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Hepatitis C Virus (HCV) genotypes distribution among hepatocellular carcinoma patients in Southern Italy: a three year retrospective study

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the major cause for cancer in the world. Aim of this case-control study was to investigate the distribution pattern of HCV genotypes among HCC patients and suggest whether infection with specific subtypes may be associated with an increased risk of progression to cancer.

Methods: 152 HCC anti-HCV positive patients, fulfilling the criteria from the Barcelona 2000 EASL conference, and 568 patients HCV chronically infected but without HCC as control group were included in the study. Serum of each patient was evaluated for viral load estimation and genotyping.

Results: Males with HCC significantly showed to have quite 2 times higher risk of exposure to HCV infection (OR = 1.72; 95% CI = 1.15–2.58). Moreover, HCC was significantly associated with older age. In fact, > 50 years older patients showed to have a higher risk of developing HCC (OR = 17.4; 95% CI = 4.24 to 71.36) compared to younger patients.
HCV RNA rate was significantly higher (83.7%) among HCC patients than in the control group (61.4%, $p < 0.001$) and the most prevalent genotype was 1b (68.0% in HCC vs 54.4% in the control group, $p < 0.005$). HCC patients significantly have a risk of exposure to HCV 1b infection almost 2 times greater than the control group (OR = 1.8; 95% CI = 1.11–2.82). The multivariate-adjusted OR (95% CI) of developing HCC for HCV 1b comparing to non-1b was 1.65 (1.16–2.33).

Conclusions: Our study detected a significantly higher rate of HCV RNA positivity and a higher rate of HCV 1b infection in HCC patients, suggesting the strict association between subtype 1b infection and HCC. A prospective study with larger number of samples would be needed to confirm our results.

Keywords: Hepatocellular carcinoma, HCV, HCV genotypes, Italy, Risk factors, Liver cancer, Viral load

Background

Hepatocellular carcinoma (HCC) is one of the most common causes of morbidity worldwide [1–3], accounting for about 7% of all cancers [4] and over 80% of primary liver cancer [5, 6]. A recent estimate indicates that it is the fifth and the seventh most common cancer in males and females respectively [2], with approximately one million deaths per year, especially in developing countries [7, 8] and represents one of the major dreaded complication of chronic liver disease, frequently associated with compensated cirrhosis. It has been reported that approximately 3–4% of HCV chronically infected patients with underlying cirrhosis will develop HCC on average 30 years after infection [9–11].

Globally, three main epidemiological zones have been defined according to the age-adjusted HCC incidence per 100,000 inhabitants per year: low (< 5%), intermediate (5% to 15%), and high (>15%) [12], with a geographical distribution that varies throughout the world, ranging from 2.1 per 100,000 in Central America to 35.5 per 100,000 in Eastern Asia [13].

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It has been suggested that a strict geographical correlation exists between the incidence of HCC and the prevalence of chronic HBV infection (especially in Eastern Asia and South East Asia where the incidence is more than 8%) and HCV infection, suggesting that these two viral infections could be the most important risk factors associated with HCC [8].

In countries where HCV infection is endemic (Japan or some areas of Southern Italy), a high prevalence of HCV infection has been reported among people with HCC [14, 15]. Several studies found a three-fold increase in HCV-related HCC, whereas the rate of HCC associated with HBV or alcohol-induced liver disease and idiopathic cirrhosis remained stable [2, 16].

It has been suggested that incidence of HCC is expected to significantly increase in the next decades, although the incidence of newly acquired HCV infections has been gradually decreasing [17]. The rate of progression from chronic hepatitis to HCC is variable and numerous factors have been identified as important predictors of progression, some related to the host (older age, longer duration of infection, male sex or alcohol consumption >50 g/day), others to environment (viral genotype/subtype or viral load) [2, 18, 19]. While alcohol and other risk factors are proposed to cause HCC because they cause cirrhosis, the carcinogenic role of HCV is still controversial, since HCV infection has been also described in HCC patients without cirrhosis [20–22].

HCV is characterized by a high degree of heterogeneity. At present, it is classified into seven recognized genotypes on the basis of sequence of the viral genome [23–26], each differing at 30–35% of nucleotide sites [27]. The geographic distribution of HCV genotypes is rather complex [28]. The so called "epidemic subtypes" —specifically 1a, 1b, 2a, and 3a— are widely distributed worldwide and account for a great proportion of the totality of HCV cases, especially in high income countries [29–32]. The so called "endemic" strains, instead, are comparatively more rare and have been restricted for long time in specific regions, as West Africa, Southern Asia, Central Africa and South Eastern Asia [33, 34].

If considered in an international context, Italy shows a moderate prevalence of anti-HCV in the population, except for the Southern regions where the infection is endemic and the HCV prevalence ranges from 6% to 12% [35, 36]. As we previously described, genotype 1b, historically the most prevalent not only in Italy but in the whole of Europe, is the most common genotype also in Southern Italy, followed by genotype 2 [37, 38].

Despite several published studies showing that patients infected with HCV genotype 1b may have a higher risk of developing HCC than those infected with other genotypes [39–41], other authors did not confirm this result [42, 43]. As a consequence, no consensus has yet emerged and the role of HCV genotypes in both accelerating the progression of the disease and as a risk factor for HCC remains to be established.

The aim of this case-control study was to investigate the distribution pattern of HCV genotypes in HCC patients and identify whether infection with specific HCV genotypes may be associated with an increased risk of HCC development.

Methods

Study population and sample collection

A total of 152 HCC cases (105 males and 47 females, with a Male/female ratio of 2.23:1), all coming from different regions of Southern Italy and collected during the period between February 2012 and November 2014, were recruited among patients referred to the Hepatobiliar and Pancreatic Unit, Department of Surgical Oncology, Istituto Nazionale Tumori – IRCCS, Fondazione "Pascale", Naples, Italy and Section of Infectious Diseases, Department of Public Health, Università "Luigi Vanvitelli", Naples, Italy. The diagnoses of HCC and chronic hepatitis were based on the criteria from the Barcelona 2000 EASL Conference and from the recommendation of AASLD 2009 updated guidelines [44]. Majority of these patients were ≥50 years (98.7% with a mean age of 73 years) and 95.4% of them had underlying cirrhosis at presentation and 83.5% serum level of alpha-fetoprotein exceeding 6 ng/mL. Cirrhosis was diagnosed based on morphological and clinical criteria, as well as ultrasound or Computed Tomography (CT), according to standard definitions [45]. All 152 patients were tested for HCV-RNA and only anti-HCV/HCV-RNA positive patients (103/152) were selected and used for the genotype characterization (73 males and 30 females, with a Male/female ratio of 2.4:1). Among them, the percentage of untreated and treated patients (with IFN therapy) were similar (48% and 52%, respectively). No sustained virological response (SVR) was described among treated patients.

Five hundred sixty eight age matched patients (320 males and 248 females, with a Male/female ratio of 1.30:1) chronically HCV infected but without HCC diagnosis were collected in the same period from the Virology Ambulatory, IRCCS "Fondazione G. Pascale", Naples, Italy and Section of Infectious Diseases, Università "Luigi Vanvitelli", Naples, Italy and used as control group. Majority of these patients were ≥50 years (81.1%, with a mean age of 65 years). Three hundred fourty nine of them (61.4%) that showed HCV-RNA positivity were selected and subsequently used for the characterization of the genotype. The percentage of untreated and treated (with IFN therapy) patients were similar (46.5% and 53.5%, respectively). Only 9% of the treated patients showed a sustained virological response (SVR).

No DAA treated patients were selected in the HCC and control groups.

The demographic details and risk factors of the two groups are described in Table 1. A large number of HCC patients (48.0%) and of control subjects (45.9%) were in the 50 to 70 years age group, while younger patients (<50 years) were most common among control subjects (18.9 vs 1.3%) and older ones (>70 years) among HCC patients (50.7 vs 35.2%).

Alcohol intake was considered significant in case of consumption of about 1 glass of wine/ day (approximately 8 g/day for men and 6 g/day for women for at least 5 years) while cigarette consumption if over 15 cigarettes/day for almost 5 years. No alcoholic patients were included in the study. Analysis of the main modality of infection (surgery, dental therapy, blood transfusion or sexual intercourses) showed no significant differences between the two groups (data not shown).

Only patients negative for HIV, HBV and HDV infections and with no clinical or serological sign of other chronic liver disease (autoimmune disease, non-alcoholic steatohepatitis, etc.) were enrolled in both groups.

A written informed consent was obtained from all the subjects and the study protocol, conformed to the ethical guidelines of the declaration of Helsinki was approved by the Ethics committee of our Institute.

Table 1 Baseline characteristics and risk factors of HCC and control group patients

Characteristics	HCC ($n = 152$)		Control Group ($n = 568$)	
	n.	%	n.	%
Male	105	69.1[A]	320	56.4[A]
Female	47	30.9	248	43.6
M/F ratio	2.23		1.30	
Median BMI (range) kg/m^2	26 (21–32)		25.5 (20–38)	
Mean Age (range), years	73 (55–91)		65 (45–85)	
< 50 years	2	1.3	107	18.9
50–70 years	73	48.0	261	45.9
> 70 years	77	50.7[B]	200	35.2[B]
Alcohol consumption*	74	48.7	289	50.8
Cigarettes smoking^	60	39.5	201	35.4
Serum levels of AST (> 45 U/L)	94	61.8	352	61.9
Serum levels of ALT (> 45 U/L)	89	58.5	325	57.2
ALP levels (> 250 U/L)	71	46.7	261	45.9
Albumin levels (< 3.5 g/L)	81	53.3	280	49.2
Bilirubin levels (> 1.2 mg/dL)	91	59.8	301	52.9

*Alcohol intake was considered only in case of consumption of 8 g/day by men and 6 g/day by women for at least 5 years

^ Cigarette consumption was considered significant if over 15 cigarettes/day for almost 5 years

[A] = χ^2:7.06; $p < 0.01$; OR: 1.72; 95% C.I = 1.15–2.58

[B] = χ^2:12.08; $p < 0.001$; OR: 1.88; 95% C.I = 1.31–2.71

Blood samples for serological and molecular analysis were collected and all the participants were interviewed using a questionnaire including biochemical, clinical and risk factors information.

Serological analysis

All the plasma samples from the HCC patients and the control group were analyzed for estimation of serum levels of liver function tests (albumin, bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and a-fetoprotein), using a Cobas c-501 automated immunoassay system (Roche Diagnostic) and the presence of anti-HCV antibodies by means of the Vitros-ECi test (Ortho Clinical Diagnostics), used according to the manufacturers' instructions. The Ortho-Clinical Vitros ECi test employs chemiluminescent technology and results are reported as signal-to-cut off (S/Co). Results greater than or equal to 1.00 were considered reactive for HCV antibodies. Specificity and sensitivity of the test were, respectively, 99.97 and 100%.

Nevertheless what we previously described [46] and the CDC recommendations [47] stating that anti-HCV positive samples with S/Co ratios of ≥8.0 can be reported as positive without further supplemental testing, we decided to include in the study only repeatedly anti-HCV reactive samples with S/Co ratios of ≥8.0 confirmed by a third generation immunoblot assay Innolia HCV Score (Fujirebio Europe N.V.) .

HCV molecular analysis

Only anti-HCV/RIBA HCV positive samples were subsequently tested for the presence of HCV-RNA and its quantification was performed via COBAS Ampliprep/ Taqman HCV 48 (Roche Diagnostics System Inc.), which exploits a polymerase chain reaction in Real time (RT-PCR). Linear range of quantification of the test was 1.50 E + 01 to 6.90 E + 07 HCV RNA IU/mL, using the accuracy acceptance criterion of +/− 0.3 $\log^{10}$. Specificity of the test was 100% and its detection limit was 15 IU/mL.

HCV genotyping was performed using the Versant HCV Genotype Assay 2.0 LiPA test (Siemens Healthcare Diagnostics), which involves the amplification and hybridization of viral genome fragments, the latter by means of genotype-specific probes adsorbed onto nitrocellulose strips. The various steps were performed by Auto-LiPA, a fully automated system for complete genotyping. Specificity and sensitivity of this test are, respectively, 96% and 99.4%, and its detection limit is 15 IU/mL.

The limitations of the test LiPA are well known, especially concerning its inability to correctly discriminate the subtypes 2a and 2c of the genotype 2 and subtype 1c of genotype 1, as demonstrated using a restriction fragment length polymorphism (RFLP) analysis [48].

Anyway, its degree of specificity is similar to that of RFLP analysis and, as previously reported, comparable to that of the direct sequencing test Trugene, with an accuracy of 76 and 74%, respectively, even though the InnoLiPA confirms its less subtype discriminating power in subtyping genotype 2 [49, 50].

Statistical analysis

For the case control study, the odds ratio (OR) with 95% confidence interval for the risk factors of HCC were calculated by logistic regression using the SAS statistical package. Statistical analysis of the data was performed using SPSS for Windows, version 16. The data are presented as mean and standard deviation and categorical variables in absolute number and percentage. Frequency tables were analysed using the χ^2 tests with the Pearson correlations being used to assess the significance of the correlation between the categorical variables. In all tests, *p*-values <0.05 were regarded as statistically significant. A two-proportion hypothesis test was applied to correct any variability between the studied groups.

To further evaluate the independence of HCV genotype on the risk of HCC, the multivariate OR (95% CI) was examined in relation to HCV genotype (1b and non-1b) and IFN responder and not responder patients.

Results

As shown in Table 1, the main risk factors (alcohol consumption and cigarette smoking) and liver function parameters (AST/ALT, albumin, bilirubin, and ALP levels) did not show significant variations between HCC and the control group. Even if our population did not include alcoholic patients, we also analysed our data excluding patients with alcohol intake and no significant differences were observed (data not shown).

A significantly higher rate of males (105/152), instead, was shown among patients with HCC (69.1%) respect to the control group (320/568, 56.4%) (χ^2:7.06; $p < 0.01$). Males with HCC showed to have quite 2 times risk of exposure to HCV infection (OR = 1.72; 95% CI = 1.15–2.58).

In addition, a significantly higher rate of older patients was observed in the HCC group both considering the threshold age at 50 years (150/152; 98.6% in HCC group and 461/ 568; 81.1% in the control group, χ^2: 28.65; $p < 0.001$) and at 70 years (77/152; 50.7% in HCC group and 200/568; 35.2% in the control group, χ^2:12.08; p < 0.001) (Table 1). This finding suggests that 50 years old patients had 17 times higher risk of developing HCC (OR = 17.4; 95% CI = 4.24 to 71.36) and 70 years old patients about 2 times higher risk (OR 1.88; 95% CI = 1.31–2.71). These data were also confirmed by using the two-proportion hypothesis test, Z test (data not shown).

Specific HCV RNA positive rate was found significantly higher (83.7%) among HCC patients (103/152) if compared to the control group (349/568; 61.4%) (χ^2: 12.49; $p < 0.001$), suggesting that HCC patients have a risk of active infection 1 and half times higher than patients without HCC (OR:1.31; 95% C.*I* = 0.90–1.92) (Fig. 1).

No significant differences in viral load (>2.0 E + 05 IU/mL) was found between HCC patients and the control group (Table 2).

The most prevalent genotype in HCC patients was 1b found in 70/103 HCC patients (67.9%) vs 190/349 (54.4%) in the control group (χ^2: 7.33 p < 0.001). HCC patients have a risk to be infected by genotype 1b quite 2 times greater than patients of the control group (OR:1.77;95% C.*I* = 1.11–2.82) (Table 3) and after adjusting for age and sex, patients HCV 1b infected showed a similar fold risk of HCC (OR:1.65; 95% C.*I* = 1.16–2.33), if compared to those with HCV non-1b infection, showing that HCV 1b subtype may be an independent risk factor for HCC (Table 4).

In order to completely remove the potential confounding effect of SVR, a separate multivariate analysis excluding patients who had achieved SVR was also provided. In this analysis, genotype 1b remained independently associated with HCC development (data not shown).

Conclusion

Hepatocellular carcinoma (HCC), with its incidence of more than 5% of all the cancers globally, is the sixth most common cancer and the third leading cause of cancer-related deaths worldwide [6, 51], especially in Japan and Eastern Asia where the age-adjusted annual death rate due to primary liver cancer has increased from approximately 10 per 100,000 in 1975 to 27.5 in 2002, probably for the increased diffusion of HCV infection [52].

Despite of the recent therapeutic efforts, HCC still remains one of the higher malignant neoplasia, with an

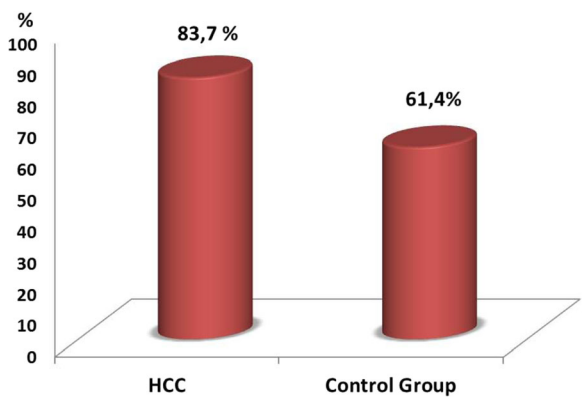

Fig. 1 HCV- RNA prevalence among HCC patients and Control group. χ2: 12.49; $p < 0.001$; OR:1.31; 95% C.*I* = 0.90–1.92

Table 2 HCV RNA viral load among HCC and control group patients

HCV RNA (IU/mL)	HCC ($n = 103$)		Control group ($n = 349$)	
	n.	%	n.	%
< 2.0 E + 10^5	24	23.7	88	25.2
2.0 E + 10^5 - 6.0 E + 10^5	17	16.5	67	19.2
> 6.0 E + 10^5	62	59.8	194	55.6

average survival rates of less than 1 year. Such a high mortality seems to be related to the fact that the majority of HCC patients are generally diagnosed at an advanced stage of the disease, especially for the complexity of the prevention and early detection [18]. It is clear, thus, how the identification of prognostic factors associated with tumor development may be extremely important for the timely referral of patients eligible for curative treatment in order to prevent HCC development.

HCV infection is well known globally as one of the main risk factor in HCC development [6, 53] and the recent increase of the incidence of this neoplasia, especially in developed countries, like the United States, is probably related to a substantial increase in HCV circulation [54]. The rate of HCC progression varies greatly among patients with chronic HCV infection and this is probably due to the existence of a complex interplay between host, viral and environmental factors [55], including older age, male gender, alcohol intake and HCV infection [2, 9, 18].

Our study confirms that males with HCC significantly show to have quite 2 times risk of exposure to HCV infection (OR = 1.72; 95% CI = 1.15–2.58) and, furthermore, that older patients have 17 times higher risk of developing HCC if older than 50 years and about 2 times higher risk of developing HCC if older than 70 years, supporting the idea that HCC is age-related and that older age acts as independent factors in HCC development [2, 18, 56, 57], probably because malignant transformation usually occurs after two or more decades from the onset of HCV infection [18]. Instead, no correlation was found between the HCC and the control group regarding liver biochemical parameters, risk factors (alcohol consumption or smoking) or other modality of infection.

Table 3 Genotype distribution among HCC patients and control group

Genotype	HCC group		Control group	
	n.	%	n.	%
1	77	74.7	212	60.8
1a	4	3.9	9	2.6
1b	70	67.9[A]	190	54.4[A]
1	3	2.9	13	3.8
2	21	20.4	123	35.2
2a/2c	16	15.5	100	28.7
2	4	3.9	23	6.5
2a	1	1.0	0	0
Other genotypes	5	4.9	14	4.0
Total	103	100	349	100

[A] χ^2:7.33; $p < 0.001$; OR: 1.77; 95% C.I = 1.11–2.82

Table 4 Odds ratios of genotypes associated with HCC

Genotype	Crude OR (95% CI)	Age–sex-adjusted OR (95% CI)
Non 1b	1.00	1.00
1b	1.77 (1.11–2.82)	1.65 (1.16–2.33)

Regarding the association between HCV infection and HCC, as recently reported [36], Italy shows a moderate prevalence of anti-HCV in the general population (approximately 2.0%), except for some Southern areas where the prevalence greatly increases ranging from 6% to 12% with an average viraemic rate estimated at over 70% [36]. As we previously reported [58], confirming several other studies [9, 59–61], anti-HCV incidence in our area is significantly greater among HCC patients, suggesting that these patients significantly have an higher risk of exposure to HCV infection if compared to the general population. Nevertheless, although some authors have suggested the central role of HCV in hepatocarcinogenesis [62–64], the mechanism of virus related carcinoma is not yet fully understood, even if it is clear that HCV, alone or in conjunction with other risk factors can contribute to the epidemiological heterogeneity of this neoplasia, as demonstrated by the findings of HCV RNA in liver tissue among HCC patients anti- HCV negative [65].

Although HCV viral load may be considered as an important prognostic variable, whose knowledge might be useful in the treatment decision to eradicate HCV infection and thus reduce or prevent HCC progression [9, 16, 17], its correlation with HCC progression still remains controversial. Even if it has been suggested a possible direct oncogenic effect of HCV, maybe related to genetic mutations [66] or to some cellular deregulation effects, no consensus has emerged yet.

Although our study is simply a retrospective analysis and prevents us to make any hypothesis about mechanisms of HCC progression, our data clearly shows that a significantly higher percentage of HCC patients (83.7%) shows HCV-RNA in their sera compared to the control group (61.4%) ($p < 0.001$), suggesting that HCC patients have a risk of HCV active infection almost 1 and half times higher than patients without HCC, even if we did not find any correlation between high levels of viremia and advanced liver stage, as reported by other authors [38, 67].

The role of genotypes 1b and 3 in increasing the risk of HCC development has been widely questioned recently and even though numerous studies have often suggested their association with the HCC carcinogenetic progression [68–74], especially in patients with underlying cirrhosis, [38–41, 75–77], probably for the strict correlation existing between these subtypes and liver damage, no consensus has emerged yet. Whether HCV 1b genotype contains specific nucleotide sequences that may be associated with direct pathogenesis or may trigger a stronger inflammatory response still needs to be extensively investigated. This has prevented the International health organizations up to now to adopt HCV genotyping as a globally prevention tool in an international program of HCC eradication [78].

Although in our area, as we previously reported [37, 38], genotype 1b is the most common subtype, our data show that its prevalence in HCC patients is significantly higher (67.9%) if compared to the control group (54.4%) ($p < 0.001$), suggesting that HCC patients have a risk to be infected by subtype 1b quite 2 times greater than patients without HCC. Although a minority of treated control group patients achieved SVR, in order to minimize the potential confounding effect of SVR, we adjusted the estimates for SVR achievement, and we also provided a separate multivariate analysis that excluded all patients who had achieved SVR. These data confirmed that genotype 1b was independently associated with the development of HCC.

In conclusion, our study detects a significantly higher rate of HCV RNA positivity in HCC patients than in control group. Furthermore, HCC patients harbours a higher rate of HCV 1b than general population, not influenced by the use of antiviral treatment as the multivariate analysis showed.

Despite of its limitations related to the absence of a prospective study and data regarding the impact of HCV subtype on the fibrosis stages, our data suggest the strict association existing between HCV genotype 1b and HCC. A prospective study with larger number of samples will be needed to confirm our results, especially considering the introduction of the new direct acting antiviral (DAA) therapies.

Abbreviations

AFP: α- fetoprotein; CDC: Centers for Disease Control; ECi: Enhanced Chemiluminescent immunoassay; HBV: Hepatitis B virus; HCC: Hepatocellular Carcinoma; HCV: Hepatitis C Virus; IU/mL: International Units per Milliliter; LiPA: Line Probe Assay; NIDD: Non-insulin-dependent diabetes; RNA: Ribonucleic Acid; RT-PCR: Reverse transcription polymerase chain reaction; S/Co: Signal-to-cutoff; SPSS: Software package used for statistical analysis; SVR: Sustained virological response

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Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Authors' contributions

AP, SM and GL acquired the data; AP drafted the article and contributed to conception and design and SM assisted him; MP and ML assisted in clinical database generation and first line interaction; RA, GB and FI contributed to critical revision for important intellectual content; all authors approved the final version to be published.

Ethics approval and consent to participate

The study received approval from the Ethic Committee and was conducted following the principles of the ICH GCP and Declaration of Helskinki. All subjects provided informed consent and all data were de-identified during data collection.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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