Porphyromonas gingivalis, the beast with two heads
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Chapter 8

Conclusion
Conclusion and final remarks

*Porphyromonas gingivalis* is a wondrous pathogen, implicated in at least two diseases widely renowned for their damaging impact on human health, periodontitis and rheumatoid arthritis (RA)\(^1\)-\(^3\). Perhaps the most striking feature of this bacterium is its status of keystone pathogen, considering the potential threat that *P. gingivalis* imposes on human health, while being a numerically underrepresented member of the oral microbiota. This bacterium, in fact, is capable of causing periodontitis while constituting only \(<0.01\%\) of the oral microbiome\(^4\). Nonetheless, as mentioned in Chapter 1 of this thesis, periodontitis contributes to \(~4\%\) of the global expenditure for oral health, making this bacterium a huge economic burden worldwide\(^5\). The main explanation for the danger posed by *P. gingivalis* potentially lies in the plethora of virulence factors that this bacterium produces. Indeed, inside its outer membrane vesicles (OMVs), molecular structures of nanometric scale that are blebbings of the outer membrane (OM), lies a cargo of virulence factors, safely encased in or within the membranous surfaces\(^6\) (Chapter 5). Allegedly, this localization has several advantages from a pathogen’s perspective. Mainly, OMVs offer a bilateral protection: to its cargo from the highly proteolytic environment of the extracellular milieu of *P. gingivalis*, and to the bacterium from the damaging effects of the cargo, enriched in potentially detrimental virulence factors. Secondly, OMVs offer a proficient and precise mechanism of delivery of cargo proteins to the human host.

Among the virulence factors shuttled by OMVs, the most notorious are probably the gingipains, the highly effective and species-specific proteases of *P. gingivalis*. Gingipains cleave proteins at arginine residues and are thought to work in tandem with another, equally infamous, OMV-shuttled virulence factor: the *P. gingivalis* peptidylarginine deiminase (PPAD). This enzyme is one of the major focuses of the present thesis and is capable, in fact, of citrullinating terminal arginine residues. This citrullinating function can have ensuing consequences leading, in genetically susceptible individuals, to the onset of RA (Chapters 1 and 2). Indeed, as discussed in Chapter 2, bacteria belonging to the oral microbiota, chiefly *P. gingivalis*, are involved in the etiology of autoimmune diseases, such as RA. More specifically, this chapter illustrates the many hypothesized mechanisms by which *P. gingivalis* can trigger or
potentiate the onset of RA. These mechanisms can be either PPAD-driven or alter elements of a physiological immune response, turning it into an aberrant phenomenon potentially leading to RA. In the first case, PPAD is assumed to cause loss of tolerance toward citrullinated proteins, a purported cause of RA, via generation of neo-epitopes or molecular mimicry. In the second case, *P. gingivalis*, through its inhibition of neutrophil apoptosis and production of the neutrophil-recruiting factor interleukin 8, promotes the persistence of neutrophils and therefore delays the clearance of NETs. NETs, or neutrophil extracellular traps, are the product of NETosis, the molecular mechanism of “programmed suicide” used by neutrophils to capture and eliminate bacteria. An aberrant NETosis, in fact, has been shown to potentiate the citrullination caused by human PADs leading, in genetically susceptible subjects, to the development of autoimmune citrullinated proteins antibodies (ACPAs), autoantibodies that have been implicated in the etiology of RA. The main foresightful remark detailed in this chapter, though, is the advancement in the field of therapies for RA.

All the findings on the influence of a dysbiotic microbiota on the onset of autoimmune diseases point out new avenues for the development of several microbiome-targeted therapies. These therapies are mainly based on the application of probiotics or small molecules that mimic the signals between beneficial bacteria and the host. Remarkably, a path toward new therapies is also paved by the results discussed in Chapter 3. In this chapter, in fact, the subcellular localizations of all the proteins of the three reference strains of *P. gingivalis* are analyzed. Identification of extracellular proteins, whether present in the outer membrane, on or within outer membrane vesicles, or secreted into the extracellular milieu, is paramount to the search for druggable targets. Localizing every protein in the proteome of *P. gingivalis* therefore provides an invaluable tool for potential therapies, especially considering that virulence factors are mainly targeted toward the host milieu. Additionally, protein localizations are also important to better understand the functions of yet uninvestigated proteins, leading both to a refinement of genomics and protein annotation studies and to an ease of selection of druggable targets. Concerning the study of PPAD, though, the main leap forward granted by the results illustrated in this chapter is the knowledge of the localization of PPAD and of the proteins that colocalize with this enzyme. Intuitively, in fact, enzymes will target only proteins from the same subcellular compartments,
Another closer look at this enzyme is presented in Chapter 4, where a large panel of *P. gingivalis* strains, isolated from patients with and without RA, is investigated for presence of PPAD. Interestingly, every clinical isolate analyzed revealed presence of the PPAD gene, shaping the hypothesis that PPAD is of paramount importance to the success of *P. gingivalis* within its ecological niche. Another insight offered in this chapter concerns the analyses of the sequences of the PPAD genes of different clinical isolates, derived from patients with or without RA. Remarkably, no significant differences were observed at the nucleotide level, hinting at both the high degree of conservation of this enzyme and at the fact that no specific mutations at the gene level influence the rheumatoid arthritis phenotype of the patients. It must be noted, though, that this does not rule out the possibility that PPAD has a role in the onset of RA, it simply marks the absence of a direct connection between the PPAD nucleotide sequence and the RA status of a patient.

Subsequently, the search for a specific correlation between PPAD and RA led to the analyses presented in Chapter 5. In this section, an ample panel of clinical isolates was probed with an antibody specifically binding to PPAD. Consistently, it was observed that the enzyme was produced in all investigated strains, corroborating the theory that PPAD is a well-conserved feature of *P. gingivalis*. More interestingly, a rather small subset of isolates, named “sorting type II isolates”, displayed an aberrant PPAD sorting profile when compared to the remaining strains of the panel (termed “sorting type I isolates”). This sorting anomaly, which occurred only for PPAD, prevented or severely reduced the attachment of the enzyme to the OM of *P. gingivalis*. This attachment is supposed to occur via modification of PPAD with a specific lipopolysaccharide: A-LPS. Remarkably, a more in-depth analysis of the sorting type II isolates identified the potential cause for the aberrant sorting phenotype in a specific amino acid substitution at residue 373 (Q373K). This finding consequently highlights the need for the presence of a glutamine, or the absence of a lysine, at that position to allow attachment of PPAD to the outer membrane.

Of note, even the PPAD of sorting type II isolates showed a remarkably high level of conservation at the amino acid level. For this reason, we decided to expand our investigation of PPAD and cross the species boundaries. Since its discovery, in fact, the PPAD enzyme, evolutionarily unrelated to the mammalian peptidylarginine
deiminases, has always been thought to be a unique feature of *P. gingivalis* that no other prokaryote possessed. The strikingly high level of conservation of this enzyme, hinting at the importance of its function, and its ubiquity in *P. gingivalis* isolates did however suggest that PPAD might also be present in closely related species belonging to the *Porphyromonas* genus. The analysis of a small panel of *Porphyromonas* species isolated from non-human hosts, documented in Chapter 6, solved the conundrum of whether PPAD is restricted to only one species. The erstwhile thought concept of the uniqueness of PPAD was, in fact, disproven by the results presented in this chapter. Indeed, PPAD homologues were shown to be produced by the species *Porphyromonas gulae* and in the recently discovered species *Porphyromonas loveana*, whose genome was firstly analyzed and published in the context of the studies for Chapter 6. Interestingly, the sorting pattern of these PPAD homologues appeared to be remarkably similar to the one of *P. gingivalis* sorting type I isolates. Additionally, more in-depth analyses revealed identical sorting behaviors for these homologues, hinting at a function similar to that of the PPAD of *P. gingivalis*. Investigation of the amino acid sequences showed an overall high degree of conservation of PPAD across species, but also an even higher degree within each species, to the point that a PPAD typing technique could be exploited to distinguish these three closely related species. As stated, a noteworthy conclusion presented in this chapter is that the hosts of the *Porphyromonas* species producing PPAD homologues can be used as novel, and perhaps more suited, animal models to better determine the etiology of rheumatoid arthritis. This would open up completely new avenues for research in this field.

Lastly, the investigation on the molecular aspects and mechanisms concerning PPAD is concluded in Chapter 7. The alleged molecular anchor that allows attachment of this protein to the outer membrane is, in fact, examined in this chapter. Heretofore, the transport system responsible for the sorting of PPAD toward the extracellular milieu, the Por secretion system, was strongly believed to secrete some of its target proteins and then, through a sortase-like mechanism, modify them with A-LPS and attach them to the outer membrane. However, no specific biochemical study investigated the molecular nature of PPAD attachment to the OM. In the study presented in Chapter 7, the anchor of PPAD to the outer membrane was therefore analyzed. The results discussed in this section further bolster the notion of the need of an A-LPS modification to allow the
PPAD attachment to the OM. Additionally, in this chapter, the sorting type II isolates discovered in Chapter 5 were further investigated. The results of these analyses showed, in this subset of samples, a production of A-LPS that was both unhindered and highly similar when compared to the scenario observed in sorting type I isolates. These findings further support our claim that the amino acid substitution Q373K is the main factor responsible for the aberrant sorting observed in PPAD sorting type II isolates. With the combined knowledge gathered in the studies for this chapter and Chapter 5, investigations on the binding of PPAD to A-LPS are both facilitated and encouraged. Albeit no clear correlation was found between sorting type II isolates and the presence or absence of RA in the human host, in fact, the absence of PPAD from the outer membrane could have a massive impact on the physiological function of this enzyme. Additionally, the lack of correlation with an RA phenotype can be explained by difficulty in the disease diagnosis, especially considering that a subject might start producing ACPAs up to, roughly, a decade prior to the development of the disease. Moreover, considering that RA is a multifactorial disease, a protective or risk factor could easily be offset by the opposite effect of other factors. Aside from the potential role that the aberrant sorting displayed by sorting type II isolates might play in the etiology of rheumatoid arthritis, these strains are a valuable tool for the study of the anchoring of PPAD to the OM. Notably, knowledge of the OM-binding of PPAD and similarly secreted proteins might be pathbreaking for the study of the association between periodontitis and RA. Indeed, additional molecular studies on PPAD, its targets, evolutionary advantages, and mechanisms of actions will most surely be sterling milestones on the open road to the discovery of a therapy for rheumatoid arthritis.

References
