

# Similarities and differences among the Opisthorchiidae liver flukes: insights from *Opisthorchis felineus*

## Review

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### Abstract

The foodborne liver trematode *Opisthorchis felineus* (Rivolta, 1884) is a member of the triad of phylogenetically related epidemiologically important Opisthorchiidae trematodes, which also includes *O. viverrini* (Poirier, 1886) and *Clonorchis sinensis* (Loos, 1907). Despite similarity in the life cycle, Opisthorchiidae liver flukes also have marked differences. Two species (*O. viverrini* and *C. sinensis*) are recognized as Group 1A biological carcinogens, whereas *O. felineus* belongs to Group 3A. In this review, we focus on these questions: Are there actual differences in carcinogenicity among these 3 liver fluke species? Is there an explanation for these differences? We provide a recent update of our knowledge on the liver fluke *O. felineus* and highlight its differences from *O. viverrini* and *C. sinensis*. In particular, we concentrate on differences in the climate of endemic areas, characteristics of the life cycle, the range of intermediate hosts, genomic and transcriptomic features of the pathogens, and clinical symptoms and morbidity of the infections in humans. The discussion of these questions can stimulate new developments in comparative studies on the pathogenicity of liver flukes and should help to identify species-specific features of opisthorchiasis and clonorchiasis pathogenesis.

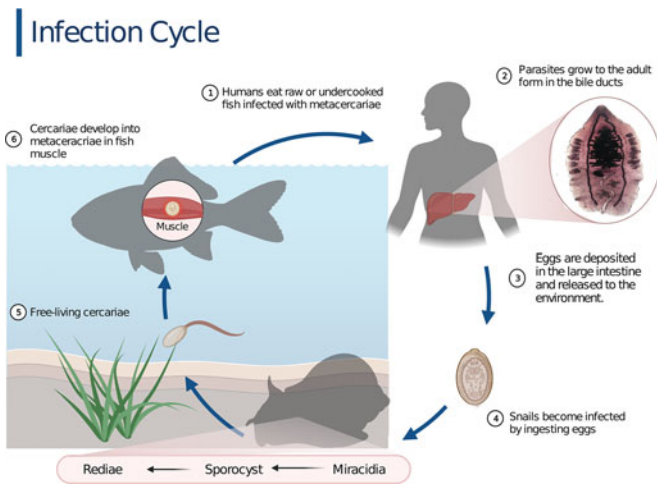
## Introduction

Three species of fish-borne trematodes belonging to the Opisthorchiidae family (Trematoda, Platyhelminthes, Digenea) rank 8th on the global list of 24 clinically important foodborne parasites (FAO/WHO, 2014). Adult worms parasitize the biliary tract of mammals, including humans, and cause damage to the hepatobiliary system, with possible serious complications.

The liver fluke *Opisthorchis felineus* (Rivolta, 1884) is a member of the triad of epidemiologically significant Opisthorchiidae parasites. The geographic range of *O. felineus* covers a major part of Eurasia from Western Siberia to Southern Europe. Natural foci of opisthorchiasis are active and tend to expand. Prevalence of human infection is highest in Russia, where up to 40 000 cases of *O. felineus* infection are diagnosed annually according to the official data from the Federal Service for Supervision of Consumer Rights Protection and Welfare (RF Rospotrebnadzor: <http://rospotrebnadzor.ru/>) [Gosudarstvennyj Doklad (The State Report), 2014]. In countries of the European Union, outbreaks of opisthorchiasis have been recorded (Pozio *et al.*, 2013; Scaramozzino *et al.*, 2018).

Other members of the triad of epidemiologically significant liver flukes are *Opisthorchis viverrini* (Poirier, 1886) and *Clonorchis sinensis* (Loos, 1907). These flukes are common in Southeast Asia and several countries of the Far East and in endemic areas are considered the main risk factors of bile duct cancer, cholangiocarcinoma (CCA) (IARC, 2012). *Opisthorchis felineus* also has carcinogenic potential (Maksimova *et al.*, 2017); however, the epidemiology of CCA in the foci of opisthorchiasis felinea is not well characterized. Nevertheless, data from long-term observations indicate that among people living in the *O. felineus* endemic area, CCA cases are registered much less frequently than among residents of *O. viverrini* or *C. sinensis* endemic areas (Mairiang *et al.*, 2006; Fedorova *et al.*, 2016; Pakharukova and Mordvinov, 2016; Petrova *et al.*, 2019). What is behind this difference? Is carcinogenic potential of *O. felineus* weaker than that of *O. viverrini* and *C. sinensis* or do other risk factors exist responsible for the comparatively high CCA prevalence in Southeast Asia? Can we compare pathogenicity of the 3 species and is there any difference in pathogenesis between opisthorchiasis and clonorchiasis?

It is not yet possible to answer these questions. The main reason, in our opinion, is the lack of comparative studies conducted on 1 model within 1 experiment and carried out without any research biases. There are only a few publications showing that histological changes characteristic of *O. felineus* and *O. viverrini* infections have much in common but are more pronounced and develop earlier in *O. felineus*-infected mammals than in *O. viverrini*-infected ones (Lvova *et al.*, 2012). Haemozoin, a haem detoxification product potentially involved in the induction of proinflammatory and immunomodulatory responses, was found in the gut of adult *O. felineus* and *C. sinensis* but not in *O. viverrini* (Lvova *et al.*, 2016). These data indicate possible differences in the scenarios of the disorders caused by *O. felineus* and *O. viverrini*.



**Fig. 1.** The infection cycle of *Opisthorchis felineus*. The life cycle is typical for trematodes with 2 intermediate hosts (freshwater *Bithynia* spp. snails and Cyprinidae fish) and 1 definitive host (mammals including humans). Created with BioRender.com.

In this review, we focus on identifying similarities and differences among the 3 liver fluke infections; in particular, we concentrate on climatic differences among the endemic areas, characteristics of the life cycle, the range of intermediate hosts, clinical symptoms and morbidity of liver fluke infections in humans, carcinogenic potential and liver fluke genomic and transcriptomic features. We believe that this discussion will facilitate comparative studies on clinically important opisthorchids. Results of such research are needed for understanding the molecular mechanisms of liver fluke pathogenicity and helminth-associated carcinogenesis.

## Materials and methods

Data on the absolute number of registered infections caused by *O. felineus* and the incidence of this infection per 100 000 people per year were retrieved from state annual reports of regional offices of Rospotrebnadzor (Russia) [Gosudarstvennyj Doklad (The State Report), 2014]. Annual reports on *O. felineus* infection are available on webpages of regional offices of Rospotrebnadzor (Russia). Data on cancer incidence, which provide both absolute numbers and the number of cases per 100 000 population per year in the Russian Federation, were analysed on the basis of annual reports issued by the Russian Center for Information Technology and Epidemiological Research in Oncology for 2011–2013 and 2017 (Kaprin *et al.*, 2013; Petrova *et al.*, 2019). In accordance with the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10), code C22 was chosen (cancer of the liver and intrahepatic bile ducts).

A large body of data has accumulated in the past several years on biomedical issues associated with *C. sinensis* and *O. viverrini* infection as well as the biology of these liver flukes. The information on *O. felineus* and opisthorchiasis felinea is still much less abundant. This review addresses the issues on these 3 species. Accordingly, we applied this filter to restrict the published data about *C. sinensis* and *O. viverrini* by the availability of corresponding published research on *O. felineus*.

A literature search for the other materials was conducted *via* a combination of a thorough manual search and searches in bibliographic databases PubMed, Elibrary (<http://elibrary.ru/defaultx.asp>) and Google Scholar (<http://scholar.google.com/>). There were no restrictions on publication date. When selecting bibliographic sources, we primarily focused on finding differences

among the 3 species of opisthorchids, in particular, differences in the climate, geographical locations, life cycle, range of intermediate hosts, morbidity of the diseases, carcinogenicity, genome and transcriptome. We tried to cover all publications containing data from comparative analysis of liver flukes *O. felineus*, *O. viverrini* and *C. sinensis*. The following keywords were used: liver flukes, *O. felineus*, *O. viverrini*, *Clonorchis sinensis*, comparative studies, reservoir animals, opisthorchiasis, clonorchiasis, prevalence, clinical symptoms, morbidity, liver cancer, bile duct cancer, CCA, animal models, genomics and transcriptomics.

Full-length articles published in peer-reviewed journals were selected for the review. All cited articles included information about a study location and description of materials and methods including statistics. Articles about animal models had to contain data from experiments with a concurrent comparison of uninfected and infected animals along with pathomorphological images.

## Results

### A historical account

The liver fluke *O. felineus* is also called the ‘European liver fluke’. Indeed, the first detailed description of *O. felineus* was made by Italian scientist S. Rivolta in 1884, who discovered these parasites in cats and dogs in Pisa (Italy) and named them ‘*Distoma felineum*’. The existing species name and systematic position of the parasite were presented by R. Blanchard in 1885.

In humans, *O. felineus* was discovered in Western Siberia in 1891 by K.N. Vinogradov. He described the parasite in detail, considering it a new species, and named it the Siberian fluke: *Distomum sibiricum* (Vinogradov, 1892). This explains another rather common name of *O. felineus*: the Siberian liver fluke (Beer, 2005; Schuster, 2010; Sripa *et al.*, 2017).

### The life cycle

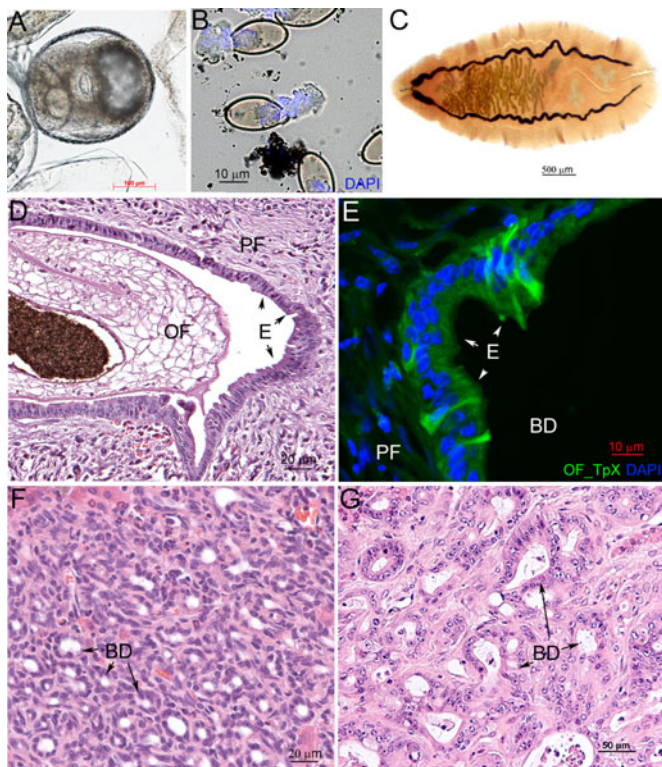
The life cycle of *O. felineus* includes 5 stages, which sequentially develop in 2 intermediate and 1 definitive host (Fig. 1). The only infectious stage for the definitive host, humans and other mammals, is metacercaria (Figs 1 and 2A). Infection occurs after ingestion of raw or undercooked freshwater fish (the second intermediate host) infested with the metacercaria. In the gastrointestinal tract, newly excysted metacercariae are released and through the Vater ampulla migrate into the hepatobiliary system. After approximately a month, juvenile worms develop into adult worms (Fedorov *et al.*, 1989; Beer, 2005).

The adult liver fluke *O. felineus* (Figs 1 and 2C) is a hermaphrodite. In the definitive host, *O. felineus* individuals self-fertilize and produce a large number of eggs: miracidia enclosed in a tight shell (Figs 1 and 2B) (Fedorov, 1979; Beer, 2005). The eggs are excreted into the environment with feces.

To continue the life cycle, the eggs of the parasite (Fig. 2B) need to be carried to a freshwater reservoir and be eaten by the first intermediate host, *Bithynia* spp. snails (Bithyniidae family) (Fedorov, 1979; Beer, 2005).

In the digestive tract of a snail, miracidia penetrate the body of the mollusc through the intestinal wall and turn into sporocysts (Fedorov, 1979). As a result of parthenogenesis, sporocysts can form their copies and motile larvae, rediae. The latter give rise to the next life stage, cercariae, the only free-living life stage. Cercariae emerge from molluscs into water and attach to the second intermediate host, freshwater Cyprinidae fish, penetrate into muscle and connective tissue, encyst and transform into metacercariae (Fedorov, 1979; Beer, 2005). It is important to note that fish species susceptible to *O. felineus* parasitism are





**Fig. 2.** Life stages of the liver fluke *Opisthorchis felineus* and histological consequences for mammalian infection. (A) Metacercariae; (B) hatching eggs with miracidia stained with 4',6-diamidino-2-phenylindole (DAPI); (C) an adult worm; (D) a liver fluke inside a hamster bile duct (E, epithelium; OF, *O. felineus*; PF, periductal fibrosis); (E) an infected hamster liver stained with an anti-OF thioredoxin peroxidase (OF\_TpX) antibody (BD, bile duct); (F) advanced cholangiofibrosis in an *O. felineus*-infected hamster at 6 months after infection initiation is recognized to be precancerous; (G) cholangiocarcinoma in an *O. felineus*-infected hamster after dimethylnitrosamine treatment at 6 months p.i.

either commercial species or recreational fishing targets. The highest infection rate is observed in the ide *Leuciscus idus*, common dace *L. leuciscus* and sunbleak *Leucaspis delineatus* (Sidorov, 1983; Beer, 2005; Pakharukova and Mordvinov, 2016).

Traditionally sun-dried fish, just as cold-smoked ones contain live metacercariae, and such fish delicacies can be a source of infection (Sidorov, 1983). Major risk factors of human opisthorchiasis are the consumption of stockfish [odds ratio (OR) from multivariate analysis (mOR) 3.2,  $P < 0.001$ ], smoked fish (mOR 1.5,  $P < 0.001$ ), frozen fish (mOR 1.6,  $P < 0.001$ ) and raw fish (mOR 1.4,  $P = 0.02$ ) (Beer, 2005; Fedorova *et al.*, 2020). A habit of eating raw freshwater fish was found to be a major contributing factor for the persistence of clonorchiasis in Korea (mOR 3.2) (Park *et al.*, 2014) and of opisthorchiasis viverrini in Thailand (OR<sub>adj</sub> = 5.17) (Chudthaisong *et al.*, 2015). Moreover, a similar habit of family members (OR<sub>adj</sub> = 3.25) and unsafe disposal of food waste (OR<sub>adj</sub> = 2.1) are significant contributing factors in Thailand (Chudthaisong *et al.*, 2015).

The life cycles of *C. sinensis* and *O. viverrini* are similar to that of *O. felineus* and also include 2 intermediate developmental stages (freshwater gastropod molluscs and fish) and a definitive (usually piscivorous mammalian) host (Choi, 1984; Harinasuta and Harinasuta, 1984; Lun *et al.*, 2005). Nevertheless, both intermediate developmental stages of the opisthorchids take place under different environmental conditions, and this state of affairs undoubtedly affects the basic metabolic characteristics of liver flukes. The following sections briefly describe natural foci of opisthorchiasis and features of the *O. felineus* life cycle in Northern Eurasia.

### *Opisthorchiasis foci*

Foci of opisthorchiasis felinea have been found in many European countries and on the territory of the former USSR (Beer, 2005; Mordvinov and Furman, 2010; Pakharukova and Mordvinov, 2016). The largest endemic area has been identified in Western Siberia in the basin of the Ob and Irtysh rivers, an area of ~3 million km<sup>2</sup>, abounding with swamps and lakes. All the factors necessary to maintain the life cycle of *O. felineus* are present in abundance here (Fedorov, 1979; Fedorov *et al.*, 1989; Beer, 2005). The prevalence of opisthorchiasis in the population of this area remains extremely high: 50–80% of rural residents were infected with *O. felineus* according to the data from the 1950 to 1980s (Sidorov, 1983; Beer, 2005; Serbina, 2012). These data were recently confirmed in a community-based cross-sectional study in 2016–2017 (Fedorova *et al.*, 2020), in particular, 60.2% of the 600 examined individuals were found to be infected. The vast majority of the infected individuals had mild infection (70.4%), but 3.1% of them had severe infection (Fedorova *et al.*, 2020).

The second largest endemic area is located in the Dnieper River basin in the north-east of Ukraine (Zavoikin *et al.*, 1989; Chemich *et al.*, 2014). The prevalence of human opisthorchiasis is 21.6 cases per 100 000 people (Chemich *et al.*, 2014). Another focus of opisthorchiasis is active and located in the basin of the Volga and Kama rivers. According to official data, the prevalence is 2.51–14.00 cases per 100 000 people.

Closer to the Ural Mountains, the prevalence of infection increases and reaches 22–52 cases per 100 000 people. In the 1960s, the problem of opisthorchiasis in this area was absent (Sidorov, 1983). Nonetheless, already in the 1970s, frequent cases of infection in cats and humans were recorded (Kanunnikova and Solovyh, 2007). Thus, it can be concluded that new territories endemic for opisthorchiasis have emerged, which may be due to the expansion of the major foci.

Over the past 20 years, quite a few original studies and literature reviews have been published confirming the existence of natural foci of opisthorchiasis in Europe (Schuster, 2010; Petney *et al.*, 2013; reviewed in Pozio *et al.*, 2013). In particular, in Portugal, Spain, Italy, Switzerland, Germany, the Netherlands, France and Belarus, infected red foxes, cats, muskrats and dogs have been found (Shimalov, 2001; reviewed by Pozio *et al.*, 2013; Skripova, 2013; Schuster *et al.*, 2021). In addition, information on the detection of *O. felineus* in 3 species of mustelids, the Eurasian otter, American mink and European polecat in Lithuania, was recently published (Nugaraitė *et al.*, 2019). The only European territory free from *O. felineus* is probably the Scandinavian Peninsula and the British Isles; we could not find any references to relevant studies.

In Italy, cases of human opisthorchiasis are recorded every now and then and are explained by amateur fishing and dishes from undercooked fish (Scaramozzino *et al.*, 2018). From time to time, patients who get ill with opisthorchiasis felinea outside the location of permanent residence are admitted to hospitals in EU countries (Tselepatiotis *et al.*, 2003). Nevertheless, in general, the circulation of *O. felineus* in Europe occurs most likely without the participation of humans.

The endemic area of *O. felineus* differs significantly from those of *O. viverrini* and *C. sinensis*. It covers several climatic zones, radically differing in the duration and temperature of the winter freezing period (Table 1). In contrast, the endemic area of *O. viverrini* located in Southeast Asia does not go beyond the boundaries of 1 climatic zone. The endemic area of *C. sinensis* is rather wide from north to south, but its northern border is located at much lower latitudes than that of *O. felineus*, and in terms of the climatic range, it occupies an intermediate position

**Table 1.** Biological similarities and differences among the liver flukes *Opisthorchis felineus*, *O. viverrini* and *Clonorchis sinensis*

Species	Climate/main foci	Duration of the freezing season	First intermediate hosts	Second intermediate hosts	Definitive/reservoir hosts other than humans	Cycle of disease	Morbidity for humans, carcinogenicity
<i>O. viverrini</i>	Southeastern Asia	–	Bithyniidae (3 spp.)	40 spp. of Cyprinidae	Domestic fish-eating mammals	Anthroponotic	1A Group
<i>O. felineus</i>	Europe, Northern Asia	Up to 7 months	Bithyniidae (3 spp.)	23 spp. of Cyprinidae	Wild/domestic fish-eating mammals	Zoonotic	3A Group
<i>C. sinensis</i>	Eastern Asia	Up to 3 months	8 major spp. of freshwater snails belonging to 5 families	132 spp., 71 belong to the Cyprinidae Crustacea 3 spp.	Wild/domestic fish-eating mammals; buffaloes	Mixture of zoo-anthroponotic	1A Group

Spp., species.

between the areas of the 2 other liver flukes (Table 1). In addition, the reservoir hosts, which maintain the circulation of *O. felineus* throughout the whole endemic area, are wild mammals living near rivers, and we should keep in mind that the human population density is low here. In contrast, the foci of *O. viverrini* are considered anthropogenic, and *C. sinensis* foci have a mixed origin: zoonotic and anthropogenic (Petney *et al.*, 2013).

### Features of the life cycle of *O. felineus*

Despite the general similarity of the life cycles of the 3 liver fluke species, it is worth noting the differences in the temperature of development of larval stages of the parasites as well as in the list of intermediate and definitive hosts.

*Opisthorchis felineus* eggs (Fig. 2B) can overwinter in lake water at 0–5°C, and 42% of eggs can survive for up to 160 days (Fedorov, 1979). The eggs may maintain viability for 10 h at temperatures ranging from –2 to 3°C (Drozdov, 1972).

*Bithynia* spp., the first intermediate host, become active at water temperatures above 15°C, which are typical for June (Fedorov, 1979). After 30–48 days, sporocysts mature and rediae are formed. Nevertheless, the formation of cercariae does not occur by autumn. Development is inhibited due to the period of winter diapause of snails for the freezing season (Table 1). The diapause of molluscs lasts from September to April (Fedorov, 1979). For the winter, molluscs burrow into silt at the bottom of a water reservoir.

The first cercariae appear in May next year. Mature cercariae are motile, emerge from molluscs in bright sunny weather and retain viability for 48–70 h (Beer, 2005; Pelgunov *et al.*, 2006; Serbina, 2012). The morphogenesis of *O. felineus* metacercariae in fish continues from 3 weeks to 2 months (Fedorov, 1979; Serbina, 2012) (Fig. 2A).

The optimum temperature for the development of *O. viverrini* and *C. sinensis* in snails differs from that of *O. felineus*. The highest level of infection of snails with *O. viverrini* eggs under experimental conditions is observed at temperatures above 30°C. When the water is cooled to 16°C, the level of infection in snails decreases 4–5-fold (Prasopdee *et al.*, 2015). It has been experimentally established that at a temperature of 24–37°C, *C. sinensis* goes through all stages of development in molluscs in ~3 months. At temperatures of 20°C and below, the emergence of cercariae has not been detected (Liang *et al.*, 2009).

The 3 liver flukes differ among themselves and in the range of intermediate hosts (Table 1). According to the mainstream opinion, only snails of the family Bithyniidae are susceptible to *O. felineus* and *O. viverrini* infestation (Serbina, 2012; Petney *et al.*,

2013). Nonetheless, species (*Melanoides tuberculatus* and *Maningila* sp.) belonging to other families were described also as the first intermediate host of *O. viverrini* (Doanh and Nawa, 2016). The range of the first intermediate hosts of *C. sinensis* is much wider and includes at least 8 species of freshwater snails, which are representatives of 5 families (Assimineidae, Bithyniidae, Hydrobiidae, Melaniidae and Thiaridae) (Lun *et al.*, 2005). It is noteworthy that except for *M. tuberculatus*, *O. viverrini* and *C. sinensis* infect distinct snail species (Doanh and Nawa, 2016).

The second intermediate host for *O. felineus* is only Cyprinidae fish (Sidorov, 1983; Beer, 2005; Pakharukova and Mordvinov, 2016). Although *O. viverrini* also preferentially infects cyprinids (Petney *et al.*, 2018), *O. viverrini* metacercariae have also been found in the snakehead fish *Channa striata* from the Channidae family (Thu *et al.*, 2007) as well as in the climbing perch *Anabas testudineus* from the Anabantidae family and in the moonbeam gourami *Trichopodus microlepis* from the Osphronemidae family (Eom *et al.*, 2015).

The list of fish species susceptible to *C. sinensis* infestation is much longer. It includes more than 130 species, of which only approximately half of the species belong to the Cyprinidae family. It is known that 3 crustacean species can also serve as the second intermediate host of *C. sinensis* (Chen *et al.*, 2013).

Overall, 15 species of wild carnivorous mammals have been identified as definitive hosts of *O. felineus* (Fig. 2C), namely the wolf (*Canis lupus*), ermine (*Mustela erminea*), corsac fox (*Vulpes corsac*), fox (*Vulpes vulpes*), arctic fox (*Alopex lagopus*), muskrat (*Ondatra zibethicus*), water vole (*Arvicola terrestris*), otter (*Lutra lutra*), bear (*Ursus arctos*), sable (*Martes zibellina*), Siberian weasel (*Mustela sibirica*), marten (*Martes* sp.), steppe polecat (*Mustela eversmanii*), wild boar (*Sus scrofa*) and mink (*Neovison vison*) (Shuteev, 1977; Safiullin and Shibitov, 2012; Siben *et al.*, 2019; Schuster *et al.*, 2021). Definitive hosts also include domestic cats and dogs fed raw fish. Nevertheless, the muskrat *O. zibethicus* is considered the main reservoir of *O. felineus* infection in Western Siberia (Shuteev, 1977; Fedorov, 1979). The muskrat is a medium-sized semiaquatic rodent settling along the banks of rivers, lakes and swamps. Plant materials constitute ~95% of its diet, but muskrats also eat small animals, such as freshwater mussels, frogs, crayfish and fish. The muskrat can get infected after consumption of weakened and dead fish accumulated near the banks of aquatic bodies after overwintering (Fedorov, 1979; Fedorov *et al.*, 1989).

The relatively low number of reservoir hosts of *O. viverrini* and *C. sinensis* is probably associated with the high population density in the endemic foci and scarcity of wild mammals. Of note, cattle



(*Bos taurus* and *indicus*) are among the definitive hosts of *C. sinensis* (Petney *et al.*, 2013); no documented cases of infection of these mammals with other opisthorchids were found.

Although any fish-eating mammals may be potential definitive hosts of *O. viverrini*, only a few, especially domestic cats and dogs, are actually known reservoir hosts for this parasite (Tangkawattana and Tangkawattana, 2018). The prevalence of *O. viverrini* infection is higher among cats (30.92%) than dogs (0.20%); accordingly, cats may play an important role in the transmission and maintenance of this disease in Thailand (Aunpromma *et al.*, 2016). Screening of wild animals in northeastern Thailand, in particular, macaques, rodents, small residential mammals and fish-eating birds, revealed the absence of *O. viverrini* infection (Tangkawattana *et al.*, 2021). This finding indicates that wild mammals and birds probably do not serve as alternate reservoir hosts of *O. viverrini* (Tangkawattana *et al.*, 2021).

### Clinical symptoms and morbidity

The clinical picture of opisthorchiasis felina varies from the absence of symptoms to pronounced manifestation. The incubation period is 2–3 weeks (Yablokov, 1984; Bronshtein *et al.*, 1991). The disease can begin acutely with the appearance of pain in the right hypochondrium, a fever lasting up to 3 weeks, symptoms of toxicity, arthralgia, myalgia, skin involvement, hepatobiliary syndrome, eosinophilia 20–40% against the background of leucocytosis up to 20 000–60 000, bilirubinemia and increased levels of transaminases and alkaline phosphatase (Yablokov, 1984; Bronshtein *et al.*, 1991).

The acute onset of opisthorchiasis is described thoroughly for *O. felineus* infection (Yablokov, 1984; Bronshtein *et al.*, 1991; Mordvinov and Furman, 2010). Although acute onset of the disease has also been mentioned regarding *O. viverrini* and *C. sinensis* infection (Ip *et al.*, 1995; Nishiura *et al.*, 2003), there are no data on such a variety of symptoms as that in opisthorchiasis felina. It seems that the acute onset of the disease is more common during *O. felineus* infection than during *O. viverrini* and *C. sinensis* infections.

The symptoms and signs of chronic opisthorchiasis and clonorchiasis are gastrointestinal (Choi, 1984; Harinasuta *et al.*, 1984; Yablokov, 1984; Bronshtein *et al.*, 1991). The presence of gallbladder stones (mOR = 2.8,  $P = 0.007$ ) is associated with *O. felineus* infection (Fedorova *et al.*, 2020), *C. sinensis* infection (OR = 7.8) (Choi *et al.*, 2008) and *O. viverrini* infection (Harinasuta *et al.*, 1984). Besides, periductal fibrosis and cholangitis are common consequences of all human liver fluke infections (Harinasuta *et al.*, 1984; Park *et al.*, 2014; Fedorova *et al.*, 2020).

### Cholangiocarcinoma

One of the possible consequences of liver fluke infection is the development of CCA, an aggressive human cancer. *Opisthorchis viverrini* and *C. sinensis* are recognized by the International Agency for Research on Cancer (IARC, 2012) as Group 1 biological carcinogens and are classified as major risk factors of CCA in endemic areas (IARC, 2012). *Opisthorchis felineus* is assigned to Group 3, which combines possible cancer-causing factors for which, according to the IARC data, there is no convincing evidence of oncogenic effects on humans and animals. Indeed, systematic studies on the carcinogenic potential of *O. felineus* and the association of opisthorchiasis felina with CCA began relatively recently. Nevertheless, there are some data on the carcinogenic potential of *O. felineus*.

A comparison of the official data on the incidence of parasitic and oncological diseases in Russia as well as the results of retrospective analysis of autopsy records and medical records confirm

the association between the *O. felineus* infection and liver cancer in residents of Western Siberia (Zubov *et al.*, 1989; Brazhnikova and Tolkaeva, 2002; Brazhnikova and Tskhai, 2004; Fedorova *et al.*, 2016; Pakharukova and Mordvinov, 2016). On the other hand, early studies do not always decipher the diagnosis of liver cancer, and additional studies are required for definitive confirmation of the association between *O. felineus* infection and CCA. More recent studies provide evidence of CCA in patients with chronic opisthorchiasis (Kovshirina *et al.*, 2019).

To indirectly assess carcinogenic potential of liver flukes, data on the incidence and prevalence of CCA in endemic areas can be used. In particular, regarding *O. viverrini*, in a hospital-based prospective, case-controlled study of 227 hepatobiliary disease patients with *O. viverrini* infection, 8 patients (3.5%) developed CCA within 2 years of the follow-up period (Kurathong *et al.*, 1985). Later, however, in a community study based on ultrasound screening of 4154 subjects residing in northeast Thailand, only 0.5% were suspected to have CCA (Mairiang *et al.*, 2006).

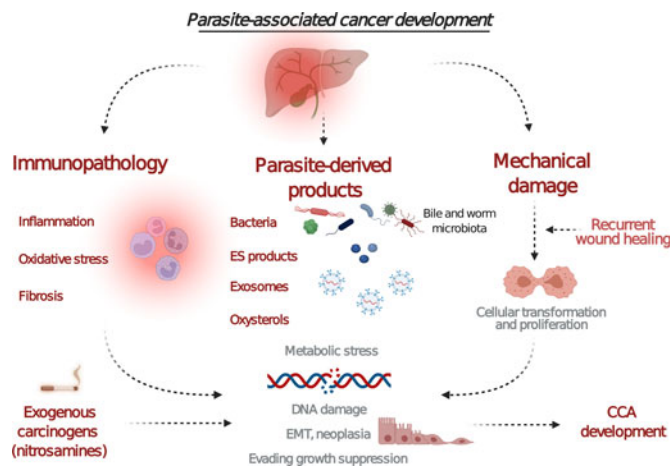
The overall OR for CCA due to *C. sinensis* infection is 4.7 ( $P = 0.000$ ) (Shin *et al.*, 2010). Moreover, a case-control study out of Korea showed that *C. sinensis* infection is significantly associated with increased risk of CCA (OR = 7.3) (Choi *et al.*, 2006). In 2016, liver cancer in Korea ranked 5th among the main types of cancer, and in terms of mortality, it took the 2nd place (Jung *et al.*, 2019).

If we compare these data with the details of cancer incidence and the main types of cancer in Russia, we can notice significant differences of that from Korea and Thailand. In 2017, 6429 cases of liver and intrahepatic bile duct cancer were registered in Russia, which is 1% of the total number of new cancer cases (Petrova *et al.*, 2019). Among newly registered cases, this type of cancer ranked 18th (Petrova *et al.*, 2019). According to official statistics, the prevalence of liver cancer in 2011–2013 was  $4.8 \pm 0.2$  cases per 100 000 people. The highest rates were noted in the Republic of Sakha and the Tomsk region (14.5 and 9.3 per 100 000 people, respectively) (Kaprin *et al.*, 2013). The incidence of *O. felineus* infection was significantly associated with liver and intrahepatic bile duct cancers in Russia in 2011 ( $r = 0.25$ ;  $P = 0.02$ ) (Fedorova *et al.*, 2016). The relatively low carcinogenicity of *O. felineus* was also evidenced by a cross-sectional study in a rural endemic region, where 600 people were examined with 60.2% prevalence of infection (Fedorova *et al.*, 2020). Those authors did not find patients with a liver mass or suspected CCA (Fedorova *et al.*, 2020). Thus, the incidence rates of CCA differ significantly between Western Siberia and Southeast Asia, even though these regions are comparable in the prevalence of liver fluke infection of the population. This observation suggests that the carcinogenic potential of *O. felineus* is lower than that of *O. viverrini* and *C. sinensis*.

Aside from liver fluke infection, the most significant risk cofactors of CCA include cigarette smoking, alcohol consumption and foods containing carcinogenic nitrosamines (Sripa *et al.*, 2012a; Steele *et al.*, 2018). Traditional dishes made from fermented fish with a high concentration of nitrosamines are extremely popular in Southeast Asia, and this practice correlates with the high prevalence of CCA in this area (Sripa *et al.*, 2012a). Inhabitants of areas endemic for *O. felineus* also have traditional dishes made from fermented vegetables; however, in our opinion, the proportion of such products in the diet of the population in Western Siberia is lower than that in Southeast Asia. It is possible that – among other reasons – this is an explanation for the low incidence of CCA in the foci of *O. felineus* infection.

### Animal models

The generally accepted model for studying opisthorchiasis caused by *O. felineus* and *O. viverrini* is the Syrian hamster *Mesocricetus*



**Fig. 3.** Proposed mechanisms of carcinogenicity induced by the liver fluke infection. Chronic inflammation leads to the activation of signalling pathways that can induce oncogenes or stimulate epithelial–mesenchymal transition. Fluke-derived substances (extracellular vesicles and proteins) and metabolites secreted into the bile duct may induce oxidative lesions that facilitate DNA damage. In addition, physical damage to host tissues together with the active wound-healing process facilitates recurrent wound healing and cell proliferation. Cofactors such as dietary nitrosamines or changes in the microbiota may promote this pathology. Combined parasite–host interaction events (chronic inflammation, parasite-derived substances and physical damage) alter cell growth, proliferation and survival thereby possibly causing carcinogenesis. Created with BioRender.com.

*auratus* (Waterhouse, 1839) (Fig. 2D–G) (Sripa *et al.*, 2012a; Mordvinov *et al.*, 2017b, 2021; Pakharukova *et al.*, 2018, 2019a, 2019b, 2019c, 2021; Arunsan *et al.*, 2019; Zaparina *et al.*, 2021). These animals are susceptible to infection by liver flukes, which is not surprising because the main reservoir animal of *O. felineus* is the muskrat, of the Cricetidae family closely related to Syrian hamsters (Fedorov, 1979; Fedorov *et al.*, 1989). It must be noted that pathomorphological, histological and biochemical characteristics of *O. felineus* infection in hamsters (experimental opisthorchiasis) are similar to the manifestations of the disease in humans (Kovner *et al.*, 2019).

Hamsters infected with *O. felineus* and treated with low doses of a carcinogenic nitrosamine (dimethylnitrosamine) develop CCA tumours (Fig. 2G) (Maksimova *et al.*, 2017; Mordvinov *et al.*, 2021): the conditions under which tumours also develop under the influence of *O. viverrini* or *C. sinensis* (Boonmars *et al.*, 2009; Sripa *et al.*, 2012a). The morphology and histological markers of *O. felineus*-promoted tumours fully match the characteristics of the CCA associated with *O. viverrini* or *C. sinensis* (Maksimova *et al.*, 2017; Kovner *et al.*, 2019; Na *et al.*, 2020). Thus, according to the hamster model, the carcinogenic properties of *O. felineus* are quite comparable to those of *O. viverrini* and *C. sinensis*.

What happens to the liver tissues when they are exposed to adult liver flukes *O. felineus* without any other damaging factors? It has been shown that during experimental opisthorchiasis in hamsters, the portal region of the liver increases, and inflammation, periductal fibrosis, cholangiofibrosis and biliary intraepithelial neoplasia take place (Figs 2D–E and 3) (Boonmars *et al.*, 2009; Gouveia *et al.*, 2017; Maksimova *et al.*, 2017; Pakharukova *et al.*, 2019b, 2019c). In general, this set of pathomorphological changes matches the precancerous lesions of liver tissue that precede the onset of CCA (Zen *et al.*, 2007).

Sometimes mice are used to study liver fluke infection, but liver fluke-associated CCA has not been obtained in this model. In particular, C3H/He mice develop extensive fibrosis rather than CCA when they are challenged by *C. sinensis*, N-nitrosodimethylamine and dicyclanil together (Uddin *et al.*,

2016). Likewise, it has not been possible to obtain liver fluke-induced CCA in rats as a model animal, although these rodents are a common model for studying *C. sinensis* infection (Rim, 2005).

### Proposed mechanisms of CCA

Long-lasting interplay between liver flukes and host responses to the parasitic infection initiates carcinogenesis. Although indirect evidence suggests that carcinogenicity is different among the 3 species of liver flukes, published results of studies on molecular events and mechanisms of liver fluke-induced carcinogenesis are insufficient for identifying species-specific features of the infections. Nevertheless, we believe that the discussion of the proposed mechanisms of CCA development, which are probably largely common for 3 opisthorchiid species, is an important part of this review and does not contradict its logic.

The potential mechanisms of carcinogenesis include damage to the biliary epithelium by flukes, prolonged immune-system-mediated pathogenesis and effects of parasite-derived biomolecules on cholangiocytes, followed by modification of the cholangiocyte proliferation (Fig. 3).

Of the parasitic biomolecules that are potentially involved in CCA development, the secreted *O. viverrini* granulin, Ov-GRN-1, has attracted much research attention. This protein is a paralog of human progranulin, which is a glycoprotein involved in the regulation of cell division, motility and migration. Mammalian progranulin is known to participate in embryonic development, wound healing and carcinogenesis (Bateman *et al.*, 2018). Ov-GRN-1 stimulates cell proliferation, angiogenesis and wound healing (Smout *et al.*, 2009, 2015; Bansal *et al.*, 2017; Dastpeyman *et al.*, 2018; Haugen *et al.*, 2018). Moreover, recombinant Ov-GRN-1 was shown to promote exosome-mediated crosstalk and a cellular microenvironment conducive to CCA (Arunsan *et al.*, 2020).

It is likely that Ov-GRN-1 is an important tool in the *O. viverrini* parasitism strategy and contributes to the pathogenesis. Indeed, hepatobiliary morbidities were less pronounced in hamsters experimentally infected with Ov-Grn-1 gene knockout *O. viverrini* than in those with wild-type flukes (Arunsan *et al.*, 2019).

*Clonorchis sinensis* granulin is localized in the tegument and testes of adult worms and is a component of the excretory–secretory product (ESP) and has been found in surrounding host tissues too. Moreover, a recombinant *C. sinensis* granulin can promote metastasis of malignant liver tumours and malignant transformation of hepatocytes (Wang *et al.*, 2017, 2021; Shi *et al.*, 2020). The properties of *O. felineus* granulin have not yet been studied.

The ESPs of all 3 species include antioxidant defence proteins thioredoxin and thioredoxin peroxidase (TPx) (Suttiaprapa *et al.*, 2008; Ju *et al.*, 2009; L'vova *et al.*, 2014). *Opisthorchis viverrini* TPx and *O. felineus* TPx have been found in the bile duct epithelium of infected animals, and this protein is located not only in the host tissues surrounding the parasites but also in small first-order bile ducts, which are substantially smaller than adult worms. Furthermore, *O. viverrini* TPx was shown to decrease apoptosis, which – when cell proliferation is activated by other parasite-derived products – may contribute to malignancy (Sripa *et al.*, 2012a).

Together with the liver fluke infection, such cofactors as environmental or exotic microbes in the biliary system that are resistant to host inflammatory responses may also contribute to carcinogenesis (Sripa *et al.*, 2012a, Chng *et al.*, 2016; Pakharukova *et al.*, 2021). Cofactors such as dietary nitrosamines could significantly facilitate the pathology in question (Fig. 3) (Boonmars

*et al.*, 2009; Sripa *et al.*, 2012a; Maksimova *et al.*, 2017; Mordvinov *et al.*, 2021).

The following mechanism can explain the association between liver fluke infection and CCA: parasite-derived substances can lead to uncontrolled growth of host cells. The parasite-derived proteins like granulin 1 of *O. viverrini* can promote proliferation of biliary cells, whereas other proteins, e.g. thioredoxin and TPx, can prevent apoptosis (Sripa *et al.*, 2012a). *Opisthorchis viverrini*- and *O. felineus*-derived substances are internalized by the bile duct epithelium (Chaiyadet *et al.*, 2015; Petrenko *et al.*, 2017; Pakharukova *et al.*, 2019c). Cholangiocytes in culture can capture exosomes of *O. viverrini*, which is followed by elevated cell proliferation, IL-6 expression and pronounced alterations of the cell phenotype (Chaiyadet *et al.*, 2015). Elevated IL-6 production was demonstrated in *O. viverrini*-infected patients with CCA as compared to those without (Sripa *et al.*, 2012b).

Additionally, an analysis of adult worm lysates has identified novel oxysterol derivatives in *O. viverrini* and *O. felineus*, which are potential promutagenic compounds (Vale *et al.*, 2013; Gouveia *et al.*, 2017, 2020).

On the other hand, the host response is also a crucial determinant of liver fluke infection outcomes and of cholangiocarcinogenesis initiation. In particular, it was demonstrated that *O. viverrini* infection downregulates RB1 (retinoblastoma 1) and p16INK4 (cyclin-dependent kinase inhibitor 2A) expression and upregulates cyclin D1 and CDK4 (cyclin-dependent kinase 4) during CCA progression (Boonmars *et al.*, 2009). There is also evidence of elevated activity of PI3K-AKT and Wnt- $\beta$ -catenin signalling pathways during chronic opisthorchiasis caused by *O. viverrini* (Yothaisong *et al.*, 2014).

Concerning *C. sinensis* infection morbidity, strong stimulation of T helper 2-associated inflammation by *C. sinensis* infection was revealed (Kim *et al.*, 2012). During *C. sinensis* infection, peroxiredoxin 6 expression inversely correlates with NF- $\kappa$ B activation due to the response to *C. sinensis*-derived ESP (Pak *et al.*, 2016).

The liver flukes have strong proinflammatory properties, which increase the risk of carcinogenesis (Maeng *et al.*, 2016; Pak *et al.*, 2016; Pakharukova *et al.*, 2019b, 2019c). During chronic inflammation, inflammatory cells are recruited to injury sites, thereby enhancing the release and accumulation of free radicals, resulting in the formation of lipid peroxidation byproducts, which can induce the formation of oxidative DNA lesions, e.g. 8-hydroxy-2'-deoxyguanosine (Maeng *et al.*, 2016; Pakharukova *et al.*, 2019b; Zapparina *et al.*, 2021).

Major factors contributing to inflammation are macrophage-derived proinflammatory cytokines. Increased mRNA expression levels of IL-1b, TGF- $\beta$  and TNF (formerly known as TNF- $\alpha$ ) were revealed in the liver of *O. viverrini*-infected hamsters (Prakobwong *et al.*, 2009). Moreover, elevated levels of TNF are reported to correlate with morbidity in *O. viverrini*-infected hamsters (Dangtakot *et al.*, 2017). The same correlation between TNF upregulation and liver problems was observed in a time course study on *O. felineus*-infected hamsters (Pakharukova *et al.*, 2019b). This cytokine is also known as an activator of fibroblast proliferation and stimulates epithelial-mesenchymal transition factor SNAIL in human *O. viverrini*-associated-CCA cells (Techasen *et al.*, 2012).

Continuous production of proinflammatory cytokines and growth factors that facilitate the activation of myofibroblasts has been revealed during *O. viverrini* infection and *O. felineus* infection (Prakobwong *et al.*, 2009; Kovner *et al.*, 2019; Pakharukova *et al.*, 2019b). Furthermore, the activation of epithelial-mesenchymal transition, extracellular-matrix deposition and fibrogenesis was demonstrated in patients and experimental animals with opisthorchiasis (Kovner *et al.*, 2019). Myofibroblasts produce  $\alpha$ -smooth muscle actin and deposit extracellular-matrix

components. In *O. felineus*-infected animals, the number of these cells is elevated during the infection as evidenced by excessive fibrogenesis (Gouveia *et al.*, 2017; Kovner *et al.*, 2019; Pakharukova *et al.*, 2019b).

In *O. felineus*-infected animals, the amount of CD163 significantly increases with time (Pakharukova *et al.*, 2019b). CD163-positive cells can infiltrate tumour tissues, in particular in CCA associated with *O. viverrini* infection, and the CD163 protein is reported to be a marker of macrophages having an alternative activated phenotype (tumour-associated macrophages) (Thanee *et al.*, 2015). Alternatively, activated CD163<sup>+</sup> macrophages contribute to the control of an inflammatory process through a release of anti-inflammatory cytokines: a process that promotes tissue regeneration (Braga *et al.*, 2015).

### Genetics and genomics

One of the intriguing features of *O. felineus* is the absence of well-defined population structure over a significant part of its range. Results of large-scale genotyping using marker DNA fragments from mitochondrial (*cox1* and *cox3*) and nuclear internal transcribed spacer sequences revealed that genetic diversity of this species is very low across Eastern Europe, Siberia and Northern Kazakhstan (Brusentsov *et al.*, 2013). In contrast, population genetic differentiation is well-pronounced in *O. viverrini* (Laoprom *et al.*, 2012). An in-depth analysis of the population genetics of *O. viverrini* and of its first intermediate host, *Bithynia siamensis*, was published recently (Saijuntha *et al.*, 2022).

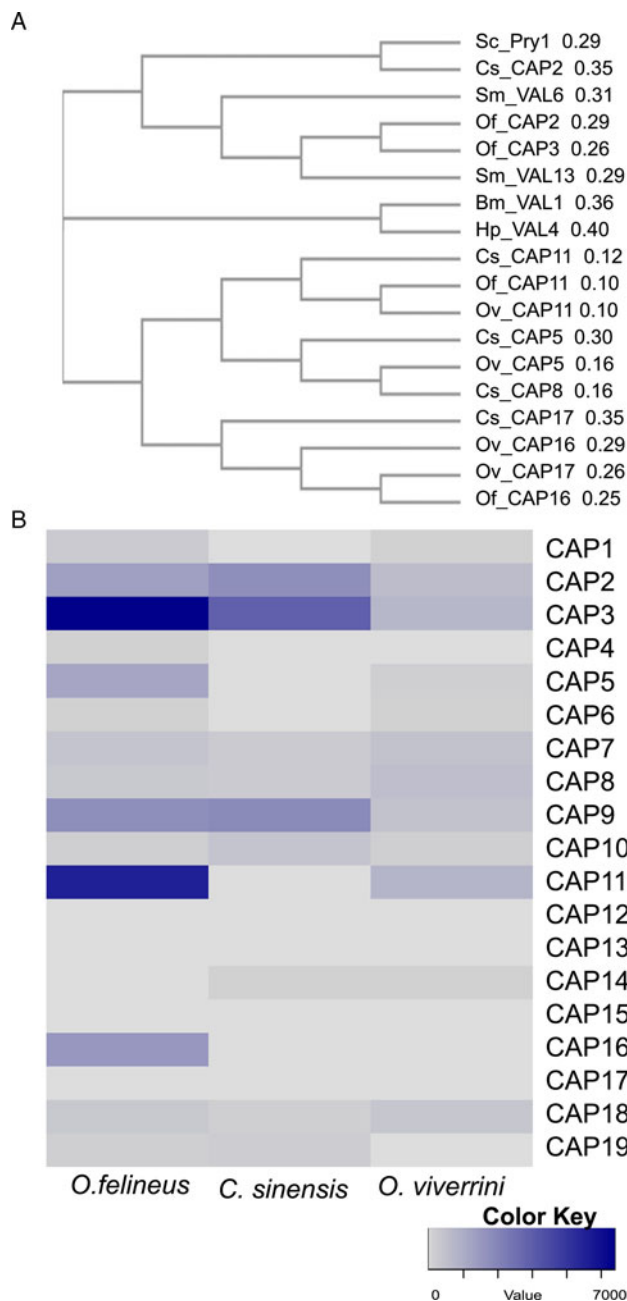
The genetic diversity of *C. sinensis* is not as pronounced as that of *O. viverrini*, but the population structure of *C. sinensis* does exist (Sun *et al.*, 2013).

Microsatellites, a powerful tool for population-genetic studies, were utilized for *O. viverrini* research (Laoprom *et al.*, 2010). It was shown that cyprinids – the second intermediate host – could contribute to the genetic diversity of *O. viverrini*. In particular, there are differences in *O. viverrini* genetic parameters between different species of fish within and between some regions (Pitaksakulrat *et al.*, 2018). Advances in the investigation into the population genetics of *O. viverrini* indicate that microsatellite DNA markers should be utilized for in-depth studies on the genetic diversity of *O. felineus*.

There are differences in the chromosome number among the 3 opisthorchids. The karyotype of *O. felineus* (Siberian isolate) and *C. sinensis* (Russian Far East isolate) consists of 7 pairs of chromosomes ( $2n = 14$ ) (Zadesenets *et al.*, 2012), whereas the karyotype of *C. sinensis* (Korea and China isolate) is represented by 28 chromosome pairs ( $2n = 56$ ) (Park *et al.*, 2000). This fact contradicts the data on the final *C. sinensis* genome assembly from a Korean isolate comprising 7 largest scaffolds corresponding to a karyotype of  $2n = 14$  (Young *et al.*, 2021). In contrast, the karyotype of *O. viverrini* is composed of 6 pairs of chromosomes ( $2n = 12$ ) (Zadesenets *et al.*, 2012).

The size of the *O. felineus* nuclear genome is 684 Mbp, and 30.3% of the genome is represented by repetitive elements, mainly retrotransposons. In the genome, 11 455 thousand protein-coding genes are annotated (Ershov *et al.*, 2019), as are 55 genes coding for microRNAs (Ovchinnikov *et al.*, 2015). In terms of the size and abundance of repetitive elements, the *O. felineus* genome is close to the genomes of *O. viverrini* and *C. sinensis*, the sizes of which are 634.5 and 558 Mbp, respectively. Nonetheless, judging by the number of annotated genes, the genomes of *O. viverrini* and *C. sinensis* surpass the genome of *O. felineus* and contain 16 379 and 13 489 genes, respectively (Young *et al.*, 2014, 2021). This is probably due to the differences in genome assembly technologies. As a result of orthology analysis, it was found that the published versions of assembly of the genomes are not exhaustive,





**Fig. 4.** The phylogenetic tree and mRNA abundance of genes encoding CAP domain-containing proteins. (A) The neighbour-joining phylogenetic tree without distance correction. A comparison of *O. felineus* venom allergen-like proteins with selected members of the CAP superfamily. The sequences were aligned with clustalw2. Pry1 (NP\_012456.1, sterol-binding protein of *Saccharomyces cerevisiae*), SmVAL-4 [XP\_018652935.1, venom allergen-like (VAL) 4 protein of *Schistosoma mansoni*], CsVAL-28 (AWV55762.1, venom allergen-like protein 28 of *Clonorchis sinensis*), BmVAL-1 (AAK12274.1, venom allergen antigen-like protein 1 of *Brugia malayi*) and HpVAL-4 (AEP82919.1, venom allergen/ancylostoma secreted protein-like 4 of *Heligmosomoides polygyrus bakeri*) were used. (B) Interspecies differences in mRNA abundance of genes coding for VAL proteins among adult liver flukes. Normalized data were taken from the paper (Ershov *et al.*, 2019) and were formatted as a heatmap using the heatmap.2 (v.2.38) R package. OF, *O. felineus*; OV, *O. viverrini*; CS, *C. sinensis*. GenBank accession numbers for sequences encoding CAP domain-containing proteins in the Opisthorchiidae liver flukes are presented in Supplementary Table ST1.

and the gene annotation for all 3 species needs to be improved (Ershov *et al.*, 2019).

Unusually high heterozygosity was found in the *O. felineus* genome in samples obtained both from single and pooled worms (Ershov *et al.*, 2019). In this regard, the *O. felineus* genome significantly differs from the *O. viverrini* genome, whose

sequencing and assembly revealed low-sequence heterozygosity (Young *et al.*, 2014). In an analysis of genome-wide synteny among the 3 opisthorchids, it was revealed that in the arrangement of homologous loci, the similarity of the genomes of *O. felineus* and *C. sinensis* is higher than that of *O. viverrini* with *O. felineus* or *C. sinensis* (Ershov *et al.*, 2019).

The data on genome synteny are confirmed by the results of phylogenetic studies involving individual genetic markers and genome-wide data from the 3 opisthorchids (Shekhovtsov *et al.*, 2009; Cai *et al.*, 2012; Pomaznoy *et al.*, 2016). Thus, according to the above findings, *O. felineus* and *C. sinensis* are closer species than *O. felineus* and *O. viverrini* are. This is inconsistent with the separation of *C. sinensis* from the genus *Opisthorchis* and suggests that this species occupies an intermediate position between *O. felineus* and *O. viverrini*. Moreover, the geographic ranges of *O. felineus* and *C. sinensis* are also closer than those of *O. felineus* and *O. viverrini*.

As mentioned above, parasitic granulins are believed to be directly involved in the development of *O. viverrini*-associated CCA (Arunsan *et al.*, 2019). Initially, 3 genes coding for single-domain granulins and 1 gene of a multidomain progranulin (PGRN) were found in the *O. viverrini* genome (Young *et al.*, 2014). Nevertheless, later, 4 genes (*grn-1* through *grn-4*) coding for single-domain granulins as well as 1 gene of PGRN have been identified in *O. viverrini*, *O. felineus* and *C. sinensis* genomes. Genes *grn-1*–*grn-4* in all 3 genomes are situated in the same chromosomal locus and constitute a syntenic group of genes. Genes *grn-4* and *grn-1* share 95% nucleotide sequence identity (Ershov *et al.*, 2019). Functional activity of the Ov-GRN-1 protein and its probable role in CCA development have been investigated (Smout *et al.*, 2009, 2015; Mulvenna *et al.*, 2010; Arunsan *et al.*, 2019). The functional activity of other opisthorchiid granulins has not yet been studied.

### Comparative transcriptomics

In a comparison of the transcriptomes of adult *O. felineus*, *O. viverrini* and *C. sinensis* worms, it was found that the expression of the overwhelming majority of genes does not have significant differences among the 3 opisthorchiid species (Ershov *et al.*, 2019). This result indicates high similarity in the mechanisms that ensure normal physiological processes of helminths in the definitive host. Nevertheless, the expression of several dozen genes proved to be species-specific. It is worth mentioning that most of these genes encode proteins of the opisthorchids' ESP, an essential component of the parasite–host interaction mechanisms (Ershov *et al.*, 2019).

In particular, a group of differentially expressed genes among the 3 species included CAP superfamily genes. Expression levels of some CAP domain-containing proteins (cysteine-rich secretory proteins, antigen 5 and pathogenesis-related 1 proteins) are higher in *O. felineus* than in *C. sinensis* and *O. viverrini* (Fig. 4B) (Ershov *et al.*, 2019). CAP domain-containing proteins represent a highly evolutionarily diversified superfamily whose members share specific sequence motifs (Gibbs *et al.*, 2008; Chalmers and Hoffmann, 2012). Ant and wasp allergens, snake venom toxins and plant antifungal proteins are included in this superfamily. Although nematodes' CAP proteins are known as *Ancylostoma*-secreted proteins, some parasitic CAP proteins are known as venom allergen-like (VAL) proteins (Fig. 4A).

VALs are ubiquitously present in ESPs of helminths that parasitize animals and seem to be highly expressed at stages of the parasite life cycle where maximal contact occurs between the parasite and host, e.g. transmission, tissue migration or feeding (Wilbers *et al.*, 2018). Roles of VALs in protease activity and modulation of the immune response during mammalian host



invasion in *Schistosoma mansoni* have been proposed too (Chalmers and Hoffmann, 2012). In particular, *S. mansoni* VAL9 affects the expression of extracellular-matrix-modifying gene products (metalloproteinases and tissue inhibitors of metalloproteinases) in both *Biomphalaria glabrata* embryonic cells and *Mus musculus* bone marrow-derived macrophage populations (Yoshino *et al.*, 2014). Two VAL proteins of *C. sinensis* (VAL13 and VAL28) are thought to hold promise for serodiagnostics; their functional motifs and structural details have been characterized *in silico* (Woo *et al.*, 2015; Lee *et al.*, 2018).

These data may form a basis for the research into functional significance of these secreted proteins in the pathogenesis of opisthorchiasis and clonorchiasis as well as for the studies on the species-specific features of the pathologies caused by these liver flukes.

In the genome of *O. felineus*, 4 genes encoding glutathione-S-transferases (GSTs) have been found, coding for xenobiotic metabolism enzymes capable of catalysing the conjugation of the reduced form of glutathione to substrates. GST sigma (molecular weight 28 kDa) has a conserved domain of prostaglandin synthase and retains its activity in the *in vitro* incubation medium of helminths (Pakharukova *et al.*, 2019c) and accumulates in the bile duct epithelium of mammals infected with *O. felineus* and patients with opisthorchiasis (Kovner *et al.*, 2019; Pakharukova *et al.*, 2019c). According to a comparative analysis of the transcriptomes of adult *O. felineus*, *O. viverrini* and *C. sinensis* worms, mRNA abundance of GST sigma in the transcriptome is many times higher in *O. felineus* than in the other opisthorchids (Ershov *et al.*, 2019). It has been hypothesized that this enzyme plays an important part in the parasite–host interaction system.

### Conclusions/future directions

In this review, we provided a recent update of our knowledge on the liver fluke *O. felineus* and drew attention to its differences from the closely related liver flukes *O. viverrini* and *C. sinensis*.

The fish-borne trematode *O. felineus* occurs across a large territory of Eurasia and poses a threat to the health of the population in a number of countries. The prevalence of opisthorchiasis in Russia remains high. Active human intervention into nature, global climate change and ever-increasing migration of the population can change the current situation, provoke the expansion of geographic foci and the resumption of outbreaks of opisthorchiasis in other regions. Awareness of the population about the risk of opisthorchiasis from eating raw or undercooked freshwater fish and about the clinical manifestations of the human infection is essential for the control and prevention of this disease.

Liver fluke infections caused by *O. felineus*, *C. sinensis* and *O. viverrini* seem to have similar clinical symptoms, and today, the same anthelmintic agents are used to treat them. This situation can give the impression that there is no difference among the disorders. Nevertheless, these disorders, in addition to similar characteristics, have distinct clinical manifestations, probably due to the biological characteristics of the causative agents. The most important issue of the liver fluke pathogenicity is their carcinogenic potential.

The carcinogenicity of *C. sinensis* and *O. viverrini* is well documented, but for *O. felineus*, there is still no convincing epidemiological data confirming the direct link of opisthorchiasis felina with cancer of the biliary tract. There are many publications pointing to relatively elevated prevalence of CCA in territories endemic for opisthorchiasis in the Russian Federation (Pakharukova and Mordvinov, 2016). In contrast to the *O. viverrini* endemic areas in Thailand, CCA in Western Siberia is not the leading type of cancer (Pakharukova and Mordvinov, 2016;

Petrova *et al.*, 2019). Although, at present, there are no comparative studies on their carcinogenicity under the same conditions, it can already be said judging by indirect signs that *O. felineus*, *O. viverrini* and *C. sinensis* have pronounced differences in carcinogenicity for humans.

On the other hand, it is necessary to take into account national traditions and dietary habits of the population in endemic areas. In addition to the traditional consumption of raw or undercooked fish, which is a major risk factor of liver fluke infection, exogenous nitrosamines in the diet significantly facilitate the initiation of CCA. Fermented foods are more popular in countries where *O. viverrini* and *C. sinensis* are common than in *O. felineus* endemic areas. It is possible that these dietary patterns contribute to CCA incidence too.

The mechanisms of biological carcinogenesis need to be investigated further. Is chronic inflammation alone a necessary and sufficient condition for the formation of CCA? If so, then 3 epidemiologically significant species of liver flukes *O. viverrini*, *C. sinensis* and *O. felineus* would cause carcinogenesis in the same way. Experimental infection caused by *O. felineus*, *O. viverrini* or *C. sinensis* represents a promising model for comparative studies of helminth-associated carcinogenesis. The adaptation of liver flukes to life cycle conditions in different climatic zones with several intermediate and final hosts may be reflected in their basic molecular and biochemical processes. Such adaptive changes may in turn be associated with the pathogenicity of certain species. Species-specific composition of parasitic ESPs components, in particular, proteins and metabolites as well as small RNAs in exosome-like vesicles, can determine carcinogenic potential of the opisthorchids. The worm microbiota and its effects on the host microbiota may also influence carcinogenic potential. Taking into account the complex life cycle and climatic differences of endemic areas, relevant differences in their microbiomes can be found as well.

In this regard, the need for comparative bias-free studies on the 3 epidemiologically significant liver fluke species is obvious. Research developments on comparative analysis – in particular, high-throughput mRNA sequencing aimed at identifying differences in host–parasite interaction and differentially expressed genes during the host response caused by closely related species with moderate and high carcinogenicity – will improve the understanding of the mechanisms behind helminth-associated carcinogenesis and will help to answer the question ‘What are the general mechanisms of biological carcinogenesis?’

To investigate the mechanisms of helminth-associated carcinogenesis, in particular, to identify key pathogenesis-related proteins, active use of functional-genomics approaches is required. These approaches have already manifested their effectiveness in studies on the xenobiotic metabolism of *O. felineus*. For instance, using RNA interference, it was shown that the genes encoding CYP and ABC proteins are important for normal physiological processes of adults *O. felineus* and can be considered drug targets (Pakharukova *et al.*, 2015; Mordvinov *et al.*, 2017a). In a recent report on *O. viverrini* granulin 1, CRISPR/Cas9 technology was utilized, which is a new genetic tool that has revolutionized the investigation of gene functions. Those authors confirmed the important role of granulin 1 in the development of *O. viverrini* opisthorchiasis (Arunsan *et al.*, 2019).

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182022000397>.

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