DUAL ROLE OF AUTOPHAGY IN PERIODONTAL DISEASE

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Abstract:

Autophagy is a self-degradative process that is important for balancing sources of energy at critical times in development and in response to nutrient stress. The induction of autophagy has been shown to have both protective and pathological effects in periodontitis. Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins, clearing damaged organelles, such as mitochondria, endoplasmic reticulum and peroxisomes, as well as eliminating intracellular pathogens. Thus, autophagy is generally thought of as a survival mechanism, although its deregulation has been linked to non-apoptotic cell death. Autophagy is an evolutionarily conserved process essential for cellular homeostasis and human health. Autophagy provides a mechanism for the turnover of cellular organelles and proteins through a lysosome-dependent degradation pathway. It also participates in various biological processes, such as cellular differentiation, cell function, and defense against pathogens. In addition, autophagic dysfunction is associated with multiple diseases such as autoimmune disease, cancer, diabetes, and oral disease. Nowadays research has ascertained the role of autophagy in Periodontal disease, especially its role in the host defence against periodontal disease drivers. A bulk of research has recognized several pharmaceuticals and nutraceuticals that can potentially modulate this kind of cell death and serve as useful therapies. However, further research is warranted in order to reach a clinical translation, which could be of help in the discovery of novel host modulation therapies for Periodontal disease.

Keywords: Autophagy, Apoptosis, Micro autophagy, immune response, Periodontitis, Periodontal pathogens, Periapical lesion

Introduction:

Autophagy is a newly recognized innate defense mechanism, acting as a cell-autonomous system for elimination of intracellular pathogens.¹ The term autophagy (from the Greek words auto meaning 'self' and phagein meaning 'to eat') was first coined by Christian de Duve over 40 years ago, which was largely based on the observation in degradation of mitochondria and other intracellular structures within lysosomes of rat liver perfused with the pancreatic hormone,

glucagon. The mechanism of glucagon-induced autophagy in the liver is still not fully understood at the molecular level, other than that it requires cyclic AMP induced activation of protein kinase-A and is highly tissue-specific.²

Autophagy is the most important and necessary pathway regulating cell growth, differentiation, and function. Autophagy and apoptosis (programmed cell death) are interrelated to each

other; autophagy provides a basis for apoptosis, and inhibition of autophagy leads to delayed apoptosis. Autophagy maintains cell balance by recycling damaged material to reuse these cytosolic components.

Autophagy is an essential cellular mechanism which plays “housekeeping” role in normal physiological processes including removing of long lived, aggregated and misfolded proteins, clearing damaged organelles, growth regulation and ageing. Autophagy is also involved in a variety of biological functions like development, cellular differentiation, defense against pathogens and nutritional starvation. The integration of autophagy into these biological functions and other stress responses is determined by the transcriptional factors that undertake the regulatory mechanism.³ Autophagy, as a strictly regulated catabolic process mediating cellular degradation and recycling, is crucial for maintaining homeostasis and development. The autophagic process is thought to be a form of endogenous defense mechanism that allows cells to survive during harsh conditions, including hypoxia, heat, starvation, and oxidative stress. Besides pro-survival effects, autophagy participates in a pro-death mechanism—previously referred to as type II programmed cell death, and now termed autophagic cell death—depending on the specific conditions.⁴

Several pathologies have been linked to this biological process since in 1999 Liang et al. discovered its beclin 1-dependant induction/inhibition by means of gene-transfer techniques in MCF-7 cells.⁵ In this vein, a significant and increasing number of diseases have been linked to autophagy (eg. Oral cancer, autoimmune diseases, metabolic disorders such as diabetes, neurodegeneration, or pathogen infection).⁶ The role of autophagy in disease is context dependent, this fact has been named as the “Autophagy paradox”. Basically, the one aspect is that defective autophagy promotes the accumulation of proteins and potentially hazardous intracellular structures providing a protective environment for several hallmarks of diseases whereas the other aspect is that

functional autophagy exerts death promoting roles to protect cells from insults. However, the underlying mechanism that regulates the autophagy paradox remains unclear.⁷ In 2004, three research articles provided the first evidences regarding autophagy role in bacterial infection.^{8,9,10}

Periodontal disease (PD) is a chronic oral disease produced by a bacterial insult that triggers an altered immune response. A recent meta-regression analysis showed that severe PD affected the 11.2% of the global population between 1990 and 2010.¹¹ PD has been strongly linked to the likelihood of suffering from different groups of human diseases such as cardiovascular diseases (CVDs), neuroinflammatory diseases, or diabetes.^{12,13} In turn, these outcomes have been heavily linked to autophagy dependant mechanisms. A possible hypothesis for this epidemiological relationship may be the effect of systemic inflammation on the danger-associated molecular patterns (DAMPs) and pathogen’s ability to build pathogen-associated molecular patterns (PAMPs). Thus, this review article has focused on elucidate the link between autophagy and PD.

THE MAJOR DEVELOPMENT IN THE FIELD OF AUTOPHAGY

More than four decades ago, Clark and Novikoff observed mitochondria from mouse kidneys within membrane-bound compartments termed ‘dense bodies’, which were subsequently shown to include lysosomal enzymes. Ashford and Porter later observed membrane-bound vesicles containing semi-digested mitochondria and endoplasmic reticulum in the hepatocytes of rats that had been exposed to glucagon, and Novikoff and Essner observed that the same bodies contained lysosomal hydrolases. One year later, in 1963, at the Ciba Foundation Symposium on Lysosomes, de Duve founded the field when he coined the term ‘autophagy’ to describe the presence of single- or double-membrane vesicles that contain parts of the cytoplasm and organelles in various states of disintegration. Autophagosomes have first been observed by

electron microscopy in 1962, and Christian De Duve coined in 1963 the name autophagy for cell organelle degradation in lysosomes.¹⁴

A new era of autophagy research began in 1990s when several groups of scientists independently discovered more than 30 autophagy-related genes (atg) using the budding yeast. In 1993 Yoshinori Ohsumi, a Japanese cell biologist, published the first screen for yeast mutants deficient in this pathway. This machinery allows membrane remodelling, vesicular fusion and substrate recruitment to autophagosomes. He pointed out that these sequestering vesicles or 'autophagosomes', were related to lysosomes and occurred in normal cells. The origin of the membrane surrounding the autophagosomes is still controversial; de Duve suggested that the sequestering membranes are derived from preformed membranes, such as smooth endoplasmic reticulum.¹⁵

In 1999, a landmark discovery connecting autophagy with cancer was published by Beth Levine's group. In 2005, Daniel J Klionsky launched "Autophagy", a scientific journal dedicated to this field.

Pioneering work by Mortimore and Schworer further demonstrated that amino acids, which are the end products of autophagic degradation, have an inhibitory effect on autophagy in rat liver cells. These early lines of evidence are consistent with our current understanding of autophagy as an adaptive catabolic and energy-generating process. Subsequently, Seglen and Gordon carried out the first biochemical analysis of autophagy and identified the pharmacological reagent 3-methyladenine as an autophagy inhibitor; they also provided the first evidence that protein kinases and phosphatases can regulate autophagy.¹⁶

NOBLE PRIZE IN 2016

It was awarded to Yoshinori Ohsumi who is a professor at Tokyo Institute of Technology's Institute of Innovative Research. He also awarded a Wiley Prize in Biomedical Sciences in 2016. In 2017, he got a Breakthrough Prize in Life Sciences for his discoveries of mechanisms for autophagy.

TYPES OF AUTOPHAGY

The general term-autophagy refers to the representative degradation pathways that deliver cytoplasmic components to the lysosome. There are 3 primary types of autophagy.¹⁷

1. Macroautophagy (MA)
2. Microautophagy (mA)
3. Chaperone-mediated autophagy (CMA)

1. Macroautophagy (MA): Macroautophagy delivers cytoplasmic cargo to the lysosome through the intermediary of a double membrane bound vesicle, referred to as an autophagosome that fuses with the lysosome to form an autolysosome.

2. Microautophagy (mA): mA is a form of cargo degradation in which targeted cytoplasmic materials are directly engulfed by the vacuole constituting multivesicular bodies (MVBs), subsequently cargo is cleaved by enzymes. Microautophagy is thought to be involved in long-lived protein turnover in mammalian cells.

3. Chaperone-mediated autophagy (CMA): Chaperone-mediated autophagy is a highly selective type of autophagy that relies on translocation of pertinent soluble cytosolic proteins across the lysosomal membrane. In the strictest and best characterized form of autophagy. It just captures protein with the (Lys-Phe-Glu-Arg-Gln) KFEQR motif. It does not require the formation of multivesicular bodies and proteins are directly internalized in lysosomal lumen.

Figure 1: three types of autophagy

The different forms of autophagy mainly differ in the mode of cargo delivery to the lysosome. Both micro- and macroautophagy have the capacity to engulf large structures through both selective and non-selective mechanisms, whereas CMA degrades only soluble proteins, albeit in a selective manner. The best characterized in macroautophagy, which in turns in frequently identified simply as “autophagy”. Macroautophagy (hereafter simply referred to as autophagy unless specified) can be divided into different phases: Initiation, elongation, transport to lysosomes and degradation (**Figure 2**).

The initiation step begins with the formation of phagophores; both ends of the membrane of phagophores elongate to engulf and enclose the targeted materials encapsulated within double-membrane autophagosomes. The outer membrane of the autophagosome fuses with lysosomes to become autolysosomes, whereas the content is degraded. Microautophagy occurs when the cytosolic cargo is directly sequestered by invagination of the lysosomal membrane. The inner membrane of autolysosomes and engulfed materials are degraded by lysosomal acid proteases.³

Figure 2: major stages in autophagic process

MOLECULAR MECHANISM OF AUTOPHAGY

The molecular machinery of autophagy was discovered in yeast genetic studies in which more than 41 ATG genes have been identified.¹⁶ Many orthologues of ATG genes have been identified in mammalian cells. The corresponding gene products are required for the dynamic processes of autophagy. Under stress conditions, the mammalian target of rapamycin (mTOR) is inactivated, which consequently activates ATG1 kinase activity. UNC-51-like kinase (ULK)/ATG1 is a key protein that initiates autophagy by forming a complex with ATG13, ATG101, and focal adhesion kinase family interacting protein of 200 kD (FIP200) to induce autophagic signals.³

The class-III phosphatidylinositol 3-kinase (PI3K) complex composed of Beclin1/ATG6, vesicular protein sorting (Vps) 34, Vps15, and ATG14, induces the production of phosphatidylinositol-3-phosphate to recruit effectors required for the formation of autophagosomes. ATG9 is a transmembrane protein and its bidirectional movement between phagophore assembly sites (PASs) and non-PASs contributes to the delivery of membranous structures to form autophagosomes.¹⁸ Two ubiquitin-like conjugation systems are also required for the vesicle elongation process.

One system is mainly formed by ATG5 and ATG12; the other involves the conjugation of

phosphatidylethanolamine (PE) to microtubule-associated protein 1 light chain 3 (LC3)/ATG8.22 LC3 is synthesized as pro-LC3, which is cleaved at the C-terminus with the help of protease AGT4 to form LC3-I. The lipid conjugation of PE leads to the conversion of LC3-I to the autophagic membrane-bound LC3-II.²³ LC3-II is recruited to the membranes of autophagosomes and remains on the completed autophagosomes until lysosomal fusion.² (Figure 3)

ULK complex is activated in response to signals such as starvation, and then binds to the PtdIns3K complex following MTOR suppression or AMPK activation. Upon induction, the orchestrated action of the ULK complex, the PtdIns3K complex and the ATG9 complex initiates the formation of the phagophore at the phagophore assembly site (PAS) in yeast or equivalent sites in more complex eukaryotes. The LC3 and ATG12 conjugation systems are key regulators in mediating the elongation of the phagophore into an autophagosome. Conversely, the major inhibitory factor MTOR suppresses autophagy under abundant nutrients conditions, which is regulated by class I PI3K and AKT signalling. The antiapoptotic proteins BCL2 and BCL2L1/BCL-xL negatively regulate autophagy by binding to BECN1. Moreover, DAPK promotes autophagy initiation through the phosphorylation of BECN1. In addition to this, autophagy mechanism can be pharmacologically inhibited or induced.

Figure 3: molecular and signalling pathways regulating autophagy

PROTEINS/GENES INVOLVED IN AUTOPHAGY MECHANISM

Macroautophagy is regulated by more than 41 autophagy-related genes (ATG) and most of genes have a role in autophagosome membrane formation.¹⁶ The complex molecular process of autophagy is primarily dependent on the ATG (autophagy-related) family proteins. Eighteen of these ATG genes (ATG1 to ATG10, ATG12 to ATG14, ATG16 to ATG18, ATG29, and ATG31) encode proteins that are required for the formation of the autophagosome and most of them are highly conserved from yeast to human.¹⁹ The “core” ATG proteins involved in autophagosome formation consist of 5 effector subgroups: the ULK1/2 (unc-51 like autophagy activating kinase 1/2)–ATG13–RB1CC1/FIP200 (RB1 inducible coiled-coil 1) complex is the initial component in the induction of autophagy.¹⁶

Briefly, the molecular events sequence in autophagy is as follows:

1. Signals such as starvation activate the ULK complex, which will bind to the PtdIns3K

complex following AMPK activation or mTOR suppression.

2. Following induction, the ULK complex, PtdIns3K complex and the ATG9 complex orchestrated action will trigger the phagophore assembly at the phagophore assembly site.

3. ATG12 and LC3 conjugation systems are key players in regulating the phagophore elongation to the autophagosome. mTOR, the major autophagy inhibitory factor, suppresses autophagy as response to abundant nutrients conditions. This suppressive action is mediated by class I PI3K and AKT signalling.

4. SQSTM1/p62 (sequestosome 1) receptor protein will consequently interact with both LC3 and ubiquitin chains.

5. Further, the autophagosome will fuse with a lysosome, resulting the autolysosome formation. Inside autolysosome, the autophagosome constituents will be hydrolytically degraded. The trapped SQSTM1 will be degraded in the autolysosome, which highlight SQSTM10s role as an autophagy flux marker.²⁰

Figure 4: possible autophagy-protein-dependent pathways of pathogen degradation

Table 1: autophagy-related genes and their function²⁰

NAME OF GENE	ROLE OF AUTOPHAGY	REFERENCE
ATG1	Induction of autophagy	Reggiori and Klionsky (2002)
ATG5	Autophagosome formation	Reggiori and Klionsky (2002)
ATG7	Autophagosome formation	Reggiori and Klionsky (2002) and Yu et al. (2004)
ATG8	Marker of autophagosome formation	Reggiori and Klionsky (2002)
ATG12	Autophagosome formation	Reggiori and Klionsky (2002)
ATG13	Induction of autophagy	Reggiori and Klionsky (2002)
Beclin -1	Initiation of autophagosome formation	Simonsen and Tooze (2009)
NBR1	Increase osteoblast differentiation and activity Increase bone mass and density.	Whitehouse et al. (2010)
Deletion of Fip200	Reduction in bone formation.	Y. Liu et al., 2013
Rapamycin	Increased osteoblast differentiation.	Oliver et al. (2012)
3-MA and chloroquine	Decreased number and size of alkaline phosphatase at Day 10 Reduction in bone mineralization at Day 21.	F. Liu et al. (2013)
Bafilomycin, chloroquine, NH ₄ CL, and shRNA-mediated knockdown of LC3-B	Blocked osteogenic Pantovic et al. (2013) siATG7 and siBCN1 Decreased osteoblast mineralization.	Nollet et al. (2014)
Mutation in SQSTM1 (P62)	Paget's disease	Helfrich and Hocking (2008)
ATG5, ATG7, ATG4B, and LC3	Generating the osteoclast-ruffled border.	De Selm et al. (2011)
Hypoxia	Increased osteoclast size and differentiation	Arnett (2010), Bozec et al. (2008), Sambandam et al. (2014), and Y. Zhao et al. (2012)
Decrease level of Beclin-1	Reduction in osteoclast differentiation and function.	Chung et al. (2014)

Autophagy: “Dual role in Periodontal Diseases” by Promoting Cell Death or Inhibitory Apoptosis in Infected Cells

It is well known that some bacteria can produce a local disease that accelerates the inflammatory response, and thus induce and enhance autophagy. Periodontal diseases, comprising periodontitis and gingivitis, are common chronic inflammatory diseases affecting tooth-supporting tissues. Numerous species of microorganisms and their virulence products compose the dental plaques in the gingival groove. Periodontal tissue and the immune system cooperatively respond to these bacterial components, especially anaerobic gram-negative perio-pathobionts, thereby

inducing inflammatory effectors in the gingival crevices and triggering destruction of periodontal tissue. It is speculated that autophagy may modulate inflammation and immune response, and could thereby play a pivotal role in regulating the initiation and progression of periodontal diseases.²¹ Nuclear HMGB1 (high mobility group box 1) released from the gingival cells is closely related to autophagy induction HMGB1 works as a chemoattractant for polymorphonuclear leukocytes, as well as inflammatory and immune stimulators in periodontal diseases.^{21,22} Studies have found elevated levels of oxidative damage products in serum, plasma and peripheral blood mononuclear cells, as well as compromised

antioxidant capacity in patients with periodontitis.²³ The oxidative stress mainly results in high levels of reactive oxygen species and thereby induces periodontal tissue damage. High levels of mitochondrial reactive oxygen species production can activate autophagy in patients with periodontitis.²¹ In addition, after treatment with the periodontal etiological agent LPS, increased expression of ATG12 and LC3 proteins as well as ATG12 and LC3 transcripts are found in human gingival fibroblasts, which suggests the involvement of autophagy in periodontal inflammation.

Autophagy has a dual role in periodontal diseases, promoting cell death or inhibiting apoptosis in infected cells.^{21,24} It is speculated that autophagy may modulate inflammation and immune response, and could thereby play a pivotal role in regulating the initiation and progression of periodontal diseases. (Figure 5)

- **In the first mode:** Autophagy can be a mode of cell death in periodontal diseases. Butyrate, produced by anaerobic bacteria and highly concentrated in mature subgingival dental plaques, activates autophagic cell death in gingival epithelial cells. This butyrate-induced cell death can be significantly reversed by the autophagic inhibitor 3-MA, but not by caspase inhibitors, which confirms the existence of

caspase-independent cell death mediated by autophagy. Of note, starvation promotes the autophagy activation in butyrate-treated gingival epithelial cells, particularly those adjacent to the mature dental plaque.

- **In the second mode:** Autophagy plays a protective role in periodontal diseases. In contrast to its pro-death mechanisms, autophagy may block apoptosis-induced cell death and maintain viability in periodontal diseases. The induction of autophagy contributes to the clearance of damaged mitochondria and prevention of unwarranted cell loss.

Bullon et al.²¹ found increased apoptosis and a decline in cell viability in peripheral blood mononuclear cells of patients with periodontitis when autophagy is suppressed. The interaction between upstream regulators of apoptosis and autophagy may arise as a result of signalling crosstalk. **Tsuda et al.**²⁴ have demonstrated the coexistence of autophagy and apoptosis in periodontal diseases. Therefore, the complex relationship between apoptosis and autophagy may be contributing factors in the pathogenesis of periodontal diseases.

Figure 5: an overview of potential roles of autophagy in the pathogenesis of periodontal disease

IMPLICATION OF AUTOPHAGY IN PERIODONTAL DISEASE RELATED DRIVERS

Autophagy has a dual role in response to periodontal pathogens. First, autophagy enhances the survival of periodontal pathogenic bacteria. Increasing evidence indicates the ability of host-adapted pathogens to exploit host autophagy for survival and persistence in the host. Some periodontal bacteria have evolved mechanisms that use the autophagic response; therefore, autophagy may provide a route for bacteria to escape from the host immune defense. It has been confirmed that the level of autophagy is higher in patients with periodontitis than in healthy individuals. Periodontal disease is characterized by a chronic infection associated with bacteria in the dental biofilm.

Autophagy in periodontal disease related drivers are:

1. *Aggregatibacter actinomycetemcomitans* (AA)
2. *Porphyromonas gingivalis* (Pg)
3. Neutrophil extracellular traps (NETs)
4. Reactive oxygen species (ROS)

1. *Aggregatibacter actinomycetemcomitans* (AA)

The Gram-negative *A. actinomycetemcomitans* is assumed to be the primary etiologic agent of LAgP and has also been implicated in chronic periodontitis and severe non-oral infections. Currently seven serotypes of this bacterium (a-g) are recognized based on the immunodominant antigen, which is an O polysaccharide of the lipopolysaccharide (LPS). Successful establishment of persistent colonization in subgingival crevices by *A. actinomycetemcomitans* may lead to periodontal destruction and development of periodontitis in

susceptible individuals.²⁵ The periodontal infection by *A. actinomycetemcomitans* is accompanied by local and systemic immune responses, and this species may invade the gingival epithelium and release virulence factors such as endotoxins and exotoxins. Endotoxin is expressed by all Gram-negative bacterial species and causes a general pro-inflammatory host response, while the two exotoxins, a cytolethal distending toxin (Cdt) and a leukotoxin (LtxA), are unique for *A. actinomycetemcomitans* within bacterial species colonizing the oral biofilm.²⁶

The bacterium moves from the initial oral colonization site to the gingival crevices and competes with other bacteria in the niche. *A. actinomycetemcomitans* has been closely associated with periodontitis in young individuals and with cases of refractory adult periodontitis. *A. actinomycetemcomitans* infection induces autophagy in human JEEKs, a cellular homeostatic process with a cytoprotective effect on this cell type, in the early stages of infection. The junctional epithelium represents the main site of the initial interaction between the dysbiotic subgingival biofilm and host. The challenge and binding of *A. actinomycetemcomitans* and its purified LPS on JEEKs surface triggers TFEB translocation to the nucleus and autophagosomes biogenesis. Bacteria and LPS internalized are sequestered to the autophagosomes in formation, through recruitment to the p62-cargo adapter protein, and interact with LC3 protein, favouring autophagy activation (upper panel). Treatment with the alkalinizing compounds of lysosomes bafilomycin A1, chloroquine, and NH₄Cl, which inhibit the late stages of autophagic flow, induced intracellular bacteria accumulation and significantly increased the infected-JEEKs number. Inhibition of autophagosome biogenesis with 3-MA induces cell death in JEEKs challenged with *A. actinomycetemcomitans* or its purified LPS in the initial stage of infection (**Figure 6**).

Figure 6: schematic representation of the junctional epithelium keratinocytes-autophagy induced by a. Actinomycetemcomitans infection

It was reported that a higher level of autophagy genes expression was found in peripheral blood mononuclear cells from patients with periodontitis, compared with those without periodontitis. In the case of PD, a few agents have been identified as autophagy modulators in several cell lines of various histotypes and animal models, establishing interesting therapeutic targets. Thus, autophagy pathway interference could be an alternative and poorly explored approach against PD. The induced autophagy in GECs provides a favourable microenvironment for its persistence and evasion of immune defense.³

2. Porphyromonas gingivalis (Pg)

Porphyromonas gingivalis, a gram-negative anaerobe, as the keystone species in the development of chronic periodontitis. This non-motile, a saccharolytic, Gram-negative bacterium is an obligately anaerobic rod which forms black-pigmented colonies on blood agar plates. It has an absolute requirement for iron in its growth. *P. gingivalis* is the major intracellular opportunistic pathogen linked to periodontal disease such as severe adult periodontitis, failing guided tissue regeneration and acute periodontal abscesses. Pathogen and its metabolic products could up-regulate the expression of many

autophagy-related proteins, and then induce the autophagy in cells.²⁵ In the oral microenvironment, inflammation is often activated by single bacteria, *P. gingivalis*, which triggers the toll-like receptor (TLR) pathway. The study conducted by Progulsk-Fox's laboratory firstly confirmed the connection between *P. gingivalis* and autophagy.²⁷

P. gingivalis can exploit autophagy to enhance its penetration into and colonization of host periodontal tissues. After internalization, *P. gingivalis* induces autophagy and suppresses apoptosis in infected host cells, which results in a microenvironment favourable to its replication and evasion of innate immunological defenses. *P. gingivalis*-related survival can be achieved by subverting the host's autophagic pathway, meaning a relevant innate immune interaction. As an inducer of PD, *P.g* and its lipopolysaccharide (LPS) have been demonstrated to enhance autophagic activity. A study found that the inhibition of autophagy by 3-methyladenine (3-MA) or ATG5 depletion significantly decreased the survival of *P.g* in gingival epithelial cells (GECs). PI3K/ protein kinase B (Akt)/mTOR signalling pathway is a critical regulator of autophagy and inactivation of it results in autophagy after *P.g* invading.³

Moreover, Pg in the cytosol is usually degraded by lysosomes, but the ratio of free Pg in the cytosol is low compared with Pg co-localized with double-membrane vacuoles. Thereby, most co-localized Pg evades host defenses by impairs the formation of autolysosomes which leads to accumulating autophagosomes that supply nutrients for bacteria survival.⁷ By contrast, autophagy also induces a type of cell death against infection by periodontal bacteria. Butyrate, a metabolic by-product of periodontal bacteria, promoted cell death via autophagy induction by increasing the conversion of LC3-I to LC3-II in GECs. The autophagy inhibitor 3-MA significantly suppressed cell death induced by butyrate. Furthermore, the induction of autophagy in immune cells enhanced intracellular bacteria killing during the antibacterial process.

Nevertheless, *P. gingivalis* is reportedly localized in early endosomes, and about half of the internalized organisms are sorted to lytic compartments including autolysosomes. A considerable number of the remaining intracellular pathogens within specific endosomes can regulate the bacterial exit from infected cells and cause further penetration into periodontal tissues. Taken together, the autophagy pathway mediates the survival, replication, and dissemination of *P. gingivalis*, thus exerting effects on periodontal diseases.

3. Neutrophil extracellular traps (NETs)

As in other bacterial assaults during PD, PMNs' efflux is a host response to pathogens. Neutrophils are especially essential elements of host resistance to PD due to their ability to disrupt PAMPs. Nevertheless, the ability of some PD drivers to evade neutrophil microbicidal machinery and to delay apoptosis can turn this defensive cell activity into unwanted immune responses. Neutrophils are key cells of the immune system and have a decisive role in fighting foreign pathogens in infectious diseases. Neutrophil extracellular traps (NETs) consist of a mesh of DNA enclosing antimicrobial peptides and histones that are released into extracellular space following neutrophil response to a wide range of stimuli, such as pathogens, host-derived

mediators and drugs. Neutrophils can remain functional after NET formation and are important for periodontal homeostasis. The pathogenesis of periodontitis includes an immune-inflammatory component in which impaired NET formation and/or elimination can be involved, contributing to an exacerbated inflammatory reaction and to the destruction of gingival tissue.²⁸

NETs are extracellular chromatin structures that can trap and degrade microbes. This feature helped in the identification of a novel form of cell death named NET cell death, or NETosis. NET-associated host damage has been linked to the onset of several immune- and infection-related conditions, such as PD. NETs exert DNA backbone activities but can also release several active proteases to the ECM, leading to bacterial lyses. On the other side, NETs' bacterial entrapment is highly variable according to the targeted bacteria, due to each bacterium's variable capacity to release nucleases into the ECM. Some bacterial virulence factors, such as the leukotoxin of *A. actinomycetemcomitans*, can induce NETosis. NETs can be stimulated by periodontopathogens and other microbes in anaerobic conditions.³

4. Reactive oxygen species (ROS): "A DOUBLE SENSE CONNECTION"

ROS are a chemically reactive chemical species containing oxygen. ROS can exert positive functions under physiological conditions. However, increased ROS levels can trigger the remodelling of cellular microenvironment, which induces the oxidative stress (OS) phenomenon. The majority of periodontal tissue damage is caused by the subversion of host immune responses, with the involvement of leukocytes, complement and reactive oxygen species (ROS).²⁸ The ROS effects (**Figure 7**) on periodontium have opened an interesting perspective on treating this outcome by using exogenous antioxidants or modulators of endogenous antioxidants. In this line, limited evidence is available regarding the usefulness of compounds with antioxidant capacity as a local adjuvant therapy or as nutraceuticals for PD. Among them, coenzyme Q10 and vitamin D are

highlighted. ROS inhibition via antioxidants delays the onset of autophagy and its related kinetics. Autophagy can be modulated by ROS via four different pathways:²⁹

1. Atg12–Atg5 complex activation, promoting autophagy elongation;
2. ROS-dependent JNK induced Bcl-2 phosphorylation triggering Beclin 1 dissociation and autophagy induction;
3. PI3K-AKT pathway initiation triggering the activation of mTOR, which, in turn, acts as an autophagy induction inhibitor;

4. The AMPK-dependent TORC1 activity inhibition leading to autophagy activation

ROS and autophagy are closely interconnected, and many key molecules are shared by the two processes. However, the available data suggest that the intricate interactions between ROS and autophagy in periodontitis remain unknown. Moreover, the mechanisms underlying how ROS participate in regulating autophagy remain to be elucidated.

Figure 7: schematic representation of autophagy mechanism modulated by ros in periodontitis

AUTOPHAGY DEPENDENT LINK WITH HUMAN ORAL DISEASES

With regard to oral disease, inappropriate autophagy can consist of either activated autophagy or impaired autophagy, because this process has dual roles in various human oral diseases. Activated autophagy may play pathogenic roles through the regulation of cell death, or prevents the development of diseases in some conditions. Impaired autophagy can exacerbate disease at different stages, or serve as a potential therapeutic target. In oral cancer, activated autophagy acts as a cytoprotective mechanism, but can enhance radiosensitivity, whereas impaired autophagy augments chemotherapy. Autophagy may dampen periodontal inflammation and promote cell vitality in periapical lesions, or exacerbate

periapical lesions and periodontal diseases through the interactions with apoptosis and the immune response. Meanwhile, the periodontopathic pathogen *Porphyromonas gingivalis* can exploit host cell autophagy for its own survival and replication. In addition, activated autophagy may promote long-term survival and invasive virulence of *Candida albicans* within the host.

Autophagy can regulate multiple cellular pathways involved in tumor suppression and promotion, inflammation, and immune response. So far, the roles of autophagy have been investigated in cancer, infections and inflammatory diseases, as a modulator of pathogenesis and as a potential therapeutic target. (Figure 8)

(Pro: beneficial effect; Cons: detrimental effect)

Figure 8: autophagy in human oral diseases

ROLE OF AUTOPHAGY IN BONE HOMEOSTATISIS

Alveolar bone homeostasis is maintained by the balance between osteoclastogenesis and osteoblastogenesis. An imbalance favouring bone resorption over formation results in alveolar bone resorption in periodontitis. Due to the large amounts of waste materials including damaged organelles, and mineral and organic components of the bone matrix during bone resorption, dynamic autophagy is required for degradation and recycling of damaged intracellular structures. Autophagy-related proteins have been demonstrated to be important mediators in the differentiation and function of bone cells in physiologic and pathologic conditions, suggesting a crucial role of autophagy in bone homeostasis.

In Osteoclast Cell

Autophagy is responsible for increasing the number of osteoclasts and for persistent osteoclastic activation during alveolar bone resorption in PD. LPS stimulates osteoclast differentiation by enhancing autophagy and increasing ROS levels in pre-osteoclasts. ROS contributes to osteoclast activation during the host response, resulting in pathological bone destruction. Overexpression of Beclin1 significantly increased the number of tartrate-

resistant acid phosphatase (TRAP)-positive osteoclasts, whereas the inhibition of autophagy inhibited osteoclastogenesis. Autophagy has a positive effect on osteoclast activity in response to pro-inflammatory cytokines. The pro-inflammatory cytokine IL-17A contributes to the pathogenesis of periodontitis, especially alveolar bone loss. Song et al found that IL-17A facilitated osteoclast differentiation and exacerbated bone resorption in vitro and in vivo and upregulated autophagy activity, including LC3 levels and autophagosome formation.³

Furthermore, the autophagy inhibitor 3-MA decreased the levels of osteoclast-related markers. ATG7-deficient osteoclast precursors did not exhibit IL-1 β -mediated upregulation of cathepsin K secretion. Thus, inhibition of osteoclast activation is a potential approach for protecting alveolar bone from excessive resorption in PD. In addition to their role in osteoclastogenesis, autophagy has been proven indispensable in the functioning of osteoclasts. When the exogenous autophagy inhibitor bafilomycin is added to the culture medium of osteoclasts, the resorptive activity is sharply decreased.³⁰ The autophagy-related proteins ATG5, ATG7, ATG4B, and MAP1LC3 have all been suggested to play critical roles in the activation of resorption function. Both in vivo and in vitro data suggest

that ATG5 and ATG7 promote osteoclastic functioning and guide lysosomes to target the actin ring. Specific knockdown of genes related to autophagosome formation (Atg5, Atg7, Atg4b, or Lc3) in mononuclear osteoclast progenitors in

mice leads to defects in lysosomal trafficking and formation of resorptive brush borders in osteoclasts and, consequently, downregulates bone resorption activity and increases bone volume.

Figure 9: role of autophagy in osteoclasts

In Osteoblast and Osteocytes cells

Autophagy plays an essential role in the differentiation and mineralization of osteoblasts. As an autophagy receptor targeting ubiquitinated proteins for degradation, neighbour of BRCA1 (NBR1) negatively regulates osteoblast differentiation and function. **Whitehouse et al**³¹ demonstrated that genetic truncation of murine NBR1 increased osteoblast differentiation and activity in vivo leading to activation of p38 MAPK. FIP200, an essential component of the autophagic process, enhanced osteoblast nodule formation and differentiation. **Nollet et al**³² found that the autophagy proteins ATG7 and Beclin1 were essential for mineralization in an osteoblastic cell line, and ATG5 deficiency in osteoblasts resulted in decreased bone volume in vivo. Inhibition of autophagy also negatively regulated MSC differentiation into osteoblasts. Coordinated AMPK-dependent autophagy and Akt/mTOR activation were crucial for

osteoblastic differentiation and maintenance of bone mass.

Osteoblast differentiation is accompanied by changes in cell morphology and intracellular organelle contents. Terminally, osteoblasts transform into osteocytes and localize to mineralized bone matrix. Upregulation of autophagic flux may be a mechanism for differentiated osteocytes to survive in the hypoxic and poor nutrient conditions within the bone matrix. The increased autophagy provides raw materials for osteocytes to adapt to a stressful environment. Using murine osteocytic cell lines, **Zahm et al** demonstrated that differentiated osteocyte-like cells exhibited elevated levels of LC3-II and autophagosomes. They also observed punctate distribution of LC3 in osteocytes, which was not observed in osteoblasts on the bone surface in rat tibia. Another study demonstrated that decreased autophagy via deletion of ATG7 led to lower bone mass.³

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Autophagy and Oral Cancer

Head and neck region cancers are one of the most common types of cancers, oral cancer being the sixth most common malignancy in the world, and is characterized by a very low five-year survival rate, about 50% due to late-stage diagnosis, high degree of invasiveness and development of therapeutic resistance. Autophagy behaves as a “double-edged sword” in oral cancer. On the one hand, at early stages of tumor development, autophagy is able to act as a tumor suppressor by increasing the damaged proteins and organelles (mostly mitochondria) degradation (**Figure 10**). In this role, autophagy acts as an efficient regulatory system that controls ROS production, insuring genomic stability. Moreover, autophagy is able to prevent necrotic cell death in apoptosis-defective cells, decreasing in this way the local inflammatory reactions’ intensity and, consequently, reducing tumor development.

Additionally, sometimes, autophagy may direct the cellular molecular events towards autophagic cell death. On the other hand, especially, at later stages of tumor evolution, under metabolic stress conditions, activated autophagy provides tumor cells nutrients for energy production and metabolic intermediates for biosynthetic pathways, in order to sustain tumoral cells survival and tumor growth. In this context, autophagy, also acts as a promotor of the resistance to cancer therapy.

The most molecular mechanisms involved in the autophagy regulation are deeply involved within signalling pathways with important roles in cancer control. The functional role of autophagy in cancer depends on tumor type, stage, genetic context, and tumor microenvironment. Many of the autophagy-inducing proteins are either oncoproteins or tumor suppressor proteins. Autophagy should be regarded as a molecular “*double-faced Janus God*”. Thus, the tumor suppressors that negatively regulate TOR (PTEN, AMPK, LKB1, and TSC1/2) will initiate autophagy machinery while, on contrary, oncogenes that activate mTOR (class I PI3K, Ras, and AKT), will inhibit autophagy.¹⁶

Figure 10: the dual character of autophagy in oral cancer

➤ **EARLY STAGE:** at early stages of tumor development, autophagy plays the role of a tumor suppressor by ensuring damaged proteins and organelles degradation. In this context, autophagy should be regarded as controlling system, able to decrease ROS production and, consequently, maintaining genomic stability. Autophagy also can prevent necrotic cell death in apoptosis defective cells, ensuring in this way the decrease of local inflammation and tumor growth. In some situations, autophagy can lead to apoptotic cell death.

➤ **LATER STAGE:** at later stages of tumor evolution, activated autophagy plays the role of cancer cell survival and tumor growth promoter, by supplying metabolic stressed tumor cells with nutrients, in order to sustain energy generation in mitochondria and biosynthetic pathways. Unfortunately, autophagy represents one of the main actors in developing the resistance to cancer therapy.³¹

Autophagy and Oral Candidiasis

Oral candidiasis is a common fungal infection mainly caused by the opportunistic pathogen *Candida albicans* (*C. albicans*), which can transform into a tissue-invasive pathogen when ho Evidence indicates that autophagy is important for *C. albicans* survival. A *C. albicans* atg9 mutant that is deficient in autophagic trafficking is not able to survive under nitrogen starvation.

Moreover, the inhibition of VMA2 in *C. albicans*, a gene that encodes vacuolar ATPase subunit ATP6V1B2, can lead to impaired autophagy and failure to survive under conditions of nitrogen starvation. The expression of ATG8 in *C. albicans* is transcriptionally upregulated in response to ER stress, which suggests a role for autophagy in alleviating ER stress and enhancing cell viability. Recent studies revealed that Atg2, Atg5, and Atg9 are required during fungal intracellular trafficking and replication. The Vps34 protein, the catalytic component of the PtdIns3K, is crucial for the protein transport and virulence of *C. albicans*. The physical interaction of Vps34 with the putative vacuolar HC-ATPase subunit Vma7 contributes to the virulence of *C. albicans*.

Dong et al.³³ recently reported that the breakdown of the autophagic clearance pathway in *C. albicans* affects filamentous development at both 30°C and 37°C and attenuates the virulence of *C. albicans*. In conclusion, autophagy may be important for commensalism by allowing long-term survival and invasive virulence of *C. albicans* within the host. The available evidence indicates that autophagy can be an alternative target for treating antifungal immunity.

Autophagy and Periapical Lesions

As a vital arbiter of cell fate decisions, autophagy plays a critical role in odontogenesis. Autophagy biomarkers LC3 and BECN1 are clearly expressed throughout all embryonic and postnatal teeth developmental stages. Furthermore, the distribution of LC3 and BECN1 is different in various developing enamel organs and epithelial cells. Autophagy can generate

ATP needed in differentiating cells, and degrade proteins that may impair differentiation and intracellular homeostasis during odontogenesis. Furthermore, autophagy might function in a complex interplay with apoptosis, and act as the antagonist, enabler or partner of apoptosis in osteogenesis. Conversely, autophagy is essential for cellular homeostasis and development of the dental pulp. Autophagy can promote the migration of dental pulp stem cells and pulp regeneration mediated by CXCL12/SDF-1a. ATG5 and LC3 are positively upregulated during CXCL12-mediated pulp revascularization of pulpectomized dog teeth with complete apical closure.

In addition, elevated expression of LC3 and BECN1 and more autophagic vacuoles are observed in senescent human dental pulp cells (HDPCs), which confirms the enhanced autophagic activity in aging dental pulp. Activated autophagy in HDPCs could be cytoprotective under hypoxic conditions, whereas suppression of autophagy through knockdown of ATG5 or treatment with 3-MA might abrogate the protective effects on HDPCs.

Despite the pivotal roles of autophagy in the tooth physiology, preliminary studies have

demonstrated the involvement of autophagy in the pathogenesis of periapical lesions. Periapical lesions begin as bacterial infection in the root canal. These lesions are characterized by both specific and nonspecific immune responses to continuous antigenic stimulation (e.g., bacteria and their products) in the infected canals and periapical tissues. Typical autophagosomes and intense staining for LC3 are observed in all samples of dental radicular cysts and periapical granulomas. Increased acidic vesicular organelle-positive dentinogenic cells and evident expression of LC3 and LAMP2 indicate the organization of a dynamic autophagic-lysosomal activity in odontoblasts.

Recent findings have suggested a dual role of autophagy in periapical lesions. The level of LC3-II reaches a peak after treatment with H₂O₂ for 4 h and then declines gradually in a MC3T3E1 murine osteoblastic cell model of apical periodontitis.^{34,35} Moreover, the LC3-II:LC3-I ratio, the levels of ATG12–ATG5, and the expression of phosphorylated (p)-ULK1 increase in mDPC6T cells (a pre-odontoblast cell line) after treatment with lipopolysaccharide (LPS) for 6 h and 12 h, but decrease after treatment with LPS for 24 h.

Besides, the viability of inflamed mDPC6T cells decreases at an early stage, but increases at a late stage of autophagy inhibition. Hence, it is presumed that autophagy can protect the inflamed odontoblasts against harsh conditions at an early stage, but promote cell death at a late stage.¹⁶

Future Direction of Autophagy Research in Periodontal Disease

A new approach of the periodontitis pathogenesis revealed that the pathogens alone are necessary but insufficient to initiate periodontal lesion development and progression. Periodontal tissue damage is caused mainly by undermining the host's immune responses with the involvement of ROS. The bacterial plaque-induced periodontal diseases are mixed infections that trigger an intense inflammatory reaction in the tissues around the teeth (site known as the periodontium) affecting its components.

The present studies on the relationship between autophagy and periodontal disease are very limited and inconsistent. In the future study, the researchers should focus on dose- and time-dependent regulative effect of pathogenic bacteria and inflammation on autophagy in various cells, for example, macrophage and periodontal cells. Furthermore, natural autophagy modulators should be noted for the novel intervention strategies in periodontal disease prevention and treatment.¹⁶

Recently, autophagy has been related to several more sophisticated issues in modern dentistry. In this sense, several authors are exploiting the autophagic properties of several materials to design scaffolds for bone tissue regeneration. A promising resource for further research is the modulation of autophagy at the level of saliva and salivary glands. Several autophagic dependent processes underlie salivary gland homeostasis and stress responses (Morgan-Bathke, Lin, Ann, & Limesand, 2015). Cutting-edge research is being done on the treatment of complex diseases with multiple target drugs. In this vein, the design of ideal molecules to fulfil the poly-pharmacologic needs of diseases such as PDs is essential. Antimicrobial salivary peptides (ASPs) play a significant role in protecting immune systems

from PD-related disease drivers in the oral cavity and in the discovery of new antimicrobial agents. Hypothetically, autophagy may be related to ASPs because it can enable cells to eliminate intracellular pathogens.

Further research should explore this mechanism in order to reach a clinical translation. Genetics should explore the possible presence of ATG-related polymorphism in the search for novel pharmacogenomics solutions. Transcriptomic studies may elucidate the ATG genes' expression profiles and uncover novel transcriptional regulatory mechanisms of autophagy. Proteomic and metabolomic profiling can help identify environmental stresses and formulate ways to improve or modify cells' fates on the basis of this type of cell death. Future integrative multi-omics approaches will unravel the precise autophagy

mechanism at a transcriptional, translational, and post-translational level.²⁹

Conclusion

Autophagy plays a dual role in the protection and elimination of periodontal pathogens in the pathogenesis of PD. Autophagy underlies the biology of a significant portion of current PD therapeutics. In recent years, researchers in this field uncovered a new layer of complexity in terms of how autophagy functions as a regulator of host inflammatory and immune responses to periodontal pathogenic bacterial stimuli. Increasing data provide evidence for an essential role of autophagy in regulating the differentiation and function of bone cells in alveolar bone resorption.

Understanding the different subtypes of cell death and their interrelationships will be a landmark in the discovery of novel PD related HMTs and in the optimization of current ones—specifically, understanding the balance between autophagy and apoptosis in periodontal tissues is a key issue.

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