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# Future research avenues for the study of fibropapillomatosis in sea turtles

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Fibropapillomatosis (FP) is a debilitating tumoral disease affecting sea turtles worldwide. While mainly afflicting immature individuals and potentially altering vital functions, the precise impact of this panzootic on turtle health and survival remains unclear. Moreover, the etiological factors implicated in the FP emergence, development and transmission are not yet definitively identified. Among them, an infection by a spreading herpesvirus and the contamination by pollutants (either organic pollutants and trace elements) are suspected. Here, we provide an overview of discoveries, knowledge and propose hypotheses related to FP within five key FP research areas, i.e., virology studies, transmission studies, contamination studies, host genomic studies, and veterinary treatment assays. Moreover, we recommend urgent research avenues to develop at the interface of virology, epidemiology, ecotoxicology, oncology, physiology, immunology, cellular and evolutionary biology, in order to characterize the dynamics of FP and to predict its consequences on sea turtle populations. Importantly, extending the implementation and development of strong collaborations between rehabilitation centers, field biologists and research laboratories at large geographical scale is required to rapidly increase our knowledge on FP and work towards its effective management.

KEYWORDS

**sea turtle, conservation, fibropapillomatosis, viral agent, pollution, veterinary medicine, host genomics**

## 1 Introduction

Fibropapillomatosis (FP) is a debilitating tumoral disease affecting sea turtles, mainly at the immature stage (Work et al., 2004). It is characterized by single or multiple internal and external fibroepithelial lesions ranging from 0.1 to 30 cm in diameter (Herbst, 1994). Internal lesions can grow on any visceral tissue (Rossi et al., 2021) and are histologically

described as fibromas, myxofibromas, and fibrosarcomas (Work et al., 2004). External FP-lesions have also been fairly found on all epithelial soft tissues, as well as on the carapace and plastron (see Figure 1 in Rossi et al., 2016). These external FP-lesions exhibit a variety of colors (black, pink, white) and gross appearances: flat plaques, pedunculated, sessile, verrucous, smooth or polypoid nodules (Page-Karjian et al., 2014; Manes et al., 2023b). Light-microscopy observation of FP-external tumors revealed orthokeratotic hyperkeratosis, epidermal hyperplasia, dermal papillary differentiation, ballooning degeneration of epidermal cells and fibroblastic proliferation in the dermis (see Figure 3 in Reséndiz et al., 2021 for a histological cross section of a skin tumor). Depending on their size, number, position, and degree of invasiveness, these lesions can interfere with vision, feeding and locomotion (Aguirre and Lutz, 2004), potentially leading to deleterious consequences on turtle survival (Work et al., 2004; Chaloupka et al., 2008; Flint et al., 2010a but see Chaloupka et al., 2009; Flint et al., 2010b; Patrício et al., 2011; Hargrove et al., 2016). Numerous molecular and epidemiological pieces of evidence point to a herpesvirus infection as a potential causal agent of FP (Quackenbush et al., 2001; Kang et al., 2008).

The first case of FP was observed in the 1930s in a captive green turtle at the New York Aquarium (Smith and Coates, 1938). Initially, the green sea turtle (*Chelonia mydas*) was then presumed to be the only species affected by this disease. However, typical FP-(external) lesions have now also been reported in all of the seven sea turtle species. The green turtle still remains the most frequently and severely affected species (see Supplementary Table 1 in Jones et al., 2016 for examples of FP prevalence in all seven sea turtle species) and the disease has reached a panzootic status in this species (see Supplementary Table S1 in Dujon et al., 2021). The full extent of the impact of the FP emergence and spread on population dynamics remains unclear (Jones et al., 2016), but is a topic of prime importance since most sea turtle species are categorized as vulnerable to critically endangered on the IUCN red list (Seminoff, 2023), because of various abiotic and biotic environmental pressures, such as bycatch, vessel strikes, loss of nesting habitat, and climate change (Hamann et al., 2010; Rees et al., 2016).

Attesting the concern of the scientific community for the conservation of sea turtle populations facing this panzootic (Aguirre and Lutz, 2004; Hamann et al., 2010; Rees et al., 2016; Mashkour et al., 2020), a growing number of scientific peer-reviewed papers studying FP have been published over the last two decades, most of them reporting FP-cases in new geographical areas (see a recent example, Origlia et al., 2023) or describing current local coastal FP prevalence (as reviewed in Buenrostro-Silva et al., 2022 for the American continent). Consequently, we now have a better overview of the disease distribution and prevalence over global coastal habitats (but a constant and concomitant effort is still needed to get a clear and updated resolution). However, there is an urgent need for a multidisciplinary, global and standardized approach at the interface of virology, epidemiology, ecotoxicology, oncology, physiology, immunology, cellular and evolutionary biology to characterize the dynamics of FP and predict the consequences of this panzootic on sea turtle health, survival and population dynamics (as mentioned for example in Hargrove et al., 2016; Mashkour et al., 2020; Jones et al., 2022). Here, we propose to apply such an interdisciplinary approach within five key FP research areas: 1) virology studies, 2) transmission studies, 3) contamination studies, 4) host genomic studies, 5) veterinary treatment assays. Finally, this paper then aims A) to present FP-related questions that need to be answered to fill the knowledge gaps and to propose standardized research guidelines and B) to stimulate the development of collaborative research projects in order to help sustain the financial, logistical and technical difficulties associated with the research on this topic.

## 2 Open questions in FP research

### 2.1 Do ChHV5 dynamics influence FP development pattern(s) and severity?

Based on the Hill's disease causation criteria and primary FP transmission studies (Hill, 1965; Herbst et al., 1995, 1996), FP seems to be primarily associated with a herpesvirus infection (Quackenbush et al., 2001; Kang et al., 2008). More specifically, the *Chelonid alphaherpesvirus 5* (hereafter designated by its widespread abbreviation ChHV5), is the most plausible viral candidate as it is frequently detected in tumoral tissue from FP-affected individuals (Jacobson et al., 1991; Quackenbush et al., 2001). This fibropapilloma-associated turtle herpesvirus, from the *Scutavirus chelonidalpha5* species, is a linear double-stranded deoxyribonucleic acid (DNA) virus from the subfamily *Alphaherpesvirinae* (Benkő et al., 2021). At this time, four ChHV5 variants have been identified in marine turtles: the eastern Pacific, western Atlantic/eastern Caribbean, mid-west Pacific and Atlantic variants (Patrício et al., 2012). Variants are geographic in nature, not being specific to different sea turtle species or rates of disease presentation (Work et al., 2020; Whitmore et al., 2021; Farrell et al., 2022). However, while the viral origin of FP might explain the rapid spread of the disease worldwide (Work et al., 2015b), its implication in the development and dynamics needs to be (molecularly) confirmed. Indeed, on one hand, this virus has already been successfully detected in FP-afflicted turtles (using molecular and microscopy techniques, Quackenbush et al., 2001 and Jacobson et al., 1991 respectively) and also cultured *in vitro* with turtle tumoral cells and organotypic skin culture (Work et al., 2017). But, on the other hand, only three of the four Koch's postulates (Rivers, 1937) have been fulfilled so far (Work et al., 2009). Specifically, the isolation of ChHV5 from FP-afflicted turtle and its *in vitro* culture using standard cell monolayers have not been successfully achieved yet, while a wide variety of validated protocols used to induce viral replication has been tested (i.e., treatment with diverse chemical modulators of replication, incubation at varying temperatures, co-cultivation with known-infected fibroblasts, Work et al., 2009; 2020). Moreover, similar to many herpesviruses, ChHV5 has the capacity to enter a state of latency after a lytic infection (Page-Karjian et al., 2017; Farrell et al., 2021). The difficulty to confirm a causal link between ChHV5 infection and tumor development is also associated with the fact that ChHV5 is near ubiquitous in sea turtle populations. Indeed, absence of FP lesions does not necessarily imply an absence of ChHV5 infection (Page-Karjian et al., 2015; Alfaro-

Núñez et al., 2016). Healthy tissue samples from both FP-afflicted and clinically healthy turtles can carry ChHV5 DNA and ribonucleic acid (RNA) (Quackenbush et al., 2001; Farrell et al., 2022). Consequently, the prevalence of individuals positive for ChHV5 is generally much higher than the prevalence of individuals with FP lesions. This observation can be explained by several factors. Firstly, cases of turtle tumor regression have been documented (Guimarães et al., 2013; Manes et al., 2023b), implying that turtles might be tested positive for the virus without showing any tumor-related symptoms. Secondly, ChHV5 has been detected in populations which have never been reported to be afflicted by FP tumors (such as Mediterranean sea turtles, Serra et al., 2023), thus ChHV5 infection alone does not seem sufficient to lead to the development of FP tumors. Thirdly, as proposed by the "viral hit and run hypothesis" (Ambinder, 2000; Niller et al., 2011), if the virus is the key initiator, its action likely happens very early in the oncogenesis process (lytic phase) and the virus might no longer be needed for tumor growth (latent phase, Farrell et al., 2021). Limited information is currently available on how the dynamics of these lytic and latent states of ChHV5 influence the emergence, growth and regression of FP tumors: A) increased viral load in high tumor burdens is due to latent viral DNA replication, rather than increased lytic activity and B) new grown tumors do not show an increase in ChHV5 expression (a proxy for lytic viral infection) compared to established tumors (Farrell et al., 2021; Yetsko et al., 2021). Finally, the development of FP lesions might also depend on the infected tissue and/or the local viral load and potentially only occurs when a certain threshold is locally reached (Jones et al., 2016; Duffy and Martindale, 2019). It should also be noted that using viral metagenomic methodologies, at least 3 additional viruses have been proposed as potential etiological agents of FP in sea turtles: the Dyozetapapillomavirus 1 (Siddell et al., 2020), also named *Chelonia mydas papillomavirus 1* – CmPV1 (Mashkour et al., 2021), the *Chelonid alphaherpesvirus 6* – ChHV6 (Page-Karjian et al., 2020a), and the *sea turtle tornovirus* – STTV1 (Ng et al., 2009). However, few little data is currently available regarding their implication into FP pathogenosis. To note, CmPV1 seems to be an especially relevant FP-associated pathogen candidate as papillomaviruses are oncogenic at a much lower load than herpesviruses, and this virus has been detected at non-negligeable prevalence in FP-affected individuals (Mashkour et al., 2018).

Based on this literature review, we strongly recommend further research to confirm the implication of a viral pathogen in FP development, and to identify the etiological agent(s) causing this pathology and its (their) role(s) in triggering FP tumor emergence, development, and spread.

First, with methodological and technical improvements, the fulfilment of the fourth Koch's postulate will definitively confirm the implication of ChHV5 in FP etiology (Work et al., 2009). This approach should also need to be considered for CmPV1 to assess its etiological implications. In this line, conducting cell culture experiments where cells collected from healthy individuals are exposed to fresh tumor extracts containing the virus(es) should be one of the main future priorities (see successful method described in Work et al., 2017 for ChHV5, and in Mashkour et al., 2018 for CmPV1). Importantly, inoculation should now be done using pure virus, but it then requires to develop laboratory techniques allowing both culture and isolation of ChHV5 and CmPV1. Moreover, to be as informative as possible, an experimental setup is also expected to include transmission electron microscopy of infected cells. Work et al. (2017) developed an innovative cell culture set up to culture ChHV5, however, it was not readily scalable to get cell-free ChHV5 at a level that could be used to fulfill Koch's postulates. Therefore, prior to ChHV5 inoculation experiments, this ChHV5 cell culture model would need to be refined further or alternative scalable models to be established. Same process should now be initiated for CmPV1.

While exploration of ChHV5 genome has already been initiated (Ackermann et al., 2012; Morrison et al., 2018; Whitmore et al., 2021), new studies of the viral genomes are needed as the complete ChHV5 reference genome remains incomplete, with a (suspected repetitive) region still not sequenced. In this line, novel long-read sequencing technologies (e.g., Pacific Biosciences or Oxford Nanopore Technologies) are ideally placed to complete the full reference genome of ChHV5. Considering the few publication already available, sequencing global ChHV5 genomes should help glean useful information to now robustly identify and confirm the virulence factors involved in the FP context. Assuming that the FP viral origin is true, such an approach will also be particularly appropriate to understand the dynamics of clinical symptoms stages (i.e., from clinically healthy to the development of tumors until complete recovery or death).

Characterizing the temporal viral dynamics (latent *vs* lytic) in relation to clinical symptoms should also be achieved. To do so, detailed transcriptomic and immunohistochemical profiling of various tumors should be achieved (see the methodology used on external new, established and postsurgical regrown tumors and internal lung and kidney tumors by Farrell et al., 2021; Yetsko et al., 2021). Notably, running these analyses for a wide range of external tumors should help determine whether the viral activity differs according to the size, colour and characteristics of the tumors as well as their anatomical position. Extending this approach should also help characterize the tumors and understand why they present such high phenotypical diversity and variety in growth rate (Manes et al., 2023b). In addition, increasing the number of individuals tested should participate in clarifying how the FP-associated differential gene expression varies according to the individual-specific clinical symptoms stage (i.e., viral infection but clinically healthy, tumor emergence, tumor growth, tumor regression, complete recovery, death). Finally, drawing the transcriptomic and immunohistochemical profiles of virus-infected but not FP-affected individuals (i.e., clinically health individuals) should be conducted. Such studies will help complete the description of the viral dynamics for every clinical symptom stage and specifically highlight the implication of the viral etiological candidates in FP emergence, which seems to actually be the most sensitive stage to the viral infection (Farrell et al., 2021). To go further, the use of innovative molecular histological approaches (such as RNAscope) should also be implemented to describe turtle cellular modifications associated with the viral etiological candidates and then characterize their modes of action.

To note, the lack of consensus on the protocols used to detect and quantify the two main viruses potentially associated with FP

makes it difficult to compare data obtained by different studies (see differences in ChHV5 detection using quantitative Polymerase Chain Reaction (PCR), nested-PCR, or singleplex PCR techniques, Alfaro-Núñez and Gilbert, 2014; Jones et al., 2020). This methodological bias may then explain the contradictory results found in the current literature (Jones et al., 2020). Therefore, in the future, we strongly recommend the use of the validated laboratory techniques for qPCR, RNA-seq and DNA-seq, regarding both DNA and RNA extraction as well as viral detection and quantification (Page-Karjian et al., 2015; Blackburn et al., 2021; Mashkour et al., 2021; Yetsko et al., 2021; Farrell et al., 2022). Importantly, we do not seek here to be proscriptive about which ChHV5 qPCR assay should be adopted as the global standard. Ideally, the decision which assay(s) to adopt should come from consensus building across the international FP research community. In our opinion, the subject of standardization should be the topic of a specific workshop e.g., at an international sea turtle conference, and based on a collaborative interlaboratory comparison of method performance conducted altogether by global FP researchers (see the conclusive study conducted for SARS-CoV-2 molecular detection, Deng et al., 2022). In our laboratories, both the Page-Karjian et al. (2015) UL30 and Mashkour et al. (2021) Dpol qPCR assays, which hit somewhat overlapping regions of the same ChHV5 gene, have worked well (Atlantic, Caribbean and Gulf of Mexico samples). The Mashkour et al. (2021) Dpol assay was originally utilized for Australian samples, indicating it would likely function well in global populations. This laboratory protocol standardization will allow the generation of comparable, consistent and reliable datasets at the global scale. Moreover, we also recommend to use standardized tissue and tumor sample collection procedures (sterile biopsy punch of 4 to 6 mm) and storage practices (RNA later or simply frozen at −80°C for DNA analyses). As far as possible, we also recommend to homogenize the samples collected as well as to collect paired samples (FP tumors and nearby healthy tissue). Importantly, we recommend to preferentially collect tissue samples rather than blood samples which have significantly lower ChHV5 loads even in heavy FP-afflicted turtles (Page-Karjian et al., 2015). Moreover, varying ChHV5 detection between fresher and historical samples has been reported by a number of laboratories, for sea turtle tissues and blood samples, and marine leech samples. Consequently, since ChHV5 may be degraded even at −80°C (Kelley, 2022), we encourage to perform analyses within one year after sample collection to avoid false negatives measurements.

## 2.2 What are the main drivers of FP epidemiology?

Fibropapillomatosis was first observed on a captive green turtle (Smith and Coates, 1938). Since then, FP has been increasingly reported in wild neritic turtles (no pelagic turtle with FP has been recorded so far for logistical constraints), and has been classified as an epizootic disease since the late 1980s (Dujon et al., 2021). Concerningly, the disease is reported in all major ocean basins occupied by sea turtles (Herbst, 1994) with drastic differences in prevalence at both spatial and temporal scales (see for instance local annual FP prevalence in Shaver et al., 2019; Jones et al., 2022; Roost et al., 2022). Several non-exclusive hypotheses have been proposed to explain the spreading of the FP causative agent(s) in turtle populations. Body fluids, tumor shedding into water, or physical contacts might be some routes of transmission in addition to external parasites, and especially *Ozobranchus* leeches, as disease vectors (Greenblatt et al., 2004; Work et al., 2015b; Farrell et al., 2021; Roost et al., 2022). Interestingly, individuals from sympatric turtle species in a given geographical area usually host the same viral variant, suggesting a viral horizontal transmission (Herbst et al., 2004; Ene et al., 2005; Greenblatt et al., 2005; Patrício et al., 2012; Rodenbusch et al., 2014; Ariel et al., 2017; Jones et al., 2020). To note, a vertical transmission from mother to offspring also seems to be plausible as ChHV5 has been detected in both hatchling tissue samples and crawl tracks from tumor free hatchlings (Farrell et al., 2021, 2022).

Ethical considerations make *in vivo* experimental approaches not desirable to study FP transmission (although successful experimental inoculations have been conducted, as described in Herbst et al., 1995). However, *ex vivo* experimental approaches may not be robust enough to infer transmission modalities. Therefore, as the next step in transmission studies, we encourage to focus on viral phylodynamics, eDNA and histological studies, as complementary avenues of research to reveal information on transmission dynamics. To do so, in-depth genomic characterization of the virus should be pursued across broader geographic areas to understand how the viral agent circulates among individuals from a given population and worldwide (Patrício et al., 2012; Ariel et al., 2017; Whitmore et al., 2021). Moreover, it will help identify how the different variants of ChHV5 vary in prevalence at both local and large geographical scales. Finally, phylogenomics will improve our understanding of transmission ability (see promising results for the moneypox virus, Yu et al., 2023, and the SARS-CoV-2, Turakhia et al., 2022), which is crucial to understand the observed variations in disease prevalence. As shedding from tumors appears to be a probable route of transmission (Work et al., 2014; Farrell et al., 2021), it is now crucial to determine the persistency of the virus (ChHV5 and others) in the environment by monitoring viral agent shedding in water from turtle rehabilitated tanks. To validate the vertical transmission hypothesis, detection and quantification of ChHV5 in hatchlings at nest emergence, using swabs, tissues from deceased hatchlings, egg shells and membrane as well as eDNA approaches from the crawl tracks of emerging hatchlings, should be conducted (Farrell et al., 2021, 2022). Such non-invasive approaches will help determine if hatchlings have already been exposed to ChHV5 by the time they are leaving the nest (without indicating exactly at which point from conception to leaving the nest that infection is occurring), a crucial information for the development of effective mitigation measures while minimizing impacts of invasive studies on these endangered species. Finally, the influence of environmental stressors (population density, diet, pathogen pressure) and individual physiological status (immune status, stress levels) should also be determined, using advanced computational models accounting for confounding factors, to understand when individuals are more susceptible to both viral transmission and viral activity. It will help characterize the time and intensity of the early immune response mounted by the host, as it is currently not understood why the early host immune response is

not sufficient to prevent tumor development and growth (Yetsko et al., 2021). Specifically, we recommend the routine measurement of white blood cell count and the determination of associated heterophil/lymphocyte ratio (H/L ratio) at the global geographical scale. Indeed, these parameters are among the easiest, cheapest, most repeatable and informative blood immune markers to collect (see detailed methodology in Kophamel et al., 2022).

## 2.3 How may pollutants trigger FP development and dynamics?

Fibropapillomatosis physiopathology is suspected to be multifactorial, with environmental abiotic and biotic variables affecting the growth dynamics of FP lesions (Herbst and Klein, 1995; Aguirre and Lutz, 2004; Foley et al., 2005; Work et al., 2015a; Dujon et al., 2021; Yetsko et al., 2021). This postulate is supported by the high differences in FP prevalence across both global and local geographical scales (Roost et al., 2022; Vanstreels et al., 2023). Exposure to immunomodulatory environmental stressors might enable ChHV5, a relatively benign opportunistic pathogen, to reach a certain viral load and cross its oncogenic threshold (Duffy and Martindale, 2019). However, such relationships between environmental triggers, turtle immunosuppression and ChHV5 infection have not been confirmed in the FP context so far (Yetsko et al., 2021). Among the abiotic and biotic potential candidates, contaminants are regularly proposed as plausible ones because of their potential influence on both the viral dynamics as well as the host physiological status and immune response (Aguirre et al., 1994). Specifically, contamination with organic pollutants and metallic trace elements are considered as a major turtle threat as they are commonly found in oceans worldwide, can accumulate in organs (Brodie et al., 2014) and have already been associated with turtle decreased health conditions (chlorinated organic pollutants, Camacho et al., 2013; trace elements, Perrault et al., 2017). In line with this, higher FP coastal prevalence have recurrently been found in juvenile and sub-adult individuals residing in anthropized areas with contaminated waters such as near-shore waters and lagoons close to high intensity agricultural, industrial and urban areas (Van Houtan et al., 2014). However, while some studies reported significant associations between FP prevalence or severity and levels of PCBs (Yan et al., 2018), and trace elements (da Silva et al., 2016; Perrault et al., 2017); others found no association (either with organic pollutants, Keller et al., 2014; Sánchez-Sarmiento et al., 2017; or trace elements, Pérez et al., 2023).

To increase our understanding of these potential associations between environmental contaminant and FP dynamics, we first encourage to standardize the methodology to measure pollutant levels, i.e., from tissue sampling to the analytical methods applied, for generating comparable data worldwide. Indeed, reported contrasting results may be explained by the different tissues (blood, heart, kidneys, liver, muscle, carapace, fat, eggs) used to measure contaminant levels: each of which has varying (lipidic) composition and therefore different bioaccumulation properties (e.g., the liver exhibits high (lipidic) bioaccumulation properties while blood has low ones, see Brodie et al., 2014). We recommend to use blood and keratinized tissues, two different but common sample matrices in ecotoxicological studies. First, it will provide insights into both the current and accumulated contamination experienced by any individual, respectively (Schneider et al., 2015). The choice of these two sample matrices is also motivated by the fact that 1) they are of limited invasiveness and can then be collected on live individuals (after obtaining appropriate ethical approbation) and 2) laboratory protocols already exist to measure numerous organic pollutants and trace elements levels on these matrices. Finally, sample processing should be standardized and contamination measurements should be conducted following appropriate laboratory techniques validated for the molecule to detect, and approved from consensus among the global FP research community. In terms of research directions, we first propose studying contamination from both FP-afflicted and healthy turtles living in the same area. Secondly, concomitant measurements of contaminant residue levels and viral presence and load between healthy and FP-afflicted turtles across several seasons (detailed in Jones et al., 2016) should also be pursued. Importantly, rather than searching for particular contaminants, future studies should implement the studied chemicals list based on local water contamination reports. Indeed, the effect of contaminants on organisms depends on their bioavailability and mixture toxicity effects resulting from chemicals' synergetic and antagonistic modes of action (cocktail effects, Benejam et al., 2010), as well as on the entire abiotic and biotic context of the studied ecosystem (see Rattner and Heath, 2002 for a review of the abiotic factors affecting contaminant toxicity). In other words, a given chemical should be identified as crucial for FP development in a given area but not in another. Thus, finding a unique or a given cocktail of contaminants associated with FP at the worldwide scale might be unlikely. However, by identifying contaminants locally associated with FP emergence and/or development, we might be able to highlight the pollutant chemical properties and modes of action playing a crucial role in FP development and to target them in other geographical regions. Ultimately, by determining the chemical features which could explain the relationship between turtle contamination and FP prevalence, we should then be able to establish whether such association is mediated by the efficiency of turtle immune system. Finally, conducting a global meta-analysis considering detailed standardized studies across multiple locations should provide an useful tool for disentangling some of the mixed ecotoxicological narrative complexity associated with FP (Dujon et al., 2021; Manes et al., 2023a).

## 2.4 How do host genomics alterations influence the FP development?

The majority of tumor types, whether initially induced by pathogens, environmental exposures or chance mutation, are ultimately driven by alterations in the host genome and transcriptome within tumor cells (Hanahan and Weinberg, 2011). Such oncogenic mutations can come in a number of forms, from small single nucleotide variants to largescale chromosomal rearrangements. These mutations can inactivate genes (such as

tumor suppressors), alter gene functioning, or increase gene expression levels, ultimately transforming cells into cancer cells by promoting rapid cell proliferation, and evasion of programmed cell death and immune system controls. Additionally, epigenetic changes can also induce the activation of oncogenic signaling pathways and the inhibition of inherent tumor suppressor defense mechanisms. Despite the prominent role of mutations and oncogenic signaling pathways in driving tumor formation and development, they were historically understudied in the FP context [e.g., tumor whole genome sequencing (WGS/genomic DNA) has only been employed for a small amount of tumor (Yetsko et al., 2021)], with the majority of research effort generally placed on viral investigations. Failing to consider host dynamics adequately has hampered our understanding of this enigmatic disease, slowing the development of effective mitigation, treatment, and prevention strategies. However, in recent years, an increasing number of studies have begun to investigate changes in host FP biology, from immune-related changes, mutational burdens and targetable perturbations to the signaling pathways driving tumor growth (Blackburn et al., 2021; Perrault et al., 2021; Yetsko et al., 2021).

The application of omics technologies, particularly those honed for human cancer research, such as transcriptomics – functionally validated in cell lines, animal models and clinical trials, are enabling the rapid elucidation of the underlying host molecular mechanistic drivers of FP, as well as the identification of diagnostic, prognostic and drug targets. As a striking evidence, the first ever sea turtle FP transcriptomics study contributed to the establishment of a post-surgical drug treatment which cut FP eye tumor regrowth rates from 67% down to 18% (Duffy and Martindale, 2019). Moreover, the few available transcriptomics studies of FP tumor tissue compared with non-tumor tissue have revealed a core oncogenic signaling network driving tumor growth identical to human pan-cancer drivers (Duffy et al., 2018; Yetsko et al., 2021). There are broad transcriptional differences between FP external tumors, and less common internal tumors. However, the oncogenic signaling pathways: mitogen-activated protein kinase (MAPK), Wingless and Int-1 (Wnt), transforming growth factor beta (TGFβ) and tumor necrosis factor (TNF) are common to both of them (Yetsko et al., 2021) and may in the future be therapeutically targeted to expand treatment options. Specifically, these FP transcriptomic signaling changes most related to patient outcome are in tumor suppressor pathways, particularly apoptotic and immune response genes. Concomitantly, sea turtles which retained higher expression of these tumor suppressor genes within their tumors had better rehabilitation outcomes (Blackburn et al., 2021). In addition to revealing viral dynamics, novel treatment options, and serving as putative rehabilitation outcome biomarkers, transcriptomic and genomic profiling of FP tumors can help identify likely contaminants contributing to FP tumorigenesis (Yetsko et al., 2021). Indeed, for other cancers, applying deep genomic sequencing to tumors revealed environmental contaminants responsible for initiating tumorigenesis (COSMIC, 2018; Stammnitz et al., 2018). Similarly, transcriptomic profiles associated with exposure to organic pollutants, trace elements, and viruses were detected in FP tumors (Yetsko et al., 2021). Such approaches should then be pursued to complement field-based environmental contaminant, and blood/tissue burden contaminant studies.

Fibropapillomatosis tumors do harbor mutations within their cancer cell genomes (Yetsko et al., 2021). To improve our fundamental understanding of the nature of FP, further research should be conducted, in larger cohorts of turtles, examining the specific mutational events driving FP tumor formation and growth. Such research can also identify shared mutational FP drivers between tumors and individuals, elucidate anti-cancer treatment options and identify DNA mutational signatures of tumor initiating environmental exposures. Epigenetic modifications have been shown to play a prominent role in many human cancer types, but epigenetic analysis has yet to be applied to FP tumor biology (Hanahan and Weinberg, 2011). FP epigenetics should be investigated as a matter of urgency, as it will likely also lead to novel insights into this panzootic disease.

Building on the outcome of gene expression prognostic biomarker discovery, with putative FP biomarkers already identified (Yetsko et al., 2021), the development of RT-qPCR assay could enable to find rapid, cost-effective gene expression-based host biomarkers (i.e., RNA) for applications in rehabilitation and field settings. Similarly, whole genome sequencing can identify recurrent FP-associated mutations. Of the identified mutations, some are likely to have prognostic value, as is the case with most human cancers. Therefore, qPCR assays could also be developed as prognostic biomarkers, targeting host genomic mutations (gDNA). When used at scale, qPCR or RT-qPCR can cost less than $10 USD *per* sample. Such outcome biomarkers could help quantify the severity of FP for free-roaming populations. Additionally, they could help inform likely outcomes, and treatment and management decisions within rehabilitation facilities. Indeed, it could contribute to classify patients into those likely to respond to intervention, and those whose tumors are too aggressive, as aggressive and responsive tumors have different underlying molecular profiles, i.e., gene expression profiles and mutational burdens. Host biomarkers for the presence of internal tumors should be particularly beneficial for early detection in rehabilitating sea turtles, as well as for providing a survey tool for monitoring the prevalence of internal tumors in wild populations. The growing awareness of the centrality of host immune and genomic alterations to the development, progression and outcome of FP will help globally tackle this panzootic.

## 2.5 How to increase treatment efficiency and prophylaxis for FP-affected turtles?

Wild FP-afflicted turtles found lethargic and floating on the sea surface or washed up on the shore are generally temporarily admitted in local rehabilitation facilities (when existing) to be cured before being released back (Manire et al., 2017). The decision to euthanize FP-afflicted turtles is justified, in most regions, by the presence of internal tumors. Indeed, these are

currently incurable through medication, and surgical removal of internal tumors is not routinely conducted because it requires specific technical expertise. In addition it is only possible for few internal tumors locations due to the turtle hard shell. However, diagnosis of internal tumors currently requires the appropriate veterinary equipment, i.e., a computed tomography (CT) scanner or X-ray, which is rarely present in rehabilitation facilities due to financial limitations (but see Li et al., 2022 for a promising platform based on ChHV5 glycoprotein B detection). Most often, the clinical cares provided to FP-affected turtles are the surgical removal of external tumors (using CO2 lasers notably, Raiti, 2008) and the treatment of secondary infections (Page-Karjian et al., 2014). However, tumor regrowth has been reported in at least 50% of the surgically treated turtles that have been released and subsequently re-stranded (Farrell et al., 2018; Page-Karjian et al., 2019). Such observation shows that the complete removal of FP tumors is not always possible and rehabilitation success (i.e., survival probability and release success) may be correlated to tumor burden for the majority of FP turtles (Stacy et al., 2019). While one could then wonder whether releasing a few cured individuals each year should have positive and significant effects on local population dynamics, there have been documented cases of previously FP-treated turtles reaching sexual maturity and successfully nesting, thereby contributing to future generations. Importantly, considering the viral FP origin as true, the non-negligible proportion of tumor regrowth should indicate that tumor-free individuals were still carrying out ChHV5 DNA. Actually, sea turtles with higher ChHV5 transcriptional loads (RNA-seq) had the best rehabilitation outcomes (Farrell et al., 2021). In other words, individuals with more active virus fared better than those with lower levels of active virus. This indicates that lytic ChHV5 virus may actually improve the likelihood of turtle survival, possibly by initiating a stronger host immune response (unlike latent virus evading the host immune system, Krump and You, 2018), which could then complement surgical tumor removal by better targeting tumors (Blackburn et al., 2021; Farrell et al., 2021). In the same sea turtle patient cohort, higher expression levels of immune-related host genes were also associated with better patient outcomes (Yetsko et al., 2021). Then, the appropriate research avenue to explore is now determining whether releasing ChHV5-positive tumor-free turtles back into their native contaminated and degraded habitats will not likely lead to re-occurrence of the disease, compromising their rehabilitation success.

With the constant progress of the veterinary medicine, new therapeutic approaches to treat these turtles are expected, with the objective to increase survival rate and reduce tumor reoccurrence. Interestingly, with the recent trend of applying advanced human oncology knowledge to wildlife medicine (Duffy and Martindale, 2019), there is hope of developing new clinical treatment in a decent timeframe. Such reasoning is particularly applicable in the FP context as similarities between human and turtle tumoral cells have been documented (Duffy et al., 2018; Yetsko et al., 2021). This approach is one of the most promising as there is already a panel of efficient orally deliverable drugs used in human oncology that may be administrated routinely in rehabilitation centers after their validation through pharmacokinetic and toxicology studies, i.e., immunomodulators or anti-cancer drugs such as Vismodegib, targeting host genetic perturbations through identified oncogenic signaling pathways, like SHH and Wnt pathways (but see also MAPK and TGF signaling, Duffy et al., 2018; Yetsko et al., 2021); or anti-cancer drugs, such as 5-Fluorouracil or Bleomycin acting as cytotoxic agents (Donnelly et al., 2019). To note, 5-Fluorouracil has already shown promising results with a significant reduction of post-surgical eye FP tumor regrowth, from 67% down to 18% (Duffy et al., 2018). Lysine may also be administrated to prevent tumor regrowth when conducting surgically FP lesions removal (Page-Karjian et al., 2014). Finally, any attempts to use anti-viral drugs for FP treatment, targeting specifically herpesvirus, such as Acyclovir or Ganciclovir, have failed so far, having no effect (D. J. Duffy, unpublished data). Such observation would corroborate with the "viral hit and run" hypothesis which implies that anti-viral treatment may only be efficient during the very early FP stages. However, when turtles are admitted in rehabilitation centers, tumors are already self-sufficient replicating due to oncogenic mutations and transcriptional changes in the host genome and transcriptome, with any remaining virus which may largely be latent. Promisingly, at the beginning of 2024, the first autogenous vaccine therapy for chelonid herpesvirus has successfully been applied in a juvenile green turtle in Colombia (Castro et al., 2024). While this finding opens a new era for FP treatment, it is important to keep in mind that there is a crucial lack of hindsight on such treatment in wild animals (see notably the case studies of avian cholera in albatross species and west Nile virus in the California condor). Then, before considering vaccination as an appropriate preventive cure, several questions must be answered. Notably, which individuals to target (clinically healthy individuals randomly captured in the wild *vs* FP-affected turtles from rehabilitation centers)? How frequently does the vaccine need to be administrated *per* individual? Is there any window of efficiency (hatchlings *vs* immature turtles *vs* breeding males and females)? Which proportion of the population to vaccine in order to ensure an efficient protection of the whole population?

The most important point to develop, related to improving treatments, is the implementation of strong collaborations between rehabilitation centers and research laboratories worldwide. Indeed, rehabilitated turtles are a unique opportunity to increase our knowledge on FP. Notably, they are regularly checked (at least once a week) which is essential in the FP context as tumors can double in size in less than two weeks (Farrell et al., 2018). Moreover, the entire removal of external tumors – as part of the therapeutic process – allows us to collect significant quantity of tumor tissue that could be used to assess oncogenic and viral dynamics according to tumor size, color, and aspect as well as to their status (regrowth, new growth, established). Moreover, this ready sample access can help follow up as well as descriptively and functionally validate detected correlations between environmental cofactors and FP. Such close collaboration between rehabilitation centers and research laboratories has already provided us numerous crucial insights on the dynamics of the

potential viral etiological agents in American FP-afflicted turtles (see for instance Duffy et al., 2018; Page-Karjian et al., 2019, 2020b, 2021; Blackburn et al., 2021; Farrell et al., 2021; Yetsko et al., 2021). It should now be globally extended to confirm these local results and draw general conclusions. Moreover, the regular health and condition record of FP-afflicted turtles should help us monitor the turtle immune status all along the recovery process (from their transfer in rehabilitation center to their release or death). This should be determinant in understanding how the viral agents interfere with host immune function, especially in the context of tumoral regrowth and new growth but also when tumor regression is observed. Such research issue is feasible by weekly monitoring selected health blood parameters (hematocrit, H/L ratio, glycemia, potassium) and comparing external aspect of turtle post-tumor removal through standardized pictures. This methodology will also provide a chance to identify secondary infections and opportunistic pathogens (Work et al., 2003), more likely due to their FP-related immunosuppression (Work et al., 2001; Page-Karjian et al., 2014). Moreover, by monitoring them after release in the wild through photo-identification (i.e., a non-invasive, cheap and standardized method to study FP prevalence, Hancock et al., 2023), it should be possible to precisely evaluate the probability of tumor development as well as their survival probability. Finally, such FP-afflicted turtles should be ideal patients for any veterinary treatment assay to conduct in the next years.

## 3 Conclusion

Fibropapillomatosis is a complex neoplastic multifactorial disease. As highlighted throughout this article, numerous and crucial knowledge gaps need to be filled, to increase our understanding of this disease. Specifically, the interplay between environmental triggers (e.g., pollutants), viral triggers (e.g., ChHV5) and host responses (e.g., immune response, gene expression changes and mutational burdens) requires further elucidation and studies focusing on the functional understand of all these three aspects and their interrelatedness, rather than attempting to examine them in isolation, should be prioritized. Several of the approaches proposed here, such as blood chemistry (e.g., white blood counts and H/L ratio), genomics and transcriptomics assays (e.g., qPCR and RT-qPCR-assay respectively) as well as veterinary treatment trials, require time but also high logistic and financial supports, limiting their advancement. Because budgets allocated worldwide to FP studies are currently quite limited, increasing local and global collaborative projects should help share costs to reach our scientific objectives in a reasonable timeframe. Finally, it is now essential to standardize the protocols used, by choosing robust and validated methods available in the literature (i.e., viral detection, tumor scoring, sampling, storage and matrix processing) to achieve powerful comparative studies at the global scale. As an ultimate step, it will contribute to improve treatment protocols and to propose appropriate conservation measures at the local scale (Mashkour et al., 2020; Jones et al., 2022).

## Author contributions

SMD: Writing – original draft, Writing – review & editing. PB: Writing – review & editing. DJD: Writing – original draft, Writing – review & editing. JF: Writing – review & editing. GLL: Writing – review & editing. PL: Writing – review & editing. DC: Funding acquisition, Project administration, Writing – review & editing. MG: Supervision, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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