Quarterly
Volume 64
Issue 1•2025
JANUARY – MARCH

CODEN:
PMKMAV 64 (1)
2025

POLISH SOCIETY OF MICROBIOLOGISTS
POLSKIE TOWARZYSTWO MIKROBIOLOGÓW

Advancements of Microbiology

formerly Postępy Mikrobiologii

Impact Factor = 0,300 (2023)
MNiSW Score = 20,00 (2024)

http://am-online.org/

JOURNAL ADDRESS

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ISBN 978 - 83 - 923731 - 3 - 1

Information about the cover photo

Adhesion of *E. coli* to line HUVEC cel cultures (primary human umbilical vein endothelial cells). Imaging was performed by scanning electron microscopy at 20 kV accelerating voltage, 13.5 mm operating distance, 3.5 k magnification

Preparation and imaging:

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REVIEW
ADVANCEMENTS OF MICROBIOLOGY
2025, 64, 1, 3–23
https://doi.org/10.2478/am-2025-0001

PORPHYROMONAS GINGIVALIS VIRULENCE FACTORS AND THEIR ROLE IN UNDERMINING ANTIMICROBIAL DEFENSES AND HOST CELL DEATH PROGRAMS IN THE PATHOBIOLOGY OF CHRONIC PERIODONTAL DISEASE

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Submitted in February 2025, accepted in April 2025

Abstract: Periodontitis (PD) is a chronic inflammatory disease that affects a significant portion of the global population. In susceptible individuals, the disease is driven by dysbiotic microbiota on the tooth surface below the gum line, progressively eroding the tooth-supporting structures of the periodontium, including the alveolar bone. Clinically, PD manifests as attachment loss and periodontal pocket formation. Influenced by environmental factors, it can ultimately lead to tooth loss and is associated with an increased risk of systemic conditions. Host cells, including oral keratinocytes, gingival fibroblasts, and monocytes/macrophages, regulate the immune response that drives chronic inflammation and tissue damage in PD. Programmed cell death pathways – apoptosis, pyroptosis, and necroptosis – are key regulators of the immune response. These pathways orchestrate the elimination of infected, activated, and/or damaged cells, which is essential for either fuelling or resolving local inflammation. However, periodontal pathogens, particularly *Porphyromonas gingivalis*, can manipulate these pathways, supporting the maintenance of highly inflammatory environment. Prolonged exposition to proinflammatory agents may induce cellular senescence. This process contributes to chronic inflammation and tissue breakdown, further exacerbating the progression of PD. In this review, we discuss the key factors contributing to the onset and progression of PD, the virulence factors of *P. gingivalis*, and their effects on immune responses and cell death in keratinocytes, gingival fibroblasts, and macrophages.

Keywords: cell death, immune response, periodontitis, *Porphyromonas gingivalis*, senescence

1. Periodontitis

Periodontal disease (PD) is a chronic inflammatory condition affecting the periodontium, the supporting apparatus that anchors teeth within the jaw. According to the latest report by the World Health Organization (WHO), PD affects approximately 19% of the global population over the age of 15 (Global oral health status report: towards universal health coverage for oral health by 2030 2022). PD is usually preceded by gingivitis, which, if left untreated, can gradually progress to periodontitis in susceptible individuals. PD is characterized by gingival recession, degradation of the periodontal ligaments, pathological periodontal pocket formation, and resorption of the alveolar bone. These pathophysiological changes result in pocket deepening, often accompanied by pain and bleeding. In advanced stages, these changes precede tooth loss.

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Cite as:
Porphyromonas gingivalis virulence factors and their role in undermining antimicrobial defenses and host cell death programs in the pathobiology of chronic periodontal disease. Plonczynska A. *et al.,* ADV MICROBIOL-NY, 2025, 64, 1, 1–21, https://doi.org/10.2478/am-2025-0001

PD is a multifactorial disease arising from the interplay of three primary factors: dysbiotic bacterial flora on the tooth surface, host susceptibility, and environmental influences that increase disease risk (Kwon *et al.* 2021). In its initial stages, pathogenic bacteria colonize a biofilm on the tooth surface, known as dental plaque, effectively out-competing Gram-positive commensals (Kwon *et al.* 2021). Among these pathobionts, *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia*, and *Treponema denticola* – collectively termed the "red complex" – play a crucial role in dysbiosis and the inflammatory response (Hajishengallis and Lambris 2012). These bacteria are strongly associated with PD severity and progression, fostering an environment within the subgingival biofilm that promotes further proliferation of pathobionts and intensifies the host's inflammatory response.

The immune response is mediated by structural cells, such as keratinocytes and gingival fibroblasts, as well as immune cells, which secrete pro-inflammatory cytokines, bactericidal enzymes, and reactive oxygen species (ROS) (Dominy *et al.* 2019). However, due to the dense structure of the biofilm and pathobionts' resistance to the bactericidal response of the host, these defence mechanisms fail to eliminate infection effectively. Instead, they contribute to host tissue degradation, supplying nutrients for bacterial proliferation and facilitating deeper penetration into periodontal tissues (Scott and Krauss 2012; Sochalska and Potempa 2017). The severity of the inflammatory response is influenced by the composition of the subgingival plaque and individual host susceptibility (Kwon *et al.* 2021). While pathobionts initiate the disease process, its progression depends on host-mediated inflammatory activity, which perpetuates periodontal tissue destruction in a positive feedback loop. *Pg* and other periodontopathogens further exploit antimicrobial defence mechanisms, redirecting them to support their growth and the survival of other pathobionts within the periodontal pockets.

The prevalence of PD is associated with age, sex, coexisting systemic conditions such as diabetes, and various environmental factors, with smoking being the most significant (Kwon *et al.* 2021). With an aging population and the increasing incidence of so-called "civilization diseases," the prevalence of PD is expected to rise further (Tonetti *et al.* 2017).

The treatment of PD primarily involves maintaining proper oral hygiene and mechanically removing dental plaque, often supplemented with antibiotic therapy and laser treatment (Kwon *et al.* 2021). These interventions focus on eliminating bacteria, the primary aetiological agents of PD. If left untreated, PD not only results in tooth loss but also increases the risk of systemic conditions, including oral and colorectal cancers, pneumonia, Alzheimer's disease, and cardiovascular diseases (Dominy *et al.* 2019; Könönen *et al.* 2019).

Beyond humans, PD is also prevalent in other primates, dogs, and cats, following a similar pattern of initiation and progression of the disease. Consequently, these animals are appropriate, but due to ethical concerns, they seldom applied models in PD research (Oz and Puleo 2011). Although the age-related periodontal bone loss in rodents – frequent models in PD studies – shares similarities with humans, this disease does not occur naturally and must be experimentally induced. This limitation affects the interpretation and translation of findings to human PD, highlighting the importance of combining rodent studies with human *in vitro* models (Oz and Puleo 2011).

2. *Porphyromonas gingivalis*

Pg is a Gram-negative, anaerobic bacterium belonging to the red complex. Although the presence of *Pg* in the oral cavity does not directly correlate with the onset of periodontitis, it is detected in up to 61% of patients with the disease (Aabed *et al.* 2023). Even a tiny number of *Pg* cells colonizing the biofilm on the tooth surface can alter the composition of subgingival plaque, favoring the proliferation of pathobionts and the development of dysbiotic bacterial flora that disrupts homeostasis and contributes to disease progression (Hajishengallis *et al.* 2012). For this reason, *Pg* is considered a "keystone pathogen".

Pg possesses numerous virulence factors, including lipopolysaccharide (LPS), adhesive proteins, fimbriae, and gingipains (Fig. 1). These factors facilitate bacterial coaggregation with other oral microbes, allowing the formation of a dense biofilm on the tooth surface below the gum line in the anaerobic environment. Additionally, they contribute to periodontal tissue destruction by triggering uncontrolled, chronic pro-inflammatory response of host cells (Sochalska and Potempa 2017).

2.1. Lipopolysaccharide (LPS)

The outer membrane of Gram-negative bacteria is composed of lipopolysaccharide (LPS), which consists of lipid A, a core oligosaccharide, and a heterogeneous O-antigen polysaccharide. Lipid A is the endotoxin's toxic and pro-inflammatory component, highly conserved among Gram-negative bacteria. It comprises two glucosamine molecules acylated with fatty acids and linked by β(1–6) D-glycosidic bonds (Darveau et al. 2004). However, species-specific variations in lipid A structure influence its immunogenicity, primarily through differences in acylation levels and attached fatty acids.

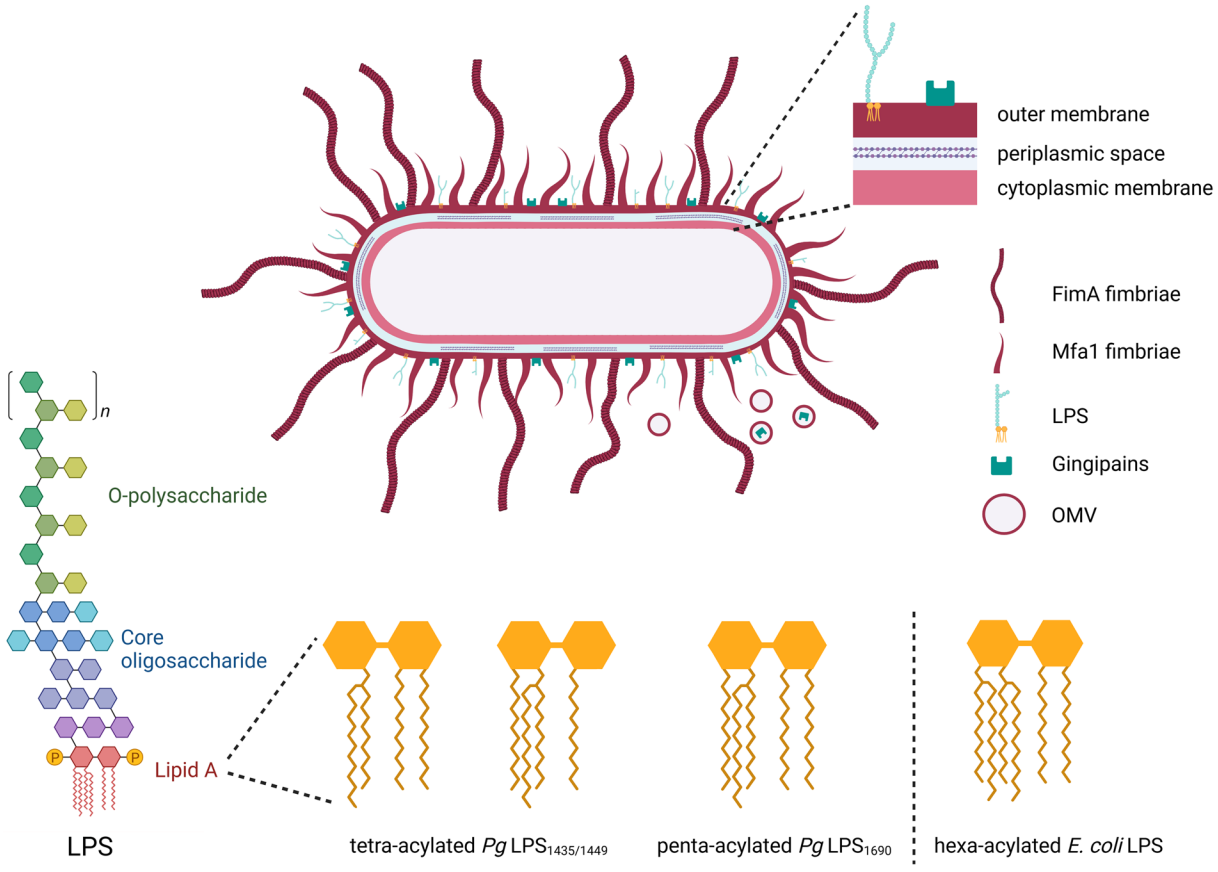

Fig. 1. *Porphyromonas gingivalis* virulence factors and LPS structure.
Pg expresses various virulence factors: long fimbriae (FimA type), short fimbriae (Mfa1), lipopolysaccharide (LPS), outer membrane vesicles (OMVs), which can contain gingipains. *Pg*-LPS exists in two forms: tetra-acylated, and penta-acylated with a different pro-inflammatory potency (Al-Qutub *et al.*, 2006; Darveau, 2010) Created in BioRender. https://BioRender.com/s67b276

In *Pg*, lipid A acylation is influenced by environmental conditions, particularly hemin availability, which serves as a growth factor. Under hemin-rich conditions, *Pg* predominantly expresses a tetra-acylated LPS variant (LPS1435/1449), while hemin scarcity favors the penta-acylated form (LPS1690) (Fig. 1). The penta-acylated variant, due to its higher affinity to TLR4, triggers a significantly stronger immune response than the tetra-acylated form, which induces only a minimal reaction in host cells (Al-Qutub *et al.* 2006; Qiu *et al.* 2021). Studies using human peripheral blood mononuclear cells (PBMCs) have shown that synthetic penta-acylated *Pg* lipid A from strain 381 strongly induces IL-6 expression compared to tri-acylated lipid A (Sawada *et al.* 2007). However, both *Pg* lipid A forms elicit weaker inflammatory responses than *Escherichia coli* lipid A in mouse macrophages (Sawada *et al.* 2007).

The ability of *Pg* to modulate its LPS structure is crucial for its survival within the periodontal environment. The tetra-acylated form suppresses inflammation, enabling *Pg* to evade immune surveillance and establish infection, whereas the penta-acylated form promotes local inflammation, providing nutrients and growth factors for inflammophilic pathobionts (Herath *et al.* 2013).

LPS interacts with host cells through Toll-like receptor 4 (TLR4), a pattern recognition receptor (PRR). TLR4 activation triggers the NF-κB signaling pathway, regulating the secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), IL-6, and chemokines like MCP1 (monocyte chemoattractant protein 1). Initially, *Pg* LPS was thought to activate both TLR4 and TLR2, unlike typical Gram-negative bacteria such as *E. coli*. However, recent findings indicate that previous studies used insufficiently purified *Pg* LPS, which contained bacterial lipoproteins capable of activating TLR2 (Nativel *et al.* 2017). Highly purified *Pg* LPS interacts exclusively with TLR4 (Ogawa *et al.* 2007). Despite this, many researchers continue to use *Pg* LPS purified by standard methods, which can activate both TLR4 and TLR2.

2.2. Fimbriae

Fimbriae are thin, filamentous structures composed of non-covalently polymerized proteins anchored in the bacterial cell wall. They play a key role in bacterial adhesion, motility, biofilm formation, and host cell invasion (Hamada *et al.* 1998). In oral bacteria,

fimbriae-mediated adhesion to the acquired pellicle on the tooth surface is a crucial initial step in plaque biofilm formation (Xu *et al.* 2020). *Pg* fimbriae also enable coaggregation with other bacterial species, supporting biofilm stability and impeding clearance by the host immune system (Maeda *et al.* 2004).

Pg produces two types of fimbriae: long fimbriae (composed of the FimA subunit) and short fimbriae (composed of the Mfa1 subunit) (Enersen *et al.* 2013). Long fimbriae primarily facilitate adhesion and invasion of host cells, particularly human epithelial cells (Nakagawa *et al.* 2002). Based on genetic variations in the *fimA* gene encoding FimA, six fimbrial types have been identified: I, Ib, II, III, IV, and V (Fujiwara *et al.* 1993). Studies on plaque samples from periodontitis patients indicate that *Pg* strains expressing type II fimbriae are most prevalent and have the highest potential for epithelial cell invasion (Nakagawa *et al.* 2002). Short fimbriae also enable the adhesion to the other bacteria species, such as *Streptococcus gordonii*, through the interaction with its surface protein SspA/B, supporting biofilm formation. Additionally, they interact directly with immune cells receptors, binding to dendritic cell specific ICAM-3 grabbing nonintegrin (DC-SIGN), facilitating the cell invasion (Lee *et al.* 2018).

Both long and short fimbriae are recognized by TLR2, leading to NF-κB-mediated secretion of pro-inflammatory cytokines such as TNFα, IL-1β, IL-8, and IL-6 (Hajishengallis *et al.* 2006) (Fig. 2). TLR2 activation requires the co-receptor CD14, commonly found on monocytes and macrophages (Eskan *et al.* 2007). Additionally, long fimbriae activate the complement system via complement receptor 3 (CR3; CD11b/CD18) on monocytes and macrophages, inducing ERK1/2 phosphorylation (Hajishengallis *et al.* 2005; Eskan *et al.* 2007). Long fimbriae may also downregulate pro-inflammatory responses by signaling through CXCR4, activating the protein kinase A (PKA) pathway, and inhibiting TLR2-mediated inflammation (Hajishengallis *et al.* 2008).

2.3. Gingipains

Gingipains are cysteine proteases essential for *Pg* survival and pathogenicity (How *et al.* 2016). They are classified based on substrate specificity: arginine-specific gingipains (Rgp), which cleave Arg-Xaa peptide bonds, and lysine-specific gingipains (Kgp), which cleave Lys-Xaa peptide bonds (How *et al.* 2016). Gingipains are localized on the bacterial surface and can also be secreted in soluble forms or within outer membrane vesicles (OMVs) (Guo *et al.* 2010).

These proteases contribute to periodontal tissue destruction by degrading extracellular matrix components, epithelial cell junctions, and adhesion molecules, facilitating bacterial invasion of connective tissues (Katz *et al.* 2000; Sheets *et al.* 2005). Gingipains also help *Pg* evade the immune system by inactivating complement proteins and degrading immune cell receptors (e.g., CD4, CD8, CD14) and pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-8, TNFα) (Sugawara *et al.* 2000; Kitamura *et al.* 2002; Guo *et al.* 2010).

2.4. Transcytosis

Beyond its ability to integrate into dysbiotic biofilms and manipulate the host immune response, *Pg* can invade subepithelial connective tissue via transcytosis. This process occurs in three stages: entry into the host cell, intracellular survival, and exit from the cell, and was demonstrated in epithelial and endothelial cells (Bélanger *et al.* 2006; Casadevall 2008; de Jongh *et al.* 2023). *Pg* can enter cells passively through phagocytosis (e.g., by macrophages) or actively via long fimbriae interactions with β1 integrins on epithelial cells (Yilmaz *et al.* 2002). Once internalized, *Pg* resides within a phagosome, which matures into an autophagosome rich in nutrients, enabling prolonged intracellular survival (Bélanger *et al.* 2006; Leea *et al.* 2018).

Inside the host cell, *Pg* prevents phagosome-lysosome fusion, evading degradation by lysosomal enzymes (Bélanger *et al.* 2006). Infected cells exhibit altered metabolic activity, favoring amino acids such as asparagine/aspartate and glutamine/glutamate, preferentially utilized by *Pg* in energy metabolism (Takahashi *et al.* 2000).

Pathogen can exit host cells through three mechanisms: inducing programmed cell death, lysing the host cell, or escaping via the plasma membrane without causing damage. Studies suggest that *Pg* exploits the endocytic recycling pathway involving Rab11 and RalA transferrin receptors to facilitate its exit (Takeuchi *et al.* 2011).

Transcytosis enables *Pg* to spread within periodontal tissues and complicates treatment, as the bacterium can evade immune defenses and persist intracellularly, making it more resistant to antibiotic therapy.

2.5. Comparison of *Pg* Strains ATCC 33277 and W83

Pg type strains ATCC 33277 and W83 are the most frequently used in both *in vitro* and *in vivo* research on the microbial and molecular basis of the pathobiology of PD. On the genetic level, differences between these strains mainly stem from the extensive rearrangement within their genomes of similar size (Chen *et al.* 2017). The same is true for other strains, including clinical isolates, whose genomes are completely sequenced and available in the NCBI database (Murugaiyan *et al.* 2024).

Based on model *in vivo* studies using mice, the ATCC 33277 strain is considered a non-invasive strain

(Murugaiyan *et al.* 2024). Similar strains are found in periodontally healthy individuals and those with PD (Murugaiyan *et al.* 2024). The laboratory ATCC 33277 possesses long fimbriae of type I, which are abundantly present on its surface (Fujiwara *et al.* 1993). These fimbriae are particularly long compared to other *Pg* strains, enhancing the bacteria's ability to adhere (Nagano *et al.* 2012). Additionally, ATCC 33277 also produces short Mfa1 fimbriae. The reduced invasiveness of ATCC 33277 is partly due to the lack of an external polysaccharide capsule (Singh *et al.* 2011; Sharaf and Hijazi 2023). The absence of the capsule makes the bacteria more susceptible to bactericidal mechanisms employed by the host, which decreases the bacterium's ability to survive in the bloodstream, a necessary stage for the systemic spread of bacteria to other organs (Singh *et al.* 2011).

Pg strains with gene arrangement resembling the W83 strain are found more frequently in individuals with PD than in healthy controls (Murugaiyan *et al.* 2024). Interestingly, the laboratory W83 strain has no long fimbriae despite the presence of the gene encoding FimA type IV (Fujiwara *et al.* 1993). The lack of fimbriae results from impaired transcription of the *fim* operon (*fimABCDE*), mainly due to the inactive histidine kinase FimS, which is essential for the activation of the translational factor for the *fim* operon (Nishikawa and Duncan 2010). Furthermore, the laboratory W83 strain does not express short Mfa1 fimbriae due to an insertion of a transposon in the promoter sequence of the *mfa1* operon (Nagano *et al.* 2012). Therefore, the *Pg* W83 strain is often described as having a non-fimbriated phenotype and, thus, a reduced ability to form biofilms (Ho *et al.* 2017). Nevertheless, it invades host cells due to a small number of FimA fimbriae on the surface (Nishikawa and Duncan 2010). W83 also possesses a polysaccharide capsule of serotype K1, which limits the phagocytosis of this strain by phagocytic cells (Sharaf and Hijazi 2023). Additionally, the capsule reduces the activation of proinflammatory responses in the host's cells, facilitating bacterial survival in the periodontal tissue (Singh *et al.* 2011). Despite both strains secreting gingipains, W83 exhibits higher proteolytic activity, which contributes to its increased virulence (measured by invasiveness) due to mechanisms that evade the host's immune response, which are dependent on the proteolytic activity of gingipains (Seers *et al.* 2021).

3. The Role of Host Cells in the Development of Periodontal Disease

The first signals of the presence of dysbiotic bacteria, including periodontal pathobionts in the dental plaque, are received by epithelial cells (oral keratinocytes) and gingival fibroblasts. These cells' primary immune response mechanism results in the production of proinflammatory cytokines and chemokines, which stimulate the host's immune system – resident immune cells in the tissue and circulating cells in the blood (Groeger and Meyle 2019; Wielento *et al.* 2023). The first cells to arrive at the site of bacterial infection are those of the innate immune response, namely neutrophils, monocytes/macrophages, and dendritic cells. The defense mechanisms employed by these cells are nonspecific and involve phagocytosis of bacteria, production of proinflammatory cytokines, release of antimicrobial peptides, generation of reactive oxygen species, and, in the case of neutrophils, degranulation and formation of neutrophil extracellular traps (NETs). Their goal is to directly eliminate pathogens and recruit other immune cells, including those involved in the adaptive immune response. As a result of their activity, proinflammatory cytokines from the IL-1, IL-6, and TNFα families dominate in the inflamed gingival tissues, driving a positive feedback loop that sustains the chronic inflammatory state in the periodontium (Pan *et al.* 2019).

During PD, the activation of both innate and adaptive immune cells, as well as structural cells in the gingiva, is observed (Pan *et al.* 2019). This section focuses on the description of three key elements involved in the host response to oral pathogens: oral keratinocytes and gingival fibroblasts, which are structural early responders to the presence of pathogens, and the monocytes/macrophages, which are crucial innate immune cells during chronic inflammation. The significant role of keratinocytes, gingival fibroblasts, and macrophages in periodontal tissues is associated with their ability to regulate other elements of both innate and adaptive immunity (Groeger and Meyle 2019; Pan *et al.* 2019; Yin *et al.* 2022; Wielento *et al.* 2023).

3.1. Keratinocytes

Keratinocytes form the oral epithelium, functioning as a protective barrier against environmental insults such as pathogens, chemicals, and physical trauma (Groeger and Meyle 2019). Thanks to transmembrane proteins, keratinocytes are interconnected, which provides the integrity of the epithelial layer. The epithelial integrity depends on transmembrane molecular complexes, which form gap junctions (GJ), tight junctions (TJ), and adherens junctions (AJ). Several studies demonstrated that during *Pg* infection, the structure and functionality of these junctions are altered. Upon *Pg* challenge, several genes coding TJ proteins, such as claudin-1, claudin-4, and occludin, are upregulated in keratinocytes (Guo *et al.* 2018). On the other

hand, *Pg* LPS stimulates the increased expression of claudin-1, claudin-15, and ZO-1 (zonula occludens-1), but decreased expression of occludin, claudin-4, and JAM- (junctional adhesion molecule)-A expression (Guo *et al.* 2018). Another study has demonstrated that both *Pg* and *Pg* LPS treatment of human oral keratinocytes (HOK-16B and OKF6) led to down-regulation of ZO, E-cadherin, claudins, and occludin, as well as GRHL2 (grainyhead-like 2), a regulator of the junction proteins (Chen *et al.* 2019). Tight junction integrity can also be directly disturbed by gingipains, which degrade members of the JAM family proteins: JAM1 and CXADR (coxsackievirus and adenovirus receptor) (Takeuchi *et al.* 2019; Takeuchi *et al.* 2021). Interestingly, gingipains do not affect claudin-1, claudin-4, occludin, ZO-1, or E-cadherin (Takeuchi *et al.* 2019). The critical role of gingipains in destroying the cell connection was demonstrated in studies comparing wild-type *Pg* with a gingipain-deficient mutant (Andrian *et al.* 2004). These studies showed that the wild-type strain had a greater ability to penetrate the gingival tissues deeply than the mutant lacking gingipains.

The permeabilization of the gingival epithelium allows penetration of pro-inflammatory agents, such as LPS, dextran, proteoglycan, and gingipains, into the subepithelial tissue (Takeuchi *et al.* 2019; Takeuchi *et al.* 2021; Takeuchi *et al.* 2022. Application of a microtissue 3D model consisting of human gingival epithelial cells and human oral fibroblasts demonstrated the *Pg* capacity to overcome the epithelial barrier and reach fibroblasts, leading to disorganization of tissue structure and fibroblast death (Bugueno *et al.* 2018). Gingipains were also shown to disturb AJ by proteolysis of N-cadherin, VE-cadherin, and β-integrin, resulting in detachment of the epithelial cells from extracellular matrix proteins (Hintermann *et al.* 2002). The ability of *Pg* to disturb keratinocyte (HOK-16) adhesion was much more potent compared to *E. coli* or *A. actinomycetemcomitans*: preincubation of cells with *Pg* at a multiplicity of infection (MOI) 1,000, corresponding to 1,000 bacteria per eukaryotic cell, reduced the adhesion capacity to laminin-5 by 50%, which could not be explained by the cell death (Hintermann *et al.* 2002). Additionally, *Pg* triggered selective proteolysis of cell-cell contact structural components in a strain-dependent manner. While ATCC 33277 and 381 were very effective, W50 almost did not affect the integrity of junctional proteins (Hintermann *et al.* 2002). These results suggest the ability of *Pg* and its virulence factors to modulate the cell-cell connections in the oral epithelium through activating defense mechanisms and destroying the protective barrier.

The role of keratinocytes goes beyond their structural functions: they are involved in the immune response, regulating it and participating directly in the fight against pathogens. Various types of TLRs are present on the surface of oral epithelial cells (Beklen *et al.* 2008). During acute or persistent gingival inflammation, the expression level of TLR2 and TLR4, the primary receptors engaged in *Pg* recognition, increases (Uehara *et al.* 2007; Beklen *et al.* 2008; Groeger and Meyle 2019; Chen *et al.* 2021). Interestingly, when the inflammation enters the chronic stage, characteristic of PD, TLR4 expression decreases in comparison to acute gingivitis. Such subversion may serve as a mechanism for preventing the excessive inflammatory response (Groeger and Meyle 2019). Keratinocytes are sensitive to *Pg* LPS treatment, which enhances the expression of pro-inflammatory factors such as IL-6, IL-8, TNFα, and IFNγ (Kim *et al.* 2018). They also respond differently to *Pg* LPS in different acylation forms. In studies using human oral keratinocytes (HOKs), penta-acylated Pg LPS increased the expression of LPS-binding protein (LBP), involved in the cell's reaction to LPS, while tetra-acylated Pg LPS did not affect the expression levels of this molecule (Ding *et al.* 2013).

Stimulated epithelial cells produce various factors, among which the most important are β-defensins (Uehara *et al.* 2007). β-defensins can directly disrupt microbial cell membranes, leading to pathogen neutralization and induce the release of pro-inflammatory cytokines and chemokines, serving as chemotactic factors for immune cell recruitment (Van Kilsdonk *et al.* 2017). The recognition of bacteria by keratinocytes also occurs through intracellular PRRs, nucleotide-binding oligomerization domain receptors (NODs), which are cytosolic proteins recognizing peptidoglycan of the bacterial wall. During PD, the expression of NOD1, which is responsible mainly for recognizing Gram-negative bacteria peptidoglycan, is notably elevated in the periodontal tissue (Chen *et al.* 2021). The activation of NOD1 induces the release of pro-inflammatory factors, such as IL-6 and IL-8 (CXCL8), as well as β-defensins (Groeger and Meyle 2019). IL-8 is one of the key regulators of the influx of immune cells into the gingiva. IL-8 is present in both healthy and diseased tissues, but its levels increase in the presence of pathogens (Ertugrul *et al.* 2013). The primary function of IL-8, which is recognized by CXCR1 and CXCR2 receptors on polymorphonuclear cells (PMNs), is to induce the influx of neutrophils to the site of inflammation (Sahingur and Yeudall 2015). Unrestricted influx of immune cells leads to increased cytokine production and progressive degradation of structural components of the periodontal tissue. Although keratinocytes are an essential source of IL-8, they also secrete CCL2 (chemokine (C-C motif) ligand 2), CCL5, CXCL10 (chemokine (C-X-C motif) ligand 10), CCL17, and CCL20, which orchestrate the influx of monocytes and T cells (Schutyser *et al.* 2003).

3.2. Gingival Fibroblasts

Human gingival fibroblasts (hGFs) make up approximately 65% of the cells in the gingival tissue (Häkkinen *et al.* 2014). As mesenchymal cells dominant in connective tissue, they produce extracellular matrix components and maintain the structural integrity of the tissue (Häkkinen *et al.* 2014). hGFs are also characterized by a high regenerative capacity as the main collagen producer for the extracellular matrix (Roman-Malo *et al.* 2019). In addition to structural functions, hGFs are involved in the inflammatory response (Wielento *et al.* 2023). As a source of pro- and anti-inflammatory factors, they influence immune cells by either promoting their activation or attenuating the inflammatory response. Gingival fibroblasts, along with keratinocytes and macrophages, are an essential source of IL-8 (Ertugrul *et al.* 2013).

GFs' response to *Pg* infection occurs primarily through the TLR2 receptor interacting with fimbriae, while sensing LPS plays a far less important role in their stimulation (Schuster *et al.* 2024). Nevertheless, similarly to keratinocytes, the hGFs response to penta-acylated LPS, but not tetra-acylated LPS, results in the increased secretion of the pro-inflammatory cytokine interleukin (IL)-6 and chemokine IL-8 (Herath *et al.* 2011). Gingival fibroblasts are also an important source of matrix metalloproteinases (MMPs), especially MMP-1, MMP-3, MMP-8, and MMP-9 (Yucel-Lindberg and Båge 2013). MMPs are responsible for extracellular matrix remodeling through the degradation of its components, including collagen. The activity of secreted MMPs is controlled by tissue inhibitors of MMPs (TIMPs), which prevent excessive matrix destruction (Yucel-Lindberg and Båge 2013). During *Pg* infection, the balance is disrupted in favor of MMP proteolytic activity due to enhanced expression of MMP-1, MMP-2, and MMP-3, as well as the decreased expression of TIMP-2 in GFs (Bozkurt *et al.* 2017). In this way, GFs contribute to excessive extracellular matrix degradation, propagating the progression of periodontal disease. Upon *Pg* infection, fibroblasts also enhance the expression of receptor activator of NF-κB ligand (RANKL), OPG, and PGE2, together with cytokines IL-1β, TNFα, and IL-6, which collectively are involved in the upregulation of osteoclastogenesis (Belibasakis *et al.* 2007). The synergistic interaction of pro-inflammatory cytokines intensifies the inflammatory response and bone destruction. GFs are also a source of reactive oxygen species (ROS). The respiratory burst is one of the strategies for combating pathogens, but at the same time, it contributes to tissue damage. Activation of the respiratory burst is regulated by the PI3K/Akt/NF-κB signaling pathway and is dependent on the activation of TLR2 and TLR4 (Vo *et al.* 2021).

A recent study showed that the pro-inflammatory activity of macrophages infected with *Pg* is reduced in the presence of fibroblasts through post-transcriptional regulation of TNFα activity (Tzach-Nahman *et al.* 2017). Therefore, fibroblasts may serve as a component of anti-inflammatory control for macrophages stimulated by periodontal pathogens. Reducing fibroblasts' inflammatory activity could lead to an overall decrease in inflammation in the periodontium by inhibiting the influx of immune cells and limiting the hyperactive inflammatory response.

3.3. Monocytes/Macrophages

Although neutrophils and macrophages together account for only 12% of all immune cells, with B lymphocytes/plasma cells (60%) and T lymphocytes (17%) constituting the absolute majority in the periodontitis lesion, the regulatory role of macrophage in the progression of PD cannot be ignored (Berglundh *et al.* 2011; Yin *et al.* 2022). In the periodontium, the macrophage pool consists of the resident cells or those differentiated from monocytes that have migrated to the site of inflammation from the bloodstream (Sun *et al.* 2021). Macrophages are functionally diverse cells, with their functional state revealed under varying environmental conditions. Due to the presence of pattern recognition receptors (PRRs) on their surface, primarily TLRs and C-type lectin receptors (CLRs), macrophages can respond to molecular patterns associated with pathogens (PAMPs) and danger signals (DAMPs) in their surroundings (Sun *et al.* 2021). Macrophages differentiation from circulating monocytes is directed by macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Ushach and Zlotnik 2016; Sun *et al.* 2021). Their primary function is the direct elimination of pathogens, antigen presentation, the mobilization and regulation of other immune cells, and maintaining tissue homeostasis. Macrophages are also responsible for the process of efferocytosis – the phagocytic clearance of apoptotic cells, mainly neutrophils, thus preventing their secondary necrosis at the site of inflammation (Greenlee-Wacker 2016). Upon stimulation, macrophages polarize into various active forms, with the most recognized being the M1 (classically polarized type), the M2 (alternatively polarized type), and various intermediate subtypes (Sun *et al.* 2021) (Fig. 2).

3.3.1. Classically Polarized Macrophages – M1

M1 macrophages present a pro-inflammatory phenotype, with polarization triggered by PAMPs (such as LPS) and inflammatory factors present at the site

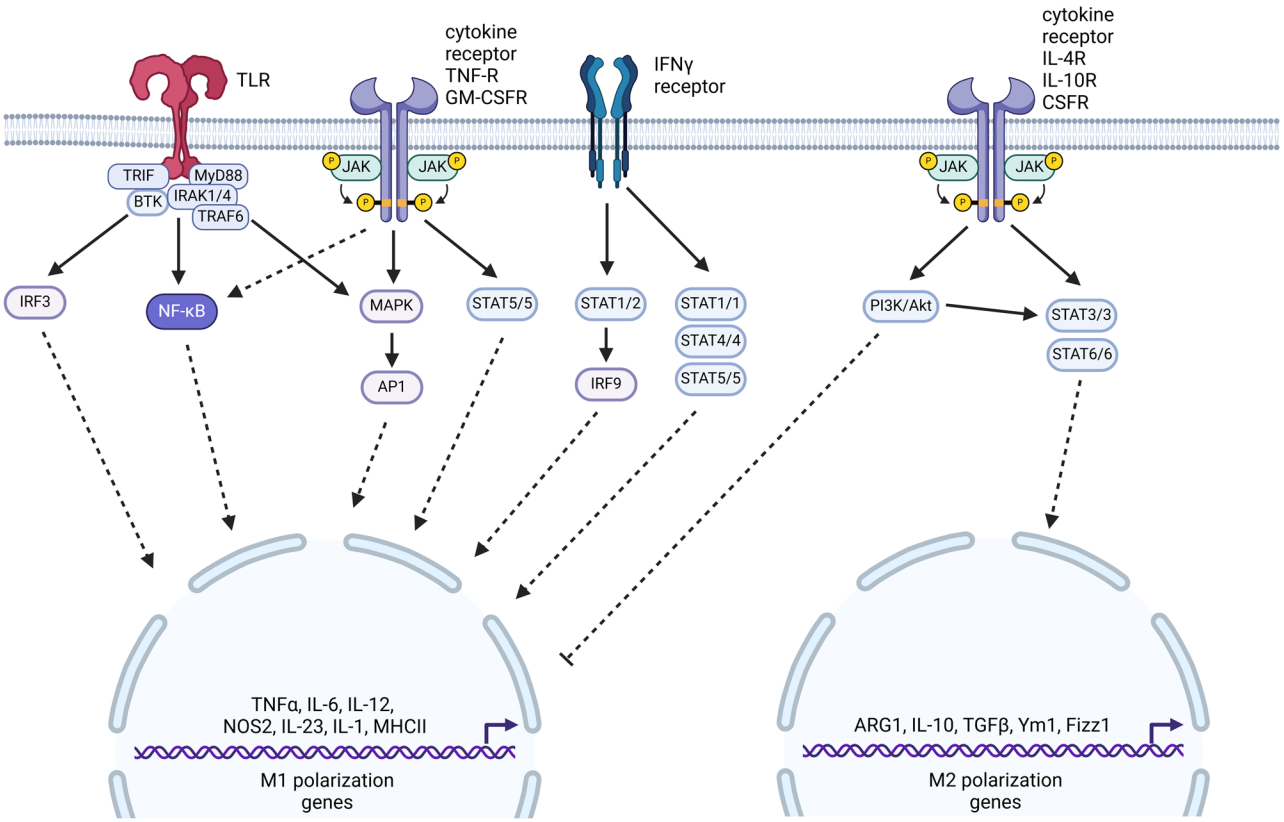

Fig. 2. Macrophage polarization pathways.
Upon various stimulus through cell surface receptors macrophage can activate M1 or M2 polarization genes. M1 polarization is related with TLRs, and cytokines receptors, such as TNF-R or IFNγ receptor. NF-κB signaling pathway is the main regulator of M1 polarization genes, but the pro-inflammatory phenotype can be also activated through IRF3, MAP kinase or STAT (members 1, 2, 4, 5). Alternatively, in response to anti-inflammatory cytokines recognized by cytokine receptors, PI3K/Akt and STAT (members 3, 6) pathways can be triggered, leading to activation of M2 polarization genes and inhibiting M1 profile (Kerneur *et al.*, 2022; Xia *et al.*, 2023). AP1, activator protein 1; ARG1, arginase 1; BTK, Bruton's tyrosine kinase; CSFR, colony-stimulating factors receptor; FIZZ1, found in inflammatory zone 1; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; IFNγ, interferon gamma; IL-4R, interleukin-4 receptor; IL-10R, interleukin-10 receptor; IRAK1/4, interleukin-1 receptor-associated kinases 1/4; IRF3/9, interferon regulatory factor 3/9; JAK, Janus activated kinases; MAPK, mitogen-activated protein kinase; MHCII, major histocompatibility complex class II; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOS2, nitric oxide synthase 2; PI3K/Akt, phosphatidylinositol 3-kinase/Akt kinase; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor β; TLR, toll-like receptor; TNFα, tumor necrosis factor alpha; TNF-R, TNF receptor; TRAF6, TNF receptor associated factor 6; TRIF, TIR-domain-containing adapter-inducing interferon-β; Ym1, chitinase-like protein 3 (Chil3); Created in BioRender. https://BioRender.com/v80l298

of inflammation, including interferon γ (IFN-γ) and TNFα (Sun *et al.* 2021). M1 macrophages are an important source of pro-inflammatory cytokines, including IL-6, TNFα, IL-12, and IL-23, and have a high bactericidal potential. They express cyclooxygenase 2 (COX2), inducible nitric oxide synthase (iNOS), and secrete extracellular proteases such as matrix metalloproteinases (MMP-1, MMP-2) (Chen *et al.* 2023). M1 macrophages trigger the inflammatory response of Th1 helper T cells, which, together with Th17 cells, drive chronic inflammation by secreting numerous cytokines, such as IL-1, IL-2, TNFα, and IFN-γ (Sun *et al.* 2021).

A key signaling pathway involved in M1 polarization is NF-κB, particularly the p65 subunit, which is the main transcription factor for genes associated with polarization (Kerneur *et al.* 2022). The cascade leading to NF-κB activation begins with TLR4 stimulation, which activates TRIF (TIR-domain-containing adapter inducing interferon-β) and MyD88 (Yin *et al.* 2022; Chen *et al.* 2023). Additionally, STAT1, PI3K, and mitogen-activated protein kinases (MAPK) are involved in regulation of M1 polarization (Chen *et al.* 2023; Xia *et al.* 2023). Bruton's tyrosine kinase (BTK) also participates in the pro-inflammatory activation of macrophages induced by LPS (Gabhann *et al.* 2014). BTK mediates the phosphorylation of TLR2, 3, 4, and 7, triggering the signaling cascade. Thus, BTK is a critical factor in regulating macrophage polarization at the early stages of signal transmission. M1 macrophages also show increased expression of the co-stimulatory molecule CD80 on their surface, which is used as a marker of polarization (Chen *et al.* 2023).

3.3.2. Alternatively Polarized Macrophages – M2

M2 polarization occurs in response to increased exposure to anti-inflammatory signals, including IL-4, IL-10, and IL-13 (Chen *et al.* 2023). M2 macrophages are characterized by increased expression of scavenger receptors (e.g., CD163), mannose receptors (e.g., CD206), and the production of anti-inflammatory cytokines such as IL-10 and TGF-β1 while having suppressed expression of IL-6 (Chen *et al.* 2023). The primary function of M2 macrophages is to prevent tissue damage and maintain homeostasis. This function is crucial, as many pro-inflammatory factors, acting via positive feedback loops, can lead to permanent tissue damage. M2 macrophages exhibit lower than M1 surface expression of CD14, TLR4, and major histocompatibility complex class II (MHC-II) molecules (Sun *et al.* 2021). Furthermore, they inhibit the secretion of MMPs and support osteoblastogenesis, leading to bone regeneration (Gong *et al.* 2016).

The polarization of macrophages towards M2 is regulated by the Janus kinase (JAK)/STAT signaling pathway: JAK1 and JAK3 activate STAT6, which translocates to the nucleus and acts as a transcription factor for M2 polarization genes (Wang *et al.* 2014). Inhibition of STAT3 and STAT6 leads to a macrophage shift towards M1 polarization.

It is important to emphasize that M1 and M2 represent two extremes of the macrophage functional phenotypes, with a spectrum of cells showing phenotypes located between M1 and M2 as displaying traits of both types of polarization.

3.3.3. Macrophage Polarization and Periodontal Disease

The complexity of macrophage polarization in the pathobiology of PD is evident from numerous studies showing an imbalance between M1 and M2 macrophage populations, with a predominance of pro-inflammatory M1 cells (Górska *et al.* 2003; Güllü *et al.* 2005; Gheren *et al.* 2008; Holden *et al.* 2014; Hussain *et al.* 2016). Disruption of homeostasis and failure to properly regulate tissue inflammation is a key element in the pathophysiology of periodontitis, in which macrophages play a significant role (Wang *et al.* 2014; Yin *et al.* 2022).

Activation of macrophages by oral pathogens occurs by stimulating CD14, TLRs, and NOD-like receptors (NLRs), leading to pro-inflammatory polarization (Hajishengallis *et al.* 2009). M1 macrophages are a source of factors stimulating osteoclastogenesis (Ahuja *et al.* 2017). Prostaglandin E2 (PGE2) produced by M1 macrophages leads to decreased bone mineralization, contributing to the resorption of the alveolar bone (Ruiz-Heiland *et al.* 2021). Other pro-inflammatory factors, such as IL-1β, TNFα, and IL-6, are also crucial for bone resorption and the development of periodontitis (Sun *et al.* 2021). TNFα interacts with B and T lymphocytes, enhancing the production of RANKL, which regulates osteoclast formation (Sima and Glogauer 2013; Sun *et al.* 2021). Additionally, as a source of MMPs in the extracellular matrix, M1 macrophages contribute to the destruction of the connective tissue, which exacerbates periodontal disease symptoms and facilitates systemic dissemination of pathogenic bacteria from the periodontal pockets (Checchi *et al.* 2020).

Macrophages are highly specialized cells in phagocytosis and subsequent pathogen elimination. However, literature data suggest that *Pg* can avoid the bactericidal mechanisms of macrophages (Lam *et al.* 2016; Werheim *et al.* 2020; de Jongh *et al.* 2023). Studies on the survival of *Pg* in macrophages (using THP-1 and RAW264.7 cell lines) showed that both ATCC 33277 and W83 strains of *Pg* can survive within naïve (unpolarized) or M2 macrophages for up to 24 hours and exit alive in greater numbers than *E. coli* or *S. gordonii* (Werheim *et al.* 2020). However, the *Pg* persistence in pro-inflammatory M1 macrophages is limited (Lam *et al.* 2016). It seems that *Pg* can exploit M2 macrophages to survive in host tissues and spread through the bloodstream (de Jongh *et al.* 2023). This strategy resembles the "Trojan horse" mechanism, used by other bacteria for systemic dissemination (Guidi-Rontani 2002).

Although many studies revealed an imbalance between M1 and M2 populations in periodontal disease patients with the predominance of M1 cells, other investigations found increased accumulation of M2 macrophages in the periodontal tissue (Gheren *et al.* 2008; Navarrete *et al.* 2014; Garaicoa-Pazmino *et al.* 2019). It was also observed that the highest accumulation of macrophages in the periodontium occurs during the acute phase of periodontitis, with a decline in numbers as the disease progresses to the chronic stage characteristic of advanced periodontitis (Garaicoa-Pazmino *et al.* 2019). These discrepancies reflect the polarization process's complexity and periodontitis's multifactorial pathophysiology. They highlight individual differences underlying varying host susceptibility to pathogens. In some individuals, the presence of chronic inflammation may be associated with local immunosuppression, while in others, a continuous attempt to eliminate bacteria results in the maintenance of the acute inflammatory phase. In this context, patient age is also significant, as it is not only a risk factor for the disease but also contributes to changes in immune system function (Huang and Dong 2022).

4. Programmed Cell Death

Programmed cell death (PCD) in immune cells is a crucial mechanism in the regulation of immune responses to bacterial pathogens. It serves two primary functions: first, limiting the pathogen's ability to survive and proliferate within the cell and exposing the bacteria to bactericidal agents present in the extracellular environment during inflammation, and second, inhibiting excessive immune activity that could lead to tissue damage (Nagata 2018).

4.1. Apoptosis

Apoptosis, the most studied form of programmed cell death, is a controlled process that does not trigger an inflammatory response, often referred to as "silent cell death". Apoptosis can be induced through two main pathways: the intrinsic (mitochondrial) and the extrinsic pathway (Kayagaki *et al.* 2024) (Fig. 3).

4.1.1. Intrinsic (Mitochondrial) Apoptosis Pathway

The intrinsic pathway is regulated by the balance between pro-apoptotic and anti-apoptotic proteins in the BCL-2 family. Under normal conditions, anti-apoptotic proteins such as BCL-2 and MCL-1 prevent apoptosis by inhibiting pro-apoptotic proteins: BAX and BAK. However, when pro-apoptotic signals dominate, BAX and BAK oligomerize in the mitochondrial membrane, leading to mitochondrial outer membrane permeabilization (MOMP) (Fig. 3). This releases cytochrome c and SMAC, activate pro-caspase-9, and trigger a cascade that results in cell death (Kayagaki *et al.* 2024).

4.1.2. Extrinsic Apoptosis Pathway

The extrinsic pathway is activated by binding death ligands to death receptors on the cell surface, such as those in the TNF receptor family. This results in the formation of the death-inducing signaling complex

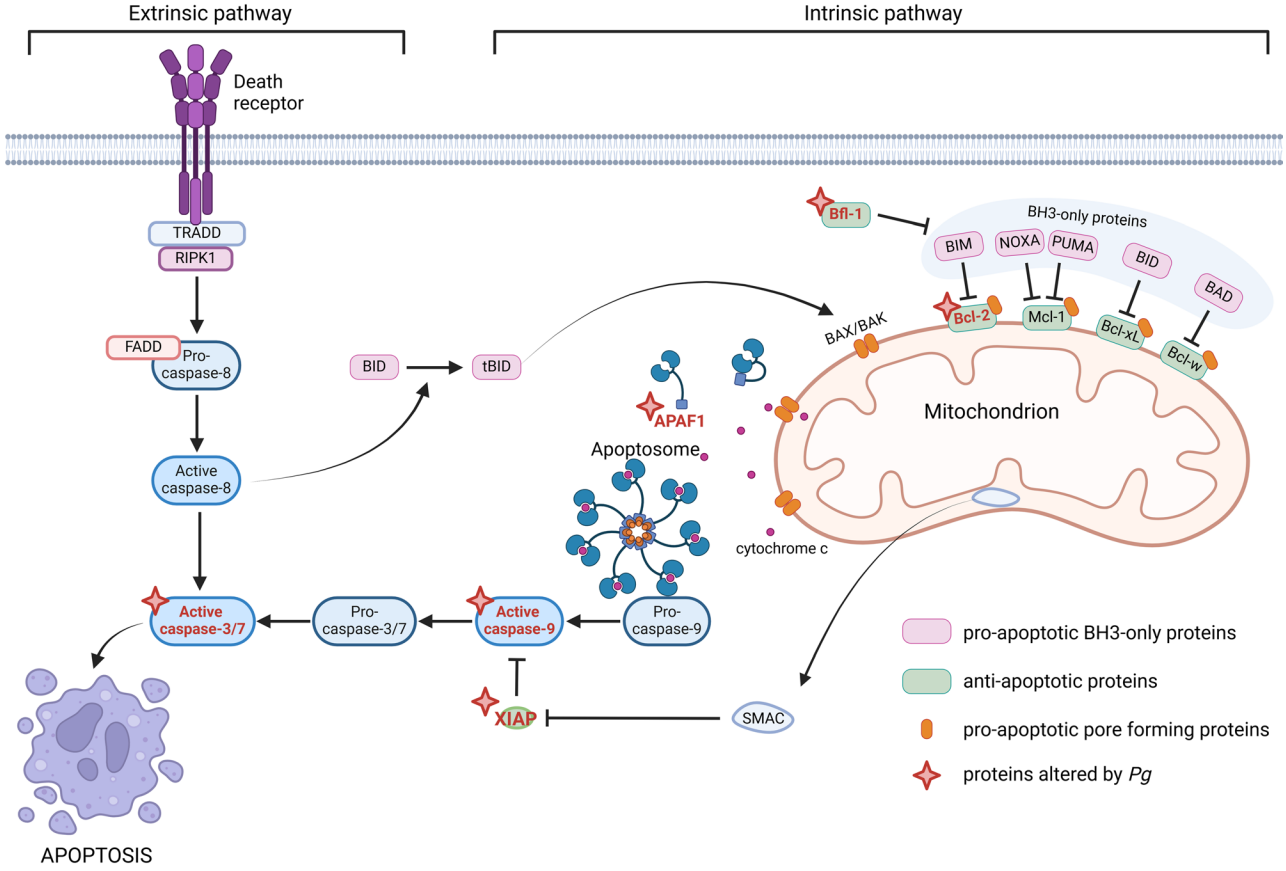

Fig. 3. Apoptosis pathways and *P. gingivalis*.
Upon pro-apoptotic signals, dominating BH3-only proteins inhibit anti-apoptotic members of Bcl-2 family (Bcl-2, Mcl-1, Bcl-xL, Bcl-w, Bfl-1), enabling BAX and BAK oligomerization, and mitochondrion permeabilization. After formation of apoptosome with APAF1, cytochrome c, and pro-capsase-9, the caspase cascade is activated, leading to apoptosis. Alternatively, the activation of death receptors leads to caspase-8 activation, and subsequently intrinsic pathway activation or directly activating caspase cascade (Kayagaki *et al.*, 2024). In sentinel cells, *Pg* can alter the expression of Bfl-1, Bcl-2, APAF1, XIAP, and caspases 3/7 and 9. APAF1, apoptotic peptidase activating factor 1; FADD, Fas-associated death domain; RIPK1, receptor-interacting serine/threonine-protein kinase 1; SMAC, second mitochondria-derived activator of caspases; TRADD, TNFR1 associated death domain protein; XIAP, X-linked inhibitor of apoptosis; Created in BioRender. https://BioRender.com/t45c156

(DISC), activating caspase-8. Next, caspase-8 activates caspase-3 and caspase-7, triggering apoptosis. Additionally, caspase-8 can cleave BID into tBID, which can either initiate the further steps of mitochondrial pathway or directly cause mitochondrial membrane permeabilization (Flores-Romero *et al.* 2022).

In both pathways, the goal is to execute cell death, but recent findings show that apoptosis can be halted at various stages if cellular integrity is not severely compromised (Kalkavan and Green 2018).

4.1.3. Apoptosis in Periodontal Disease

Govindaraj *et al.* (2011) were the first to demonstrate that 60% of patients suffering from periodontitis exhibit various forms of mitochondrial abnormalities in gingival tissue cells. Furthermore, inflammation was found to promote mutations within mitochondrial DNA (mtDNA) and mitochondrial dysfunction, which may lead to the activation of apoptosis (Govindaraj *et al.* 2011). In tissue samples isolated from the gums of individuals with periodontitis (PD), significantly higher activation levels of key apoptotic regulators were detected compared to healthy donors. These included APAF1 (19.2-fold higher expression in PD), caspase-9 (14.5-fold), and caspase-3 (6.8-fold), highlighting the importance of apoptosis in PD progression (Bugueno *et al.* 2018).

The complexity of apoptosis regulation by *Pg* virulence factors is apparent from the study by O'Brien-Simpson *et al.* (2009), which showed that both *Pg* and the gingipain RgpA-Kgp complex induce apoptosis in human epithelial cells (KB line) and fibroblasts (MRC-5 line) in a dose-dependent manner. When present in low numbers within connective tissue, the RgpA-Kgp complex stimulated the release of pro-inflammatory factors. However, at higher concentrations, it interacted with host cells near the plaque, inducing apoptosis and reducing pro-inflammatory factor secretion (O'Brien-Simpson *et al.* 2009). Infection of human gingival fibroblasts with high doses of live *Pg* (MOI: 100, 300, and 900) triggered apoptosis via a caspase-independent pathway, with the pro-apoptotic effect increasing in response to higher bacterial inoculum levels (Desta and Graves 2007). A morphological shift in fibroblasts to a spherical shape was also observed, which was linked to extracellular matrix degradation by bacterial enzymes (Desta and Graves 2007).

Additionally, in human gingival fibroblasts (hGFs) isolated from PD patients, both mitochondrial structure and function were impaired, with this effect being further exacerbated upon stimulation with *Pg* lipopolysaccharide (LPS) (5 μg/ml) (Liu *et al.* 2022). Similar mitochondrial abnormalities were observed in fibroblasts from healthy donors following stimulation with *Pg* LPS (Liu *et al.* 2022). These mitochondrial changes may amplify the cells' pro-inflammatory response. Furthermore, *Pg* can activate fibroblast apoptosis by interacting with APAF1, XIAP, caspase-3, and caspase-9 while simultaneously inhibiting apoptosis in epithelial cells through interactions with the same apoptotic regulators (Bugueno *et al.* 2018). Another study demonstrated that *Pg* can inhibit epithelial cell apoptosis by inducing BCL-2 protein expression, which supports bacterial survival within eukaryotic cells (Nakhjiri *et al.* 2001). In contrast, infection of human primary gingival epithelial cells (keratinocytes) with *Pg* or *Fusobacterium nucleatum* induced apoptosis (Li *et al.* 2013; Bhattacharya *et al.* 2014). Notably, adding the protease inhibitor leupeptin reduced *Pg*-induced apoptosis, likely due to the inhibition of gingipain RgpA/B activity (Li *et al.* 2013).

Interestingly, *Pg* has only a limited impact on macrophage apoptosis. Infection of the mouse macrophage line RAW264.7 with *Pg* at MOI 10 demonstrated that these cells exhibit low sensitivity to apoptosis activation by *Pg* (Lam *et al.* 2016). The susceptibility of macrophages to apoptosis depends on their polarization – M1 macrophages undergo apoptosis to a small but significant extent compared to the more resistant M2 or naïve macrophages at MOI 10. However, exposure to high bacterial loads (MOI 1,000) induces apoptosis in all macrophage phenotypes, with M2 populations being the most susceptible (Lam *et al.* 2016). Notably, *Pg* phagocytosis by neutrophils enhances their survival caused by increased expression of the anti-apoptotic protein BCL2-A1/BFL-1 (Prucsi *et al.* 2023).

A major inducer of apoptosis in the periodontium is *Aggregatibacter actinomycetemcomitans*, which promotes macrophage apoptosis by stimulating cell death receptors and the co-receptor CD14 during phagocytosis while secreting leukotoxin (LTX), a pro-apoptotic factor (Kato *et al.* 1995). Conversely, *F. nucleatum*, another periodontal pathogen, can inhibit macrophage apoptosis by activating the PI3K/Akt pathway and suppressing pro-apoptotic BCL-2 family proteins, as demonstrated in macrophages derived from the human monocyte line THP-1 (Xue *et al.* 2018). This strategy enables bacterial replication within host cells and evasion of the inflammatory response.

4.2. Pyroptosis

In contrast to apoptosis, pyroptosis is a highly immunogenic and lytic form of cell death (Kayagaki *et al.* 2024). It is closely linked to the biology of immune cells and serves as a key mechanism of the inflammatory response. Initially described in macrophages, pyroptosis can also occur in dendritic cells, neutrophils, and epithelial cells (Kayagaki *et al.* 2024). A hallmark

of pyroptosis is cell membrane permeabilization caused by incorporating gasdermin (GSDM) polymers into the membrane. This compromised membrane allows inflammatory factors and danger-associated molecular patterns (DAMPs) to leak into the extracellular space. Depending on the activation source, pyroptosis can proceed via either the canonical or the alternative pathway (Fig. 4).

The canonical pyroptosis pathway is triggered by receptors that recognize pathogen-associated molecular patterns (PAMPs) and DAMPs, such as toxins, double-stranded DNA (dsDNA), proteases, ubiquitin ligases, and bacterial proteins (Kayagaki *et al.* 2024). The binding of these factors to specific receptors induces conformational changes that promote the assembly of an active inflammasome complex within the cytoplasm. The inflammasome consists of intracellular NLR receptors (nucleotide-binding domain and leucine-rich repeat-containing receptors), pro-caspase-1, and the adaptor protein ASC (apoptotic speck protein containing a caspase recruitment domain). The best-characterized inflammasome is NLRP3 (nucleotide-binding domain, leucine-rich repeat-containing family, and pyrin domain-containing-3/caspase recruitment domain) (Shao *et al.* 2015).

NLRP3 activation is tightly regulated by BTK kinase, which directly binds to the inflammasome complex. BTK phosphorylates tyrosine residues during activation in the NACHT domain of NLRP3, enhancing its interaction with ASC, which is crucial for full inflammasome activation (Bittner *et al.* 2021). Once assembled, the inflammasome facilitates the auto-activation of pro-caspase-1, which then proteolytically processes the precursors of pro-inflammatory cytokines IL-1β

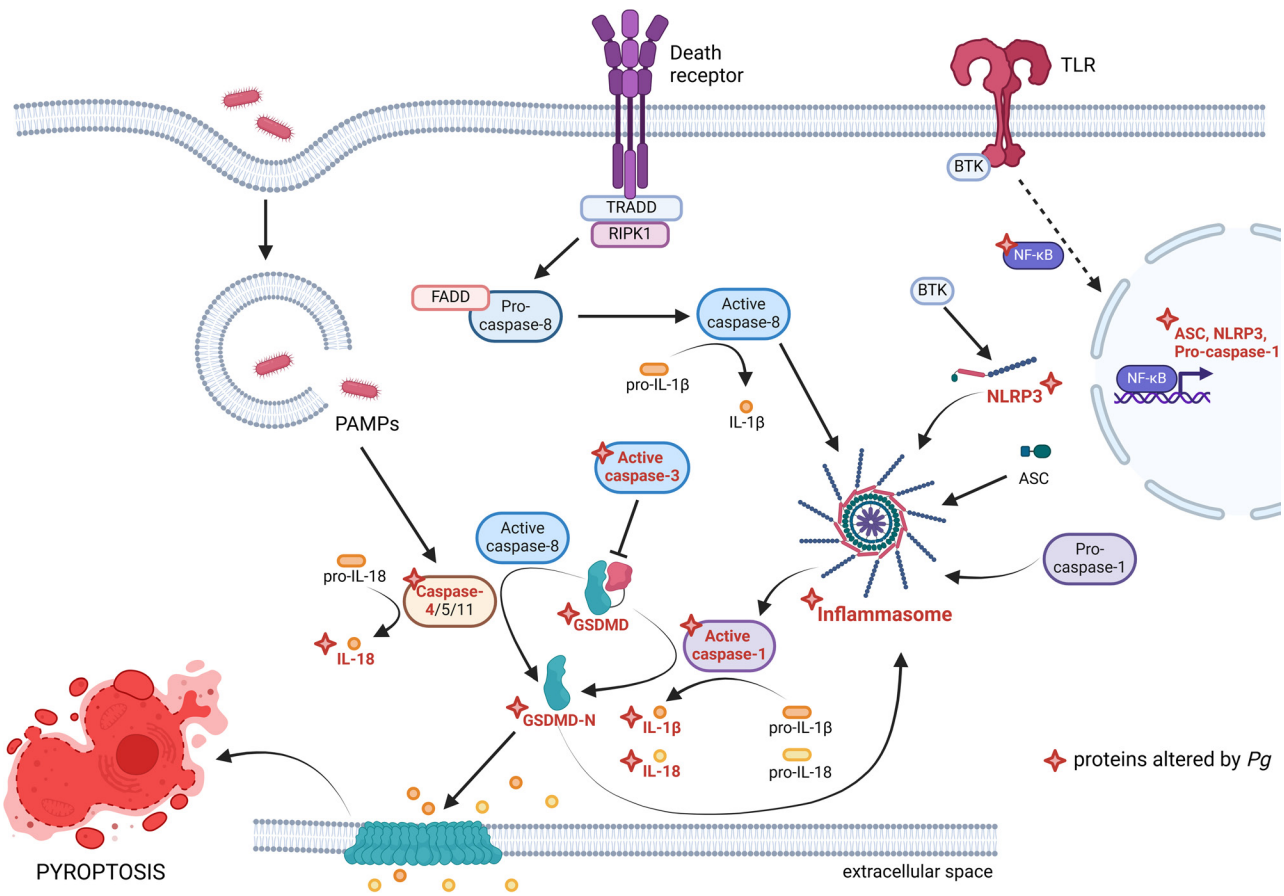

Fig. 4. Pyroptosis pathways and *P. gingivalis*.
Upon TLRs stimulation, NF-κB upregulates the expression of ASC, NLRP3, and pro-caspase-1, which subsequently form inflammasome. Caspase-1 activates IL-1β, IL-18, and cleaves GSDMD into pore forming domains, which oligomerize in cell membrane, creating pores. Alternatively, caspase-8 can activate inflammasome, and IL-1β. Intracellular presence of PAMPs, especially LPS, is recognized by caspase-4/5/11, which upon oligomerization can cleave GSDMD and trigger pore formation, and activate IL-18. Pro-apoptotic caspase-3 acts as a GSDMD inhibitor (Kayagaki *et al.*, 2024). *Pg* can modulate pyroptosis through changes in the activation of NF-κB, caspase-1, 4, and 3, the expression profile of ASC, NLRP3, pro-capsase-1, inflammasome, GSDMD, and secretion of IL-1β and IL-18. ASC, apoptotic speck protein containing a caspase recruitment domain; BTK, Bruton's tyrosine kinase; FADD, Fas-associated death domain; GSDMD, gasdermin D; GSDMD-N, cleaved N-terminal GSDMD; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, nucleotide-binding domain, leucine-rich repeat-containing family, and pyrin domain-containing-3/caspase recruitment domain; PAPMs, pathogen associated molecular patterns; RIPK1, receptor-interacting serine/threonine-protein kinase 1; TLR, toll-like receptor; TRADD, TNFR1 associated death domain protein; Created in BioRender. https://BioRender.com/l81p084

and IL-18 into their active forms (Kayagaki *et al.* 2024). In addition, activated caspase-1 cleaves gasdermin D (GSDMD), releasing its N-terminal pore-forming domain (PFD) from the C-terminal inhibitory domain. GSDMD polymers embed into the cell membrane to form pores that allow the release of IL-1β and IL-18 into the extracellular space, thereby amplifying inflammation. These GSDMD pores also induce non-selective ion flux, disrupting the electrochemical gradient and leading to cell death. This process is accompanied by the release of cellular contents, including lactate dehydrogenase (LDH), commonly used as a marker of pyroptosis in culture media (Wang *et al.* 2022).

In the alternative pyroptosis pathway, intracellular lipopolysaccharide (LPS) is the primary signal for inflammasome activation. Intracellular LPS is bind by caspase-4 and -5 (in human cells) or caspase-11 (in murine), which leads to activation and oligomerization of these caspases (Gabarin *et al.* 2021). Subsequently, they initiate inflammasome assembly and cleave GSDMD, forming membrane pores and the induction of pyroptosis (Gabarin *et al.* 2021).

4.2.1. Pyroptosis in Periodontal Disease

Several pyroptosis markers – such as NLRP3, caspase-1, caspase-4, and IL-18 – are elevated in tissues obtained from PD patients. Additionally, reduced E-cadherin expression suggests compromised epithelial cell-cell junctions due to cell death (Li *et al.* 2021). Another study demonstrated that epithelial gingival cells isolated from PD patients and the human keratinocyte cell line HaCaT express a different type of inflammasome complex, NLRP6, upon infection with *Pg* (Liu *et al.* 2023).

Similarly, human gingival fibroblasts (hGFs) challenged with a combination of *Pg* LPS and ATP showed increased expression of NLRP3, GSDMD, and IL-1β at both the transcript and protein levels. These changes were accompanied by caspase-1 activation and LDH release, indicating pyroptosis's involvement in fibroblast death (Lv *et al.* 2021). Other studies have revealed that a high dose of *Pg* LPS (above 50 μg/ml) reduces hGF survival, a process accompanied by LDH release and caspase-1 activation (Li *et al.* 2021). Likewise, a study on hGFs and human periodontal ligament fibroblasts (hPDLFs) demonstrated that *Pg* LPS (10 μg/ml) induces caspase-1 activation and NF-κB pathway activation. This effect was linked to the regulation of the transcription factor Dec2 (differentiated embryo chondrocyte 2), as silencing *Dec2* expression with siRNA enhanced *Pg* LPS-induced pyroptosis, accompanied by IL-1β secretion (Oka *et al.* 2021).

Interestingly, other studies on hGFs have shown that lower doses of *Pg* LPS (1 μg/ml) do not induce inflammasome activation under normoxic conditions. However, when hGFs were stimulated under hypoxic conditions (1% oxygen), increased activation of the NLRP3 protein complex was observed, along with elevated IL-1β and GSDMD expression and increased cell mortality (Yang *et al.* 2021).

Human primary monocyte-derived macrophages (hMDMs) and THP-1-derived macrophages infected with *Pg* (strain A7436) can also undergo pyroptosis via an NLRP3- and ASC-dependent pathway (Huang *et al.* 2009). Stimulation with *Pg* LPS and *E. coli* LPS led to similar effects. However, in both cases, macrophage mortality was not linked to IL-1β levels, suggesting that cytokine secretion can occur independently of cell death (Huang *et al.* 2009). Conversely, in other studies on hMDMs and bone marrow-derived macrophages (BMDMs), *Pg* infection did not induce caspase-1 activation, IL-1β or IL-18 release, LDH release, or other cell death markers (7-AAD) (Fleetwood *et al.* 2017). Interestingly, the pro-pyroptotic effect – accompanied by high levels of caspase-1 and cytokine release – was triggered by outer membrane vesicles (OMVs) produced by *Pg* (Fleetwood *et al.* 2017). This finding highlights pathogens' diverse strategies to manipulate the host's inflammatory response.

A study on RAW264.7 mouse macrophages demonstrated that *Pg* LPS enhances IL-1β expression and induces pyroptosis via GSDMD activation (He *et al.* 2021). Another periodontal pathogen, *A. actinomycetemcomitans*, previously discussed in the context of apoptosis, can also contribute to pyroptosis in RAW264.7 mouse macrophages through activation of the alternative caspase-11 pathway, triggered by β-glucan in the bacterial cell wall (Yang *et al.* 2021). Additionally, leukotoxin (LTX) secreted by *A. actinomycetemcomitans* has been shown to induce pyroptosis in human macrophages (Huang *et al.* 2009).

Based on studies involving human and mouse macrophages, epithelial cells, and gingival fibroblasts, it can be concluded that pyroptosis plays a significant role in shaping the inflammatory response in periodontal tissues during PD progression. However, the exact mechanisms remain incompletely understood.

4.3. Necroptosis

Necroptosis is a form of programmed cell death exhibiting apoptosis and necrosis characteristics. This pathway is initiated by receptors such as Fas, TNF, and TRAIL, which, upon binding to their respective ligands, activate receptor-interacting protein kinases 1 and 3 (RIPK1 and RIPK3) (Dhuriya and Sharma 2018) (Fig. 5). Caspase-8 negatively regulates necroptosis by cleaving and inactivating RIPK1 and CYLD (cylindromatosis) proteins, thereby preventing necroptotic signaling (O'Donnell *et al.* 2011). Consequently, for

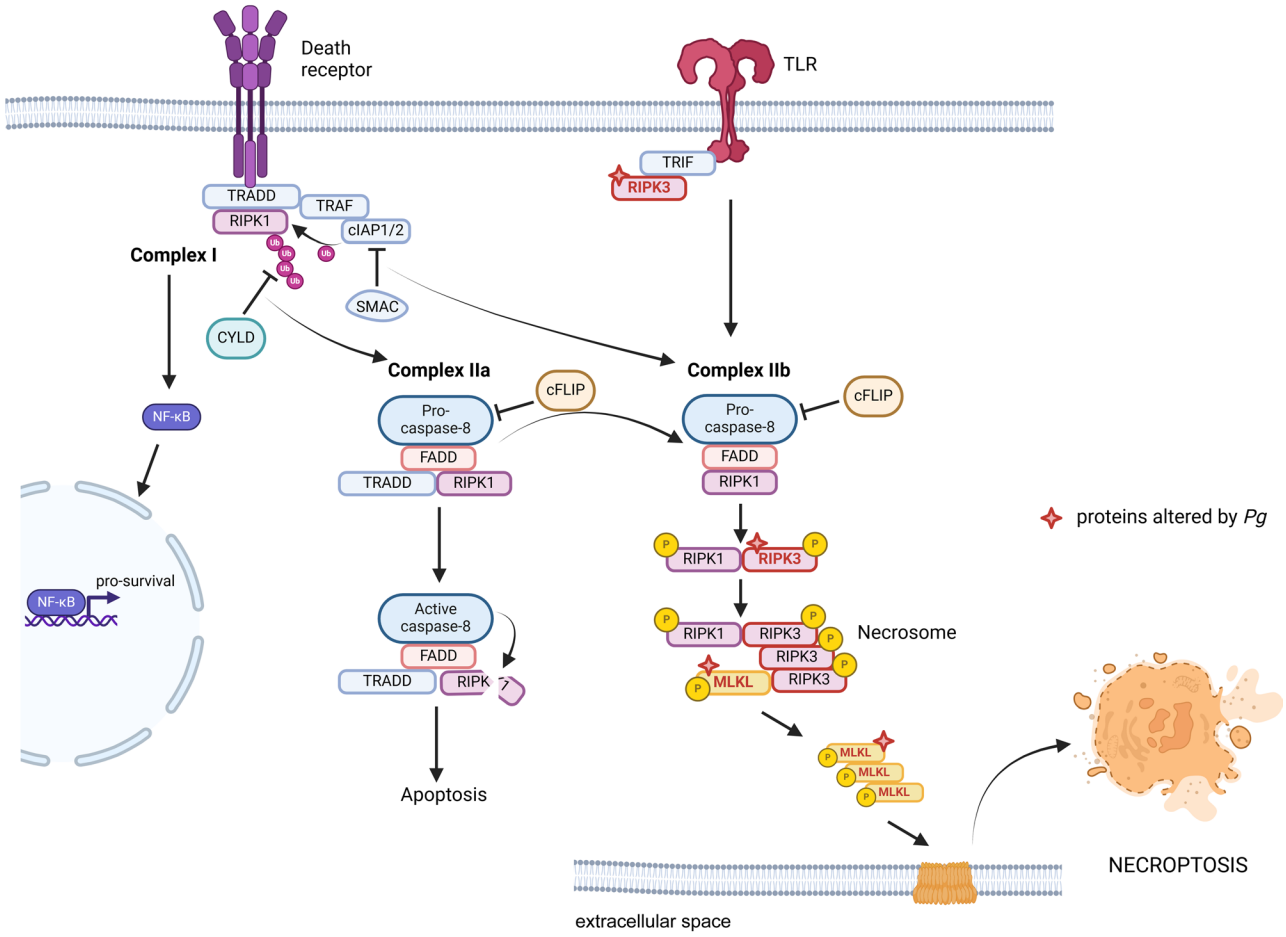

Fig. 5. Necroptosis pathways and *P. gingivalis*.
The activation of death receptors triggers formation of complex I, which activates NF-κB pathway and pro-survival genes. Upon inhibition of RIPK1 ubiquitination by CYLD, complex IIa is formed. The cleavage of RIPK1 by caspase-8 activates apoptosis pathway. If pro-caspase-8 remains inactive by cFLIP, complex IIb is formed. Subsequently, phosphorylation of RIPK1, and RIPK3, and their oligomerization, together with phosphorylated MLKL leads to necrosome formation. Necrosome translocates to cellular membranes, forming pores, which leads to necroptosis (Dhuriya & Sharma, 2018; Holler *et al.*, 2000; O'Donnell *et al.*, 2011). *Pg* modulates necroptosis pathways targeting RIPK3, and MLKL. cIAP1/2, cellular inhibitor of apoptosis protein 1/2; CYLD, cylindromatosis; FADD, Fas-associated death domain; MLKL, mixed lineage kinase domain-like; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RIPK1/3, receptor-interacting serine/threonine-protein kinases 1/3; SMAC, second mitochondria-derived activator of caspases; TRADD, TNFR1 associated death domain protein; TRAF, TNF receptor-associated factor; TRIF, TIR-domain-containing adapter-inducing interferon-β; Created in BioRender. https://BioRender.com/r22c556

necroptosis to proceed, caspase-8 must be inhibited in addition to RIPK3 being expressed in the cell (Dhuriya and Sharma 2018).

RIPK1 interacts with RIPK3 through the receptor homology domain (RHD), leading to the formation of the necrosome complex. This complex subsequently activates the pseudokinase MLKL (mixed lineage kinase domain-like) (O'Donnell *et al.* 2011). Like in pyroptosis, activated MLKL translocates to the cell membrane, facilitating the accumulation of ion channels transporting Na^+ and Ca^{2+} or initiating the formation of membrane pores by interacting with phosphoinositol molecules in the membrane. During necroptosis, pro-inflammatory cytokines are produced and released, particularly in response to TNFα pathway activation (O'Donnell *et al.* 2011).

4.3.1. Necroptosis in Periodontal Disease

Analysis of biopsy samples from PD sites confirmed a significantly increased expression of RIPK3, MLKL, and phosphorylated MLKL (pMLKL) compared to samples from healthy donors (Shi *et al.* 2019). Similarly, studies in mice demonstrated elevated pMLKL levels in the periodontium of *Pg*-infected groups. Interestingly, alveolar bone loss was more pronounced in mice with pharmacologically inhibited MLKL than in control animals, suggesting that pro-inflammatory necroptosis may be beneficial in combating periodontal pathogens (Ke *et al.* 2016).

Other studies using a mouse periodontitis model found that *Pg* infection increases the phosphorylation levels of RIPK3 and MLKL in periodontal ligament

fibroblasts. This aligns with *in vitro* data showing that *Pg* LPS activates RIPK3, leading to necroptosis in mouse fibroblasts (L929 cell line) (Yue *et al.* 2024). Analysis of samples from patients with untreated PD revealed that periodontal ligament fibroblasts (PDLFs) undergo necroptosis only at high bacterial loads (MOI 400). However, in contact with *Pg*-infected monocytes, PDLFs succumb to RIPK1- and RIPK3-dependent necroptosis, triggered by the extracellular release of monocyte cytoplasmic contents (Shi *et al.* 2019).

Human oral epithelial cells stimulated with *Pg* LPS (1 μg/ml) also undergo necroptosis, as indicated by RIPK3 and MLKL activation and LDH release. This suggests that *Pg* can compromise epithelial barrier integrity (Geng *et al.* 2022). Additionally, the release of large amounts of DAMP signals from necroptotic epithelial cells acts as a pro-inflammatory stimulus for naïve macrophages, inhibiting the Mincle/SYK signaling pathway, which otherwise promotes M2 macrophage polarization (Geng *et al.* 2022).

In contrast, mouse bone marrow-derived macrophages (mBMDMs) and L929 cells stimulated with *Pg* LPS (1 μg/ml) did not undergo necroptosis (Yang *et al.* 2022). This process was only activated when *Pg* LPS stimulation was combined with pharmacological caspase inhibition using zVAD, a pan-caspase inhibitor. Reduced cell survival was associated with decreased p65 protein levels, suggesting that the NF-κB signaling pathway regulates necroptosis initiation (Yang *et al.* 2022).

In human THP-1 monocytes, *Pg* infection triggers necroptosis through the TLR/RIPK3/MLKL signaling pathway, accompanied by TNFα and IL-6 secretion (Ke *et al.* 2016). Another murine study, in which periodontitis was experimentally induced by tooth ligation, showed that MLKL deficiency reduced alveolar bone loss and weakened osteoclast activation (Yang *et al.* 2022).

Although these studies highlight the role of necroptosis in PD pathogenesis, they do not definitively clarify whether this process primarily contributes to pathogen elimination or exacerbates disease progression.

4.4. Interactions Between Types of Programmed Cell Death

Each previously described cell death type follows a well-characterized and distinct process. However, while danger-associated molecular patterns (DAMPs) serve as common activators across these pathways, they do not solely dictate which form of cell death will occur. Instead, programmed cell death is the response to many competing signals, with regulatory proteins either promoting or inhibiting different pathways.

Some proteins play roles in multiple cell death mechanisms, while others exert mutually inhibitory effects. For example, caspase-3, a key apoptosis regulator, also negatively regulates pyroptosis by proteolytically cleaving gasdermin D (GSDMD) into a form incapable of pore formation (Dai *et al.* 2023). Paradoxically, caspase-3 can also trigger pyroptosis by activating gasdermin E (GSDME), implying that pyroptosis may be a sequel of apoptosis (Jiang *et al.* 2020). Similarly, caspase-8, another critical apoptosis regulator, inhibits necroptosis (Dhuriya and Sharma 2018).

Conversely, RIPK3, a central protein in necroptosis, can directly activate the NLRP3 inflammasome, promoting pyroptosis and the secretion of IL-1β and IL-18 (Bertheloot *et al.* 2021). These interactions highlight the complex crosstalk between cell death pathways.

5. Cellular Aging – Senescence

The preceding chapters discussed how periodontal pathogens contribute to chronic inflammation and disrupt programmed cell death pathways. The persistent presence of inflammatory factors, such as cytokines and reactive oxygen species (ROS), can induce cellular senescence phenotype (Gorgoulis *et al.* 2019). Additionally, sub-lethal activation of apoptotic pathways results in mitochondrial damage, further disrupting cellular metabolism (Kalkavan and Green 2018). Despite a decline in functional mitochondria, cells remain viable due to the upregulation of anti-apoptotic proteins from the BCL-2 family.

Under continuous cellular stress, key cell cycle regulators, p21 and p16 – both inhibitors of cyclin-dependent kinases (CDKs) – become elevated, leading to cell cycle arrest. However, rather than becoming quiescent, these senescent cells adopt a distinct secretory profile known as the senescence-associated secretory phenotype (SASP). This phenotype is characterized by the heightened release of pro-inflammatory mediators, including IL-6, IL-8, and matrix metalloproteinase-1 (MMP-1), further amplifying inflammation in the surrounding tissue. Significantly, SASP factors can influence neighboring cells, promoting their senescence and contributing to the progression of tissue dysfunction.

Morphological changes, including increased cytoplasmic volume and a reduced nuclear-to-cytoplasmic ratio, accompany the development of cellular senescence (Gorgoulis *et al.* 2019). In addition to inflammatory stimuli, ROS, and sub-lethal apoptotic signaling, cellular aging can also be triggered by DNA damage. Damaged DNA in the nucleus activates the DNA damage response (DDR) pathway, further reinforcing the senescent state (Gorgoulis *et al.* 2019).

5.1. Senescence in Periodontal Disease

Cellular aging influences various pathophysiological processes and has been implicated in aging-related and inflammatory diseases, including atherosclerosis,

cardiovascular disorders, type 2 diabetes, and Alzheimer's disease (Tchkonia *et al.* 2010; Ritzel *et al.* 2019). Recent research also suggests a role for cellular senescence in periodontal disease (PD) (Albuquerque-Souza *et al.* 2022; Rattanaprukskul *et al.* 2024).

Rattanaprukskul *et al.* (2024) demonstrated that PD patients exhibit significantly elevated senescence markers, including p16, lipofuscin, β-galactosidase, and SASP. Notably, these senescence markers were present regardless of patient age, indicating that periodontitis-related senescence can occur even in young individuals. Senescent cells were identified in both the epithelium and connective tissue, particularly within fibroblast and macrophage populations derived from PD patient biopsies (Rattanaprukskul *et al.* 2024).

Interestingly, a case study followed by longitudinal research in a mouse model provided evidence that periodontitis-induced senescence can be triggered via TLR9-dependent signaling (Albuquerque-Souza *et al.* 2022). Another study revealed that prolonged exposure to *F. nucleatum* led to increased expression of senescence markers (p16, p21, and β-galactosidase) alongside decreased levels of p14 and p53, impairing gingival epithelial wound healing (Albuquerque-Souza *et al.* 2024). Additionally, senescence-associated changes in the human periodontal ligament (PDL) have been linked to increased expression of microRNA (miR)-34a, which promotes inflammation and tissue destruction through SASP-related protein secretion (Ikegami *et al.* 2023).

Emerging evidence also suggests that senescence can be initiated by sub-lethal apoptotic signaling and the release of cellular contents from cells undergoing pro-inflammatory cell death (Zhao *et al.* 2021). For instance, human gingival fibroblasts (hGFs) exposed to pyroptotic RAW264.7 macrophages displayed increased expression of senescence-associated markers. A similar effect was observed in an *in vivo* mouse model of hyperglycemia, suggesting that initial macrophage pyroptosis triggered by hyperglycemia can act as a secondary signal, promoting cellular senescence in periodontal tissues (Zhao *et al.* 2021).

These findings highlight a growing interest in the role of senescence in PD pathogenesis. Given the extensive evidence for the co-occurrence of multiple cell death pathways in PD and their ability to drive cellular senescence, it is likely that both processes are linked to the disease progression.

6. Closing remarks

In this review, we have summarized the current understanding of key regulatory and sentinel cells – keratinocytes, fibroblasts, and macrophages – in orchestrating inflammation in PD. We highlighted the significance of various forms of programmed cell death as critical mechanisms influencing disease progression. Additionally, we identified key areas requiring further investigation, particularly those that could inform the development of novel therapeutic strategies for PD treatment.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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REVIEW
ADVANCEMENTS OF MICROBIOLOGY
2025, 64, 1, 24–38
https://doi.org/10.2478/am-2025-0002

CHEMOTHERAPY RESISTANCE STATUS OF COMMON HUMAN PATHOGENIC PROTOZOA

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Submitted in October 2024, accepted in February 2025

Abstract. Protozoal infections exert significant health, community, and economic impact globally, primarily in tropical and subtropical regions. Due to the absence or inefficacy of vaccines for deadly protozoal illnesses, chemotherapy is a primary means for preventing such diseases. Growing drug resistance, rising cross-resistance, and a lack of new agents with novel modes of action all significantly reduce the effectiveness of current antiprotozoal treatments. Society seems to be ignorant of the extent and repercussions of drug resistance associated with anti-infective agents, even though it is a reality. Evidence suggests that reduced drug uptake, reshaped drug targets, genetic modifications resulting in loss of drug activity, and decreased drug export from parasites contribute to resistance development. Recently, there has been a significant gain in our understanding of drug resistance by isolating and characterizing genes and proteins associated with resistance. This fact has also paved the way for the discovery of potential new drugs. This review focuses on drug resistance in the most common vector and foodborne human-recovered parasites.

Keywords: bloodborne parasites, foodborne parasites, parasite drug resistance, parasite treatment, protozoa

1. Introduction

Protozoan infections continue to pose a significant global health challenge, particularly in regions with limited access to medical resources. These parasitic infections are quite common, especially in less wealthy nations, causing illnesses ranging from mild to severe impairment and mortality. Previous records show a significant influence on the emergence and spread of infections in high-income countries, and this pattern is likely to continue (Steverding 2020). Vector-borne *Plasmodium* spp., *Leishmania* spp., *Trypanosoma* spp., and foodborne *Toxoplasma* spp., *Entamoeba* spp., and *Giardia* spp. are among the prevalent protozoans that pose a continuous threat to human life (Fig. 1). Across historical records and other current investigations, they have ranked among the most frequently recovered parasites (Gibb *et al*. 2015; Naghavi *et al*. 2024). The absence of safe and affordable medications and effective vaccines for preventing and treating human protozoan infections has further contributed to the significant impact of these parasites and the diseases they cause. The emergence of drug resistance (DR) in parasites progressively challenges the efficacy of existing medications. Therefore, the need for novel antiparasitic medications motivates research efforts worldwide, spurring the development of innovative approaches to ensure the continuous discovery of promising drugs. This review will provide an update on the possible key elements associated with DR in commonly encountered parasites.

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Cite as:
Chemotherapy Resistance Status of Common Human Pathogenic Protozoa. Baakdah F.M., ADV MICROBIOL-NY, 2025, 64, 1, 22–36
https://doi.org/10.2478/am-2025-0002

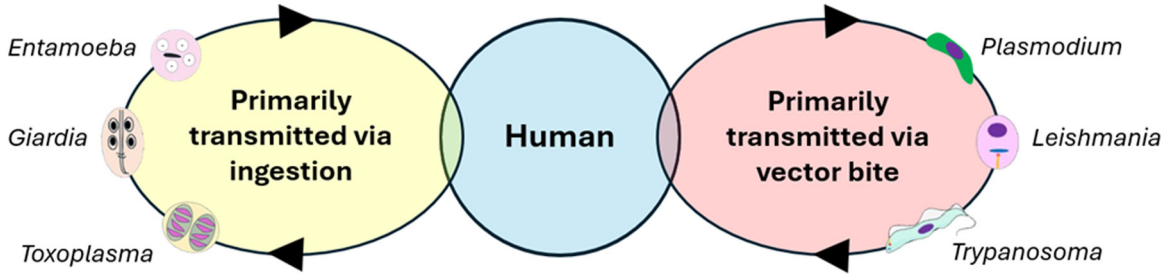

Fig. 1. The link between humans and six parasitic genera. *Plasmodium*, *Leishmania*, and *Trypanosoma* are transferred to humans mainly through the bite of an infected vector, while *Toxoplasma*, *Giardia*, and *Entamoeba* are transmitted via ingestion.

2. *Plasmodium* overview

Among all human parasites, those belonging to the genus *Plasmodium* are responsible for the devastating malaria disease and are the most lethal. Malaria continues to be a significant public health issue in the majority of tropical regions. According to the 2023 World Malaria Report, global estimates for 2022 indicated over 200,000,000 cases and 608,000 fatalities. Nigeria, the Democratic Republic of Congo (DRC), and Uganda have the highest reported incidence of malaria cases among African countries, totalling over 100,000,000 cases combined. Five *Plasmodium* species, namely *P. falciparum, P. vivax, P. ovale, P. malariae*, and *P. knowlesi* are responsible for the etiology of this medical condition. Infections caused by *P. falciparum* and *P. vivax* account for the vast majority of cases, with the most serious symptoms and the highest rates of DR (WHO 2023). The *Plasmodium* parasite's life cycle is intricate, involving a succession between their vector *Anopheles* mosquito host and their human host (CDC 2024a). These apicomplexan parasites adjust their structure and metabolic requirements to suit the various environments found in their diverse hosts. The different shifting forms of each species are helpful in species laboratory identification. Depending on its developmental stage, it can reside within or outside cells particularly during transmission and between infection cycles. Most *Plasmodium* parasites reside in the host's liver or red blood cells, shielded from host defenses, posing a significant obstacle to developing effective vaccinations (Rénia and Goh 2016). Malaria may not exhibit any diagnostic clinical characteristics; however, certain patients may develop classical periodic febrile paroxysms that occur every 48 or 72 hours as a part of uncomplicated malaria situations. Moreover, *P. vivax* and *P. ovale* during the liver stage can form dormant structures responsible for the disease's recurrence (Balaji et al. 2020). Pregnant women are as vulnerable as many people are, and so they are considered high-risk, as well as young children, HIV+, and immunocompromised individuals. Delays in treatment or delayed treatment responses may result in life-threatening consequences for the patient, such as organ dysfunction or failure; such clinical signs are referred to as severe malaria, usually caused by *P. falciparum*. One of the main reasons why *P. falciparum* infection is so dangerous and often fatal is that the parasites can hide in the tiny blood vessels of several organs, including the brain, causing cerebral malaria (Moreira *et al.* 2025). The course of malaria treatment depends mainly on the availability and efficacy of antimalarial chemotherpy.

2.1. *Plasmodium* treatment and resistance

For thousands of years, people have used the bark, roots, and leaves of plants such as the bark of the cinchona tree, which contains quinine (QN) as remedies for malaria (Noronha *et al.* 2020). However, the extraction and employment of their active components as pharmaceutical drugs did not occur until the last century (Semedo *et al.* 2021). Throughout malaria treatment history, pharmacological research derived the most field appreciated compound, chloroquine (CQ), from QN (Berberian 1947). Incomplete patient compliance exposed *Plasmodium* parasites to intense drug selection pressure, leading them to develop resistance mechanisms (Waithera *et al.* 2023). In addition to vector control, emerging antimalarial resistance is one of the current obstacles to malaria prevention. Some of the available anti-malaria drug arsenals include QN, CQ, mefloquine (MQ), halofantrine (HF), lumefantrine (LF), quinacrine, sulfadoxine-pyrimethamine (SP), atovaquone (AQ), proguanil (PR), primaquine (PRQ), amodiaquine (ADQ), piperaquine (PPQ), clindamycin, doxycycline, tetracycline, tafenoquine (TFQ), artemisinin (ART), artesunate (ARN), artemether (ARM), and other ART derivatives (Meshnick and Dobson 2001; Tse *et al.* 2019). Moreover, with the emergence of resistance to CQ, researchers initiated investigations on CQ resistance (CQR) in the 1960s, which led to the adoption of MQ and HF, two more drugs that met a similar fate to CQ in the 1980s (Amelo and Makonnen 2021). A genetic-cross experiment revealed that the membrane transport protein PfCRT (*P. falciparum* CQ resistance transporter) is essential for CQR and localizes to the food vacuole (FV), where hemoglobin is degraded (Fidock

et al. 2000). Researchers have investigated the protein for its ability to transport numerous synthetic compounds. Still, they have not yet identified the physiological substrate. PvCRT, the homolog of PfCRT in *P. vivax*, is not believed to be connected to CQR, whereas PfCRT in *falciparum* is (Marques *et al*. 2014). Instead, PvMDR1 (*P. vivax* multidrug resistance protein 1), a homolog of human p-glycoprotein, is the suggested implicated gene (Schousboe *et al*. 2015). The ABC (ATP binding cassette) transporter PfMDR1 in *P. falciparum*, located on the FV membrane, is also believed to influence CQR (Reed *et al*. 2000). Mutations in the PfMDR1 gene (N86Y) and the PfCRT gene (K76T and A220S, respectively) confer substantial resistance to CQ (Nsobya *et al*. 2007). In addition to CQR, mutant PfCRT was suggested to confer resistance to PPQ and ADQ, while PfMDR1 to MQ, ART, and QN (Wicht *et al*. 2020). These and other changes impact the relative abundance of parasite populations by conferring fitness costs (Pulcini *et al*. 2015). The K76T substitution in PfCRT exhibits notable regional variability across Africa, with particularly high frequencies observed in Ethiopia (91.6%) and Mali (72.9%), compared to lower frequencies in DRC (26%) and non-African nations like India (78%) and Turkey (12.1%) (Patel *et al*. 2017; Hassen *et al*. 2022; Avcı *et al*. 2024; Baina *et al*. 2024; Dama *et al*. 2024). Meanwhile, the N86Y mutant in the PfMDR1 gene shows significant geographic variation as well: it occurs at 6.8% in DRC and 10.2% in Niger and rises dramatically to 78.4% in Oceania (Issa *et al*. 2022; Moss *et al*. 2022; Baina *et al*. 2024). Remarkably, CQ-sensitive strains have returned to countries like Ghana and Cote d'Ivoire after switching to ART-based combination therapy (ACT) instead of CQ for some years. This fact has raised the possibility of CQ returning to the field as it used to be (Asare *et al*. 2021). Furthermore, the parasite expresses PfMRP1, an ABC transporter located on membrane-bound vesicles and its plasma membrane. This protein promotes the transport of organic anionic substrates, such as oxidized glutathione, sulfate conjugates, and drugs. Two mutations in the PfMRP1 locus at positions Y191H and A437S were identified as being associated with resistance to QN and CQ (Gil and Fançony 2021). Although CQR remains a persistent issue, CQ is currently employed to treat uncomplicated CQ-sensitive (CQS) malaria caused by any of the five human pathogenic *Plasmodium* species (CDC 2024b). Some compounds, such as SP, stop the production of folate by blocking enzymes called *P. falciparum* dihydropteroate synthase (PfDHPS) and dihydrofolate reductase-thymidylate synthase (PfDHFR-TS). Biochemical and genetic investigations of *P. falciparum* reveal that mutations in these genes diminish the drug sensitivity of antifolates (Pacheco *et al*. 2020). When the S108N mutation is present, other changes in the inhibitor binding region of PfDHFR, like A16V, N51I, C59R, and I164L, make antifolate resistance even worse. *P. falciparum's* resistance to cycloguanil is linked to the PfDHFR double mutations A16V and S108T (Staines *et al*. 2018). In Thailand and other Southeast Asian regions, *P. vivax* is naturally resistant to sulfadoxine and has obtained resistance to pyrimethamine (Imwong *et al*. 2001). In a study, SP treatment failure occurred in over 50% of *P. vivax* patients due to mutations in PfDHFR and PfDHFR-TS homologs in *P. vivax* PvDHPS and PvDHFR-TS, which are reserved with mutations described to cause resistance in *P. falciparum*, suggesting a possible association (Tjitra *et al*. 2002). AQ is a mitochondrial electron transport inhibitor that disrupts parasite respiration by binding to cytochrome b (cyt-b) and inhibiting ubiquinol oxidation. *P. falciparum* isolates with a single mutation in the Pfcyt-b gene, specifically in the Y268N/S/C codon, exhibited AQ resistance (Staines *et al*. 2018). It is usually combined with PR (Malarone™) for uncomplicated malaria caused by *P. falciparum*. Moreover, ARTs have been documented to bind to many parasite proteins and influence various cellular and organellar processes. Once activated, the carbon-centred radical of the heme drug accelerates the production of additional cytotoxic reactive oxygen species (ROS) through a cluster bomb-like effect, ultimately leading to cell death (O'Neill *et al*. 2010). The first-line treatment for malaria, known as ACTs, has been widely used since the early 2000s (CDC 2024b). It is based on the idea of combining two drugs, one with a shorter half-life (such as ART or an ART derivative) and one with a longer one (such as a quinolone). ACTs are most frequently administered in the following combinations: (ARM + LF), (ARN + MQ), (ARN + AM), and (dihydroartemisinin + PPQ) (Pluijm *et al*. 2021; CDC 2024b). Whole-genome sequencing of an ART-resistant parasite linked the *P. falciparum* Kelch 13 (K13) to ART resistance mutations in both clinical and field isolates of *P. falciparum* (Xie *et al*. 2020). The C580Y mutation in K13 has been strongly linked to reduced susceptibility to artemisinin-based treatments, with a particularly high prevalence in Southeast Asia, notably within the Greater Mekong Subregion, including Cambodia, Myanmar, and Thailand, where it occurs in 35.5% of cases (Takala-Harrison *et al*. 2015; Yoshida *et al*. 2021). In contrast, the K189T mutation in K13 is the dominant variant in Africa, with a prevalence of 22.8% (Hung *et al*. 2024). On the other hand, *in vitro* investigations have shown a probable link between a Y976F mutation in the PvMDR1 gene and resistance to ARN and MQ; however, additional clinical trials are necessary to understand this association fully (Cowell and Winzeler 2019). Moreover, mutations in the Na^+/H^+ exchanger gene Pfnhe-1 have been reported to confer resistance to QN, but this is still a debatable matter of research (Andriantsoanirina *et al*. 2013). For relapse prevention because of *P. vivax* and

P. ovale liver stages, either PRQ or a newer drug called TFQ is used. While some studies indicate the effectiveness of a single dose of TFQ against *P. vivax*, testing on patients with blood disorders or young children remains to be done, necessitating further research (Rodrigo *et al.* 2020). A summary of the medications to be administered to malaria-infected patients is illustrated in Table I. The table illustrates the 1st line of common malaria treatment regimens based on the Centers for Disease Control and Prevention (CDC) guidelines and displays the status of DR (CDC 2024b). Researchers are currently investigating, testing, or inventing various potential vaccines against malaria.

2.2. *Plasmodium* vaccines

On October 6, 2021, the World Health Organization (WHO) recommended RTS,S/AS01 (Mosquirix™), a malaria vaccine developed by GlaxoSmithKline Biologicals, for broad usage. The sporozoite surface protein (CSP) is the basis of the subunit vaccine RTS,S/AS01. The vaccine comprises CSP fused with hepatitis B surface antigen (HBsAg) and other virus-like particles of extra HBsAg that have not been fused (Hammershaimb and Berry 2024). The WHO recommended RTS,S/AS01 for children aged 5 months and older living in regions with moderate to high malaria transmission. Currently, available malaria vaccines reduce the risk of uncomplicated malaria by approximately 40%, severe malaria by approximately 30%, and mortality by approximately 13% (WHO 2023). The CDC recommends administering malaria medicines in conjunction with other control measures.

3. *Leishmania* overview

The obligate intracellular parasite *Leishmania* is the primary cause of leishmaniasis, predominantly transmitted by sand fly vectors. In both the Old and New Worlds, this parasite, a member of the Trypanosomatidae family, causes skin, mucous membrane, and internal organ diseases. Multiple subspecies of leishmaniasis cause the complex disease with many symptoms. The disease has spread to many parts of the world, including Asia, Africa, Central and South America, and the Middle East. The WHO anticipates approximately one million new cases annually, impacting 12,000,000 individuals globally (WHO 2024a). Poverty, migration, hunger, lack of personal hygiene, and an impaired immune system are all contributors to the development of leishmaniasis. The amastigotes (diagnostic stage of *Leishmania*) undergo proliferation within specific host immune cells, such as macrophages, and then spread to several tissues in the body, eliciting diverse immunological responses. Visceral leishmaniasis (VL) is a life-threatening systemic infection that affects the internal organs and can lead to fatalities during epidemics. Several species, including *L. donovani*, *L. infantum*, and *L. chagasi* cause it. In countries such as India, Bangladesh, and Nepal, these species are responsible for an estimated three hundred thousand to five hundred thousand cases of VL globally each year (Perry *et al.* 2013). Mucocutaneous leishmaniasis (MCL) is a clinical manifestation associated with several *Leishmania* species, including *L. braziliensis*, *L. amazonensis*, and *L. panamensis*. MCL is characterized by visible physical disfigurement resulting from mucosal involvement. It has been reported that 9% of cases in Brazil and 2.3% of cases in France present with mucosal involvement (Camuset *et al.* 2007; Faucher *et al.* 2011). Cutaneous leishmaniasis (CL) is generally considered a non-lethal, self-limiting skin infection. However, it can lead to significant, stigmatizing skin lesions. The disease is caused by several *Leishmania* species, including *L. major*, *L. tropica*, and *L. aethiopica*, which are endemic in regions such as Yemen and Ethiopia, with reported prevalence rates of 29.4% and 22.4%, respectively (Bisetegn *et al.* 2020; Mann *et al.* 2021; Alshahethi *et al.* 2024). Animal reservoirs such as dogs and rodents affect the control efforts by maintaining the parasite's survival (Tripathi and Nailwal 2021). Human treatment has depended on chemotherapy since the early 1920s due to the absence of an effective vaccine. Although vaccines for dogs have been developed, there is still ongoing debate regarding their effectiveness (Sasidharan and Saudagar 2021).

3.1. *Leishmania* treatment and resistance

There are only a handful of anti-*Leishmania* drugs available, and they all have serious drawbacks such as toxicity, expensive manufacturing costs, and low efficiency. Sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) are two examples of the several pentavalent antimonials (Sb^V) that are available as systemic therapy. Amphotericin B (AMB), pentamidine (PMD), fluconazole (FLZ), and miltefosine (MT) are additional compounds that are currently in use. Sb^V compounds are typically used in many countries as the first-line treatment (Herwaldt and Berman 1992). In Latin America, Sb^vs represent the gold standard for CL treatment (Mann *et al.* 2021). Several regions have seen the emergence of resistance in the past few years, including India, Europe, the Middle East, and South America (Wijnant *et al.* 2022). The resistance pathway is not clear yet. However, several ABC transporters, such as ABCI4 and ABCG2, were thought to contribute to drug efflux mechanisms (Ponte-Sucre *et al.* 2017). It was also suggested that MRP1 in Sb^v-resistant *L. donovani* strains reduces drug accumulation by reduc-

ing drug import functions (Mukherjee *et al*. 2007). The aquaglyceroporin (AQP1) transporter is another potential factor in Sb^V resistance; its inactivation may decrease Sb^V absorption, and AQP1 mutations may increase resistance (Potvin *et al*. 2020). The ergosterol antagonist AMB is utilized in regions where Sb^V treatment is hindered by resistance (Pinart *et al*. 2020). No resistance has been reported except in laboratory-pressured isolates that showed mutations in CYP51, SC5D, and SMT (Pountain *et al*. 2019). Moreover, an oral medication known as MT is thought to block *Leishmania* parasites' phospholipid metabolism. MT resistance has been found in both *in vitro* and *in vivo* tests in *L. donovani* when the MT translocation pathway has mutations or deletions (Carnielli *et al*. 2019). Experimentally induced mutations in the *L. donovani* MT transporter (LdMT) and LdRos3 have been reported to affect drug transport (Srivastava *et al.* 2017). Another suggested resistance mechanism to MT is reduced drug accumulation by overexpression of ABCB4, ABCG4, and ABCG6 (Pérez-Victoria *et al*. 2011). Moreover, another drug that has shown promise against both CL and VL is paromomycin (PMM), which has a cure rate of up to 80%. The widespread application of PMM in VL treatment is challenged by the relatively high post-treatment relapse rates, which call for proper implementation and monitoring of the development of resistance (Sosa *et al.* 2019). The underlying causes of resistance and the specific mutations responsible remain largely unresolved and require further investigation. Table II summarizes the status of *Leishmania* treatment and DR status in general.

4. *Toxoplasma* overview

The apicomplexan intracellular parasite known as *Toxoplasma gondii* (*T. gondii*), the agent responsible for toxoplasmosis, is an intracellular parasite that infects humans and a broad range of animals. The three transmissible stages of *T. gondii* are as follows: the fast-replicating tachyzoites (involved in the acute phase of infection), which are found in clusters or clones; the slow-replicating bradyzoites (involved in the chronic phase of infection), which are found in tissue cysts, and the sporozoites, which are found in oocysts (Black and Boothroyd 2000). The parasite can be acquired through ingesting tissue cysts through raw or uncooked meat or consuming contaminated water, food, fruits, and vegetation, with mature oocysts usually originating from feline feces. It can also be transplacental, passing from the mother to the fetus via the congenital pathway. Transmission through blood transfusion (tachyzoites) and organ transplantation (bradyzoites) have been reported as well. *T. gondii* can transition between tachyzoites and bradyzoites within the host, adding complexity to its life cycle. *T. gondii* cysts may develop in various organs, such as the brain, eyes, liver, lungs, heart, kidneys, and skeletal muscles. The parasites are exclusively capable of sexual breeding in felines, which is why they are regarded as the definitive host (CDC 2024c). The parasite infection remains asymptomatic or manifests as moderate symptoms similar to the flu in most immunocompetent individuals and is often self-limiting. That said, in individuals with immunological deficiencies, relatively severe pathology may develop, including encephalitis, myocarditis, hepatitis, pneumonia, and eye disease. Suppose a mother contracts *T. gondii* while she is pregnant; in that case, her unborn child may suffer from congenital toxoplasmosis, a condition characterized by severe neurological deficits, retinal lesions, or even stillbirth or miscarriage (Johnson 1990). According to estimates, congenital toxoplasmosis imposes a staggering annual disease burden of 1.20 million DALYs (disability-adjusted life years), with 190,100 new cases reported globally each year (Torgerson and Mastroiacovo 2013). These figures are alarming when considering the wide-ranging impact of the disease. The *T. gondii* antibody prevalence remains relatively low in the United States at approximately 11.14%. However, in regions such as Europe, Central, and South America, the infection rate soars to an astonishing 30–90% of the population (Dubey and Jones 2008; Minbaeva *et al*. 2013; Jones *et al*. 2018; Dubey 2021). Even more concerning is the finding from a recent study on pregnant women in Africa, where the seroprevalence of *T. gondii* reached 42.89%. Countries like Ethiopia, Tanzania, Nigeria, and Morocco, where the study was conducted, reflect some of the highest infection rates globally (Gelaw *et al*. 2024).

All these findings emphasize the urgent need to address toxoplasmosis as a critical public health issue, with significant consequences for both morbidity and mortality worldwide. The data also paints a stark picture of the disease's disproportionate burden in certain regions, calling for targeted interventions to mitigate its impact. Treatment of *T. gondii* depends on the administration of drugs due to the lack of an effective vaccine, but many studies are optimistic.

4.1. *Toxoplasma* treatment and resistance

Anti-toxoplasma medications primarily target the folate pathway, an enzyme complex including the dihydrofolate reductase (DHFR) and the dihydropteroate synthetase (DHPS). This complex is involved in DNA synthesis (Lapinskas and Ben-Harari 2019). It is noteworthy to mention that no medication available today can eliminate *T. gondii* tissue cysts from the infected host; instead, they remain dormant within the host as long as the immune system is robust enough to

prevent the cysts from reactivating and becoming tachyzoites (Konstantinovic *et al*. 2019). Spiramycin (SPI) is the preferred treatment for acute *T. gondii* infection during pregnancy (at least for the 1st trimester) due to the potential for PYR to result in congenital abnormalities (Bogacz 1954). In cases when fetal toxoplasmosis is identified beyond week 16 of gestation, the treatment is typically substituted with PYR and sulfadiazine (SDZ) because SPI has trouble crossing the placenta barrier (Robert-Gangneux *et al*. 2011). Regrettably, there are significant adverse effects associated with the combination. Both PYR and SDZ suppress the DNA synthesis in tachyzoites of *T. gondii* and may have a similar impact on specific host organs, even bone marrow. Hence, incorporating folinic acid (FA) into the drug combination can prevent these negative consequences, which are restored when the medication is stopped, as seen in previous studies (Prusa *et al*. 2015). Various combinations are being investigated to find a better treatment option with fewer adverse effects since treatment failure has been linked to either DR, malabsorption, or intolerance of the current combination (Silva *et al*. 2017). Clarithromycin, cotrimoxazole, AQ, dapsone, and azithromycin are other medications used to treat toxoplasmosis. However, these treatments do not work against the bradyzoites form (Dunay *et al*. 2018). Because of the lack of conclusive evidence from *in vitro* research, identifying genes imparting resistance to PYR and SDZ is an area of dispute (Meneceur *et al*. 2008; Doliwa *et al*. 2013). However, treating clinical toxoplasmosis with AQ is possible if SPI is unavailable (SA Maternal & Neonatal Clinical Network 2015). Like *P. falciparum*, AQ kills *Toxoplasma* at its chronic bradyzoite stage by blocking the mitochondrial electron transport chain. Evidence suggests that AQ binds to the Qo domain of cyt-b, hence targeting the *Toxoplasma* cyt-bc1 enzyme (Alday *et al.* 2017). Significant mutational alterations in M129L and I254L on the Qo domain are thought to be responsible for *T. gondii's* resistance to AQ. However, this theory has not been validated by further research (McFadden *et al.* 2000). Furthermore, the treatment of immunocompromised individuals is based on the same medications used to treat congenital toxoplasmosis, demonstrating how limited our existing ammunition is and emphasizing the importance of searching and testing for more and safer compounds (Hajj *et al*. 2021). Table III summarizes the status of *T. gondii's* standard treatment and DR status in general.

5. African *Trypanosoma* overview

The African sleeping sickness kinetoplastids parasites, *Trypanosoma brucei* (*T.b.*), are human African trypanosomiasis (HAT) causative agents. The blood-sucking tsetse fly of the *Glossina* species transmits HAT to humans and animals through its bite. The two hemoflagellate sub-species, *T.b. gambiense* (*T.b.g*) and *T.b. rhodesiense* (*T.b.r*), are the causative agents of HAT in rural regions of sub-Saharan Africa (Büscher *et al*. 2017). HAT prevalence correlates with the vector distribution, which lives near plants and sources of water. The predominant cause of infections in West and Central Africa is *T.b.g*, which is endemic in 24 countries (CDC 2024d). In recent reports, DRC and Côte d'Ivoire reported a prevalence of 0.3% and 0.06%, respectively (Koné *et al*. 2021; Franco *et al*. 2024). The decline in cases is credited to the hard work of national control programs and other agencies that aided the WHO mission (WHO 2020). In Eastern and Southern Africa, *T.b.r* infects humans but also domestic and wildlife species. Identifying HAT at the early stages can be challenging because the significant symptoms might take months or even years to manifest, depending on the sub-species. Infections caused by *T.b.r* are acute, whereas infections caused by *T.b.g* are chronic (CDC 2024d). The first stage of HAT symptoms includes headaches, fever, and joint discomfort caused by parasites proliferating in the circulation and lymphatic system, commonly known as the hemolymphatic phase. The second stage of the disease is characterized by severe neurological abnormalities, such as meningoencephalitis, caused by parasites crossing the blood-brain barrier (BBB) to the central nervous system, and, if left untreated, it can lead to death. Recently, the implementation of control measures and eradication efforts by the WHO and other organizations has decreased the overall impact of the diseases (WHO 2020). The severity of the illness dictates the course of treatment. No vaccine is available for human use yet.

5.1. African *Trypanosoma* treatment and resistance

Several anti-trypanosomal drugs are available to use in clinics, including PMD, suramin (SUR), melarsoprol (Mel^b), eflornithine (EFL), fexinidazole (FXZ), and nifurtimox (NFX). PMD is a drug administered to treat the initial phase of the illness caused by *T.b.g* (Bouteille *et al*. 2003). There are several routes via which the medication enters parasite cells. Partial transport is facilitated by the aminopurine transporter P2, also known as *T.b.* aminopurine transporter 1 (TbAT1). This has been demonstrated by observing reduced sensitivity to PMD when the TbAT1 gene is knocked out and partial suppression of PMD transport by adenine, an established substrate of TbAT1 (de Koning *et al*. 2004). In 2001, the presence of two additional channels, a high-affinity PMD transporter (HAPT1) and a low-affinity PMD transporter (LAPT1), were identified, which facilitated the majority of PMD transport (Bridges *et al.* 2007). The synthesis of DNA and RNA is believed to be inhibited

by PMD, which interferes with nuclear mechanisms (Sands *et al*. 1985). It is reported that PMD resistance in HAT results from the loss of TbAT1 function (Matovu *et al*. 2003). Even with PMD resistance, treatment effectiveness is over 93% (WHO 2024b). If PMD is not available to treat the initial stage of *T.b.g,* FXZ is recommended (CDC 2024d). Also, NECT (NFX/EFL Combination Therapy), a therapy created in 2009, works just as well at treating *gambiense* types of disease in Central and West African countries. It is considered much safer for patients and is usually used for second-stage *gambiense* disease (CDC 2024d). Moreover, since the 1920s, SUR has been employed as the primary therapy for the hemolymphatic early phases of HAT caused by *T.b.r* (Zoltner *et al*. 2020). Research indicates that it is conveyed by the invariant surface glycoprotein 75 (ISG75) and the major facilitator superfamily transporter (MFST). SUR interacts synergistically with the second-stage medicines EFL, NFX, and Mel^{b} (Makarov *et al*. 2023). By contrast, PMD's action is inhibited by SUR (Guimaraes and Lourie 1951). Apart from having a half-life of around 44–54 days and being in use for nearly a century, there have been no instances of SUR resistance in human pathogenic trypanosomes (Babokhov *et al*. 2013). Moreover, Mel^{b} is an organic medicine containing melaminophenyl arsenic. It was developed in the late 1940s as a treatment for second-stage HAT because of its capacity to cross the BBB. It continues to be the primary treatment for second-stage *T.b.r* infection (Nok 2003). TbAT1, as well as aquaglyceroporin 2 (AQP2), are suggested as the transporters responsible for the selective uptake of Mel^{b} by the parasite (Graf *et al*. 2013). Research indicates that each of TbAT1 and TbAQP2 have significant involvement in the transport of Mel^{b} and PMD (Ungogo *et al*. 2022). Furthermore, Mel^{b} and PMD cross-resistance (MPXR) are two of the most distinct patterns from *T.b.* DR research (Munday *et al*. 2015). This phenomenon has since been linked to the decreased uptake of these two drugs from MPXR *Trypanosoma* cells, which may result from genetic modifications or the loss of essential transporter proteins (Munday *et al*. 2014). Table IV summarizes the status of *T.b.* current treatment and DR status in general.

6. *Giardia* overview

Giardia lamblia (G. lamblia), also known as *G. intestinalis* or *G. duodenalis*, is a flagellate enteric protozoan parasite that thrives in low-oxygen environments. Approximately 200,000,000 individuals in Asia, Africa, and Latin America experience symptomatic giardiasis, with around 500,000 new cases reported each year, despite the existence of current programs for surveillance (Certad *et al*. 2017). A recent study identified a prevalence rate of 6.8% in India (Ghosal *et al*. 2023). Additionally, a pooled prevalence of 18.3% was observed among children across several African countries, including Niger and Cameroon (Kalavani *et al*. 2024). Giardiasis is spread by ingesting its cyst stage, which is achieved primarily through the fecal-oral route. It can be transmitted directly from person to person, indirectly through food or water, or zoonotically from animal to human or animal to animal. The trophozoite, the active feeding stage, is responsible for the destruction of the enterocytes, the loss of the brush boundary of the epithelial cells of the intestine, the shortening of microvilli, and the alteration of epithelial barrier function, all of which contribute to the human disease (Allain *et al*. 2017). The majority of the time, a *Giardia* infection resolves on its own. Still, if it gets worse, it can cause a variety of symptoms, including malabsorption and weight loss, and in cases of chronic disease, gas, bloating, steatorrhea, nausea, and vomiting (Cernikova *et al*. 2018). Veterinary studies using phylogenetic analysis identified eight *G. lamblia* assemblages from A to H (Monis *et al*. 2009). Even though assemblages A and B have been demonstrated to infect humans, controversy remains (Zajaczkowski *et al.* 2021). Although *Giardia* vaccines are commercially available for animals like cats and dogs, not humans, their efficacy results remain debated and unclear.

6.1. *Giardia* treatment and resistance

Metronidazole (MTZ), albendazole (ALB), and tinidazole (TNZ) are the most commonly prescribed drugs for giardiasis treatment (CDC 2024e). MTZ monotherapy and ALB have shown effectiveness in most cases, but there have been reported cases of treatment failure (Krakovka *et al*. 2022). Although the precise process by which MTZ kills anaerobic microbes is still unresolved, a potential explanation is that it disrupts their double-strand DNA. The compound is also thought to inhibit the function of thioredoxin reductase, a redox enzyme, in *G. lamblia* by targeting its disulfide reductase activity. These mechanisms induce severe oxidative stress (Riches *et al*. 2020). The reduced cellular concentrations of pyruvate, ferredoxin oxidoreductase and downregulation of ferredoxin pathways in *G. lamblia* are believed to contribute to its resistance to MTZ. Resistance leads to decreased MTZ uptake into the protozoa lumen due to the low-redox-potential anaerobic metabolism. The proportion of treatment failures attributable to actual resistance is unknown (Adam 2021). ALB affects β-tubulin, which is a cytoskeleton subunit. The resistance to ALB is believed to be caused by changes in the cytoskeleton of *G. lamblia*, specifically in the ROD domain of the β-tubulin structure (Lagunas-Rangel *et al*. 2021). Alternatively, other research suggests an efflux mechanism where an ABC-C1 actively transports less ALB, resulting

in a lower ALB concentration (Ángeles-Arvizu *et al.* 2021). Another theory for ALB resistance is to reduce ROS generated by ALB by activating an antioxidant response (Argüello-García *et al.* 2015). Table V summarizes the general treatment and DR status of *Giardia*.

7. *Entamoeba* overview

Amebiasis is a condition induced by unicellular intestinal parasites of the *Entamoeba* genus. In humans, the estimated prevalence of *Entamoeba* infection is 3.55%, making it the third most prevalent parasitic disease associated with mortality on a global scale (Cui *et al.* 2019). Numerous species of the genus *Entamoeba*, including *E. histolytica*, *E. dispar*, *E. hartmanni*, *E. moshkovskii,* and *E. coli,* are known to parasitize the human intestine. Despite its long-held reputation as the sole pathogenic *Entamoeba* species, *E. histolytica* is indistinguishable in appearance from *E. dispar* and *E. moshkovskii*. So, even under a microscope, it's difficult to tell them apart (Singh *et al.* 2009). To contract amebiasis, one must drink water or consume food contaminated with the parasite's infectious cysts. After the parasite excystation, they either colonize the large intestine symptomlessly (which happens in most cases) or cause bloody diarrhea. The trophozoites can become virulent and invasive, causing amebic dysentery and migrating through the portal veins to the liver, damaging the hepatocellular layer. Ulcers that resemble colonic flasks are diagnostic for *E. histolytica* (Tharmaratnam *et al.* 2020). *E. histolytica* infections display significant regional variation in prevalence across the globe. In Asia, India is recognized as one of the countries with the highest burden, with prevalence rates ranging from 3% to 23% (Gupta *et al.* 2022). In Africa, particularly in Ethiopia, a notable 19.8% prevalence rate has been reported (Roro *et al.* 2022). Similarly, a study involving 30 countries in the Americas estimated an overall prevalence of 9%, with 22 countries showing varying infection rates (Servián *et al.* 2022). These regional differences highlight the widespread nature of *E. histolytica* infections and the need for focused public health strategies. Chemotherapy is the only therapeutic option for amoebiasis since no vaccination is currently available for humans.

7.1. *Entamoeba* treatment and resistance

Although several medications are available to treat amoebiasis, MTZ has long been considered the gold standard for *E. histolytica*. It has been well-established that it is safe and effective against amoebiasis (CDC 2019). Thus far, there has been no evidence of resistance from *E. histolytica*. Other systems, including bacteria such as *E. coli*, suggest the drug exerts its effects through DNA damage and oxidative stress. However, the *in vitro* mechanisms of resistance and the existence of resistant clones have yet to be fully established (Jackson *et al.* 1984). A recent investigation found that treating clinical isolates with MTZ increased their IC^{50} (Singh *et al.* 2023). Moreover, like MTZ, TNZ is a second-generation nitroimidazole that acts on several protozoa. In addition to effectively eliminating amoebiasis, its greater half-life (12 to 14 hours vs. 8 hours) makes it possible to shorten the duration of treatment (Sawyer *et al.* 1976). Table VI summarizes the current *Entamoeba* treatment status.

Table I. *Plasmodium* species treatment and possible resistance proteins summary

Plasmodium
Uncomplicated malaria by CQS *P. vivax, P. ovale, P. malariae, P. knowlesi,* and *P. falciparum*
CQ phosphate; is also the drug of choice for pregnant women
Uncomplicated malaria by CQR *P. falciparum*, *P. vivax*, and *P. ovale*
An ACT combination such as ARM-LF; is also the drug of choice for pregnant women
Anti-relapse treatment *P. vivax* and *P. ovale*
PRQ phosphate or TFQ; Anti-relapse is risky during pregnancy
Complicated malaria regardless of the species or drug susceptibility
Administer intravenous ARN
Possible proteins involved in resistance
P. vivax: PvMDRI (CQ, ARN, and MQ), PvDFHR-TS, and PvDHPS (SP)
P. falciparum: PfCRT (CQ, PPQ, ADQ), PfMDRI (CQ, QN, ART, and MQ), PfMRPI (CQ and QN), PfK13 (ART), Pfcytb (AQ), Pfnhe-1 (QN), PfDHPS, and PfDHFR-TS (SP)
Available human vaccine
RTS,S/AS01 (Mosquirix™) by GlaxoSmithKline Biologicals

Sources: Fidock et al. 2000; Reed et al. 2000; Tjitra et al. 2002; Nsobya et al. 2007; O'Neill et al. 2010; Andriantsoanirina et al. 2013; Schousboe et al. 2015; Pacheco et al. 2020; Wicht et al. 2020; Gil and Fançony 2021; CDC 2024a; Hammershaimb and Berry 2024

Table II. *Leishmania* treatment and possible resistance proteins summary

Leishmania
Compounds usually used for treatment
Sb^V, AMB, PMD, FLZ or MT
Possible proteins involved in resistance
- Sb^V: gene products of ABCI4, ABCG2, MRP1 and AQP1 - MT: gene products of LdMT gene and LdRos, and ABCB4, ABCG4, and ABCG6 - AMB: gene products of CYP51, SC5D, and SMT
Available human vaccine
No human vaccine is available

Sources: Herwaldt and Berman 1992; Mukherjee et al. 2007; Pérez-Victoria et al. 2011; Ponte-Sucre et al. 2017; Srivastava et al. 2017; Pountain et al. 2019; Potvin et al. 2020

Table III. *Toxoplasma* treatment and possible resistance proteins summary

Toxoplasma
Commonly used compounds for treatment
- If fetal illness was negative: SPI (in some cases AQ) - If fetal illness was positive: PYR + SDZ + FA
Proteins involved in resistance
AQ: *Toxoplasma* cyt-bc1
Available human vaccine
No human vaccine is available

Sources: Bogacz 1954; McFadden et al. 2000; Robert-Gangneux et al. 2011; Prusa et al. 2015; SA Maternal & Neonatal Clinical Network 2015; Alday et al. 2017; Lapinskas and Ben-Harari 2019

Table IV. African *Trypanosoma* treatment and possible resistance proteins summary

African *Trypanosoma*
Commonly used compounds for treatment
T.b.g: 1st stage: PMD or FXZ 2nd stage: NECT or FXZ *T.b.r.* 1st stage: SUR 2nd stage: Mel[b]
Possible proteins involved in resistance
PMD and Mel[b]: TbAT1 and TbAQP2
Available human vaccine
No human vaccine is available

Sources: Bouteille et al. 2003; Nok 2003; Munday et al. 2015; Zoltner et al. 2020; CDC 2024b

Table V. *Giardia* treatment and possible resistance proteins summary

Giardia
Commonly used compounds for treatment
MTZ, ALB or TNZ
Possible proteins involved in resistance
ALB: ROD domain of the β-tubulin and ABC-C1
Available human vaccine
No human vaccine is available

Sources: Argüello-García et al. 2015; Ángeles-Arvizu et al. 2021; Lagunas-Rangel et al. 2021; CDC 2024c

Table VI. *Entamoeba* treatment and possible resistance proteins summary

Entamoeba
Commonly used compounds for treatment
MTZ or TNZ
Proteins involved in resistance
No clear evidence yet
Available human vaccine
No human vaccine is available

Sources: Sawyer et al. 1976; CDC 2019

8. Perspective

The unavoidable global issue of DR in parasitic microorganisms has impeded progress in human health over the past 50 years. This review investigated six pathogenic human parasites and determined that at least two (*Giardia* and *Entamoeba*) did not develop resistance to their current first-line chemo treatments (or at least it is not clear if it is yet). At the same time, the rest have varying degrees of resistance, and some

suspected resistance pathways were identified. There appears to be a proportional relationship between the level of resistance in a system (which stems from the proportion of people suffering from it) and the amount of effort, money, and human power invested in developing novel therapeutics, effective vaccines, and fast and reliable diagnostics, as seen in the case of *Plasmodium* compared to the others. Unfortunately, the bulk of these diseases and resistance occur in less developed countries, and as such, it necessitates the support of developed nations in disease elimination for sustainable living. Moreover, to get a cure and stop protozoans from getting resistant, everyone must do their bit, especially patients who must take their medicine exactly as prescribed. Managing vectors can reduce the strain on chemotherapy treatments for protozoans transmitted by vectors. Using insecticide-coated bed nets, insecticide spray, and improved home construction and fortification can prevent arthropod-transmitted parasites from succumbing to drug pressure selection, as recommended by the WHO. Furthermore, administering treatment as a single dose, similar to certain medications for bacterial urinary tract infections like Fosfomycin (Monural), would benefit the field (Keating 2013). This approach would prevent drug misuse, provide the prescribed dose in a single shot, and would help to monitor DR. Also, the gut microbiome has the potential to be a novel approach to fighting enteric parasites, provided that we have a comprehensive understanding of the interactions and behaviors between parasites and the microbiome (Sharpton *et al*. 2020). Not only humans but also animals are treated using a variety of antiparasitic treatments to treat the same parasites and non-parasitic agents. MTZ is well established in many reports as a treatment for some protozoan diseases, including amoebiasis, giardiasis, and other parasites and pathogenic bacteria. Dogs with giardiasis are also treated with MTZ (Ciuca *et al*. 2021). Examining the library of accessible, efficient medications for various systems and testing them on some of the most prevalent ailments caused by new pathogens would be helpful; these medications would serve as a great alternative if the current ones were ineffective. Hence, to maintain successful, cost-effective, and sustainable control of protozoal diseases, key sectors must work together within the "one health" strategy (Kaplan *et al*. 2009). The WHO, UN Environment Programme, Food and Agriculture Organization of the United Nations, and World Organisation for Animal Health recently launched the "One Health joint plan of action (2022–2026)" to implement strategies on the various forms of life on the planet to prevent health risks and enhance the quality of life. This initiative is based on the fact that our lives are inextricably linked, whether directly or indirectly. However, it is crucial to closely monitor the execution of field plans to assess the effectiveness of the chosen method and develop more efficient strategies. Previously, it was believed that developing a vaccine for parasites was unrealistic. Nevertheless, the WHO approval of the first malaria vaccine in 2021 has renewed optimism that chemotherapy is not the sole viable option for combating malaria and potentially other parasitic diseases. Various institutions are currently developing vaccines to reduce infection rates and improve life sustainability.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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CASE REPORT
ADVANCEMENTS OF MICROBIOLOGY
2025, 64, 1, 39–45
https://doi.org/10.2478/am-2025-0003

RARE CASE OF *PSYCHROBACTER SANGUINIS* BACTEREMIA IN A HOMELESS PATIENT WITH THIGH PHLEGMON

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Submitted in January 2025, accepted in March 2025

Abstract. *Psychrobacter* sanguinis is an emerging opportunistic pathogen predominantly isolated from cold environments that is increasingly recognized for its clinical relevance. This case report documents a rare instance of *P. sanguinis* bacteremia in a 69-year-old homeless patient presenting with thigh phlegmon. The patient was admitted with elevated inflammatory markers and treated empirically with antibiotics. Blood cultures identified *P. phenylpyruvicus*, later confirmed as *P. sanguinis* via 16S rRNA sequencing. Despite initial antibiotic therapy, the patient's condition necessitated surgical intervention for phlegmon drainage. The antibiotic regimen was adjusted based on susceptibility profiles, leading to gradual clinical improvement. This case underscores the significance of accurate microbial identification in managing infections caused by less common pathogens. The severity of infection was doubtlessly influenced by the patient's homeless status and associated risk factors, such as poor hygiene and potential environmental exposure. This report highlights the clinical implications of *P. sanguinis* and the importance of considering environmental pathogens in atypical presentations of bacteremia.

Keywords: antimicrobial therapy, blood infection, clinical microbiology, opportunistic infection

1. Introduction

Microorganisms have an incredible ability to thrive in diverse environments and under extreme, rare, and challenging conditions and cause infection in humans. The genus *Psychrobacter* comprises a variety of Gram-negative chemoheterotrophic, non-motile strict aerobes, which are osmotolerant, spherical or cylindrical in shape, and adapted to life at low temperatures. *Psychrobacter* spp. are primarily isolated from various sources, including ecosystems with wide variations in temperature and salinity, and from glacial ice, chilled meat and fish, and human clinical materials, such as blood or cerebrospinal fluid. *Psychrobacter* spp. may also be a component of the human microbiota – studies have shown the presence of *P. arenosus*, *P. faecalis*, *P. phenylpyruvicus* and *P. pulmonis* in the human intestine (Lager *et al.* 2016).

The genus *Psychrobacter* has only recently become better known after intensive 16S rRNA sequencing studies on their presence in various environments. The findings indicate that *Psychrobacter* spp. is a common and evolutionarily successful taxon whose biology can provide important information about environmental adaptation and survival. In the laboratory, it can be cultured at 30–37°C on brain heart infusion

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Cite as:
Rare case of Psychrobacter sanguinis bacteremia in a homeless patient with thigh phlegmon. Bielec F. et al., ADV MICROBIOL-NY, 2025, 64, 1, 37–43, https://doi.org/10.2478/am-2025-0003

(BHI) medium or nutrient agar enriched with 5% blood (Lager *et al.* 2016).

The genus *Psychrobacter* was described initially by Juni and Heym in 1986 (Juni and Heym 1986), and at that time included only the species *Psychrobacter immobilis*. Since then, the genus has rapidly acquired further species, mainly due to the increasing exploration of marine and polar ecosystems (Brenner *et al.* 2005). Currently, The International Code of Nomenclature of Prokaryotes (ICNP) recognizes 44 valid species within the genus that belong to the family *Moraxellaceae* (LPSN 2024).

Based on the limited number of published case reports, *Psychrobacter* spp. are considered opportunistic pathogens (Lager *et al.* 2016; Deschaght *et al.* 2012). Clinical manifestations include bacteremia (Caspar *et al.* 2013; Leung *et al.* 2006; Guttigoli and Zaman 2000), meningitis (Ortiz-Alcántara *et al.* 2016; Le Guern *et al.* 2014), surgical wound infection (Stepanovic *et al.* 2007), and eye infection (Gini 1990), depending on the site of infection. Of these cases, only one was related to exposure to a marine environment; in this case, the patient developed bacteremia caused by *P. phenylpyruvicus* after eating raw mussels (Leung *et al.* 2006).

Psychrobacter sanguinis is an opportunistic pathogen with limited documented cases of human infection. While *Psychrobacter* spp. are commonly isolated from cold environments, their role in human bacteremia remains poorly understood. This case report highlights the challenges associated with identifying and treating *Psychrobacter sanguinis* infections, particularly in vulnerable populations such as homeless individuals with compromised immunity.

2. Case report

2.1. Patient presentation and initial assessment

A 69-year-old man was brought to the emergency room by the emergency medical team. The patient had been lying by a dumpster gazebo on the street for a long time, which had alarmed a police patrol. During the medical interview, the patient was conscious and in average general condition; despite being intoxicated (Glasgow Coma Scale = 15), he displayed preserved logical-verbal contact and responded to questions in a slurred manner. The patient reported dizziness, periodic shortness of breath and pain in the lower limbs, preventing him from walking. He did not report any other ailments, chronic diseases, allergies or long-term medication.

Medical examination revealed the presence of trophic changes, numerous abrasions, old scabs, and scratches on the lower limbs. A cut wound about 4 cm long with clotted blood, after washing, without signs of bleeding, was noted in the left-side occiput area; the patient did not consent to it being dressed. Otherwise, the man was generally hygienically neglected, his skin was dirty, and his clothes were contaminated with urine.

About a week earlier, the patient had fallen and suffered a head injury on the left side of the skull, which was confirmed by the stitches. The patient could not give the exact circumstances of the injury. He also had no medical records related to this event. No abnormalities were found in the rest of the general examination, including a neurological exam.

2.2. Diagnostic workup

The laboratory tests showed elevated parameters of inflammation (CRP = 356 mg/L, WBC = 28×10^3/μL, NEU = 26×10^3/μL), which provoked the decision to admit the patient to the internal medicine department of the local hospital. Suspecting an infectious cause of inflammation, blood samples for culture were taken from two new independent punctures on the upper limbs. Empirical antibiotic therapy was started: the course comprised amoxicillin with clavulanate (1.2 g) every 12 hours intravenously and ciprofloxacin 400 milligrams every 12 hours intravenously. On day 3 of hospitalization, the parameters of inflammation remained elevated (CRP = 279 mg/L, WBC = 27×10^3/μL, NEU = 24×10^3/μL), and an additional plasma procalcitonin test (PCT = 12.56 μg/L) confirmed infection. Laboratory tests on day 5 of hospitalization confirmed the effectiveness of empiric antibiotic therapy (CRP = 190 mg/L, WBC = 13×10^3/μL, NEU = 8×10^3/μL, PCT = 2.55 μg/L). The trends in laboratory values are summarized in Fig. 1.

2.3. Treatment and clinical course

Due to persistent pain in the lower limbs and their ulceration, the patient was consulted surgically. A phlegmon of the left thigh was diagnosed with an indication for incision and drainage. The procedure was performed on day 6 of hospitalization in the local surgical clinic. Drains were left in the phlegmon wounds, and the wounds were rinsed with sodium hypochlorite, and a sterile dressing was applied. The patient's condition after the procedure was assessed as good. The next day, a swab was taken from the wound for culture. On day 8 of hospitalization, while the downward trend in C-reactive protein concentration continued (CRP = 157 mg/L), the other inflammatory parameters increased (WBC = 17×10^3/μL, NEU = 12×10^3/μL).

On the same day, the blood culture result confirmed the presence of *P. phenylpyruvicus*. The antibiogram is presented in Table I. Bacteria identification was performed on the VITEK MS system (bioMerieux,

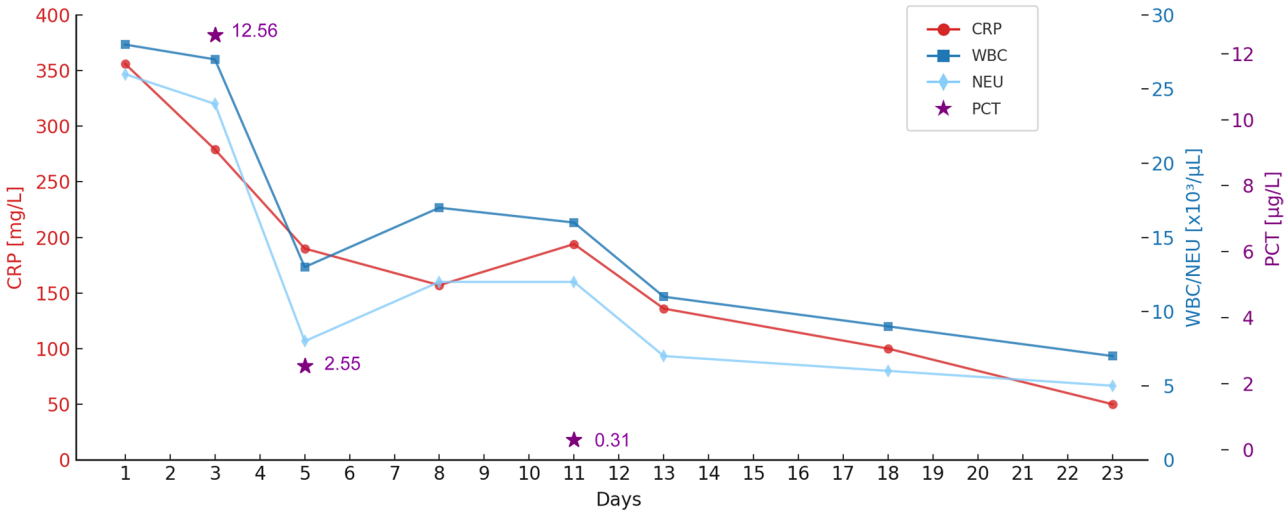

Fig. 1. C-reactive protein (CRP), white blood cells (WBC), neutrophils (NEU), and procalcitonin (PCT) patient's levels over time.

France), and drug susceptibility was assessed using the BD Phoenix system (Becton, Dickinson and Company, USA). Subsequent identification by 16S rRNA sequencing revealed that the bacterium was indeed *P. sanguinis*, but this had no impact on clinical management. After analyzing the antibiogram (Table I), it was decided to modify the antibiotic therapy to include piperacillin with tazobactam 4.5 g every six hours intravenously.

Table I. Susceptibility profile of *Psychrobacter sanguinis* obtained from blood culture during hospitalization.

Antibiotic	Susceptibility
Amoxicillin/ clavulanic acid	S
Piperacillin/ tazobactam	S
Cefotaxime	S
Cefepime	S
Imipenem	S
Meropenem	S
Ciprofloxacin	R

S – susceptible, R – resistant

On day 11 of hospitalization, the parameters of inflammation remained elevated (CRP = 194 mg/L, WBC = 16×10^3/µL, NEU = 12×10^3/µL), but procalcitonin was low (PCT = 0.31 µg /L), indicating the effectiveness of antibiotic therapy. The next day, a wound swab was cultured for *Streptococcus pyogenes*; the antibiogram is shown in Table II. The bacterium was successfully cultured from broth cultures of the swab culture but not the direct cultures on solid media, which most likely indicates a small population of this bacteria in the wound. Bacterial identification was performed using a VITEK MS system (bioMerieux, France), and drug susceptibility was assessed using a BD Phoenix system (Becton, Dickinson and Company, USA). After analyzing the antibiogram (Table II), on day 12 of hospitalization, it was decided to modify the antibiotic therapy to clindamycin 600 milligrams every 12 hours intravenously.

Table II. Susceptibility profile of *Streptococcus pyogenes* as on the wound swab culture during hospitalization.

Antibiotic	Susceptibility
Benzylpenicillin	S
Erythromycin	S
Clindamycin	S
Linezolid	S
Vancomycin	S

S – susceptible, R – resistant

Decreasing levels of inflammatory markers were noted on day 13, indicating that the infection was receding (CRP = 136 mg/L, WBC = 11×10^3/µL, NEU = $7 \times$ $\times 10^3$/µL). Due to the oozing of blood and purulent content from the wounds after the phlegmon incision, the patient was again consulted surgically. It was decided to re-incise and drain the left thigh area. The patient initially did not want to consent to the procedure; however, after informing the patient about the possible consequences of non-treatment, he gave his consent, and a secondary incision and drainage of the phlegmon was performed at the local surgery clinic on day 18.

2.4. Outcome

On day 23, after removing the drains and finding no leakage from the wounds after the procedure, the patient was discharged from the hospital in good general condition for further follow-up at the surgical

clinic. The patient was advised to continue antibiotic therapy with clindamycin 300 mg every eight hours orally for five days on an outpatient basis.

During the entire period of hospitalization, the patient was undisciplined, refused to take medications, pulled out intravenous lines and removed the dressing several times, which could have had a significant impact on the course of treatment.

3. Discussion

The present case study describes the presence of *P. sanguinis*, first described by Wirth *et al.* (2012) as the etiological factor of bacteremia in an 84-year-old man in the USA, in bacteremia in a 69-year-old homeless man. The bacterium was cultured from a positive blood sample in a BacT/ALERT 3D system (bioMerieux, France). A microscope preparation from the blood confirmed the presence of Gram-negative cocci (Fig. 2), and the growth of *P. sanguinis* colonies was noted on Columbia Agar w 5% Sheep Blood after incubation at 35°C for 24 hours (Fig. 3). An isolate was primarily identified as *P. phenylpyruvicus* using a VITEK MS matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (bioMerieux, France). Only the analysis of the 16S rRNA sequence, ordered for this case report, confirmed that the bacterium was *P. sanguinis*, or the new species *Psychrobacter raelei* proposed in 2024 (isolated only from a dog with peritonitis) (Manzulli *et al.* 2024).

The present case highlights a rare instance of *P. sanguinis* bacteremia, with only a limited number of previously documented cases in the literature (Wirth *et al.* 2012; Le Guern *et al.* 2014). A comparison with these reports underscores the unique aspects of this case, particularly the presence of a severe soft tissue infection in a homeless patient with multiple risk factors, including trauma, poor hygiene, and likely chronic immunosuppression due to lifestyle-related factors. In contrast to previously reported cases, which have often involved nosocomial settings, this case suggests that *Psychrobacter* spp. may be capable of causing severe infections outside of hospital environments, possibly through environmental exposure.

The *Psychrobacter* sp. antibiogram was assessed based on the disc diffusion method. Due to the lack of appropriate European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations, the results of *P. sanguinis* were interpreted using the breakpoint values recommended for non-fermenting Gram-negative bacilli (Table I). Previous studies have shown that *Psychrobacter* spp. clinical isolates were susceptible to the following antibiotics: amoxicillin, amoxicillin with clavulanate, ticarcillin with clavulanate, piperacillin, piperacillin with tazobactam, cefalotin, cefotaxime, ceftazidime, cefpiroa, cefepime, imipenem, gentamicin, trimethoprim/sulfamethoxazole, nalidixic acid, ofloxa-

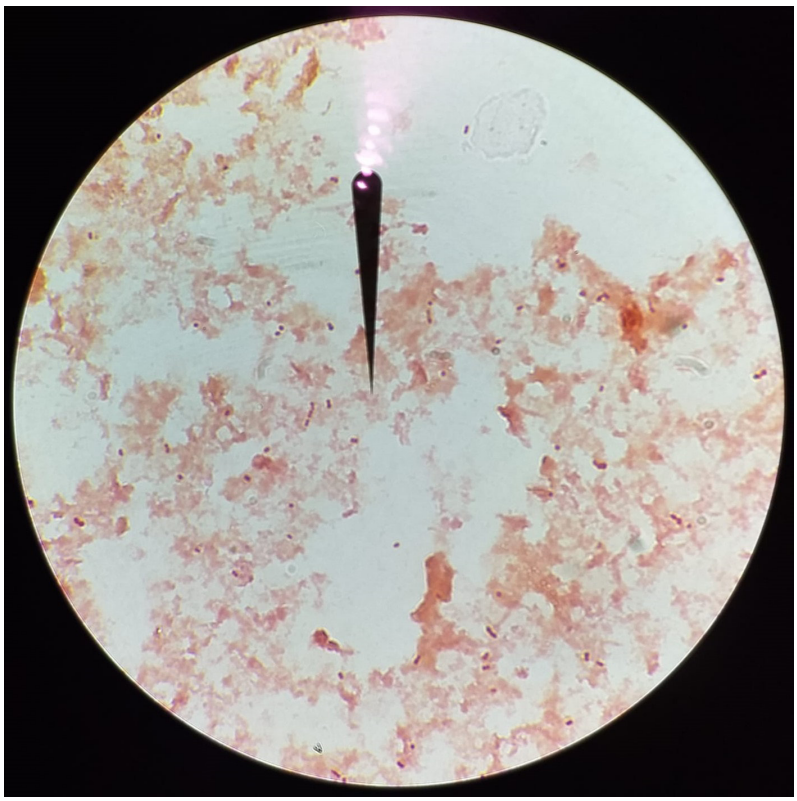

Fig. 2. Microscope view of gram-stained *Psychrobacter sp.* described in the report.

Fig. 3. The growth of *Psychrobacter* sp. described in the report on Columbia Agar with 5% sheep blood.

cin, ciprofloxacin and fosfomycin (Caspar *et al.* 2013; Leung *et al.* 2006; Guttigoli and Zaman 2000).

The molecular identification was performed by analysis of the 16S rRNA gene to identify the bacterium. In the PCR reaction, the 16S rRNA gene was amplified using the following primer pair: forward, 27F: 5'-AGA GTT TGA TCM TGG CTC AG-3' and reverse, 1492R: 5'-TAC GGY TAC CTT GTT ACG ACT T-3'. The obtained sequences were quality-checked and trimmed using FastQC and Chromas v. 2.6.6 software. A contig sequence of 1101 base pairs was obtained using the DNA Base Assembler v. 5.15.0 tool and deposited in GenBank under accession number PP892960.

The sequence was analyzed using the GenBank Basic Local Alignment Search Tool (BLAST). The sequence shared 99.91% homology with a reference sequence of *P. sanguinis* (GenBank: KR232928.1) and *P. raelei* (GenBank: MK771149.1). Our sequence showed a point mutation at T1074A compared to *P. raelei* (GenBank: MK771149.1) and at G514A compared to *P. sanguinis* (GenBank: KR232928.1).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree is shown in Fig. 4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown below the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and given as the base substitutions per site. This analysis involved 22 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). A total of 1109 positions were included in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.* 2021).

The identification of *S. pyogenes* in the wound culture highlights the complexity of wound infections. *S. pyogenes* is a well-known pathogen responsible for various soft tissue infections, including cellulitis, necrotizing fasciitis, and phlegmon (Brouwer *et al.* 2020). Given the patient's poor hygiene and prolonged exposure to unsanitary conditions, the risk of polymicrobial contamination was significantly increased (Lavigne *et al.* 2019). The presence of *S. pyogenes* may have weakened the immune response, creating an environment conducive to secondary infections, such as *Psychrobacter sanguinis* bacteremia. Opportunistic pathogens like *Psychrobacter* spp. typically exploit immune system dysregulation, which can arise due to prior infections, trauma, or prolonged antibiotic therapy (Brouwer *et al.* 2020). This underscores the importance of comprehensive microbiological evaluation and targeted antimicrobial treatment in complex, polymicrobial infections.

From a clinical perspective, the detection of *P. sanguinis* in a bloodstream infection raises the question of whether this species should be considered a true

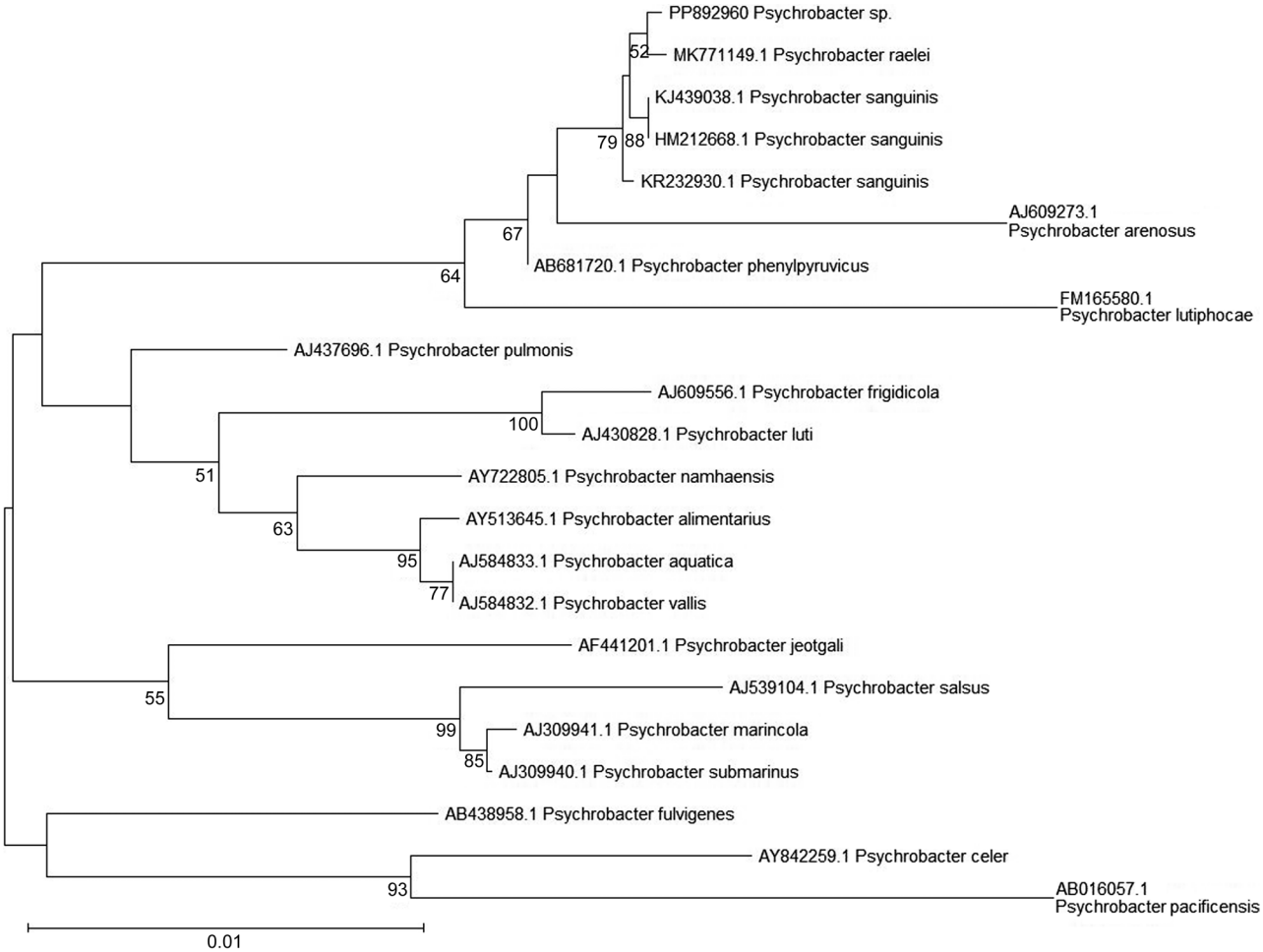

Fig. 4. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences from *Psychrobacter* spp. type strains, *Psychrobacter* sp. isolate PP892960 from our study, and two extra *Psychrobacter sanguinis* strains. Bootstrap values (>50%) are indicated at branch nodes.

pathogen or an incidental environmental contaminant. While previous reports have suggested that *Psychrobacter* spp. are opportunistic rather than primary pathogens, their repeated isolation from human infections, including bacteremia and meningitis, supports their potential role as emerging infectious agents (Caspar *et al.* 2013; Ortiz-Alcántara *et al.* 2016). This case suggests that *P. sanguinis* should be considered in differential diagnoses in laboratories equipped with modern bacterial identification technologies, such as MALDI-TOF mass spectrometry or next-generation sequencing (NGS). Further epidemiological studies are needed to determine the prevalence and pathogenic potential of *Psychrobacter* spp. in human infections.

4. Conclusion

In conclusion, this case report emphasizes the need for targeted antibiotic therapy and surgical management in complex infections. Our findings contribute to the limited but growing body of literature on *Psychrobacter* spp. as human pathogens and illustrate the challenges in diagnosing and treating infections caused by rare bacteria. Given the increasing detection of *Psychrobacter* spp. in human infections, further research is warranted to define better its clinical relevance, pathogenic potential, and optimal therapeutic approaches.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Nucleotide sequence accession number

The *Psychrobacter* sp. 16S rRNA gene sequence data described in this article was deposited in GenBank under accession number PP892960.

Funding

This research was supported by the Medical University of Lodz.

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CONTENTS