellulose membranes. Membranes were blocked with 5% *W*/*V* BSA for 1 h. After overnight incubation with primary antibodies (1 : 1.000) at 4°C, membranes were incubated with the corresponding peroxidase-labeled secondary antibodies (1 : 10.000) for 2 h at ambient temperature. For signal development, membranes were incubated for 1 min with ECL substrate solution. Chemiluminescent signals were quantified using the ImageJ software [24].

2.11. Catalase Activity Assay. The catalase activity was measured with the fluorometric catalase activity kit OxiSelect (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Fluorescence was measured at 544 nm (λex) and 590 nm (λem) [31].

2.12. Superoxide Dismutase Activity Assay. In order to measure the SOD activity, SOD from HepG2 cells and a commercially available kit (Sigma-Aldrich, Taufkirchen, Germany) were used according to the manufacturer's protocol. Absorbance was measured at 450 nm every 5 min over 30 min [31].

2.13. Glutathione Peroxidase (GPx) Activity Assay. The measurement of GPx activity was performed using cumene hydroperoxide as a substrate for GPx. 5 μl of 1 U/ml GPx was mixed with 5 μl of each sample, 15 μl of 4 mM NADPH, and 75 μl of GPx assay solution (1.33 U/ml glutathione reductase, 1.33 mM L-glutathione reduced in 0.05 mM potassium phosphate buffer (pH = 7.0) containing 1.1 mM EDTA and 1.1 mM NaN_3) and incubated at RT for 5 min. Then, 10 μl of 15 mM cumene hydroperoxide solution was added and the decrease in absorbance at $\lambda = 340$ nm was measured within a 15 min time interval [30, 32].

2.14. Glutathione Reductase (GR) Activity Assay. The GR activity was measured by the increase in the absorbance due to the reduction of 5′-dithiobis-(2-nitrobenzoic acid) to 5-thio-(2-nitrobenzoic acid). 2.5 μl of 1 U/ml GR and 2.5 μl of

each sample were mixed with 185 μl reaction mixture (0.8 mM DTNB, 0.1 mM NADPH, and 1 M EDTA (1 M) in 0.2 M potassium phosphate buffer (pH = 7.5)) and 10 μl of 20 mM L-glutathione (oxidized). Then, the increase in absorbance at $\lambda = 412$ nm was measured of a time interval of 15 min [30, 33].

2.15. Statistics. Results are expressed as the bar chart (mean ± SEM) of at least 4 independent experiments ($N \geq 4$) measured as triplicate or more ($n \geq 3$). Data sets were compared by the Kruskal-Wallis H test followed by Dunn's multiple comparison test (GraphPad Software Inc., La Jolla, CA, USA). $p < 0.05$ was taken as the minimum level of significance.

3. Results

3.1. Effect of Nicotine and Its Primary Metabolite Cotinine on Osteogenic Differentiation of SCP-1 Cells. In order to determine which components present in cigarette smoke are responsible for impaired osteogenic differentiation of MSCs exposed to CSE [11], SCP-1 cells were treated with nicotine and its first metabolite cotinine. Nicotine and cotinine were applied to the cells at concentrations ranging from 50 ng/ml to 320 ng/ml and 100 ng/ml to 300 ng/ml, respectively. These concentrations were chosen based on reported blood levels of nicotine and cotinine from smokers and the calculated amount from our CSE. Nicotine concentration in arterial blood from smokers ranks between 20 and 60 ng/ml and rises up to 100 ng/ml after smoking one cigarette [9]. The average of cotinine in blood from smokers is 250–300 ng/ml [9]. The theoretical concentration of nicotine and cotinine in our CSE is 160 ng/ml and 150 ng/ml, respectively [11]. Therefore, SCP-1 cells were osteogenically differentiated for 21 days, in the presence of nicotine and cotinine, with concentrations up to 320 ng/ml and 300 ng/ml, respectively. Based on our previous work, 5% CSE was used as control. After osteogenic differentiation, effects on the mitochondrial activity (an indirect indicator of viability and proliferation) were measured by Resazurin conversion (Figure 1(a)). Mitochondrial activity of SCP-1 cells exposed to nicotine and cotinine was not significantly affected (Figure 1(a)). The differentiation status of the SCP-1 cells was evaluated by AP activity and matrix mineralization: AP activity, an early marker of osteogenic differentiation [34], after 14 days and the production of matrix mineralization, a late marker of osteogenic differentiation [34], after 21 days. Similar to the viability of the cells, the AP activity and the matrix production were not affected by nicotine and cotinine (Figures 1(b) and 1(c)).

Primary cilia, a microtubule-based organelle, have been shown to play an important role in the initiation of osteogenic differentiation of MSCs and also in the maintenance of function in the differentiated cells [35]. Therefore, the primary cilium structure was assessed by immunofluorescence staining of acetylated α-tubulin on SCP-1 cells differentiated in the presence of nicotine or cotinine for 21 days. Primary cilia on SCP-1 cells exposed to CSE showed a reduction of 62.5% in the length. However, nicotine and cotinine exposure did not affect the primary cilia structure of SCP-1 cells (Figure 1(d)). Representative immunofluorescence staining pictures of primary cilia are shown in Supplementary Figure 1.

Since the production of ROS is one of the best known negative consequences of cigarette smoking, we were interested in evaluating the effect of nicotine and cotinine in ROS production by SCP-1 cells. After exposure to CSE, the production of ROS by SCP-1 cells significantly increased; however, ROS levels did not increase after nicotine exposure. Surprisingly, its first metabolite showed a slight (not significant) increase in ROS production by SCP-1 cells, which seemed to be dose-dependent, concluding that nicotine and its first metabolite, cotinine, are not the direct effectors inducing ROS production in SCP-1 cells exposed to CSE. However, this data does not exclude that both substances might interfere with the cells' antioxidative defense mechanisms and thus indirectly favor the accumulation of ROS in the presence of CSE.

3.2. CSE Induced Oxidative Stress by Increasing $^{\cdot}O_2^-$ and Reducing GST Activity in SCP-1 Cells. In order to better identify the ROS formed by CSE exposure, a DCFH-DA assay with several radical scavengers was performed [28, 31]. Exposure to CSE significantly (2 fold) induced ROS levels, measured by DCFH-DA assay. Incubation with 25 μM α-tocopherol, which traps $^{\cdot}O_2^-$, significantly reduced ROS levels in SCP-1 cells exposed to CSE. However, scavengers of H_2O_2 (10 mM sodium-pyruvate), $HO^{\cdot}$ (250 mM DMSO), and $ONOO^-$ (100 μM uric acid) could not significantly reduce ROS levels in SCP-1 cells exposed to CSE (Figure 2(a)). In order to confirm that $^{\cdot}O_2^-$ is induced by CSE in SCP-1 cells, a dihydroethidium (DHE) assay was performed. CSE exposure, but not nicotine and cotinine, significantly increased (2.5 fold) the level of $^{\cdot}O_2^-$ (Figure 2(b)). Since GSH is the master antioxidant present in mammalian cells to prevent damage caused by ROS, the total GSH was measured by Ellman assay. SCP-1 cells exposed to CSE significantly decreased total GSH levels; nevertheless, nicotine and cotinine did not affect total GSH levels (Figure 2(c)). Thus, increased levels of $^{\cdot}O2^-$ and decreased total GSH affect the antioxidant capacity of SCP-1 cells exposed to CSE.

3.3. Antioxidants Rescued CSE-Impaired Osteogenesis in SCP-1 Cells. The overproduction of ROS as well as the decrease of intracellular GSH, beyond the antioxidant scavenging capacity of the cells, causes oxidative stress that disrupts the primary cilia structure, which in turn impairs osteogenic differentiation of SCP-1 cells [11]. Previous studies have demonstrated the positive effects of NAC and L-ascorbate on osteogenesis [17, 36–42]. Therefore, to protect SCP-1 cells from oxidative stress generated by CSE, the effect of NAC and L-ascorbate during osteogenic differentiation with CSE was evaluated. In order to determine the concentrations of NAC and L-ascorbate that were not toxic to the cells, SCP-1 cells were osteogenically differentiated in the presence of NAC (1 mM–30 mM) or L-ascorbate (200 μM, 1 mM) for 14 days. According to the mitochondrial activity and the total protein staining of the cells, the concentrations of 1 mM NAC and 200 μM L-ascorbate were used in the following

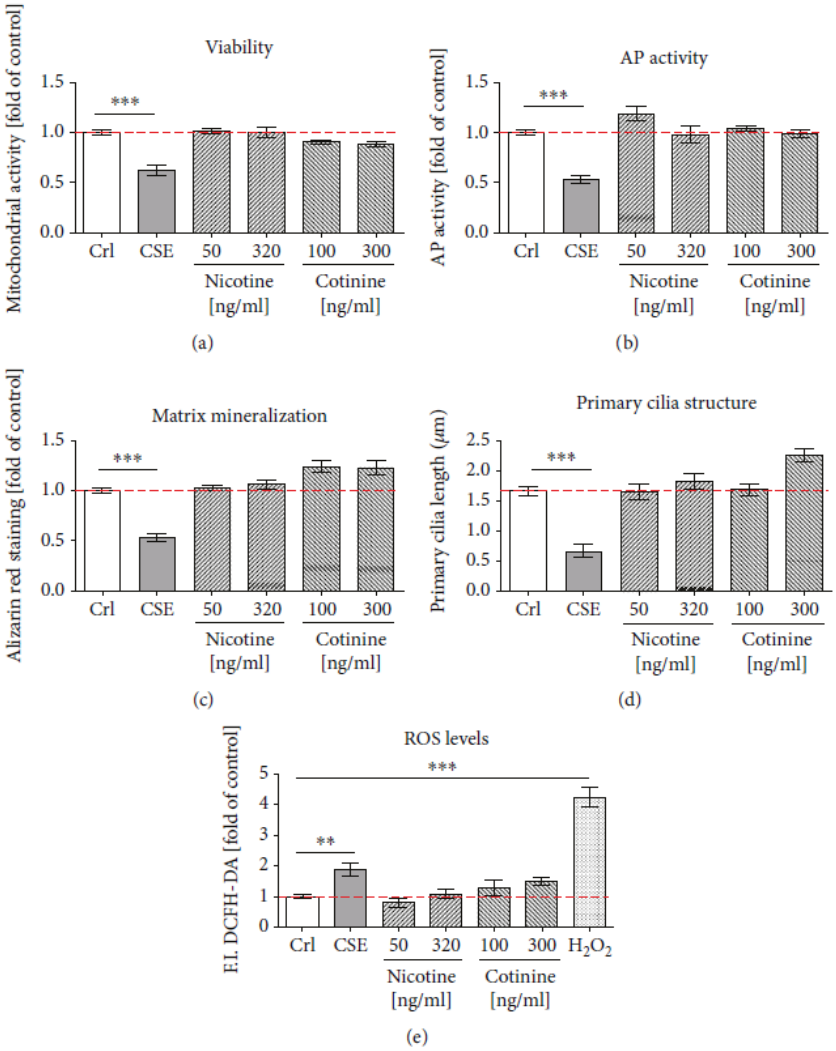

FIGURE 1: Nicotine and cotinine do not affect osteogenic differentiation of SCP-1. SCP-1 cells were osteogenically differentiated with nicotine (50 and 320 ng/ml) or its primary metabolite cotinine (100 and 300 ng/ml). Cell viability by Resazurin conversion (a) and AP activity (b) was measured on day 14. (c) Matrix mineralization was evaluated by Alizarin red after 21 days. (d) Primary cilium length was measured on day 21. (e) DCFH-DA assay was used to detect ROS in SCP-1 cells exposed to nicotine and cotinine. 0.01% *V*/*V* H_2O_2 was used as a positive control. Each experiment was conducted at least four times independently with triplicate. The statistical significance was determined by the Kruskal-Wallis H test followed by Dunn's posttest. Data are represented as the mean ± SEM, and the significance was represented as $^{***}p < 0.001$ vs the control group.

experiments (Supplementary Figure 2). NAC and L-ascorbate significantly increased the mitochondrial activity of SCP-1 cells after 14 days of coincubation with CSE (Figure 3(a)). Their addition upregulated the AP activity and the matrix mineralization of SCP-1 cells differentiated with CSE after 14 and 21 days, respectively (Figures 3(b) and 3(c)). NAC and L-ascorbate restored the primary cilium structure on SCP-1 cells that was altered by CSE exposure (Figure 3(d)). Moreover, treatment with NAC or L-ascorbate significantly decreased ROS levels in SCP-1 cells exposed to CSE (Figure 3(e)). These results suggest that antioxidant treatment enhances primary cilium integrity and improves the osteogenic differentiation of SCP-1 cells exposed to CSE by decreasing oxidative stress.

3.4. Nrf2 Signaling Was Activated by NAC and L-Ascorbate in SCP-1 Cells during Osteogenic Differentiation with CSE. Several studies have shown that redox-sensitive transcription factor Nrf2 plays an important role in cellular defense against oxidative stress by inducing the transcription of antioxidative enzymes [43, 44]. Therefore, the protective effect of the Nrf2 signaling pathway on SCP-1 cells, which were osteogenically differentiated and treated with antioxidants, was investigated. Western blot for phospho-Nrf2 and phospho-p38 MAPKinase was performed from SCP-1 cells differentiated with 5% CSE and 1 mM NAC or 200 μM L-ascorbate. CSE exposure significantly increased the active form of Nrf2 in response to the oxidative stress (Figure 4(a)). Costimulation with NAC or L-ascorbate increased the levels of phospho-

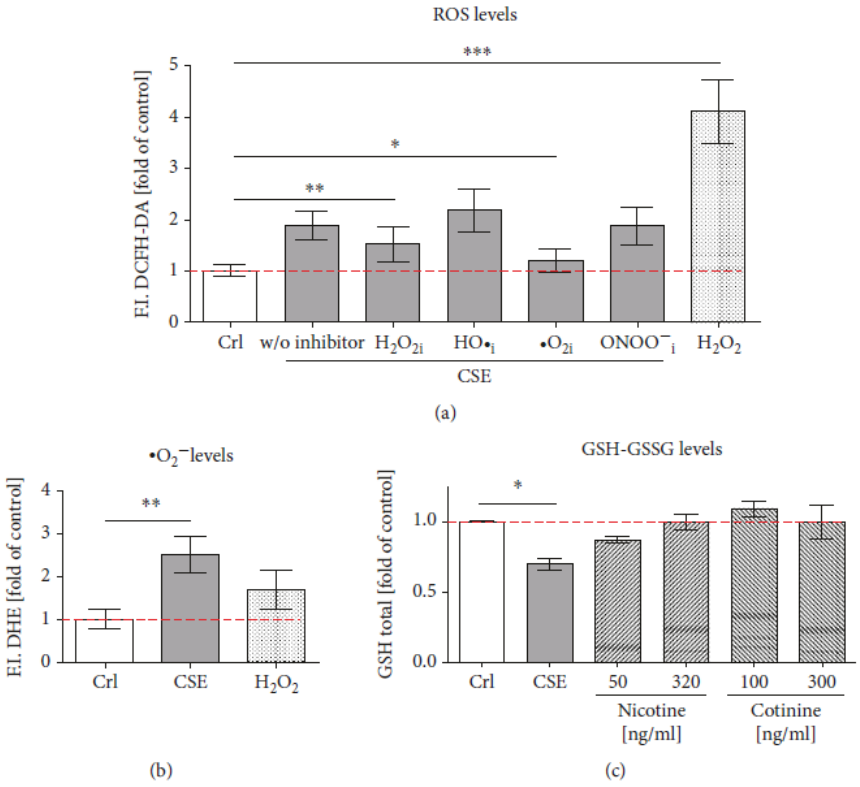

FIGURE 2: CSE induces oxidative stress with an increase in $^{\cdot}O_2^-$ and GSH reduction in SCP-1. SCP-1 cells were exposed to 5% CSE, and intracellular ROS and GSH levels were measured with different fluorescent probes: (a) DCFH-DA assay was used to detect $^{\cdot}O_2^-$, H_2O_2, $HO^{\cdot}$, and $ONOO^-$. To trap different ROS, SCP-1 cells were coincubated with 25 μM α-tocopherol ($^{\cdot}O_2^-$ i), 10 mM sodium-pyruvate (H_2O_2i), 250 mM DMSO ($HO^{\cdot}$i), or 100 μM uric acid ($ONOO^-$ i); (b) DHE assay was used to detect $^{\cdot}O_2^-$, and (c) Ellman assay was used to detect total GSH levels. Results were normalized to control SCP-1 cells (Crl). 0.01% V/V H_2O_2 was used as positive control (a) or negative control (b) of the assays. Each experiment was conducted at least four times independently with triplicate. The statistical significance was determined by the Kruskal-Wallis H test followed by Dunn's posttest. Data are represented as the mean ± SEM, and the significance was represented as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs the control group.

Nrf2 compared with SCP-1 cells exposed to CSE alone (Figure 4(a)), suggesting that the NAC and L-ascorbate protective effect is through an Nrf2-dependent mechanism and not only due to their radical scavenging properties. Besides, NAC coincubation with CSE significantly increased the levels of phospho-p38 MAPKinase (Figure 4(b)). Therefore, NAC-dependent activation of Nrf2 could be via the activation of protein kinases such as p38, causing phosphorylation and subsequent release of Nrf2 from its inhibitory protein Keap-1 (Figure 5). However, coincubation of CSE with L-ascorbate had no effect on phospho-p38 MAPKinase levels (Figure 4(b)), suggesting that L-ascorbate might react with the thiol residues in Keap-1, increasing the cellular availability of Nrf2 (Figure 5). Since the activation of the transcription factor Nrf2 was induced by antioxidant treatment, it was interesting to investigate the expression of SOD-1 and catalase, the target enzymes of this pathway. SOD-1 catalyzes the dismutation of two molecules of $^{\cdot}O_2^-$ to H_2O_2 and molecular oxygen O_2 for further processing. Catalase catalyzes the reduction of H_2O_2 to water and O_2, completing the detoxification process initiated by SOD-1 [45]. Similarly, to Nrf2 activation, SOD-1 and catalase protein expression was upregulated by NAC and L-ascorbate coincubation with CSE (Figure 4(c) and 4(d)). Thus, an increase of SOD-1 and catalase initiated by Nrf2 might be involved in the protective effect of NAC and L-ascorbate during osteogenic differentiation of SCP-1 cells exposed to CSE. Representative Western blot pictures are shown in Supplementary Figure 3.

3.5. CSE, Nicotine, and Cotinine Generated an Imbalance in the Antioxidative System. Since CSE exposure activated Nrf2 signaling and induced the protein expression of antioxidative enzymes, it was interesting to evaluate the effect of CSE on the activity of enzymes involved in $^{\cdot}O_2^-$ detoxification and GSH recycling. Thus, we investigated the effect of CSE, nicotine, and cotinine on the activity of the isolated enzymes involved in mitochondrial antioxidative defense, namely, SOD, catalase, GPx, and GR. Interestingly, the presence of CSE significantly decreased the activity of catalase (Figure 6(a)) and slightly decreased the enzymatic activity of SOD (Figure 6(b)). This might explain the observed accumulation of $^{\cdot}O_2^-$ and H_2O_2 in the CSE-treated cells.

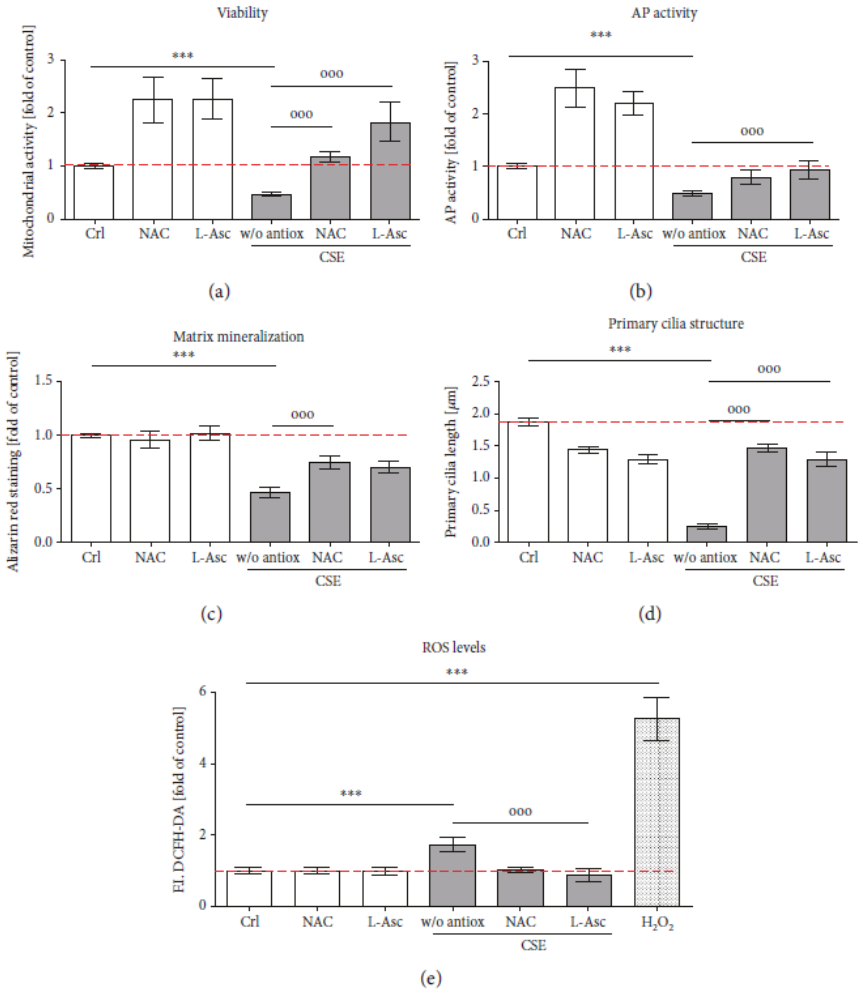

FIGURE 3: Antioxidants rescue CSE-impaired osteogenesis in SCP-1. SCP-1 cells were osteogenically differentiated with coincubation of antioxidants NAC 1 mM or L-Asc 200 μM and CSE 5%. After 14 days of treatment, (a) the viability of the cells was measured by Resazurin conversion. The differentiation status of the cells was evaluated by (b) AP activity at day 14 and (c) Alizarin red staining at day 21. (d) Primary cilium length was measured at day 21. (e) DCFH-DA assay was used to detect ROS in SCP-1 cells exposed to 5% CSE and coincubation of antioxidants NAC 1 mM or L-Asc 200 μM. 0.01% V/V H_2O_2 was used as positive control. Each experiment was conducted at least four times independently with triplicate. The statistical significance was determined by the Kruskal-Wallis H test followed by Dunn's posttest. Data are represented as the mean ± SEM, and the significance was represented as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs control and $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, and $^{\circ\circ\circ}p < 0.001$ vs CSE.

However, GPx can also catalyze the reduction of H_2O_2 to H_2O via oxidation of reduced GSH into its disulfide form. The presence of CSE significantly increased GPx enzymatic activity (Figure 6(c)). At the same time, the presence of CSE significantly decreased the total GSH (Figure 2(c)) and significantly decreased the GR activity (Figure 6(d)), causing that there is no GSH availed in cells exposed to CSE. Nicotine and cotinine, despite not affecting the osteogenic differentiation of the cells, evidenced significant inhibitory effects on the enzymatic activity of catalase and GR. Therefore, an imbalance in the antioxidative system induced by the most addictive substance, its first metabolite, and other molecules present in cigarette smoke affects the osteogenic differentiation of SCP-1 cells.

4. Discussion

Cigarette smoke contains more than 150 of 6000 molecular substances that are considered toxic compounds [3, 4]. Nicotine is known as the major active and addictive compound associated with smoking. The role of nicotine on osteogenic differentiation is still unclear, as different effects have been shown dependent on the concentration and exposure time. On the one hand, it has been shown that nicotine induces apoptosis in human osteoblasts via increased ROS levels [8]. Moreover, reduced matrix formation was observed with SaOS-2 cells exposed to nicotine for 14 days [7]. On the other hand, it has been reported that nicotine increases osteoblast activity in bone marrow stromal cells [5]. In our experiments,

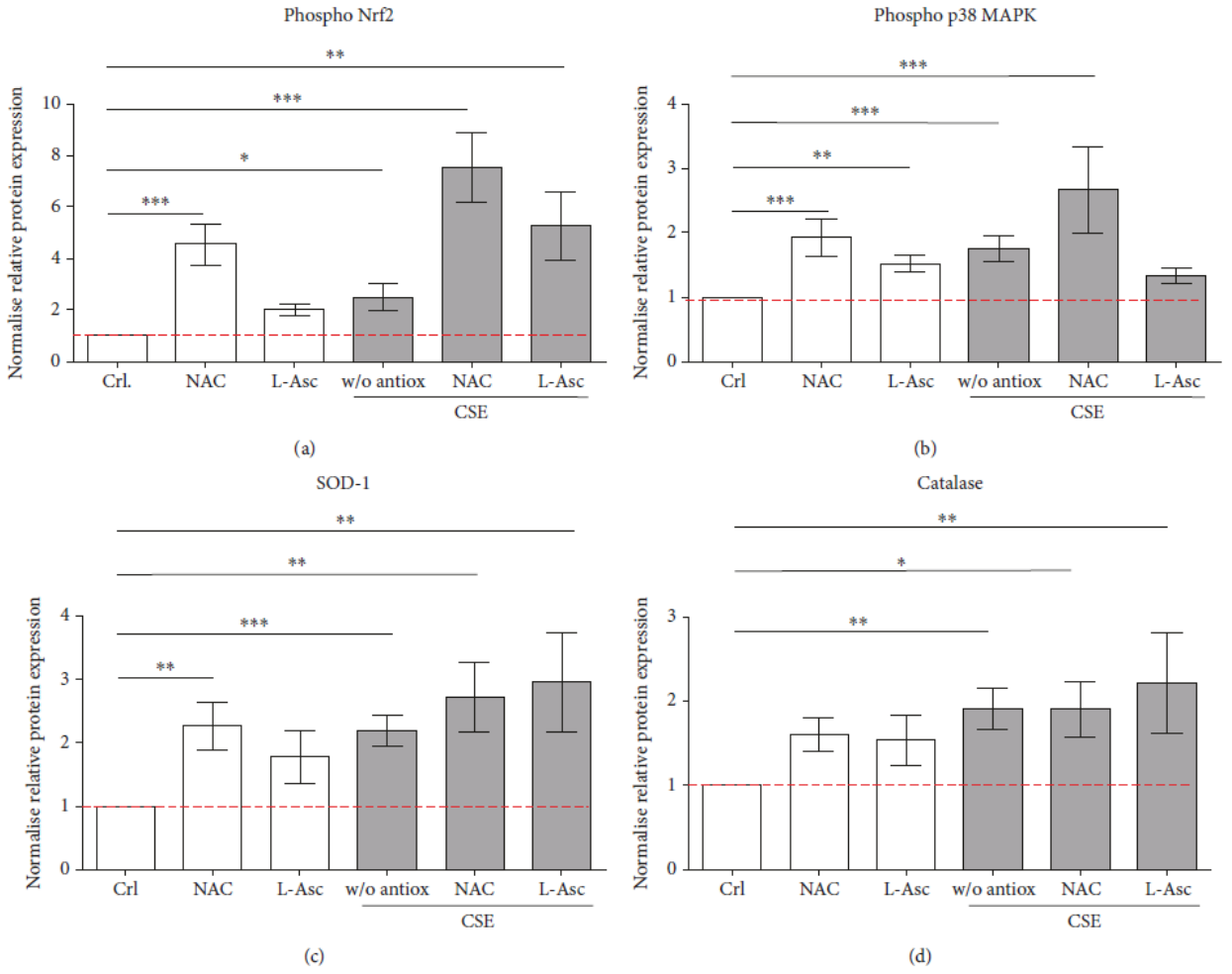

FIGURE 4: Nrf2-related signaling was activated by NAC and L-ascorbate in SCP-1 during osteogenic differentiation with CSE. SCP-1 cells were osteogenically differentiated with coincubation of antioxidants NAC 1 mM or L-Asc 200 μM and CSE 5%. After 14 days of treatment, phosphorylated Nrf2 (a), p38 MAPKinase (b), SOD-1 (c), and catalase (d) protein expression levels were detected by Western blot. GAPDH was used as internal control. Each experiment was conducted at least three times independently with triplicate. The statistical significance was determined by the Kruskal-Wallis H test followed by Dunn's posttest. Data are represented as the mean ± SEM, and the significance was represented as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs the control group.

blood nicotine concentrations did not affect the osteogenic differentiation of SCP-1 cells; besides, its first metabolite cotinine also did not influence the osteogenic differentiation of SCP-1 cells. These results point out the negative role of ROS induced by CSE during osteogenic differentiation. In the clinic, smokers often show delayed fracture healing, increased frequency of complications, and prolonged hospital stays [1, 2]. This led to the assumption that devices that administer nicotine, e.g., e-cigarettes, tobacco heat systems, nicotine sprays, or tapes, could be a less harmful alternative for smoking in orthopedic patients.

In line with this assumption, our results clearly show that only CSE exposure, but not nicotine and cotinine, increases the levels of $^{\cdot}O_2^{-}$ while reducing the total GSH available in the cells. From these results, we endorse previous suggestions that an imbalance in the antioxidative system could be responsible for the impaired osteogenesis observed in SCP-1 cells after CSE exposure [46–48].

Since we observed a significant reduction in total GSH levels and a significant increase in ROS levels upon CSE exposure, we decided to use NAC as a precursor for GSH to support the osteogenic differentiation of SCP-1 cells exposed to CSE. NAC is a derivative of the amino acid L-cysteine, in which the thiol (sulfhydryl) group exhibits antioxidative effects by scavenging free radicals [49]. Previous studies have shown the beneficial effects of NAC on osteogenic differentiation [37–39]. Moreover, NAC has been shown to reverse the negative effects of H_2O_2 on osteogenic differentiation of MC3T3-E1 cells [17]. We used L-ascorbate as a potential treatment strategy due to the fact that smokers present lower levels of L-ascorbate in the blood than nonsmokers [50, 51]. Therefore, smokers require a higher

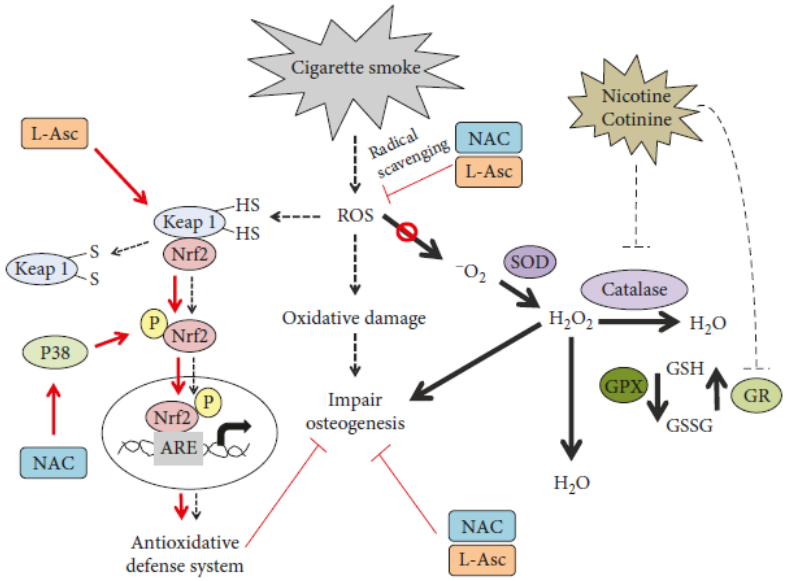

FIGURE 5: NAC and L-ascorbate enhance the osteogenic differentiation in SCP-1 cells exposed to CSE by activation of Nrf-2 signaling and through radical scavenging. Proposed mechanisms for oxidative stress impair osteogenic differentiation under CSE exposure and potential roles of antioxidant. High-level oxidative stress generated by CSE resulted in oxidative damage and impaired SCP-1 cells' osteogenic differentiation. ROS induced through CSE exposure can oxidize the Cys residues on Keap-1, leading to the conformational change and releasing Nrf2. Phospho-Nrf2 can translocate to the nucleus and activates the antioxidant response element (ARE) leading to an activation of antioxidant genes. However, activation of Nrf2 in CSE exposure cells may not be enough to protect the cells from the oxidative stress generated by CSE. NAC activates upstream p38 MAPKinase, which is required to activate Nrf2 and transactivate antioxidant genes that may reduce oxidative stress induced by CSE. L-Asc might act with thiol residues of Keap-1, increasing the levels of Nrf2 available. CSE inhibited catalase activity being not able to process H_2O_2. GR activity is also affected by CSE to a decrease of total GSH. NAC and L-Asc treatment decreased CSE-induced ROS production by increasing the biosynthesis of GSH via Nrf2 signaling and also by radical scavenging. CSE decreased the enzymatic activity of SOD and catalase, leading to accumulation of $^{\cdot}O_2^{-}$ and H_2O_2 in the cells. Additionally, CSE decreased total GSH and decreased GR activity causing that there is no GSH availed. Therefore, GPx cannot catalyze the reduction of H_2O_2 to H_2O. Nicotine and cotinine, despite not affecting the osteogenic differentiation of the cells, evidenced negative inhibitory effects on the enzymatic activity of catalase and GR. Nicotine and cotinine imbalance the antioxidative system contributing in part to the negative effects in the osteogenic differentiation of SCP-1 cell exposure to CSE.

daily intake of L-ascorbate to reach similar blood levels than nonsmokers due to an increased metabolic demand and a defective L-ascorbate recycling [51, 52]. Furthermore, L-ascorbate is known for stimulating proliferation of osteoblasts as well as for possessing free radical scavenging properties [41, 42]. Besides its antioxidative properties, L-ascorbate has been shown to support bone formation by stimulating the production of collagen [40]. Indeed, concentrations of 1 mM NAC and 200 μM L-ascorbate improved the differentiation of SCP-1 cells coincubated with CSE, resulting in an increased AP activity on day 14 of differentiation and an increased amount of mineralized matrix formed after 21 days of differentiation. The concentrations of L-ascorbate and NAC used in this study were higher than those found in blood plasma that can be obtained by oral supplementation (L-ascorbate 3 g every 4 h administered orally produces a peak plasma concentration of 200 μM [53]; NAC 600 mg oral administration produces a peak plasma concentration of 15 μM [54]). However, intravenous doses of L-ascorbate and NAC can produce plasma concentrations up to 800 μM [53] and 121 μM [55], respectively, suggesting the possibility of implementing intravenous applications of antioxidant treatment in smokers during the surgical reposition of fractures or even for a limited time during the process of fracture healing.

However, ROS levels are necessary to induce cellular responses. ROS induced by CSE exposure can oxidize the Cys residues on Keap-1, causing its conformational change and releasing Nrf2 for activation. The active phospho-Nrf2 can then translocate to the nucleus and, upon binding to the antioxidant response element (ARE), lead to the transcription of antioxidant enzymes like SOD, catalase, GPx, and others [56]. The impaired osteogenic function observed in the CSE-exposed SCP-1 cells suggests that a mechanism in this process fails or that the activation of Nrf2 in CSE-exposed cells may not be enough to protect the cells from the oxidative stress generated by CSE.

Addition of antioxidants like NAC and L-ascorbate partly restored the osteogenic differentiation of CSE-exposed SCP-1 cells. NAC and L-ascorbate treatment decreased CSE-induced ROS production. However, despite a strong activation of p38 MAPKinase signaling, which is

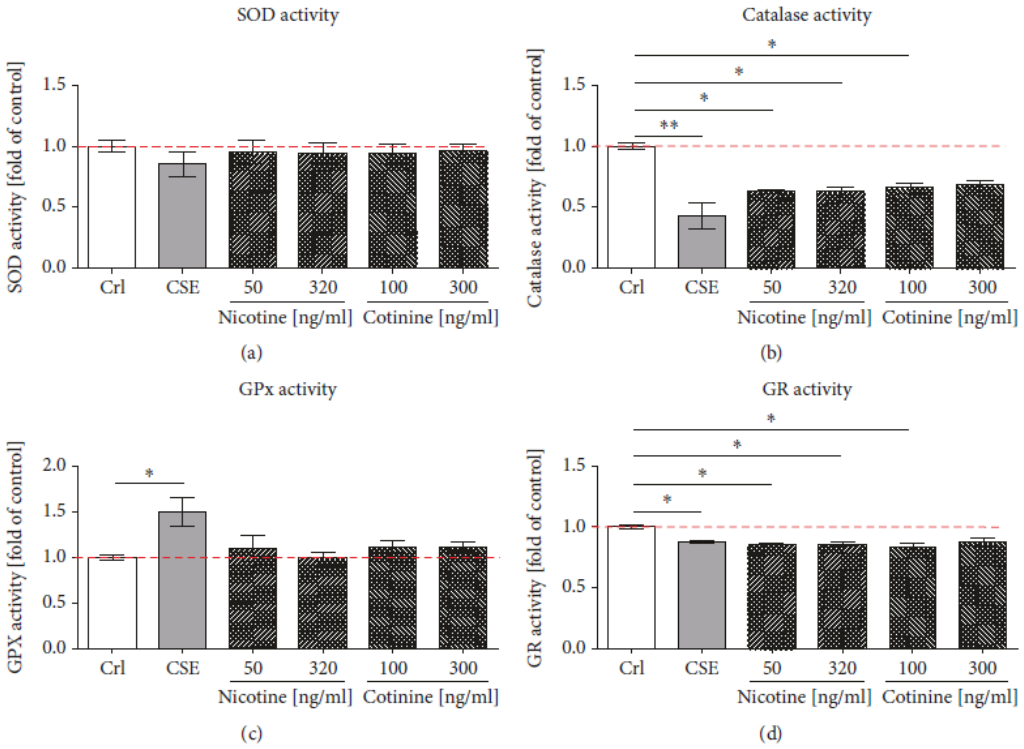

FIGURE 6: CSE generated an imbalance in the antioxidative system. Enzyme activities SOD (a), catalase (b), GPx (c), and GR (d) were determined with and without exposure to 5% CSE, 50 ng/ml or 320 ng/ml nicotine, and 100 ng/ml or 300 ng/ml cotinine. The enzymatic activity was expressed as the fold of control. Each experiment was conducted at least three times independently with triplicate. The statistical significance was determined by the Kruskal-Wallis H test followed by Dunn's posttest. Data are represented as the mean ± SEM, and the significance was represented as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs the control group.

required for the activation of Nrf2 [57, 58], NAC treatment could not significantly increase protein levels of activated Nrf2 or antioxidative enzymes, when compared to CSE exposure alone. Similarly, addition of L-ascorbate which might react with the thiol residues in Keap-1, increasing the Nrf2 availability, could not further increase the cellular levels of phospho-Nrf2 and antioxidative enzymes, when compared to CSE exposure alone.

In response to the oxidative stress induced by CSE exposure, we have shown that CSE can induce the antioxidant signaling pathways via its transcription factor phospho-Nrf2 and increase the protein expression of SOD-1 and catalase, suggesting that the cells react adequately to the stress stimuli. SOD and catalase are strongly upregulated during osteogenic differentiation of MSCs [59], suggesting key regulatory roles of these enzymes. In low amounts, H_2O_2 is involved in many cellular processes such as activation of signaling pathways involved in cell migration, proliferation, and differentiation [60]. Additionally, H_2O_2 has been shown to induce osteogenic differentiation of vascular smooth muscle cells through the increase of Runt-related transcription factor 2 (Runx2) a key transcription factor for osteogenesis [61]. However, with increasing amounts, H_2O_2 exerts damage to cellular macromolecules including proteins and DNA consequently, causing cell death [62–64].

Even so, SOD and catalase protein expression was induced upon stimulation with CSE, an impaired osteogenic function was still observed, suggesting that the function of the antioxidative enzymes during the differentiation with CSE fails. We could show that CSE as well as nicotine and cotinine strongly inhibits catalase activity, suggesting that the inhibitory effect of CSE on the enzymatic activity of catalase and SOD, contributed in part from nicotine and cotinine. This supposedly generated a feedback where the cells produce more antioxidative enzymes. However, in the presence of nicotine and cotinine, these enzymes cannot perform their function properly; thus, both substances seem to indirectly participate in the observed ROS accumulation by CSE. SOD being less affected converts $^{\cdot}O_2^-$ and H_2O_2 which then accumulates in the cells as catalase is not able to further process the H_2O_2. Nicotine and cotinine, despite having no effect on osteogenic differentiation, also decreased catalase activity contributing to the negative effect observed with CSE, supporting previous results that demonstrated lower enzymatic activity of antioxidant enzymes in blood of smokers [65]. As GPx activity is not negatively affected by CSE, nicotine, or cotinine, it is conceivable that upregulation of GPx could compensate for the inhibition of catalase in the CSE-exposed cells. However, similar to catalase, GR activity is also affected

by CSE, nicotine, and cotinine. Thus, these substances interfere with glutathione recycling, limiting the substrate for GPx and thus leading to a decrease in total GSH.

The observation that nicotine and cotinine inhibit catalase and GR function critically challenges the assumption that devices administering nicotine could be a less harmful alternative for smoking in orthopedic patients, as the trauma itself and the associated surgical intervention represent (oxidative) stress the body has to cope with.

5. Conclusions

In summary, our study shows for the first time that nicotine and cotinine do not directly affect osteogenic differentiation of MSCs; however, these compounds negatively affect the function of the antioxidative enzymes. Therefore, the most addictive compound present in cigarette smoke and its first metabolite contribute in part to the negative effects on osteogenic differentiation observed in MSC following CSE exposure.

Data Availability

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

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Romina H. Aspera-Werz and Sabrina Ehnert contributed equally to this work.

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Supplementary Materials

Supplementary File 1. Supplementary Figure 1: nicotine and cotinine not affected hMSC primary cilia structure. Immunostained images of primary cilia in SCP-1 cells osteogenically differentiated at day 14 without CSE (a), with 5% CSE (b), 320 ng/ml nicotine (c), and 300 ng/ml cotinine (d) exposed are shown. Primary cilium is shown by acetylated α-tubulin (green) and nucleus by Hoechst staining.

Supplementary File 2. Supplementary Figure 2: toxicity test of NAC and L-Asc in hMSC. SCP-1 cells were osteogenically differentiated with increasing concentrations of NAC (a, b) and L-Asc (c, d). The toxicity was measure by Resazurin conversion (mitochondrial activity) (a–c) and SRB staining (total protein measure) (b–d) after 14 days. Each experiment was conducted at least four times independently with triplicate. The statistical significance was determined by the Kruskal-Wallis H test followed by Dunn's posttest. Data are represented as the mean ± SEM, and the significance is represented as $^{***}p < 0.001$ vs the control group.

Supplementary File 3. Supplementary Figure 3: protein expression analysis of antioxidative enzymes. Representative Western blot pictures from phosphorylated Nrf2, phosphorylated p38 MAPKinase, SOD-1, catalase, and GAPDH are shown. SCP-1 cells were osteogenically differentiated with coincubation of antioxidants NAC 1 mM or L-Asc 200 μM and CSE 5%. After 14 days of treatment, protein expression level was detected.

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3.4 Cigarette Smoke Induces the Risk of Metabolic Bone Diseases: Transforming Growth Factor Beta Signaling Impairment *via* Dysfunctional Primary Cilia Affects Migration, Proliferation, and Differentiation of Human Mesenchymal Stem Cells.

Aspera-Werz, R.; Chen, T.; Ehnert, S.; Zhu, S.; Frohlich, T.; Nussler, A. *Int J Mol Sci*, 2019.

3.4.1 Summary and Major Findings

Orthopedic patients who smoke present lower TGF-β serum-levels compared with non-smokers (Poniatowski *et al.*, 2015, Moghaddam *et al.*, 2010). When a fracture occurs, cytokine levels rise during the inflammatory courses with the aim of attract MSCs to the fracture gap. Beside, TGF-β positively influences MSC chondrogenic differentiation, a process that produces a cartilage intermediate that is replaced by bone during fracture healing (Bahney *et al.*, 2015). Previous research has reported a direct association between retarded fracture repair and lower TGF-β levels at this stage in smokers (Poniatowski *et al.*, 2015, Zimmermann *et al.*, 2005). The following paper aimed to elucidate the role of cigarette smoke on TGF-β signaling as well as to identify at which step the pathway was disrupted (Aspera-Werz *et al.*, 2019).

An adenoviral reporter assay displayed that in MSCs, CSE downregulated TGF-β signaling *via* Smad2/3 dose-dependently. Additionally, chemical abrogation of primary cilia structure with CH decreased TGF-β signaling similarly to CSE exposure (Aspera-Werz *et al.*, 2019). A rescue experiment with resveratrol reversed the CSE-mediated detrimental effects by protecting the primary cilia integrity and, consequently, enhancing TGF-β signaling. Interestingly, the reduces TGF-β signaling *via* CSE correlated with decreased protein synthesis and activation of downstream effectors (phospho-Smad2/3) as well as associated cofactors (Smad4). Furthermore, CSE exposure and pharmacological abrogation of primary cilia downregulated the transport to the nucleus of the active phospho-Smad3/4 complex (Aspera-Werz *et al.*, 2019). This outcome emphasizes that any intranuclear translocation mechanisms counteract for the reduced phospho-Smad2/3 and Smad4 protein levels and further distribute the signal to the nucleus. We confirmed that impair TGF-β signaling during CSE treatment was circumstantiated by the lack in the kinase activity of the receptor ALK5 and not

due to the unsuccessful interaction of the ligand to the receptor (Aspera-Werz *et al.*, 2019).

Additionally, CSE-modulated TGF-β signaling disruption negatively influenced MSC migration and proliferation. However, TGF-β treatment improved MSC migration, but it could not reverse the negative influence of CSE on MSC proliferation (Aspera-Werz *et al.*, 2019). We then investigated transcription levels of genes associated with chondrogenic phenotype on MSCs differentiated with CSE under TGF-β signaling induction or CH treatment. CSE downregulated *collagen II* (*COL2A1*), a major ECM protein in cartilage, and upregulated the hypertrophic marker *collagen X* (*COLXA1*), the transcriptional factor *SOX9*, and the main component of the cartilage ECM, *aggrecan* (*ACAN*). Deteriorated TGF-β signaling *via* primary cilia structure disturbance displayed an analogous expression pattern to CSE exposure (Aspera-Werz *et al.*, 2019).

Our results demonstrated that MSCs differentiation to chondrogenic linage was negatively regulated by impair TGF-β signaling with CSE (Aspera-Werz *et al.*, 2019). Consequently, MSCs migration and proliferation were also affected (Aspera-Werz *et al.*, 2019). Identification of treatment options that improve the canonical TGF-β signaling pathway or activate non-canonical Smad phosphorylation might open up new targets for the development of therapeutics to promote fracture repair in smokers compared to TGF-β supplementation.

3.4.2 Personal Contribution

I was responsible for the experimental setup and helped perform the experiments. I analyzed the data and was responsible for its visual presentation. I was responsible for writing the manuscript. I carried out experiments and critically revised the manuscript according to the reviewers' suggestions

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Cigarette Smoke Induces the Risk of Metabolic Bone Diseases: Transforming Growth Factor Beta Signaling Impairment via Dysfunctional Primary Cilia Affects Migration, Proliferation, and Differentiation of Human Mesenchymal Stem Cells

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Abstract: It is well established that smoking has detrimental effects on bone integrity and is a preventable risk factor for metabolic bone disorders. Following orthopedic surgeries, smokers frequently show delayed fracture healing associated with many complications, which results in prolonged hospital stays. One crucial factor responsible for fracture repair is the recruitment and differentiation of mesenchymal stem cells (MSCs) at early stages, a mechanism mediated by transforming growth factor β (TGF-β). Although it is known that smokers frequently have decreased TGF-β levels, little is known about the actual signaling occurring in these patients. We investigated the effect of cigarette smoke on TGF-β signaling in MSCs to evaluate which step in the pathway is affected by cigarette smoke extract (CSE). Single-cell-derived human mesenchymal stem cell line (SCP-1 cells) were treated with CSE concentrations associated with smoking up to 20 cigarettes a day. TGF-β signaling was analyzed using an adenovirus-based reporter assay system. Primary cilia structure and downstream TGF-β signaling modulators (Smad2, Smad3, and Smad4) were analyzed by Western blot and immunofluorescence staining. CSE exposure significantly reduced TGF-β signaling. Intriguingly, we observed that protein levels of phospho-Smad2/3 (active forms) as well as nuclear translocation of the phospho-Smad3/4 complex decreased after CSE exposure, phenomena that affected signal propagation. CSE exposure reduced the activation of TGF-β modulators under constitutive activation of TGF-β receptor type I (ALK5), evidencing that CSE affects signaling downstream of the ALK5 receptor but not the binding of the cytokine to the receptor itself. CSE-mediated TGF-β signaling impaired MSC migration, proliferation, and differentiation and ultimately affected endochondral ossification. Thus, we conclude that CSE-mediated disruption of TGF-β signaling in MSCs is partially responsible for delayed fracture healing in smokers.

Keywords: cigarette smoke; TGF-β signaling; MSCs; smokers; fracture; primary cilia; bone metabolic diseases; osteoporosis; Smad signaling

1. Introduction

Cigarette smoking (CS) continues to be the leading global cause of preventable death. In 2030, CS will cause 8 million deaths per year worldwide, according to the World Health Organization. Tabaco consumption denotes a major health risk that affects the entire human body and is linked to

various health disorders, including coronary heart disease, chronic obstructive pulmonary disease, cerebrovascular disease, and cancer [1,2].

Interestingly, CS also affects bone integrity, with a positive correlation between years of exposure and the number of cigarettes smoked per day [1,3]. Furthermore, smokers submitted to orthopedic surgery have an increased risk of delayed fracture healing, complications (e.g., infections and non-union fractures), and longer hospital stays [1,3]. CS is considered one of the main social risk factors for developing metabolic bone diseases [3–6]. Metabolic bone diseases describe a diverse group of pathologies (e.g., osteoporosis, Paget disease, Rickets, osteomalacia, diabetic osteopathy) that impair bone remodeling for different reasons: impaired bone formation by osteoblasts, enhanced bone degradation by osteoclasts, or a combination of both. Osteoporosis is the most prevalent metabolic bone disease, characterized by decreased bone strength and increased risk of fractures [7]. CS significantly increases the likelihood of developing osteoporosis [8].

Fractures in patients suffering from metabolic bone diseases are associated with delayed or non-union fracture healing. In these patients there is an imbalance in bone-forming and bone-resorbing cells [9,10]. Transforming growth factor β (TGF-β) is a multifactorial regulatory protein that has several effects on mesenchymal stem cells (MSC), e.g., migration, proliferation, and differentiation [11,12].

Once TGF-β is released from the bone matrix, the signaling pathway commences with binding of the ligand to the TGF-β receptor complex. This action activates the canonical (Smad-dependent) TGF-β signaling pathway, which regulates the transcription of several target genes [13]. The TGF-β receptor complex can also activate a non-canonical, Smad-independent pathway that involves other factors, such as mitogen-activated protein kinase pathways (extracellular-signal-regulated kinases, c-Jun N-terminal kinase, and p38), Rho-like Guanosine triphosphate hydrolase enzymes signaling pathways, or phosphatidylinositol-3-kinase/Protein kinase B pathways [14].

Besides the direct stimulation of MSCs, osteoblasts, and chondrocytes, and inhibition of osteoclasts, TGF-β can boost the effect of other factors, like bone morphogenetic proteins (BMPs) and insulin-like growth factors that assist in fracture healing [15–17].

Canonical TGF-β signaling is reportedly partially controlled by the microtubule base organelle (primary cilia) in stem cells that differentiate into cardiomyocytes; proper downstream activation is reliant on clathrin-dependent endocytosis at the cilia pocket region [18]. This organelle can coordinate the activity of multiple signaling pathways during tissue development and homeostasis [19]. Mutations, as well as small interfering RNA (siRNA) that affect the intraflagellar transport system (IFT) in primary cilia, impair signaling and the primary cilia structure [20,21]. Mutations in the *IFT88* gene reduce TGF-β-mediated Smad2/3 activation, results that demonstrate the primary cilia structure is indispensable for the correct functioning of the pathway [22]. Additionally, depleted *IFT88* in MSCs reduces TGF-β-induced migration [23]. Our previous studies demonstrated that exposure to cigarette smoke extract (CSE) affects osteoblast function and impairs MSC osteogenic differentiation. Interestingly, CSE exposure also affects the primary cilia structure in these cells during differentiation [24–28].

Surprisingly, smokers present lower serum TGF-β concentrations than non-smokers [13,29]. After a fracture, TGF-β levels increase during endochondral ossification in order to attract MSCs to form the cartilage callus, which is later systematically replaced with mineralized tissue by differentiated MSCs [13]. At this stage, smokers show a positive correlation between decreased TGF-β levels and delayed fracture healing [13,15].

However, it is still not known how CS affects the TGF-β signaling pathway. Therefore, the purpose of this study was to elucidate the effects of CSE on TGF-β signaling and how it influences the migration, proliferation, and appropriate differentiation of MSCs.

2. Results

2.1. CSE Downregulated TGF-β Signaling Through Disruption of Primary Cilia on SCP-1 Cells

Previous studies revealed that exposure to CSE disrupts the primary cilia structure and therefore impairs the osteogenic differentiation of the human telomerase reverse transcriptase immortalized single-cell human mesenchymal cell line (SCP-1 cells) [24,25].

SCP-1 cells infected with an adenoviral-based reporter construct (Ad5-CAGA9-MLP-Luciferase) were exposed to CSE for 24 h, followed by induction of the TGF-β pathway with rhTGF-β1. These cells exhibited a dose-dependent reduction in TGF-β signaling; there was statistical significance at 10% *v/v* CSE (Figure 1a). Induction of Smad2/3 signaling was evaluated by measuring luciferase activity in protein lysates from SCP-1 cells.

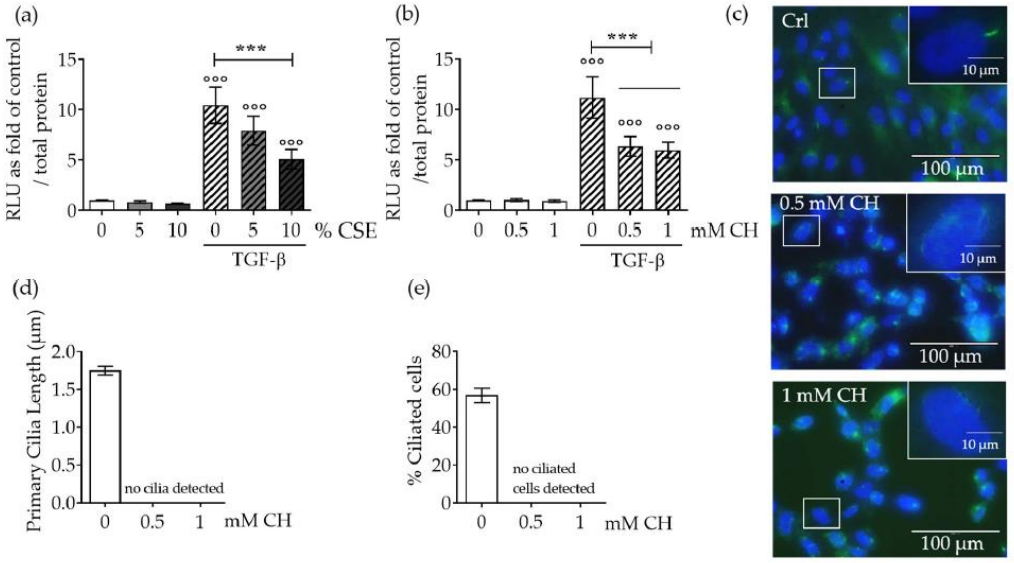

Figure 1. CSE exposure decreased TGF-β signaling by disrupting MSC primary cilia. Single-cell-derived human mesenchymal stem cell line (SCP-1 cells (N = 4, n = 8)) infected with an adenoviral Ad5-CAGA9-MLP-Luciferase reporter constructs (Smad2/3 reporter) were exposed overnight, either with or without Cigarette smoke extract (CSE) (**a**; 5–10%) or chloral hydrate (CH) (**b**; 0.5–1 mM). Next, cultures were incubated with recombinant human transforming growth factor beta one (rhTGF-β1) 10 ng/mL for 48 h, and luciferase activity was measured in cell lysates. The results were normalized to total protein content and expressed as relative luminesce units (RLU). Results represent mean ± standard error of the mean (SEM). Statistical significance was determined by the Kruskal–Wallis H test, followed by Dunn's post-test. Significance was established as *** $p < 0.001$ compared to TGF-β-treated cells and °°° $p < 0.001$ compared to untreated cells. (**c**) Representative immunostaining images of SCP-1 cells stained for acetylated α-tubulin (green), and nuclei (blue), after CH exposure. (**d**) Primary cilia length quantification of SCP-1 cells treated with and without CH. (**e**) Percentage of ciliated SCP-1 cells following CH treatment.

To emphasize the role of primary cilia on TGF-β signaling, we also investigated the effect of the chemical disruption of primary cilia on TGF-β signaling. SCP-1 cells treated with chloral hydrate (CH, 0.5–1 µM) showed disrupted primary cilia structure (Figure 1c–e), a result that confirmed earlier published results with CSE [24,25]. Following the same line of evidence, pharmacological disruption of primary cilia significantly reduced TGF-β signaling (Figure 1b). However, TGF-β signaling was not entirely abolished after primary cilia disruption, a finding that evidenced receptors located in this organelle contributed to the pathway, but receptors localized in the membrane also activated the basal TGF-β pathway.

2.2. Protection of Primary Cilia Structure with Resveratrol rescues TGF-β Signaling Suppressed by CSE

In order to confirm that the disruption of the primary cilia structure leads to aberrant TGF-β signaling, primary cilia structures were protected from the deleterious effects of CSE with resveratrol. Resveratrol is a polyphenol found in grapes with antioxidant properties [30]. Resveratrol administration in mice exposed to CS reduced cilia loss in trachea epithelia [31]. Moreover, co-incubation with

resveratrol protected primary cilia against the deleterious effects of CSE via a reduction of oxidative stress in SCP-1 cells [24].

SCP-1 cells infected with an adenoviral Smad2/3 reporter construct (Ad5-CAGA9-MLP-Luciferase) were co-incubated with resveratrol (1 μM) and CSE for 24 h, followed by the induction of the TGF-β pathway with rhTGF-β1In *Proceedings of the* . These cells co-incubated with resveratrol and CSE exhibited an increase in TGF-β signaling in comparison to CSE exposure alone (Figure 2a). To confirm the protective effects of resveratrol on the primary cilia structure, immunofluorescence analysis showed that co-incubation with resveratrol significantly increased the cilia length and the number of ciliated SCP-1 cells (Figure 2b–d). These results support the evidence that dysfunctional primary cilia affect the propagation of TGF-β signaling.

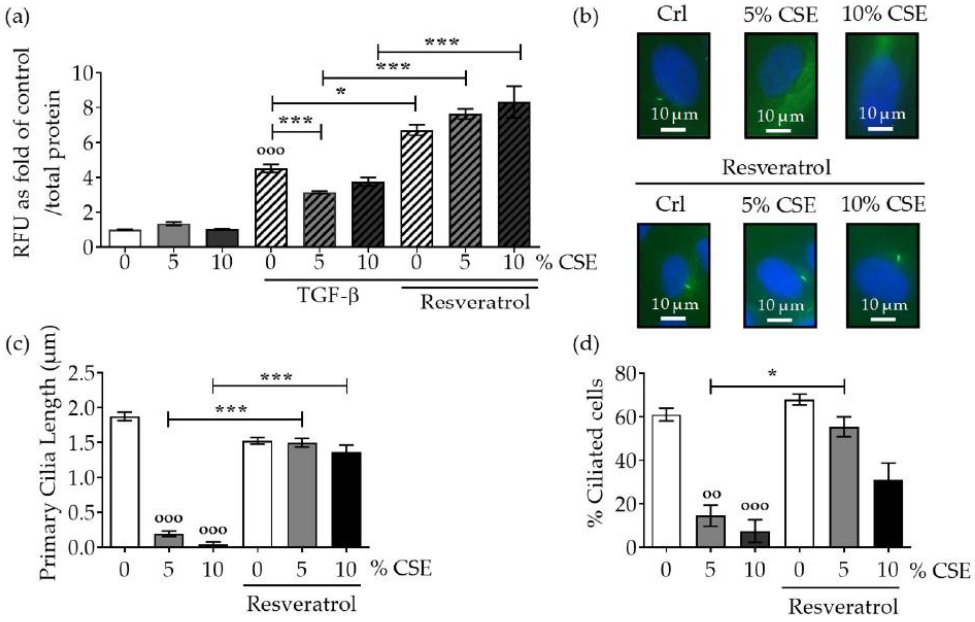

Figure 2. Resveratrol preserves the primary cilia structure from CSE and reestablishes TGF-β signaling. SCP-1 cells ($N = 3$, $n = 6$) infected with Ad5-CAGA9-MLP-Luciferase reporter constructs (Smad2/3 reporter) were co-incubated overnight, either with or without CSE (5–10%) and resveratrol (1 μM). Next, cultures were incubated with rhTGF-β1 (10 ng/mL) for 48 h, and (**a**) luciferase activity was measured in cell lysates. The results were normalized to total protein content and expressed as relative luminesce units (RLU). Results represent mean ± standard error of the mean (SEM). Statistical significance was determined by the Kruskal–Wallis H test, followed by Dunn's post-test. Significance was established as * $p < 0.05$, *** $p < 0.001$ compared to TGF-β-treated cells and °°° $p < 0.001$ compared to untreated cells. (**b**) Representative immunostaining images of SCP-1 cells stained for acetylated α-tubulin (green), and nuclei (blue), after incubation with CSE and resveratrol. (**c**) Primary cilia length quantification of SCP-1 cells treated with and without CSE and resveratrol. (**d**) Percentage of ciliated SCP-1 cells following resveratrol treatment.

2.3. CSE Reduced the Levels of Downstream TGF-β Pathway Mediators and the Nuclear Translocation of the Active Complex

We next investigated the effect of CSE on the protein levels of active TGF-β signaling modulators. SCP-1 cells were exposed to CSE (5% *v/v*) for 14 days in order generate chronic damage in the primary cilia structure. To avoid indirect effects due to cytotoxicity or additive effects of long term exposure to CSE, SCP-1 cells were only treated with 5% *v/v* CSE. After 14 days, TGF-β signaling was induced by the addition of rhTGF-β1 (10 ng/mL) for 1 h. Downstream TGF-β signaling mediator protein expression levels were analyzed by Western blot. rhTGF-β1 increased phospho-Smad2 and phospho-Smad3 levels. However, the induction of phospho-Smad2 was higher than phospho-Smad3, a result that

indicates Smad2 more dominantly mediated TGF-β signaling in SCP-1 cells (Figure 3a,b). As expected, CSE decreased the levels of active mediators; phospho-Smad2 downregulation was more pronounced (Figure 3a). Furthermore, CSE significantly reduced Smad4 protein (Figure 2c), a cofactor necessary to assemble the active complex with phospho-Smad2/3, that propagates the signaling complex to the nucleus. Thus, it is feasible to speculate that the concentration of the active complex in the nucleus was reduced. Therefore, we investigated whether the nuclear translocation of the active complex was affected by CSE. SCP-1 cells were treated with CSE (5–10% *v/v*) or CH (0.5–1 mM), and then signaling was induced with rhTGF-β1 (10 ng/mL) for 1 h. Nuclear translocation of the active complex was analyzed by Smad3 immunofluorescence. CSE (5–10% *v/v*) exposure significantly reduced Smad3 nuclear localization after TGF-β signaling induction (Figure 3e,f). This finding suggests that there are not any compensatory mechanisms in the nuclear translocation system that compensate for the lower phospho-Smad2/3 and Smad4 protein levels and consequently propagate the signal to the nucleus. Nuclear localization of the phospho-Smad3/4 complex was also reduced in SCP-1 cells with CH-stunted primary cilia (Figure 3e,f), a result that indicates TGF-β signaling is associated with primary cilia structure. These results suggest that defective primary cilia could lead to aberrant cell signaling coordination under smoking conditions. Possible regulations may be due to failures in receptor–ligand interactions, impaired internalization of the ligand–receptor complex, or affected the kinase receptor function.

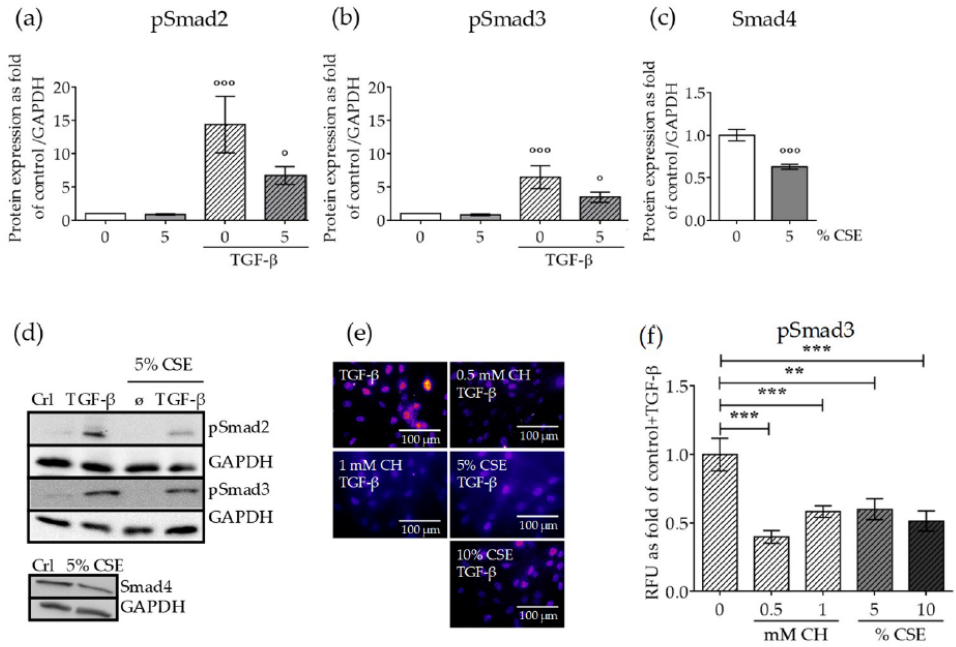

Figure 3. CSE exposure affected protein expression levels of canonical TGF-β signaling mediators and their nuclear translocation. SCP-1 cells ($N = 3$, $n = 3$) were exposed to CSE (5%) twice a week. After 14 days, the cells were treated with rhTGF-β1 (10 ng/mL) for 1 h. Protein expression of phospho-Smad2 (**a**), phospho-Smad3 (**b**), and Smad4 (**c**) was measured in cell lysates by Western blot and normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**d**) A representative Western blot of the measured proteins is shown. SCP-1 cells ($N = 3$, $n = 3$) were treated overnight with or without CSE (5–10%) or CH (0.5–1 mM). Next, cultures were incubated with rhTGF-β1 (10 ng/mL) for 24 h and then stained for Smad3. (**e**) Representative immunostaining images of nuclear localization of Smad3. The immunofluorescence signal was pseudocolored for better visualization using the fire tool in ImageJ. (**f**) Quantification of Smad3 nuclear translocation was performed with ImageJ. The results are expressed as mean ± SEM. Statistical significance was determined by the Kruskal–Wallis H, test followed by Dunn's post-test. Significance was established as ** $p < 0.01$ or *** $p < 0.001$ compared to TGF-β and ° $p < 0.05$ or °°° $p < 0.001$ compared to untreated cells.

2.4. CSE Perturbed Normal TGF-β Receptor Type I Function

Since CSE reduced active Smad2/3 protein levels, and consequently reduced nuclear translocation of the active pSmad3/4 complex, we evaluated the effect of CSE on the function of TGF-β receptor kinase type I (ALK5). ALK5 is responsible for phosphorylating and activating TGF-β signaling mediators (e.g., Smad2/3) with its serine/threonine kinase activity. SCP-1 cells were infected with Ad5-caALK5 virus particles. The expressed ALK5 was genetically modified to constitutively activate Smad2/3 phosphorylation and the associated signaling, independent of TGF-β binding to the receptor. Protein expression levels of phospho-Smad2 were evaluated in protein lysates from SCP-1 cells infected with Ad5-caALK5 and treated with CSE (5–10% *v/v*) for 48 h. Constitutive ALK5 activation increased the phospho-Smad2 protein level (Figure 4). The addition of CSE significantly decreased the phospho-Smad2 protein in a dose-dependent manner (Figure 4). Our data clearly suggest that the observed effects could be mediated by failure in the TGF-β mediator activation by the ALK5 receptor and not from unsuccessful binding of the ligand to the receptor.

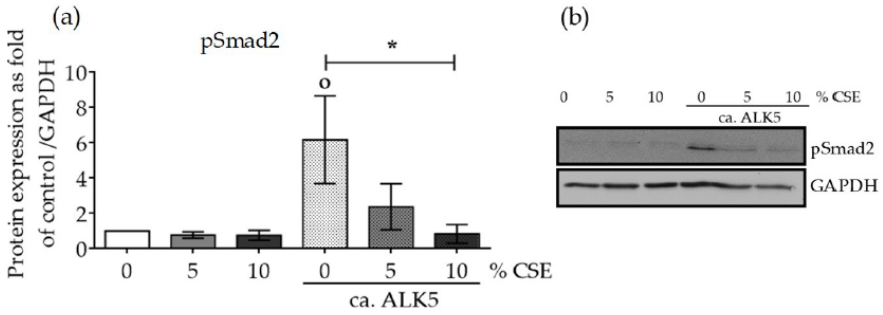

Figure 4. CSE reduced the protein expression of phospho-Smad2, despite a constitutively active TGF-β receptor type I (ca. ALK5). SCP-1 cells (N = 3, n = 3) infected with Ad5-ca. ALK5 were treated with CSE (5–10%). After 24 h, the total protein expression of phospho-Smad2 (**a**) was measured in cell lysates by Western blot and normalized to GAPDH. (**b**) A representative Western blot is shown. The results are expressed as mean ± SEM. Statistical significance was determined by the Kruskal–Wallis H test, followed by Dunn's post-test. Significance was established as * $p < 0.05$ compared to circa ALK5-infected cells and ° $p < 0.05$ compared to untreated cells.

2.5. CSE Affected SCP-1 Cell Migration and Proliferation

MSC migration to the fracture site is an important step for successful healing in patients with delayed fracture healing or non-union fractures due to bone metabolic diseases. TGF-β was proposed as a key chemokine for MSCs, and smokers present lower serum TGF-β levels after fracture than non-smokers [29]. Therefore, we examined the effect of CSE on MSC migration using a scratch assay. A wound was generated in SCP-1 cell monolayers with following exposure to CSE (5–10% *v/v*) and rhTGF-β1 (10 ng/mL). After 16 h, the wound closure was evaluated. SCP-1 cells exposed to CSE (5–10% *v/v*) for 16 h exhibited significantly reduced wound closure (Figure 5a,b), which evidenced that CSE negatively and dose-dependently affected cell migration. However, supplementing the cells with rhTGF-β1 (10 ng/mL) accelerated SCP-1 migration to the wound (Figure 5a,b), a finding that supports the TGF-β chemokine function for MSCs [32]. TGF-β also induces cell proliferation. To directly determine whether CSE affects cell proliferation, SCP-1 cells were treated with CSE (5% *v/v*) for 24 or 48 h, with or without co-incubation with rhTGF-β (10 ng/mL), to induce proliferation. An assessment of the protein expression levels of the proliferation marker proliferating cell nuclear antigen (PCNA) by Western blot indicated that CSE did not affect the proliferation rate after 24 h (Figure 5c,d). However, 48 h CSE exposure decreased the proliferation rate. TGF-β pathway induction increased the PCNA level after 24 h under control conditions. However, this positive effect was not reproduced in cells exposed to CSE (Figure 5c,d). These results indicate that TGF-β directly promoted

cell migration under CSE exposure, but TGF-β addition could not compensate for the detrimental SCP-1 cell proliferation caused by CSE.

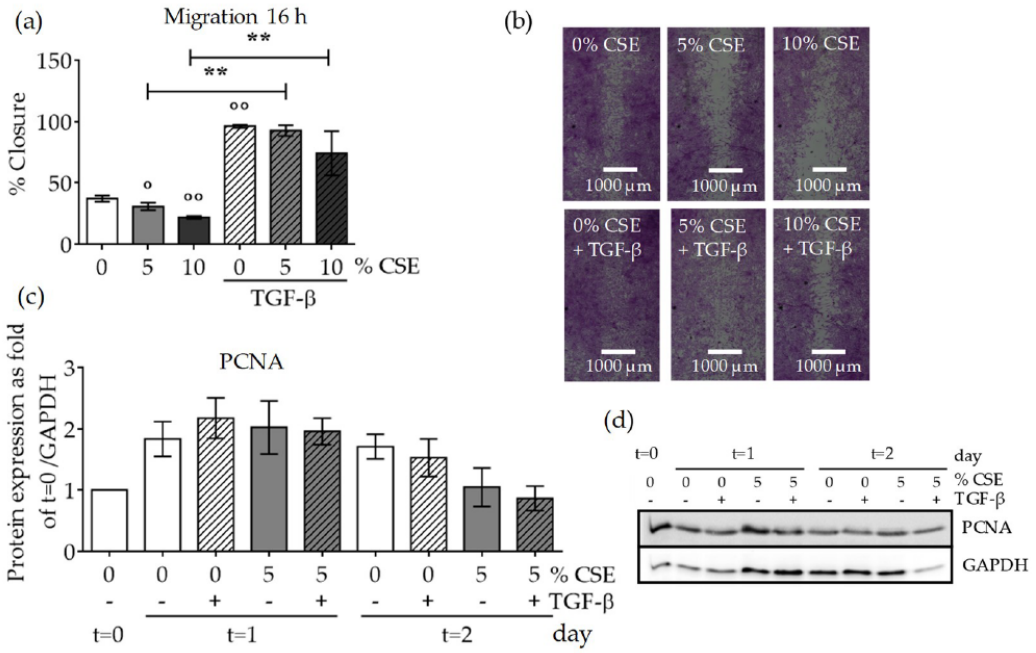

Figure 5. CSE exposure decreased MSC migration and proliferation. In order to investigate the effect of CSE on SCP-1 cell migration, a scratch assay was performed. SCP-1 cells ($N \geq 3$, $n \geq 3$) were co-incubated with CSE (5–10%) and rhTGF-β1 (10 ng/mL). Wound closure was determined from microscopic pictures (equation: (100 − wound area at 16 h/wound area at 0 h) × 100) with ImageJ software. (**a**) SCP-1 cell migration after 16 h. (**b**) Representative migration pictures. Cells were visualized with sulforhodamine B (SRB) staining. (**c**) Normalized proliferating cell nuclear antigen (PCNA) protein expression in SCP-1 cells at day 0 ($t = 0$) and after 24 h ($t = 1$) or 48 h ($t = 2$) stimulation with rhTGF-β1 (10 ng/mL) and with or without CSE (5% *v/v*). (**d**) A representative PCNA Western blot. The results are expressed as mean ± SEM. Statistical significance was determined by the Kruskal–Wallis H test, followed by Dunn's post-test. Significance was established as ** $p < 0.01$ compared to TGF-β1-treated cells and ° $p < 0.05$ or °° $p < 0.01$ compared to untreated cells.

2.6. Impaired TGF-β Signaling by CSE Negatively Affected Osteochondral Progenitor Cell Differentiation

During endochondral bone fracture repair, new bone is formed through a cartilage intermediate (callus) produced from chondrogenicaly differentiated MSCs [33]. Several studies demonstrated that TGF-β plays an essential role during MSC chondrogenic differentiation [34–36]. Thus, we investigated whether CSE-modulated TGF-β signaling disruption affected the expression of chondrogenic markers on MSCs differentiated into chondrocytes. SCP-1 cells were exposed to CSE during chondrogenic differentiation. Since after 14 days of chondrogenic differentiation, SCP-1 cells showed a chondrogenic phenotype (positive staining for glycosaminoglycan and proteoglycan (data no shown)), this time the point was selected to determine effects of CSE on this TGF-β mediated mechanism. At day 14, total RNA Semi-quantitative gene expression analysis revealed that *Collagen II*, a major extracellular matrix protein in cartilage, was significantly downregulated in SCP-1 cells exposed to CSE and treated with TGF-β for 14 days (Figure 6a,e). Surprisingly, the expression of *Aggrecan*, a specific marker for the cartilage extracellular matrix, was upregulated in cells exposed to CSE and TGF-β, a result that suggests a compensatory mechanism from the cells in response to decreased Collagen II with CSE treatment (Figure 6d,e). Intriguingly, the hypertrophic chondrogenic phenotype marker *Collagen X* was upregulated with CSE treatment and TGF-β induction (Figure 6b,e). The transcriptional factor *Sox9* induces the differentiation of MSCs into pre-chondrocytes. As expected, TGF-β treatment

downregulated this transcription factor. However, the expression of *Sox9* was upregulated in cells exposed to CSE (Figure 6c,e). Following the same line of results, impaired TGF-β signaling via CH-mediated primary cilia disruption exhibited a similar expression pattern to CSE exposure. Taken together, CSE exposure affects the expression of chondrogenic markers, which may disturb MSC chondrogenic differentiation and result in a hypertrophic phenotype. Consequently, these results could explain the altered endochondral ossification observed in smokers during long bone fracture healing [37,38].

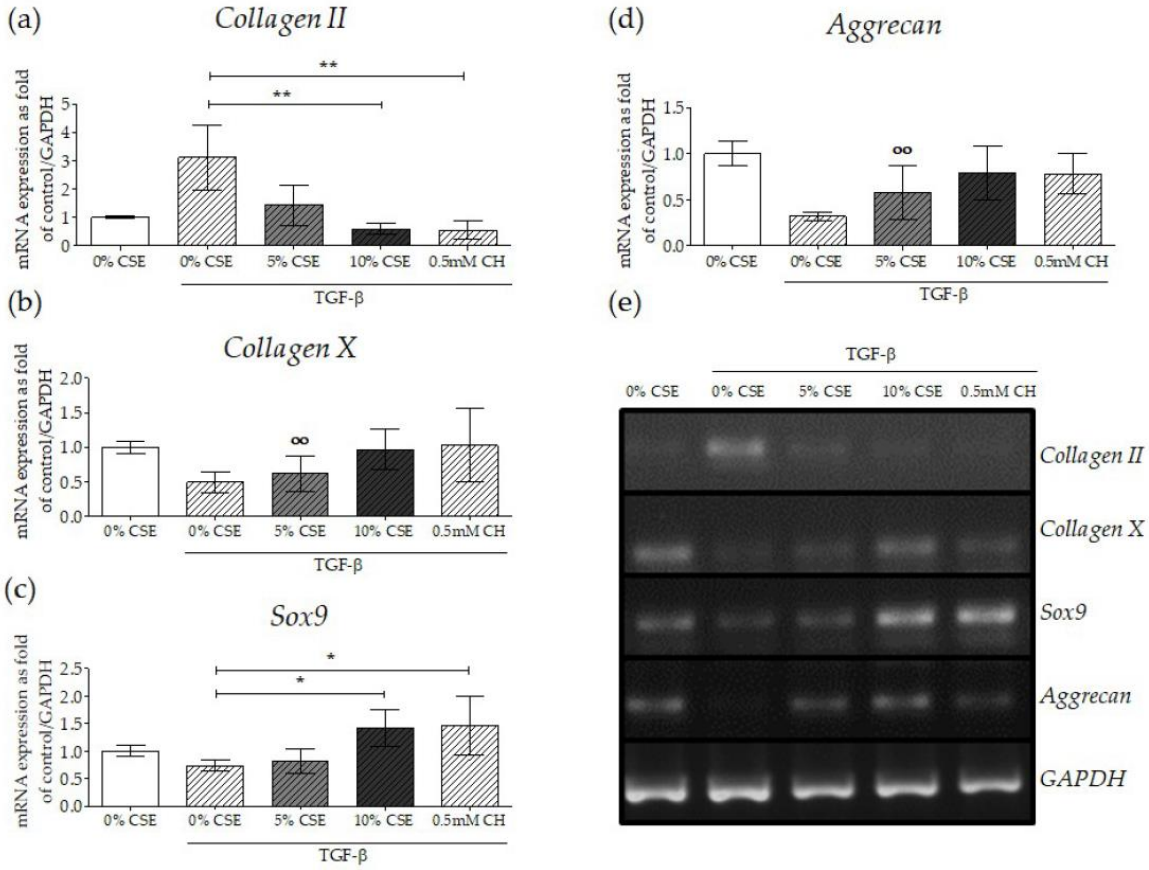

Figure 6. Disruption of TGF-β signaling with CSE-mediated primary cilia disruption affected MSC chondrogenic differentiation. In order to evaluate the gene expression of chondrocyte markers under impaired TGF-β signaling, SCP-1 cells ($N = 3$, $n = 2$) were differentiated with CSE (5–10%) and rhTGF-β1 (10 ng/mL) for 14 days. Gene expression analysis was performed with semi-quantitative RT-PCR from 10 ng cDNA. The graph represents gene expression, normalized to the *GAPDH* (housekeeping gene), of (**a**) *Collagen II*, (**b**) *Collagen X*, (**c**) *Sox9*, and (**d**) *Aggrecan*. The results are expressed as mean ± SEM. Statistical significance was determined by the Kruskal–Wallis H test, followed by Dunn's post-test. Significance was established as ** $p < 0.01$ or * $p < 0.05$ compared to TGF-β1-treated cells or °° $p < 0.01$ compared to untreated cells. (**e**) A representative semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) gel picture.

3. Discussion

Smoking is considered one of the main risk factors for developing metabolic bone diseases [3–6]. Metabolic bone diseases are characterized by impaired bone remodeling for different reasons: disrupted bone formation by osteoblasts, enhanced bone degradation by osteoclasts, or a combination of both. Fractures in patients suffering from metabolic bone diseases are clearly associated with delayed or non-union fracture healing regarding imbalances between bone-forming and bone-resorbing cells [9,10].

TGF-β1 is a multifunctional signaling protein that significantly affects bone cells and plays an essential role in the maintenance of appropriate bone remodeling [12,13,39,40]. Furthermore, TGF-β induces the expression of extracellular matrix compounds like collagen type I, fibronectin, osteopontin, osteonectin, thrombospondin, and proteoglycans, all of which contribute to fracture healing [13,29,41]. Animal studies have revealed that a lack of TGF-β causes defects in bone strength and microarchitecture. Additionally, the local presence of cytokines (TGF-β, BMP, Insulin-like growth factor 1, etc.) enhances the fracture healing process [42].

Interestingly, smokers present lower serum TGF-β concentrations compared to non-smokers [15,29]. After a fracture, TGF-β increases during the inflammatory processes in order to guide immature progenitor cells to invade the fracture area. At this stage, patients with delayed fracture healing (as well as smokers) exhibit a marked decrease in systemic TGF-β1 levels [15]. However, the effects of CSE on the TGF-β signaling pathway of bone cells are still unclear. The current study demonstrated that CSE directly inhibited canonical TGF-β signaling in MSCs. CSE-mediated downregulation of TGF-β signaling was dose dependent, and the disruption of primary cilia by pharmacological treatments presented similar results. Furthermore, we performed a rescue experiment confirming the central role of the primary cilia structure on TGF-β signaling. We observed that protection of the primary cilia structure from CSE deleterious effects by co-incubation with resveratrol significantly reversed the negative effects on TGF-β signaling. Several studies associated primary cilia with different signaling pathways and postulated that the organelle is a key player in signaling translation [18,22,43]. Although CSE detrimentally affected TGF-β signaling, the pathway was not totally abolished. This fact highlights that receptors localized in the primary cilia propagate signaling activation, but receptors in the cell membrane could also propagate, contributing to the pathway activation.

Since we observed significant CSE-mediated inhibition of canonical TGF-β signaling, we evaluated changes in the levels of active canonical TGF-β signaling downstream modulators (Smad2 and Smad3). CSE exposure reduced phospho-Smad2 and phospho-Smad3 levels. We detected that TGF-β signaling induction had a greater effect on phospho-Smad2, a result that suggests this mediator is more affected by CSE. Smad2 knockout is embryonically lethal in mice, while Smad3-deficient mice are viable. Thus, Smad2 function can apparently compensate for Smad3, but Smad3 cannot compensate for Smad2 [44].

Smad4 is the central cofactor mediator of TGF-β signaling. Smad4 binds to phosphorylated Smads to form the active complex that translocates into the nucleus and triggers target gene transcription. Smad4 is required to maintain normal bone homeostasis [45]. In vitro studies proposed that Smad4 interacts with transcriptional factors, such as Runx2 and AP-1 (c-Fos-JunD), that influence MSC osteogenic differentiation [46]. Moreover, conditional Smad4 knockout in chondrocytes amplifies the hypertrophic phenotype [47]. Fascinatingly, CSE exposure downregulated basal MSC Smad4 expression, which caused the cells to have less available cofactor to form the activated complex, and consequently, the activation was not adequately propagated to the nucleus.

Nuclear translocation of the active complex differs for Smad2 and Smad3. For Smad3/Smad4, the activated complex translocates in an importin-dependent manner (mediated by importin-β1) [48,49] or by associating with nuclear pore proteins (NUP214/NUP153) [50]. However, for Smad2/Smad4, nuclear translocation occurs only through nuclear pore proteins [51]. Since Smad3 has multiple translocation mechanisms, it is an attractive target to investigate in order to elucidate whether there is a compensatory mechanism that balances the lack of phosphorylated Smads under CSE exposure. As expected, CSE-mediated TGF-β signaling disruption impaired Smad3/Smad4 translocation into the nucleus. This result demonstrates that the reduced levels of active protein are not compensated by the nuclear import machinery.

Next, we investigated whether CSE exposure affected the TGF-β ligand–receptor interaction, since it is a critical step in pathway initiation. A constitutively activated TGF-β receptor type I (ALK5) did not abolish the dose-dependent reduction of phospho-Smad2 induced by CSE. This result suggests that the observed CSE effects were mediated by impaired TGF-β downstream mediator (Smad2/Smad3) activation through ALK5 and not by inhibition of the ligand–receptor interaction.

Accurate internalization of the active TGF-β receptor–ligand complex enhances the activation of downstream Smad mediators. Clathrin-dependent endocytosis of the ligand–receptor complex is reduced at the cilia pocket region in cells with truncated primary cilia structure [52]. We suggest that CSE exposure could affect endocytosis of the activated receptor–ligand complex in the cilia pocket region via a defective primary cilia structure, a phenomenon that would lead to aberrant cell signaling coordination by this organelle. Inappropriate endocytosis affects the interaction between TGF-β receptor type I and a cofactor, such as the Smad anchor for receptor activation protein (SARA), which enhances the association with Smad mediators and improves their phosphorylation [53]. It is possible that CSE exposure either affects binding of cofactors (to interfere in proper Smad phosphorylation) or inhibits the serine/threonine kinase function of the receptor type 1.

For bone tissue, TGF-β1 plays a crucial role in MSC migration, regulating osteoblast function (induce proliferation and inhibit late differentiation), appropriate chondrogenic differentiation, and consequently influences the bone healing process [13,32,34,40,41,54–58]. Cell migration is also coordinated by primary cilia via appropriate TGF-β signaling. In this regard, defects in the formation or sensory function of primary cilia are associated with a series of migration-related disorders and diseases [59]. Interestingly, CSE dose dependently reduces MSC migration, as previously reported [60]. However, TGF-β signaling induction through the addition of rhTGF-β1 reversed the detrimental effects of CSE on cell migration. These results suggest that topic TGF-β1 application could enhance MSC recruitment in the fracture place. Nevertheless, TGF-β1 supplementation did not reverse the decreased proliferation (denoted by PCNA expression) observed with 48 h CSE exposure, a finding that challenges the use of rhTGF-β in smokers to enhance fracture healing.

After long bone fracture, MSCs migrate to the fracture, condensate, differentiate into chondrocytes, and begin to produce cartilage. Cartilage is later systematically replaced with mineralized tissue by osteoblasts derived from the recruited MSCs during the process of endochondral ossification [61,62]. Since endochondral ossification is an essential process during long bone fracture healing, and TGF-β regulates the behavior and function of the cells involved in the process [61], we investigated the effect of CSE on MSC chondrogenesis via TGF-β signaling induction. Fourteen-day exposure to TGF-β significantly increased the gene expression of *Collagen II*, a major extracellular matrix protein in cartilage, and downregulated *Sox9*, a marker of pre-chondrocytes, and *Collagen X*, a marker of hypertrophic chondrocytes. Interestingly, CSE dose dependently decreased *Collagen II* expression independent of TGF-β signaling induction. This result demonstrates that the cartilage structure produced by chondrocytes exposed to CSE is feeble. Moreover, the hypertrophic marker *Collagen X* increased with CSE. Increased *Sox9* gene expression (a transcription factor necessary to activate the earliest chondrogenic-specific genes) demonstrated an improper MSC chondrogenic differentiation under CSE exposure. These results are supported by the fact that human adipose-derived MSCs treated with CSE show decreased *Collagen II* and increased *Sox9* [60]. Furthermore, alcian blue staining (which stains glycosaminoglycans in cartilage) is decreased in human periodontal ligament-derived stem cells isolated from smokers [63]. We previously showed that CSE exposure downregulates Smad4 protein levels. Smad4 deletion in chondrocytes causes a disorganized growth plate, reduces chondrocyte proliferation, increases apoptosis, and accelerates hypertrophic differentiation [23]. Similar effects were reported in Smad4 mutant mice, results that demonstrate Smad4 is necessary for maintaining sequential chondrocyte differentiation [47]. The lack of Smad4 could partly explain the impaired CSE-exposed MSC chondrogenic differentiation. Surprisingly, CSE induced Aggrecan expression, which is a major component of the cartilage extracellular matrix. Nevertheless, one study questioned the use of Aggrecan as a chondrocyte marker since it is constitutively expressed by MSCs [64]. Additionally, increased Aggrecan expression may also be associated with chondroid accumulation in response to damage [65]. Moreover, a disruption of the primary cilia from chondroprogenitor cells leads to reduced Collagen II, Collagen X, and BMP-2 expression and cannot be reestablished by mechanical activation. However, the role of primary cilia in hypertrophic chondrocytes is more restricted, since primary cilia disruption only reduces Collagen X in response to mechanical activation. These results

demonstrated the role of primary cilia in regulating the chondrogenic profile of progenitor cells [66]. Furthermore, CH-mediated primary cilia disruption impaired TGF-β signaling and downstream activation. This expression pattern was similar to CSE-exposed MSCs, which affect chondrogenic differentiation and led to the hypertrophic differentiation profile. These results partially support the in vivo observations that CSE affected endochondral ossification and consequently delayed or impaired long bone fracture healing.

Based on our findings, we consider that topic rhTGF-β1 application could provide positive effects at early stages of endochondral ossification among smokers (inducing progenitor cell migration). However, proliferation and appropriate osteochondral progenitor cell differentiation will be compromised due to the block in the canonical TGF-β signaling pathway. Therefore, therapies that enhance or reestablish the canonical pathway or activate non-canonical Smad phosphorylation (e.g., riluzole, which activates glycogen synthase kinase 3 for Smad2/3 activation [67]) may be a more promising alternative for smokers who suffer from delayed fracture healing compared to rhTGF-β1 application.

Additionally, the findings of this study have to be seen in the light of some limitations. The effect of CSE on the canonical TGF-β signaling through primary cilia structure were only evaluated in MSCs. However, during bone fracture, several cells play an important role in the success of the fracture healing. Therefore, CSE's detrimental effects in TGF-β signaling could also influence the function of immune cells (at early stages of the healing process) or osteoclast (at the remodeling stage of the healing process) [62], whereby, co-culture systems might be useful for screening purposes to study different effects of CSE on various bone cells during fracture healing.

4. Materials and Methods

4.1. Materials

Cell Culture Medium and supplements were purchased from Life Technologies (Darmstadt, Germany). Chemicals were obtained from Sigma (Munich, Germany). Recombinant human active TGF-β1 was obtained from Peprotech (London, UK).

4.2. SCP-1 Cells Culture and Chondrogenic Differentiation

Human immortalized bone marrow mesenchymal stem cells (SCP-1 cells, provided by Dr. Matthias Schieker [68]) were cultured in Minimum Essential Medium Eagle alpha (MEM α) supplemented with 10% *v/v* fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin, in a water-saturated atmosphere of 5% CO_2 at 37 °C [69]. Chondrogenic differentiation was induced with Dulbecco's Modified Eagle Medium (DMEM) high glucose medium containing 100 nM Dexamethasone, 1 mM sodium pyruvate, 220 µM L-ascorbic acid-2 phosphate, 347 µM L-proline, 1.25 g/mL bovine serum albumin (BSA), 625 µL liquid media supplement ITS 100x stock (mixture of recombinant human insulin, human transferrin, and sodium selenite), 20 ng/mL linolic acid, 100 U/mL penicillin, and 100 mg/mL streptomycin. Chondrogenic differentiation was confirmed with glycosaminoglycan and proteoglycan positive staining (Alcian blue and Safranin O). Chondrogenic differentiation was adapted from a previous publication with SCP-1 cells [68]. For experiments, 5% CSE, 10% *v/v* CSE, 0.5 mM chloral hydrate, or 10 ng/µL rhTGF-β1 were added to the media. The medium was changed twice a week during chondrogenic differentiation, which was sustained for 14 days.

4.3. Cigarette Smoke Extract Generation

The smoke of the combustion of two commercial cigarettes (Marlboro, Philip Morris, New York City, USA), containing 0.8 mg nicotine and 10 mg tar each, was continuously bubbled through a 50 mL pre-warmed MEM α medium (0% *v/v* FCS) in a standard gas wash bottle, at a speed of 95 bubbles/min, as described before [24]. The concentration of CSE was normalized by its optical density at 320 nm (OD320), with an OD320 of 0.7 considered 100% *v/v* CSE [26]. CSE was freshly prepared for every experiment and sterile filtered (0.22 µm pores filter) before diluted to reach 5% and 10% *v/v* CSE, which

corresponds to exposures associated with smoking 10 cigarettes (0.5 pack) a day to 20 cigarettes (1pack) a day [70].

4.4. Transient SCP-1 Cells Infections and Reporter Assay

For the TGF-β reporter assay, SCP-1 cells were stably infected with an adenoviral vector system expressing luciferase under the control of Smad2/3-responsive element (Ad5-CAGA9-MLP-Luciferase, kindly provided by Professor Peter ten Dijke [71,72]) 1:7 (*v/v*), as described before [73]. After 24 h, the cells were washed with phosphate-buffered saline (PBS) and treated with 0.5–1 mM chloral hydrate (16 h) or 5–10% CSE (24 h). Subsequently, the cells were exposure to 10 ng/mL rhTGF-β1 for 24 h. Upon binding of phosphorylated Smad3/4 (induced by rhTGF-β1), luciferase was expressed by the cells. Cell lysate preparation and luciferase measurement were performed according to the manufacturer's instructions, using the Steady-Glo Luciferase Assay System (Promega, Madison, USA), and normalized to total protein content, measured with Lowry. In order to investigate TGF-β effects independent of substrate binding, SCP-1 cells were infected with adenoviral particles, resulting in the expression of constitutive active ALK5 (Ad5-caALK5, kindly provided by Professor Peter ten Dijke [74]) 1:50 *v/v*. The expressed ALK5 was genetically modified in a way to constitutively activate Smad2/3 phosphorylation and associated signaling independent of subtract binding. After 5 h, cells were washed with PBS and treated with 5–10% CSE (24 h). Protein expression levels of active Smad2/3 was evaluated by Western blot. Adenovirus infection efficiency was >90%, as shown by the fluorescent microscopy of cells infected with Ad5-green fluorescent protein (GFP) after 24 h (Appendix A Figure A1) [73].

4.5. Immunofluorescence Staining

After treatment, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 4% (*w/v*) paraformaldehyde for 10 min at room temperature. This was followed by permeabilization with 0.2% Triton-X-100 solution for 20 min at room temperature and treatment with 2% (*w/v*) paraformaldehyde for 10 min at room temperature. Unspecific binding sites were blocked with 5% (*w/v*) BSA for 1 h at room temperature, followed by incubation with the first antibody overnight at 4 °C (Table 1). After washing three times with PBS, cells were incubated with Alexa-fluor labeled secondary antibody (1:1000) for 2 h at room temperature (Table 1). Nuclei were stained with Hoechst 33,342 (1:1000). Images were taken with an epifluorescence microscope (EVOS FL, life technologies, Darmstadt, Germany). Pictures were analyzed with the ImageJ software (Version 1.5, NIH, Bethesda, MD, USA) by two independent investigators in a blinded fashion. Based on the microscopic pictures taken, cilia length was determined by the maximum intensity projection method [58].

Table 1. Antibodies used in Western blot and immunofluorescence staining.

Antibody	Catalog No.	Company	Dilution
phospho-Smad2	3108	Cell Signaling	1:1000
phospho-Smad3	9520	Cell Signaling	1:1000
Smad4	9515	Cell Signaling	1:1000
PCNA	ab92522	Abcam	1:1000
HRP antirabbit IgG	sc-2004	Santa Cruz	1:10000
Smad3	9523	Cell Signaling	1:50
Alexa 488 antirabbit IgG	A21206	Invitrogen	1:1000
Acetylated αTubulin (6-11b-1)	sc-23950	Santa Cruz	1:100
Alexa 488 antimouse IgG	A10667	Invitrogen	1:1000

4.6. Western Blot Analysis

Cells were lysed in a freshly prepared ice-cold radioimmunoprecipitation assay buffer (RIPA) with protease and phosphatase inhibitors. After quantification with micro Lowry, 30 µg total protein was separated by sodium dodecyl sulfate–polyacrylamide gel (SDS page) and transferred to nitrocellulose

membranes. Membranes were blocked with 5% *v/v* BSA in Tris-buffered saline with Tween20 (TBS-T) for 1 h at room temperature. After overnight incubation with primary antibodies in TBS-T (1:1.000) at 4 °C, membranes were incubated with the corresponding peroxidase-labeled secondary antibodies in TBS-T (1:10.000) for 2 h at room temperature (Table 1). For signal development, membranes were incubated for 1 min with an electrogenerated chemiluminescence (ECL) substrate solution. Chemiluminescent signals were detected by a charge-couple device camera (INTAS Science Imaging, Göttingen; Germany) and quantified using the ImageJ software [25].

4.7. SCP-1 Cells Migration Assay—Scratch Assay

SCP-1 cells were plated at high density in 24-well plates. After 24 h, the cell monolayer was mechanically wounded with a 200 µL pipet tip. Immediately after setting the wound, the medium was changed to remove detached cells and start stimulation with 5–10% CSE. The "scratches" were documented by taking microscopic images directly after wounding (0 h) and after 16 h. For better visualization, cells were stained with Sulforhodamine B (SRB) [25]. Wound closure was quantified with the ImageJ software by using the following formula: 100 − (area16h × 100/area0h) [32].

4.8. Semi-Quantitative Reverse-Transcription Polymerase Chain Reaction RT-PCR

Total RNA was isolated from the SCP-1 treated cells using Trifast (Peqlab, Erlangen, Germany) according to the manufacturer´s protocol and quantified using a spectrophotometer (Omega plate reader, BMG Labtech GmbH, Germany). cDNA was synthesized using the First Strand cDNA Synthesis Kit from 2500 ng total RNA (Fermentas St, Leon-Rot, Germany). Afterwards, semi-quantitative RT-PCR was performed from the 10 ng cDNA template using KAPA2G Fast Ready Mix (Peqlab, Erlangen, Germany). Primers and PCR conditions were previously optimized with increasing amounts of cDNA, in order to analyzed the PCR product obtained from the logarithmic phase. Primer sequences and PCR conditions are shown in Table 2. GAPDH was used as an internal control for normalization. PCR products were resolved using a 1.5% agarose gel, with ethidium bromide for visualization. Densitometric analysis was performed using the ImageJ software.

Table 2. Primer sequences and PCR conditions for the investigated genes.

Gene	Accession Number	Forward Primer (5′–3′)	Reverse Primer (3′–5′)	Product Length (bp)	Annealing Temperature (°C)	Cycles. (N°)
Aggrecan	NM_001135.3	CTTGGACTTGGGCAAACTGC	CACTAAAGTCAGGCAGGCCA	143	60	35
Collagen II	NM_001844.4	TGGATGCCACACTCAAGTCC	GCTGCTCCACCAGTTCTTCT	254	60	35
Collagen X	NM_000493.3	AAACCTGGACAACAGGGACC	CGACCAGGAGCACCATATCC	124	60	35
SOX9	NM_000346.3	GAAGGACCACCCGGATTACA	GCCTTGAAGATGGCGTTGG	120	60	35
GAPDH	NM_002046.4	GTCAGTGGTGGACCTGACCT	AGGGGTCTACATGGCAACTG	420	56	30

4.9. Statistic Analysis

Results were represented as bar diagrams (mean ± SEM) of at least three independent experiments (biological replicates, $N \geq 3$) measured as triplicate or more (technical replicates, $n \geq 3$). The comparison of multiple groups was done using the Kruskal–Wallis H-test, followed by the Dunn's multiple comparison test. The Mann–Whitney U-test (two-sided) was used to compare two single groups with each other. Statistical analysis was performed using the GraphPad Prism Software (Version 5, El Camino Real, CA, USA). $p < 0.05$ was considered as the minimum level of statistical significance.

5. Conclusions

In summary, our study demonstrated for the first time that TGF-β signaling was impaired in CSE-exposed MSCs. Moreover, the disruption of primary cilia with CSE and CH affected proper TGF-β signal transmission thought the cell. Therefore, TGF-β signaling dysregulation contributes in part to the adverse effects observed in MSC migration, proliferation, and differentiation, which could explain

the effect of endochondral ossification, and consequently, impair or delay long bone fracture healing in smokers.

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Abbreviations

ALK5	TGF-β type I receptor kinase
BSA	Bovine serum albumin
CAGA	Promotor region of TGF-β target genes
CH	Chloral Hydrate
CS	Cigarette smoke
CSE	Cigarette smoke extract
ECL	Electrogenerated chemiluminescence
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
IFT	Intraflagellar transport system
MSCs	Mesenchymal stem cells
NUP	Nucleoporin protein
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
RIPA	Radioimmunoprecipitation assay buffer
RLU	Relative fluorescent units
SARA	Smad anchor for receptor activation
SCP-1	Single-cell-derived human mesenchymal stem cell line
SDS page	Sodium dodecyl sulfate–polyacrylamide gel
SRB	Sulforhodamine B
TBS-T	Tris-buffered Saline-Tween
TGF-β1	Transforming growth factor β1

Appendix A

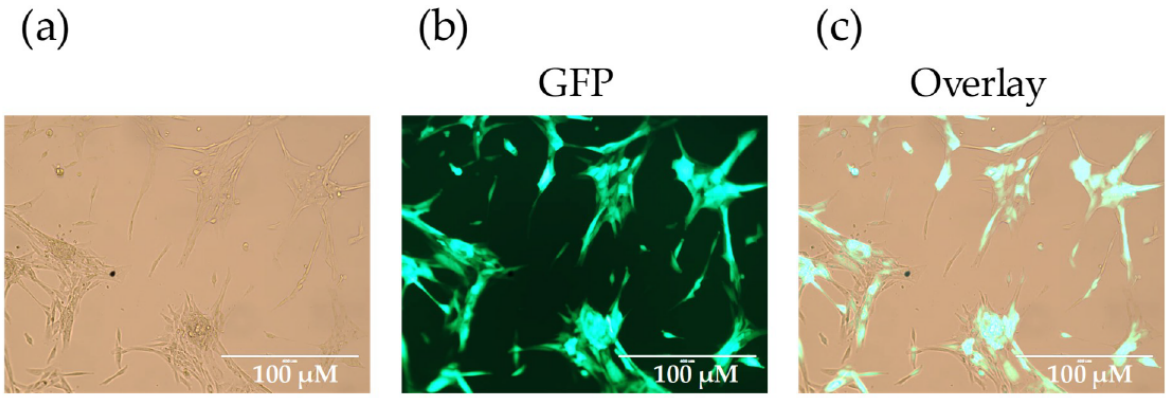

Figure A1. Adenovirus infection efficiency was approximately 90%. Micrographs of SCP-1 cells infected with Ad5-green fluorescent protein (GFP) after 24 h are shown. Micrographs represent (**a**) bright field, (**b**) GFP fluorescence, and (**c**) the merged picture.

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4. Discussion

It has been hardy demonstrated that CS is a hazard for second osteoporosis and bone fractures, alters bone balance, and correlates with a raised risk of post-surgical complications such as delayed or impaired bone healing and infections (Rudang *et al.*, 2012, Sloan *et al*., 2010, Scolaro *et al*., 2014, Hess *et al*., 2018, Kanis *et al.*, 2005). However, the mechanisms that underlie the detrimental effects of CS on bone homeostasis have been poorly defined. Understanding the mechanisms will allow developing treatment strategies to improve bone quality or treat orthopedic patients who smoke to enhance the surgical outcome. Thus, we aimed to characterize bone homeostasis in orthopedic patients who smoke and establish an *in vitro* model that represents the clinical situation of smokers with impaired bone-forming cell function to investigate possible underlying mechanisms and potential treatment strategies.

Our study confirms that CS is an accute danger for suffering an infection, delayed healing, and revision surgery in comparison to malnutrition and regular alcohol consumption in orthopedic patients (Ehnert *et al*., 2019). Interestingly, a recent report revealed a relation between smoking habits and the probability of develop complications (Knapik and Bedno, 2018). Unexpectedly, our orthopedic patients who smoke were on average 5.4 years younger compared with non-smokers, demonstrating the harmful effect of cigarette consume on bone quality, with an increasing probability of bone fracture and required joint replacement at younger ages (Ehnert *et al*., 2019). This finding supports the lower bone quality for young smokers reported by Rudang *et al.* (Rudang *et al.*, 2012).

In order to better comprehend the molecular mechanisms behind the detrimental effect of CS on bone homeostasis, we evaluated the serum expression levels of cytokines associated with bone healing and bone cell function on serum samples from orthopedic patients classified as heavy smokers, moderate smokers, and non-smokers. OPG and RANKL levels were upregulated after surgery in heavy smokers; thus, there was no change in the RANKL/OPG ratio. Additionally, there were similar levels of TRAP in the blood from heavy smokers and non-smokers after surgery (Ehnert *et al.*, 2019). These results demonstrated that in long bone damage, osteoclast function is not strongly affected by CS. This finding differs from the reports on oral cavity bones (where an increased RANKL/OPG ratio leads to elevated osteoclast activity (Behfarnia *et al.*, 2016, Belibasakis and Bostanci, 2012, Ozcaka *et al.*, 2010)).

These differences may be because cigarette smoke is in direct contact in the oral cavity in comparison with long bones, where the harmful components of cigarette smoke reach the bone through the bloodstream. Additionally, RANKL is upregulated after orthopedic surgery due to surgery-induced oxidative stress (Kapasa *et al.*, 2017). We noted this effect in our study *via* the upregulation of oxidative stress marker MCP-1. Furthermore, the 'bone bridge' glycoprotein OPN was upregulated in smokers (Ehnert *et al.*, 2019). This protein favors osteoclast attachment to the ECM and resorption activity (Bishop *et al.*, 2012). This result is consistent with the induced levels of the bone turnover marker C-terminal telopeptide of type I collagen (CTX-1) observed in heavy smokers (Ehnert *et al.*, 2019). Thus, we conclude that CS does not negatively affect bone-resorbing cell (osteoclast) in orthopedic patients with total joint replacement.

Our study showed that CS has detrimental effects on osteoblasts. Decreased serum levels of AP and the bone formation marker type IC-terminal collagen pro-peptide (CICP) decidedly associated with the number of cigarettes consumed in a dose-related manner (Ehnert *et al.*, 2019). The adverse effects of CS on osteoblast function may be due to increased oxidative stress produced by smoking (which was previously demonstrated *via* increased serum MCP-1).

Early diagnosis of impaired bone homeostasis is essential to reduce the risk of fracture and improve delayed fracture healing in orthopedic patients who smoke. The use of bone remodeling markers associated with collagen degradation/formation (CTX-1 and CICP) should be carefully used and individually evaluated because smokers frequently suffer from lung or liver fibrosis (Morse and Rosas, 2014, Oh *et al*., 2012, Corpechot *et al*., 2012, Zein *et al*., 2011). These diseases may influence collagen synthesis, an outcome that may result in inaccurate diagnosis (Liu *et al*., 2012, Organ *et al.*, 2019). Therefore, the application of bone cell function markers (*e.g.* AP and TRAP) in the blood to identify impaired bone homeostasis in the clinic would be more accurate for smokers Additionally, our results showed the immunosuppression status of smokers (reduced levels of pro-inflammatory markers IL-1β, IL-6, and TNF-α) (Ehnert *et al*., 2019). This reduction is consistent with the reported risk of infection as a complication in smokers compared with non-smokers (Chen *et al*., 2007, Goncalves *et al*., 2011). Identifying potential treatment strategies and alternatives for smokers that improve bone healing and protect bone-forming cells from the deleterious effects of

CS, it is essential to understand the role of oxidative stress induced by CS on bone-forming cells.

Given the negative effect of CS on bone-forming cells in orthopedic patients who smoke (Ehnert *et al*., 2019), we employed an *in vitro* model with human bone marrow MSCs immortalized SCP-1 cell line exposure to CSE to investigate the above issue. This *in vitro* model successfully represents the clinic condition, and thus we can elucidate the molecular mechanisms involved and develop potential treatment strategies for orthopedic patients who smoke to improve bone-forming cells function. Our *in vitro* system confirmed that CSE exposure affected cell viability and delayed MSC osteogenic differentiation, denoted by a significant downregulation in early and late osteogenic markers (AP activity and matrix formation, respectively (Huang *et al*., 2007)) (Sreekumar *et al.*, 2018). In our model, there was increased oxidative stress in MSCs exposed to CSE (Sreekumar *et al*., 2018), a finding that is consistent with our cohort orthopedic patients who smoke (Ehnert *et al.*, 2019) and data from other groups (Church and Pryor, 1985, Pappas, 2011).

Primary cilia are microtubule-based sensory organelles that play an essential role during the osteogenic differentiation of osteoprogenitor cells. Several studies have demonstrated the leading role of primary cilia in skeletal development – to maintain the phenotype of differentiated cells – and have shown a correlation between damaged primary cilia and skeletal abnormalities (Temiyasathit and Jacobs, 2010, Xiao *et al*., 2008, Tummala *et al.*, 2010). Interestingly, osteoblasts and osteocytes with damaged primary cilia present downregulation of osteogenic markers (Delaine-Smith *et al*., 2014). A reduced cilia length in the epithelial airway of smokers is associated with reduced mucociliary clearance (Leopold *et al.*, 2009). Our results demonstrated that CSE exposure reduced the number of ciliated cells as well as the primary cilia length on osteoprogenitor cells; these alterations correlated with an increment in free radical. Moreover, H_2O_2-treated cells showed the same primary cilia structure pattern as with CSE exposure. Additionally, with a sequential time point experiment and chemical disassembly of primary cilia structure (with CH treatment), we demonstrated that removed primary cilia was the cause of impaired MSC differentiation to functional osteoblast (Sreekumar *et al.*, 2018).

At the molecular level, CSE-exposed MSCs showed a decreased gene expression of the Hh pathway transcriptional factor *Gli2*, the downstream protein *BMP-2*, and osteogenic master transcriptional factor *RUNX2* (Shimoyama *et al*., 2007, Zhao

et al., 2006, Sreekumar *et al*., 2018). This finding supports the downregulation of *BMP-2* transcript reported by Chassanidis *et al.* in human periosteum samples from smokers (Chassanidis *et al*., 2012) and Giorgetti *et al*. during alveolar bone healing in rats exposed to cigarette smoke (Giorgetti *et al.*, 2010). It is known that Hh and BMP pathways promote MSC osteogenic differentiation and contribute to bone homeostasis (Zhao *et al*., 2006); therefore, their decreased levels in our system support the delayed fracture healing observed in smokers. Additionally, *RANKL* and *OPG* were downregulated in our model (Sreekumar *et al.*, 2018), and thus the RANKL/OPG ratio did not change, as previously observed in blood from smokers (Ehnert *et al*., 2019). Consequently, bone-resorbing cell function is not negatively affected by bone-forming cells *via* RANKL pathway in smokers. Thus, we shall be considered that increased oxidative stress from smoking may be a leading cause for the damaged primary cilia structure and delayed differentiation on bone-forming cells. These dysfunctions impair fracture repair in smokers.

Resveratrol is a potent antioxidant and anti-inflammatory with protective and positive effects on the skeletal system (Alarcon De La Lastra and Villegas, 2005, Gülçin, 2010, Jiang *et al.*, 2020, Murgia *et al*., 2019). We demonstrated that 1 µM resveratrol reduced CSE-induced oxidative stress in MSCs (Sreekumar *et al.,* 2018). Accordingly, resveratrol protected the primary cilia structure, improved osteogenic differentiation (increases AP activity and matrix production), and re-established gene expression of osteogenic transcriptional factors and inducers (*Gli2*, *RUNX2*, and *BMP-2*) on MSCs co-incubated with CSE (Sreekumar *et al*., 2018). Thus, resveratrol might positively affect bone homeostasis in smokers and enhance fracture healing. Our results are supported by several studies that have demonstrated the positive effect of resveratrol on *in vivo* and *in vitro* bone oral cavity models (Franck *et al*., 2018, Andreou *et al*., 2004, Ribeiro *et al*., 2017, Lameira Jr *et al*., 2018, Şahin *et al*., 2016). However, the use of resveratrol to improve bone formation in smokers with delayed fracture healing cannot be easily implemented *via* dietary intake. Although we used a physiological resveratrol concentration in our study, it is not reachable in the human system without causing toxicity (Zunino and Storms, 2015). Indeed, a daily intake of approximately 8 kg of grapes or 20 L of red vine would be required to achieve this concentration. However, oral supplementation with 13 mg resveratrol pill provides the blood plasma concentration used in the study.

Our results provide evidence that protection and restoration of primary cilia arrange from the harmful impact of CSE improve osteogenesis (Aspera-Werz *et al*., 2018). Treatment strategies that protect this essential organelle and detect and translate signals into an appropriate response are promising alternatives to improve bone cells respone to external clues (*e.g*. mechanical stimulation, pulsed electromagnetic field, *etc.*). These strategies should enhance osteogenic differentiation and function and, hence, contribute to bone homeostasis.

While it is known that more than 150 of the 6500 molecular compounds contained in cigarette smoke from tobacco combustion are toxic (Rothem *et al*., 2009, Pappas, 2011), the exact compounds that induce oxidative stress and impair osteogenesis of osteoprogenitor cells remain unknown. According to reports, the osteosarcoma cell line Saos-2 treated with nicotine enlarge oxidative stress, a phenomenon that leads to apoptosis (Marinucci *et al*., 2018). Additionally, nicotine exposure decreases matrix formation by this cell type (Tanaka *et al.*, 2005a). Nevertheless, another study indicated that nicotine upregulates bone marrow stromal cell osteogenic activity (Daffner *et al*., 2012). In line with this result, our system – treated with nicotine blood concentrations its primary metabolite (cotinine) that are found in smokers – neither induced nor affected MSC osteogenic differentiation (Aspera-Werz *et al.*, 2018). These controversial findings regarding the role of nicotine on osteogenic differentiation can be explained by the concentrations used in the studies: high nicotine concentrations show detrimental effects on bone-forming cells and lower concentrations (associated with blood concentrations of nicotine in smokers) positively affect the osteogenesis of bone-forming cells. Our study highlights the detrimental effect of the molecular compounds originated from tobacco combustion (upregulated $\cdot O_2^-$ and reduced total GSH) (Aspera-Werz *et al*., 2018) on osteoprogenitor cell oxidative stress levels. These data lead us to hypothesized that homeostasis dysregulation in the antioxidative system may underlie the negative compromise osteogenic differentiation of MSCs chronically treated with CSE (Bai *et al*., 2004, Lee *et al*., 2006, Romagnoli *et al.*, 2013, Aspera-Werz *et al*., 2018).

Based on the above findings, we examined the effect of co-incubation with NAC (1 mM), as a precursor for GSH, or L-ascorbate (200 µM), due to its free radical scavenger properties and the lower levels present in the blood of smokers (Kelly, 2003, Smith and Hodges, 1987), as a treatment strategy to enhance osteogenic differentiation of MSCs exposed to CSE. NAC and L-ascorbate reversed the CSE-

mediated detrimental effect on MSC osteogenic differentiation (reduced ROS, reestablished primary cilia structure, increased AP activity, and matrix formation) (Aspera-Werz *et al*., 2018). Interestingly, the addition of NAC and L-ascorbate neither induced the antioxidant signaling pathway *via* its transcription factor phospho-Nrf2 nor augmented the expression of antioxidative enzymes (SOD-1 and catalase) compared with CSE treatment (Aspera-Werz *et al*., 2018). Our findings are consistent with several studies that have also shown the beneficial effect of NAC and L-ascorbate on osteogenic differentiation and bone formation (Yamada *et al*., 2013, Ji *et al*., 2011, Jun *et al*., 2008, Ganta *et al*., 1997, Alcain and Buron, 1994, Aspera-Werz *et al*., 2018). Lee *et al.* demonstrated the protective effect of NAC on MC3T3-E1 cells treated with H_2O_2 (Lee *et al*., 2015) and Takamizawa *et al*. demonstrated that L-ascorbate enhances osteogenesis of MG-63 osteosarcoma cells (Takamizawa *et al*., 2004).

In contrast to resveratrol, the L-ascorbate and NAC concentrations that promoted beneficial effects in our system are unobtainable in the blood plasma by oral supplementation. The peak plasma level of 200 µM L-ascorbate occurs with oral intake of 3 g L-ascorbate every 4 hours (Padayatty *et al*., 2004). For NAC, the mean peak plasma concentration of 15 µM occurs after 600 mg administrated orally (Borgstrom *et al*., 1986). Nevertheless, intravenous administration of L-ascorbate or NAC may be considered as a possible treatment for smokers during the fracture healing process because this modality achieves plasma concentrations up to 800 µM for L-ascorbate (Padayatty *et al*., 2004) and 121 µM for NAC (Olsson *et al*., 1988). While our study demonstrated that co-incubation with CSE and antioxidant positively correlated with enhanced MSCs differentiation to bone-forming cells compared to cells treated with CSE alone. Therefore, the use of antioxidants in the clinic as a promising treatment for orthopedic patients should be limited to smokers due to prooxidative effects of antioxidants in non-smokers with physiological ROS levels. Additionally, our system reacts adequately to the oxidative stress stimuli, given that CSE induced the Nrf2 antioxidant signaling pathway (releasing Nrf2 for phosphorylation and nuclear translocation) an antioxidative enzyme synthesis (SOD-1 and catalase) (Aspera-Werz *et al*., 2018). However, the trigger of the antioxidative signaling pathway may not be sufficient or fail to reverse the inhibitory role of CSE-induced oxidative stress on MSCs.

Although our data revealed that nicotine and cotinine did not directly increase free radical productions by osteoprogenitor cells, these substances showed inhibitory effects on SOD and catalase activities (Aspera-Werz *et al*., 2018). These results are

supported by the lower enzymatic activity of antioxidant enzymes found by Raddam *et al*. in smokers' blood (Raddam *et al.*, 2017). Furthermore, nicotine and cotinine also inhibited GR enzymatic activity, interfering with GSH recycling and reducing the total GSH level (Aspera-Werz *et al*., 2018). Thus, nicotine and cotinine partially contribute to the elevated oxidative stress and the adverse effects observed on MSC primary cilia structure and osteogenic differentiation following CSE treatment.

Quitting smoking is the most effective method to alleviate the adverse effect of cigarette smoke on human health (Mills *et al.*, 2011). However, many smokers cannot, wish not to, or fail to quit (Malarcher *et al*., 2011). In the past, several smoke-less nicotine substitute products have been assessed as an alternative to smoking conventional cigarettes (*e.g.* nicotine mouth or nasal spray, transdermal patch, gum or lozenge) (Aspera-Werz *et al.*, 2020). Unfortunately, these therapies fail in most smokers due to a lack of the smoking ritual. Consequently, new technologies are based on preserving the smoking ritual while providing less harmful constituents and maintaining the same nicotine levels found in conventional cigarettes. To achieve this goal, the new technologies have concentrated on developing electronic nicotine delivery systems (*e.g*. e-cigarette, tobacco heating systems [THS]) that avoid tobacco burning and consequently minimize the production of unhealthy compounds formed in mainstream cigarette smoke (Aspera-Werz *et al*., 2020). E-cigarettes are heating device that vaporize a liquid solution (based on propylene glycol, glycerin, flavors, and nicotine [optional]). The resulting aerosol is inhaled. THS are designed for electronically heating rolled tobacco leaves (Heat-stick) up to 350°C in place of igniting or burning (to prevent tobacco combustion, generation of ashes and release of toxic compounds) (Aspera-Werz *et al*., 2020). Interestingly, a current study from our group showed that e-cigarette aerosol does not affect bone morphology, structure, and strength compared with CS in a mouse model exposed to these compounds for 6 months (Reumann *et al.,* 2020). Additionally, we demonstrated that MSC and human osteoblast exposure to aqueous extract from THS showed less impact on cell viability and function, primary cilia structure, and oxidative stress levels compared with CSE (Aspera-Werz *et al*., 2020).

The fact that MSC differentiation to functional bone-forming cells was not directly influenced by nicotine and cotinine led us to supposition that smoke-less nicotine substitute products (with alternatives that administer nicotine such as sprays, patches, gums, e-cigarettes, or THS) may be a promising alternative to maintain

appropriate bone homeostasis and, consequently, reduce fracture risk and prevent or delay secondary osteoporosis in smokers (Aspera-Werz *et al*., 2020). Nonetheless, nicotine and cotinine inhibit the antioxidative cell system function and after trauma or orthopedic surgery, oxidative stress levels rice. These findings critically challenge the supposition that nicotine substitute products are a more propitious replacement for cigarette consume in orthopedic patients (Aspera-Werz *et al*., 2018).

Until now, we focused on the role of CS and the molecular compounds from tobacco combustion on MSC osteogenic differentiation. However, through fracture repair of long bone, chondrocytes play a vital role. MSCs invade the fracture hematoma, condense, and give rise to functional chondrocytes that synthesize the soft callus, which will be substituted with mineralized tissue by osteoblast *via* intramembranous or endochondral ossification (Goldhahn *et al.*, 2012, Dangelo *et al.*, 2001, Einhorn and Gerstenfeld, 2015). In skeletal tissue, TGF-β1 is among the principal and abundant cytokines that modulates the migration, behavior, and function of MSCs, chondrocytes, osteoblasts. Consequently, this protein influences endochondral ossification during fracture healing (Dallas *et al*., 2005, Harris *et al*., 1994, Ignotz and Massague, 1987, Robey *et al*., 1987, Noda and Camilliere, 1989, Ehnert *et al*., 2018, Tang *et al*., 2009, Xia *et al*., 2017, Ehnert *et al*., 2017b, Poniatowski *et al.,* 2015). Through the inflammatory phase of the fracture healing, the TGF-β concentration increases; this signal acts as a chemoattractant for MSCs to migrate to the fracture hematoma. Interesting, Moghaddam *et al*. and Zimmerman *et al.* demonstrated that smokers and patients with delayed fracture healing present lower TGF-β concentrations in their serum (Moghaddam *et al.*, 2010, Zimmermann *et al.,* 2005).

In addition to reducing serum TGF-β levels, our study revealed that CS inhibited TGF-β canonical signaling by demolishing primary cilia, the key organelle involved in the signaling pathway in MSCs. There was a similar outcome with chemical removal of primary cilia with CH. These findings support the core function of the primary cilia integrity on TGF-β signaling (Aspera-Werz *et al.*, 2019). However, CSE did not completely abolish TGF-β signaling; thus, there are receptors localized in the cell membrane that can propagate the signal. Nevertheless, receptors in primary cilia are unable to contribute to activation of the pathway. Additionally, as previously shown, resveratrol-mediated protection of primary cilia integrity enhanced CSE-impair TGF-β signaling pathway. The analysis of canonical TGF-β signaling downstream regulators

and cofactors revealed reduced protein synthesis and activation of phospho-Smad2, phospho-Smad3, and Smad4 upon CSE influence (Aspera-Werz *et al*., 2019). Interestingly, recombinant TGF-β1 treatment had a more pronounced effect on phospho-Smad2 expression; thus, Smad2 is more affected by CSE compared with Smad3 (Aspera-Werz *et al.*, 2019). Therefore, the detrimental effects of CS on TGF-β signaling are more pronounced because the function of Smad2 cannot be compensated or rescued by Smad3. This fact was demonstrated *in vivo* with Smad2 deficient mice that result in early embryonic lethality; however, Smad3 knockout mice are viable and fertile (Brown *et al.,* 2007). Additionally, as a consequence of CSE-reduced Smad4 synthesis, less cofactor is available to form the active complex (binding to phosphorylated Smads), and subsequently could not translocate to the nucleus and activate the transcription of target genes (Aspera-Werz *et al*., 2019). Moreover, under smoking conditions, Smad4 will not be available to interact with the transcriptional factors Runx2 and AP-1 (c-Fos/JunD), and thus MSC osteogenic differentiation is delayed (Lai and Cheng, 2002).

Our study also demonstrated that cigarette smoke impaired nuclear import of phospho-Smad3/4 complex contributes to impair TGF-β signaling (Aspera-Werz *et al.*, 2019). The nuclear translocation system of the cells could not compensate the deficiency of phospho-Smads. Moreover, our results proved that the negative CSE effect on TGF-β signaling was not related to inhibition of the ligand-receptor interaction since a ligand-independent trigger of TGF-β receptor type I (ALK5) did not compensate the down regulation of phospho-Smad2 caused by CSE (Aspera-Werz *et al.*, 2019). Thus, our results suggest that cigarette smoke may lead to inappropriate TGF-β signaling through nonfunctional primary cilia (Aspera-Werz *et al.*, 2019). Therefore, internalization of the ligand-receptor assemblage could fail at the base of the primary cilia (pocket) under primary cilia ablation (Clement *et al*., 2013). Thus, we suggest that CSE negatively influences the endocytosis of the activated complex through dysfunctional primary cilia. Consequently, a lack of ligand–receptor endocytosis directly affects the interaction of ALK5 with cofactors like Smad anchor for receptor activation (SARA), which improves the interaction of Smad mediators and enhances their activation (Hill, 2009). Overall, cigarette smoke may either inhibit the kinase function of the ALK5 or interfere with the binding of cofactors and affect the activation of Smad mediators. However, further studies should be performed to evaluate the

direct effect of CS on clathrin-dependent endocytosis, TGF-β receptor type I serine/threonine kinase activity, and interaction with cofactors.

In our model, CSE exposure destroyed the primary cilia structure and impaired TGF-β signaling (Aspera-Werz *et al.*, 2019). Given that the primary cilium is supposed to coordinate cell migration *via* TGF-β signaling, CSE reduced MSC migration (Aspera-Werz *et al.*, 2019), consistent with findings from Wahl and colleagues (Wahl *et al*., 2016). Moreover, migration-related disorders are linked to deterioration and loss of the primary cilia structure and function (Veland *et al.*, 2014). Interestingly, induction of TGF-β signaling with human recombinant TGF-β1 treatment enhanced MSC migration exposure to CSE. However, human recombinant TGF-β1 treatment did not improve the detrimental effect of CSE on MSC proliferation (Aspera-Werz *et al.,* 2019). Thus, we suggest that direct topical application of human recombinant TGF-β1 may promote MSC infiltration into the area of bone injury, but the appropriate proliferation will be jeopardized in orthopedic patients who smoke (Aspera-Werz *et al.*, 2019). Moreover, human recombinant TGF-β1 induces early and inhibits late MSC osteogenic differentiation (Alliston *et al.*, 2001, Maeda *et al.*, 2004). Therefore, to enhance fracture healing in smokers, the use of human recombinant TGF-β is challenged.

To identify the role of CS on MSC chondrogenic differentiation through TGF-β signaling mediated by primary cilia, we evaluated the expression of chondrogenic markers on MSC differentiated with TGF-β1 in combination with CSE primary cilia disruption. As expected, CSE downregulated *COL2A1* (encodes a major ECM protein in cartilage) expression, which is normally induced by TGF-β signaling. This finding suggests that there is weak cartilage synthesis in CSE-treated chondrocytes. Additionally, *COLXA1* (encodes a hypertrophic chondrocyte marker) as well as *SOX9* (encodes a transcription factor that activates early chondrogenic genes) were upregulated after CSE treatment. These changes indicate impaired MSC chondrogenic differentiation with CSE primary cilia disruption (Aspera-Werz *et al.*, 2019). In line with these data, it was also reported that CSE induces downregulation of COL2A1 and upregulation of SOX9 transcript levels in human adipose-derived MSCs (Wahl *et al*., 2016). Moreover, *in vitro* cell culture of human periodontal ligament-derived stem cells from smokers showed reduced glycosaminoglycan (evaluated by alcian blue staining) deposition in comparison to non-smokers (Ng *et al.*, 2015). Deren and colleges also demonstrated the close association between primary cilia structure and chondrogenic profile (Deren *et al.*, 2016). In that study, biological (small interfering RNA technology)

and pharmacological (CH treatment) disruption of primary cilia downregulated *COL2A1*, *COLXA1*, and *BMP-2* transcript levels in chondrocytes. Nevertheless, only *COLXA1* was reduced in hypertrophic chondrocytes with truncated primary cilia. Moreover, mechanical induction could not reestablish the gene expression due to lack of primary cilia structure, likely because primary cilia in chondrocytes have multiple functions. Indeed, primary cilia disruption affects the expression of more chondrogenic marker genes in comparison with hypertrophic chondrocytes (Deren *et al.*, 2016). Unexpectedly, our results showed increased *ACAN* (encodes a major proteoglycan in cartilage ECM) transcript levels in MSCs under chondrogenic differentiation with CSE (Aspera-Werz *et al.*, 2019). Interestingly, Mwale *et al.* challenged the use of *ACAN* as a chondrogenic marker due to its constitutive expression in MSCs (Mwale *et al.*, 2006). Moreover, *ACAN* upregulation positively correlates with chondroid cells accumulation in response to tendon damage (Bell *et al.*, 2013). Interestingly, there was a similar gene expression profile in MSCs that underwent chondrogenic differentiation with primary cilia disrupted by CH treatment (Aspera-Werz *et al.*, 2019). Thus, we demonstrated that impaired primary cilia structure due to CSE or CH treatment disrupted TGF-β signaling propagation and the expression of target genes.

Smad4 deficiency may partly explain the impaired MSC chondrogenesis during CSE exposure: a lack of Smad4 in chondrocytes negatively affects proliferation, increases cell apoptosis, and induces a hypertrophic phenotype (Labour *et al.,* 2016). Similarly, Smad4 abrogation in mouse cartilage impairs cell grow, activates programmed cell death, and promotes hypertrophic differentiation, all of which lead to a disorganized growth plate (Zhang *et al.*, 2005).

The observations that harm primary cilia organization via CSE induced oxidative stress, negatively impacted TGF-β signaling propagation, and impaired MSC chondrogenic differentiation relatively explains that smokers with delayed fracture repair experience perturbed endochondral ossification (Aspera-Werz *et al.*, 2019). In addition to the preservation of the primary cilia integrity from CSE-induced ROS, therapies that reestablish Smad dependent (canonical) TGF-β signaling or induce non-canonical Smads activation, such as riluzole that induces Smad2/3 phosphorylation *via* glycogen synthase kinase-3 activation (Abushahba *et al.*, 2012), may represent treatment strategies to reestablish TGF-β signaling and enhance or improve delayed fracture repair in smokers (Aspera-Werz *et al.*, 2019).

Given that human recombinant TGF-β1 improves MSC migration and enhanced early stages of endochondral ossification under CSE exposure, direct topical administration of TGF-β1 may improve the early stages of endochondral ossification during the reparative phase of fracture repair in smokers (Aspera-Werz *et al.*, 2019). When using this treatment, some additional factors should be considered. Systemic administration of TGF-β1 is uncomplicated; however, undesired side effects will develop in other tissues due to its wide distribution. Therefore, direct topical application is strongly suggested. Moreover, the active TGF-β1 half-life is about 2–3 min (Wakefield *et al.*, 1990). Thus, fusion of the cytokine with longer-lived proteins (*e.g.* albumin) or conjugation with high molecular weight polymers should be considered to increase stability and reduce clearance, respectively. Furthermore, TGF-β1 enhances MSC proliferation and early osteogenic differentiation; however, late osteogenic differentiation is inhibited and, consequently, appropriate bone formation is affected (Alliston *et al*., 2001, Maeda *et al.*, 2004). Nevertheless, in smoking context the application of TGF-β1 will not positively influence osteoprogenitor cells grow and differentiation to functional chondrocytes as a consequence of inhibition in the TGF-β signaling demonstrated in our study. So, these factors challenge the implementation of human recombinant TGF-β1 as a treatment strategy to support bone healing in smokers orthopedic patients. (Aspera-Werz *et al.*, 2019).

In summary, our study supports the hypothesis that increased oxidative stress due to compounds from tobacco combustion disrupt primary cilia and hence lead in impaired osteoprogenitor cell differentiation. Our data suggest an imbalance between bone-forming and bone-resorbing cells in smokers. We showed several effects of cigarette smoke on MSC osteogenic differentiation as well as chondrogenic differentiation, including alterations in cell function and oxidative stress levels, dysregulation of central signaling pathways, and changes in the expression of proteins or target genes. More precisely, in smokers a functional primary cilium in a bone cell seems to be essential for appropriate bone homeostasis and fracture healing. However, to evaluate potential treatment strategies for orthopedic patients who smoke, we must elucidate the role of CS on other cell types that are also involved in bone homeostasis and fracture healing. Thus, bone-resorbing cells (osteoclasts), osteocytes, immune cells, and/or endothelial cells should be added to our system to better represent the fracture healing phases.

5. Outlook

Several advances have been made in this study regarding the mechanisms involved in delayed fracture healing in orthopedic patients who smoke. However, there are still several challenges that should be addressed with the aim of developing treatment strategies to improve fracture healing in smokers.

We demonstrated an immunosuppressed status in heavy smokers (Ehnert *et al.*, 2019), and previous studies have indicated that the communication and interaction between immune cells and bone cells is crucial for appropriate bone remodeling and repair (Mori *et al*., 2013, Ponzetti and Rucci, 2019). Therefore, explore the influence of cigarette smoke on immune cell function and its interaction with MSCs in the frame of the inflammatory phase during fracture healing is mandatory to improve the initial phases of fracture repair in smokers. Besides, develop therapies that enhance the immune system response may prevent further complications (*e.g.* infections) that are frequently associated with orthopedic surgery in smokers.

Our system only included MSCs under osteogenic or chondrogenic differentiation with cigarette smoke exposure. This system represents, in part, endochondral ossification in the reparative and remodeling phases. During these phases, osteoclasts also play also an important role. Therefore, co-culture systems that represent the interaction between bone-forming and bone-resorbing cells might be useful for screening the effects of CS as well as antioxidants, TGF-β, or other treatment strategies. The interplay, mediated *via* paracrine and systemic factors, and function of bone cells may be analyzed with an *in vitro* model that better represents human bone metabolism.

There is growing evidence that bone cells interact with the bone matrix, which also regulates bone cell functions (Green *et al.*, 1995, Florencio-Silva *et al.,* 2015). Additionally, culture in a three-dimensional structure provides a close resemblance to the *in vivo* environment of bone cells. Therefore, evaluating the effects of potential treatment strategies for smokers on a three-dimensional structure co-culture could be performed with the use of scaffolds. Scaffolds should represent a structure and composition that is similar to bone matrix. Additionally, this three-dimensional system may be coupled to another tissue representing the function of different organs to closely mimic human physiology. For example, paracrine cross-talk between different

organs may be assayed and potential toxicity or side effects of the additional metabolites form CS on bone cells could be investigated. However, one should consider that the addition of complexity to the model increases the limitation regarding functional test that could be achieved.

Nicotine replacement therapies seem to be a promising alternative for smokers because they do not directly affect bone homeostasis (Reumann *et al.*, 2020). Nevertheless, it is necessary to evaluate the outcome of nicotine replacement therapies on bone cells (co-culture osteoblast/osteoclast) under stress conditions, such as those generated after a trauma (*e.g.* increased oxidative stress by fracture or orthopedic surgery). Additionally, little is known regarding the role of CS on osteocytes. Osteocytes are the furthermost numerous cell type in bone (Johannesdottir and Bouxsein, 2018). They regulate the remodeling of surrounding matrix and thus modulate the production of factors that can recruit or suppress osteoclasts/osteoblasts (Nakashima *et al.*, 2011, Poole *et al.*, 2005, Standal *et al.*, 2014). It would be attractive to analyze changes in the production pattern of osteocyte factors under smoking conditions and how this influences bone homeostasis.

It remains unknown whether enhancing MSC osteogenic differentiation through protection of primary cilia structure with antioxidant treatment would also improve bone healing in orthopedic patients who smoke. Consequently, a clinical trial to assess the outcome of smokers follow antioxidant treatment is needed.

Given that a limited blood supply to the fracture site is an additional aggravating condition in smokers due to harmful effects of CS on vascular tissue, vascularization to the fracture site must be improved. Therefore, it would be interesting to incorporate endothelial cells in our model in order to analyze strategies to improve vessel formation and, consequently, nutrient and oxygen supply to the fracture site.

6. Summary

Since 1976, several studies have demonstrated the correlation between cigarette smoking, reduced bone mass, and impaired fracture healing. Cigarette smoking can affect bone homeostasis directly through harmful effects in bone-forming and bone-resorbing cells or indirectly *via* disturbed hormonal and immune responses. Following orthopedic surgery, smokers frequently show an increased complication rate, which results in augmented health system costs. As a main risk factor for osteoporosis, the underlying mechanisms behind impaired bone remodeling in smokers remain unclear. The purpose of this dissertation was to link the delayed fracture healing observed in orthopedic patients who smoke with the effects of cigarette smoke–induced oxidative stress on primary cilia structure with regard to osteoprogenitor cells signaling and differentiation.

Our study cohort confirmed cigarette consumption as a hazard for developing complications compared to malnutrition and daily alcohol consume. Interestingly, smokers were 5.4 years younger compared with non-smokers but with comparable levels of comorbidities. Additionally, the complication rate positively correlated with the number of cigarette packs consumed per years. Subsequently, smokers' hospitalizations were on average 3 days longer compared with non-smokers. Analysis of blood plasma levels of bone formation markers showed downregulation in smokers. However, bone resorption markers were not affected, and oxidative stress markers were upregulated in comparison with non-smokers. Thus, these results support the impaired fracture healing observed in orthopedic patients who smoke. Regarding the increased complications observed in smokers, blood plasma levels of immune modulator cytokines demonstrated an immunosuppressive status for smokers. Therefore, cigarette consumption negatively affects bone-forming cell function and inhibits the immune response in orthopedic patients. Consequently, investigating treatment strategies that stimulate bone-forming cell function, decrease oxidative stress, or boost the immune system are mandatory to improve the outcome of smoker orthopedic patients.

We developed an *in vitro* system that represents the clinical condition observed in smokers to investigate the underlying mechanisms behind the deleterious effects of cigarette smoking on bone-forming cells. In addition to delayed MSC osteogenic differentiation, cigarette smoke exposure increased oxidative stress and affected the

integrity of the cellular sensor (primary cilia). Interesting, we out results have shown that cigarette smoke exposure impairs MSC differentiation to bone forming cells was not associated with nicotine as well as its principal metabolite cotinine. As a protective approach, resveratrol prevented free radical production, decreased oxidative stress levels, preserved the primary cilia integrity, and improved osteogenic differentiation in response to cigarette smoke exposure. Accordingly, cigarette smoke–induced oxidative stress *via* impaired primary cilia structure negatively affected osteoprogenitor cell osteogenic differentiation and function. Moreover, we demonstrated that cigarette smoke–induced oxidative stress by an accumulation of superoxide radicals and reduced levels of intracellular glutathione. While nicotine and cotinine did not increase oxidative stress, these substances inhibited the function of antioxidative enzymes, contributing indirectly to the adverse outcome observed on MSCs by cigarette smoke exposure. Furthermore, treatment with N-acetylcysteine and L-ascorbate reversed the compromised osteogenic differentiation generated by cigarette smoke *via* upregulation of the cellular antioxidative system and free radical scavenging abilities.

TGF-β is a crucial cytokine that promotes osteoprogenitor cell motility, growth, and suitable differentiation to the bone injury area. There were lower levels of TGF-β in smokers along with orthopedic patients with disturbed fracture repair. Additionally, our results demonstrated that the nuclear import of downstream TGF-β signaling activated effector complex was negatively affected by cigarette smoke, namely due to abrogated primary cilia. Furthermore, the abolition of primary cilia with CH and the protection of primary cilia structure with resveratrol supported the link between TGF-β signaling and primary cilia. Besides, cigarette smoke-blocking TGF-β signaling perturbed osteoprogenitor cell motility, growth, and chondrogenic differentiation. TGF-β treatment did not improve osteoprogenitor cell proliferation and chondrogenic differentiation due to the lack of functional TGF-β signaling.

For the first time, we showed the detrimental effects of cigarette smoke–induced oxidative stress on the primary cilium structure and associated signaling pathways. Antioxidant treatment provided scavenging properties, activated the cellular antioxidative defense system, reduced oxidative stress levels, protected primary cilia structure, and reestablished TGF-β signaling. Consequently, osteoprogenitor cell migration, proliferation, and differentiation are improved. These results suggest that preserving the primary cilia structure may represent a therapeutic goal to support the reparative and remodeling phase of fracture healing in smokers.

7. Zusammenfassung

Seit 1976 haben mehrere Studien den Zusammenhang zwischen Zigarettenrauchen, reduzierter Knochenmasse und beeinträchtigter Frakturheilung gezeigt. Zigarettenrauchen kann die Knochenhomöostase direkt durch schädliche Wirkungen auf knochenbildenden und knochenresorbierenden Zellen oder indirekt durch gestörte hormonelle und Immunantworten beeinflussen. Nach orthopädischen Eingriffen weisen Raucher häufig eine erhöhte Komplikationsrate auf, was zu erhöhten Kosten für das Gesundheitssystem führt. Als ein Hauptrisikofaktor für Osteoporose bleiben die zugrundeliegenden Mechanismen eines gestörten Knochenumbaus bei Rauchern weiterhin unklar. Ziel dieser Dissertation war es, die beobachtete Verzögerung in der Frakturheilung bei orthopädischen Patienten, die rauchen, mit den Auswirkungen von durch Zigarettenrauch induziertem oxidativem Stress auf die primäre Zilienstruktur im Hinblick auf die Signalübertragung und Differenzierung von Osteoprogenitorzellen in Verbindung zu bringen.

Unsere Studienkohorte bestätigte das Rauchen von Zigaretten ein größerer Risikofaktor für die Entwicklung von Komplikationen ist als Unterernährung und täglicher Alkoholkonsum. Interessanterweise waren Raucher im Vergleich zu Nichtrauchern 5,4 Jahre jünger, zeigten aber vergleichbare Komorbiditäten. Zusätzlich korrelierte die Komplikationsrate positiv mit der Anzahl der pro Jahr konsumierten Zigarettenschachteln. In der Folge waren die Krankenhausaufenthalte von Rauchern im Vergleich zu Nichtrauchern durchschnittlich 3 Tage länger. Die Analyse der Blutplasmaspiegel von Knochenbildungsmarkern zeigte eine Herunterregulierung bei Rauchern. Knochenresorptionsmarker waren jedoch nicht betroffen, und oxidative Stressmarker waren im Vergleich zu Nichtrauchern hochreguliert. Somit zeigen diese Ergebnisse die Ursachen für die beeinträchtigte Frakturheilung auf, die bei rauchenden orthopädischen Patienten beobachtet wird. In Bezug auf die bei Rauchern beobachteten erhöhten Komplikationen weisen die Blutplasmaspiegel von immunmodulatorisch wirksamen Zytokinen einen immunsuppressiven Status für Raucher auf. Daher beeinflusst der Zigarettenkonsum die knochenbildende Zellfunktion negativ und hemmt die Immunantwort bei orthopädischen Patienten. Folglich ist die Untersuchung von Behandlungsstrategien, die die knochenbildende

Zellfunktion stimulieren, oxidativen Stress verringern oder das Immunsystem stärken, obligatorisch, um die Genesung von rauchenden orthopädischen Patienten zu verbessern.

Wir haben ein *in vitro*-System entwickelt, das den bei Rauchern beobachteten klinischen Zustand darstellt, um die zugrundeliegenden Mechanismen zu untersuchen, die hinter den schädlichen Auswirkungen des Zigarettenrauchens auf knochenbildende Zellen stehen. Zusätzlich zur verzögerten osteogenen Differenzierung von MSC erhöhte die Exposition gegenüber Zigarettenrauch den oxidativen Stress und beeinträchtigte die Integrität des zellulären Sensors (primäre Zilien). Interessanterweise haben wir gezeigt, dass Nikotin und sein primärer Metabolit Cotinin die osteogene Differenzierung von MSC nicht direkt beeinflussen. Als Behandlungsstrategie, konnten wir zeigen, dass Resveratrol die Produktion freier Radikale verringerte, und so die primäre Zilienstruktur schützt was zu einer verstärkten osteogenen Differenzierung als Reaktion auf die Exposition gegenüber Zigarettenrauch führt. Dementsprechend beeinflusste Zigarettenrauch-induzierter oxidativer Stress über eine beeinträchtigte primäre Zilienstruktur die osteogene Differenzierung und Funktion der Osteoprogenitorzellen negativ. Darüber hinaus haben wir gezeigt, dass Zigarettenrauch oxidativen Stress durch eine Anreicherung von Superoxidradikalen und reduzierte intrazelluläre Glutathionspiegel induziert. Während Nikotin und Cotinin den oxidativen Stress nicht erhöhten, hemmten diese Substanzen die Funktion von antioxidativen Enzymen und trugen indirekt zu den schädlichen Wirkungen bei, die durch Zigarettenrauchexposition auf MSCs beobachtet wurden. Darüber hinaus kehrte die Behandlung mit N-Acetylcystein und L-Ascorbat die durch Zigarettenrauch verursachte beeinträchtigte osteogene Differenzierung durch Aktivierung des zellulären Antioxidationssystems und Radikalfängereigenschaften um.

TGF-β ist ein entscheidendes Zytokin, das die Migration, Proliferation und angemessene Differenzierung von Osteoprogenitorzellen zur Frakturstelle fördert. Es gab niedrigere TGF-β-Spiegel bei Rauchern sowie bei orthopädischen Patienten mit verzögerter Frakturheilung. Zusätzlich zeigten unsere Ergebnisse, dass die nukleare Translokation von nachgeschalteten TGF-β-Signalmodulatoren durch Zigarettenrauch negativ beeinflusst wurde, und zwar aufgrund einer gestörten primären Zilienstruktur. Darüber hinaus unterstützten die pharmakologische Störung der primären Zilien mit Chloralhydrat sowie der Schutz der primären Zilienstruktur mit Resveratrol die

Verbindung zwischen der TGF-β-Signalübertragung und den primären Zilien. Außerdem störte die Blockierung der TGF-β-Signalübertragung durch Zigarettenrauch die Migration, Proliferation und chondrogene Differenzierung von Osteoprogenitorzellen. Die TGF-β; -Behandlung verbesserte die Proliferation von Osteoprogenitorzellen und die chondrogene Differenzierung aufgrund des Fehlens einer funktionellen TGF-β; -Signalweges nicht.

Zum ersten Mal zeigten wir die schädlichen Auswirkungen von durch Zigarettenrauch induziertem oxidativem Stress auf die primäre Zilienstruktur und die damit verbundenen Signalwege. Die antioxidative Behandlung lieferte abfangende Eigenschaften, aktivierte das zelluläre antioxidative Abwehrsystem, reduzierte den oxidativen Stress, schützte die primäre Zilienstruktur und stellte die TGF-β-Signalübertragung wieder her. Folglich werden die Migration, Proliferation und Differenzierung von Osteoprogenitorzellen verbessert. Diese Ergebnisse legen nahe, dass die Erhaltung der primären Zilienstruktur ein therapeutisches Ziel darstellen kann, um die Reparatur- und Umbauphase der Frakturheilung bei Rauchern zu unterstützen.

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9. Appendix

9.1 Ethic statement

All studies involving human participants were performed in accordance with the 1964 Helsinki declaration and its later amendment. Collection of the clinically relevant data was performed in accordance with the ethical vote 193/2014BO2. Serum samples were harvested only after medical consultation and participant have signed a written informed consent in agreement with the ethical vote 538/2016BO2. Corresponding ethical votes (193/2014BO2 and 538/2016BO2) were approved by Ethik-Kommission an der Medizinischen Fakultät der Eberhard-Karls-Universität und am Universitätsklinikum Tübingen.

10. Declaration

I declare that the submitted doctoral thesis has been composed solely by myself under the guidance of Prof. Dr. Andreas Nüssler. Only manuscripts for which I was significantly involved in the planning, execution, and evaluation of the experiments, as well as in the writing of the manuscripts and visualization of the results, were included in this thesis. This thesis has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own. This thesis was linguistically edited by a commercial Professional English Proofreading Service (Proof-Reading-Service.com).

Herrenberg, 10 September 2020

Romina Haydeé Aspera-Werz

11. Author contribution

Smoking Dependent Alterations in Bone Formation and Inflammation Represent Major Risk Factors for Complications Following Total Joint Arthroplasty.

J Clin Med. 2019

The conceptualization of the study was performed by Dr. Sabrina Ehnert, Dr. Christoph Ihle, Romina Aspera-Werz and Prof. Dr. Andreas Nüssler.

The formal analysis was done by Dr. Sabrina Ehnert, Dr. Christoph Ihle and Romina Aspera-Werz.

The investigation was carried out by Romina Aspera-Werz, Markus Trost and Barbara Zirn

Resources were provided by Dr. Christoph Ihle, Dr. Ingo Flesch and Dr. Steffen Schröter.

The manuscript was written and prepare by Dr. Sabrina Ehnert and Dr. Christoph Ihle.

Additional writing, review and editing of the manuscript was performed by Romina Aspera-Werz, Markus Trost, Barbara Zirn, Dr. Ingo Flesch, Dr. Steffen Schröter, Prof. Dr. Borna Relja and Prof. Dr. Andreas Nüssler.

Visualization was carried out by Dr. Sabrina Ehnert.

The supervision was achieved by Dr. Sabrina Ehnert and Dr. Christoph Ihle

Prof. Dr. Andreas Nüssler was responsible for the project administration

Dr. Sabrina Ehnert, Romina Aspera-Werz and Prof. Dr. Andreas Nüssler were involved in the funding acquisition.

Resveratrol Protects Primary Cilia Integrity of Human Mesenchymal Stem Cells from Cigarette Smoke to Improve Osteogenic Differentiation *In Vitro*

Arch Toxicol. 2018

The conceptualization of the study was performed by Dr. Vrinda Sreekumar, Romina Aspera-Werz, Dr. Sabrina Ehnert and Prof. Dr. Andreas Nüssler.

The formal analysis was done by Dr. Vrinda Sreekumar, Romina Aspera-Werz, Julius Strobel and Daniel Heid.

The investigation was carried out by Romina Aspera-Werz, Dr. Vrinda Sreekumar, Julius Strobel and Daniel Heid.

Resources were provided by Dr. Anna Schreiner and Dr. Christian Arnscheidt.

The manuscript was written and prepare by Dr. Vrinda Sreekumar, Romina Aspera-Werz.

Additional writing, review and editing of the manuscript was performed by Dr. Sabrina Ehnert, Julius Strobel, Dr. Gauri Tendulkar, Daniel Heid, Dr. Anna Schreiner, Dr. Christian Arnscheidt and Prof. Dr. Andreas Nüssler.

Visualization was carried out by Dr. Vrinda Sreekumar, Romina Aspera-Werz

The supervision was achieved by Dr. Vrinda Sreekumar, Dr. Sabrina Ehnert and Prof. Dr. Andreas Nüssler.

Prof. Dr. Andreas Nüssler was responsible for the project administration

Dr. Vrinda Sreekumar, Dr. Sabrina Ehnert and Prof. Dr. Andreas Nüssler were involved in the funding acquisition.

Nicotine and Cotinine Inhibit Catalase and Glutathione Reductase Activity Contributing to the Impaired Osteogenesis of SCP-1 Cells Exposed to Cigarette Smoke.

Oxid Med Cell Longev. 2018

The conceptualization of the study was performed by Romina Aspera-Werz, Dr. Sabrina Ehnert, Dr. Vrinda Sreekumar and Prof. Dr. Andreas Nüssler.

The formal analysis was done by Romina Aspera-Werz, Dr. Sabrina Ehnert and Daniel Heid.

The investigation was carried out by Romina Aspera-Werz, Dr. Sabrina Ehnert and Daniel Heid.

Resources were provided by Dr. Christian Arnscheidt.

The manuscript was written and prepare by Romina Aspera-Werz.

Additional writing, review and editing of the manuscript was performed by Dr. Sabrina Ehnert, Daniel Heid, Sheng Zhu, Tao Chen, Bianca Braun, Dr. Vrinda Sreekumar, Dr. Christian Arnscheidt and Prof. Dr. Andreas Nüssler.

Visualization was carried out by Romina Aspera-Werz and Dr. Sabrina Ehnert,

The supervision was achieved by Dr. Sabrina Ehnert, Romina Aspera-Werz and Prof. Dr. Andreas Nüssler.

Prof. Dr. Andreas Nüssler was responsible for the project administration

Dr. Sabrina Ehnert, Romina Aspera-Werz and Prof. Dr. Andreas Nüssler were involved in the funding acquisition.

Cigarette Smoke Induces the Risk of Metabolic Bone Diseases: Transforming Growth Factor Beta Signaling Impairment *via* Dysfunctional Primary Cilia Affects Migration, Proliferation, and Differentiation of Human Mesenchymal Stem Cells.

Int J Mol Sci. 2019

The conceptualization of the study was performed by Romina Aspera-Werz, Dr. Sabrina Ehnert, and Prof. Dr. Andreas Nüssler.

The methodology was done by Romina Aspera-Werz, Dr. Sabrina Ehnert and Prof. Dr. Andreas Nüssler.

Data curation was carried out by Romina Aspera-Werz, Tao Chen, Sheng Zhu, and Theresa Fröhlich.

The formal analysis was performed by Romina Aspera-Werz, Tao Chen and Dr. Sabrina Ehnert.

The investigation was carried out by Romina Aspera-Werz, Tao Chen and Dr. Sabrina Ehnert.

The manuscript was written and prepare by Romina Aspera-Werz.

Additional writing, review and editing of the manuscript was performed by Dr. Sabrina Ehnert, Tao Chen, Sheng Zhu, Theresa Fröhlich and Prof. Dr. Andreas Nüssler.

Visualization was carried out by Romina Aspera-Werz, Dr. Sabrina Ehnert, and Prof. Dr. Andreas Nüssler.

The supervision was achieved by Romina Aspera-Werz, Dr. Sabrina Ehnert, and Prof. Dr. Andreas Nüssler.

Prof. Dr. Andreas Nüssler was responsible for the project administration

Dr. Sabrina Ehnert, Romina Aspera-Werz and Prof. Dr. Andreas Nüssler were involved in the funding acquisition.

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