Vascular endothelial growth factors and receptors - from normal dental pulp to apical pathology

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Scientific environment

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Contents

Abbreviations

ACTB	β-Actin
APC	Antigen Presenting Cell
BEC	Blood endothelial cell
B2M	Beta-2 microglobulin
BMP	Bone Morphogenic Protein
CCL	Chemokine ligand
CD	Cluster of differentiation
µCT	Micro-Computed Tomography
CT-1	Cardiotrophin-1
D2-40	Human podoplanin antibody
DAB	3, 3'-diaminobenzidine
DICOM	Digital imaging and communication in medicine
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ehtylenediaminetetraacetic acid
ELA-2	Neutrophil elastase
ELISA	Enzyme-linked immunosorbant assay
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HEV	High endothelial venule
H&E	Hematoxylin and eosin
HIF	Hypoxia-inducible factor
HIS48	Granulocyte antibody
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
IFN	Interferon
IGF	Insulin-like growth factor

IL	Interleukin
KC	Keratinocyte chemoattractantMCF
LEC	Lymphatic endothelial cell
LPS	Lipopolysaccharide
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
MHC II	Major histocompatibility complex class II
MKC	Murine keratinocyte chemoattractant
MIP	Macrophage inflammatory protein
NFAT	Nuclear factor activated T-cells
NOD	Nucleotide binding oligomerization domain receptors
NRP	Neuropilin
OPG	Osteoprotegerin
PDL	Periodontal ligament
PECAM-1	Platelet endothelial cell adhesion molecule -1
Pl3K	Phosphoinositide kinase
PFA	Paraformaldehyde
PlGF	Placental growth factor
PKC	Protein kinase C
PLA2G6	Phospholipase A2 group VI
PMN	Polymorphonuclear leukocyte
Prox-1	Prospero homebox-1
RANK/L	Receptor activator of nuclear factor kappa-B/ligand
RANTES	Regulated on activation, normal T cell expressed and secreted
RPL13A	Ribosomal Protein L13a
SHC2	Src Homology 2 Domain Containing Transforming Protein 2
Tc	T-cytotoxic cell
Th	T-helper cell
TGF	Transforming growth factor

TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TRAP	Tartrate resistant acid phosphatase
VEGF	Vascular endothelial growth factors
VEGFR	Vascular endothelial growth factor receptor
vWF	von Willebrand factor

Abstract

The VEGFs -A, -B, -C, -D are important signaling molecules with pivotal implication in the growth of blood and lymphatic vessels, the so-called angio- and lymphangiogenesis. They exert their activities by binding to their receptors, VEGFRs-1, -2 and -3. Their involvement in pathologic processes such as tumor growth or inflammatory disorders has been thoroughly described, rheumatoid arthritis being just one example. The VEGF family represents the link between angiogenesis and bone turnover, as seen in cancer metastasis to the bone or arthritis. Furthermore, they are involved in the differentiation of dendritic cells and lymphocytes, while macrophages and PMNs have been identified as sources of VEGFs, showing a mediatory function of these molecules in immune responses.

The presence of VEGF-A and its main angiogenic receptor VEGFR-2 in well-vascularized normal dental pulp has been previously described. Apical periodontitis, a common inflammatory disease caused by the interaction of root canal bacteria with the host immune response, is characterized by bone resorption. VEGF-A is also known to be present in these lesions of endodontic origin.

However, the picture of VEGF family and their receptors with respect to location and function in the dental pulp and in periapical pathology have so far been incomplete.

The aims of this thesis were to identify and map the presence of VEGF family and their receptors VEGFR-2 and -3 in apical periodontitis and dental pulp and to investigate their role in periapical disease development.

In normal rat apical periodontium (Paper I), VEGF-A, -C, -D and VEGFR-2 and -3 were present on blood vessels. Upon endodontic exposure for periapical disease development, an intensification of immunohistochemically stained vascular structures was noticed. Macrophages and neutrophils expressed all VEGFs and VEGFRs in the lesions, with macrophages being an important source of VEGF-C and -D. Osteoclasts were the source for VEGFR-2 and -3. The gene expression of VEGF-A and VEGFR-3 was significantly up-regulated following pulp exposure. The results suggest the

implication of VEGF family and their receptors in the periapical immune response, vascular remodeling and in bone resorptive activities.

In human periapical lesions (Paper II) VEGFs and VEGFRs were expressed on blood vessels and on macrophages, PMNs, B- and T-lymphocytes. At the gene level, significant up-regulations were recorded for genes involved in VEGF-mediated angiogenic activity, such as phosphatidylinositol-3-kinases (*Pl3K*), protein kinase C (*PKC*), mitogen-activated protein kinases (*MAPK*) and phospholipases (*PL*). These findings suggest the implication of VEGF family in ongoing immune reactions along with vascular remodeling in human established periapical lesions.

The normal dental pulp (Paper III) presented with blood vessels, macrophages and T-lymphocytes positive for the same VEGFs and VEGFRs. Furthermore, VEGF-B was only seen at cellular level in the dental pulp. Twenty-six of 84 VEGF signaling genes, including *VEGFR-3* were significantly altered in the dental pulp compared with control PDL. The pulpal tissue has high VEGF signaling capacity with respect to immune responses and vascular activity.

Using specific markers for lymphatic vessels, we confirmed the absence of lymphatic vessels from both apical periodontium and the dental pulp. Macrophages expressing LYVE-1 were found in human periapical lesions and the dental pulp, with an assumed angiogenic role.

Upon inducing periapical lesions we systemically blocked VEGFR-2 and/or -3 in order to investigate their signaling patterns with respect to lesion size, angiogenesis, local inflammatory response and lymphangiogenesis in the draining lymph nodes (Paper IV). We have found that VEGFR-2 reduces inflammation whereas combined VEGFR-2 and -3 signaling causes an increase of the process, seen in amounts of PMNs and osteoclasts, as well as different cytokines expression. In the regional lymph nodes, lymphangiogenesis is dependent on VEGFR-2 and/or VEGFR-3 signaling.

The results of these studies provide evidence on the presence of VEGFs and VEGFRs in dental pulp and apical periodontitis, with implications in immune responses and vascular remodeling. VEGFR-2 and/or -3 signaling influences inflammatory reactions during periapical disease development.

List of papers

I. **Bletsa A, Virtej A, Berggreen E (2012): "Vascular endothelial growth factors are up-regulated during development of apical periodontitis", Journal of Endodontics; 38(5):628-35.**

II. **Virtej A, Løes SS, Berggreen E, Bletsa A (2013): "Localization and signaling patterns of vascular endothelial growth factors and receptors in human periapical lesions", Journal of Endodontics; 39(5):605-11.**

III. **Virtej A, Løes S, Iden O, Bletsa A, Berggreen E (2013): "Vascular endothelial growth factors in normal human dental pulp: a study of gene and protein expression", European Journal of Oral Sciences; 121(2):92-100.**

IV. **Virtej A, Bletsa A, Papadakou P, Pytowski B, Sasaki H, Berggreen E (2015): "VEGFR-2 reduces while combined VEGFR-2 and -3 signaling increases inflammation in apical periodontitis". Manuscript.**

1. Introduction

1.1 Vascular endothelial growth factors (VEGFs) and receptors (VEGFRs)

First organ system to develop during embryogenesis is the circulatory system. The major regulators of blood and lymphatic vessel development are the members of the vascular endothelial growth factor (VEGF) family. The initial formation of the primitive vascular plexus is known as vasculogenesis [1], whereas the process of blood and lymphatic vessel development from preexisting vessels, as occurring in adult tissues, is known as angio- and lymphangiogenesis, respectively. The essential cells from which the vessels are built are the endothelial cells (ECs) [2].

The VEGF family in mammals includes five glycoproteins: VEGF-A (or VEGF/vascular permeability factor), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) [3]. These bind with differing affinities to three endothelial transmembrane tyrosine kinase receptors, the VEGFRs: VEGFR-1 (Flt1), VEGFR-2 (human KDR/mouse Flk1) and VEGFR-3 (Flt4). Neuropilins (NRP-1 and NRP-2) can also work as co-receptors to the various VEGFRs in ECs [4].

VEGF-A is essential for development of blood vessels and exerts this activity via VEGFR-2, but is also to bind VEGFR-1, as well as NRP-1 and NRP-2 [5]. The angiogenic effect is mainly driven by VEGFR-2, while VEGFR-1 acts as a negative regulator of the process [6].

Under physiologic conditions, ECs in adult organisms are mostly quiescent. Exceptions, during which VEGF-A and its receptors increase in expression, include exercise-induced angiogenesis in heart and skeletal muscle [7], the hair cycle [8], the female reproductive cycle and placental growth [9] and wound healing [10]. VEGF-A mostly exerts its' activities on ECs, but it can also bind to its' receptors on hematopoietic stem cells, neurons or monocytes [11]. Osteoblasts and chondrocytes produce VEGF-A [12, 13], linking it to skeletal growth and endochondral bone formation [14, 15]. The presence of VEGFR-2 on both osteoblasts and osteoclasts indicates the involvement of VEGF-A in bone remodeling [16]. Activation of

angiogenesis starts as VEGF-A engages with VEGFR-2 thereby inducing the proliferation, survival, sprouting and migration of ECs and increasing endothelial permeability [3].

Under homeostatic conditions, VEGF-B is highly expressed in heart myocardium, skeletal muscle, brown fat, vascular smooth muscle, brain, kidney and parietal cells of the stomach [17-20], while PlGF is found in placenta, heart, lungs, skeletal muscle and adipose tissue [21-23]. Both are specific ligands to VEGFR-1 [3, 24], but can also transduce their signals via NRP-1 [25]. While PlGF has an angiogenic role [26, 27], VEGF-B seems to be related to metabolic functions [28, 29] neuroprotection [30] and primarily connected to inflammatory settings [31].

VEGF-C and -D normally bind to VEGFR-3 and are responsible for lymphatic vessel development. However, upon proteolytic activation, VEGF-C and -D also bind to VEGFR-2, thus being involved in angiogenic, as well as lymphangiogenic activities [32]. NRP-2 is a co-receptor for both VEGF-C and -D and required for efficient sprouting of lymphatic capillaries [33]. During embryogenesis, VEGF-C is expressed around areas of lymphatic development, along with its receptor VEGFR-3 [34, 35]. The expression decreases thereafter, however remaining high in lymph nodes [36]. VEGF-D is expressed in various locations in murine embryos, while in humans it is strongly detected in skeletal muscle, colon, small intestine, heart and lung [37-39]. Although VEGF-C is essential for lymphatic development, VEGF-D seems to be dispensable, with uncertain physiological role during embryogenesis, as seen in knockout mice models [40]. VEGFR-3 is widely expressed in ECs during mouse embryogenesis, but later on it is mostly restricted to lymphatic endothelium [41, 42]. It seems to also play a role in mouse embryonic angiogenesis [43]. In human adult tissues, VEGFR-3 is mostly found on lymphatic ECs, exerting a protective role at this level, but could also be identified on fenestrated blood capillaries [44], monocytes, macrophages and some dendritic cells [45, 46]. Figure 1 gives an overview of VEGF family and their binding affinities to different receptors.

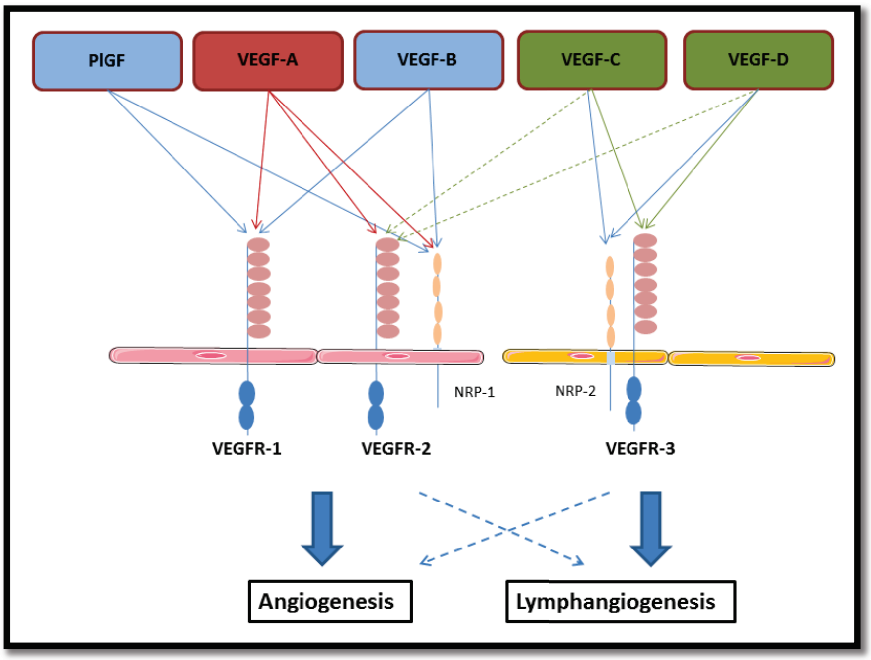

Figure 1. Schematic representation of VEGF binding affinities to the different receptors and their involvement in angio- and lymphangiogenesis. VEGF-A is the main angiogenic factor. VEGF-C and -D transduce lymphangiogenic signals, and upon proteolytic activation bind to both VEGFR-2 and -3, thus also promoting angiogenesis.

1.2 Blood and lymphatic vasculature

The vascular system must supply tissues with nutrients, growth factors and hormones as well as clear waste products.

Therefore, a certain vascular permeability is required in order to allow small molecules to extravasate under homeostatic conditions. [47]. Lymphatic ECs (LECs), just like blood ECs (BECs) are also quiescent under resting conditions. During homeostasis, lymphatics are essential for regulation of interstitial pressure, lipid metabolism and immune surveillance [48]. They absorb water and macromolecules from the interstitium, uptake vitamins and lipids in the intestine and provide an important traffic route for immune cells [49]. Lymphatics start as blind-ended vessels in the interstitium and have the primary aim to drain excess interstitial fluid, returning it into the blood stream. Hereby, the lymph passes through collecting ducts and

lymph nodes, and in the latter scanning for foreign antigens occurs – hence the importance of the lymphatic system for the immune defense [24].

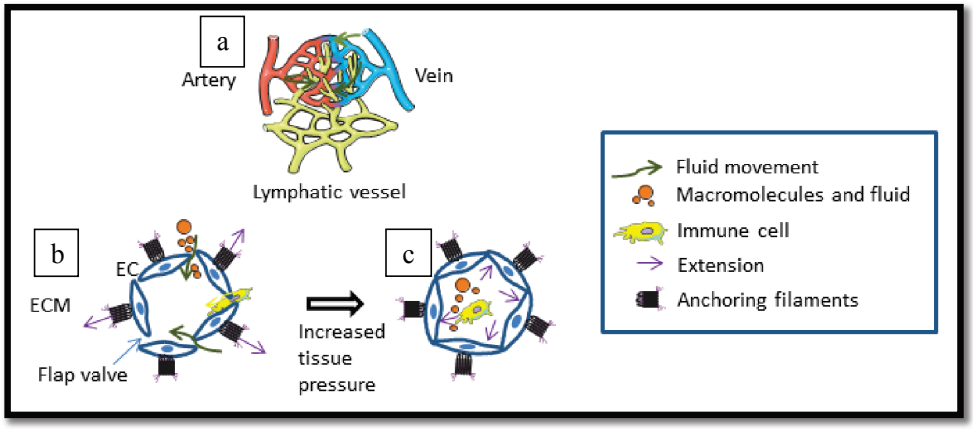

Figure 2. Lymphatic vessels collect macromolecules, cells and fluid provided by blood vessels from the interstitium (a, modified image from Servier Medical Art, Laboratoires Servier, Suresnes, FR). These enter the lymphatic capillaries via valve-like structures between the LECs. These portals also prevent the return of lymph to the intersitium (b). The anchoring filaments attach the ECs to the extracellular matrix (ECM) and also prevent the collapse of vessels under conditions of increased tissue pressure (c).

1.2.1 Distinguishing blood from lymphatic vasculature

Lymphatic and blood capillaries are structurally different. With the exception of fenestrated and discontinuous endothelia, BECs interconnect via tight junctions, resulting in a continuous basement membrane. In contrast, lymphatic capillaries lack the surrounding connective tissue cells of the blood vessels, the pericytes, and present with loosely connected LECs and a discontinuous basement membrane. The LECs attach to the ECM via anchoring filaments [50], which regulate the valve-like opening of the ECs in the lymphatic lumen [48]. These portals allow fluid, particles and immune cells, such as dendritic cells and some leukocytes, to enter the vessel lumen [51-53] (Figure 2).

Both BECs and LECs carry pan-endothelial markers, such as Platelet endothelial cell adhesion molecule-1 (PECAM-1), even though this is only weakly expressed on

lymphatics [54]. The differentiation can be difficult regarding their VEGFRs expression. BECs express VEGFRs-1 and -2, while VEGFRs-2 and -3 are found on LECs. Furthermore, VEGFR-3 is also expressed by fenestrated blood endothelium, high endothelial venules (HEVs) of the lymph nodes, as well as abnormal tumor vessels [41, 44, 55]. LECs are intensely positive for specific molecules, such as prospero homebox-1 (Prox1), the membrane glycoprotein podoplanin (D2-40) and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) [34, 56-59], which can be used in order to distinguish between BECs and LECs. A selection of blood- and lymphatic markers is presented in figure 3.

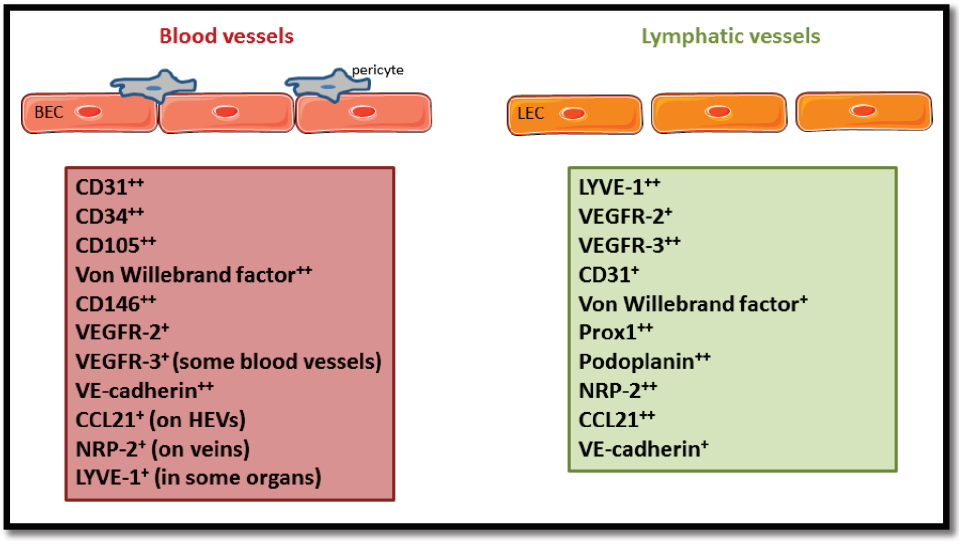

Figure 3. Selected markers for blood and lymphatic endothelium, reviewed in [60] (BEC, blood endothelial cell; LEC, lymphatic endothelial cell). "+"sign represents staining intensity.

1.2.2 Angio- and lymphangiogenesis in disease

ECs maintain the ability to divide and proliferate in response to different stimuli, such as hypoxia (insufficient oxygen concentration), often found during tissue expansion. Activation of hypoxia-inducible factor (HIF), especially HIF-1α, stimulates vasculo- and angiogenesis through induction of VEGF-A. However, hypoxia is also characteristic of both inflammation and cancer [61, 62]. Ferrara N. [63] reported that

hormones, several growth factors, inflammatory cytokines and oncogenes can induce VEGF-A expression in various tissues and cells. Consequently, excessive angiogenesis is used as a hallmark of many disorders – cancer, chronic inflammations like arthritis [64] or psoriasis [65], atherosclerosis [66, 67], infectious diseases [68, 69], diabetic retinopathy [70], asthma [71] or obesity [72] (Table 1).

Diseases related to increased or abnormal angiogenesis

Organ	Pathological process
Multiple organs	Cancer Autoimmune disorders Infectious diseases, including periodontal disease
Blood vessels	Atherosclerosis, hemangiomas
Skin	Warts, pyogenic granulomas, psoriasis, Kaposi's sarcoma, scar keloids
Female reproductive system	Endometriosis, uterine bleeding, ovarian cysts/hyperstimulation
Adipose tissue	Obesity
Bone, joints	Rheumatoid arthritis, synovitis, osteomyelitis
Eye	Prematurity - and diabetic retinopathy
Digestive system	Inflammatory bowel disease, ascites

Table 1. Diseases related to abnormal and increased angiogenesis, modified after Carmeliet P. [73].

The VEGF family and their receptors have been identified in a variety of immune cells involved in different disorders. Mast cells, which infiltrate chronic inflammatory sites and tumors, can express VEGF-A, -B, -C and -D and enhance the expression of VEGF-A [74]. VEGF-A induces monocyte migration through the activation of VEGFR-1 [75]. Monocytes, progenitor cells of macrophages and dendritic cells, express VEGFR-1 in a high majority [76], whereas PlGF, ligand to VEGFR-1,

induces cytokine and chemokine gene expression in these cells [77]. Macrophage colony stimulating factor (M-CSF), a pro-inflammatory cytokine, can induce VEGF-A expression by monocytes [78], suggestive of the angiogenic role of monocytes during inflammation.

Macrophages, with important roles in phagocytosis, are known for their increased presence in chronic inflammatory settings and tumors. Thereby they produce a range of growth factors, cytokines, such as interleukin 1β (IL-1β) or tumor necrosis factor α (TNFα), and proteolytic enzymes according to their microenvironment [79]. TNFα sustains tumor and blood vessel growth by stimulating angiogenic factors like VEGF-A or IL-8, a major angiogenic chemokine [80]. IL-1 enhances tumor invasiveness and induces angiogenesis by stimulating TNFα and HIF-1α release [80, 81]. Tumor cells and macrophages express VEGF-C and -D, thus having an important role in lymphangiogenesis [82]. Increased VEGF-C expression is related to lymph node metastasis for thyroid, lung or gastro-intestinal cancers [83]. Thus, both blood and lymphatic vessel proliferation occur under pathologic conditions, such as inflammation or tumor growth.

Polymorphonuclear leukocytes (PMNs) are known to be involved in the physiological progress of menstrual cycle angiogenesis [84]. However, they can also stimulate an increased release of angiogenic factors within tumors and be the source of these factors upon TNFα stimulation arising during tumor or chronic inflammation progression [85]. In addition, PMNs contribute to inflammatory lymphangiogenesis, by increasing VEGF-A bioavailability and secreting VEGF-D [86].

Lymphangiogenesis occurs as a response to inflammatory stimuli with the aim of facilitating clearance of antigens, inflammatory cells, and cytokines from inflamed sites and of establishing the resolution of inflammation. The sprouting from pre-existing lymphatics, the characteristic of adult lymphangiogenesis, is mediated by VEGF-C and -D signaling upon binding to the main lymphatic endothelial receptor, VEGFR-3 [87]. Pro-inflammatory cytokines and bacterial lipopolysaccharide (LPS), encountered during inflammation caused by bacterial infections, generate VEGF-C expression in many cell types [51]. During chronic inflammation, VEGF-A induces lymphangiogenesis via VEGFR-2 expressed on lymphatic endothelium. VEGF-A

mediated lymphangiogenesis is also seen in the draining lymph nodes representing a contrast to the angiogenic drive seen during development [88, 89]. Thus, lymph node inflammatory lymphangiogenesis can be induced both via VEGF-A/VEGFR-2 and VEGF-C and-D/VEGFR-3 signaling [90].

Some chronic inflammatory diseases are actively maintained through lymphangiogenesis [91], as demonstrated in chronic airway inflammation [51], psoriasis [92], ulcerative colitis [93], as well as rejected renal transplants [94]. Experimental blocking of lymphangiogenic factors during inflammation suppresses lymhangiogenesis, as well as reactive lymphadenitis, and consequently edema persists [51]. A study performed on K14-VEGF-A transgenic mice showed that in chronic skin inflammation, blocking VEGFR-2 inhibits angiogenesis and inflammatory reactions. VEGFR-3 inhibition however prolonged inflammatory edema [95]. In a model of transgenic TNFα mice with chronic inflammatory arthritis, blocking of VEGFR-3 resulted in a decrease of lymph node lymphangiogenesis and an augmentation of inflammation [96]. On the other hand, in the same disease setting, anti-VEGFR-2 treatment reduced both lymphangiogenesis and the severity of inflammation [96]. These findings suggest that induced lymphangiogenesis and lymphatic drainage are related to the severity of inflammatory lesions and that adequate lymphatic drainage may be beneficial during chronic inflammatory conditions.

1.3 The tooth biology

The tooth is a highly complex organ comprising organized structures with various and specific shapes. It develops within the alveolar bone and after it erupts in the oral cavity, the root(s) firmly anchors to surrounding bone. The tooth structure comprises of three outer mineralized tissues – coronal enamel, radicular cementum and corono-radicular dentin which surround the inner non-mineralized soft tissue, the dental pulp.

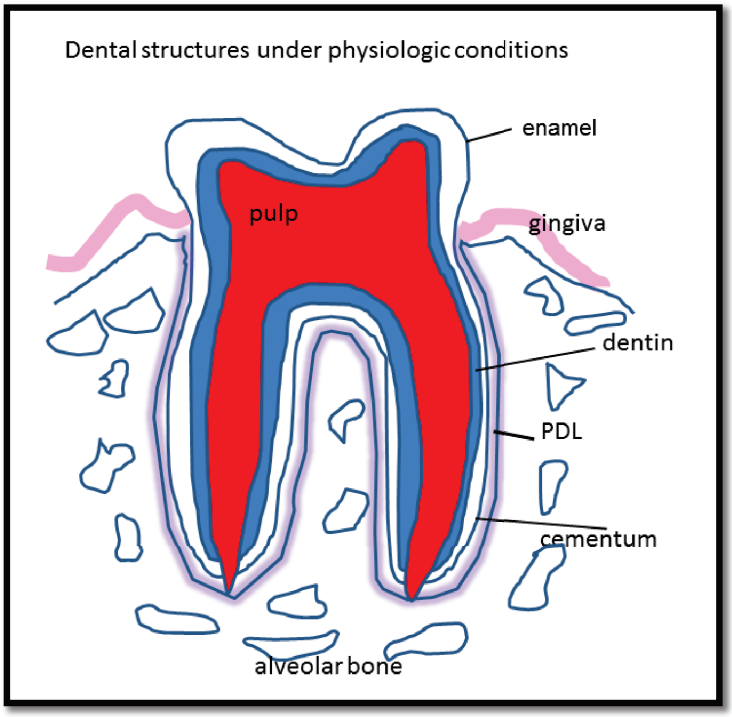

Figure 4. Schematic representation of the tooth and supporting tissues.

1.3.1 The dental pulp

The dental pulp is a unique tissue confined within rigid walls. It exhibits specialized cellular components – the odontoblasts, known for their dentin-deposition activity throughout life and upon external aggression - such as caries, and for their involvement in sensory transduction [97, 98]. Additionally, the pulp has rich sensory innervation [99] and is well supplied with blood vessels [100], which contribute to its' defensive functions. It is also equipped with cellular components necessary for antigen recognition and immune reaction, such as T lymphocytes, macrophages and the major antigen-presenting cells (APCs), the dendritic cells [101].

Dental caries and traumatic injuries may provide entry-ways for bacteria to the pulpal space, hence causing inflammation which may evolve to pulpal necrosis if left unattended. It has been shown that teeth with viable dental pulp are more resistant to

bacterial invasion of the dentine than teeth with root canal fillings [102], thus the important role of the pulp in defense processes.

1.3.2 Pulp vascularization

Like in other tissues, pulp blood flow brings oxygen and nutrients to the dental pulp, while also removing waste products thereafter. The pulp microcirculation is supplied by the maxillary artery, which divides into dental arteries and further arterioles that enter the teeth. These have a central orientation and give capillary branches towards pulp periphery. The blood drains into venules, which are also centrally located. The vasculature differs in the crown and root of the tooth. In the root, blood vessels penetrate the apical area of the pulp and form tiny branches. Dental crown capillaries form successive glomerular individualized structures that supply areas of 100-150 μm in subodontoblastic and odontoblastic areas [103, 104]. Furthermore, some coronal capillaries have been described with a fenestrated structure [105].

An increased blood supply during inflammation leads to angiogenic capillary sprouting regulated by hypoxia. Under normal conditions, pro- and anti-angiogenic factors and neuropeptides control pulp vascularization [106, 107]. Dentin and pulp fibroblasts as well as ECs express pro-angiogenic factors, such as VEGF and fibroblast growth factor (FGF-2) [106-108]. Immunohistochemical analysis of normal and inflamed pulp has revealed the presence of VEGF under both conditions [109]. Dental pulp stem cells have the capability of differentiation into ECs [110]. This indicates that the pulp has the capacities of healing and regeneration processes which require angiogenesis.

Even though a lymphatic system is required for fluid and macromolecules transport especially during inflammatory conditions, the presence of lymphatics in the dental pulp remains controversial. Initial observations suggested that the pulp contains lymphatic capillaries [111-113]. This fact was later on disputed, as specific lymphatic markers (Prox1) did not reveal any lymphatics in the dental pulp [114]. Berggreen *et al.* [115] found LYVE-1^+ immune cells in mouse dental pulp, and VEGFR-3^+ vessels which were interpreted as lymphatics. And yet, another study partially contradicts these findings, as by use of multiple specific lymphatic markers (LYVE-1, D2-

40/Podoplanin and Prox-1) no lymphatic vessels were identified in human dental pulp [116].

1.3.3 The periodontium

The tooth supporting and surrounding structures enclose gingiva, periodontal ligament (PDL), cementum and alveolar bone.

The gingiva is the part of the alveolar mucosa covering the cervical part of the tooth and the tooth-carrying portion of the alveolar bone [117].

The PDL contains mainly collagen fibers, anchoring the root surface to the tooth socket of the maxilla or mandible, but also various cells – fibroblasts, osteoblasts, cementoblasts, progenitor stem cells, epithelial cell rests of Malassez, neural cells, and ECs, associated with the vasculature as well as ECM [118-120]. The PDL fulfills several functions – tooth support, stability, nutrition and protection, as well as mobility and adaptation to mechanical forces.

Cementum covers the root surface and continues deposition throughout life after tooth eruption [121]. There are three types of cementum in human teeth- acellular afibrillar cementum that covers minor areas of enamel; acellular extrinsic fiber cementum located mostly in the cervical and middle root portions, with apical exceptions in front teeth; and cellular intrinsic fiber cementum, mostly covering the rest of areas, with a predilection for the furcation and apical parts of the roots. Sometimes they overgrow each other, resulting in mixed stratified cementum [121, 122]. It is mainly the cellular intrinsic cementum type which participates in root repair processes [121].

Alterations in adult cementum structure and organic as well as inorganic composition occur due to pathological changes – bacteria-caused long-lasting inflammation being a major example [121, 123, 124]. Even though it is considered that root surface is more resistant to resorption than alveolar bone, radicular resorption of cementum and dentin does occur upon pulpal pathology, trauma or orthodontic treatment [125, 126]. The key players of this process are odontoclasts, similar cells to osteoclasts concerning multinucleated structural and functional characteristics [127].

The alveolar bone consists of the outer buccal, lingual or palatal cortical plates, and the central part containing a large amount of trabecular (spongious) bone with bone marrow in between trabeculae. The cortical bone is covered on the external side by the periosteum, which contains fibroblasts, osteoclast and osteoblast progenitor cells, nerve cells, blood vessels and bone-forming osteoblasts at the innermost part. The endosteum covers the inner side of cortical bone and external surface of the spongiosa. It also contains osteoblasts, as well as flat, bone-lining cells with no active bone secretory function [128, 129]. Other cells of the bone are the osteocytes which participate in bone remodeling processes along with osteoblasts and osteoclasts and actively maintain the bone matrix [130, 131]. Osteoclasts, multinucleated giant cells, are present on bone surfaces or intracortically and are responsible for bone resorptive activity [132].

1.3.4 Blood supply of the periodontium

The periodontium is highly vascularized. Alveolar arteries supply the PDL with three different branches: the dental, the interradicular and the interdental. Before entering the tooth apex, the dental artery emerges from the bone and gives off afferent subdivisions that form a vascular network in the apical third of the PDL [128]. While the interdental and interradicular arteries give mostly lateral branches, all these vessels within the PDL intercommunicate [128]. The apical and coronal thirds of the PDL are generally more vascularized than the middle third. Arteries are commonly accompanied by larger venous channels. Lymphatics exist in the gingiva, where they start as blind-ended vessels and pass along the external surface of the alveolar bone [133]. Gingival lymphangiogenesis occurs during periodontal disease development [134] and a recent study has shown the protective role of lymphatics in periodontal inflammation [135]. While no lymphatic vessels were detected at the border between gingiva and PDL [133], some LYVE-1^+ vessels have been described to transverse interdental bone [115]. The lymphatic drainage of the PDL is still debated [136].

1.4 Bone remodeling

Bone is a dynamic tissue with constant forming and resorption activity in order to maintain mineral homeostasis and structural integrity [137]. During the so-called coupling sequence, osteoclasts resorb the mineralized matrix, while osteoblasts form new bone matrix [138]. This process is regulated by local and systemic elements, such as mechanical load, calcium levels, paracrine and endocrine factors.

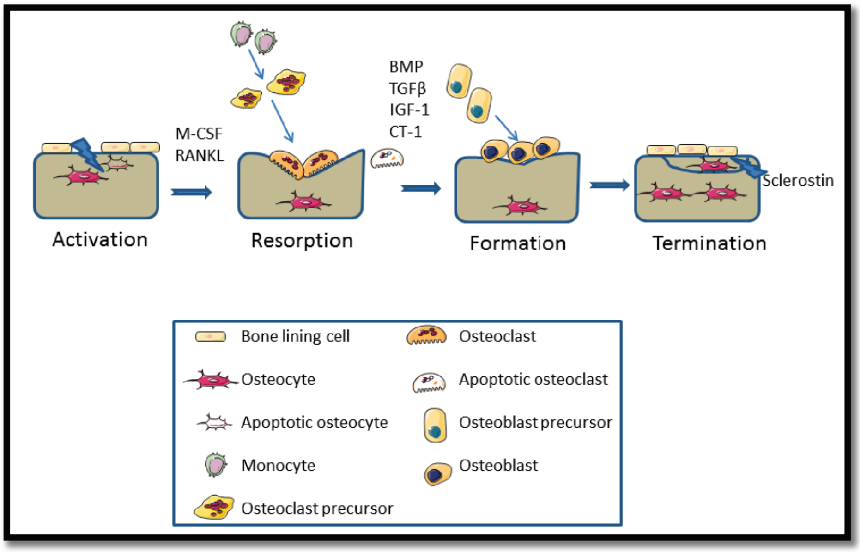

Figure 5. Schematic illustration of bone remodeling cycle. Micro-cracks lead to osteocyte apoptosis which determines osteoclast precursors' recruitment. M-CSF and receptor activator of nuclear factor kappa-B ligand (RANKL) are produced by bone-lining cells and activate osteoclasts. Bone morphogenic proteins (BMP), transforming growth factor-β (TGF-β), insulin growth factor-1 (IGF-1), cardiotrophin-1 (CT-1) are osteoclast-derived molecules which control bone apposition by osteoblasts. Newly-formed osteocytes secrete sclerostin, which terminates the bone secretion. (Shapes modified from Servier Medical Art, Laboratoires Servier, Suresnes, FR).

The activation of resorption occurs via apoptosis of osteocytes around a micro-fracture [137]. This leads to osteoclast precursor recruitment, osteoclastogenesis and bone resorption. The osteoclasts then die by apoptosis, which recruits osteoblast precursors. The resorbed area is filled with bone formed by osteoblasts, concluding

the cycle [137]. Alterations of this process can disturb the balance with pathological consequences [138].

Bone remodeling is controlled by signals between bone cells [139, 140], but also via sympathetic nervous system, hematopoietic stem cells, inflammation and the immune system, and vasculature [141-143]. In the 1990's the RANKL/RANK/osteoprotegerin (OPG) system was discovered and made it possible to understand more of the mechanism of bone resorption and remodeling [144, 145]. RANKL, the major osteoclast differentiation factor, is expressed by osteoblasts upon stimulation by a multitude of factors, but also by activated T cells, an indication of the immune system influencing bone resorption [146]. Under homeostatic conditions, RANKL binds to RANK, the receptor on osteoclast surface, thus activating the NF-kB pathway and resulting in bone resorption. Expression of RANKL can be regulated by glucocorticoids, Vitamin D3, IL-1, TNFα or LPS [147-150]. The RANKL/RANK binding is inhibited by OPG, a decoy receptor of the osteoblasts for RANKL, thus limiting bone resorption [144]. Under pathologic conditions, such as rheumatoid arthritis, periodontal disease, osteoporosis, metastatic bone tumors or multiple myeloma, the ratio RANKL/OPG increases in favor of bone breakdown [151].

1.5 Dental inflammation

As a response to injuries, living tissues respond with inflammation. The causes include microbial infections, trauma, chemical injuries, tissue necrosis and hypersensitivity reactions. Dental inflammation caused by microorganisms has been well established [152-154].

Gingival inflammation, a common disease with high prevalence, is caused by microorganisms; it can be reversed through treatment, but can also evolve to chronic periodontal breakdown. Dental caries is the most prevalent infectious disease, affecting most individuals, with both functional and economic impacts [155]. These lesions, along with tooth fractures or iatrogenic maneuvers are the portal of bacteria to the dental pulp, causing pulpal inflammation. If untreated, pulpitis evolves to pulp necrosis and apical periodontitis, with destructive processes of the bone. The human

oral cavity is host to approximately 700 bacterial species [156, 157]. It is mostly anaerobic gram-negative bacteria and their virulence factors that take part in dental disease development [158, 159].

The inflammatory response consists of a vascular and a cellular component. Vasodilation and increased blood flow at sites of inflammation go along with increased vascular permeability, resulting in plasma and protein extravasation into injury site and movement of leukocytes from vessels into the inflamed tissue [160]. The initial recognition of bacteria and their by-products is mediated by tissue-resident macrophages, mast cells, fibroblasts or dendritic cells via their toll-like (TLRs) and nucleotide binding oligomerization domain receptors (NODs) [161, 162]. In turn, this activation of the innate immune response leads to production of a variety of inflammatory mediators, including chemokines and cytokines. The vasodilation and extravasation of neutrophils represent the immediate effects of these mediators. When reaching the affected site, activated PMNs try to eliminate pathogens by releasing toxic contents of their granules [163]. If successful, resolution of inflammation occurs. If not resolved, a chronic inflammation takes place, where the PMN infiltrate is replaced with macrophages, B- and T- lymphocytes – characteristic of the adaptive immune response. Macrophages usually predominate in chronic inflammation, being responsible for the release of multiple cytokines and inflammatory mediators with both pro- and anti-inflammatory roles, thus also affecting resident tissue. Along with dendritic cells, they present antigens via their major histocompatibility complex (MHC) class II to T-helper (Th) cells in the regional lymph nodes, activating these cells. This leads to production of more cytokines that contribute to increased macrophage activation and to formation of memory B- and Th-cells. Furthermore, B lymphocytes also become activated, developing into antibody-producing plasma cells against the presented antigens. Thus, lymph node enlargement seen during inflammation, the lymphadenitis, is the result of immune cell reactions which try to prevent the spread of a local aggressor throughout the organism.

Little is known on lymphangiogenesis and lymphadenitis occurring during dental inflammation. Lymphangiogenesis in the gingiva during periodontal disease has previously been described [134]. Clinical findings have shown that during acute

phases of marginal and apical periodontitis mainly caused by bacteria, local and regional lymphadenitis arise [164]. Within the dental pulp, the presence of lymphatics is still disputed. A study on normal and inflamed pulps suggests that during inflammation, lymphangiogenesis occurs as more $CD31^{+}$/VEGFR-3^{+} vessels have been seen [165]. Knowing that VEGFR-3 is also expressed on some fenestrated blood vessels and that no other lymphatic markers have been employed in that study, this evidence [165] remains controversial. An interesting recent finding shows the migration of pulpal dendritic cells to regional lymph nodes as soon as 16 hours upon cusp trimming and bacterial exposure. This may lead to further immune cell recruitment [166]. The exact migratory pathways of pulp APCs to regional lymph nodes are in need of further investigation.

1.5.1 Apical periodontitis

The periapical lesion is in fact the result of the host's immune response upon constant bacterial aggression from the root canal aiming at confining the infection to the endodontic space. Apical bone loss in the forms of granulomas or cysts, which dental practitioners notice on radiographs as radiolucent areas, represents a main indicator of periapical disease (Figure 6).

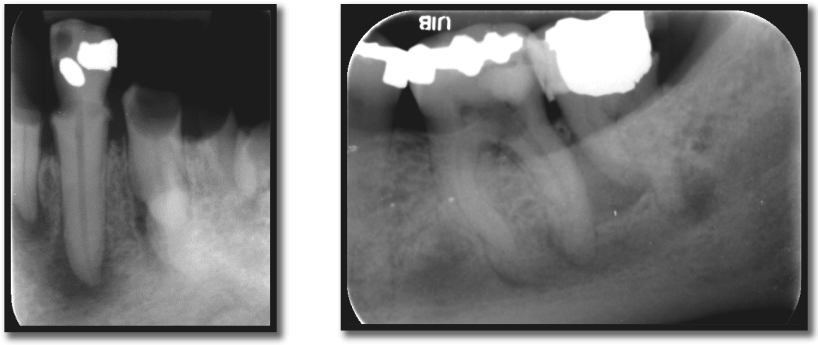

Figure 6. Radiographic exemplifications of teeth with deep, untreated or secondary caries and periapical lesions (unpublished images, Department of Endodontics, University of Bergen).

A necrotic dental pulp lacks blood supply and subsequently inflammatory cells that would eliminate bacteria [167]. Thus, bacteria penetrating the root canal towards the apex trigger a periapical immune defense with a rich cellular component consisting of PMNs, macrophages, B- and T-lymphocytes or plasma cells [168-171]. Fibroblasts, osteoblasts, osteoclasts, eosinophils, mast cells and epithelial cells are also among cells found in periapical lesions [172, 173]. Figure 7 exemplifies cellular infiltrates in mouse (A and B) and human (C) periapical lesions, as well as presence of osteoclasts (D) in a human apical periodontitis lesion.

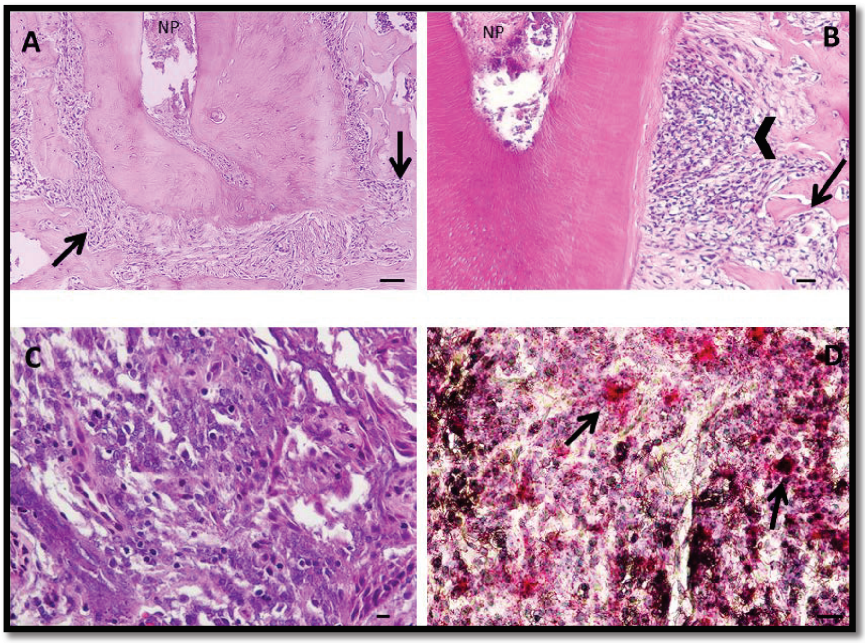

Figure 7. A and B. Periapical lesions induced in mice, following 21 days of pulpal exposure, (H&E staining). Necrotic pulp (NP) remnants are visible in the root canals. Periradicular cellular infiltrates of different intensities (arrowhead), along with areas of bone resorption (arrows) are observed. C. H&E staining of human periapical lesions revealing different cellular and fibrous infiltrates. D. TRAP staining of human periapical lesion exemplifying pre-osteoclasts or osteoclasts (arrows, intense red staining) in the body of human periapical lesion (Unpublished data).

The inflammatory response cannot occur without its' vascular component. The extravasation of PMNs, of high importance in periradicular disease development,

follows vascular hyperemia, congestion and local edema [172]. Though mostly protective, PMNs also degrade host tissues, not just microorganisms, due to release of their cytotoxic granules, especially in the acute phases of apical periodontitis where they predominate [172].

Reports vary considering the dominating type of cells in established periapical lesions with either macrophages [170, 174] or lymphocytes [175, 176] taking the lead. Macrophages have central roles in innate, as well as acquired immunity [177]. In rat models of apical periodontitis, it has been shown that their numbers increase in the first 10 days of lesion development, maintaining similar levels for up to 60 days and decreasing thereafter [178]. Through their phagocytic role, they prevent dissemination of bacteria from the root canal to the surrounding bone. By being APCs, they process the antigen, present it to Th cells, which they also activate, thus triggering the adaptive immune response. Along with IL-1 and TGF-β production, macrophages also release metallo-proteases (elastase, collagenase) and prostaglandins, which participate in the bone destructive process [177].

Usually T cells are more numerous than B cells [175]. Of the T-cells, Th1, Th2 and Th17 as well as T-cytotoxic (Tc) subtypes were identified in periapical lesions [171, 179]. Th1 cells produce IL-2 and Interferonγ (IFNγ), which control cell-mediated immunity, while Th2 cells secrete IL-4, -5, -6, -9, -10 and -13 that regulate plasma cells and humoral immune response [180-182]. Out of these cytokines, IL-6 and IL-10 may exert anti-inflammatory effects in bone inflammation [183, 184]. Th17 cells are known for IL-17 production, a cytokine, which so far has been described with both pro- and anti-inflammatory functions [179, 185]. They also release IFNγ, with protective effects in periapical inflammation [186]. Upon cytokine activation, B-cells proliferate into plasma cells, which produce antibodies to bacterial antigens [187]. An overview of periapical cellular interactions during apical periodontitis development is given in figure 8.

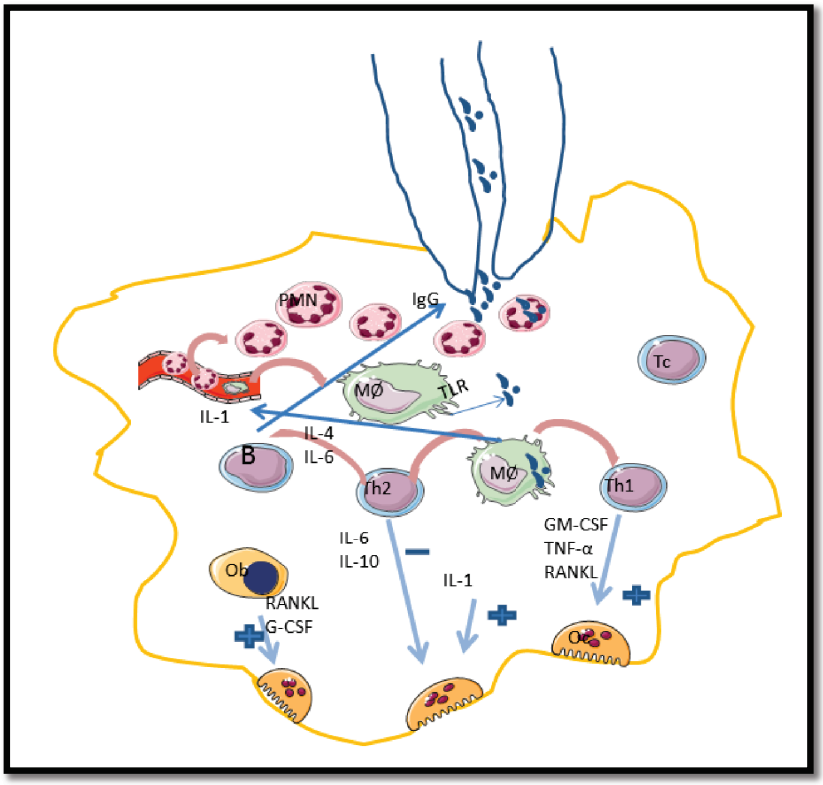

Figure 8. Schematic representation of major cellular interactions during periapical disease development. Bacteria and their products from the root canal are attacked by extravasated PMNs from blood vessels. Macrophages (MØ) recognize antigens via TLRs, engulf these and present the information to T cells, activating the adaptive immune response. Activated macrophages enhance migration of monocytes and PMNs from vessels in the area via IL-1. B-cells activation determines antibody production (IgG) against specific antigens. Many mediators are being produced, some with osteoclastic stimulating effect (Granulocyte macrophage colony-stimulating factor (GM-CSF), TNFα, IL-1), others with anti-inflammatory roles (IL-6, IL-10). Osteoclasts (Oc) are also activated due to release of RANKL and Granulocyte colony-stimulating factor (G-CSF) by osteoblasts (Ob) in the periapical bone (Shapes modified from Servier Medical Art, Laboratoires Servier, Suresnes, FR).

The interplay between ECs, PMNs, macrophages, lymphocytes and osteoclasts characterize these endodontic lesions, along with the main consequence of this process – bone resorption [167, 172, 188]. Osteoclastic bone breakdown is stimulated through the induction of the major pro-inflammatory cytokines such as IL-1α, IL-1β,

RANKL or TNFα [189, 190]. IL-1α and IL-1β are produced in apical periodontitis lesions by several types of cells – macrophages, PMNs, fibroblasts and osteoclasts [191, 192]. The role of IL-1 in apical periodontitis development was demonstrated by inhibiting IL-1 receptor and showing a 60% reduction of bone destruction [187]. Additional to bone resorption enhancement, IL-1 can stimulate T- and B-cells [193], enhance leukocyte adhesion to endothelial walls or activate the production of prostaglandins and proteolytic enzymes [172].

Fibroblasts, macrophages and PMNs are also the source of TNFα, which mediates RANKL stimulation of osteoclast differentiation through autocrine mechanism [194], hence stimulating bone resorption.

Although osteoblasts are the main source of RANKL, B- and T-cells [195] as well as fibroblasts [196] also express this factor. Furthermore, it has been suggested that other cells, like macrophages, PMNs, ECs and epithelial cells express RANKL in periapical lesions [197].

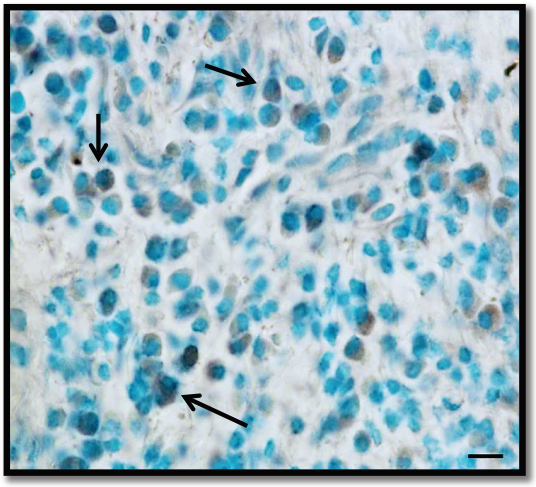

Figure 9. Scattered cells (arrows) in a human periapical lesion stained positive for RANKL (Unpublished data).

Microorganisms, inflammatory molecules such as IL-1, TNFα and IFNγ or even chemokines themselves stimulate the production of chemokines at sites of apical

periodontitis [198]. Osteoclast differentiation and activation is also induced by chemokines, such as IL-8/CXCL8 [199], macrophage inflammatory protein-1α (MIP-1α)/CCL3 or regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5 [200, 201]. Monocyte chemotactic protein (MCP-1)/CCL2, abundantly produced in chronic inflammation, is associated with osteoclast chemotaxis and differentiation [202, 203], while mediating monocyte recruitment to bone inflammatory sites and being involved in bone remodeling [204, 205]. Dentin proteins stimulate PMN migration by inducing the release of keratinocyte chemoattractant (KC)/CXCL1 and MIP-2/CXCL2 [206]. In granulomas, RANTES is associated with recruitment of $CD4^+$ Th cells and $CD68^+$ macrophages, whereas MIP-1α and MIP-1β/CCL4 are related to the $CD8^+$ Tc population. Moreover, the increased expression of RANTES and MCP-1, in cysts versus granulomas is indicative of augmented osteoclast recruitment and thus may be of importance in periapical disease development [207].

Cytokines and chemokines are also important mediators of angiogenesis. IL-1 and TNFα are known inducers of angiogenic responses [80, 81], while IL-6 has also been shown to promote VEGF-dependent angiogenesis in tumor settings [208]. MCP-1 can trigger EC migration and vessel sprouting [209], whereas mice lacking MCP-1 have experienced delayed wound healing, indicative of the angiogenic involvement of this chemokine [210]. Apart from its' role in osteoclastogenesis, IL-8 is also a potent angiogenic factor [211], acknowledged for intensifying the local vascular response in apical periodontitis [172].

Additional to angiogenic cytokines and chemokines, recent studies have demonstrated the presence of the angiogenic factors FGF-2 and VEGF in these lesions [212, 213]. Microvessel density counts have revealed increased amounts of blood vessels in periapical lesions with intense inflammatory infiltrates [214]. During periods of exacerbation of chronic periapical inflammation, vasodilation and increased vascular leakage can occur, seen as regional mucosal swelling. This process is often accompanied by local lymphadenitis. The lymphangiogenesis mechanism in the draining lymph nodes during acute and chronic apical periodontitis is still in need of further investigations.

All of the above indicate the complex development of periapical disease, with intricate vascular and cellular processes taking place.

2. Aims of this study

The dental pulp is a well vascularized tissue, very often under inflammatory insults. Pulpal inflammation, when untreated, further evolves to necrosis of the tissue and periapical inflammation. The general aims of this study were to identify and map the presence of the VEGF family and receptors in normal dental pulp, as well as in apical periodontitis and to establish their roles in periapical disease development.

Specific aims

- To determine the existence and localization of VEGFs and VEGFRs during development of apical periodontitis in a rat model (Paper I).
- To investigate the presence of VEGFs and VEGFRs in human dental tissue with focus on normal dental pulp and periapical lesions (Papers II and III).
- To examine VEGF signaling at the gene level in normal dental pulp versus normal PDL (Paper III).
- To investigate the existence of lymphatics in periapical tissues and human pulp (Papers I-III).
- To study the roles of VEGFR-2 and -3 signaling during development of apical periodontitis in a murine model by individual or combined blocking of these receptors (Paper IV).
- To investigate inflammatory reactions in regional draining lymph nodes with respect to lymph node size and lymphangiogenesis upon pulpal exposure and systemic inhibition of VEGFR-2 and/or -3 (Paper IV).

3. Materials and methods

Animal experiments (Papers I and IV)

Female Wistar rats (Paper I) and male C57BL/6 mice (Paper IV) were used. The animals were housed in polycarbonate cages at the animal facility at least one week prior to experiments. They were fed standard pellet diet and given water *ad libitum*. The experiments were approved by the Regional Committee for Animal Research Ethics, University of Bergen, under the supervision of the Norwegian Experimental Animal Board.

Anesthesia: The rats were anesthetized with intramuscular injections of medetomidine/Domitor® (Orion Pharma, Espoo, FI), 0,4 mg/kg body weight and Ketamine/Ketalar® (Pfizer, Solentuna, SWE), 60 mg/kg body weight (Paper I). The mice were anesthetized with ketamine/Ketalar® 100 mg/kg body weight and Xylazine/Rompun Vet® (Bayer, Leverkusen, DE) 10 mg/kg body weight (Paper IV).

Pulp exposure (Paper I): First right maxillary and mandibular molars were drilled with a round bur until the pulp was exposed. Thereafter they were left open to the oral environment for either 10 days or 3 weeks for periapical lesions development.

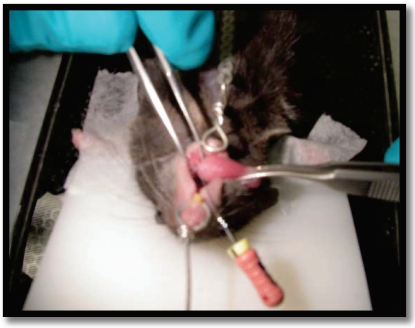

Figure 10. Picture taken during pulp exposures induced in mice. The anesthetized animals were placed on a jaw retraction board. Instrumentation of root canals in first mandibular molars was done upon pulp exposure with a 0.6 K-file.

Pulp exposure (Paper IV): Bilateral first mandibular molar pulps were exposed and removed with round burs. The mesial and distal canals were filed with a 0.06 K-file. The teeth were left open to the oral cavity for either 10 days or 3 weeks during which periapical lesions were developed.

Systemic treatments (Paper IV): Administration dosage and frequency of each substance are presented in the manuscript. Briefly, upon pulp exposures, the mice of both observation groups were injected intraperitoneal throughout the experimental periods with either normal IgG (controls), anti-VEGFR-2 (anti-R2), anti-VEGFR-3 (anti-R3) or combined anti-VEGFR-2 and-3 (anti-R2/R3) antibodies.

Upon experiment completion and euthanasia of all animals with anesthesia overdose, rats and mice jaws (Papers I and IV) and mice lymph nodes (Paper IV) were collected for further analysis.

Human tissue collection (Papers II and III)

Periapical lesions were collected after endodontic surgery of teeth diagnosed with chronic apical periodontitis (Paper II). Normal PDL (Papers II and III) and dental pulps (Paper III) were extracted from surgically removed healthy wisdom teeth. All human tissue was provided by the Department of Maxillofacial Surgery, Haukeland University Hospital, Bergen, upon patient written consent. The samples were stored in an authorized bio bank (REK 3.2008.1750) at -80˚ until further analysis.

RNA extraction and quantitative real-time PCR analysis (Papers I-III)

Rat jaws were freed of gingival remnants and bone blocks containing the first molar with the periapical lesion were dissected. The dissected tissue (Paper I), human granulomas (Paper II), normal PDL (Paper II and III) and dental pulp (Paper III) were placed in RNAlater® (Ambion, Austin, TX, USA) and frozen until further handling. Total RNA was extracted using commercially available RNAeasy minikit (Qiagen, Chatsworth, CA, USA) following manufacturer's protocol. Upon homogenization

and an on-column DNase digestion with RNase-free DNase set (Qiagen), quality was assessed by gel electrophoresis to ensure that 28S and 18S ribosomal RNA were clearly evident. A spectrophotometer (Nanodrop, Wilmigton, DE) was used for RNA quantity measurements. First strand cDNA synthesis was performed with M-MLV Reverse Transcriptase (Ambion) and Random Hexamer Primer (Fermentas GmbH, St Leon-Rot, DE) with 2 µg total RNA for rat samples (Paper I) and RT^2 First Strand Kit (Qiagen) using 1 µg RNA for human tissue samples (Papers II and III).

Gene expression analysis of rat periapical lesions was performed by use of predesigned TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) for *VEGF-A, -C* and *VEGFR-3* with *ACTB* as a control gene (Paper I). Human samples (Papers II and III) were screened for 84 genes contained in the Human VEGF Signaling RT^2 Profiler PCR Array System (Qiagen). Both human pulp and periapical lesion samples were normalized to calibrator PDL and 5 genes served as references (*GAPDH, B2M, ACTB, HPRT1, RPL13A*). An RT-PCR thermocycler (LightCycler 480, Roche Diagnostics AG, Rotkreuz, SUI) was used for all analyses. Expression profiles of the target genes were measured relative to the mean critical threshold (Ct) of the reference genes by using the $\Delta\Delta C_t$ method described by Livak and Schmittgen [215].

Histology (Papers I, II and IV)

Representative rat jaws (Paper I) and periapical lesions (Paper II) were embedded in paraffin, sectioned (5µm) and stained with H&E. Cryosections (12-14µm) from exposed and control mice lymph nodes (Paper IV) were also stained with H&E.

Immunohistochemistry (Papers I-IV)

Rats deeply anesthetized with sodium pentobarbital (Mebumal) were transcardially perfused with heparinized saline, followed by 4% PFA with 0,2% picric acid in 0.1M phosphate-buffered saline, pH 7,4 (Paper I). The jaws were removed and decalcified in 10% EDTA, saturated in 30% sucrose and stored at -80° until cryosectioning (20

μm). In paper IV, mice mandibles were removed without prefixation. Hemi-mandibles exposed for 10 days were decalcified in 10 % EDTA and post-fixed with 4% PFA upon cryosectioning during staining protocols. Mice hemi-mandibles exposed for 21 days were fixed with 4% PFA, stored in 50% ethanol, used for μCT analysis and thereafter followed the same treatment for immunohistochemical (IHC) analysis. Lymph node cryosections were PFA fixed prior to staining.

In papers II and III, frozen human periapical lesions and dental pulps were cryosectioned (12-16μm) and stored at -80° until further handling. They were used either for single staining with the avidin-biotin peroxidase (ABC) method and nickel-enhanced 3, 3'-diaminobenzidine (DAB) as the chromogen (Papers I-IV) or for immunofluorescent procedures (Papers I-IV). In papers I-III the staining aimed at identifying the presence and localization of VEGFs and their receptors in periapical lesions and dental pulp. In paper IV, IHC was used for identification and quantification of immune cells (neutrophils and macrophages) and blood vessels in induced apical periodontitis, as well as lymphangiogenesis occurring in draining lymph nodes. Details on the used primary and secondary antibodies, as well as protocols are given in papers I-IV.

The specificity of all immune reactions was tested by omission of the primary antibodies and/or substitution with isotype controls. Sections were visualized in a photomicroscope (Nikon Eclipse E600; Nikon Instruments, Kanagawa, Japan) or a fluorescent microscope (Axio Imager; Carl Zeiss Microimaging Inc, Jena, Germany) connected to AxioCam Mrm camera (Carl Zeiss) that used the AxioVision 4.8.1 (Carl Zeiss) imaging system.

TRAP staining (Paper IV)

In order to identify and quantify osteoclasts, sections from exposed mice jaws were stained with tartrate resistant acid phosphatase (TRAP) according to manufacturer's (Sigma-Aldrich, USA) instructions.

Quantification of osteoclasts, neutrophils, macrophages and blood vessels (Paper IV)

Immunolabeled cells ($TRAP^+$ osteoclasts, $Ly\text{-}6.B2^+$ neutrophils, $F4/80^+$ macrophages) and $CD31^+$ vessels were counted in the periapical lesions from the apical constriction to the bone periphery on 2-4 sections from each mouse under a photomicroscope, quantified with Lucia imaging software (Lucia, v. 480; Laboratory Imaging, Hostivař, Czech Republic) and averaged for each animal.

Lymph nodes analysis (Paper IV)

Lymph node cryosections from the 21 days exposed mice and untreated animals (negative controls) were subjected to histological (H&E staining) and LYVE-1 immunofluorescent analyses. Histological evaluations were used for total area measurements (mm^2), while $LYVE\text{-}1^+$ sinus areas were measured in the hilum and medulla in 4-6 sections from each mouse in a 350μmx350μm grid. All evaluations were done with Lucia imaging software upon image capture in either a photomicroscope (H&E sections) or fluorescent microscope (LYVE-1 stained sections).

Micro-computed tomography (μCT) imaging analysis (Paper IV)

PFA fixed hemi-mandibles from each group exposed for 21 days were washed with phosphate buffer, stored in 50% ethanol, number coded for blinded analysis and sent for μCT evaluation to the Forsyth Institute, Boston, MA, USA. The analysis was performed by means of a compact fan-beam-type tomograph (μCT40, Scanco Medical, Basserdorf, SUI). The data were exported into DICOM format, re-sliced in a standardized manner using the ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) to obtain pivotal sections containing the entire length of the root canals and periapical lesions.

Lesion size measurement (Paper IV)

Periapical lesion sizes (mm^2) of the distal roots were measured on 2-4 sections from each mouse in mesio-distal (both observation times) and bucco-lingual (after 21 days only) directions on cross-sectional areas containing root canal and lesions, using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA) and Lucia imaging software. A standardized device was placed through the apical constriction and the lesions were outlined to the bone periphery. All technical details are given in paper IV.

Protein extraction (Paper IV)

Dissected bone blocks containing periapical lesions developed after 21 days, and also from negative controls were homogenized in 400μL cell lysis buffer mix, the supernatants collected and stored at -80˚ until further analysis.

Multiplex and ELISA analysis (Paper IV)

A 96-well Milliplex MAP mouse cytokine/chemokine magnetic bead panel (MCYTOMAG-70K, Millipore, MA, USA) was used for the simultaneous detection of 25 cytokines in mouse bone blocks containing periapical lesions. G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, MKC, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES and TNFα were the analyzed cytokines according to manufacturer's instructions. A range of 3.2-10.000 pg/ml recombinant cytokines was used to establish the standard curves. The levels of inflammatory molecules were measured using the multiplex array reader from Luminex™ Instrumentation System (Bio-Plex Workstation from Bio-Rad Laboratories, Hercules, CA, USA) and concentrations were calculated with software provided by the manufacturer (Bio-Plex Manager Software). The RANKL concentration in the same samples was measured with a Quantikine Enzyme Linked Immunosorbent Assay (ELISA) kit (R&D Systems,

Minneapolis, MN, USA), presented with a 96-well plate, following manufacturers' protocol. The concentrations were calculated and presented as pg/mg tissue – corresponding to samples' weights.

Statistical analyses (Papers I-IV)

In papers I-III, qRT-PCR results were statistically analyzed. The *p* values were calculated based on an unpaired Student's t-test comparing the replicate $2^{-\Delta Ct}$ values for each gene.

In paper IV, all data was subjected to statistical analysis and the following tests were employed: One-way ANOVA and Bonferroni post-hoc test for normally distributed populations; Kruskal-Wallis and Dunn's post-hoc test for non-normally distributed populations; Two-way ANOVA and Bonferroni post-hoc test for grouped analysis.

A p value of <0.05 was considered statistically significant.

4. Summary of results

4.1 Expression of VEGFs and their receptors in rat apical periodontitis (Paper I)

Immunohistochemical analysis demonstrated that VEGF-A, -C, -D and VEGFR-2 and -3 were expressed on cells and vessels in non-exposed control teeth PDL. Some PDL cells with fibroblastic appearance and others lining the bone or the root surface were positive for VEGFR-2 and -3.

All the investigated factors and receptors showed more intense staining on cells and vessels in induced periapical lesions at both 10 days and 3 weeks exposure times. With increased exposure time, the degrees of pulpal necrosis, periapical inflammatory infiltrate, root- and bone resorption were also amplified.

Within the infiltrates, $CD68^+$ macrophages and $HIS48^+$ neutrophils were sources of investigated VEGFs. Multinucleated $CD68^+$ cells lining the bone, indicative of osteoclasts, were also positive for VEGFR-2 and-3.

At gene level, a significant up-regulation of *VEGF-A* and *VEGFR-3* was observed after induction of periapical lesions.

4.2 Presence of VEGFs and VEGFRs in human dental tissues (Papers II and III)

Periapical lesions: All lesions presented with heterogeneous morphologies. VEGF-A, -C, -D and VEGFR-2 and -3 were found on vWF^+ blood vessels, $ELA\text{-}2^+$ neutrophils, $CD19^+$ B- and $CD3^+$ T-lymphocytes, as well as $CD68^+$ macrophages.

Normal dental pulp: $CD68^+$ macrophages and $CD3^+$ T-cells were the major cellular sources of VEGF-A, -B, -C, -D and VEGFR-2 and -3. $CD31^+$ blood vessels were positive for the same VEGFs and their receptors with the exception of VEGF-B. No $ELA\text{-}2^+$ neutrophils or $CD19^+$ B lymphocytes could be identified in the analyzed normal dental pulp.

Gene expression analysis: Analysis with the Human VEGF Signalling RT² Profiler™ PCR Array System revealed that out of 84 investigated genes, 27 and 67 were more than 2-fold altered in periapical lesions and dental pulp respectively, compared to control PDL. *VEGF-A, -D* and *VEGFR-3* showed a non-significant up-regulation in periapical lesions. A significantly increased expression of *VEGFR-3* was seen in dental pulp samples, while *VEGFR-1, -2* and *VEGF-D* were non-significantly amplified in the same tissue.

4.3 Existence of lymphatics in dental pulp and periapical tissues (Papers I-III)

No lymphatic vessels could be identified in human dental pulp, while double immunofluorescent staining revealed the presence of LYVE-1^+/$CD68^+$ cells with dendritic appearance. No LYVE-1^+ vessels could be localized in rat or human periapical lesions, indicative of all vessels being of angiogenic origin in both species. Some $CD68^+$ macrophages were also positive for LYVE-1 in the investigated human periapical lesions.

4.4 The effects of VEGFR-2 and -3 signaling during development of murine apical periodontitis (Paper IV)

Lesion sizes: Systemic inhibition of either VEGFR-2 or -3 resulted in a fast lesion size development until day 10. Thereafter, a slowed progression was recorded. Combined inhibition of VEGFR-2 and -3 or IgG-treated controls showed slower growth of lesions until day 10, but all four groups reached similar levels on day 21.

Immune cells, blood vessels and osteoclasts: Blocking of VEGFR-2 significantly increased Ly-$6.B2^+$ neutrophils, along with $TRAP^+$ osteoclasts upon 10 days of exposure compared with the other test groups ($p<0.05$). After 21 days, neutrophil infiltrates were substantially increased upon either VEGFR-2 or VEGFR-3 blocking. Anti-R2/R3 treatment resulted in fewer $F4/80^+$ macrophages and $TRAP^+$ osteoclasts on day 10. The latter systemic treatment also reduced angiogenesis seen on both observation times as fewer $CD31^+$ blood vessels within the lesions.

Cytokine expression: In addition to RANKL, 17 out of 25 cytokines and chemokines were expressed in periapical lesions. The pro-inflammatory cytokines IL-1β, RANTES, TNFα, IL-17 and MCP-1 showed the highest values upon VEGFR-2 inhibition and the lowest expression in the anti-R2/R3 group. A tendency to down-regulation of many cytokines (RANKL, IL-1 β, IL-6, IL-17, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES and TNFα) was observed in the anti-R2/R3 group, statistically significant for MCP-1 and RANKL compared to the IgG group ($p<0.05$).

Lymphadenitis and lymphangiogenesis in regional lymph nodes: Significantly bigger lymph nodes were found upon anti-R2 compared with anti-R2/R3 treatment and controls. The LYVE-1^+ area fraction in the lymph nodes hilum and medulla, indicative for lymphangiogenesis, was greater in the IgG treated group compared to the other groups, yet only significant in the medulla ($p<0.05$).

5. General discussion

The processes of angio- and lymphangiogenesis, mediated through the VEGF family are of high importance in physiological processes, such as growth or wound healing, but also in major pathological settings of tumor progression, inflammation and bone resorption.

VEGF-A has been previously found in normal dental pulp where bacteria can up-regulate its' expression [216, 217]. The main angiogenic receptor, VEGFR-2, has been described in primary and permanent dentition pulps [218]. The presence of VEGFR-3^{+} vessels in mouse and human dental pulp has been reported [115]. An association between the presence of VEGF-A and the development of apical periodontitis has also been made [212, 214, 219].

However, other members of the VEGF family had not yet been addressed in the context of location and signaling patterns in periapical disease or dental pulp.

The focus of this thesis was to investigate the presence of VEGF family and their receptors in periapical lesions and in normal dental pulp. The existence of lymphatics was also evaluated in these tissues. The implication of VEGFR-2 and/or -3 signaling in murine periapical disease development was studied with respect to lesion size, local inflammatory and vascular reactions as well as regional lymphangiogenic responses of the lymph nodes.

5.1 Presence of VEGFs and VEGFRs in apical periodontitis (Papers I and II)

By using an animal model of apical periodontitis, the disease development can be tracked from its acute phase (until day 7), through an active phase between days 7 and 20 and slowing down thereafter as it enters a chronic stage [187]. Human samples of periapical lesions, even though essential for providing further information, give no data on the time aspect of disease progression, nor on the surrounding structures that are known to be affected by the infection, like dentin, cementum and bone.

In the rat experimental model, immunohistochemical analysis revealed the presence of VEGF-A, -C, -D and VEGFR-2 and -3 on cells and vessels in both normal PDL and bone and in inflamed areas. Inflammation led to increased staining of the analyzed factors and receptors over the entire observation period. The involvement of immune cells in triggering periodontal and periapical bone resorption is well known [188]. We therefore sought at identifying the cells expressing VEGFs and VEGFRs, thus also finding their possible involvement in periapical inflammation.

$CD68^+$ macrophages and $HIS48^+$ PMNs expressed the investigated factors and receptors. Double immunofluorescent staining of B- and T- cells in human periapical lesions added these cells to the VEGFs and VEGFRs sources. Taken together, these findings indicate that immune cells produce VEGFs and VEGFRs which are involved in periapical disease development and that these cells may communicate with each other and with ECs.

Interestingly, our results showed the expression of all investigated VEGFs and VEGFRs only on vessels of angiogenic origin, thus contradicting the presence of lymphatics in the apical periodontium. Former studies have revealed the presence of VEGFR-3 on lymphatic ECs, but also on fenestrated blood capillaries, blood vessels in inflammatory tissues, macrophages and dendritic cells [44, 46, 220]. Our results find VEGFR-3 positive blood vessels in rat normal and inflamed areas of PDL and periapical bone and also in inflamed human periapical lesions. We confirm earlier data that identified macrophages as the source of LYVE-1 in a pathologic context [221, 222]. However, in our investigated tissues, these cells couldn't be related to growth of lymphatic vessels. Their role requires further investigation.

Another novel finding was the presence of $CD68^+$ multinucleated cells, indicative of osteoclasts, in the periphery of rat periapical lesions, cells which also expressed both VEGFR-2 and -3. The role of osteoclasts in periradicular diseases remains undisputed. *In vitro* studies have shown that VEGF-A enhances osteoclastic bone resorption via VEGFR-2 [223] and that the interaction between VEGF-C, -D and RANKL mediates osteoclast differentiation [224]. Due to their binding affinities to both VEGFR-2 and -3 we can hereby assume that VEGF-C and -D are involved in osteoclast regulation *in vivo.*

At the gene level, the up-regulation of *VEGFR-3* and *VEGF-A* upon rat pulp exposure was significant at both observation times. These genes, along with *VEGF-D* were non-significantly up-regulated in human periapical lesions compared with control PDL. *PKC,* a gene related to increased endothelial activity, *PI3K*, involved in EC migration and survival and *PLA2G6,* which induces pathologic VEGF-A angiogenesis, were all significantly up-regulated in the analyzed periapical lesions. In contrast, *SHC2*, which participates in VEGF-induced signaling by influencing VEGFR-2 activation, was found significantly down-regulated. Accordingly, *VEGFR-2* was also down-regulated. In another rat study where apical periodontitis was induced and $CD31^+$ BECs were isolated, the gene expression of *VEGFR-2* decreased after day 14 of pulp exposure [225]. We can therefore assume that in human chronic apical periodontitis, where time of lesion development is unknown, the gene expression of certain angiogenic factors may decrease over time. Further investigation is however required to determine the exact roles of angiogenic dynamics in periapical disease development.

5.2 The normal human dental pulp – reservoir of angiogenic factors (Paper III)

It is mostly upon pulp pathologic conditions that periapical tissues exhibit inflammatory disorders. We therefore sought to continue the investigation on the presence of VEGF family and receptors in normal dental human pulp.

The existence of VEGF and VEGFR-2 in well-vascularized pulp has been described before [217, 218]. In our study we demonstrate for the first time the presence of VEGF-B, -C, -D at gene and protein level in the normal human dental pulp. We identified $CD68^+$ macrophages and $CD3^+$ T lymphocytes as the sources of VEGF-A, -B, -C and -D, as well as VEGFR-2 and -3. Thus, cells involved in the pulp immunosurveillance [226] are also the source of angiogenic factors. No $ELA\text{-}2^+$ neutrophils or $CD19^+$ B-cells were identified in the analyzed tissue. Other investigations have also reported the absence of neutrophils and B-lymphocytes from normal pulp tissue, which is in accordance with our findings [227, 228]. However, a

new study using flow cytometry as method of analysis has identified a small percentage of approximately 2% B-lymphocytes, as well as a general population of $CD16^+$ granulocytes in dental pulp [229]. This discrepancy of results may be due to the technique employed by Gaudin *et al*. and furthermore, the authors did not use specific markers for neutrophils [229].

VEGF-B was only found on immune cells in our analyzed samples. This factor is involved in blood ECs and pericyte survival, with no angiogenic activity [230]. It seems to be related to metabolic and neuroprotective functions [28-30]. We assume that VEGF-B may also exert a protective action on pulpal neuronal and ECs, possibly also under inflammatory conditions.

Matsushita *et al.* found that VEGF-A produced by dental pulp cells acts directly on these cells via autocrine mechanisms, promoting chemotaxis and cellular differentiation, action which mostly occurs via VEGFR-2 signaling [231].

The presence of the investigated VEGFs and VEGFRs on ECs, immune and stromal cells denotes possible cellular autocrine and paracrine communications, which may also be involved in vascular activities occurring in the dental pulp. Dental pulp stem cells are a known source of VEGF-A [232], however the presence of VEGF-B, -C and -D on these potent cells remains an open research topic.

We confirm the presence of vessels of angiogenic origin ($CD31^+$) expressing VEGF-A, -C, -D and VEGFR-2 and -3 in normal dental pulps. Negative immunostaining with specific lymphatic markers, such as LYVE-1 and D2-40 exclude the presence of lymphatic vascular structures in the analyzed tissue. Even though formerly disputed, this is in accordance with another observation stating that human pulp does not present with lymphatic vessels [116]. However, fenestrated blood capillaries, a known source of VEGFR-3, have been described in the dental pulp [105]. Other studies have shown that VEGFR-3 expression is induced in ECs during angiogenesis in developing cornea and that it forms heterodimers with VEGFR-2 in blood vessels [233, 234]. The important up-regulation of the *VEGFR-3* gene encountered in our study, as well as its' roles along with the VEGF-C and -D presence on cells and vessels in the normal dental pulp require further investigations.

In normal human dental pulp sections we commonly found LYVE-1$^+$ macrophages, as also seen in mouse pulp [115]. In epididymal adipose tissue, LYVE-1$^+$ macrophages play an angiogenic role [235]. This epididymal tissue is highly hypoxic, thus promoting increased VEGF-activity via VEGFR-2, which in turn recruits bone-marrow derived LYVE-1$^+$ macrophages. At gene level, *VEGF*, *VEGFR-1* and *VEGFR-2* were significantly up-regulated in the dental pulp. VEGFR-1 has been shown to be necessary for survival of ECs and macrophages [236]. Furthermore, the inhibition of VEGFR-1 resulted in reduced macrophage recruitment, as well as impaired angiogenesis via MCP-1 signaling [237]. These findings may support an angiogenic role of macrophages in the dental pulp.

Interestingly, *HIF1A,* known for its ability to induce VEGF-signaling during hypoxia, showed a significant up-regulation in the pulp compared with control PDL, another highly vascularized tissue. Additionally, HIF-1α influences self-renewal and differentiation of stem cells by specific gene regulation and transcription factors important for stem-cell quiescence [238]. It is also known for regulating bone formation by osteoblasts [239]. Since the dental pulp rests in a low-oxygen environment, it is likely that the level of *HIF1A* may influence the stem cells numbers of the dental pulp and extrapolating its' known effects on bone development, it may also control odontoblast function. A similar role in stem-cell quiescence is demonstrated by *NFATc1* [240], which also exhibited an increased gene expression in the current study.

Furthermore, genes of the *MAPK* and *Pl3K* families, involved in ECs migration and survival [241, 242], were also highly expressed in normal dental pulp, fact which suggests an intense vascular activity of this tissue.

Our findings show that the dental pulp is an angiogenic reservoir, with vascular and immune cell interplay. However the exact roles of VEGF family members and genes involved in VEGF signaling, along with the roles of LYVE-1$^+$ macrophages in the dental pulp still require further investigations.

5.3 The roles of VEGFR-2 and/or -3 signaling in apical periodontitis

Our results on the presence of VEGFs and VEGFRs in dental tissues and in periapical lesions of endodontic origin raised numerous questions on the action mechanisms of these molecules. We aimed at looking into the VEGFR-2 and/or -3 signaling pathways during the development of apical periodontitis by individual or simultaneous blocking of these receptors. There were two major findings: blocking VEGFR-2 increased periapical inflammation characterized by amplified immune cell recruitment and inflammatory cytokine levels, while combined inhibition of VEGFR-2 and -3 showed a decreased inflammatory response.

Lesion sizes increased rapidly during the active phase of development until day 10 upon single VEGFR-2 and -3 blocking, compared to positive controls (IgG) and combined anti-VEGFR-2 and -3 treatments. In the chronic stage of disease progress, on day 21, they have reached similar levels in all four experimental groups. Even though no statistically significant differences were found between the test groups, time was of the essence regarding the overall lesion progression from day 0 to day 21 within each test group. The strong inflammatory response seen upon VEGFR-2 inhibition with significantly higher amounts of neutrophils and $TRAP^+$ osteoclasts may have resulted in rapid lesion growth. In a study conducted on osteopetrotic mice, VEGFR-3 was found to participate in osteoclast differentiation, with the inhibition of this receptor resulting in a reduction of osteoclasts [243]. We assume that in the context of periapical disease, VEGFR-3 signaling may have a direct inhibitory effect on osteoclast activity independent of their cellular numbers. After blocking VEGFR-3, an increased initial resorptive activity occurs, translated as faster lesion size expansion until day 10.

Osteoclasts, immune cells and blood ECs were found positive for both VEGFR-2 and -3 in papers I and II. In the current investigation, in order to evaluate the effects of VEGFR-2 and/or -3 signaling on these cells, we quantified $TRAP^+$ osteoclasts, Ly-$6.B2^+$ PMNs and $F4/80^+$ macrophages, along with $CD31^+$ blood vessels within the developed periapical lesions.

The numbers of PMNs and osteoclasts significantly increased upon VEGFR-2 blocking on day 10, whereas anti-VEGFR-3 treatment and combined inhibition of VEGFR-2 and -3 resulted in substantially fewer PMNs and osteoclasts than after VEGFR-2 blocking. This may indicate that PMN recruitment and osteoclast differentiation in apical periodontitis are under VEGFR-2 inhibitory signaling. Macrophages seem to be influenced by combined VEGFR-2/-3 signaling, as their numbers significantly decreased following combined systemic treatment on day 10. Blocking VEGFR-2 alone or simultaneous with VEGFR-3 decreased blood vessel counts, whereas the opposite effect was noticed under VEGFR-3 inhibition. This indicates that the intense inflammatory response seen in the VEGFR-2 treatment group is independent of angiogenesis. Lymphangiogenesis in the regional draining lymph nodes seems to depend on both VEGFR-2 and -3, since an inhibition of the process occurred by individual as well as combined blocking of these receptors. The intense apical inflammatory reaction that follows the VEGFR-2 inhibition is also seen as lymphadenitis of the lymph nodes.

Blocking both VEGFR-2 and -3 in the current study resulted in reduced periapical inflammation and angiogenesis, also seen in a model of corneal inflammation [244]. The same study [244] reports the inhibition of lymphagiogenesis under combined VEGFR-2/-3 blocking, which we have also seen in the draining lymph nodes.

The lack of lymphatics in the apical periodontium that we observed in papers I and II may result in different outcomes of the current investigation. Inhibition of VEGFR-2 decreased the severity of skin inflammation and arthritis [95, 96]. However, in those disease models, lymphangiogenesis does happen and is affected by both VEGFR-2 and -3 [95]. In apical periodontitis, no lymphangiogenesis can take place due to absence of lymphatic vessels. In contrast, we also report outcomes from an infectious disease with continuous antigenic stimulation, while the used models of skin inflammation and arthritis occurred in a sterile manner. This may explain our contradictory results.

Immune cells encountered in periapical lesions are the source of highly potent cytokines that activate bone resorption. We therefore investigated the expression of a wide panel of cytokines and chemokines involved in inflammation in well-established

periapical lesions. The results provided evidence for the important role in augmentation or attenuation of inflammatory reactions via VEGFR-2 and combined VEGFR-2/-3 signaling, respectively.

A tendency to down-regulation of many pro-inflammatory cytokines and chemokines, involved in the complex network of osteoclast differentiation and bone resorption, such as RANKL, IL-1α, IL-1β, IL-6, TNFα, IL-17, MCP-1, MIP-1α, MIP-1β, MIP-2 and RANTES was observed upon combined inhibition of both receptors. Furthermore, most cytokines showed highest levels upon VEGFR-2 blocking, supportive of the intense inflammatory reaction. Even though IL-1α, IL-1β, TNFα or MCP-1 levels are significantly increased after VEGFR-2 treatment, their pro-angiogenic effect does not occur, as seen in the low numbers of blood vessels counted on day 21. The VEGFR-2 is known as the main angiogenic receptor. The increased expression of these cytokines can be attributed to both the intense inflammatory immune response and to a compensatory reaction following VEGFR-2 inhibition.

MKC, a chemokine involved in neutrophil chemotaxis, increased in all experimental groups. A collaborating effect of MKC with G-CSF has been described with respect to PMN mobilization during acute inflammation [245]. G-CSF seems to have beneficial roles during inflammatory conditions, as it down-regulates LPS-induced TNFα production [246]. Our findings show that upon VEGFR-2/-3 blocking, TNFα exhibits lowest values, while MKC and G-CSF are significantly increased. This supports the anti-inflammatory and MKC-collaborative effects of G-CSF, but cannot explain the intense inflammation occurring upon VEGFR-2 inhibition.

The exact mechanisms through which VEGFR-2 signaling reduces, while simultaneous VEGFR-2 and -3 activation boosts inflammatory reactions during apical periodontitis is in need of further investigation.

6. Conclusive remarks

1. The VEGFs-A, -C, - D and VEGFRs- 2 and -3 are well represented on blood vessels in normal rat periodontium. Cells lining the bone and on root surface as well as cells with fibroblastic appearance express VEGFR-2 and -3 under normal conditions.

2. Inducing apical periodontitis in rats augments the presence of cells and vessels positive for VEGFs and VEGFRs in the inflamed areas. The sources of VEGF-A, -C and -D are macrophages and neutrophils. An important finding is that multinucleated osteoclasts express VEGFR-2 and -3 in inflamed apical tissue. The presence of VEGF family and their receptors on immune cells, blood vessels and osteoclasts during development of apical periodontitis suggests their implication in periapical disease development with respect to immune reactions, bone remodeling and angiogenesis.

3. Macrophages, PMNs, B- and T-lymphocytes, along with blood vessels express VEGF-A, -C, -D and VEGFR-2 and -3 in human periapical lesions. Gene expression of several pathways involved in VEGF signaling was altered in diseased periapical tissue compared with healthy PDL. This suggests ongoing vascular remodeling with immune cell implication in human periapical pathology.

4. In the well-vascularized dental pulp, macrophages and T-lymphocytes are the sources of VEGF-A, -B, -C, -D and VEGFR-2 and-3. In accordance with previous reports, no PMNs and B-lymphocytes reside in the normal dental pulp. While VEGF-B is only found at immune cellular level, the rest of investigated factors and receptors are expressed on pulp blood vessels. Furthermore, 67 out of 84 investigated genes involved in VEGF signaling were altered in normal dental pulp vs PDL. *HIF1A* and *NFATc1* expression is

likely induced by hypoxia in the pulp and these probably represent important factors for stem-cell quiescence. Thus, the dental pulp represents an angiogenic reservoir with high vascular activity and remodeling potential.

5. Our studies confirm that lymphatic vessels are absent from periapical tissues and from the dental pulp. Macrophages expressing the lymphatic marker LYVE-1 are present in both human normal dental pulp and periapical inflamed tissues. Even though their exact role remains to be determined, their main activity is assumed to be angiogenic.

6. In a murine model of apical periodontitis we show that VEGFR-2 inhibits, while combined VEGFR-2 and -3 signaling enhances periapical inflammation. Evidence for this finding is provided by the initial fast growth of the lesion sizes along with an increased presence of PMNs and osteoclasts upon VEGFR-2 inhibition, while numbers of macrophages, neutrophils and blood vessels are lowered after combined VEGFR-2/-3 systemic blocking. Furthermore, in the well-established periapical lesions, major pro-inflammatory cytokines like IL-1 or TNFα exhibit highest levels under anti-VEGFR-2 systemic treatment. In contrast, blocking both VEGFR-2 and -3 decreases expressions of inflammatory cytokines, significant for RANKL and MCP-1. We also conclude that lymphangiogenesis in the draining lymph nodes seems to be equally dependent on VEGFR-2 and -3.

7. Even though the exact interplay between cells expressing ligands to VEGFR-2 and/or -3 involved in periapical disease pathogenesis still needs further investigations, we provide important initial evidence of their signaling contribution to periapical disease development.

7. Future perspectives

Since our initial investigations for mapping VEGF family members and their receptors at protein level are mainly descriptive, a quantification of these molecules in animal and human dental tissues should be performed. Both normal and inflamed pulp as well as periapical tissues can be subjected to quantification of VEGFs and VEGFRs expression by counting immunohistochemically stained structures and by use of enzyme-linked immunosorbent assays or flow cytometry analysis of the respective tissues.

We assume that the high gene expression levels of *HIF1A* and *NFATc1* in normal dental pulp are important for stem cell quiescence. It has been shown that dental pulp stem cells can induce VEGFR-2 dependent angiogenesis [247]. Investigations on the expression of all VEGFs and VEGFRs by stem cells in the normal dental pulp could provide more information on their possible involvement in pulp angiogenesis.

Pulp repair and revascularization of immature necrotic teeth require angiogenic processes. Understanding the molecular mechanisms underlying angiogenesis can lead to new therapeutic strategies. It has been previously suggested that topical application of VEGF-A enhances pulp neovascularization [248]. Knowing that both VEGFR-2 and -3 are present on vessels of angiogenic origin in the dental pulp, tissue engineering and possible therapeutic intervention on injured dental pulp could also be expanded to VEGF-C and -D.

Since we could not confirm the presence of lymphatic vessels in dental pulp or periodontium there are still open questions on the exact roles of VEGF-C, -D and their receptors VEGFR-2 and -3, along with their possible angiogenic involvement.

Macrophages expressing LYVE-1, a lymphatic marker, were found both in normal human dental pulp, as well as inflamed apical tissue. This implies that they are resident cells of the dental tissues, but also recruited at sites of dental inflammation, where they may have a role in immune response. Due to the absence of lymphatics in the dental pulp and apical periodontium, we assume an angiogenic role of these

macrophages. However, information on their kinetics and exact function is still in need of further investigation.

Lymphatic responses were found in the draining lymph nodes in apical periodontitis settings. However, the localization of peripheral lymphatic vessels remains unknown. Further research is needed to clarify lymphatic mechanisms.

Research on macrophages has focused on the polarization of these cells during inflammatory conditions, describing different expression of pro-inflammatory cytokines and chemokines by 2 distinct types of macrophages, M1 and M2, and accordingly with diverse immune effects [249]. The M1 form expresses pro-inflammatory, whereas anti-inflammatory cytokines are produced by the M2 subtype. In our studies we used antibodies that generically stain cells of monocytic origin. Thus, more research is needed in order to understand the macrophage types present in dental tissues and their involvement in the pathogenesis of apical periodontitis and possibly in vascular remodeling.

In murine apical periodontitis we show that VEGFR-2 reduces, while combined VEGFR-2 and -3 signaling increases inflammation. Yet, further investigations are needed in order to elucidate the exact mechanisms of VEGFR-2 and/or -3 signaling in periapical inflammation and it remains to be seen to what extent these findings can offer therapeutic strategies.

8. References

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