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Endoparasite diversity of the main wild ungulates in Portugal

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Wild ungulates have expanded their geographical range across Europe and Portugal is no exception. Despite the known benefits associated with the increase of these populations (e.g. increased prey for wild carnivores), the negative impacts also need to be taken into account (e.g. damages in agriculture and forestry, ungulate–vehicle collisions). Additionally, their role as reservoirs of zoonotic agents has gained scientific relevance due to the potential human health risks, impact on livestock and food safety. In northeast Portugal, Montesinho Natural Park, three species of ungulates occur in sympatry, the wild boar *Sus scrofa*, the red deer *Cervus elaphus* and the roe deer *Capreolus capreolus*. Considering their close association with humans and livestock, it is essential to understand their role as reservoirs of infectious diseases, namely as vectors for parasitic infections. In order to achieve this, 112 fresh faecal samples were collected to assess, by means of coprological analyses, their parasite diversity, prevalence and mean intensity. In total, 88 (78.60%, ±69.81–85.76) samples were infected with at least one parasite species. Parasite prevalence was different among the three species, with the red deer showing higher prevalence values (83.6%), then the wild boar (80.2%) and the roe deer (46.7%). The results have revealed that these species carry parasites that not only represent a health problem for domestic ruminants and domestic pigs (e.g. *Muellerius* sp., Trichostrongylidae, Strongylidae, *Metastrongylus* sp., *Moniezia benedeni*, *Eimeria* spp. and *Cystoisospora* sp.) but they can also pose a potential public health risk (e.g. *Balantidium coli*). The implementation of surveillance programs must include regular monitoring protocols of wild ungulates.

Keywords: *Balantidium coli*, *Cystoisospora*, Portugal, wild ungulates, zoonoses

Over the last decades, wild ungulate populations have expanded their geographical range all across Europe (Apollonio et al. 2010, Torres et al. 2015, Carvalho et al. 2018). Populations that once suffered a decrease in number and distribution during the 19th century are now facing a great increase, which, in some cases, is leading to overabundant populations (Apollonio et al. 2010). This growth has changed the management and conservation paradigm (Apollonio et al. 2010), and recently a lot of attention has been given to the negative impacts of such uprise, particularly the damages caused to agriculture and forestry, through overbrowsing and bark stripping (Côté et al. 2004, Gill and Morgan 2010), increment of ungulate–vehicle collisions (Langbein et al. 2010, Lagos et al. 2012) and infectious diseases, which have emerged as a scientific priority (Daszak et al. 2000, Cleaveland et al. 2001, Thompson et al. 2009, Hassell et al. 2017).

The majority of infectious diseases have its origin from wildlife (Jones et al. 2008), representing a potential health risk for humans and livestock (Ferroglio et al. 2010). Moreover they can have a negative economic impact, putting at risk food security through repercussions from livestock, leading to loss of income (Böhm et al. 2007). As humans transform landscapes (Hassell et al. 2017), the consequent contact between livestock and wildlife is also intensified which is particularly concerning when there is spatial overlap between them, causing disease spillover (Böhm et al. 2007). Diseases, such as parasitic infections, can be transmitted by direct contact, through social interactions or by indirect contact, through the ingestion of contaminated water, soil, faeces or meat, accounting for a route of transmission between wildlife, livestock and human populations (Böhm et al. 2007). Migration and colonization of urban areas (Podgórski et al. 2018) have fostered the spread of diseases and their persistence in the wildlife–livestock–human interface (Machackova et al. 2003, Böhm et al. 2007). Host abundance,

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aggregation and wildlife populations health status are closely related, which will enhance the risk of infection (Böhm et al. 2007, Putman et al. 2011), facilitate disease transmission, and ultimately affect the conservation of endangered species (Gortázar et al. 2006).

In mainland Portugal, there are three native species of wild ungulates: the wild boar *Sus scrofa*, the red deer *Cervus elaphus* and the roe deer *Capreolus capreolus*. Wild ungulate expansion scenario experienced across Europe also occurred in Portugal as a result of natural re-colonization of the territory, multiple reintroductions and naturally dispersion from Spain (Vingada et al. 2010). In northeast Portugal, Montesinho Natural Park, inhabits one of the most representative and diverse wild ungulate populations in Portugal, not only because it is one of the last places where the three ungulate species co-exist (Vingada et al. 2010), but also because they are a cross-border population in close contact with the Spanish population of wild ungulates. These wild species are considered the preferred food items of the endangered Iberian wolf *Canis lupus signatus*, having an important role in its conservation (Figueiredo et al. unpubl.). Furthermore, these populations also have an important role in the epidemiology of several parasites, representing a potential risk of transmission for livestock (Santos 2015). Additionally, people living in rural communities are at higher risk of becoming infected owing to the close contact with freely roaming wild ungulates around the villages. These communities can also be indirectly affected due to livestock losses caused by parasitic infections, as animal husbandry is the main source of income in this region. However, there is still a lack of information regarding the parasitic fauna of these three wild ungulate species in this area. To fill in this gap, this study is aimed to assess the prevalence and mean intensity of parasites in the red deer, the roe deer and the wild boar populations by means of coprological analyses. Such knowledge will provide important baseline data that will aid future surveillance programs.

Material and methods

Study area

Our study was performed in Montesinho Natural Park (MNP) (41°43′–41°59′N, 6°30′–7°12′W), one of European Union's Natura 2000 Network sites (Fig. 1). The total prospected area was 35 000 ha; the landscape is characterized by the presence of mountains, ranging from 438 to 1481 m. It is a Mediterranean climate, with an annual average temperature ranging from 3°C in the coldest month to 21°C in the warmest, and precipitation between 600 and 1500 mm (Castro et al. 2010). The area exhibits a mosaic of deciduous and coniferous forest, characterized by oaks, *Quercus pyrenaica*, *Q. rotundifolia*, *Q. suber*, sweet chestnut *Castanea sativa* and maritime pine *Pinus pinaster*; shrub vegetation, dominated by heather *Erica* spp., gum rockrose *Cistus ladanifer* furzes *Ulex europaeus* and *U. minor*, and fragmented by small cultivated fields (Valente et al. 2014, Torres et al. 2015). Animal densities are 1.23 roe deer 100 ha^{-1} (Valente et al. 2014) and 5.81 red deer 100 ha^{-1} (Torres et al. 2015), but no data is available for wild boar density. Livestock density estimation throughout the study area is 0.54 bovines 100 ha^{-1}, 0.01 domestic pigs 100 ha^{-1}, 9.21 sheep 100 ha^{-1} and 0.93 goats 100 ha^{-1} (Instituto Nacional de Estatistica (INE) 2011, Direção-Geral de Alimentação e Veterinaria (DGAV), unpubl.).

Sample collection and coprological analyses

Between September 2017 and August 2018, 112 fresh faeces were collected from red deer (n = 73), wild boar (n = 24) and roe deer (n = 15) by prospecting several well-distributed transects across the study area (Fig. 1). Samples were collected based on its morphology (e.g. shape, size, content of the faeces) and deposition site, by two experienced and field-trained staff, which helps to narrow down the observer error. Collected samples were stored at 4°C up to a maximum of one month, in order to avoid degradation of parasitic forms until examination in the laboratory (Zajac and Conboy 2012).

In each collected sample, egg/larvae parasite prevalence and mean intensity was evaluated by means of one quantitative technique and four different qualitative techniques: 1) modified McMaster test (quantitative technique) and 2) Willis flotation technique, performed with saturated sugar solution and used to isolate gastrointestinal nematode/cestode eggs and coccidia oocysts (Thienpont et al. 1986, Zajac and Conboy 2012); 3) sedimentation technique with methylene blue dye to select the trematode eggs (Domínguez and de la Torre 2002); 4) modified Baermann technique with 24 h-reading (Paradies et al. 2013), was used to detect L1 lungworms nematodes (Zajac and Conboy 2012); and 5) faecal culture, for undifferentiated strongylid eggs, evolving to a third larval stage (L3), possible to identify at genus level (Zajac and Conboy 2012).

Statistical analysis

Parasite prevalence was calculated based on Bush et al. (1997), as the percentage of hosts infected by that parasite species, and the intensity of the infection as the mean number of parasite eggs or oocysts, obtained with the quantitative McMaster technique, per infected hosts (Rózsa et al. 2000). This quantitative test has a sensitivity of 50 eggs per gram (EPG) of faeces (Zajac and Conboy 2012).

Using a binomial distribution, the prevalence (proportion of infected/no infected hosts) was calculated with the 'stats' package in R software (<www.r-project.org>) with the function 'binom.test', and confidence limits were established with 95% confidence intervals (CI). χ^2-test was calculated using the same package 'stats' in R with the function 'chisq.test' to define significant differences between parasite prevalence found in at least two wild ungulate species, for a p-value $\leq$0.05. Mean intensity and range were calculated using Microsoft Excel 2018.

Results

From a total of 112 samples, 88 (78.60%, ±69.81–85.76) were infected with sixteen different helminths (15 pulmonary and gastrointestinal nematodes and one cestode) and three protozoa (Table 1). In total, 83.6% (61/73) red deer

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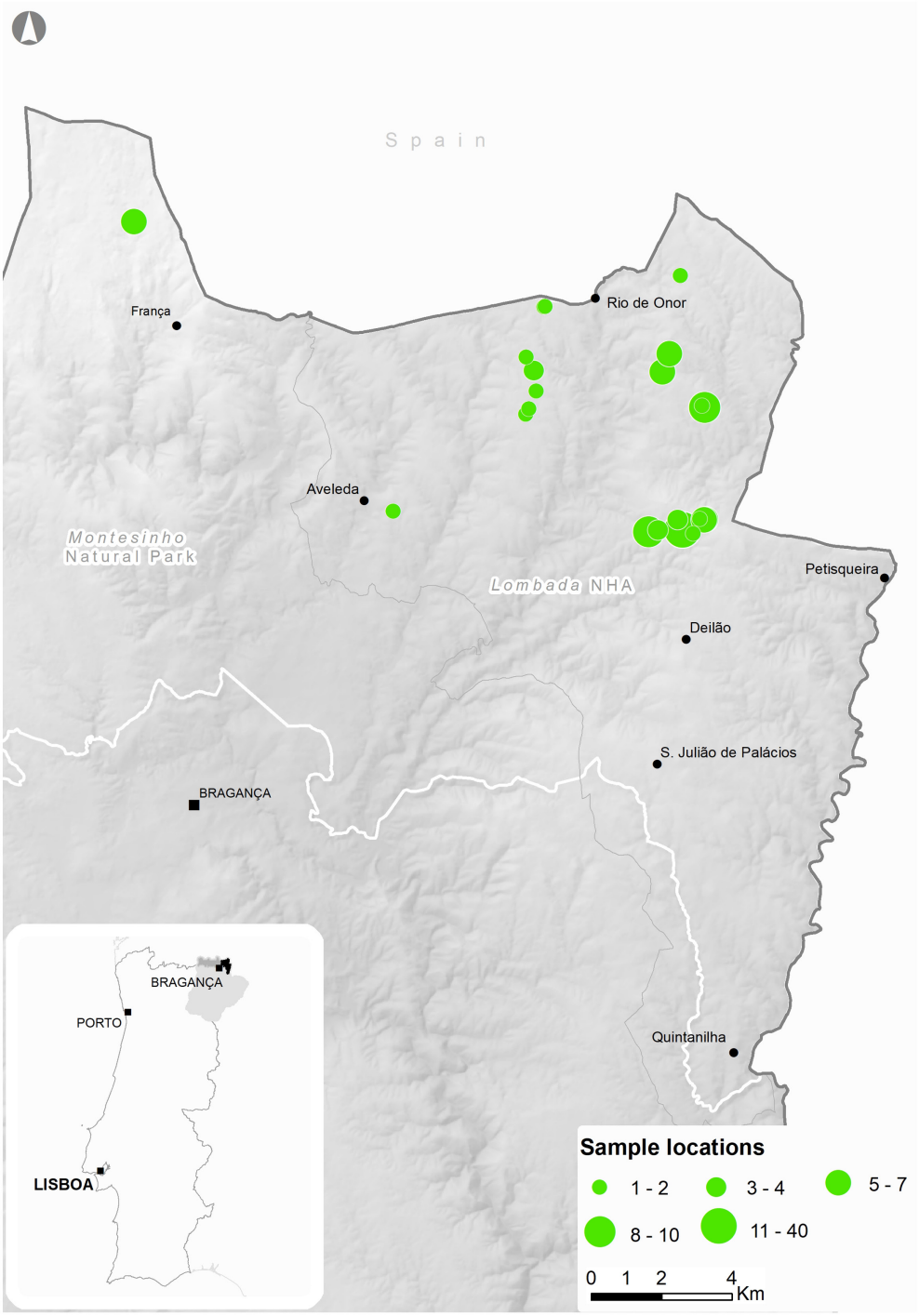

Figure 1. Location of the study area in Portugal. The green circles represent the number and location of the faeces collected from the three wild ungulates for coprological analysis (Montesinho Natural Park).

samples were positive for at least one parasite, while in roe deer and wild boar, 46.7% (7/15) and 83.3% (20/24) of the analysed samples showed parasitic forms, respectively. Mixed infections were found in 48 (42.86%) of the total analysed samples from the three ungulate species; Strongylida and *Muellerius* sp. were found in 30.14% (22/73) of red deer samples and Strongylida and *Metastrongylus* sp. in 54.17% (13/24) of wild boar samples. No commonly mixed infections were found in roe deer. Pulmonary nematodes were the parasites found with the highest prevalence among the three ungulate species: *Muellerius* sp. was found in 56.16% (41/73) of total red deer samples analysed, and in 26.67% (4/15) of roe deer, whereas *Metastrongylus* sp. was present in 66.67% (16/24) samples of wild boar.

Significant differences were calculated for *Nematodirus* sp., Strongylida, *Trichostrongylus* sp., *Muellerius* sp. and *Eimeria* sp., parasites found in at least two of the three wild ungulates. However, we only found significant differences regarding

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Table 1. Number of infected animals and parasite prevalence mean and confidence intervals (CI, 95%) found in red deer, roe deer and wild boar in Montesinho Natural Park.

Species		Red deer *Cervus elaphus*		Roe deer *Capreolus capreolus*		Wild boar *Sus scrofa*
Total samples		73		15		24
Parasites	n	% (CI 95%)	n	% (CI 95%)	n	% (CI 95%)
Gastrointestinal nemotodes						
Cooperia sp.	1	1.37 (0.03–7.40)	–	–	–	–
Globocephalus sp.	–	–	–	–	1	4.17 (0.11–21.13)
Haemonchus sp.	2	2.74 (0.33–9.55)	–	–	–	–
Hyostrongylus sp.	–	–	–	–	2	8.33 (1.03–27.00)
Nematodirus sp.	1	1.37 (0.03–7.40)	1	6.67 (0.17–31.95)	–	–
Oesophagostomum sp.	8	10.96 (4.85–20.46)	–	–	3	12.50 (2.66–32.36)
Strongylida	25	34.25 (23.53–46.28)	3	20.00 (4.33–48.09)	14	58.33 (36.64–77.89)
Teladorsagia sp.	1	1.37 (0.03–7.40)	–	–	–	–
Trichostrongylus sp.	3	4.11 (0.86–11.54)	–	–	2	8.33 (1.03–27.13)
Pulmonary nematodes						
Dictyocaulus sp.	2	2.74 (0.33–9.55)	–	–	–	–
Elaphostrongylus cervi	14	19.18 (10.90–30.08)	–	–	–	–
Metastrongylus sp.	–	–	–	–	16	66.67 (44.68–84.37)
Muellerius sp.	41	56.16 (44.05–67.76)	4	26.67 (7.79–55.10)	–	–
Protostrongylus sp.	2	2.74 (0.33–9.55)	–	–	–	–
Cestodes						
Moniezia benedeni	3	4.11 (0.86–11.54)	–	–	–	–
Protozoa						
Balantidium coli	–	–	–	–	1	4.17 (0.11–21.13)
Cystoisospora sp.	2	2.74 (0.33–9.55)	–	–	–	–
Eimeria spp.	1	1.37 (0.03–7.40)	2	13.33 (1.66–40.46)	1	4.17 (0.11–21.13)

parasite prevalence for Strongylida ($\chi^2 = 6.73$, df = 2, p = 0.034) and for *Muellerius* sp. ($\chi^2 = 6.39$, df = 2, p = 0.041).

Table 2 shows the mean intensity (eggs per gram (EPG)) and the respective parasite ranges, obtained with the McMaster quantitative technique. Strongylida was present in the three ungulate species, and the cestode *Moniezia benedeni* was the one with the highest EPG (500), showing that these and the other parasites present on Table 2 were not spurious infections found in our samples.

Discussion

By analysing the parasite community of the most widespread wild ungulates in Portugal, we confirmed the presence of several helminth species (pulmonary and gastrointestinal nematodes and cestodes) and three protozoa species. All the parasites found in the red deer and the wild boar were already described in the same species in mainland Portugal (Maia 2001, Bruno de Sousa et al. 2004, 2014, Calado 2009, Santos 2013, Bernardino 2017), except for *Teladorsagia* sp. and *Eimeria* sp., which were previously described in domestic, feral and wild goats in northwest Portugal (Peneda-Gerês National Park) (Figueiredo 2011) and in livestock e.g. sheep (Anastácio 2012). Likewise, *Cystoisospora suis* and *Eimeria* spp., to our knowledge, were only described in domestic pig on extensive regime, southern Portugal (Gomes 2009).

Comparingly, previous studies performed in Portugal have recorded higher parasite prevalences (Maia 2001, Bruno de Sousa et al. 2004, Santos 2013, Bernardino 2017), probably explained by the large proportion of samples that were collected directly from dead animals (namely from lungs, intestines, rectum). Whilst our results are based only on non-invasive sampling technique (e.g. fresh faeces collected from the ground), these findings are important to consider as they also reflect the health status of these populations, and can

Table 2. Mean intensities and ranges of parasitic excretion for the different parasitic infections found in red deer, roe deer and wild boar, expressed in eggs per gram of faeces (EPG).

Species		Red deer *Cervus elaphus*		Roe deer *Capreolus capreolus*		Wild boar *Sus scrofa*
Parasites	n	Mean intensity (range)	n	Mean intensity (range)	n	Mean intensity (range)
Gastrointestinal nematodes						
Strongylida	12	133,33 (50–450)	2	50 (50)	1	50 (50)
Oseophagostomum sp.	1	50 (50)	–	–	–	–
Hyostrongylus sp.	–	–	–	–	1	50 (50)
Haemonchus sp.	1	50 (50)	–	–	–	–
Cestode						
Moniezia benedeni	1	500 (500)	–	–	–	–
Protozoa						
Cystoisospora sp.	1	100 (100)[a]	–	–	–	–

[a] Oocysts.

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be used as a baseline for future studies. Although wild boar and red deer samples could have been collected from dead animals, since both species are hunted in this area, they are only hunted seasonally, diminishing the possibility to assess the full spectrum of the parasites that can infect these populations. Moreover, roe deer can only be hunted in a few areas in Portugal, given the narrow distribution and still increasing numbers, which may explain why no study have assessed the parasitic fauna of the roe deer in Portugal so far.

Regarding the parasite prevalence in the wild boar, lower prevalence for *Metastrongylus* spp. has been found, while higher values were found for e.g. *Globocephalus* sp. and Strongylida (Bruno de Sousa et al. 2004, Santos 2013, Bernardino 2017). In the red deer e.g. *Elaphostrongylus cervi*, and Strongylida were both found in higher prevalence by Maia (2001), Santos (2013) and Bernardino (2017); *Muellerius* sp. was the lungworm more frequently found in both the previously mentioned studies and in our study, and we did not find any other description of this parasite elsewhere in Europe. To our knowledge, this was the first description of *Cystoisospora* spp. in the red deer.

Average intensity for EPG was found with higher infection rates in Bruno de Sousa et al (2014) for the wild boar (2142 EGP) and in Bernardino (2017) for the red deer (534 EPG), compared to ours. *Elaphostrongylus cervi* larvae per gram (LPG) in the red deer was calculated for our study area and Sierra de la Culebra (Spain) by Santos (2015), and a significant intensity was found for both areas, 1079.2 ± 279.77 LPG. Although we did not calculate LPG in our study, it is important to mention it, since this is the only available data regarding ungulate parasites in our study area. Our lower EPG counts obtained for the wild boar, compared to the higher ones found by Bruno de Sousa et al (2004), may be related to our free-range animals and lower density in MNP compared with Bruno de Sousa et al (2004) study area, which was performed in a hunting enclosure with higher animal density.

All the parasites found in this study represent a potential health risk for livestock, especially for domestic ruminants and pigs (Gortázar et al. 2006), but they can also represent a significant public health risk (Schuster and Ramirez-Avila 2008), which is the case of *Balantidium coli* parasite, that was found in the wild boar. Nematodes were the most common species found among our three ungulate species, maybe because most gastrointestinal nematodes have a direct route of transmission, requiring no intermediate host. However, their life cycles do involve a partial development outside the host (Walker and Morgan 2014). On the other hand, pulmonary nematodes, like *Muellerius* sp. and *Metastrongylus* sp., that were the most prevalent among the three ungulate species, have indirect life cycles, using snails and slugs as intermediate hosts. Changes in the livestock industry from an intensive to an extensive system, coupled with the ungulate expansion, have increased the potential risk of pathogen transmission between wild ungulates and livestock, which already experience ecosystem overlapping (Gortázar et al. 2007, Hoberg et al. 2008, Putman et al. 2011). When these pathogens are found in high prevalence and intensity, they can cause great economic losses, hampering parasite control attempts in livestock (Gortázar et al. 2007, East et al. 2010). Even though we cannot be sure whether the transmission route occurred from these wild ungulates to livestock or the other way around without further studies, it is stated by Jones et al (2008) that nearly 60–80% of newly emerging infectious diseases have a zoonotic origin, and 70% are from a wildlife source. Therefore, these wild ungulate populations can be important reservoirs of parasitic infections and the increasing contact with livestock/humans can represent a potential risk for their health.

Free-ranging ungulate populations are prone to co-infections, involving normally a combination of diverse micro- and macropathogens (East et al. 2010). High pathogeny may affect individuals reproductive success since body resources are required to fight the infection, instead of directed to reproduction. Furthermore, partners with lower parasite loads are chosen for mating, ultimately affecting fitness and genetic diversity on the population (Hamilton and Zuk 1982, East et al. 2010). Despite recognizing that these populations are growing and expanding (Valente et al. 2014, Torres et al. 2015, Valente et al. unpubl.) it is mandatory to continue monitoring them since the risk of a stochastic event that may jeopardize the population or subpopulations cannot be discarded.

Conclusion

All the parasites found in our study represent a potential health risk for livestock, namely to domestic ruminants and pigs. Additionally, *Balantidium coli*, which was found in the wild boar, may constitute a significant health risk for human populations, particularly when the wild boar is considered a link between natural and humanized areas. The implementation of wildlife disease monitoring will allow to identify hotspots of diseases in order to minimize parasitic transmission to livestock and humans. Furthermore, to understand the role of parasitic infections in natural systems, especially at a local level, baseline data is vital. We suggest that surveillance programs on wild ungulates must also include regular monitoring protocols, considering the density-dependent relationship with parasitic infections.

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Author contributions – AMF: conceptualization, investigation, formal analysis, writing – original draft, writing – review and editing. AMV: investigation, writing – review and editing. CF: writing – review and editing, resources, supervision, funding acquisition. LMC: writing – review and editing, resources, supervision, funding acquisition, methodology, validation. RTT: conceptualization, writing – review and editing, supervision, methodology, validation.

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