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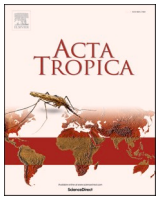

West Nile fever upsurge in a Greek regional unit, 2020

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ABSTRACT

During the 2020 West Nile virus (WNV) transmission season, Greece was the most affected EU Member State. More than one third of human cases occurred in Serres regional unit in northern Greece, which is characterized by the presence of a major wetland (Kerkini lake and Strimon river). A total of 2809 *Culex pipiens* mosquitoes collected in Serres were grouped into 70 pools and tested for WNV. Ten (14.3%) pools were found positive, and all WNV sequences belonged to the Central European subclade of WNV lineage 2. The first human case occurred in a village nearby the lake, and all following cases occurred across the connected river and its tributaries. Similar distribution presented the sites where WNV-positive mosquitoes were detected. The number of *Culex* spp. mosquitoes per trap per night was higher in 2020 than in previous years (2017-2019). The spatial and temporal distribution of human cases and WNV-positive mosquitoes in 2020 in Serres regional unit suggest that the upsurge of the virus circulation was probably related with factors that affected the ecosystem of the wetland.

West Nile virus (WNV, *Flaviviridae* family) infection in humans causes an asymptomatic or mild illness to severe neuroinvasive disease (WNND). The virus circulates in nature between birds and ornithophilic mosquitoes (mainly of the *Culex* genus) (Campbell et al., 2002). Therefore, the life cycle of the virus relies on the abundance of competent mosquitoes and susceptible birds, suggesting that the emergence and the following incidence of WNV infections are driven by several biotic and abiotic factors. Wetlands where migrating birds rest and mosquito vectors are abundant are considered hotspots for WNV introduction (Jourdain et al., 2007).

WNV emerged in Greece in 2010 and caused a large outbreak of WNV infections in humans; most cases occurred close to Axios river delta, a major Mediterranean wetland (Ramsar site no. 59) in Central Macedonia region (NUTS 2) in northern Greece (Papa et al., 2010). WNV lineage 2 sequences from *Culex pipiens* showed that the Greek strain (Nea Santa-Greece-2010) clustered within the Central European clade of WNV lineage 2 (Papa et al., 2011; Papa et al., 2013). Soon after, the virus was isolated from a blood donor who was living in a village located between two lakes in northern Greece (Ramsar site no. 57) (Papa et al., 2012). Since then, cases were recorded every year (except 2015 and 2016) with different geographic distribution. The year 2018 was a record year for human WNV infections in Greece (along with other EU countries), with 317 reported cases, including 243 WNND cases (Pervanidou et al., 2020).

During the 2020 transmission season, 316 human cases of WNV infection, including 37 deaths, were reported by EU Member States to ECDC (European Centre for Disease Prevention and Control (ECDC), 2020). Greece was the most affected country with 144 reported human cases (116 WNND, 23 fatal), and all, except two, occurred in the northern part of the country (Hellenic National Public Health Organization, 2020). Especially Serres, was the most affected regional unit (NUTS3 level), with 56 cases of WNV infection, including 42 WNND cases, which represent 55.2% of the WNND cases in Central Macedonia region, and 36.2% of the total number of WNND cases in Greece (Hellenic National Public Health Organization, 2020). Such high incidence was never observed previously in Serres; even in 2018 (WNV record year) only three cases were reported in this area (Hellenic National Public Health Organization. Available at: https://eody.gov.gr/en/epidemiological-statistical-data/annual-epidemiological-data/). The overload on the health care system and the laboratories was even worse in 2020 as it coincided with the COVID-19 pandemic.

Serres regional unit (population reaching 175,000) is characterized

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Table 1
. Detection of West Nile virus in humans and mosquitoes per municipality of Serres regional unit, 2020.

Municipality	No WNND cases	No WNND cases per 100.000*	No Non-WNND cases	No *C. pipiens*	No Pools	No Positive pools	Detection rate
Sintiki	11	49.6	3	782	22	4	18.2
Irakleia	8	37.8	5	649	17	4	23.5
Serres	10	13.0	3	588	13	1	7.7
Visaltia	6	30.0	2	731	16	1	6.3
N. Zichni	2	16.1	0	9	1	0	0.0
Amphipoli	3	32.7	0	0	0	0	0.0
E. Pappa	2	13.6	1	50	1	0	0.0
Total	42	23.8	14	2809	70	10	14.3

* Population data (2011 census) from Hellenic Statistical Authority (EL.STAT.)

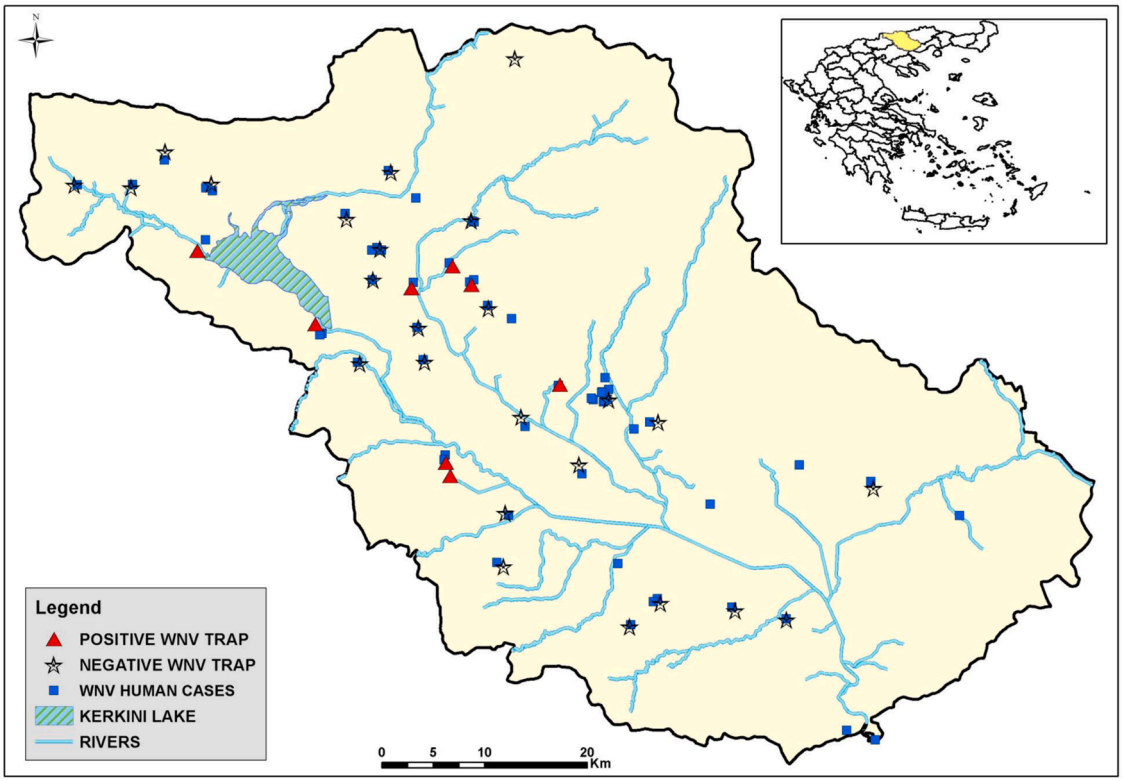

Fig. 1. Spatial distribution of WNV-positive mosquito pools and probable places of human infections, Serres regional unit, 2020.

by the presence of a major wetland complex. Strimon River flows from Bulgaria throughout the valley of Serres, and empties into the Aegean Sea, and, together with the related artificial Kerkini lake (area 10,996 hectares), constitute one of the ten wetlands of international importance in Greece (Kerkini lake and Strimon river, Ramsar site no. 58). The valley of Serres is one of the largest irrigated arable lands in Greece, with a variety of crops (mainly wheat, barley, corn, cotton, and rice). Aim of the present study was to investigate the spatial and temporal distribution of WNV human cases and WNV-positive *C. pipiens* mosquitoes in Serres regional unit and discuss the probable factors that might contributed to the WNV upsurge in the area.

The exact date of symptoms' onset and the probable place of patients' infection were identified through telephone interviews of the treating physicians and the patients (or close relatives) by the Vector-borne Diseases Department of the Hellenic National Public Health Organization (NPHO). Week numbers were assigned using the International Organization for Standardization (ISO) 8601 standard (EpochCoverter, 2020). Human cases of WNV infection were recorded in 2020 in all seven municipalities of Serres regional unit, but the highest incidence was observed in the municipalities of Sintiki and Iraklia (Table 1) (Hellenic National Public Health Organization, 2020). Kerkini lake is shared by these two municipalities. The location of residence and work of the patients was nearby wetlands, streams, canals, or rice fields with permanent presence of water (Fig. 1).

The first human case was reported in a village at the east side of Kerkini lake on July 22, 2020, with neurological symptoms onset on July 10, 2020 (week 28/2020). Additional cases were recorded close and around the lake, while the following cases presented a southward dispersion across the Strimon river route. The date of symptoms' onset of the patients ranged from week 28 to week 40, and the week numbers are depicted in Fig. 2. The peak of cases occurred in weeks 31-33. Up to week 36, cases were already spread nearby the river estuaries, while the last case of the year was reported in week 40.

During May-September 2020, a total of 2,809 *C. pipiens* mosquitoes, collected using CO_2 light traps at 33 sites of Serres regional unit, were transported in dry ice to the National Reference Centre for Arboviruses for WNV testing. Especially three sites (Lithotopos, Kerkini and Ampeloi) were being tested at 2-week interval basis starting from week 21 to week 39. The coordinates of the collection sites were recorded using a GPS device. The location of human cases and the WNV-positive and

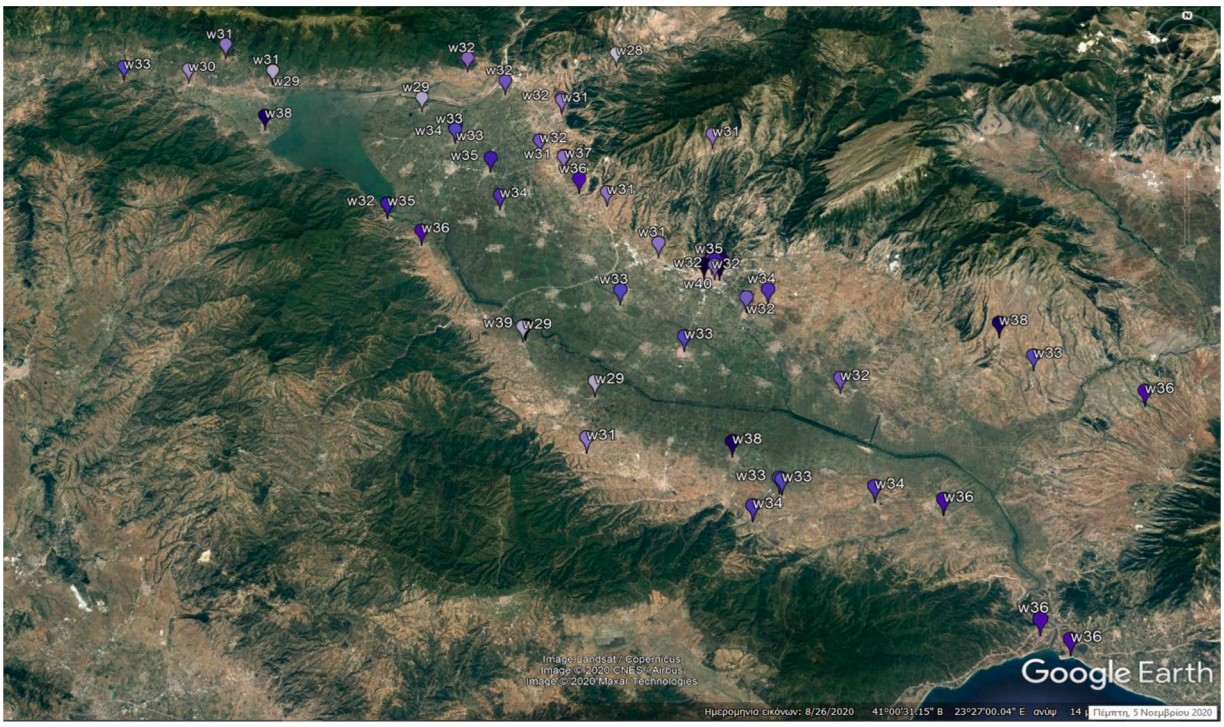

Fig. 2. Temporal distribution of WNV human cases, Serres regional unit, 2020; the number of week (w) when the onset of patients' symptoms occurred are shown (source: Hellenic National Public Health Organization).

WNV-negative mosquitoes were mapped using the geographic information system (GIS) technology (ArcPro 2.7 software). Based on the date and site of collection, mosquitoes were grouped into 70 pools (range 3-50 mosquitoes per pool). RNA was extracted from mosquito pools using the RNeasy Mini kit (Qiagen GmbH, Hilden, DE), and WNV RNA was amplified using a commercial real-time RT-PCR (RealStar WNV RT-PCR Kit 2.0, altona Diagnostics GmbH, Hamburg, DE). WNV positive mosquitoes were further tested by a nested RT-PCR (Papa et al., 2012), and PCR products were sequenced. WNV was detected in 10 pools (detection rate 14.3%) (Table 1). All sequences belonged to WNV lineage 2 (Fig. 3). The sequences were submitted to the GenBank database and received the accession numbers MZ333220-MZ333226. For comparison, the detection rates in the rest six regional units of Central Macedonia in 2020 ranged from 0% to 2.5%. The first WNV positive pools were detected on July 23 and 29 in two villages located at the southern corner of Kerkini lake. The highest detection rates were observed in the municipalities where the highest incidence in humans was recorded (Table 1). Regarding the three regularly tested sites, WNV positive mosquitoes were first detected on July 29 (Lithotopos, week 31) and August 10 (Kerkini and Ampeloi, week 33). Human cases were recorded in two of these sites (Lithotopos and Kerkini), one and five weeks later, respectively, suggesting that the regular mosquito testing can serve as early warning system. Since these sites are regularly tested every year, a comparison on mosquito abundance showed that the number of *Culex* spp. mosquitoes per trap per night was higher in 2020 than in previous years (2017-2019) (Fig. 4).

The 2020 WNV transmission season in Serres presented a clear difference with what happened in previous years. The first cases in 2020 in Serres occurred in the eastern part of Kerkini lake which supports the largest mixed nesting colony of water birds in Greece, including numerous species of migratory birds (https://rsis.ramsar.org/ris/58). Bird migration begins in early March and lasts up to early May (https://birdwing.eu/sites-of-northern-greece/lake-kerkini). This was the time period of the lockdown during the first wave of COVID-19 in Greece (23 March to 4 May). A recent study in USA showed that the measures taken during the lockdown in March and April of 2020 had an effect on birds that rely on sound for survival and reproduction as the decrease of the auditory pressure improved the song performance and communication of urban white-crowned sparrows (Derryberry et al., 2020). Since Kerkini lake is one of the most touristic peri-urban wetlands in Greece, it attracts numerous visitors for bird watching and other leisure activities (boating, canoeing, riding, cycling, hiking). To control the COVID-19 outbreak in Greece, the human mobility was restricted during the lock down, leaving quiet places in the wetland, thus, a favorable environment for the birds.

In addition, the travel out of the regional units was prohibited during the lockdown, and even after the lockdown, the leisure trips were limited due to the fear of contracting COVID-19. Thus, several country houses remained closed with gardens neglected for a long time, while the mosquito control company had no access to the backyards and gardens, neither to the cesspools of private households to perform larvicide and/or residual adulticide spraying applications during their door – to door campaigns. It has to be noted that in most settlements in Serres (25/42, 59.5%) where human cases occurred, the sewage disposal is still performed through cesspools, which are known breeding sites of *C. pipiens*.

Further studies, e.g. correlation analysis combined with bird population studies, could estimate the exact impact of the potential anthropogenic drivers. Citizen science data about mosquitoes and birds could also assist the modelling and prediction of risk for WNV infections (Eritja et al., 2019; Kain and Bolker, 2019). The spatial and temporal distribution of WNV human cases and WNV-positive *C. pipiens* mosquitoes in Serres regional unit in 2020, combined with the higher *Culex* spp. populations, suggest that the upsurge of WNV infections was probably related with factors that affected the ecosystem of the wetland. A further study on phylogeography of the virus based on whole genome sequences will provide information on whether the WNV strain in Serres was a new introduction or evolution of previously circulating strains in Greece.

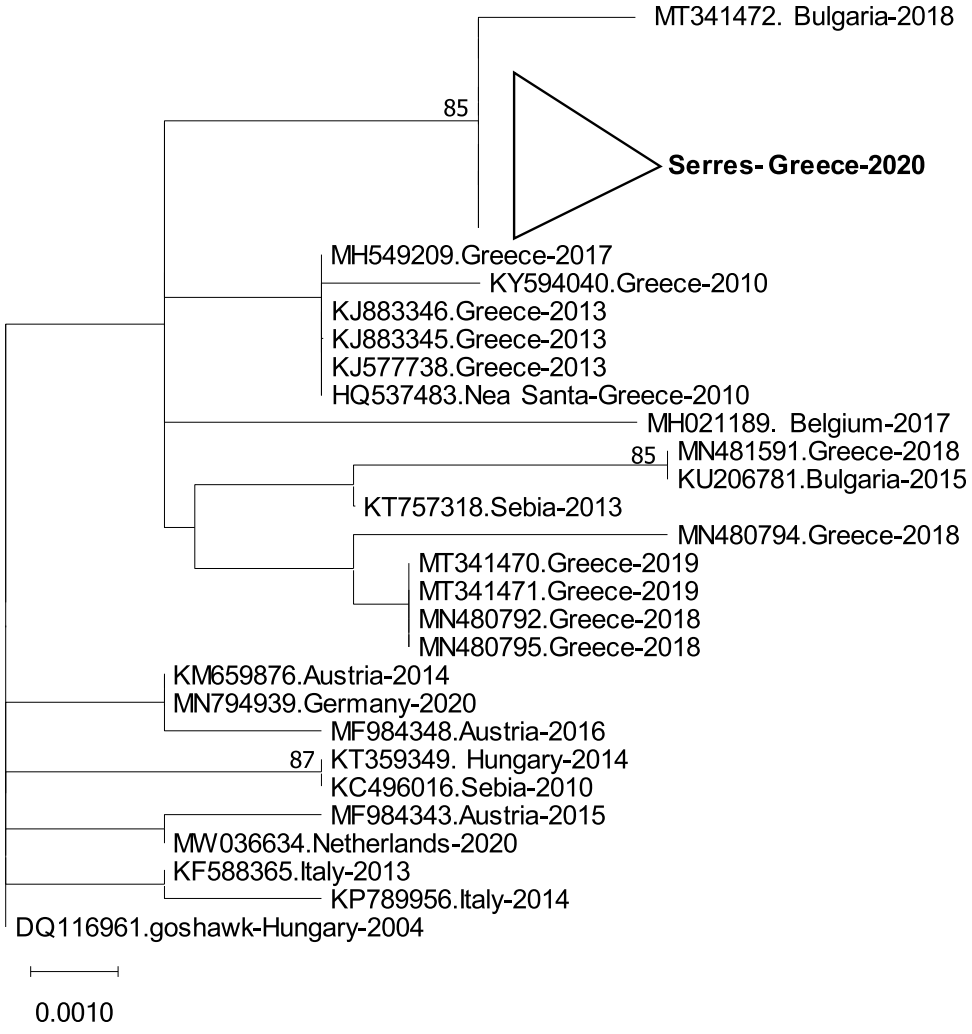

Fig. 3. Maximum Likelihood phylogenetic tree based on partial sequences of NS3 protein gene of West Nile virus. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale. The evolutionary distances were computed using the Kimura 2-parameter method. Sequences from Serres obtained in 2020 are shown with a triangle. Sequences are shown as Accession number, strain, country, year. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018)

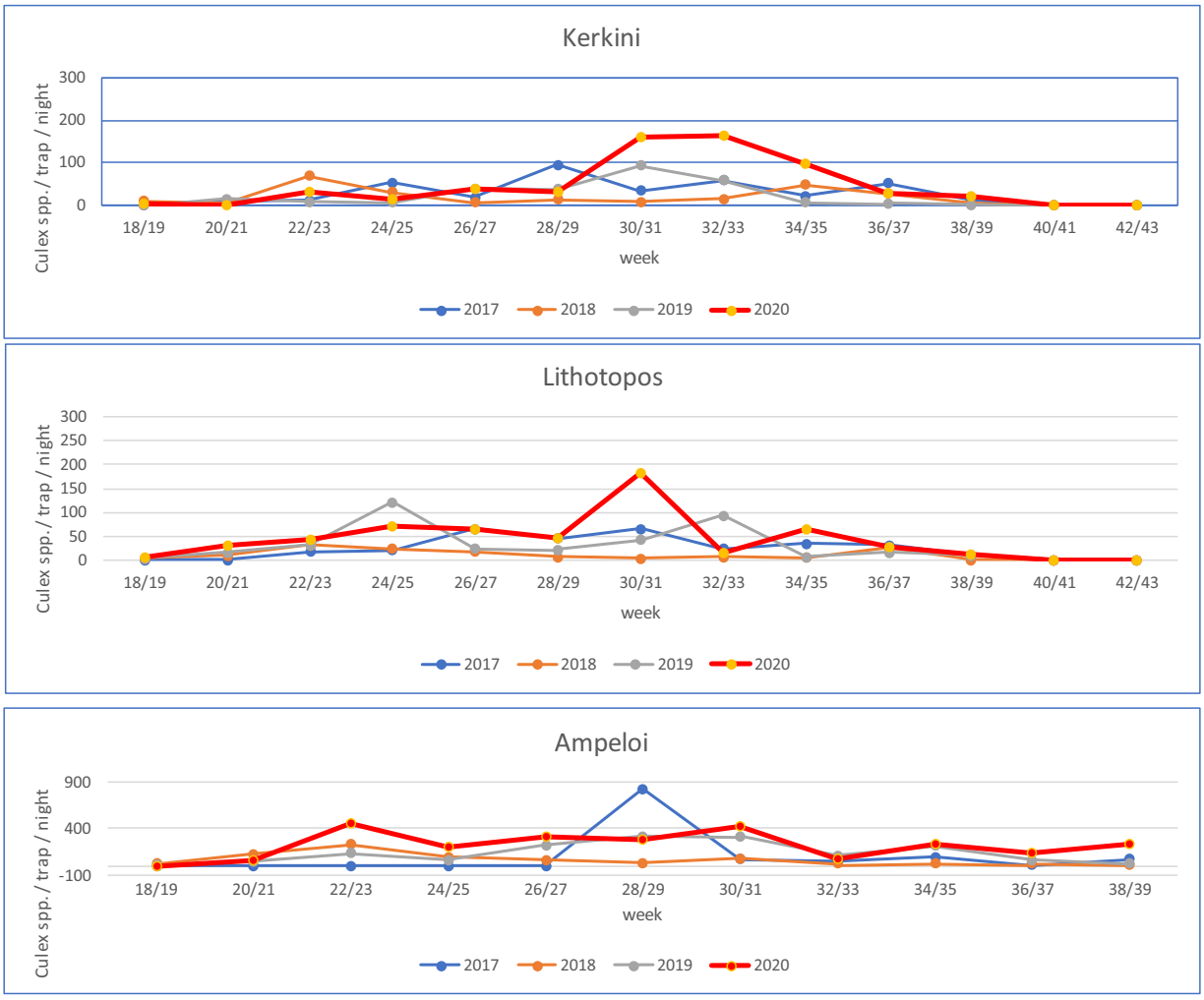

Fig. 4. Number of *Culex* spp. mosquitoes per trap per night in three sites (Kerkini, Lithotopos, Ampeloi) in Serres regional unit, 2017-2020.

CRediT Authorship contribution statement

Anna Papa: Conceptualization, Supervision, Validation, Writing original draft. Katerina Tsioka: Methodology. Sandra Gewehr: Investigation, Data curation. Stella Kalaitzopoulou: Investigation, Visualization. Danai Pervanidou: Investigation, Validation. Anna Vakali: Investigation, Visualization. Chrysovalantou Kefaloudi: Investigation, Visualization. Styliani Pappa: Methodology. Xanthoula Louka: Data curation. Xanthoula Louka: Data curation. Spiros Mourelatos: Conceptualization, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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