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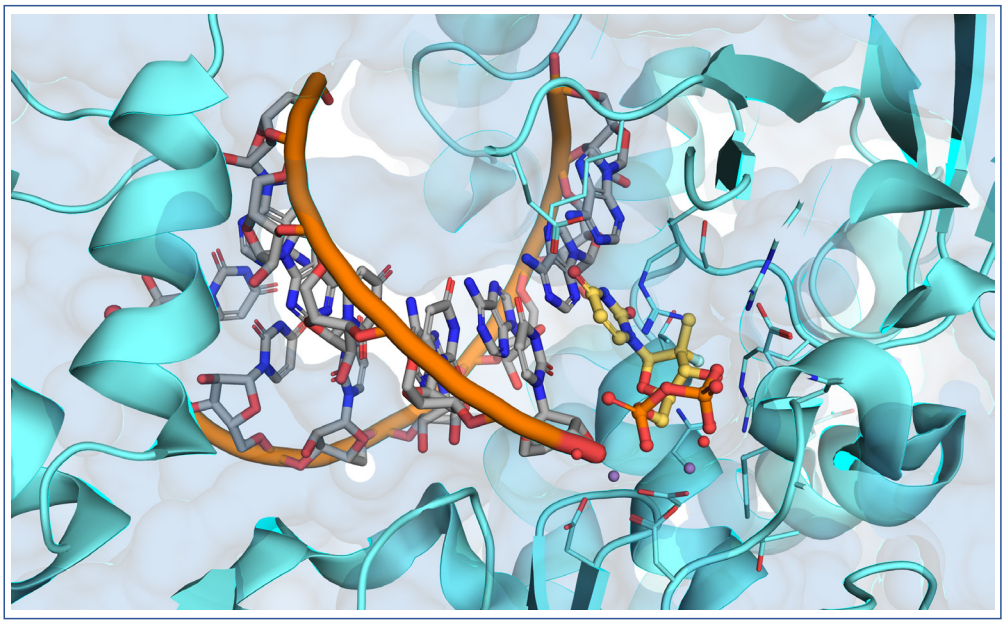

Crystal structure of HCV's NS5B ternary complex reveals
the mechanism of sofosbuvir inhibition of HCV replication (Page iv)

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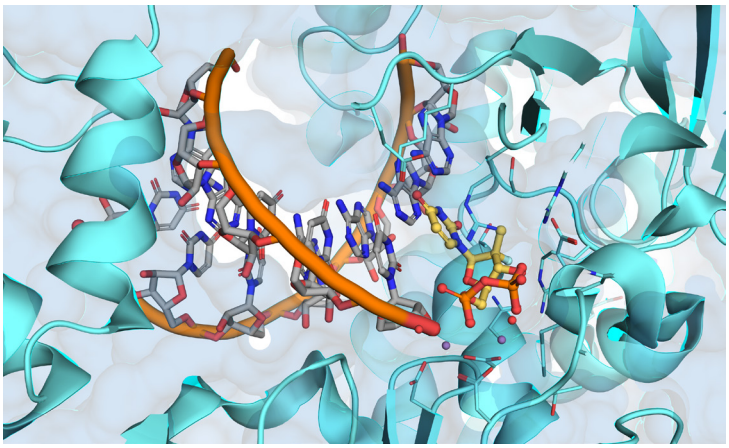

The crystal structure of an NS5B ternary complex (PDB: 4WTG) reveals the mechanism of sofosbuvir inhibition of hepatitis C virus replication. Sofosbuvir is a prodrug of the Protide type and has favorable cell permeability and oral bioavailability. Sofosbuvir, a nucleotide analog, intracellularly becomes its triphosphate form (sofosbuvir-DP), is incorporated into the growing viral RNA chain being mediated by NS5B, the virally-encoded RNA-dependent RNA polymerase, and is thought to act as a chain terminator because the 2'-methyl group of the nucleotide analogue causes a steric clash with an incoming NTP. Cyan ribbons depict the NS5B polymerase, with the RNA template shown as gray sticks colored by atom types. Two purple spheres represent catalytic Mn^{2+} ions. Yellow-colored sofosbuvir occupies the UDP entry site, mimicking the in-line conformation needed for RNA chain elongation. Watson-Crick base pairing with the template enables this conformation, leading to non-obligate chain termination by sofosbuvir.

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Developments and current challenges in the process of cell culture-based seasonal influenza vaccine manufacture in Japan

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Abstract: Seasonal influenza is an acute respiratory infection primarily caused by influenza A and B viruses, which circulate annually and cause substantial morbidity and mortality worldwide. Annual influenza vaccination is currently the most effective measure for preventing influenza and greatly reduces the risk of disease severity and the incidence of complications and death. Annual seasonal influenza vaccines are traditionally produced in Japan and many other countries using viruses propagated in embryonated chicken eggs. However, at present, the effectiveness of the seasonal influenza vaccines has some significant limitations, partly because of egg-adaptive mutations in the antigenic sites of the influenza virus haemagglutinin, which are caused by the continued evolution of seasonal influenza viruses. To overcome the limitations of egg-based influenza vaccine production, a mammalian cell culture-based influenza vaccine production system has been developed in Japan in the past decade as an alternative to the current production method. In this review, I have summarised the progress in the development of cell-based seasonal influenza vaccines and discussed the technological challenges encountered in the development of influenza vaccines.

***Keywords*:** MDCK cell-based influenza vaccine, vaccine development, seasonal influenza candidate vaccine virus, mutation, cell culture techniques

Introduction

The World Health Organization (WHO) estimates that annual influenza epidemics worldwide cause one billion cases of influenza, three to five million cases of severe illness, and approximately 290,000-650,000 respiratory deaths (*1*). Influenza vaccines are the most effective preventive measures against influenza infection and generally reduce the risk of influenza disease in the general population by 40-60% during influenza season, particularly when the circulating viral strains are antigenically well matched to the vaccine strains (*2,3*).

However, every year, the influenza virus evolves rapidly worldwide; therefore, propagation of the influenza virus in eggs is most likely a significant cause of an antigenic mismatch between the circulating influenza virus strains and the vaccine strains, reducing vaccine effectiveness (*3*). A sustainable and continuous cell-based influenza vaccine production system was developed in Japan to produce and supply vaccines that are effective and sufficient for pandemics and seasonal influenza preparedness. Compared to the advantages of egg-derived influenza vaccines, cell-based influenza vaccines offer additional advantages, including: *i*) a large-scale continuous supply process; *ii*) low risk of microbial contamination; *iii*) no risk of severe allergic reactions; *iv*) enhanced vaccine immunogenicity; *v*) ease of manufacture, manipulation, and production; *vi*) manufacturing flexibility (*e.g.*, strengthening of manufacturing capacity and procurement of influenza vaccines during pandemics); and *vii*) a lack of egg-adaptive mutations in the antigenic sites of haemagglutinin (HA) (*4,5*).

Currently, two manufacturers produce cell-based influenza vaccines: Flucelvax Quadrivalent (Flucelvax Tetra in various markets; CSL Seqirus Ltd., Melbourne, Australia) and SKYCellflu trivalent and quadrivalent subunit inactivated vaccines (SK Bioscience Co., Ltd., Seongnam-si, South Korea) (*6*). Since the 2019/2020 influenza season, all four influenza strains (2009 H1N1 Pandemic [A{H1N1}pdm09], A[H3N2], B[Yamagata], and B[Victoria]) of the Flucelvax Quadrivalent formulation have been isolated and propagated exclusively in a Madin-Darby Canine Kidney (MDCK) 33016-PF cell line (*7*). A cell-based quadrivalent inactivated influenza vaccine has been approved in the European Union (Flucelvax Tetra) and the United States (Flucelvax Quadrivalent) for the prevention of influenza in adults and children (*8*).

In 2019, SKYCellflu trivalent and quadrivalent vaccines, produced in MDCK-Sky3851 cells, were accorded WHO prequalification status for being the

first cell-based influenza vaccines, which certifies the safety and the protective efficacy of the vaccines that were assessed by monitoring the manufacturing process, quality, and clinical trial results of the vaccines (*9,10*). Currently, the SKYCellflu trivalent and quadrivalent vaccines have been approved in 11 countries (*10*). Since the 2017/2018 influenza season, the WHO has provided strain recommendations for seasonal influenza candidate vaccine viruses (CVVs) derived from both eggs and cells (*11*). One of the major developments in cell-based influenza vaccines is the potential to use cell-derived seed viruses that can avoid egg-adaptive mutations and produce viruses that are antigenically similar to the circulating viruses, thereby improving vaccine effectiveness (*6*).

As part of the ongoing strategies to provide a continuous long-term supply of effective cell-based seasonal influenza vaccines, the following fundamental research studies have been undertaken: *i*) development of a cell line substrate for the isolation of influenza viruses from clinical specimens; *ii*) assessment of isolation rates, growth properties, and antigenic and genetic characteristics of influenza viruses in the cell lines; *iii*) establishment of an assay for detection and identification of viral adventitious agents in biological materials, including vaccines and clinical specimens; *iv*) assessment of the viral clearance capacity of the cell line during the process of the isolation of influenza viruses; and *v*) development of *in-vitro* methods for assessing the potency of cell-derived seasonal influenza vaccines. In this review, I have described the progress made until now and the current challenges in the practical use of cell-based seasonal influenza vaccines in Japan.

Efforts towards the practical application of cell-based influenza vaccines in Japan

Establishment of a certified National Institute of Infectious Diseases (NIID)-MDCK cell line

MDCK cells are the most suitable substrates for isolation and propagation of a broad range of influenza viruses (*12*); however, they are not suitable for propagating certain influenza viral strains. When seasonal influenza vaccines are manufactured using cell-based technology, effective cell-derived influenza viruses of the WHO-recommended strains with high growth capacity and antigenic consistency are selected (*3*) and distributed to vaccine manufacturers to establish vaccine seed viruses. To address these challenges, a novel adherent NIID-MDCK cell line was developed from the parental MDCK line (ATCC CCL-43) that can isolate influenza viruses from original clinical specimens. The viral growth medium must be free from extraneous agents at all stages of virus preparation for vaccine production (*13,14*). MDCK cells that were grown in serum-free medium in the presence of trypsin showed high titres of influenza virus (*15*). Therefore, the NIID-MDCK cell line was grown in both serum- and protein-free cell culture media. Because cell substrates used in the manufacturing process have an impact on the safety and the purity of vaccines (*13,16*), the biological characteristics of the NIID-MDCK cell line were assessed, and the quality was ensured in the context of cell-substrate use for producing cell-derived influenza vaccines by a global comprehensive biosafety testing company, BioReliance (Glasgow, UK).

Isolation efficiency of influenza viruses in the NIID-MDCK cell line

Following influenza virus isolation from clinical specimens using the NIID-MDCK cell line, appropriate NIID-MDCK cell-derived isolates were adapted and propagated in vaccine manufacturer-owned cells to establish seed viruses for cell-based seasonal influenza vaccine production. If the NIID-MDCK cell-derived isolates have low growth capacity and are antigenically mismatched to the WHO-recommended vaccine prototype viruses, they cannot be distributed to vaccine manufacturers as they may have a significant impact on manufacturing volume, leading to a risk of vaccine shortage. To evaluate the growth properties and the isolation rates of influenza viruses grown in the NIID-MDCK cell line, clinical specimens were directly inoculated into the cell line. Five clinical samples of each type/subtype/lineage with various Ct values were inoculated into MDCK cell lines. Following each passage, the growth characteristics of the influenza viruses isolated from the NIID-MDCK and the conventional MDCK cell lines were determined by HA tests. Influenza A viruses (H1N1pdm09 and H3N2) and influenza B viruses (Yamagata and Victoria lineages) were isolated when the HA titres were greater than two after serial passages. Similar to that in the conventional MDCK cell line, the NIID-MDCK cell line allowed for the successful propagation of A(H3N2) and influenza B viruses (Yamagata and Victoria lineages) at high HA titres (*17*) (Table 1). Although A(H1N1)pdm09 viruses showed limited propagation and lower HA titres in the NIID-MDCK cell line than that in the conventional MDCK cell line, it was possible to isolate A(H1N1) pdm09 viruses with high efficiency by selecting clinical specimens that contained high virus titres (*17*) (Table 2). The A(H1N1)pdm09 viruses were efficiently isolated from clinical specimens with cycle threshold (Ct) values of 24 or below, and the A(H3N2) viruses were isolated from clinical specimens with Ct values of 27 or below after multiple passages (*17*). All influenza B viruses (Victoria and Yamagata lineages) were successfully isolated from clinical specimens after two passages, with Ct values close to 35 (*17*). Therefore, the NIID-MDCK cell line is considered a suitable substrate for isolating influenza A and B viruses from clinical

Table 1. Virus isolation efficiency and haemagglutinin (HA) titres of the isolates in the National Institute of Infectious Diseases-Madin-Darby Canine Kidney (NIID-MDCK) and conventional cell lines

Type/Subtype	Cell line	Specimen ID	Passage number			
			1	2	3	4
A(H1N1)pdm09	NIID-MDCK	TA77	< 2	< 2	< 2	< 2
		TA78	8	32	64	ND
		TA79	< 2	< 2	< 2	< 2
		TA95	< 2	< 2	< 2	< 2
		TA108	< 2	< 2	< 2	< 2
	Conventional MDCK	TA77	< 2	4	16	ND
		TA78	32	32	16	ND
		TA79	< 2	8	4	2
		TA95	< 2	4	8	16
		TA108	2	16	4	4
A(H3N2)	NIID-MDCK	TA232	< 2	< 2	64	256
		TA233	< 2	< 2	8	256
		TA234	2	16	128	ND
		TA235	< 2	128	128	ND
		TA236	< 2	128	128	ND
	Conventional MDCK	TA232	< 2	64	512	ND
		TA233	< 2	< 2	256	ND
		TA234	8	16	256	ND
		TA235	4	128	256	ND
		TA236	< 2	128	512	ND
B(Victoria)	NIID-MDCK	TA318	256	512	256	ND
		TA322	128	512	256	ND
		TA327	128	512	256	ND
		TA330	< 2	512	256	ND
		TA331	64	1,024	512	ND
	Conventional MDCK	TA318	128	512	512	ND
		TA322	128	512	512	ND
		TA327	128	512	1,024	ND
		TA330	16	512	512	ND
		TA331	128	512	1,024	ND
B(Yamagata)	NIID-MDCK	TA336	4	512	512	ND
		TA338	2	512	512	ND
		TA351	64	512	512	ND
		TA376	512	512	256	ND
		TA381	128	512	512	ND
	Conventional MDCK	TA336	128	512	512	ND
		TA338	32	512	512	ND
		TA351	512	512	512	ND
		TA376	512	512	512	ND
		TA381	512	1024	512	ND

Data source: Ministry of Health, Labour and Welfare. The 8th Health Science Council Immunisation and Vaccine Sectorial Committee (5th September, 2014) (*17*). MDCK, Madin–Darby Canine Kidney; ND, Not Done; NIID, National Institute of Infectious Diseases.

Table 2. Virus isolation efficiency of A(H1N1)pdm09 isolates in the NIID-MDCK cell line

Specimen ID	Passage number				Ct value
	1	2	3	4	
TA71	4	4	16	32	19.77
TA72	2	2	16	16	19.71
TA73	< 2	8	16	16	21.86
TA75	< 2	< 2	32	32	23.44
TA84	< 2	< 2	4	64	24.81
TA95	< 2	< 2	< 2	< 2	25.16
TA87	< 2	< 2	< 2	< 2	26.03
TA85	< 2	< 2	< 2	< 2	27.73
TA83	< 2	< 2	< 2	< 2	30.13
TA79	< 2	< 2	< 2	< 2	35.15

Data source: Ministry of Health, Labour and Welfare. The 8th Health Science Council Immunisation and Vaccine Sectorial Committee (5th September, 2014) (*17*). Ct, cycle threshold.

specimens. The A(H1N1)pdm09 viruses isolated from the MDCK33016-PF cell line also had lower HA titres than those isolated from the conventional MDCK cell line (*18*).

Recently, clinical strains of influenza A(H3N2) viruses have been observed to replicate poorly in MDCK cells and rapidly acquire HA or neuraminidase (NA) mutations, leading to viral antigenic alternation (*19-21*). Due to the fact that these findings are prevalent in various MDCK cell lines, and that the NIID-MDCK cell line has similar isolation and propagation efficiency to other MDCK cell lines, these major issues of enhanced virus isolation and propagation are not necessarily improved by propagation in the NIID-MDCK cell line. Therefore, an alternative strategy should be utilised to isolate and propagate a wide range of influenza viral

strains in the NIID-MDCK cell line.

Antigenic analyses of NIID-MDCK cell isolates

Influenza vaccine strains may undergo genetic changes at antigenic and glycosylation sites during the process of egg-based seasonal influenza vaccine production, resulting in antigenic changes in the original vaccine strain. To assess genetic and antigenic variations in the influenza virus during repeated passages in the NIID-MDCK cell line, clinical specimens were inoculated onto confluent monolayers of the cell line, and the HA and NA genes of the NIID-MDCK cell isolates were sequenced and analysed. The impact of amino acid substitutions on the antigenic properties of influenza viruses was assessed using a haemagglutination inhibition (HI) assay with post-infection ferret antisera raised against the cell culture-propagated vaccine prototype viruses. NIID-MDCK cell isolates were considered antigenically similar to each other if their HI titres were within two-fold of the reference virus titre. Diverse amino acid substitutions were observed among NIID-MDCK cell isolates, similar to that in conventional MDCK cell isolates (*18*); however, most of the isolates were antigenicity equivalent with prototype viruses (*22*) (Table 3). After isolating and propagating the influenza viruses in the NIID-MDCK cell line, the suitability of the NIID-MDCK cell isolates for the establishment of a vaccine seed virus whose antigenicity matches that of the prototype viruses was assessed using the one-way HI or the viral neutralisation (VN) tests. If vaccine manufacturers develop their own vaccine seed viruses from appropriate NIID-MDCK cell isolates using their own cell lines, the antigenic similarity between these seed viruses and the prototype viruses can be confirmed by the two-way HI or VN tests.

Development of a multiplex real-time polymerase chain reaction (PCR) assay for the detection of adventitious viruses

Vaccine safety concerns regarding potential contamination with adventitious viruses have arisen from the incidental introduction of viruses into a vaccine through starting materials, such as cell substrates, porcine trypsin, bovine serum, or any other source materials of animal or human origin (*23,24*). The influenza virus was propagated and isolated using the qualified NIID-MDCK cell line that was free of serum and animal components. However, adventitious agents can be introduced into the production process *via* clinical specimens and human intervention. Real-time PCR is one of the most common laboratory methods for detecting pathogens as it is highly sensitive, even for low-level detection of pathogens. Therefore, a rapid and highly sensitive multiplex real-time PCR detection system was developed for the detection of 42 different viruses that are frequently found in clinical specimens and commonly grown in MDCK cells.

Using this assay, 34 NIID-MDCK isolates were tested to detect the presence of 27 different respiratory viruses. No adventitious viral genome was detected in the NIID-MDCK cell isolates, whereas the viral genome of human enterovirus D68 (HEV-D68) was detected when it was present at a concentration below the limit of detection (LOD) in two NIID-MDCK cell isolates (*25*). HEV-D68 did not proliferate in the NIID-MDCK cell line (*25*). Furthermore, the susceptibility of the NIID-MDCK cell line to eight representative human respiratory viruses, namely, human adenovirus serotype 4, human coronavirus OC43, HEV-D68, human metapneumovirus A, human respiratory syncytial virus A, human rhinovirus A, herpes simplex virus 1 (HSV-1), and severe acute respiratory syndrome coronavirus 2, which are frequently co-detected in influenza clinical specimens, was evaluated. The NIID-MDCK cell line was infected with individual viruses, and the viral genome copy number was determined using the assay after each passage. Similar to the findings of a previous study showing that the MDCK33016-PF cell line only supports the growth of a limited spectrum of viruses (*26*), the results of this analysis showed that all the tested respiratory viruses were effectively eliminated through passages in the NIID-MDCK cell line (*25*) (Table 4). A previous study demonstrated that HSV-1 could propagate in the MDCK33016-PF cell line (*27*), whereas

Table 3. Characterisation of viruses isolated in the NIID-MDCK cell line

Type A subtype; B lineage	Specimen ID	Passage number	HI test (one-way)	MDCK cell culture-propagated vaccine prototype viruses
A(H1N1)pdm09	TA73	5	equivalent	A/Wakayama/153/2013 (C1/C1)
	TA78	3	2-fold difference	
A(H3N2)	TA232	4	equivalent	A/Victoria/361/2011 (C2/C2)
	TA233	4	equivalent	
B(Victoria)	TA318	3	equivalent	B/Brisbane/60/2008 (CX/C1/C2)
	TA322	3	equivalent	
B(Yamagata)	TA336	3	equivalent	B/Wisconsin/1/2010 (C1/C1/C2)
	TA338	3	2-fold difference	

Data source: Ministry of Health, Labour and Welfare. The 11th Health Science Council Immunisation and Vaccine Sectoral Committee (19th February, 2016) (*22*). HA, haemagglutinin; HI, haemagglutination inhibition; MDCK, Madin-Darby Canine Kidney.

the number of HSV-1 genomes decreased with each passage and reduced to a level below the LOD at the third passage in the NIID-MDCK cell line, indicating the low susceptibility of the NIID-MDCK cell line to HSV-1. Overall, the NIID-MDCK cell line is less susceptible to most respiratory viral infections, suggesting that it is a suitable substrate for the isolation and propagation of influenza viruses from a safety perspective.

Development of a modified NIID-MDCK cell line with high productivity of influenza viruses

As described above, the isolation efficiency and the growth characteristics of influenza A and B viruses in the NIID-MDCK cell line did not differ significantly from those in the conventional MDCK cell isolates. A modified NIID-MDCK cell line, which could be used for isolating a wide range of high-yield influenza viruses, was further developed for cell-based seasonal influenza vaccine production. The human interferon regulatory factor 7 (IRF7) gene was identified using a small interfering RNA library targeting 78 human genes involved in the type I interferon signalling pathway (*28*), the first line of immune defence against influenza virus infection (*29*). The HA titres of both influenza A and B viruses in the NIID-MDCK-shIRF7 cell line, expressing short hairpin RNA (shRNA) against IRF7, displayed a two to eight-fold increase compared to that of the NIID-MDCK-control shRNA cell line at 48-96 h post-infection (hpi) (*28*) (Table 5). A recent study also demonstrated that influenza A and B viruses showed an approximately four to five-fold increase in numbers at 48 hpi in IRF7-knockout-MDCK cells, using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 technology (*30*). Based on these findings, the NIID-MDCK cell line with low IRF7 expression levels can be used to improve the production capacity of cell-based seasonal influenza vaccines.

Table 4. Detection of the virus genomes in the supernatant of the infected culture[1)]

Virus	Copy number[2)] of each viral genome at different passage in NIID-MDCK cell isolates			
	Input	P1	P2	P3
FluAV	2.5×10^{8}	2.5×10^{9}	5.0×10^{9}	
HAdV4	1.0×10^{9}	5.0×10^{5}	— [3)]	
FluAV	2.5×10^{8}	3.2×10^{9}	1.6×10^{9}	
HCoV-OC43	1.3×10^{9}	1.3×10^{5}	—	
FluAV	2.5×10^{8}	5.0×10^{9}	1.3×10^{9}	2.0×10^{9}
HEV-D68	1.0×10^{10}	1.6×10^{6}	1.3×10^{4}	—
FluAV	2.5×10^{8}	5.0×10^{9}	3.2×10^{9}	
HMPVA	7.9×10^{8}	1.0×10^{4}	—	
FluAV	2.5×10^{8}	4.0×10^{9}	3.2×10^{9}	
HRSVA	3.2×10^{8}	1.3×10^{8}	—	
FluAV	2.5×10^{8}	2.5×10^{9}	2.0×10^{9}	
HRVA	2.5×10^{8}	3.2×10^{4}	—	
FluAV	2.5×10^{8}	1.0×10^{10}	6.3×10^{9}	5.0×10^{9}
HSV-1	6.3×10^{8}	2.0×10^{6}	4.0×10^{3}	—
FluAV	2.8×10^{7}	3.1×10^{9}	2.2×10^{9}	
SARS-CoV-2	3.3×10^{9}	1.8×10^{5}	—	

[1)] NIID-MDCK cell isolates were co-infected with influenza A virus and one of the common respiratory viruses. The supernatant of the infected culture was used for the serial passages in NIID-MDCK cell isolates. [2)] Copy numbers were determined *via* the improved assay using viral nucleic acids extracted from the supernatant of the culture medium, as described in the text. Copy numbers were shown per 1 mL of the supernatant of the infected culture. [3)] The copy number was below the limit of detection. This table was modified from Hamamoto I, *et al*. Microbiol Immunol. 2022 (*25*). HAdV4, human adenovirus serotype 4; HCoV-OC43, human coronavirus OC43; HEV-D68, human enterovirus D68; HMPVA, human metapneumovirus A; HRSVA, human respiratory syncytial virus A; HRVA, human rhinovirus A; HSV-1, herpes simplex virus 1; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; NIID-MDCK, National Institute of Infectious Diseases-Madin-Darby Canine Kidney.

Development of in-vitro potency testing methods for cell-based seasonal influenza vaccines

The single radial immunodiffusion (SRD) assay is the only internationally recognised quality control method for determining influenza vaccine potency and stability. The SRD assay requires strain-specific reference sera and calibrated standard reference antigens to determine the number of influenza HA antigens in the vaccine (*31,32*). In Japan, the SRD assay is routinely used to measure the HA content of egg-cultured seasonal influenza vaccines; however, no method has been established to measure the HA content of cell-cultured seasonal influenza vaccines. To establish a method for determining the HA content of a cell-culture-derived quadrivalent vaccine using the SRD assay, a reference serum and a purified primary liquid standard (PLS), which were used to calibrate the antigen reference reagent in SRD, were prepared from cell-derived influenza CVVs. The proportion of HA protein in the PLS was determined from a densitometric analysis of the viral proteins separated by sodium dodecyl sulphate-poly-acrylamide gel electrophoresis. Although a small amount of cell debris, including nucleic acids,

Table 5. HA titres of influenza viruses produced from the NIID-MDCK cell line

Influenza virus	Subtype/Lineage	shRNA-Control	shRNA-IRF7	Input virus titre/well	Time after infection (hour)
A/Puerto Rico/8/1934	H1N1	8	16	100 pfu	48
A/Narita/1/2009	H1N1pdm09	4~8	16~32	1000 $TCID_{50}$	96
A/Victoria/361/2011	H3N2	4	16~32	100 $TCID_{50}$	72
B/Florida/4/2006	Yamagata	4~8	16~32	100 pfu	72

This table was modified from Hamamoto I, *et al*. PLoS One. 2013 (*28*). shRNA, short hairpin RNA.

was detected using electron micrographs, virtually pure PLS (less than 5% non-viral proteins) was successfully prepared (*33*). As the purity of PLS is important for the accurate determination of vaccine potency (*34*), it is necessary to devise a reliable alternative method for measuring the HA content by improving the technique for increasing the degree of HA purification. The antiserum is prepared by immunising sheep with HA prepared from prototype viruses or their derivatives, whereas the standard reference antigens, which are used to determine the HA content of the vaccine, must be derived from the strain used in the vaccine. The standard reference antigens containing a known amount of diluted HA (15 μg HA per 0.5 mL) and a cell-derived vaccine solution were placed in the wells of a serum-spiked agarose gel to determine the relative titres against the standard reference antigens. The HA content of each antigen in the quadrivalent vaccines that were isolated in the NIID-MDCK cell line, determined using SRD assay reagents derived entirely from cell-cultured vaccine viruses, were virtually equivalent to each other (approximately 15 μg HA per 0.5 mL) (*33*). Overall, the SRD assay can also be used for the determination of HA content in seasonal cell culture-derived influenza vaccines using cell-derived SRD standards.

Further challenges for the practical application of cell-based seasonal influenza vaccines

To establish a basis for producing seed viruses used in cell-based seasonal influenza vaccines, a certified NIID-MDCK cell line and its cell bank, a modified MDCK cell line with improved virus propagation, and an adventitious virus detection method were developed. The isolation rates and genetic and antigenic stability of the NIID-MDCK isolates were assessed. Moreover, the SRD method for the assessment of the vaccine quality was developed. Even though the cell-based production process has been established, there remains a technical challenge, including efficiently isolating a wide range of seasonal influenza viruses without introducing MDCK cell-adapted mutations. Beyond the technical issue, further challenges include: *i*) relatively high manufacturing cost versus egg-based influenza vaccines (*35*); *ii*) seasonal influenza is lower in priority than other health emergency risks; *iii*) limited function, human resources, and collaboration between industry and academia in research institutes capable of developing the vaccines; *iv*) insufficient strategic research funds for vaccine development; *v*) financial risk related to investment in vaccine manufacturing equipment; and *vi*) insufficient evidence demonstrating the benefits and effectiveness of developing cell-based influenza vaccines. It is essential to encourage participation by Japanese pharmaceutical companies, universities, and research institutions in vaccine development, as well as collaboration with overseas institutions, to make cell-based seasonal influenza vaccine development practical in Japan.

Conclusions

Over the last decade, several new platforms that do not use egg-based technologies have been developed to produce effective seasonal influenza vaccines in many countries worldwide, and efforts are being made to make cell-based seasonal influenza vaccines available in more regions. In Japan, effective cell-cultured seasonal influenza vaccine production strains have been developed, and a quality control system for cell-cultured seasonal influenza vaccines has been established in collaboration with vaccine manufacturers. Furthermore, Japan has substantially contributed to the field globally by not only originally establishing the certified NIID-MDCK cell line for vaccine production, but also by listing the cell line on the WHO-recommended list since the southern hemisphere influenza season of 2017 (*36*). Therefore, it can be used globally for human cell culture-derived CVV isolation for the development and production of annual seasonal influenza vaccines. In addition, Japan is the sole nation promoting cell-based seasonal influenza vaccine production as a national project, and its advances and achievements have been drawing international attention. Although there are theoretical benefits of producing cell-based influenza vaccines, some drawbacks remain in the practical use of cell-based seasonal influenza vaccines in Japan. Limited isolation rates, poor growth, and antigenic changes in seasonal influenza viruses grown in cell lines reduce the selection and availability of viruses for the establishment of seed viruses and vaccine manufacture. In addition, even if highly proliferative isolates were obtained after passages in the NIID-MDCK cell line, antigenic mutations were introduced in the isolates. Therefore, it is necessary to focus on more effective alternative strategies using cell lines to propagate primary influenza isolates and distribute effective influenza viruses to vaccine manufacturers in Japan.

Numerous clinical studies have shown that cell-based inactivated influenza vaccines are more effective than egg-based inactivated influenza vaccines (*37,38*). Hence, it is expected that the use of cell-based seasonal influenza vaccines will further improve the efficacy of the vaccine in future years.

One of the challenges identified in this study was that previous research has demonstrated that the antigenic changes that occur during egg acclimation can be avoided by growing cultured cells. A further challenge was avoiding mutations caused by egg culture, but it became clear that antigenic mutations occur even in MDCK cell lines due to cell adaptation. We need to demonstrate the advantages of this manufacturing strategy, such as the ability to avoid changes in antigenicity that would otherwise occur, and promote research and development

through industry-academia-government collaboration.

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Prevalence, characteristics, and virologic correlations of hepatitis delta (D) among patients with hepatitis B surface antigen in Mongolia

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Abstract: Clinical and biochemical features of hepatitis delta virus (HDV) infections in Mongolia remain largely unknown. We aimed to investigate the clinical characteristics of HDV patients in Mongolia using several markers. The 143 hepatitis B surface antigen (HBsAg)-positive patients were divided into 122 HDV-positive and 21 HDV-negative patients by HDV RNA positivity. Subgroup analysis was performed between hepatitis B e antigen (HBeAg)-positive and -negative HDV-positive patients. Liver function, quantitative HBsAg (qHBsAg), anti-HDV Immunoglobulin (Ig) M, Mac-2 binding protein glycosylation isomer (M2BPGi), hepatitis B virus (HBV) DNA level, and HDV RNA level were tested. HDV RNA was positive in 85.3% (122/143) of patients showing anti-HDV IgG. Liver disease activity was higher in HDV-positive patients than in HDV-negative patients. The HDV-positive group included a higher proportion of patients with high qHBsAg and M2BPGi levels ($p < 0.001$). The positivity rate for anti-HDV IgM was significantly higher in the HDV-positive group ($p < 0.001$). HDV RNA levels showed an inverse correlation with qHBsAg levels in HBeAg-positive-HDV-positive patients ($r = -0.49$, $p = 0.034$), and a positive correlation with qHBsAg levels in HBeAg-negative patients ($r = 0.35$, $p < 0.001$). Hepatitis B virus (HBV) DNA and HDV RNA levels did not show any correlation. M2BPGi levels likewise did not correlate with HDV RNA levels. A high positivity rate for HDV RNA was observed for HBV patients in Mongolia using the highly sensitive HDV RNA assay. The positivity rate for anti-HDV IgM was high in HDV RNA-positive patients. Severity of liver disease and M2BPGi levels were both high in the HDV RNA-positive group.

***Keywords*:** highly sensitive HDV RNA assay, anti-HDV IgM, M2BPGi, hepatitis B virus, quantitative HBsAg, HBeAg

Introduction

Four decades ago, Rizetto *et al.* discovered the hepatitis delta virus (HDV), which requires the hepatitis B virus surface antigen (HBsAg) helper function for viral assembly and propagation (*1*). Mongolia is known as an endemic country with a high prevalence of viral hepatitis. The studies reported the seroprevalence of HDV to be approximately 60–65% among Mongolian patients with chronic hepatitis B (*2,3*).

Sodium taurocholate co-transporting polypeptide (NTCP) was recently identified as an entry receptor for both hepatitis B virus (HBV) and HDV (*4*). HBV uses a retrograde trafficking route for infection that is facilitated by dedicator of cytokinesis 11 (DOCK11) and serves to maintain HBV covalently closed circular DNA (*5*).

The presence of anti-HDV immunoglobulin (Ig) M antibodies is used in clinical practice not only to differentiate between acute and chronic HDV infection (*6*), but also to identify persistent viremia during chronic hepatitis delta (*7*).

Polymerase chain reaction (PCR) is the most reliable diagnostic tool for detecting HDV RNA, but commercial assays have been unavailable because of the genetic heterogeneity of HDV (*8*). In 2017, the World Health Organization (WHO) approved the standard method for quantitative determination of HDV RNA levels (*9*), which was recently validated as a highly sensitive method for accurately evaluating

disease and antiviral treatment outcomes (*10*). Mac-2 binding protein glycosylation isomer (M2BPGi) is a new glyco-biomarker for liver fibrosis found by Japanese researchers (*11-13*) They reported that serum M2BPGi level offers a reliable, non-invasive marker of liver fibrosis in patients with chronic hepatitis B, C, non-alcoholic fatty liver disease, and primary biliary cholangitis (*14*).

Although the prevalence of HDV infection is reportedly high, data is lacking for anti-HDV IgM, M2BPGi, and highly sensitive HDV RNA testing in chronic hepatitis delta patients in Mongolia. We therefore aimed to investigate the clinical characteristics of HDV patients using a combination of sensitive biomarkers such as anti-HDV IgM, HDV RNA testing, qHBsAg, and M2BPGi levels.

Materials and Methods

Patients

Study subjects were selected from among 363 HBV patients being followed-up at Intermed Hospital and the University Hospital of the Mongolian National University of Medical Sciences between June 2015 and August 2016. All patients were negative for HCV infection. The flowchart for study participation is displayed in Figure 1. Among these 363 hepatitis B surface antigen (HBsAg)-positive patients, 306 patients (84.3%) tested positive for anti-HDV IgG. The 158 patients who were not tested for HDV RNA were excluded from the present study. Another five patients who responded to pegylated interferon-α therapy were excluded from this study. All the remaining 143 patients were tested using the highly sensitive HDV RNA assay and were selected for further investigation. Of these 143 patients, 71 (49.7%) had chronic hepatitis and 72 (50.3%) had cirrhosis. We then compared clinical, virologic, and biochemical findings between groups according to HDV status, comprising 122 HDV-positive (HDV RNA-detectable) patients and 21 HDV-negative (HDV RNA-undetectable) patients. We also compared detailed characteristics between hepatitis B e antigen (HBeAg)-positive and HBeAg-negative HDV-positive patients. Liver biopsy was not performed.

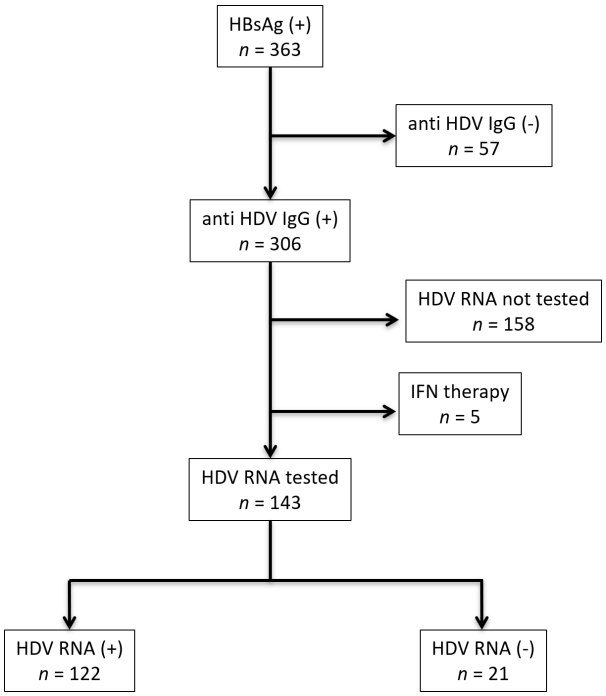

Figure 1. Subjects included in this study.

Clinical laboratory methods

Complete blood counts were determined using commercially available tests (XS-800i analyzer; Sysmex Co., Hyogo, Japan). An automatic biochemistry analyzer Chemix-180 (Sysmex Co.) was used for liver function tests, including aspartate aminotransferase (*10*), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), albumin, and total bilirubin. Upper limits of normal (ULNs) were considered to be 30 U/L for ALT and 33 U/L for AST in women and 40 U/L for ALT and 41 U/L for AST in men.

Serologic methods

HBV markers, α-fetoprotein (AFP), and serum M2BPGi were measured by chemiluminescent enzyme immunoassay using an HISCL-5000 analyzer (Sysmex Co.). The diagnostic range for the quantitative determination of HBsAg titer was 0.03–2,500 IU/mL. Samples showing qHBsAg > 2,500 IU/mL were retested at a higher dilution. M2BPGi level was expressed as a cut-off index. Anti-HDV IgG level was determined by HDV-IgG enzyme immunoassay (Fortress Diagnostics, Antrim, UK). Anti-HDV IgM testing was performed using HDV IgM enzyme-linked immunosorbent assay (DRG Instruments GmbH, Marburg, Germany), according to the manufacturer's instructions. All samples with a signal-to-cutoff ratio (S/Co) > 1.1 were considered positive.

Detection of HBV DNA and HDV RNA

Detection of HDV RNA was performed using a RoboGen HDV RNA quantification kit 2.0 (Roboscreen GmbH, Marburg, Germany). This kit references the first WHO standard for HDV RNA quantification (*9*). Real-time PCR was performed using a Lightcycler 480 (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions. All samples with a detection threshold for viral load >14 IU/mL were considered positive for HDV RNA.

Following DNA extraction from 500 μL of serum

using a High Pure System Viral Nucleic Acid Kit (Roche Diagnostics), quantitative detection of HBV DNA was performed using the Cobas TaqMan HBV test kit 2.0 (Roche Diagnostics) according to the recommendations from the manufacturer on using the Real-Time PCR Cobas TaqMan 48 instrument (Roche Diagnostics).

Statistical analysis

Statistical analyses were performed using SPSS version 22.0 software (SPSS Inc., Chicago, IL). All continuous variables are described as mean ± standard deviation or median and range, as appropriate. Categorical variables are expressed as frequency and percentage. Continuous variables were analyzed using Student's *t*-test or the Mann–Whitney *U*-test. A chi-square test was used to compare discrete variables. Logistic regression models were used to calculate odds ratio and 95% confidence intervals (CIs). Spearman's correlation test was used to determine the correlation between HDV RNA level and other continuous variables. Values of $p < 0.05$ were considered statistically significant.

Ethical considerations

The study design and protocols were reviewed and approved by the Ethics Review Committee of the Mongolian National University of Medical Sciences, in accordance with the 1975 Declaration of Helsinki (as revised in 2013). Written informed consent was obtained from each study subject prior to blood testing.

Results

Comparison of HDV-positive and HDV-negative groups

First, based on HDV RNA assay results, subjects were divided into the two main groups (Figure 1), namely, the HDV-positive and HDV-negative groups. Of the 143 patients, 122 patients (85.3%) tested positive and only 21 tested negative for HDV RNA. Comparisons of main laboratory and clinical characteristics were then performed between the two study groups (Table 1). Study groups showed a similar median age (40 years *vs.* 43 years) and sex distribution (53% *vs.* 52% in men). Compared with the HDV-negative group, the HDV-positive group showed more severe biochemical activity of liver disease, measured as the mean ratio of ALT to ULN (2.0 *vs.* 0.9, $p = 0.003$); mean ratio of AST to ULN (1.6 *vs.* 0.8, $p = 0.005$); GGT level (45 U/L *vs.* 21 U/L, $p = 0.002$); and albumin level (44 g/L *vs.* 48 g/L, $p = 0.025$). Platelet counts were lower in the HDV-positive group (189 $\times 10^9$/L *vs.* 235 $\times 10^9$/L, $p = 0.004$). On the other hand, mean levels of bilirubin and ALP did not differ between groups.

Table 2 shows a significantly higher mean qHBsAg level in the HDV-positive group (3.7 ± 0.7 log IU/mL *vs*. 2.9 ± 1.0 log IU/mL, $p = 0.001$). The HDV-positive group also showed a significantly higher frequency of patients testing positive for HDV IgM (68.1% *vs.* 14.3%, $p < 0.001$). The positivity rate for HBeAg did not differ significantly between groups (15.6% *vs.* 4.8%, $p = 0.31$). The rate of detectable HBV DNA did not differ significantly between the HDV-positive group (30.3%) and HDV-negative group (9.5%, $p = 0.06$). A higher proportion of patients with M2BPGi > 1 of the COI was observed in the HDV-positive group (84.4% *vs.* 38.1%, $p < 0.001$).

Subgroup analysis of HDV-positive patients with and without HBeAg

Since qHBsAg levels depend on HBV viral load and HBeAg positivity, we performed subgroup analyses of HDV-positive patients. Patients were divided into two subgroups: an HBeAg-positive HDV-positive; and an HBeAg-negative HDV-positive group. Table 3 shows the comparison between HBeAg-positive and HBeAg-negative patients with HDV RNA. Median age, sex distribution, and basic laboratory features did not differ

Table 1. Comparison of clinical and biochemical characteristics between HDV-positive and HDV-negative groups

Variables	HDV-positive ($n = 122$)	HDV-negative ($n = 21$)	*p* value
Age, y, median (range)	40 (21–70)	43 (26–65)	0.8
Sex, male, *n* (%)	65 (53.3%)	11 (52.4%)	0.5
WBC, $\times 10^{12}$/L, median (range)	4.8 (2.1–9.6)	5 (2.5–9.0)	0.48
PLT, $\times 10^9$/L, median (range)	189 (57–354)	235 (123–384)	0.004*
ALT, ratio to ULN, mean ± SD	2.0 ± 1.5	0.9 ± 0.7	0.003*
AST, ratio to ULN, mean ± SD	1.6 ± 1.1	0.8 ± 0.4	0.005*
Total bilirubin, μmol/L, median (range)	17 (4–49)	18 (8–39)	0.81
Alkaline phosphatase, U/L, median (range)	300 (128–982)	250 (154–541)	0.06
GGT, U/L, median (range)	45 (11–444)	21 (11–158)	0.002*
Albumin, g/L, median (range)	44 (28–52)	48 (39–54)	0.025*

*p value < 0.05. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; HDV, hepatitis delta virus; PLT, platelet count; SD, standard deviation; ULN, upper limit of normal; WBC, white blood cell count; HDV-positive, HDV RNA-positive; HDV-negative, HDV RNA-negative.

Table 2. Comparison of serologic and virologic features between HDV-positive and HDV-negative groups

Variables	HDV-positive (*n* = 122)	HDV-negative (*n* = 21)	OR 95% CI	*p* value
qHBsAg log IU/mL, mean ± SD	3.7 ± 0.7	2.9 ± 1.0	-	0.001*
qHBsAg > 1,000 IU/mL, *n* (%)	108 (88.5%)	10 (47.6%)	8.5 (3.0–23.5)	< 0.001*
HBeAg-positive, *n* (%)	19 (15.6%)	1 (4.8%)	3.7 (0.4–29.1)	0.31
Anti-HBe-positive, *n* (%)	107 (87.7%)	20 (95.2)	2.8 (0.5–22.4)	0.47
Anti-HDV IgM-positive, *n* (%)	83 (68.1%)	3 (14.3%)	12.7 (3.5–45.9)	< 0.001*
HBV DNA (positive PCR), *n* (%)	37 (30.3%)	2 (9.5%)	4.1 (0.9–18.7)	0.06
AFP, ng/mL, median (range)	4.3 (0.6–78.8)	2.3 (1.0–7.5)	-	0.024*
M2BPGi, COI, median (range)	1.90 (0.1–12.4)	0.77 (0.4–2.7)	-	0.001*
M2BPGi > 1 COI, *n* (%)	103 (84.4%)	8 (38.1%)	8.8 (3.2–24.1)	< 0.001*

*p value < 0.05. AFP, α-fetoprotein; anti-HBe, antibody against hepatitis B e antigen; anti-HDV IgM, immunoglobulin M against hepatitis delta virus; CI, confidence interval; COI, cut-off index; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; HDV, hepatitis delta virus; M2BPGi, Mac-2 binding protein glycosylation isomer; PCR, polymerase chain reaction; OR, odds ratio; qHBsAg, quantitative hepatitis B surface antigen; HDV-positive, HDV RNA-positive; HDV-negative, HDV RNA-negative.

Table 3. Comparison of clinical and biochemical characteristics between HBeAg-positive and HBeAg-negative subgroups in HDV-positive patients

Variables	HBeAg-positive (*n* = 19)	HBeAg-negative (*n* = 103)	*p* value
Age, y, median (range)	42 (26–65)	40 (21–70)	0.8
Sex, male (%)	11 (57.9%)	54 (52.4%)	0.8
WBC, $\times 10^{12}$/L, median (range)	4.4 (2.1–9.6)	4.8 (2.1–8.9)	0.8
PLT, $\times 10^{9}$/L, median, (range)	189 (76–306)	188 (57–345)	0.9
ALT, ratio to ULN, mean ± SD	1.8 ± 1.3	2.0 ± 1.5	0.6
AST, ratio to ULN, mean ± SD	1.6 ± 1.0	1.6 ± 1.1	0.9
Total bilirubin, μmol/L, median (range)	20 (4–41)	17 (4–49)	0.1
Alkaline phosphatase, U/L, median (range)	303 (223–634)	297 (128–982)	0.6
GGT, U/L, median (range)	45 (11–413)	44 (11–444)	0.7
Albumin, g/L, median (range)	48 (39–54)	44 (28–52)	0.2

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; HBeAg, hepatitis B e antigen; PLT, platelet count; SD, standard deviation; ULN, upper limit of normal; WBC, white blood cell count.

Table 4. Comparison of serologic and virologic features between HBeAg-positive and HBeAg-negative subgroups among HDV-positive patients

Variables	HBeAg-positive (*n* = 19)	HBeAg-negative (*n* = 103)	*p* value
qHBsAg, log IU/mL, mean ± SD	4.2 ± 0.3	3.6 ± 0.7	0.006*
Anti-HDV IgM-positive, *n* (%)	13 (68.4%)	70 (67.9%)	0.6
HBV DNA, log IU/mL	2.5 ± 1.8	1.8 ± 0.8	0.023*
HDV RNA, log IU/mL	5.2 ± 1.1	5.2 ± 1.2	0.9

*p value < 0.05. Anti-HDV IgM, immunoglobulin M against hepatitis delta virus; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; HDV RNA, hepatitis delta virus ribonucleic acid; qHBsAg, quantitative hepatitis B surface antigen.

between groups. As predicted, mean qHBsAg level was significantly higher in HBeAg-positive patients (4.2 log IU/mL) than in HBeAg-negative patients (3.6 log IU/mL, $p = 0.006$) (Table 4). The proportion of patients with anti-HDV IgM positivity was around 68% in both groups (Table 4). HBV DNA was detectable in 52.6% of HBeAg-positive patients and 26.2% of HBeAg-negative patients. Mean HBV DNA level was significantly higher in HBeAg-positive patients (2.5 ± 1.8 log IU/mL) than in HBeAg-negative patients (1.8 ± 0.8 log IU/mL, $p = 0.023$). On the other hand, HDV RNA level did not differ between groups (5.2 ± 1.1 and 5.2 ± 1.2 log IU/mL, $p = 0.9$).

On correlation analyses, serum HDV RNA level correlated negatively with qHBsAg levels in HBeAg-positive HDV patients ($r = -0.49$, $p = 0.034$) and positively in HBeAg-negative HDV patients ($r = 0.35$, $p < 0.001$) (Table 5). In HBeAg-positive subjects, serum GGT level was significantly associated with HDV RNA level ($r = -0.52$, $p = 0.028$). In the HBeAg-negative group, ALT level was associated with HDV RNA level ($r = 0.35$, $p < 0.001$). No association was found between HBV DNA and HDV RNA levels in this study. M2BPGi level also did not correlate with HDV RNA level.

Table 5. Correlation of quantitative HDV RNA level with other variables in HBeAg-positive and HBeAg-negative HDV-positive patients

Variables	HBeAg-positive (n = 19)		HBeAg-negative (n = 103)	
	r	p value	r	p value
Age	0.11	0.64	-0.07	0.4
PLT	-0.14	0.56	0.02	0.78
ALT	-0.23	0.33	0.35	< 0.001*
GGT	-0.52	0.028*	0.09	0.32
ALP	-0.35	0.13	0.09	0.34
M2BPGi	-0.13	0.58	-0.53	0.59
qHBsAg log IU/mL	-0.49	0.034*	0.35	< 0.001*
HBV DNA	-0.25	0.40	-0.09	0.47

*p value < 0.05. ALP, alkaline phosphatase; AFP, α-fetoprotein; GGT, gamma-glutamyltransferase; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; HDV RNA, hepatitis delta virus ribonucleic acid; M2BPGi, Mac-2 binding protein glycosylation isomer; PLT, platelet count; qHBsAg, quantitative hepatitis B surface antigen; r, correlation coefficient.

Discussion

Interest in HDV infection has been re-emerging during recent years. Several reports on topics including HDV virology, immune control of HDV infection, and defining the clinical benefits of interferon therapy have been published (*15-18*). Reliable global epidemiologic data on the prevalence of HDV and clinical data are required in light of these new findings.

Mongolia stands out with one of the highest recorded prevalence rates of HDV globally (*2,3*). This unique epidemiological scenario may contribute to distinctive drivers identified in Mongolian HCC, including noteworthy genomic factors associated with hepatitis D viral infection (*19*). Recently published data have revealed that 65% of HBsAg carriers in the country were co-infected with HDV, with 74% of these cases detectable for HDV RNA (*3*).

In our study, we found that 84.3% of HBsAg-positive patients were positive for anti-HDV IgG. Moreover, 85.3% of patients positive for the anti-HDV IgG antibody also tested positive for HDV RNA. This observation may be related to the specific characteristics of the study population in our research.

The HDV RNA assay used in this study was validated by the WHO in recent years. This highly sensitive assay is the standard for HDV quantification (*9, 10*).

Hepatitis severity was higher in the HDV-positive group, supporting previous findings from Western countries (*20,21*). However, a report from Taiwan found no relationship between severity of hepatitis and anti-HDV status (*22*). The less severe course of HDV infection in Taiwan compared to Western countries may be explained by differences in the distribution of HDV genotypes between geographic regions. HDV genotype 2 has been shown to be predominant in Taiwan and is associated with a milder disease course. In contrast, HDV genotype 1 is predominant in Western countries and Mongolia, with a more pronounced severity of disease compared to genotype 2 (*3,23*).

In this study, a positive correlation was found between the HBsAg levels and the HDV RNA load in HBeAg-negative group. This was similar to a previous report, and was attributed to the ease with which HDV proliferates (*24*). Liver inflammation and fibrosis were also more pronounced in the HDV-positive group. These findings were also similar to previous reports (*7,24*). M2BPGi was higher in the HDV-positive group and was considered to reflect fibrosis and inflammation. The HDV-positive group had a high anti-HDV IgM-positivity rate indicating active hepatitis delta infection (*7*). The HDV-negative group may have included cases in which only HDV had been eliminated after co-infection with HBV.

HDV has been reported to suppress HBV replication (*25-27*).However, no correlation between levels of HDV RNA and HBV DNA was shown in this study. This may be due to differences in the combinations of HDV and HBV genotypes between reports (*7*). In Mongolia, HBV genotype D and HDV genotype 1 are reportedly dominant (*3,23,28,29*). Another report found that HBV genotype B and HDV genotype 2 were unrelated in Taiwan (*30*). Unfortunately, the present study could not consider the genotypes of HBV and HDV because it was not possible to examine the virus genotypes this time.

Comparing the HBeAg-positive and HBe-negative groups revealed that severity of hepatitis delta was not significantly related to HBeAg status. Similar findings have been reported previously (*31*). Consistent with previous reports, no significant difference in HDV RNA levels was evident between HBeAg-positive and -negative groups (*30*). Likewise, no difference was found in anti-HDV IgM prevalence, probably because HDV RNA levels were comparable (*7*). Notably, our study explores separate examinations for correlations between HDV RNA levels and liver disease markers in both HBeAg-positive and HBeAg-negative cases. ALT levels showed a weak positive correlation with HDV RNA levels in the HBeAg-negative group. GGT showed an inverse correlation with HDV RNA levels in the HBeAg-positive group. The clinical implications of these findings require further investigation.

In our study, we measured M2BPGi in Mongolian patients with HDV. M2BPGi serves as a marker for inflammation and fibrosis in liver diseases of various origins (*14*). Previous assessments included measurements in healthy individuals (*32*), patients with HBV and HCV (*33*), and HDV-positive patients (*34*). The latter demonstrated significantly elevated M2BPGi levels in HDV patients compared to healthy individuals (6.04 ± 5.8 COI *vs.* 0.8 ± 0.49 COI), particularly in those with high HDV viral loads as opposed to low viral loads.

A noteworthy discovery in our study was the lack of correlation between M2BPGi and HDV RNA levels in HDV RNA-positive patients. Nonetheless, M2BPGi levels were higher in the HDV-positive group compared to the HDV-negative group. Taken together, these findings suggest that M2BPGi could serve as a potential predictor of disease progression in HDV infection. Further investigation is necessary to fully understand the significance of M2BPGi in HDV infection, and this remains an avenue for exploration in future research.

The routes of infection are thought to include mother-to-child transmission, iatrogenic incidents, folk remedies, tattoos, intravenous drug use (IVDU), and sexual contact including that among men who have sex with men. IVDU is well recognized as a risk factor for HDV co-infection (*35*), but medical records in the present study did not consistently contain such information.

Taiwan is considered to be endemic for hepatitis B, but HBV prevalence has decreased markedly following the implementation of effective immunization schedules (*36*). In Mongolia, however, HDV prevalence still appears high. This continues to be a public problem of medical importance.

The data in this study were obtained from among HBV patients who visit hospitals in Mongolia and will be important in the future. A common standard measurement is ideal for obtaining epidemiologic data. Use of a common measurement kit would thus be indispensable for measuring HDV RNA in anti-HDV-positive individuals around the world.

This study aims to compare HDV-positive and HDV-negative groups among anti-HDV-positive subjects in Mongolia using a high-sensitive HDV RNA assay to diagnose active HDV infection. However, several limitations should be considered. Firstly, the study is constrained by a small sample size, which may affect the generalizability of the findings. Additionally, the availability of data on liver biopsies and genotyping of HBV and HDV is limited, limiting a comprehensive understanding of the disease dynamics. Future studies should address these limitations and delve into investigating the risk factors, clinical and virologic characteristics, as well as host factors influencing HDV viral clearance in Mongolian patients with chronic hepatitis D.

In conclusion, we measured sera of HDV patients in Mongolia and confirmed a high positivity rate using a highly sensitive HDV RNA assay. The anti-HDV IgM-positivity rate was high among HDV RNA-positive patients. HDV RNA-positive patients showed more severe hepatitis and higher M2BPGi levels, but no correlation was seen between HDV RNA levels and M2BPGi levels.

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Knowledge, attitudes, practices and prevalence of hepatitis B and C and hepatitis B vaccination coverage among public sector healthcare workers in Cambodia

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Abstract: Healthcare workers (HCWs) are a key population at high risk for hepatitis B (HBV) and hepatitis C (HCV) infections. We aim to study HBV vaccination coverage, seroprevalence, knowledge, attitudes, and practices towards HBV and HCV infections among HCWs in public sector in Cambodia. A nationally representative cross-sectional study was implemented in 2019, among Cambodian HCWs. A standardized questionnaire was administered to randomly selected HCWs whose blood was then sampled. We used univariate and multivariate regression to determine predictors of outcomes. Among 755 participants, we found 4.9% positive HBsAg and 2.3% positive anti-HCV Ab. HBV vaccination coverage was 59.3%. Lack of knowledge was found on the route of transmission, HBV vaccination, diagnosis and treatment of HBV and HCV. 67% of HCWs thought that all patients should be screened for HBV and HCV and about 30% of them would refuse to take care of infected patients. 58% of HCWs always recapped the needle after use. In univariate analysis, older age-group (> 50 years) is more likely to have positive anti-HCV (OR: 9.48; 95% CI: 2.36–38.18). HCWs who were younger, female or having higher education or having ever been tested, were more likely to have gotten HBV vaccinated. Multivariate analysis reconfirmed these predictors of getting vaccinated. Study findings indicated an urgent need of a national policy for Cambodian HCWs given the high prevalence of hepatitis among this group. Policy should include an effective in-service training program to improve knowledge and practices, a testing and vaccination program for HCWs and it should emphasize stigma intervention towards people living with HBV/HCV.

***Keywords*:** HBV, HCV, healthcare workers, vaccination, Cambodia

Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infection is a major global public health problem responsible for liver damage including cirrhosis and hepatocellular carcinoma. World Health Organization estimated that 257 million people were living with chronic HBV infection and 75 million were living with chronic HCV infection, and 1.34 million died from HBV and HCV related complications, such as liver cirrhosis and hepatocellular carcinoma (HCC) (*1*). In Cambodia, viral hepatitis is responsible for 44.3% of HBV-associated primary liver cancer, and 43% of HCV-associated primary liver cancer among HCC patients (*2*). Epidemic modelling in 2017 estimated 3% prevalence of chronic HBV infection and 1.6% prevalence of chronic HCV infection in general Cambodians (*3*). HBV vaccination coverage among children is high in Cambodia with 81.5% of newborns having received birth dose administration during the first 24h and 98.3% got 3rd dose vaccination in 2021 (*4*). A Cambodia nationwide HBV serosurvey conducted in 2017 revealed 0.56% HBsAg positivity among children aged 5–7 years (*5*), emphasizing the success of the HBV immunization program held by the National Immunization Program (NIP).

Although Cambodia has made significant progress with hepatitis B immunization for infants, no national policy for vaccinating healthcare workers (HCWs) has been developed to date. HCWs are at higher risk for HBV or HCV infection because of exposure to blood and body fluids, usually through needle-stick

injuries, with an estimated range of 5% to 36% of HBV infections and 27% to 41% of HCV infections attributed to contaminated sharp injuries among HCW in the Western Pacific Region (*6*). The Regional Action Plan for Viral Hepatitis in the Western Pacific 2016–2020 calls for establishment of national policies for vaccinating HCWs against HBV in over 80% of countries by 2017 and of HCWs, medical students in all countries and areas by 2020 (*7*). Furthermore, direct-acting antivirals (DAAs), which effectively cure HCV infection provide a great opportunity to control and eventually eliminate HCV as a public health threat. However, the rate of HBV vaccination for Cambodian HCWs and their HBV and HCV prevalence remains unknown. To inform the national responses to properly address viral hepatitis prevention in health facilities in Cambodia, we undertook a study to assess data and policy gaps in HBV vaccination for Cambodian HCWs and determine knowledge, attitude and practice (KAP), and seroprevalence of HBV and HCV among HCWs.

Materials and Methods

Study setting and population

A nationally representative cross-sectional survey was conducted from July to September 2019 among HCWs in public healthcare services of Cambodia. A two-stage cluster sample was designed to first randomly select public healthcare institutions including Operational District and National hospital (OD/NH) geographical clusters, and then select participants among HCWs from those healthcare facilities within each selected OD/NH clusters. Among 100 Operational Districts and 8 National Hospitals across Cambodia, 26 clusters were selected to enroll in the study. HCWs were randomly selected from the most up to date staff lists of each selected cluster. HCWs in the study included medical doctors, nurses, midwifes, pharmacists, dentists, laboratory technicians, radiology technicians, physiotherapists, administration staffs, janitorial, security and all other staffs registered in staff lists.

Sample size

Sample size calculations assumed 5% prevalence of HBV and 50% HBV vaccination coverage among HCWs at a 95% level of significance, a design effect of 1.5 and 10% nonresponse rate. The target sample size was 761. Thirty HCWs were recruited from each of the 26 selected clusters.

Data collection

After obtaining informed consent, data on KAP towards HBV vaccination, HBV and HCV infection, prevention and treatment were obtained through face-to-face interviews using structured questionnaires by twenty teams (1 team supervisor, 2 interviewers and 1 phlebotomist).

Laboratory testing and interpretation

Blood samples were collected from each participant and tested on site for the presence of HBV surface antigen (HBsAg), HBV surface antibody (anti-HBs), and HCV antibody (anti-HCV) using SD Bioline rapid tests (Ref. 01FK10, 01F20 and 02FK0, respectively). Remaining sera were sent to Rodolphe Mérieux laboratory of the University of Health Sciences in Phnom Penh capital for further laboratory analyses to detect the presence of IgM antibody to HBV core antigen (IgM anti-HBc) and total HBV core antibody (total anti-HBc) by ELISA Biocentic Kits (Ref. 4CME3 and 4CBE3, respectively) and for HCV RNA viral load by Real-time PCR using HCV PUMA PCR Kit (Omunis, ref. OPK2-100).

HBV and HCV infection status was interpreted as follows: susceptible to HBV infection (negative for HBsAg, total anti-HBc and anti-HBs); immune due to natural infection (HBsAg negative, total anti-HBc positive, and anti-HBs positive); immune due to vaccination (HBsAg negative, total anti-HBc negative, and anti-HBs positive); acute HBV infection (positive for HBsAg, total anti-HBc and IgM anti-HBc and negative for anti-HBs); chronic HBV infection (positive for HBsAg and total anti-HBc and negative for anti-HBs and IgM anti-HBc); past HCV infection (anti-HCV positive and HCV RNA negative) and chronic HCV infection (positive for anti-HCV and HCV RNA). Overall HBV vaccination coverage was defined as self-reported to having completed three doses of hepatitis B vaccination or having obtained immunity from natural infection or vaccination.

Statistical analysis

Data were analyzed by weighing statistics using STATA v.15 and presented as percentage or odd ratio (OR) with 95% confidence intervals (CI). We conducted a univariate analysis to measure the association of the outcomes with socio-demographic and KAP factors. Only risk factors with significant association (p-value < 0.05) were further analyzed using a Binary logistic regression model to check the association between HBV/HCV prevalence or HBV vaccination coverage with other important predictors including age, sex, education level, current profession and working exposure.

Ethical considerations

Ethical clearance was obtained from the National Ethic Committee for Health Research (NECHR, N°. 320) of the Cambodia Ministry of Health and the Ethical

Review Panel of the World Health Organization Regional Office for the Western Pacific (WPRO-ERC, N°.19.7.Cam.1.HIS). This study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from each participant regarding the blood sample and the use of his or her data for research purposes.

Results

Sociodemographic characteristics of study participants

A total of 755 HCWs consented to participate in the study while 745 HWCs accepted providing blood samples. Twenty-five HCWs refused to participate in the study. Table 1 describes study participant characteristics. The gender proportion is very similar to the whole HCWs population in Cambodia. The majority of participants were nurses (42.7%), followed by midwives (30.2%) and medical doctors (10.0%). With respect to the highest education attained, 44.4% of participants hold an associate degree and 12.6% a bachelor's degree. Most participants (60.9%) reported a monthly income between 250–500 USD. With respect to years spent in the current job position, 23.1% reported 2–5 years, 25.6% reported 5-10 years and 27.8% reported over 20 years.

Knowledge of HBV and HCV

HCWs have ever heard of HBV for 97.2% (95% CI: 95.9–98.1) and of HCV for 95.7% (95% CI: 94.1–96.9). Participants answered correctly for up to more than 90% on transmission not through mosquito or insect bites, on transmission route through syringe or needle sharing, or blood transfusion (Table 2). Only 36.3% and 42.6% of participants knew that HBV and HCV respectively could not be transmitted by just eating with

Table 1. Sociodemographic and occupational characteristics of HCWs participants

Participant characteristics	*n* = 755	%	(95% CI)
Gender			
Male	340	45.0	(40.1–50.0)
Female	415	55.0	(50.0–59.9)
Age (Years)			
20–34 Year	380	50.4	(43.6–57.2)
35–49 Year	215	28.2	(24.4–32.4)
50–64 Year	154	20.7	(16.6–25.5)
≥ 65 Year	6	0.7	(0.3–1.9)
Marital Status			
Single	127	16.7	(13.2–20.9)
Currently married	589	78.0	(73.5–81.9)
Living together, not married	4	0.5	(0.2–1.8)
Separated/Divorced	5	0.7	(0.2–1.9)
Widowed	28	3.8	(2.3–6.2)
No response	2	0.3	(0.1–1.3)
Profession			
Medical Doctor	77	10.0	(6.8–14.5)
Nurse	320	42.7	(42.7–48.0)
Midwife	224	30.2	(24.3–36.9)
Dentist	5	0.7	(0.3–1.7)
Pharmacist	8	1.0	(0.5–2.2)
Laboratory technician	17	2.3	(1.5–3.4)
Radio technician	4	0.6	(0.2–1.5)
Physiotherapist	2	0.3	(0.1–1.2)
Administrator	16	2.1	(1.1–3.9)
Janitorial	22	2.9	(1.3–6.6)
Security and Labor Unit	16	2.0	(1.1–3.5)
Unknown (other)	44	5.4	(3.5–8.4)
Highest education attained			
Doctoral degree	14	1.9	(1.1–3.3)
Master's degree	10	1.3	(0.7–2.6)
Specialized doctoral degree	25	2.8	(1.2–6.5)
General Practitioner/General Dentist	44	6.0	(3.9–9.1)
Bachelor's degree	98	12.6	(9.8–16.1)
Associate degree	332	44.4	(37.2–51.9)
Primary level Professional degree	97	13.2	(9.1–18.8)
High school	39	5.1	(2.6–9.7)
Secondary school	32	4.2	(2.8–6.3)
Primary school	25	3.3	(1.8–5.9)
No education	22	2.9	(1.0–8.4)
Don't know	5	0.7	(0.2–1.9)
No response	12	1.5	(0.7–3.1)

Table 2. Proportion of HCWs correctly answering questions on transmission of HBV and HCV, HBV vaccination, diagnosis and treatment and guideline

Correctly answered	HBV		HCV	
	%	(95% CI)	%	(95% CI)
Knowledge on transmission and prevention of HBV/HCV				
1. HBV/HCV is a viral infection.	77.9	(73.3–81.8)	74.1	(67.9–79.4)
2. Someone infected with HBV/HCV can look healthy.	70.7	(64.5–76.3)	66.7	(60.4–72.5)
3. Eating with HBV/HCV infected people, will not get him/her also infected.	36.3	(31.9–40.9)	42.6	(38.2–47.2)
4. HBV/HCV can be transmitted through unprotected sex.	80.1	(75.7–83.9)	77.8	(72.8–82.1)
5. HBV/HCV cannot be transmitted through sneezing, coughing & spitting.	57.1	(52.4–61.7)	58.8	(53.6–63.9)
6. HBV/HCV cannot be transmitted through Mosquito / insect bite.	91.2	(88.0–93.5)	89.4	(85.7–92.3)
7. HBV/HCV can be transmitted through sharing needles and syringes.	95.7	(93.8–97.1)	95.0	(92.9–96.5)
8. HBV/HCV can be transmitted through blood transfusion.	96.6	(95.0–97.8)	96.4	(94.3–97.7)
9. HBV/HCV can be transmitted from mother to child through breastfeeding, during birth process and in utero.	17.4	(11.7–25.1)	16.0	(10.9–22.7)
10. HBV/HCV infection is preventable.	96.7	(94.5–98.0)	75.0	(68.7–80.4)
11. HBV/HCV infection could be prevented by using condom when having sexual intercourse.	77.5	(73.7–80.9)	79.1	(75.4–82.4)
12. We cannot prevent HBV/HCV by just avoiding touching HBV/HCV infected person.	73.9	(67.0–79.8)	73.7	(66.7–79.6)
13. HBV/HCV infection can be prevented by not sharing needle & syringe.	91.4	(87.4–94.3)	91.1	(86.7–94.2)
Knowledge on HBV vaccination				
14. Ever heard about HBV vaccination.	98.1	(96.9–98.8)		
15. Vaccination can protect a person from HBV infection.	92.2	(88.7–94.7)		
16. HBV vaccine cannot protect all types of liver diseases.	86.5	(83.0–89.4)		
17. An adult should receive 3 doses of HBV vaccination to be effective.	56.6	(50.2–62.8)		
18. A child should receive 3 doses of HBV vaccination to be effective.	40.2	(33.8–47.1)		
19. An infant should receive their first dose of hepatitis B vaccine within 24 hours after birth.	91.5	(85.7–95.1)		
20. A full dose of hepatitis B vaccine can protect someone for ≥ 20 years.	37.1	(30.2–44.6)		
Knowledge on HBV/HCV diagnosis and treatment				
21. Symptoms of HBV infection are jaundice, nausea, vomiting, loss of appetite, no symptom.	7.3	(4.2–12.3)	7.1	(4.1–12.1)
22. We can tell that someone is infected by HBV/HCV by testing blood.	98.4	(95.8–99.4)	97.8	(95.8–98.9)
23. There is a treatment available for HBV/HCV infection.	72.7	(64.8–79.4)	81.2	(74.7–86.4)
24. HBV is not curable / HCV is curable.	36.9	(32.2–41.8)	71.4	(65.5–76.7)
25. A treatment for chronic HBV infection is a long-life treatment.	72.9	(68.8–76.5)		
Knowledge on HBV/HCV guideline				
26. There is a national guideline for treatment of HBV/HCV infection in Cambodia.	41.5	(33.7–49.7)	44.7	(36.8–52.8)
27. Has Cambodia got a national immunization program that includes vaccination for HBV? vaccination for HCV?	93.6	(88.0–96.7)	71.6	(65.2–77.2)

HBV/HCV infected people. Only 57.1% and 58.8% knew that HBV and HCV respectively could not be transmitted through sneezing, coughing and spitting. Less than 20% answered correctly for the possible transmission route from mother to child by breast feeding, during birth process and in utero, with 66.3% (95% CI: 58.2–73.5) of respondents not knowing that HBV can be transmitted in utero (data not shown) and being mostly medical staffs (69% of medical doctors, 69% of nurses and 65% of midwifes). Around 78% answered correctly that using a condom during sexual intercourse is an infection protection. More than 90% knew that the vaccination could protect someone from HBV infection. But only 56.6% and 40.2% answered correctly that 3 doses of HBV vaccine were effective to protect an adult and a child respectively. Only 37.1% knew that a full dose of HBV vaccine could protect someone for > 20 years. For the diagnosis and treatment theme, more than 97% knew that blood testing is a mean to detect an infected patient. Less than 10% knew all correct clinical symptoms of HBV/HCV infection with 67.2% and 69.4% of respondents not knowing that HBV and HCV respectively would have no symptoms. 36.9% knew that HBV was not curable while 71.4% knew that HCV was curable. Less than 50% of participants knew there was a national treatment guideline for HBV and HCV infection.

Among the 755 respondents, up to 87% thought that their current workers were at risk of being infected with HBV/HCV and more than 95% answered that they

would join the profession even though knowing the risk. Almost all participants thought that HCWs should be tested for HBV/HCV and they were also willing to test for it. Around 67% thought that all patients should have been screened for HBV/HCV and 51% thought that infected patients should have been separated from others. Around 67% answered that they would accept taking care of infected patients. Around 88% thought that HCWs infected with HBV/HCV should be allowed to work. For vaccination, 16% of participants still thought that HBV vaccine was slightly effective but 99% were willing to get the vaccination. There was a very high demand (> 99%) for getting treatment and up to 85% answered they would go to a public hospital (Table 3).

Around 57.7% responded that they always recapped needles after uses, putting them at a higher risk of needle stick injury. About half (46.7%) reported ever been vaccinated for HBV and 80% got a vaccination at private facilities (Table 4).

Prevalence of HBV and HCV infection

Among 745 participants who accepted providing blood samples, 4.9% (95% CI: 3.6–6.6) and 2.3% (95% CI: 1.6–3.2) were positive for HBsAg and for anti-HCV respectively but there was no coinfection. We found 3.8% (95% CI: 2.7–5.2) chronic HBV infection, 1.1%

Table 3. Attitude answers on HBV and HCV

Questions on attitude	"yes" for HBV		"yes" for HCV	
	%	(95% CI)	%	(95% CI)
Attitude towards HBV/HCV risk exposures / susceptibility				
1. Think that his/her current work is at risk of being infected with HBV/HCV.	88.0	(84.5–90.8)	87.3	(83.4–90.4)
2. He/she would join this profession though knowing the potential risks of exposure to HBV/HCV.	95.8	(93.2–97.5)	95.7	(93.2–97.3)
Attitude towards HBV/HCV Testing and infected patients				
3. He/she think that all healthcare workers (HCWs) should be tested for HBV/HCV infection.	96.8	(95.2–97.9)	96.3	(94.7–97.4)
4. He/she is willing to be tested for HBV/HCV infection.	98.8	(97.0–99.6)	98.8	(97.0–99.5)
5. To avoid contamination to/from patients, it is important to screen HBV/HCV on all patients.	67.0	(60.1–73.2)	67.1	(60.3–73.3)
6. He/she would accept if being asked to take care of HBV/HCV infected patients.	67.6	(61.6–73.1)	66.8	(60.9–72.2)
7. He/she think that an HBV/HCV infected patient should be taken care separately from other patients.	51.9	(45.5–58.2)	51.7	(45.3–58.0)
8. Think that if a HCW was infected with HBV/HCV, he/she would be at risk of transmitting HBV/HCV to patients.	56.8	(50.5–62.8)	55.4	(49.3–61.3)
9. Think that HCWs infected with HBV/HCV should be allowed to work.	88.4	(84.5–91.3)	88.3	(84.8–91.2)
Attitude towards HBV vaccination				
10. Think that all HCWs who are working directly with patients should be vaccinated for HBV.	98.0	(95.9–99.0)		
11. He/she is willing to get vaccination, if not yet infected.	99.0	(97.8–99.6)		
12. He/she think that, in protecting someone against HBV infection, HBV vaccination is:				
Very effective	78.3	(74.1–81.9)		
Slightly effective	16.0	(12.9–19.6)		
Not effective	0.6	(0.6–1.8)		
13. He/she think that all infants shall get HBV vaccine.	99.6	(98.6– 99.9)		
Attitude towards HBV treatment/ seriousness				
14. If found infected with HBV/HCV, he/she is willing to go for further investigation and treatment.	99.6	(98.6–99.9)	99.7	(98.7–99.9)
15. For HBV/HCV treatment, he/she would go to:				
Public hospital	84.8	(78.4–89.5)	85.1	(79.3–89.5)
NGO clinics	7.5	(4.6–12.0)	7.8	(5.1–11.6)
Private hospital/clinic	4.3	(2.9–6.4)	3.8	(2.4–6.0)
In another country	2.7	(1.5–4.8)	2.5	(1.4–4.2)
16. If found chronically infected with HBV/HCV, he/she would be worried the most about:				
Developing liver cancer	46.5	(40.1–53.1)	47.4	(41.1–53.8)
Transmission to family	27.2	(21.8–33.4)	26.5	(20.9–32.9)
Death	12.3	(8.3–17.7)	12.2	(8.4–17.6)
No effective treatment	6.3	(4.4–9.1)	5.8	(3.9–8.6)
High cost of treatment	5.8	(4.0–8.2)	5.2	(3.5–7.6)

Table 4. Practice answers on HBV and HCV

Questions for Practice	Response "yes"	
	%	(95% CI)
Practice on prevention and vaccination		
1. He/she has ever tested for HBV infection.	72.0	(66.1–77.2)
2. He/she has ever tested for HCV infection.	55.8	(48.3–63.1)
3. Ask his/her barber to change to new blade, every time he/she has haircut:		
Always	77.1	(70.4–82.6)
Never	13.1	(9.2–18.3)
Sometimes	6.2	(3.8–9.9)
4. Ask beauty salon staff to use clean nail cutter, every time she has her nail cut:		
Always	66.7	(57.1–75.1)
Never	7.3	(4.7–11.2)
Sometimes	4.6	(2.9–7.3)
5. He/she recap needle after usage in everyday practice:		
Always	57.6	(49.4–65.4)
Never	29.9	(23.3–37.4)
Sometimes	9.3	(6.5–13.2)
6. He/she has ever been vaccinated against HBV.	46.7	(40.5–53.1)
7. If vaccinated, he/she got injection of:		
≥ Three doses	81.6	(77.1–85.5)
Two doses	11.0	(8.0–14.9)
One doses	5.9	(4.0–8.6)
8. After receiving the last dose of HBV vaccine, he/she has checked anti-HBs Ab.	73.5	(68.2–78.2)
9. He/she received the last HBV vaccine at:		
Private hospital/clinic	79.7	(71.2–86.2)
Public facility	18.6	(12.1–27.5)

(95% CI: 0.6–2.0) acute HBV infection and 0.9% (95% CI: 0.4–2.1) active HCV infection among all HCWs. Among positive HBsAg HCWs, HBV acute infection was 22.2%.

HBV vaccination coverage

Serological markers showed 21.6% (95% CI: 18.3–25.4) immunity due to natural infection, 24.9% (95% CI: 20.7–29.7) immunity due to vaccination and 38.8% (95% CI: 33.0–45.0) immunity on 3 doses reported. The overall HBV vaccination coverage was 59.3% (95% CI: 53.1–65.2).

Factors associated with HBV and HCV infection and HBV vaccination coverage

Univariate analysis found no significant association between HBsAg positive with socio-demographic variables, nor with work exposure, nor with KAP outcomes in the study. However, the prevalence of anti-HCV positive was seven times higher in the old age group (≥ 50 years old) compared to other age groups (OR = 9.5, 95% CI: 2.4–38.2, p = 0.003). For HBV vaccination coverage, the study showed that the young age group (20–34 years old) was more likely to have gotten vaccinated compared to old age groups (35–49 and ≥ 50 years old) (Table 5). Females are also more likely to have gotten vaccinated compared to males (OR = 1.7; 95% CI: 1.2–2.5, p = 0.008). Participants with education above high school were more likely to have gotten vaccinated compared to those with high school level and below. For professions, nurses, janitorial and security were less likely to have gotten vaccinated if compared to medical doctors (Table 5). No statistically significant association was found between HBV vaccination coverage and work exposure to blood and other body fluids. When analyzing with KAP variables, participants who answered being ever tested for HBV infection were seven times more likely to have gotten vaccinated when compared to other participants (OR = 7.2, 95% CI: 4.9–10.7, p = 0.0001). Multivariate analysis reconfirmed that younger age group, females, and higher professional categories are predictors of getting vaccinated (Table 5).

Discussion

Study results reveal low knowledge on HBV/HCV route of transmission. A quarter of HCWs wrongly knew that eating and touching were routes of transmission, similar to a recent finding among HCWs in Vietnam regarding HBV transmission (*8*). This might potentially lead to discrimination of HCWs from providing care to HBV/HCV infected patients as previously seen in HIV infected patients (*9*). Route of transmission from mother to child in utero was also not well known by HCWs, in particular medical doctors, nurses and midwifes who should have been well trained about this transmission route, so that they would take preventive measures of testing, and treatment prior to antennal care (*10*). However, they are HCWs at the front line

Table 5. Univariate and multivariate analysis with significant associated outcomes

Variables	*n*	%	Crude association		Adjusted association	
			OR	(95% CI)	AOR	(95% CI)
Outcome: Vaccination coverage						
Association with socio-demographic variables						
Age	754					
20-34		66.9	1.0	-	1.0	-
35-49		54.6	**0.6**	(0.4–0.9)	**0.7**	(0.5–1.0)
≥ 50		47.4	**0.5**	(0.3–0.7)	**0.5**	(0.3–0.7)
Sex	754					
Male		52.2	1.0	-	1.0	-
Female		65.0	**1.7**	(1.2–2.5)	**2.1**	(1.4–3.2)
Education level	737					
High School and Below		41.9	1.0	-		
Above High School		62.8	**2.3**	(1.4–3.9)		
Current profession	754					
Medical Doctor		68.6	1.0	-	1.0	-
Nurse		57.7	**0.6**	(0.4–0.9)	**0.6**	(0.3–1.0)
Midwife		64.1	0.8	(0.4–1.6)	**0.4**	(0.2–0.7)
Dentist		81.2	1.9	(0.2–21.6)	1.4	(0.1–13.3)
Pharmacist, RadioTech, Physio		84.6	2.5	(0.6–11.3)	2.1	(0.4–10.4)
Laboratory technician		66.2	0.9	(0.3–2.6)	0.8	(0.2–2.4)
Administrator		51.2	0.5	(0.2–1.2)	0.5	(0.2–1.5)
Janitorial		27.8	**0.2**	(0.1–0.5)	**0.1**	(0.0–0.4)
Security & Labor		22.3	**0.1**	(0.0–0.5)	**0.2**	(0.0–0.5)
Other (unknown)		46.5	**0.4**	(0.2–0.7)	**0.3**	(0.2–0.7)
Work exposure	747					
Minimum Exposure = 1		57.3	1.0	-		
Medium blood exposure = 2		57.8	1.0	(0.7–1.5)		
High blood exposure = 3		65.3	1.4	(0.8–2.4)		
Association with KAP variables						
Have you ever tested for HBV infection?	754	72.3	**7.2**	(4.9–10.7)		

Note: OR and AOR numbers in bold indicate significant association between outcomes and predictors.

with low knowledge of this transmission route and would put infants at high risk of becoming HBV infected ranging from 70% to 90%, especially when mothers have high HBV load or are HBeAg positive (*11*). While almost all HCWs knew that HBV was preventable, only a quarter responded that HCV could be prevented. This is possibly because respondents were attributing prevention of transmission only to the existence of vaccines, rather than considering all potential preventative measures of blood-borne diseases. The results surprisingly showed that only around 80% of HCWs knew that unprotected sex was a route of transmission while 20% did not know, and those included 6% of medical doctors, 22% of nurses and 15% of midwifes (data not shown). There were still up to 30% of HCWs who did not know that HCV is treatable but fortunately, 90% of medical doctors got the correct answer (data not shown). To improve the knowledge of HCWs, the 1st national strategic plan (2020–2024) for viral hepatitis B and C has set specific action plans, which include increasing awareness of hepatitis infection to HCWs by integrating the prevention and treatment of viral hepatitis into the existing training curriculum for all healthcare providers, providing in-service training and integrating content on prevention and treatment of viral hepatitis into the National Infection Prevention Control (IPC) program (*12*).

Regarding HCW's attitude, positive responses to their wanting for HCWs to be tested for HBV/HCV and willingness to be tested and vaccinated, indicate that a national policy would be welcomed by Cambodian HCWs. However, we are concerned that a smaller number of HCWs want all patients to be tested that may indicate some stigma (*13*) to be addressed in the national policy. So awareness needs to be raised that HCWs can be protected without compromising equal treatment of patients, to prevent potential stigma and discrimination of patients in the health care setting.

The national immunization program (NIP) provides free HBV vaccine to newborns in public healthcare facilities up to the 3rd dose. The NIP began in 2001 and became universal in the country in 2005 (*14*). This indicates that all HCWs participants in this study were not covered by this Universal coverage immunization program, because they were older than 20 years old at the study time, 2019. Today HBV vaccination for 3 doses could cost around 30$, so it appears expensive if compared to their monthly incomes between $250–500 for 61% (95% CI: 52.5–68.8), so there would be a need for free vaccination or at least an affordable price in the national policy. This vaccination policy would apply

across the public and private health workforce, given that 25% of HCWs work also in the private sector (data not shown). The HBV vaccination coverage found is similar to some recent studies among HCWs in Asian countries (*15,16*).

High acute HBV prevalence indicates IPC problems. As expected, the HBV prevalence among HCWs is higher than among the general population (4.9% *vs.* 3.0%, respectively). However, this prevalence was slightly lower compared to studies among HCWs in neighboring countries (*17-19*). Risk factors associated with HBV/HCV prevalence was not found. The lack of significance associated with a positive HBsAg was not surprising, because the infection may occur during childhood (*20*). The seroprevalence of HCV among HCWs and among the Cambodian general population was similar (2.3 *vs.* 2.6%, respectively) (*21*). Surprisingly, the HCV viremic prevalence was even lower among HCWs than among Cambodian general population (0.9 *vs.* 1.9%, respectively) (*21*). The older ages (≥ 50 years) seemed to be the main risk factor associated with the HCV seroprevalence in HCWs addressing that DAA medicines shall be included in the national policy for HCWs and make the medicine available to protect and cure HCWs.

As another IPC concern, the study still found risks of infection due to HCWs practicing recapping needles after use. This inappropriate behavior might be a result of the lack of sharp boxes available in the setting in remote areas as noticed on site by the study teams. These IPC issues might be supported by high acute HBV infection (22%) among positive HBsAg HCWs, addressing IPC is still very critical for the health care setting given the high proportion of those who are recently infected.

The study provides first-time data of HBV and HCV well representative for general HCWs based on HCWs' data of the Ministry of Health in 2019. However, it has several limitations. Some parts of IPC questionnaires were missing, so we could not conclude the actual link between HBV high prevalence and practical issues of HCWs. Finally, overall HBV vaccination coverage included self-reported data of having completed three doses of HBV vaccine without proof of vaccination card, which might be subject to recall bias.

Conclusion

Findings from the study indicated an urgent need of a national policy for Cambodian HCWs given the high prevalence of hepatitis among this group and an annual action plan to implement that policy in order to strengthen the capacity of HCWs through in-service training programs for health professionals. The policy should also include a testing strategy and vaccination program for HCWs. There is need to ensure a strong policy on stigma and discrimination towards people living with HBV/HCV, including in healthcare settings.

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Prevalence of transmitted drug resistance and phylogenetic analysis of HIV-1 among antiretroviral therapy-naïve patients in Northern Vietnam from 2019 to 2022

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Abstract: Since the rapid expansion of antiretroviral therapy (ART) for HIV, transmitted drug resistance (TDR) has become a major concern in Vietnam. HIV services there are transitioning to be covered by social insurance. Access to pre-exposure prophylaxis (PrEP) is being expanded to tackle the growing HIV epidemic among men who have sex with men. Therefore, a cross-sectional study was conducted at 10 ART facilities in Northern Vietnam from 9th December 2019 to 9th June 2022 to investigate the prevalence and pattern of TDR among ART-naïve people living with HIV (PLWH). TDR mutations were defined according to the World Health Organization 2009 List of Mutations for Surveillance of Transmitted Drug Resistant HIV Strains. Mutation transmission dynamics and TDR clusters were investigated *via* phylogenetic analysis. We enrolled 391 ART-naïve PLWH. The overall TDR prevalence was 4.6%, with an annual prevalence of 6.0% in 2019/2020, 4.8% in 2021, and 1.3% in 2022. TDR mutations to non-nucleoside reverse transcriptase inhibitors (2.8%), including K103N were the most common. Less commonly, the protease inhibitor-associated mutation M46I and mutations to nucleoside reverse transcriptase inhibitors, including M184V/I, were observed. CRF01_AE was the most common subtype (77.0%). CRF07_BC (14.3%), which had been rare in Vietnam, was also observed. No genetic association was observed between HIV-1 sequences with TDR mutations. In conclusion, the overall prevalence of TDR was stably low in this region. The phylogenetic tree suggests that TDR clusters have not formed. Continuous monitoring of HIV TDR and strains is crucial to maintaining ART and PrEP efficacy.

***Keywords*:** transmitted drug resistance, mutations, phylogeny, HIV, Vietnam

Introduction

The extensive expansion of access to antiretroviral therapy (ART) and pre-exposure prophylaxis (PrEP) are key components to controlling and preventing HIV infection and ending the HIV/AIDS epidemic. However, with the increased use of antiretroviral drugs, HIV drug resistance (HIVDR) has emerged, impacting the drugs' ability to block viral replication. In particular, transmitted drug resistance (TDR), which occurs when individuals are infected with an HIV strain that is already resistant to one or more drugs, compromises the efficacy of ART and PrEP, and poses a potential threat to the success of HIV elimination. A recent review found that the prevalence of TDR among ART-naïve people living with HIV (PLWH) varied regionally from 4.1% in South/Southeast Asia to 14.2% in North America between 2014 and 2019, with a rising trend in some regions (*1*).

Vietnam has made considerable progress in responding to the HIV epidemic over the past 30 years. By 2021, approximately 70% of an estimated 240,000 PLWH were receiving ART in Vietnam (*2*). The number of new infections decreased from 210,000 in 2003 to 5,700 in 2021 (*2*). In addition, according to nationally representative surveillance, the prevalence of TDR in Vietnam remained low at 5.8% (the prevalence of having any pre-treatment HIVDR) in 2017–2018 (*3*). The prevalence has been maintained below the threshold of 10% beyond which the World Health Organization (WHO) recommends changing the first-line treatment regimen (*4*).

However, in recent years, there has been a growing concern that the incidence of TDR could rise and these

gains could be reversed in Vietnam's unique context, which would hinder achievement of the 95-95-95 target (*5*) by 2025. Vietnam is currently facing a transition in the funding of HIV services, from international donor support to domestic funding using social health insurance (SHI) (*6*). This transition imposes a series of important changes on beneficiaries and providers. In particular, decentralization of HIV services and out-of-pocket costs can be barriers to continuing ART for PLWH (*7*), with potential subsequent increases in the risk of developing and transmitting HIVDR.

Increased TDR levels could have a negative impact on the efficacy of PrEP. Recently, elevated HIV prevalence in men who have sex with men (MSM) has been noted in Vietnam; HIV prevalence among MSM increased from 5.1% in 2015 to 13.2% in 2020 (*8*). To address HIV prevention in this emerging population with elevated HIV infection rates, Vietnam introduced PrEP for MSM and transgender women in 2017 (*9*). In addition, drug resistance testing is not covered by SHI due to limited testing capacity, and it is rarely performed in Vietnam for purposes other than surveillance. In this context, there is a considerable need to evaluate TDR in Vietnam. However, relevant data are limited, especially from real-world clinical settings.

HIV is a highly diverse and rapidly evolving pathogen, with multiple subtypes and recombinant forms that can differ in their transmission dynamics, virulence, and drug resistance profiles. Phylogenetic analysis is a powerful tool to study the evolutionary relationships among different HIV strains, as well as infer transmission networks and patterns within specific populations. Amid the rapidly changing dynamics of the HIV epidemic, this analysis can help identify circulating HIV strains and the distribution of TDR in ART-naïve PLWH in Vietnam. This information will be crucial for the development of targeted HIV infection prevention and control strategies.

Conducted during the transition to SHI-based HIV services and the emerging HIV epidemic among MSM in Northern Vietnam, this study aimed to investigate the prevalence and pattern of TDR among PLWH with no previous exposure to ART. We also analyzed the phylogeny of HIV strains to identify their transmission dynamics and the distribution of TDR.

Patients and Methods

Study design and participants

From 9th December 2019 to 9th June 2022, a cross-sectional study was conducted among ART-naïve PLWH from 10 provincial- or district-level facilities in Northern Vietnam. These facilities were registered in a program titled "Science and Technology Research Partnership for Sustainable Development (SATREPS)". Eligible participants were ART-naïve PLWH aged ≥16 years with confirmed HIV infection according to local guidelines. Participants were excluded if their physician deemed them likely to have a history of ART use, which would render them ineligible to participate in the study. The convenience sampling technique was applied and all patients who met the inclusion criteria were invited to take part in the study. Well-trained healthcare staff at the study sites collected the baseline characteristics of the participants.

Drug resistance testing

Drug resistance testing was performed for those with an HIV viral load (HIV-VL) > 1,000 copies/mL, following a guideline issued by the Vietnam Ministry of Health (*10*). To measure HIV-VL, two EDTA tubes of whole blood were collected from each participant to extract plasma. All plasma samples were stored at −20 °C or lower at the study sites, then transferred on dry ice or gel ice to the National Hospital for Tropical Diseases in Hanoi. The quantitative measurement of HIV-VL was carried out using two automated systems, COBAS® AmpliPrep/COBAS® TaqMan and cobas® 6800 (Roche Diagnostics, Switzerland).

First, for drug resistance testing, HIV-1 RNA purification was conducted with the QIAamp Viral RNA Mini Kit (Qiagen, Germany). Next, the PrimeScript II High Fidelity RT-PCR Kit and the PrimeSTAR GXL DNA Polymerase Kit (Takara Bio, Japan) were used to amplify the protease (1–99) and reverse transcriptase (1–560) regions of HIV-1. Then, Sanger sequencing was conducted using the 3500 Genetic Analyzer (Thermo Fisher Scientific, USA). All primers used for polymerase chain reaction and sequencing are listed in Supplemental Table S1 (*https://www.globalhealthmedicine.com/site/supplementaldata.html?ID=77*) (*11,12*). Finally, the sequence results were analyzed with ATGC software (Genetyx Corporation, Japan) to confirm the mixture of bases and construct consensus sequencing. The Stanford HIV Drug Resistance Mutations Database (*https://hivdb.stanford.edu/*) was used to evaluate relevant sequences associated with resistance to nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitor (PIs). TDR was determined using the World Health Organization 2009 List of Mutations for Surveillance of Transmitted Drug Resistant HIV Strains (hereafter referred to as the WHO 2009 Mutation List) (*13,14*); HIV strains with at least one TDR mutation on the list were defined as resistant.

Phylogenetic analyses

To evaluate the transmission dynamics and distribution of TDR, a phylogenetic tree was constructed. Sequences were aligned with the HIValign tool of the Los Alamos

database (*https://www.hiv.lanl.gov/content/index*) and altered by the GENETYX Parallel Editor program (Genetyx Corporation, Japan). MEGA 7 software was used to construct the phylogenetic tree from the aligned data, with the neighbor-joining method using the Kimura two-parameter model. The bootstrap replication was set as 1,000, and the clustering of sequences with a bootstrap value > 90% was considered significant.

Statistical analyses

The overall and class-specific TDR prevalence by year were calculated from 2019 to 2022. However, only nine samples were collected in December 2019. These were combined with the samples collected in 2020 for annual analysis. In addition, chi-square tests were performed to assess associations between any TDR mutation and demographic factors (*i.e*., gender, age, facility level, facility location, and route of HIV transmission). The pattern of class-specific TDR mutations was also descriptively analyzed. All statistical analysis was performed using Stata software (version 16, StataCorp, College Station, TX, USA).

Ethical considerations

The study was approved by both the Human Research Ethics Committee of the National Center for Global Health and Medicine in Japan (reference: NCGM-G-003124-02, NCGM-G-003124-03) and the Bio-medical Research Ethics Committees of the National Hospital for Tropical Diseases in Vietnam (reference:12/HDDD-NDTU, 17/HDDD-NDTU). All study participants provided written informed consent for participation and for the use and publication of their clinical and laboratory data for research. This study was conducted in accordance with the principles of the Declaration of Helsinki.

Results

Study participants

In total, 391 ART-naïve PLWH from 10 study sites participated in this study. Of these, 55.8% were from provincial-level hospitals and 44.2% were from district-level facilities. Their median age was 33 years old (inter-quantile range: 26–44), and the majority were male (80.0%). The main route of HIV transmission was heterosexual contact (44.0%), particularly contact with a spouse or long-term partner, followed by male homosexual contact (39.6%). The median baseline HIV-VL was 95,300 copies/mL (inter-quantile range: 37,100–273,000) with approximately half having an HIV-VL > 100,000 copies/mL (Table 1). There was no association between the baseline characteristics of

Table 1. Characteristics of study participants and prevalence of transmitted drug resistance mutations

Characteristics	*n* (%)	any TDR *n* (%)	*p* value*
Gender			0.413
Male	312 (80.0)	13 (4.2)	
Female	79 (20.0)	5 (6.3)	
Age (years)			
Median (IQR)	33 (26–44)		0.385
< 30	155 (39.6)	6 (3.9)	
30 to < 40	103 (26.3)	6 (5.8)	
40 to < 50	85 (21.8)	2 (2.4)	
≥ 50	48 (12.3)	4 (8.3)	
Facility level			0.986
Provincial	218 (55.8)	10 (4.6)	
District	173 (44.2)	8 (4.6)	
Facility location			0.150
Hanoi	319 (81.6)	17 (5.3)	
Other provinces	72 (18.4)	1 (1.4)	
Route of HIV transmission			0.838
Heterosexual contact	172 (44.0)	9 (5.2)	
MSM	155 (39.6)	6 (3.9)	
IDU	34 (8.7)	1 (2.9)	
Other/Unknown	30 (7.7)	2 (6.7)	
Baseline viral load (copies/mL)			
Median (IQR)	95,300 (37,100–273,000)	-	
200–999	2 (0.5)	-	
1,000–9,999	29 (7.4)	-	
10,000–99,999	168 (43)	-	
100,000–999,999	162 (41.4)	-	
≥ 1,000,000	30 (7.7)	-	

*Chi-squared test. TDR: transmitted drug resistance; IQR: inter-quantile range; MSM: men who have sex with men; IDU: injection drug use.

Table 2. The prevalence of transmitted drug resistance

TDR prevalence	Total *n* (%)	2019[#]/2020	2021	2022
Study participants (*n*)	391	150	165	76
Any DRM	18 (4.6)	9 (6.0)	8 (4.8)	1 (1.3)
Any NRTI DRM	5 (1.3)*	2 (1.3)	2 (1.2)*	1 (1.3)
Any NNRTI DRM	11 (2.8)*	4 (2.7)	7 (4.2)*	0 (0.0)
Any PI DRM	3 (0.8)	3 (2.0)	0 (0.0)	0 (0.0)

*One patient had drug resistance mutations to both NRTIs and NNRTIs and was classified in two categories. [#]Only nine samples were collected in December 2019, so they were combined with the 2020 samples for annual analysis. TDR: transmitted drug resistance; DRM: drug resistance mutation; NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: nonnucleoside reverse transcriptase inhibitor; PI: protease inhibitor.

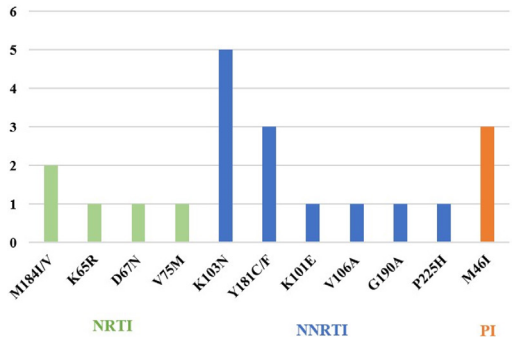

Figure 1. Drug class-specific transmitted drug resistance mutations. NRTI: nucleotide reverse transcriptase inhibitor; NNRTI: nonnucleoside reverse transcriptase inhibitor; PI: protease inhibitor.

the participants and the presence of any TDR mutation (Table 1).

Overall and annual prevalence of TDR

The overall prevalence of having any TDR mutation was 4.6%, with an annual prevalence of 6.0% in 2019/2020, 4.8% in 2021, and 1.3% in 2022. In addition, TDR mutations for NNRTIs were the most prevalent throughout the study period, whereas TDR mutations for NRTIs and PIs were less prevalent. One patient carried TDR mutations for both NRTIs and NNRTIs. No patient was resistant to all three drug classes (Table 2).

Drug class-specific TDR mutations are listed in Figure 1. Single mutations occurred the most frequently. The most common NNRTI-associated mutations was K103N followed by Y181C/F, K101E, V106A, G190A, and P225H. As for the NRTI-associated mutations, M184I/V was the most frequently observed, followed by K65R, D67N, and V75M. The M46I mutation, the only one associated with PI resistance, was detected in three participants.

Phylogenetic tree analysis

Phylogenetic tree analysis was conducted for the protease and reverse transcriptase regions of HIV-1 (HXB2: 2253-4229). No genetic link was observed among HIV-1 sequences with any TDR mutation in this study. The analysis revealed that the most common subtype of HIV-1 was CRF01_AE (n = 301, 77.0%), followed by CRF07_BC (n = 56, 14.3%), CRF08_BC (n = 5, 1.3%), A (n = 1, 0.3%), C (n = 1, 0.3%), and F (n = 1, 0.3%). In addition, other recombinant subtypes (n = 26, 6.6%) formed a cluster at the bottom of the phylogenetic tree (Figure 2). Among the recombinant subtypes, some sequences were nearly identical to those of CRF109_0107, whereas other sequences had a slightly different pattern of recombination. In the upper right region of the phylogenetic tree, heterosexual female participants, heterosexual male participants, and those with a history of injection drug use were abundant. This region had more TDR cases than the other regions of the phylogenetic tree. However, sequences in the upper right were diverse and could not be defined as one group (bootstrap value ≤ 90). The CRF07_BC group made a significant cluster, which was composed of mainly MSM. Sequence data are available in DDBJ/EMBL/GenBank (accession number LC765998-LC766388).

Discussion

Against the backdrop of the transition to SHI-based HIV services and the emerging HIV epidemic among MSM in Vietnam, our study evaluated the prevalence of TDR mutations among ART-naïve PLWH in Northern Vietnam from 2019 to 2022. The TDR prevalence was 4.6% overall, with a preponderance of resistance mutations to NNRTIs (2.8%) over NRTIs (1.3%) and PIs (0.8%), according to the WHO 2009 Mutation List. Our findings are consistent with previous data from Vietnam on pre-treatment PLWH (3.5% between 2009 and 2010 in three major cities (*15*); 6.4% between 2005 and 2008 in the south (*16*), and 5.8% in a nationally representative surveillance between 2017 and 2018 (*3*)). However, the stable trend in TDR prevalence observed in this study contrasts with the trend for rising TDR prevalence in Asia and low-income countries (*17,18*). One explanation for such a difference may be the successful maintenance of HIV treatment during the coronavirus disease 2019 (COVID-19) pandemic in Vietnam (*19*). The effective responses to COVID-19 at all HIV service levels to ensure the preservation of the HIV continuum of care may have contributed to stable treatment outcomes during the pandemic and prevented the development and transmission of HIVDR among PLWH.

The most common TDR mutation observed in this study was K103N in the NNRTI resistance class. This finding is consistent with those of other studies

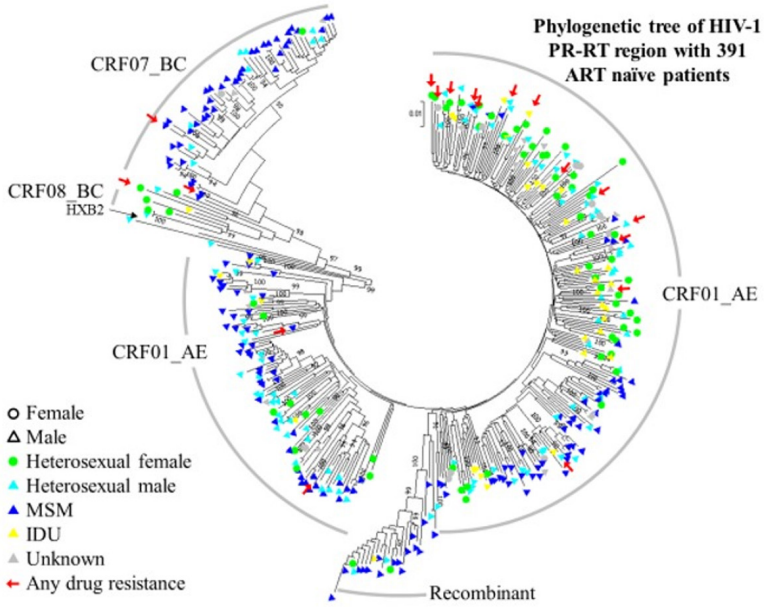

Figure 2. Phylogenetic analysis of HIV strains from antiretroviral therapy-naïve patients in Northern Vietnam from 2019 to 2022. MSM: men who have sex with men; IDU: injection drug users.

conducted in Vietnam (*3,12*) and similar to findings from other low- and middle-income countries (*20-25*). K103N and Y181C/F are drug resistance mutations that can develop in response to treatment with NNRTIs, such as nevirapine or efavirenz. These drugs have been commonly used as the first-line regimen for ART-naïve PLWH in Vietnam for many years (*26*). However, the recent introduction of integrase strand transfer inhibitors (INSTIs) such as dolutegravir (DTG) has provided a new option for the management of HIV infection. DTG has a high genetic barrier to the development of HIV drug resistance. Therefore, the expanded use of a DTG-based regimen may have contributed to the stabilization of TDR prevalence in recent years and may further reduce the prevalence in the future (*27*).

It is also noteworthy that an NRTI-related mutation, M184V/I, and a PI-related mutation, M46I, were found in 2/391 (0.5%) and 3/391 patients (0.8%), respectively. The M184V/I mutation is selected by lamivudine (3TC) and emtricitabine (FTC), one of the combination drugs (TDF-FTC) in PrEP. Therefore, while the use of PrEP is rapidly expanding in Vietnam, the M184V/I mutation may offset its benefits. However, PIs are the major class of drug used in second-line and alternative regimens for those who experience treatment failure. Given that options for second-line and third-line regimens are limited in Vietnam, the emergence of PI-associated mutations could pose challenges for the selection of an optimal ART regimen. Although the prevalence of these TDR mutations is still low, continuous monitoring of HIVDR in PLWH is necessary to ensure effective clinical management and maximize the efficacy of ART and PrEP.

In line with previous reports from Vietnam, in which CRF01_AE predominated (≥ 97%) over other subtypes (*3,12,16,28-30*), CRF01_AE was the most prevalent HIV-1 subtype observed in this study, accounting for infection in 77.0% of the participants. However, in contrast to the findings of these reports, we found that the prevalence of CRF07_BC was much higher (14.3%) and the CRF07_BC cluster consisted primarily of MSM. Notably, a similar trend was observed in a recent study in China, where the prevalence of CRF07_BC increased from 24.1% in 2007 to 40.3% in 2022 (*31*). Furthermore, in addition to the prevalent subtypes CRF07_BC and CRF08_BC in China, we also found subtype CRF109_0107, which has recently been reported in Shenzhen, China (*32*). This is the first report of the migration and expansion of these new strains from China to Northern Vietnam, especially in MSM. Further studies are needed to understand the transmission dynamics of new strains such as CRF07_BC, not only in Northern Vietnam but throughout the country. Furthermore, there was no genetic association observed between TDR cases in our study, indicating that there is no current outbreak of HIV-1-resistant strains. This may be one reason for the low prevalence of TDR in this country.

When assessing the findings of our study, one major limitation should be taken into consideration. We collected data from 391 ART-naïve PLWH in Northern Vietnam. This relatively small sample size from a specific geographical region could limit the generalizability of the findings. A larger sample size would have provided the statistical power to investigate an association between TDR and the characteristics of the study participants.

In conclusion, we found that the prevalence of

TDR was stable at a low level (4.6%) among ART-naïve PLWH in Vietnam from 2019 to 2022, during the transition to SHI-based HIV services and the emerging HIV epidemic among MSM. However, given the prevalence of M46I and M184V/I, and the emerging risk of the development of INSTI-associated mutations during the expansion of DTG use, continuous monitoring of HIVDR is crucial to maintain the efficacy of ART and PrEP and to meet the 95-95-95 target (*5*) by 2025 in Vietnam. An increase in CRF07_BC infection among MSM observed in this study may indicate rapid HIV-1 migration from Southern China to Northern Vietnam, especially among MSM.

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Coronary artery stenosis in Japanese people living with HIV-1 with or without haemophilia

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Abstract: An extremely high prevalence (12.2%) of moderate-to-severe coronary artery stenosis (CAS) was documented in asymptomatic Japanese haemophiliacs living with HIV-1 (JHLH) in our previous study. The cause of this phenomenon remains unknown. We conducted the CAS screening in people living with HIV-1 without haemophilia (PLWH without haemophilia) to compare the prevalence of CAS in JHLH and PLWH without haemophilia and to identify the risk factors including inflammation markers. Ninety-seven age-matched male PLWH without haemophilia who consulted our outpatient clinic between June and July 2021 were randomly selected, and 69 patients who provided informed consent were screened for CAS using coronary computed tomography angiography (CCTA). The number of JHLH cases was 62 in this study. The prevalence of moderate (> 50%) to severe (> 75%) CAS was significantly higher in JHLH [14/57 (24.6%) *vs.* 6/69 (8.7%), $p = 0.015$], and the ratio of CAS requiring urgent interventions was significantly higher [7 (12.3%) *vs.* 1 (1.4%), $p = 0.013$] in JHLH than in PLWH without haemophilia. Among the inflammatory markers, serum titres of intercellular adhesion molecule-1 ($p < 0.05$) and interleukin-6 ($p < 0.05$) in JHLH were significantly higher than those in PLWH without haemophilia. Although some patient demographics were different in the age-matched study, it might be possible to speculate that intravascular inflammation might promote CAS in JHLH.

Keywords: HIV, haemophilia, coronary artery stenosis, coronary computed tomography, Japanese

Introduction

Generally, HIV-infected patients have higher mortality rates due to ischaemic heart disease compared with the general population (*1*). In our previous study, coronary artery stenosis (CAS) screening was performed using coronary computed tomography angiography (CCTA) in 57 almost asymptomatic Japanese haemophiliacs living with HIV-1 (JHLH), with an average age of 47 years (*2*). The study documented that seven patients (12.2%) had severe CAS that required urgent interventions. Five patients underwent percutaneous coronary intervention (PCI), while one patient underwent coronary artery bypass graft surgery (CABG). One patient refused any coronary intervention. The prevalence of CAS was unexpectedly high, suggesting an unknown aetiology that caused CAS in JHLH.

This prevalence was higher than that generally reported for HIV-1-infected patients. The possibility that the presence of haemophilia may have contributed to this higher prevalence was hypothesized. Studies on haemophiliacs without HIV-1 infection and people living with HIV-1 (PLWH without haemophilia) without haemophilia will clarify this issue. In this study, we examined PLWH without haemophilia because we did not encounter haemophiliacs without HIV-1 infection in our clinic. We performed the same screening tests on the asymptomatic randomly selected age-matched male PLWH without haemophilia because all the JHLH in the previous study were male and the results were compared with those of the previous study. Inflammatory markers, such as interleukin-6 (IL-6), highly sensitive C-reactive protein (hs-CRP), and D-dimer, have been reported to be associated with increased CAS risk in HIV-infected patients (*3*). We hypothesized that JHLH shows more severe coronary ischemic lesions with high levels of inflammatory markers.

Patients and Methods

Patients

Ninety-seven, age-matched male PLWH without haemophilia were selected at our outpatient clinic

between June and July 2021 and asked to participate in the study. They had no symptoms suggestive of ischemic heart disease, such as chest pain on exertion or at rest. Sixty-nine patients provided informed consent and underwent CAS screening using CCTA. The median age of patients who participated during the period was 47 years (range, 35–68). JHLH was the 57 participants in the previous study their median age of the participants was 47 years (range, 36–69 years) (Table 1).

Data collection

The CAS screening study included medical interviews, blood tests for inflammatory markers, physiological function tests, and CCTA. The medical interview included information on age, height, weight, history of hypertension, diabetes, dyslipidaemia, medications, smoking history, alcohol consumption history, history of cerebral and cardiovascular disease (CVD), allergies, and family history. Blood laboratory tests included biochemistry, blood counts, nadir and current CD4 lymphocyte counts, coagulation markers including fibrinogen and D-dimer, and inflammatory markers including hs-CRP, tumour necrosis factor-a (TNF-α), intercellular adhesion molecule-1 (ICAM-1), and IL-6. Coagulation and inflammatory markers were measured using frozen stored serum obtained from the 57 JHLH who participated in the previous study. Physiological function tests, such as electrocardiography, echocardiography, and pulse wave velocity (PWV) testing (Omron Healthcare, Kyoto, Japan) were performed in the physiological function testing laboratory. The laboratory has obtained ISO 15189 certification.

Diagnosis of CAS by CCTA and coronary angiography

Patients considered to have no severe renal dysfunction or allergy to the contrast agent underwent CCTA with 320-row multidetector computed tomography angiography (Aquilion ONE, Canon Medical System, Otawara, Japan). In CCTA, CAS is judged by visual appearance. If the coronary artery was 50–69% stenosed in appearance, it was considered as moderate, and if it was 70% or more stenosed, it was considered as severe (*4*). The result was evaluated by a cardiologist and radiologists with expertise in image reading.

Patients with moderate or greater stenosis on CCTA underwent coronary angiography (CAG). Generally, a stenosis of more than 75% at CAG is considered significant stenosis. If CAG showed stenosis of 75% or greater, evaluation of intravascular pressure measurements was performed if necessary to determine the appropriate indication for treatment (*5*). Then appropriate treatment such as PCI or CABG was performed. If those patients refused to undergo CAG, we performed myocardial perfusion scintigraphy to evaluate cardiovascular blood flow.

Classification of coronary artery calcium score

The coronary artery calcium score (CACS) was weighted by CT value as the cross-sectional area according to Agatston *et al.* (*6*). Based on previous study, CACS was classified into five categories: no calcification (score =

Table 1. Comparison of patient demographics between JHLH and PLWH without haemophilia

Demographics and variables	JHLH (*n* = 57)	PLWH without haemophilia (*n* = 69)	*p*
Age, median year (range)	47 (36–69)	47 (35–68)	0.62
BMI kg/m^2, median (IQR)	23.0 (22.0–25.0)	25.1 (22.3–27.7)	< 0.05
CAS risk factors			
SUITA score, median (IQR)	38 (31–45)	38 (31–45.5)	0.91
Smoking history, *n* (%)	30 (52.6)	43 (62.3)	0.24
Hypertension, *n* (%)	24 (42.1)	8 (11.6)	< 0.05
Diabetes mellitus, *n* (%)	8 (14.0)	2 (9.3)	< 0.05
Dyslipidaemia, *n* (%)	22 (38.6)	41 (59.4)	< 0.05
Family history of CAS, *n* (%)	13 (22.8)	12 (17.4)	0.45
LVEF, % (IQR)	65.0 (62.0–68.0)	64.3 (61.5–66.1)	0.23
PWV cm/sec, median (IQR)	1,512 (1,396–1,631)	1355 (1230–1474)	< 0.05
HIV-related indicators			
Nadir CD4/μL, median (IQR)	129 (74–175)	194 (109–277)	< 0.05
Current CD4/μL, median (IQR)	457 (370–627)	587 (482–723)	< 0.05
Duration of undetectable VL, median year (IQR)	16.1 (11.8–17.7)	9.3 (5.5–13.0)	< 0.05
Duration of treatment for HIV, median year	25 (22–28)	11 (7–15.5)	< 0.05
Duration of PI use, median year (IQR)	10 (3–17)	2 (0–7)	< 0.05
Duration of d-drug use, median year (IQR)	6 (1–9)	0 (0–0)	< 0.05
Hepatitis B, *n* (%)	6 (10.5)	36 (52.2)	< 0.05
Hepatitis C, *n* (%)	55 (96.5)	4 (5.8)	< 0.05
Treponema pallidum, *n* (%)	0 (0)	34 (49.3)	< 0.05

JHLH, Japanese haemophiliacs living with HIV-1; PLWH without haemophilia, Japanese people living with HIV-1; BMI, body mass index; CAS, coronary artery stenosis; *n*, number of patients; IQR, interquartile range; LVEF, left ventricular ejection fraction; PWV, pulse wave velocity; VL, plasma viral load; PI, protease inhibitor; d-drug, any of didanosine (ddI), zalcitabine (ddC), and stavudine (d4T).

0), minimal risk (score: 1–10), low risk (score: 11–100), moderate risk (score: 101–400), and high risk (score > 400) (*7,8*). It is reported that as CACS increases, cardiovascular events also increase (*9,10*). In particular, a cardiovascular event rate of 1.5% per year was reported for CACS of 100 or higher (*11*).

Statistical analysis

Categorical and continuous variables were evaluated using the Mann–Whitney *U* test, and categorical variables were evaluated using Fisher's exact test. *P*-values were two-sided, and a significance level of $p < 0.05$ was used. Odds ratios (OR) are presented with 95% confidence intervals (95% CI). The inflammatory markers in both groups were compared using a two-sample *t*-test. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS version 26, SPSS Inc., Chicago, IL).

Ethics statement

The study protocol was reviewed and approved by the Human Ethics Committee of the National Centre for Global Health and Medicine (NCGM) (#NCGM-G-003468-01) on 9 April 2021. Written informed consent was obtained from all participants at study entry in accordance with the Declaration of Helsinki. This study was registered with the University Hospital Medical Information Network Clinical Trial Registry (registry number #UMIN000044328).

Data availability statement

This study was registered with the UMIN ---(#UMIN 000044328), and the previous study with the registry (#UMIN 000035307).

Results

CAS study in JHLH and PLWH without haemophilia

Among the 69 PLWH without haemophilia who underwent CCTA, six patients (8.7%) had moderate-to-severe CAS (Figure 1A). Among the six patients, four patients further underwent CAG, and severe stenosis was found in one patient (1.4%) who underwent PCI after CAG. Two patients underwent cardiac perfusion scintigraphy instead because they refused to undergo CAG. The scintigraphy showed that there were no perfusion defects. Fourteen of 57 JHLH (24.6%) who underwent CCTA was found moderate-to-severe CAS. Twelve of the 15 patients underwent CAG, and seven had significant stenosis requiring treatment. Five patients underwent PCI, and one underwent CABG (one refused both PCI and CABG) (Figure 1B) (*2*).

In patients who underwent PCI, intravascular characteristics were confirmed by intravascular ultrasound (IVUS) or optical frequency domain imaging (OFDI). In JHLH, three patients had fibrocalcific plaque lesions and the other two patients had lipid plaques. The one patient who underwent PCI for PLWH without haemophilia had a fibrocalcific plaque lesion.

JHLH has decreased or defective blood coagulation ability, and bleeding complications caused by antiplatelet drugs are a major problem. In this study, blood products were adjusted, and attention was paid to bleeding complications during dual antiplatelet therapy. No cases of major bleeding complications in the perioperative period were observed.

Comparison of patient demographics between JHLH and PLWH without haemophilia

Table 1 shows the patient demographics of the two age-matched groups. Regarding CAS risk factors, no significant differences existed in smoking or family history. Hypertension and diabetes mellitus were significantly higher in the JHLH group, whereas dyslipidaemia was significantly higher in the PLWH without haemophilia group. Additionally, the PWV in JHLH was significantly higher than that in PLWH without haemophilia, indicating that arteriosclerosis was more advanced in JHLH (Figure 2). However, there was no significant difference in the Suita score in predicting CVD in healthy Japanese subjects (*12*). As for HIV-1-related indicators, nadir CD4 and current CD4 were significantly lower in JHLH than in PLWH without haemophilia. The duration of HIV-1 treatment and undetectable viral load (VL) of JHLH were longer than those of PLWH without haemophilia, indicating a longer history of HIV infection. Regarding HIV treatment-related issues, the duration of protease inhibitor (PI) and d-drug use was longer in JHLH than in PLWH without haemophilia. The higher rates of hepatitis B and C infections in JHLH might have been caused by frequent transfusions of blood products before 1986 (*13*). Generally, in the two age-matched groups, HIV-related indicators were favourable to PLWH without haemophilia, whereas CAS risk factors varied and did not have a favourable tendency in either group.

Comparison of CAS studies between JHLH and PLWH without haemophilia

Table 2 presents a comparison of CAS studies between the two groups. The ratio of moderate-to-severe CAS on CCTA in the JHLH group (24.6%) was significantly higher ($p = 0.015$, OR [95% CI]: 3.42 [1.22, 9.60]) than that in the PLWH without haemophilia group (8.7%). The ratio of severe stenosis requiring urgent interventions in the JHLH group (12.3%) was also significantly higher ($p = 0.013$, OR [95% CI]: 9.52 [1.14, 79.86]) than that in the PLWH without haemophilia group (1.4%). At

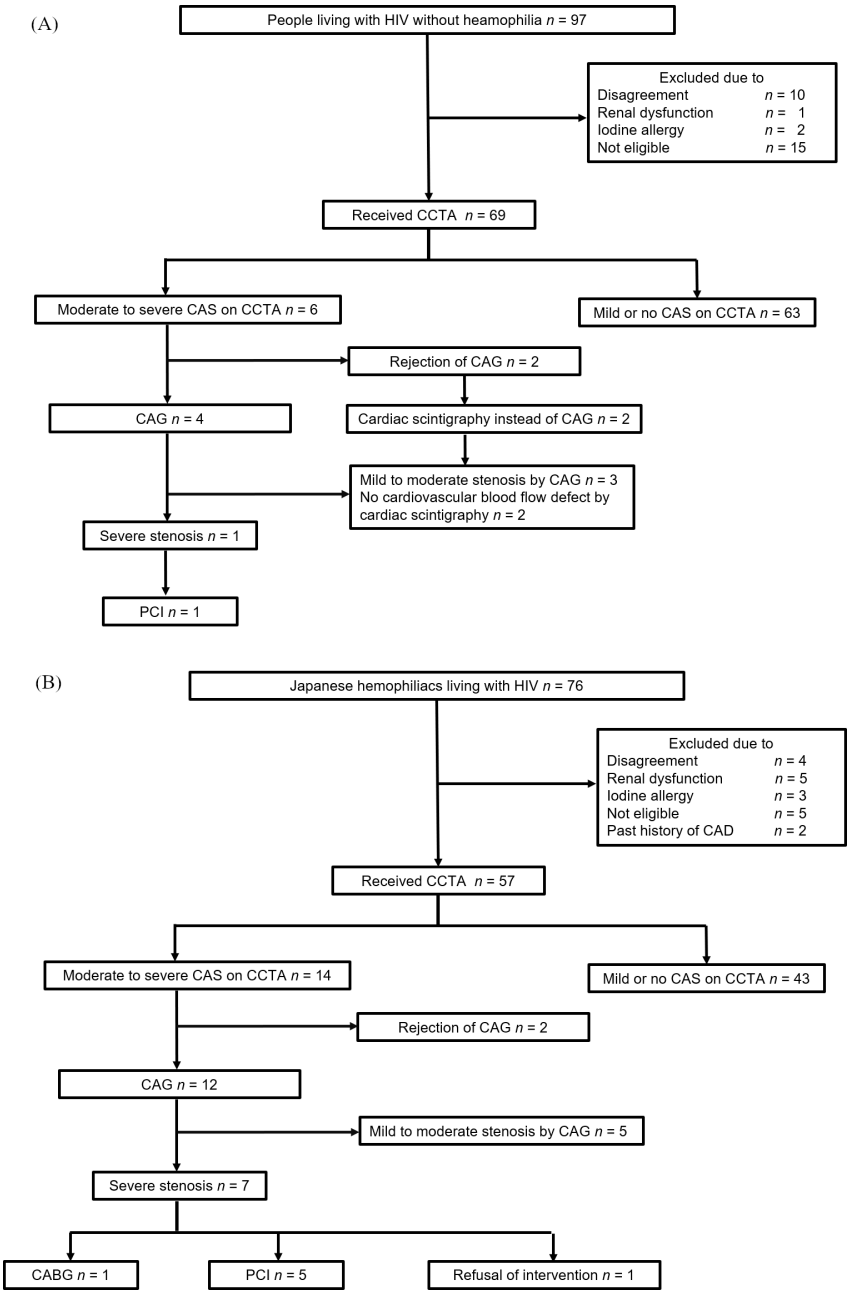

Figure 1. Study flow and patient selection. (A) Ninety-seven age- and gender-matched Japanese non-haemophiliacs living with HIV-1 in the previous study were randomly selected at AIDS Clinical Centre (ACC), National Centre for Global Health and Medicine, Tokyo, Japan. Among them, 69 patients gave written informed consent and received CCTA. If moderate-to-severe CAS were suspected on CCTA, CAG or cardiac perfusion scintigraphy was performed. Patients with severe stenosis who required urgent treatment underwent PCI. **(B)** After completion of the previous study, 11 new Japanese haemophiliacs living with HIV-1 (JHLH) consulted ACC. Subsequently, there were 87 JHLH in ACC during the study. Among them, five additional cases participated in the study and received CCTA. Thus, 62 cases received CCTA in JHLH. CCTA, coronary computed tomography angiography; CAS, coronary artery stenosis; CAG, coronary angiography; PCI, percutaneous coronary intervention.

this step, the prevalence of CAS in the JHLH group was higher than that in the PLWH without haemophilia group.

CACS were classified as follows: No classification (0) 33 (57.9%) *vs.* 53 (76.8%), Minimal risk (1–10) 6 (17.5%) *vs.* 1 (1.4%), Low risk (11–100) 10 (17.5%) *vs.* 6 (8 .7%), Moderate risk (101–400) and High risk (> 400) were 4 (7.0%) *vs.* 1 (1.4%). The rate of CACS of 101 or greater, which meant a moderate to high risk of CVD, did not differ between the two groups.

Comparison of coagulation factors and intravascular inflammation markers between the JHLH and PLWH without haemophilia

We measured coagulation factors and inflammatory markers in both groups. The levels of fibrinogen and D-dimer did not show any significant difference between the two groups (Table 3). The levels of the inflammatory markers IL-6 ($p < 0.05$) and ICAM-1 ($p < 0.05$) were significantly higher in JHLH than in PLWH

without haemophilia indicating higher intravascular inflammation in JHLH.

Discussion

This study demonstrated that the prevalence of CAS in JHLH was significantly higher than that in PLWH without haemophilia, indicating that JHLH had a higher risk of CVD compared with PLWH without haemophilia, namely HIV-1 infected individuals. Generally, only HIV-1 infection is associated with a high prevalence of CVD (*14*). The incidence of acute myocardial infarction in Japanese men has been reported to be 0.3–0.6/1,000 person-years (PY) in one study (*15*) while another reported a rate of 0.12–2.56/1,000 PY in middle-aged men (*16*). Triant *et al.* (*17*) reported that the incidence of acute myocardial infarction in HIV-1 patients was significantly higher at 11.13/1,000 PY than at 6.98/1,000 PY in non-HIV-1 patients. This is possibly because of high intravascular inflammation in this population (*18,19*). The SMART

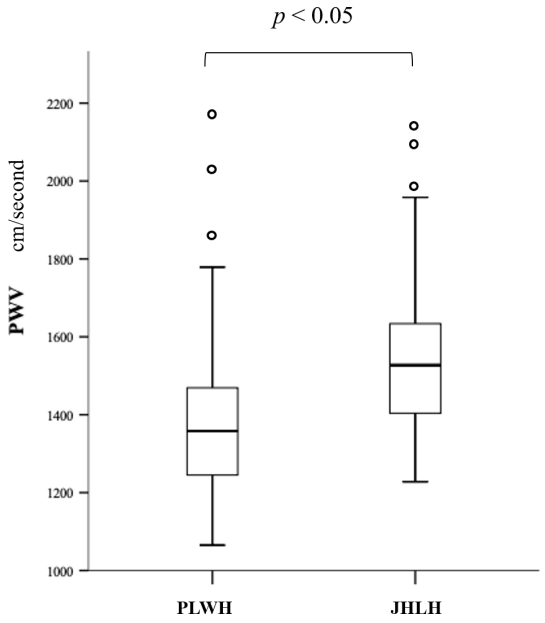

Figure 2. Box plot of PWV. PWV was measured in PLWH without haemophilia in this study, and the results were compared with those of JHLH in the previous study. PWV, pulse wave velocity; JHLH, Japanese haemophiliacs living with HIV-1; PLWH without haemophilia, Japanese non-haemophiliacs living with HIV-1.

Table 3. Comparison of coagulation factors and inflammatory markers between JHLH and PLWH without haemophilia

Characteristics	JHLH	PLWH without haemophilia	*p*
Coagulation factor			
Fibrinogen, mean (SD) mg/mL	249.1 (66.3)	255.2 (58.7)	0.62
D-dimer, positive rate %	7 (12.3%)	4 (5.8%)	0.20
Inflammatory marker			
hs-CRP, mean (SD) mg/mL	1.43 (2.4)	1.33 (1.3)	0.98
IL-6*, mean (SD) pg/mL	4.61 (13.2)	2.10 (6.52)	< 0.05
TNF-a, mean (SD) pg/mL	0.80 (0.47)	0.68 (0.26)	0.35
ICAM-1, mean (SD) pg/mL	239.7 (86.1)	201.2 (62.9)	< 0.05

JHLH, Japanese haemophiliacs living with HIV; PLWH without haemophilia, Japanese people living with HIV-1; SD, standard deviation; hs-CRP, highly sensitive C-reactive protein, IL-6; interleukin-6, TNF-, tumour necrosis factor-; ICAM-1, intercellular adhesion molecule-1; *$\log_{10}$ transformed, was used for this analysis.

Table 2. Comparison of CAS study between JHLH and PLWH without haemophilia

Characteristics	JHLH	PLWH without haemophilia	*p*, OR (95% CI)
Receive CCTA, *n*	57	69	
Moderate to severe stenosis by CCTA	14	6	*p* = 0.015 OR 3.42 (1.22, 9.60)
Further examinations, *n*			
CAG	12	4	
Cardiac perfusion scintigraphy	0	2	
Refused further examinations	2	1	
CAS with urgent interventions required, *n* (%)	7 (12.3%)	1 (1.4%)	*p* = 0.013 OR 9.52 (1.14, 79.86)
Interventions, *n*	6	1	
PCI	5	1	
CABG	1	0	
Refused coronary intervention	1	0	
CACS greater than 100, *n* (%)	8 (14.0%)	9 (13.0%)	*p* = 0.87
Risk classification by CACS on CCTA, *n* (%)			OR 1.09 (0.39, 3.03)
High risk (> 400)	4 (7.0%)	1 (1.4%)	
Moderate risk (101–400)	4 (7.0%)	8 (11.6%)	
Low risk (11–100)	10 (17.5%)	6 (8.7%)	
Minimal risk (1–10)	6 (17.5%)	1 (1.4%)	
No classification (0)	33 (57.9%)	53 (76.8%)	

CAS, coronary artery stenosis; JHLH, Japanese haemophiliacs living with HIV-1; PLWH without haemophilia, Japanese people living with HIV-1; CCTA, coronary computed tomography angiography; *n*, number of patients; CAG, coronary angiography; PCI, percutaneous coronary intervention; CABG, coronary artery bypass graft surgery; OR, odds ratio; 95%CI, 95% confidence interval; CACS, coronary artery calcification score.

study documented that intravascular inflammation caused by circulating HIV-1 in plasma plays a major role in CVD (*20*). Duprez *et al.* reported in a sub-study that inflammatory markers of hs-CRP and IL-6 in HIV-1-infected patients with CVD were higher than those without CVD [hs-CRP 3.34 mg/mL (1.47–7.51 mg/mL) and IL-6 3.07 pg/mL (1.87–4.83 pg/mL) in CVD (+), while hs-CRP 1.67 mg/mL (0.70–4.02mg/mL) and IL-6 1.72 pg/mL (1.07–2.29 pg/mL) in CVD(-)], concluding that intravascular inflammation was associated with an increased risk of CVD independent of other CVD risk factors (*3*). Notably, CVD risks in JHLH were higher than those in PLWH without haemophilia in this study. In support of this result, serum titres of IL-6 and ICAM-1 were significantly higher in JHLH than in PLWH without haemophilia. It might be possible to speculate that the regular injection of blood products induced intravascular inflammation.

Patients with haemophilia have reduced vascular endothelial function compared to the general population (*21,22*). Thus, the haemophilia-associated risks added to HIV-1 infection might increase the prevalence of CAS. The detailed mechanism is not known so far. Future study of patients with haemophilia without HIV infection will help us to understand the mechanism.

We had believed that patients with haemophilia had a low incidence of ischaemic heart disease, attributed to their bleeding tendency. Van Der Valk *et al.* reported reduced cardiovascular events in patients with haemophilia (*23*). Nagao *et al.* also reported ischemic events are rare among Japanese adults with haemophilia (*24*).

However, advances in blood coagulation products have improved life expectancy of haemophilia patients, resulting in an increased risk of CAS (*25-27*). Thus, different results have been reported on the prevalence of ischemic heart disease in haemophilia patients. The results of this study might indicate that the combination of both HIV-1 infection and haemophilia synergistically increases the prevalence of ischemic heart disease. The same study of CAS screening for non-HIV-1 infected haemophiliacs will address this speculation in the future. The use of PI has been reported to increase dyslipidaemia, which in turn increases the incidence of myocardial infarction (*28*). However, in this study, the prevalence of dyslipidaemia was significantly lower in JHLH with a longer history of PI use. The use of d-drugs, such as stavudine, has been reported to increase the risk of cardiovascular events and was used significantly longer in JHLH (*29*). Therefore, the effects of d-drugs could not be ruled out.

In this study the rate of CACS of 101 or greater, which meant a moderate to high risk of CVD, did not differ between the two groups. Therefore, it is difficult to predict CVD based on CACS alone. Some cases required treatment even with a calcification score of 0, therefore, attention should be paid not only to calcified lesions but also to plaque lesions.

We speculated that both haemophilia and HIV-1 infection (JHLH) might be responsible for the high prevalence of CAS. JHLH has significantly higher coronary risks, such as hypertension and diabetes mellitus, which may cause atherosclerosis and increase the prevalence of CAS. Additionally, hepatitis C infection rates were significantly higher in the JHLH group [13]. Hepatitis C infection has been reported to be a risk factor for CAS (*30,31*). However, there were conflicting reports (*32*), and its involvement in CAS was unknown (*33*). Therefore, in this study, the effect of hepatitis C infection could not be ruled out.

This study had some limitations. This was a single-centre study and the number of participants was limited. Although all JHLH who provided informed consent in the previous study and PLWH without haemophilia randomly selected age- and sex-matched individuals in the current study were included, participants in the two groups were significantly different in some coronary risk factors, duration of HIV-1 infection, and other viral infections. When we matched age in the two groups, differences in factors, such as the duration of HIV-1 infection and co-infection with hepatitis C could not be avoided because of the unique history of the JHLH group. If we could match CAS risk factors, such as hypertension and diabetes mellitus, to age and sex, there would be a faint possibility that we would have a different result. The lack of studies on patients with haemophilia without HIV infection is a limitation.

Conclusion

The JHLH group had a significantly higher prevalence of CAS compared with the PLWH without haemophilia group. Despite differences in some patient demographics in the age-and sex-matched studies, it has been suggested that the coexistence of haemophilia and HIV infection may have a synergistic effect, contributing to an increased prevalence of CVD. The significantly higher intravascular inflammation in JHLH might also be involved.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Decreased resting-state functional connectivity and brain network abnormalities in the prefrontal cortex of elderly patients with Parkinson's disease accompanied by depressive symptoms

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Abstract: This study aimed to explore the brain network characteristics in elderly patients with Parkinson's disease (PD) with depressive symptoms. Thirty elderly PD patients with depressive symptoms (PD-D) and 26 matched PD patients without depressive symptoms (PD-NOD) were recruited based on HAMD-24 with a cut-off of 7. The resting-state functional connectivity (RSFC) was conducted by 53-channel functional near-infrared spectroscopy (fNIRS). There were no statistically significant differences in MMSE scores, disease duration, Hoehn-Yahr stage, daily levodopa equivalent dose, and MDS-UPDRS III between the two groups. However, compared to the PD-NOD group, the PD-D group showed significantly higher MDS-UPDRS II, HAMA-14, and HAMD-24. The interhemispheric FC strength and the FC strength between the left dorsolateral prefrontal cortex (DLPFC-L) and the left frontal polar area (FPA-L) was significantly lower in the PD-D group (FDR $p < 0.05$). As for graph theoretic metrics, the PD-D group had significantly lower degree centrality (aDc) and node efficiency (aNe) in the DLPFC-L and the FPA-L (FDR, $p < 0.05$), as well as decreased global efficiency (aEg). Pearson correlation analysis indicated moderate negative correlations between HAMD-24 scores and the interhemispheric FC strength, FC between DLPFC-L and FPA-L, aEg, aDc in FPA-L, aNe in DLPFC-L and FPA-L. In conclusion, PD-D patients show decreased integration and efficiency in their brain networks. Furthermore, RSFC between DLPFC-L and FPA-L regions is negatively correlated with depressive symptoms. These findings propose that targeting DLPFC-L and FPA-L regions *via* non-invasive brain stimulation may be a potential intervention for alleviating depressive symptoms in elderly PD patients.

***Keywords*:** Parkinson's disease, depressive symptoms, functional near-infrared spectroscopy, functional connectivity, prefrontal lobe

Introduction

Parkinson's disease (PD) is a common chronic, progressive, and neurodegenerative disorder, which is common in the middle-aged and elderly groups and characterized by motor dysfunction and various non-motor symptoms. The pathological process of PD primarily involves dopamine deficiency in the substantia nigra striatum and abnormalities in other neurotransmitter systems, such as noradrenergic, serotonergic, and cholinergic systems (*1*). Depressive symptoms represent a common and heterogeneous non-motor manifestation in the prodromal and diagnosed stages of PD, particularly occurring frequently in the early and late phases of PD (*2*). The reported prevalence of depression in PD varies widely (*3*), ranging from 2.7% to over 90% (*4*), because of differences in survey methods, inconsistent diagnostic criteria for depressive symptom, and diverse assessment scales. A systematic review and meta-analysis reported that the subtypes of depressive disorders in PD patients were similar to those with other diseases, including major depressive disorder (MDD) and non-major depressive disorders (mild and persistent depressive disorders). They found a global frequency of depressive disorders of 30.7% and MDD of 14.0% (*5*). Studies suggest that depressive symptoms have a greater impact on patients' quality of life than severe motor symptoms (*6*). Depressive symptoms are associated with increased disability (such as dementia), rapid progression of motor symptoms, and increased

mortality (*7*). Early assessment and intervention for depressive symptoms can prevent patients from sliding into MDD or experiencing relapses after depression treatment, ultimately improving their quality of life. However, the depressive symptoms of PD are often underestimated and inadequately treated in clinical practice, which is partly due to the overlap of some symptoms in PD patients with depressive symptoms (such as psychomotor retardation, reduced facial expression, insomnia, decreased appetite, anhedonia, fatigue) (*8*), the insidious nature of symptoms, lack of active reporting symptoms, cognitive impairment hindering cooperation with examinations, and the absence of objective biomarkers (*9*).

The pathophysiology and etiological mechanisms of depression in PD is multifactorial and complex, primarily associated with underlying neurodegenerative processes, in particular dysfunction in neurotransmitter systems and disturbances of striato-thalamic-prefrontal, cortico-limbic, mediotemporal-limbic networks; other hypothesized mechanisms include neuroinflammation, neuroimmune imbalance, stress hormones, neurotrophic factors, and toxic or metabolic factors (*10*). The advancement of brain imaging technology and the development of graph theory offer possibilities to explore potential mechanisms of PD with depressive symptoms. A wide range of brain regions and networks have been proven involved in the occurrence and development of depression in PD. Still, there is no unified conclusion, and more research is needed to investigate changes in brain anatomical structures and functional networks during the dynamic progression of the disease. Functional near-infrared spectroscopy (fNIRS) is a non-invasive, ecologically valid, and cost-effective neuroimaging technique. The wavelength of near-infrared light ranges from 650 to 950 nm and fNIRS travel several centimeters through the scalp and skull. fNIRS is able to measure changes in cortical oxyhemoglobin (HbO_2) and deoxyhemoglobin concentrations in real time because of the differences in optical properties of brain tissues (*e.g.*, absorption and scattering coefficients) (*11*). While fMRI has been often used in previous studies to measure metabolic activities in the brains of PD patients, fNIRS, with its higher temporal resolution than fMRI, has been widely employed in clinical studies of various chronic neurological and psychiatric disorders (*12*).

Functional connectivity (FC) is essentially an "unmodelled" description of the joint state of multiple brain elements (*e.g.*, neurons, regions) (*13*), providing a time-dependent representation of the activation patterns of anatomically separated brain regions (*14*). Resting-state functional connectivity (RSFC) manifests as slow spontaneous oscillations during quiet rest or sleep (*15*). Compared to task-related data, resting-state data can effectively eliminate individual differences when performing specific tasks. Therefore, this study collected data on the hemodynamic changes in the frontal lobe cortex of elderly Chinese PD patients during the resting state and compared the differences in FC and brain networks between patients with and without depressive symptoms.

Materials and Methods

Subject

In this study, primary PD patients were recruited from October 2022 to June 2023 from outpatients of the Department of Neurology of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine (XH-SJTUSM). The study followed the Declaration of Helsinki and was reviewed and approved by the Ethical Review Committee of XH-SJTUSM (approval no.: XHEC-C-2022-134-1). All patients made their own decision to participate in this study and gave verbal consent after being fully informed of the potential benefits and risks of participating in the study.

Inclusion criteria were as follows: *i*) Chinese residents aged 60 to 80 years, *ii*) met the Chinese diagnostic criteria (2016 version), currently undergoing dopaminergic medication for at least one month with a favorable response, *iii*) without any apparent cognitive impairment (Mini-mental State Examination scale [MMSE] cu-toff values: illiterate > 17, elementary school > 20, junior high school and above > 24), and *iv*) right-handed.

Exclusion criteria included: *i*) history of deep brain stimulation surgery, *ii*) severe mental illness, *iii*) combined with severe diseases affecting the heart, brain, kidneys, *etc.*, and *iv*) suffered from a major negative life event (*e.g.*, bereavement) within one year.

Clinical assessment

All patients were examined in a practically defined "ON" state by healthcare professionals specializing in PD. General Demographic and Disease Information including gender, age, years of education, the onset of motor symptoms, and usage of anti-Parkinsonian medications were collected.

The Hoehn-Yahr (H-Y) stage and Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) part II and III scores were used to evaluate the disease severity. H-Y stage is a single-item assessment to evaluate the symptoms and severity of PD, which ranges from 0 (no symptoms) to stage 5 (complete dependence on a wheelchair or bedridden without assistance). MDS-UPDRS II and MDS-UPDRS III consist of 13 and 18 items respectively, and evaluate the impact of motor symptoms on daily life and motor function respectively, with scores ranging from 0 to 4 for each item. Higher scores of MDS-UPDRS indicate more severe motor symptoms in PD patients. The MMSE was a 30-item questionnaire to assess the cognitive function

of each subject based on their cultural background. The Hamilton Anxiety Rating Scale (HAMA-14) was used to assess the anxiety state of each subject. The Hamilton Depression Rating Scale (HAMD-24) was used to evaluate depression severity, and all patients with a score of at least 8 points were considered depressive.

fNIRS signal acquisition of resting state

In a resting state, patients sit on a chair in a quiet, temperature-appropriate room. Patients were asked to stay awake, not to speak, and not to think about anything as much as possible. A 53-channel BS-7000 fNIRS system (Wuhan Znion Technology Co., Wuhan, China) was used to monitor the hemodynamic oxygenation changes in the cerebral cortices. A helmet with 16 light-emitting probes and 16 optical receivers covered the patients' prefrontal and temporal lobes. Distribution of near-infrared sensors followed the international 10-20 system, with the lowest channel positioned at Fp1-Fp2.

The region is divided into the bilateral Pre-Motor and Supplementary Motor Cortex (PreM and SMC) (Channel 1,4,10,40,47,52), Broca's area (Broca) (Channel 2,3,5,7 ,8,13,44,46,49,50,51,53), dorsolateral prefrontal cortex (DLPFC) (Channel 6,11,14,17,18,20,31,32,34,39,42,4 5), frontal eye fields (FEF) (Channel 12,24,26,38) and frontopolar area (FPA) (Channel 9,15,16,19,21,22,23,2 7,30,33,35,36,37,41,43,48) according to the Brodmann areas (BA) (Figure 1). The fNIRS data collection lasted for 6 min for each subject.

Data processing and statistical analysis

Descriptive and analytical statistics were conducted using SPSS 25.0. Statistical descriptions of measurement data were expressed as mean, standard deviation, median, and quartiles; statistical descriptions of count data were expressed as frequency, percentage, rate, and constitutive ratio. Continuous data were analyzed using *t*-tests, and categorical data were analyzed using the χ^2 test to compare differences in FC and brain network topological properties between PD with depressive symptoms (PD-D, HAMD-24 scores ≥ 8) group and PD without depressive symptoms (PD-NOD, HAMD-24 scores ≤ 7) group. Pearson correlation analysis was employed for a two-sided test to examine the correlation between RSFC and self-reported depressive symptoms. Statistical significance was set at $p < 0.05$.

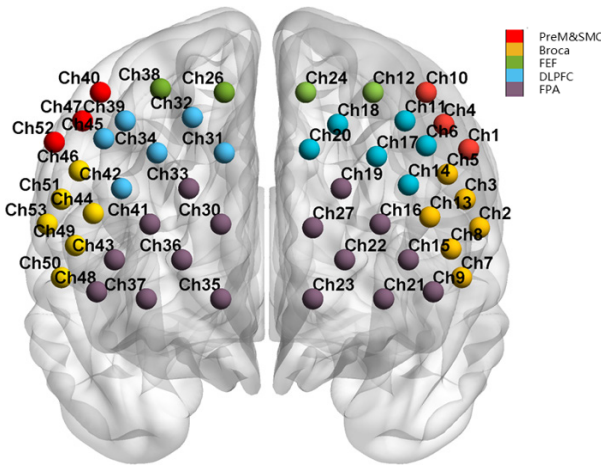

Figure 1. The arrangement of channels of the 53-channel near-infrared spectroscopy system according to Brodmann's map of the cortex. Ch, channel; PreM & SMC, Pre-Motor and Supplementary Motor Cortex; Broca, Broca's area; DLPFC, dorsolateral prefrontal cortex; FEF, frontal eye fields; FPA, frontopolar area.

The data processing and statistical analysis for fNIRS in this experiment were primarily conducted in the Matlab (2014b) environment. The brain network analysis of all fNIRS data was performed using the Homer2_UI, NIRS-SPM, and GRETNA2.0 toolkits, and visualized by NIRS_KIT and BrainNet Viewer.

Preprocessing of fNIRS data involved converting raw data into hemoglobin concentration data. The first and last 10 seconds of resting-state data were discarded to obtain stable signals. The raw data were then converted to the .nirs format, and bad channels were removed based on the coefficient of variation value. Homer2 was used for preprocessing, converting raw intensity data to optical density (OD) data. Motion artifacts were identified by using a moving time window with a time window of 0.5 seconds and a standard deviation threshold of 20 for each channel, and were calibrated and removed; and then low-pass filtering (0.1Hz) was used to eliminate physiological noise (*e.g.*, heart, respiration and blood pressure fluctuations), and finally, the OD was converted to HbO_2 concentration.

In this study, each of the 10 brain regions was defined as a node, corresponding to a measurement channel. Functional connectivity matrices were obtained by calculating Pearson correlation coefficients (*r* values) of the time series for each channel. Fisher's r-to-z transformation was then applied to convert the obtained *r* values to z scores, creating a 50 × 50 correlation matrix. RSFC graphs specific to each group were generated using single-sample *t*-tests. RSFC strength between ROIs in the PD-D and PD-NOD groups was compared using two-sample *t*-tests.

In this study, the overall and local topological properties of prefrontal resting-state brain networks were calculated within the level of threshold 0.4 to 0.65 and step size 0.05.

The overall topological properties: *i*) Small-world parameters (Sigma), clustering coefficient (Cp), and shortest path length (Lp). Sigma value > 1 is considered to be a small-world property of the network. The Cp measures the likelihood its neighborhoods are connected to each other. The Lp quantifies the mean distance or routing efficiency between this node and all the other nodes in the network. *ii*) Network efficiency parameters, including global efficiency (Eg) and local

efficiency (Eloc), both of which measure the network's ability to transmit information at the global and local levels, respectively; the higher the value, the faster the transmission speed.

The local topological parameters include five node indicators: *i*) the nodal degree centrality (Dc) for a given node reflects its information communication ability in the functional network; *ii*) the betweenness centrality (Bc) for a given node characterizes its effect on information flow between other nodes, and regions with higher Bc have a role of hub for the network; *iii*) the node efficiency (Ne) refers to the efficiency of parallel information transfer of network nodes, and regions with high Ne has a higher efficiency in communicating with other brain regions; *iv*) the nodal local efficiency (NLe) is a measure of functional segregation, higher NLe suggest a higher degree of interconnectedness in local networks consisting of direct neighbors of the region; *v*) the nodal clustering coefficient (NCp) measures the extent to which nodes tend to cluster together. The area under the curve (AUC) is a scalar that is highly sensitive to the topology of brain disease anomalies and does not depend on the selection of a specific threshold, so we calculated the AUC of each network metric as the final metric; this study included the AUC of Sigma(aSigma), the AUC of Cp(aCp), the AUC of Lp(aLp), the AUC of Eg(aEg), the AUC of Eloc(aEloc), the AUC of Dc(aDc), the AUC of Bc(aBc), the AUC of Ne(aNe), the AUC of NLe(aNLe), the AUC of NCp (aNCp) as the final indicators. The false discovery rate (FDR) was utilized to correct for multiple comparisons.

Results

A total of 83 elderly primary PD patients were recruited in the neurology outpatient clinic, and 64 patients' data of resting-state fNIRS were collected after initial screening.. One patient with drowsiness, one patient with cough, and six patients with bad channels in the data collection were deleted. A total of 56 patients' data were analyzed.

Demographic and clinical characteristics

The age of the patients ranged from 60 to 79 (69.39 ± 4.58) years and 30 (53.6%) were male. A comparison of demographic and clinical characteristics between the two groups is shown in Table 1. There was no significant difference in gender, age, education years, MMSE score, course of PD disease, H-Y stage, daily levodopa equivalent dose (LEDD), and MDS-UPDRS III score between the PD-D group and PD-NOD group (all $p > 0.05$). The MDS-UPDRS II (14.07 ± 8.78 *vs.* 9.04 ± 4.26), HAMA (14.07 ± 7.99 *vs*. 3.96 ± 3.16) and HAMD (17.43 ± 7.69 *vs.* 3.58 ± 2.45) scores of PD-D group were higher than those in the PD-NOD group (all $p < 0.05$).

Functional connectivity

fNIRS quantified the strength of the interaction of HbO_2 signals between the various brain regions covered by the channel. There was a significant difference in the RSFC matrix between the two groups of patients (Figure 2).

Interhemispheric FC strength of patients in the PD-D group is lower than that of the PD-NOD group and the difference was significant (0.19 ± 0.26 *vs.* 0.39 ± 0.33, $p = 0.015$); the FC strength of the DLPFC-L and FPA-L (0.19 ± 0.24 *vs*. 0.50 ± 0.26) is lower than that of the PD-NOD group and the difference was significant (FDR, $p < 0.001$) (Figure 3). There was no statistically significant difference in FC strength between the other brain regions of the two groups.

Pearson correlation analysis found a significant negative correlation between interhemispheric FC strength and HAMD scores ($r = -0.327$, $p = 0.014$); and a significant negative correlation between the FC strength of DLPFC-L and FPA-L, and the HAMD scores ($r = -0.458$, $p < 0.001$).

Table 1. Comparison of demographic and clinical characteristics of patients in the PD-D and PD-NOD groups

Variable	Total ($n = 56$)	PD-D ($n = 30$)	PD-NOD ($n = 26$)	t/χ^2-value	p value
Male (%)[a]	30 (53.6%)	14 (46.7%)	16 (61.5%)	1.239	0.266
Age (y)[b]	69.39 ± 4.56	69.20 ± 5.11	69.62 ± 3.96	-0.336	0.738
Years of education (y)[b]	10.29 ± 2.10	10.6 ± 2.22	9.92 ± 1.94	1.206	0.233
MMSE scores[b]	27.64 ± 2.02	27.4 ± 2.36	27.92 ± 1.55	-0.965	0.339
Disease duration (y)[b]	5.35 ± 4.76	5.96 ± 4.0	4.64 ± 5.51	1.039	0.303
H-Y stage[a]	2 (2,3)	2 (2,3)	2 (2,2)	4.151	0.246
LEED (mg/day)[b]	484.19 ± 268.61	536.97 ± 314.77	423.29 ± 191.33	1.656	0.104
MDS-UPDRS II scores[b]	11.73 ± 7.44	14.07 ± 8.78	9.04 ± 4.26	2.659	0.010
MDS-UPDRS III scores[b]	26.52 ± 13.68	29.27 ± 14.74	23.35 ± 11.85	1.639	0.107
HAMA scores[b]	9.38 ± 8.0	14.07 ± 7.99	3.96 ± 3.16	6.379	< 0.001
HAMD scores[b]	11.0 ± 9.09	17.43 ± 7.69	3.58 ± 2.45	9.336	< 0.001

Note: [a]Data were performed for group differences with the chi-squared test. [b]Data were performed for group differences with *t*-test. PD-D, Parkinson's disease with depressive symptoms; PD-NOD, Parkinson's disease without depressive symptoms; MMSE, Mini-mental State Examination scale; H-Y stage, Hoehn-Yahr stage; LEDD, levodopa equivalent daily dosage; MDS-UPDRS II, Movement Disorder Society Unified Parkinson's Disease Rating Scale part II; MDS-UPDRS III, Movement Disorder Society Unified Parkinson's Disease Rating Scale part III; HAMA, Hamilton Anxiety Rating Scale; HAMD, Hamilton Depression Rating Scale.

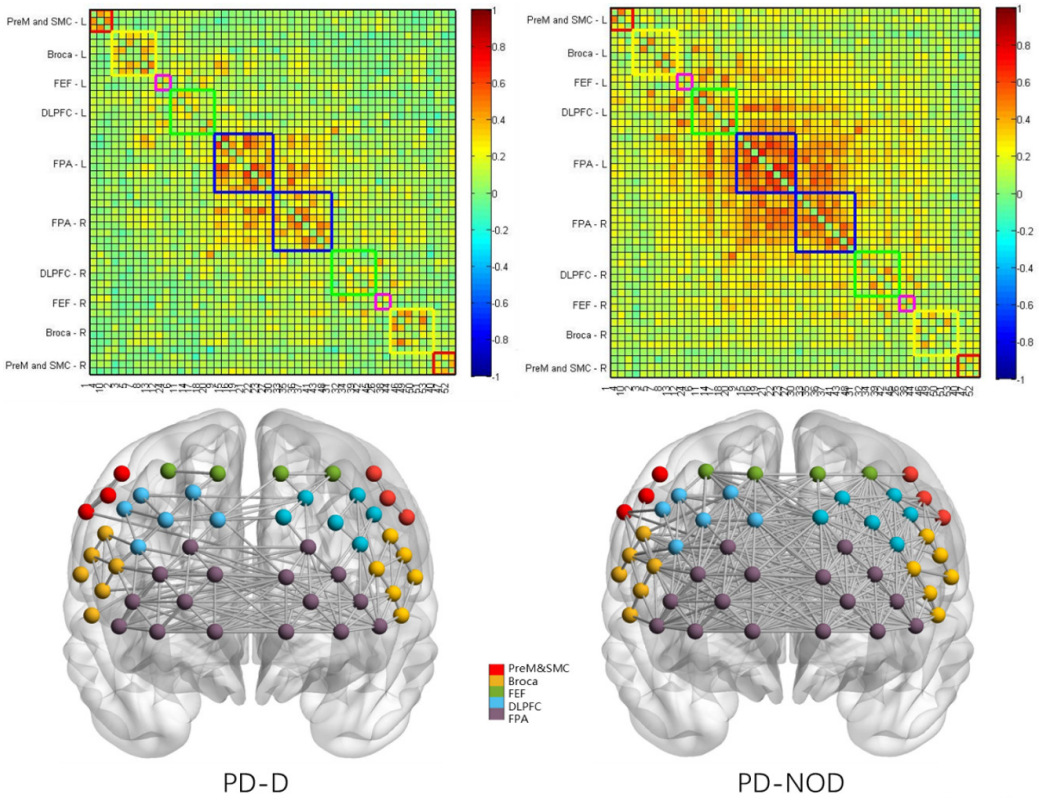

Figure 2. RSFC strength between regions of interest is lower in PD-D than in the PD-NOD group patients. PD-D, Parkinson's disease with depressive symptoms; PD-NOD, Parkinson's disease without depressive symptoms; RSFC, Resting-state functional connectivity; PreM and SMC-L, left Pre-Motor and Supplementary Motor Cortex; Broca-L, left Broca's area; FEF-L, left frontal eye fields; DLPFC-L, left dorsolateral prefrontal cortex; FPA-L, left frontal polar area; FPA-R, right frontal polar area; DLPFC-R, right dorsolateral prefrontal cortex; FEF-R, right frontal eye fields; Broca-R, right Broca's area; PreM and SMC-R, right Pre-Motor and Supplementary Motor Cortex; PreM & SMC, Pre-Motor and Supplementary Motor Cortex; Broca, Broca's area; DLPFC, dorsolateral prefrontal cortex; FEF, frontal eye fields; FPA, frontopolar area.

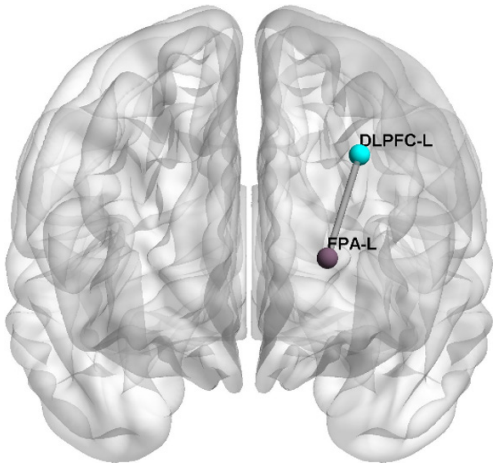

Figure 3. Low strength of FC between DLPFC-L and FPA-L in PD-D group patients compared to PD-NOD. PD-D, Parkinson's disease with depressive symptoms; PD-NOD, Parkinson's disease without depressive symptoms; FC, functional connectivity; DLPFC-L, left dorsolateral prefrontal cortex; FPA-L, left frontal polar area.

Brain network topology properties

The Sigma values of the PD-NOD and PD-D groups were > 1 at each absolute threshold, which suggests that the brain networks of the patients in both groups have small-world networks. Compared with the PD-NOD group, aSigma (0.56 ± 0.31 *vs*. 0.46 ± 0.26), aCp (0.12 ± 0.02 *vs*. 0.13 ± 0.04), aLp (1.36 ± 0.8 *vs*. 1.07 ± 0.86), and aEloc (0.14 ± 0.02 *vs*. 0.15 ± 0.04) were not statistically different in the PD-D group (all $p > 0.05$), whereas aEg (0.07 ± 0.02 *vs*. 0.09 ± 0.04, $p = 0.003$) in PD-D group was significantly lower; this suggests that the brain network of elderly PD patients with depressive symptoms is less efficient in information transmission.

Pearson correlation analysis showed that there was no correlation between aSigma, aCp, aLp, and aEloc and the HAMD scores (r = -0.037~0.101, all $p > 0.05$), whereas there was a significant negative correlation between aEg and the HAMD scores (r = -0.294, $p = 0.028$).

Compared to the PD-NOD group, aDc was significantly lower in the PD-D group in the DLPFC-L (1.61 ± 1.02 *vs*. 2.80 ± 1.86), FPA-L (1.98 ± 0.73 *vs*. 3.68 ± 2.19), and FPA-R (1.88 ± 0.97 *vs*. 3.0 ± 2.0); aBc was significantly lower in the FEF-L (2.73 ± 2.61 *vs*. 6.52 ± 5.14); aNe in the PreM and SMC-L (0.06 ± 0.03 *vs*. 0.08 ± 0.04), Broca-L (0.06 ± 0.03 *vs*. 0.08 ± 0.03), FEF-L (0.06 ± 0.03 *vs*. 0.09 ± 0.05), DLPFC-L (0.07 ± 0.03 *vs*. 0.10 ± 0.04), FPA-L (0.08 ± 0.02 *vs*. 0.12 ± 0.04), FPA-R (0.08 ± 0.02 *vs*. 0.11 ± 0.04), and DLPFC-R (0.07 ±

Table 2. Comparison of aDc of patients in the PD-D and PD-NOD groups

Region	PD-D (n = 30)	PD-NOD (n = 26)	t value	p value
DLPFC-L	1.61 ± 1.02	2.80 ± 1.86	-2.895	0.030
FPA-L	1.98 ± 0.73	3.68 ± 2.19	-3.778	0.010
FPA-R	1.88 ± 0.97	3.0 ± 2.0	-2.592	0.047

PD-D, Parkinson's disease with depressive symptoms; PD-NOD, Parkinson's disease without depressive symptoms; aDc, area under the curve of degree centrality; DLPFC-L, left dorsolateral prefrontal cortex; FPA-L, left frontal polar area; FPA-R, right frontal polar area.

Table 3. Comparison of aBc of patients in the PD-D and PD-NOD groups

Region	PD-D (n = 30)	PD-NOD (n = 26)	t value	p value
FEF-L	2.73 ± 2.61	6.52 ± 5.14	-3.391	0.020

PD-D, Parkinson's disease with depressive symptoms; PD-NOD, Parkinson's disease without depressive symptoms; aBc, area under the curve of betweenness centrality; FEF-L, left frontal eye fields.

Table 4. Comparison of aNe of patients in the PD-D and PD-NOD groups

Region	PD-D (n = 30)	PD-NOD (n = 26)	t value	p value
PreM and SMC - L	0.06 ± 0.03	0.08 ± 0.04	-2.323	0.040
Broca-L	0.06 ± 0.03	0.08 ± 0.03	-2.357	0.040
FEF-L	0.06 ± 0.03	0.09 ± 0.05	-2.266	0.040
DLPFC-L	0.07 ± 0.03	0.10 ± 0.04	-3.596	0.005
FPA-L	0.08 ± 0.02	0.12 ± 0.04	-4.213	< 0.001
FPA-R	0.08 ± 0.02	0.11 ± 0.04	-3.096	0.013
DLPFC-R	0.07 ± 0.03	0.09 ± 0.04	-2.450	0.040

PD-D, Parkinson's disease with depressive symptoms; PD-NOD, Parkinson's disease without depressive symptoms; aNe, area under the curve of node efficiency; PreM and SMC-L, left Pre-Motor and Supplementary Motor Cortex; Broca-L, left Broca's area; FEF-L, left frontal eye fields; DLPFC-L, left dorsolateral prefrontal cortex; FPA-L, left frontal polar area; FPA-R, right frontal polar area; DLPFC-R, right dorsolateral prefrontal cortex.

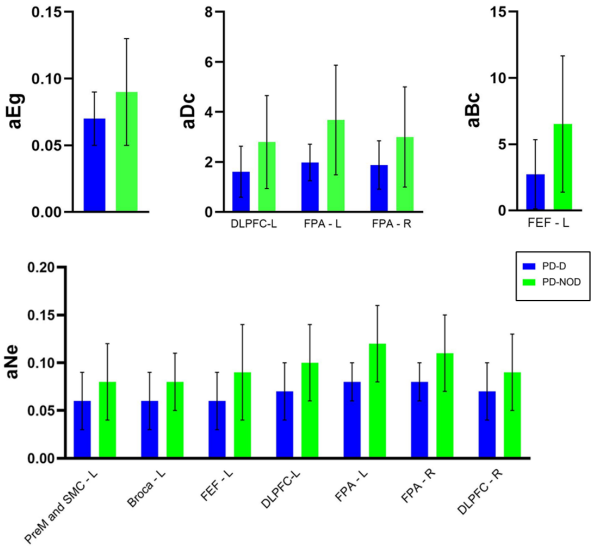

Figure 4. PD-D group patients' brain network properties are different from PD-NOD. PD-D, Parkinson's disease with depressive symptoms; PD-NOD, Parkinson's disease without depressive symptoms; aEg, area under the curve of global efficiency; aDc, area under the curve of degree centrality; aBc, area under the curve of betweenness centrality; aNe, area under the curve of node efficiency; PreM and SMC-L, left Pre-Motor and Supplementary Motor Cortex; Broca-L, left Broca's area; FEF-L, left frontal eye fields; DLPFC-L, left dorsolateral prefrontal cortex; FPA-L, left frontal polar area; FPA-R, right frontal polar area; DLPFC-R, right dorsolateral prefrontal cortex.

0.03 *vs.* 0.09 ± 0.04) were significantly reduced (FDR, all $p < 0.05$) (Tables 2–4, and Figure 4). aNLe and aNcp in 10 brain regions of the two groups had no statistical differences (all $p > 0.05$). A comparison of the local topological properties between the two groups illustrated that the elderly PD patients with depressive symptoms had abnormal topological parameters in several brain regions of the prefrontal lobe, and the brain network of PD-D patients were more sparse compared to the PD-NOD patients.

Pearson correlation analyses of the AUC of nodal metrics in each brain region and the HAMD scores showed significant negative correlations for aDc in FPA-L (r = -0.374, p = 0.004) and none of the other brain regions (p = 0.067~0.733); significant negative correlations for aBc in FEF-L (r = -0.330, p =0.013) and none of the other brain regions (p = 0.353 ~ 0.944); significant negative correlations for aNe in DLPFC-L (r = -0.311, p = 0.019) and FPA-L (r = -0.419, p = 0.001), and no correlation existed in any other brain regions (p = 0.064~0.400); significant negative correlations for aNLe in FPA-L (r = -0.267, p = 0.047); and aNcp did not correlate in any of the brain regions (p = 0.079~0.905). This suggests that the more severe the depressive symptoms in elderly PD patients, the more pronounced the local topological abnormalities in multiple brain regions.

Discussion

The relatively fixed anatomical structure of the brain serves as the physical basis for dynamic functional networks. The concept that a brain region may participate in multiple networks transcends structural constraints. The frontal lobe cortex, being the latest mature structure during brain development, is topologically central and plays a crucial role in emotion and cognition (*16*). The small-world network is an attractive model for complex brain networks, initially proposed by Watts and Strogatz in 1998 (*17*). Distinguished from regular networks (higher Cp and longer Lp) and random networks (lower Cp and shorter Lp), small-world networks exhibit higher Cp and shorter Lp, enabling the brain to achieve efficient local information segregation and global information integration with minimal wiring and energy consumption (*18*).

This study observed that the FC strength between

DLPFC-L and FPA-L was lower in the PD-D group compared to the PD-NOD group. Additionally, there was a significant negative correlation between the FC strength of DLPFC-L and FPA-L and the severity of depressive symptoms in the PD-D group. In the DLPFC-L (BA9, 46) and bilateral FPA (BA10, anterior portions of the superior and middle frontal gyrus), both aDc and aNe values in the PD-D group were lower than those in the PD-NOD group. Furthermore, the severity of depressive symptoms in patients was negatively correlated with the aNe value of DLPFC-L and the aDc, aNe, and aNLe values of FPA-L.

These findings are consistent with previous studies that identified four networks closely related to depression (*19-21*): the default mode network (DMN), which mainly includes the medial prefrontal cortex (mPFC) and posterior cingulate cortex (PCC), and which is negatively activated when there is an extraneous attention task, and positively activated when engaging in activities such as introspection, wandering, situational memory, and future envisioning; and the frontal-parietal network (FPN, also known as the executive control network, ECN), including the DLPFC, inferior parietal sulcus (IPS), and posterior parietal cortex (PPC), which is responsible for top-down regulation of attention and emotion; the salience network (SN), consisting mainly of the bilateral anterior insula (aINS) and dorsal anterior cingulate cortex (dACC), which is a bottom-up processor of salient experiences and can switch between the DMN and the FPN; the dorsal attentional network (DAN), associated with top-down control of attention activated by endogenous stimuli.

A Meta-analysis of RSFC in patients with MDD reported that hyper-connectivity within the DMN and hypo-connectivity within the FPN, increased connectivity between the FPN and DMN, and decreased connectivity between the FPN and the DAN may reflect patients' descent into ruminative thinking without attention to the external world (*19*). Sezer reviewed the mechanisms of mindfulness improving depression, in which the pathways of decreased connectivity between DMN and ACC, increased connectivity between PCC and DLPFC, increased connectivity between DMN and DAN, and increased connectivity between FPN and SN have been confirmed by several studies (*22*).

Although the location of lesions associated with depression is highly heterogeneous, these lesions map to connective brain circuits centered on the DLPFC-L (*23*). DLPFC is involved in cognitive or executive functioning and plays a role in the episode buffer of the depression schema feedback loop (*24*). Depressive PD patients were found the loss of grey matter in the prefrontal lobe, temporal lobe and some limbic regions and the reduction of white matter (*25*). Wei *et al.* (*26*) found that elevated FC in the left FPN and SN, and decreased FC in the DMN in depressed PD patients compared to non-depressed PD patients and that FC abnormalities in DMN, left FPN, and SN were correlated with depression severity in PD patients. Previous studies have also found abnormalities in the Dc of brain networks in depressed PD patients. Wang *et al.* (*27*) found that Dc in patients with depression (HAMD-17 scores ≥ 7) was abnormal in the right frontal middle gyrus (including BA9,10), bilateral anterior cingulate cortex, SMC, and cerebellar VI lobules, and the Dc of the right frontal middle gyrus was negatively correlated with the depression scores.

Currently, transcranial magnetic stimulation (TMS) is a well-established, non-invasive brain stimulation therapy for treating depressed patients with PD (*2*). A large number of studies have demonstrated that high-frequency stimulation on the left side of the DLPFC (≥ 5.0 Hz) or low-frequency stimulation on the right side of the DLPFC (≤ 1.0 Hz) can alleviate depressive symptoms (*28*). Notably, previous studies have varied but still demonstrated consistency. Our results reinforce the critical role of the DLPFC-L in depression and also suggest that depressive symptoms in elderly PD patients may share similar pathological changes with primary depression.

Our findings suggest that RSFC was sparser in the PD-D group, where the interhemispheric FC strength was significantly lower in the PD-D group than in the PD-NOD group; and the higher the depressive symptoms, the lower the interhemispheric FC strength. Interhemispheric coordination is the basis of coherent cognition, emotion regulation, and behavior, and the presence of imbalanced interhemispheric functional coordination in patients with MDD may be related to the impairment of the corpus callosum (the largest white matter connection between the hemispheres (*29*). Zhu *et al.* (*25*) found that middle-aged and elderly PD patients accompanied by depression had impaired interhemispheric synchrony.

Besides, Zheng *et al.* (*30*) found that impairment of interhemispheric FC in patients with recurrent MDD was associated with the severity of clinical depression in patients. All of the elderly PD patients in this study had economic small-world characteristics. The differences were not statistically significant compared with the PD-NOD group, although the PD-D group had higher aSigma, lower aCp and higher aLp. However, the brain network information transfer efficiency of the patients in the PD-D group was significantly lower than that of the PD-NOD group at the global level, and the more severe the depressive symptoms were, the lower the global transfer efficiency of the brain was. This suggests that elderly PD patients with depressive symptoms may have poorer network local interconnectivity and lower overall network routing efficiency. The global integration ability of elderly PD patients with depressive symptoms has been weakened, and the balance between local separation and global integration of brain networks has been impaired, and early intervention to avoid deterioration is necessary.

Chinese patients with MDD over 50 years of age

have been found to have significantly different overall brain network indices than normal controls, including decreased Eg, Eloc, Cp, and Sigma as well as increased Lp (*31*). Interestingly, a study that included resting-state fMRI scans of 30 first-episode, unmedicated MDD patients and 63 healthy control subjects found that MDD patients had shorter Lp, no change in Eloc, and higher Eg (*32*). These inconsistent and conflicting findings must be interpreted with caution because of the large differences between patients with first-episode unmedicated MDD and older PD patients with concomitant depressive symptoms.

In this study, patients in the PD-D group had lower aBc and aNe on the FEF-L (BA8, which belongs to the DAN) than those in the PD-NOD group, suggesting a decrease in both node influence and speed of information transfer. There was a significant negative correlation between the aBc value on the FEF-L and the severity of depressive symptoms. The pleasure deficit in depressed PD patients is related to the lack of attention to normal sensory perceptions, which in turn fails to activate reward loops (*25*). Froeliger *et al.* (*33*) compared the resting-state network of 7 experienced meditators and 7 beginners and found that the experienced meditators exhibited higher connectivity within the DAN (right anterior IPS and FEF-L, and right MT and FEF-L), between the DAN and DMN, and between the ECN and SN; this is related to the ability of experienced meditators to focus on sensory stimuli in the present moment.

Moreover, patients in the PD-D group in this study had significantly lower aNe values on PreM and SMC-L, Broca-L, and DLPFC-R than the PD-NOD group. These results are not surprising, as a large number of studies have demonstrated the presence of extensive brain function abnormalities in PD patients with depression. However, the variance in the results of different studies stems from different sample sizes, heterogeneity of PD patients (especially disease duration and medication status), dopaminergic treatment status, different brain imaging techniques and statistical methods. The homogeneity of the two groups in this study in terms of gender, age, cognitive function, duration of PD, H-Y stage, LEDD, and patients' motor function enhances the reliability of the findings.

This study has several limitations. Firstly, the fNIRS signals in this study were only collected from the prefrontal cortex, and regions such as the posterior cingulate gyrus, amygdala, and caudate nucleus, which are relevant to depression, were not investigated. Future studies of the whole brain will be useful in unveiling brain network characteristics and will contribute to a more in-depth understanding of the neural mechanisms of depressed PD. Secondly, considering the cultural sensitivity of the elderly population in China (such as being reluctant to express emotions and societal expectations), it is challenging to completely avoid false positives and false negatives in the assessment of depressive symptoms. Moreover, cultural values in East Asia foster an interdependent self-construal and shape the brain network, which may impose restrictions on the cross-cultural extrapolation of our conclusions. A more extensive testing approach could be beneficial in evaluating depression symptoms in PD patients. Finally, the relatively small sample size might impact the accuracy of the current analysis results, and further studies with larger samples are needed to validate our findings. Additionally, empirical studies targeting potential therapeutic targets would contribute to obtaining more reliable conclusions.

Conclusion

Overall, our data revealed that in elderly PD patients with depressive symptoms, the FC strength between the DLPFC-L and FPA-L was weakened, and the FC strength of DLPFC-L and FPA-L in elderly PD patients is negatively correlated with depressive symptoms. Additionally, abnormalities were observed in multiple nodal parameters of several brain regions, such as bilateral DLPFC and FPA. DLPFC-L and FPA-L are candidates for use as biological markers and preventive targets for the occurrence and development of depressive symptoms in elderly PD patients. Furthermore, elderly PD patients with depressive symptoms are less integrated and less efficient. Our findings provide new clues for exploring the pathogenesis of elderly PD patients with depressive symptoms and developing neural regulation methods.

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Patient satisfaction with nursing care in infertility patients: A questionnaire survey

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Abstract: Infertility remains a persistent global reproductive health challenge, with causative factors encompassing abnormalities in both the male and female reproductive systems. Typically, female partners seek initial consultations for infertility concerns, often within the context of routine annual well-woman check-ups. Nurses providing preventive care play a crucial role, conducting initial diagnostic assessments, and addressing certain causes of infertility. Patient satisfaction serves as a vital indicator of care quality. Identifying factors contributing to patient satisfaction with nursing services is crucial, yet research in this area has been limited. This study aimed to compare infertility patients' assessments of nurse quality and satisfaction with hospital services. The findings could offer valuable insights for healthcare providers, hospitals, and policymakers, guiding improvements in nursing care delivery and enhancing patient satisfaction in China's infertility treatment sector. By understanding patients' perspectives and experiences, healthcare providers can make necessary adjustments to improve care quality and patient outcomes. The sample included 1200 patients, and data collection utilized a self-assessment questionnaire, with percentages employed for analysis. Nurses are integral to caring for infertility patients during visits and conducting research to advance fertility care practices.

***Keywords*:** patient satisfaction, healthcare quality, infertility

Introduction

Infertility is commonly defined as the inability to achieve pregnancy for a duration of 12 months or more despite regular, unprotected sexual intercourse, leading a significant number of individuals to seek services that can provide them with the possibility of pregnancy (*1*). Given the potential long-lasting and devastating emotional and psychological impact of infertility on couples and individuals, nurses are advised not to overlook the social and psychological complexities of infertility (*2*). In the context of infertility consultations, nurses play multiple roles, including providing support, education, coordinating care, and advocating for individuals struggling with infertility. This necessitates an understanding of the complexity of infertility issues and care (*3*). An increasing number of women are in search of fertility treatment options (*4*), underscoring the importance of healthcare professionals, particularly nurses, possessing an up-to-date knowledge of contemporary infertility treatments and available options. As such, the general public can benefit from seeking guidance from nurses on all aspects of healthcare-related matters (*5,6*). The incidence of infertility can take a toll on both women and their partners, potentially causing significant grief and disappointment, particularly when faced with potential pregnancy loss (*7,8*). Therefore, providing professional nursing care is a vital component in addressing the unique requirements of such patients (*9*).

In light of all the complexities that can arise in women's reproductive health, specialized nurses have a critical function to fulfill in supporting infertility care and management (*10*). With the increasing number of women seeking infertility treatment, the demand for skilled nurses with expertise in this field continues to rise. Irrespective of whether they are employed in direct care roles for infertile patients or other facets of the healthcare industry, nurses must possess comprehensive knowledge of the infertility experience, including the various stages of treatment and appropriate avenues for care (*11*). To develop tailored care plans that suit individual needs, a systematic, step-by-step approach is necessary, allowing healthcare providers to understand each woman's situation and apply general nursing theory to her specific circumstances (*12*). The impact of infertility on mental well-being has been a subject of keen research interest. Infertility can be regarded as

a prolonged source of stress, which may contribute to a broad range of psychological issues (*13,14*). Several studies have demonstrated that women who receive counselling and actively participate in nurse-led support groups have better chances of achieving successful pregnancies compared to those who do not receive such support (*15*). In addition, some evidence suggests that stress has a direct impact on the outcome of infertility treatment (*16*). Nurses who work with infertility patients must be prepared to conduct comprehensive assessments of patients, assist in reducing discomfort, and provide optimal counselling. Every woman's journey of experiencing and managing infertility is unique, and healthcare professionals must pay close attention to her narrative to provide the best care. It is important for nurses to avoid making assumptions based on factors such as occupation, education level, or financial status. Even those with medical or healthcare backgrounds may have limited knowledge about infertility and may require empathetic explanations (*17,18*).

This article delineates the systematic approach of assessing, planning, implementing, and evaluating the care process for women undergoing infertility assessment and intervention. The primary objective of the study was to evaluate the satisfaction levels of infertility patients with the care provided by nurses. To address the research aim and objectives, a descriptive research design was adopted to determine patients' contentment with the quality of care administered.

Materials and Methods

A descriptive research design

The study population consisted of patients who met the diagnosis of infertility and patients were surveyed and interviewed about their satisfaction with the care they received. The inclusion criteria for the study were: patients who had been married for at least 1 year (*19*), having normal sex life, not using any contraception and unable to pregnancy. The final sample consisted of 1,200 patients. All research content and methodologies strictly adhere to the Helsinki Declaration and have received approval from the Ethics Committee of the Obstetrics & Gynecology Hospital affiliated with Fudan University (2021-154-X1). Clinical data for statistical analysis were derived exclusively from cases meeting diagnostic criteria with complete information. Informed consent was obtained from all patients involved in the study.

Procedures

This study focuses on the satisfaction of infertility patients with outpatient and inpatient healthcare services, covering data collected from July 2022 to July 2023. The focus of the research was the voluntary assessment of satisfaction among patients aged 20 to 45 during their recent outpatient and inpatient treatments. Data collection was conducted through questionnaire surveys, with respondents having the option to complete electronic versions of the questionnaire either during outpatient visits or within hospital wards. This voluntary assessment approach helps to comprehend patients' subjective perceptions of healthcare services, providing a comprehensive evaluation of both outpatient and inpatient treatments. Firstly, a questionnaire was used to measure the quality of nursing care. The satisfaction instrument was modified from the LaMonica-Oberst Patient Satisfaction Instrument to collect data (*20*). The study utilized a rating system that was adapted from a Likert scale (*21*), providing the participants with a range of responses from "strongly agree" to "strongly disagree". A 5-point scale was used, 1) for "Strongly agree", 2) for "Agree" and 3) for "Neither" and 4) for "Disagree" and for 5) "Strongly disagree".

The study applied a multidimensional method of Structural Equation Model (SEM) to estimate and verify the relationships between the variables (*22*). SEM is a statistical technique that integrates Confirmatory Factor Analysis (CFA) and Path Analysis (PA) approaches to analyze the relationships between multiple variables, both latent traits and observable variables (*23*). The CFA technique was employed to deduce the underlying factors or latent traits, such as patient attitudes and satisfaction levels towards selected factors, alongside the corresponding manifest variables. Meanwhile, the PA approach was employed to identify the causal relationships between the latent variables by producing a path diagram in the form of an SEM model (*24*).

Statistical analysis

A chi-squared test result with $p < 0.05$ was considered as indicating a good model fit for the SEM. $P < 0.05$ indicates a statistically significant difference. Acceptable levels of fit were defined by Comparative Fit Index (CFI) and standardized root mean square residual (SRMR) values greater than or equal to 0.90 and less than 0.80, respectively (*25*). All the collected data were subjected to descriptive analysis and SEM. Specifically, the SPSSPRO software was utilized to evaluate the data structure and establish the equation models. The SEM parameters were computed using the maximum likelihood approach and validated based on the established models. Through this process, the SEM approach helped to determine the factors that influence infertility patient satisfaction, and establish the connection between patient adherence, satisfaction, and their degree of impact.

Results

Analysis of overall satisfaction with care services

The number of completed questionnaires was 1,225

(98%). Twenty-five patients were deemed unusable due to incomplete questionnaires. Therefore, the sample of 1,200 infertile patients for analysis were conducted. Table 1 presents the Likert scale questions used to assess infertility patient satisfaction with care, totaling 5 questions. According to the research design, the respondents were requested to evaluate their satisfaction level using a rating scale of 1 to 5. The survey questions presented to the respondents encompassed various aspects relating to the quality of care and the nursing staff's performance. These questions aimed to obtain feedback on several factors influencing patient satisfaction with nursing care, such as the provision of emotional support, communication, explanations of medical procedures, accessibility, and promptness of care, among other aspects. By collecting and analyzing the data from these questions, healthcare providers can assess patient satisfaction levels, identify areas for improvement, and develop strategies to enhance overall patient satisfaction (Table 1).

Structural equation model path diagram

In this study, we used SEM path diagrams to investigate infertility patient satisfaction factors and the correlation between patient adherence and satisfaction. They outline connections among variables such as patient medical adherence, satisfaction, healthcare service quality, and communication effectiveness. Arrows indicate directional relationships, showing if one variable predicts changes in another (*26*). The path diagrams help identify variables directly influencing satisfaction and reveal indirect pathways through other mediating variables, contributing to understanding the complex mechanisms behind satisfaction formation (*27*). They also represent the researcher's hypotheses about causal relationships among variables, clarifying which variables are considered as drivers in the model. Moreover, the path diagram aids in identifying variables directly influencing satisfaction and reveals pathways of indirect influence through other mediating variables. This contributes to the understanding of the intricate mechanisms underlying the formation of satisfaction. The path diagram expresses the researcher's hypotheses regarding causal relationships among variables. By examining the directionality of paths, it elucidates which variables are considered the drivers of causal relationships in the model.

According to the results of the fit test summaries and the application of the SEM method, the final SEM model presented in Figure 1 was deemed to be the most appropriate solution for the study. This model was able to effectively estimate and quantify the relationships between the latent variables and their corresponding manifest variables and establish both significant indirect and direct connections between the variables. Figure 1 depicts the weighted structure path diagram, consisting of the model's standardization coefficients, which provides insight into the influence of the structural pathways on the relationships among the variables. The SEM model for infertility patient satisfaction with nursing consists of four latent variables (Professionalism, Emotional Care, Satisfaction, Medical Adherence) and 11 observed variables. The relationships between the final SEM model and variables are depicted in Figure 1.

A hypothesized direct relationship

The Table 2 represents the factor loading coefficient table of the model, encompassing latent variables, observed items, non-standardized loading coefficients,

Table 1. Likert scale for assessing infertility patient satisfaction with care (*n* = 1,200)

Categories	Questions	Strongly Agree	Agree	Neither	Disagree	Strongly Disagree
Professional knowledge and skills of nurses	To what extent are you satisfied with the professional knowledge and skills of the nurses?	80%	10%	5%	5%	0%
Execution quality	Does the care team follow a well-established treatment plan and process?	83%	12%	2%	3%	0%
Decision-making transparency and clarity	How satisfied are you with the transparency and clarity of decision-making by both nurses and doctors?	70%	22%	4%	4%	0%
Quality of communication and explanation of information related to infertility	How satisfied are you with the quality of communication with nurses throughout your care process?	30%	35%	10%	22%	3%
Attitude and performance of nursing staff	How satisfied are you with the timeliness and thoroughness of the medical advice and counseling provided by the nursing staff?	45%	25%	10%	20%	0%
	How satisfied are you with the quality of communication and the clarity of information provided regarding infertility during your care?	78%	12%	5%	5%	0%
	How satisfied are you with the responsiveness and timeliness of communication regarding the support and recognition you receive?	45%	32%	13%	5%	5%

Z-test results, and related information. In the assessment of measurement relationships, the first item was utilized as the reference point. The findings from Table 2, based on the path coefficient table of the model, indicate the rejection of the null hypothesis for both Professional Knowledge and Execution Quality, as their standard load coefficients surpass 0.4 (***$p < 0.001$). This signifies a satisfactory level of explained variance, suggesting that each variable exhibits a meaningful degree of interpretability within the same factor. Comparable observations are applicable to the rejection of the null hypothesis at the level of Human Care and Service Attitude variables, where each variable's standard load coefficient exceeds 0.4 (***$p < 0.001$), underscoring the substantial influence of these latent variables on their respective factors. Furthermore, at the levels of Attitude and Dependency, the null hypothesis was also rejected. This implies a significant impact of these latent variables on their associated factors, thereby contributing meaningfully to the overall satisfaction level of infertility patients. The standardized loading coefficients for both Attitude and Medical Adherence exceed 0.4 (***$p < 0.001$), indicating a substantial effect on their respective factors. This underscores a sufficient level of interpretability, suggesting the potential for each variable to manifest on the same factor.

The assessment of standardized regression coefficients

The Table 3 presents the regression coefficients of the path nodes, employing the least squares single linear regression method. The path coefficient table of the model indicates that the pairing of Professionalism to Satisfaction was statistically significant, with a *p*-value of 0.025 (*$p < 0.05$), and an associated influence coefficient of 0.144. Similarly, for the pairing of Emotional Care to Satisfaction, the null hypothesis is rejected, with a *p*-value of 0.024 (*$p < 0.05$), signifying a statistically significant and valid path, characterized by an influence coefficient of 0.144. Furthermore, the pairing of Satisfaction to Medical Adherence demonstrates a statistically significant path with a *p*-value of 0.007 (**$p < 0.01$),

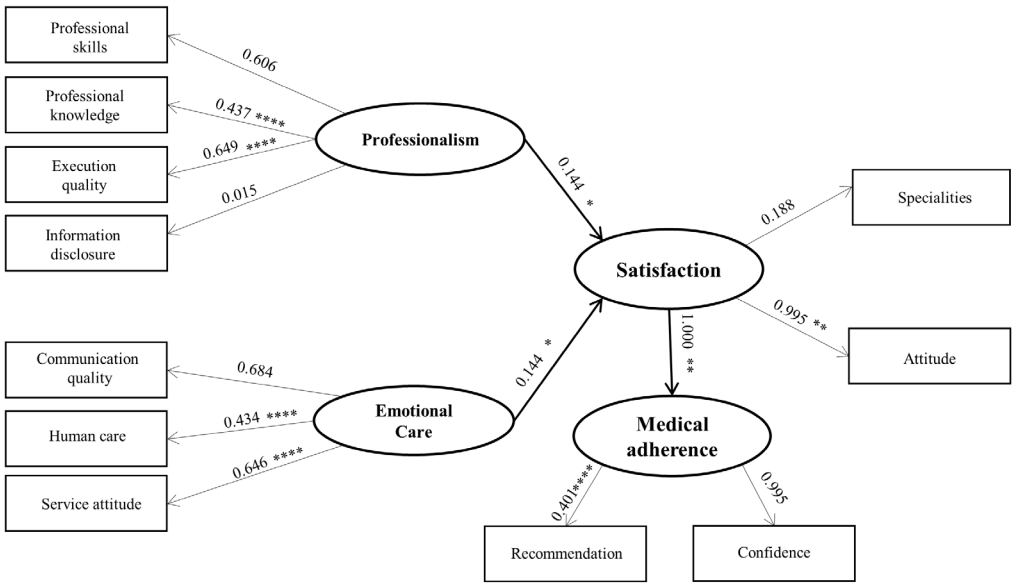

Figure 1. Structural equation model path diagram. This is a path diagram of a SEM illustrating the associations among Professionalism, Emotional Care, Satisfaction, and Medical Adherence. **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Table 2. Factor loading coefficient

Factor	Variables	Unchanged factor loading	Standardized factor loading	*Z*	S.E.	*p*
Professionalism	Professional skills	1	0.606	-	-	-
	Professional knowledge	0.765	0.437	8.93	0.086	0.000****
	Execution quality	1.158	0.649	10.634	0.109	0.000****
	Information disclosure	0.026	0.015	0.956	0.027	0.339
Emotional Care	Communication quality	1	0.604	-	-	-
	Human care	0.762	0.434	8.896	0.086	0.000****
	Service attitude	1.157	0.646	10.592	0.109	0.000****
Satisfaction	Specialities	1	0.188	-	-	-
	Attitude	4.104	0.995	2.702	1.519	0.007**
Medical Adherence	Confidence	1	0.995	-	-	-
	Recommendation	0.459	0.401	6.166	0.074	0.000****

Abbreviations: S.E., standard error. **$p < 0.01$, ****$p < 0.0001$.

confirming its validity, and an associated influence coefficient of 1.0.

Evaluation of model fit indicators

In Table 4, the model was deemed well-fitted, with a RMR < 0.1. The CFI index is employed in the comparison of hypothetical and independent models, with a value closer to 1 signifying a better fit. With a CFI of 0.9 in these results, the model is considered well-fitted. The fit indices in Table 4 suggest that the final SEM has a good fit with the observed data. CFI and Tucker-Lewis Index (TLI) values above 0.95 indicate a good fit, while the Root Mean Square Error of Approximation (RMSEA) value of 0.06 and the Standardized Root Mean Residual (SRMR) value of 0.07 are both below the acceptable thresholds of 0.08, indicating a reasonable fit. Additionally, both the Goodness of Fit Index (GFI) and Adjusted Goodness-of-Fit Index (AGFI) values were above 0.90, indicating a good model fit. All these fit indices collectively suggest a satisfactory alignment between the conceptual model and the data, supporting the overall validity and reliability of the model. Therefore, the final SEM model can be considered a robust fit for the data employed in this study.

A path-node covariance matrix

The Table 5 presents the results of the factor covariance analysis, including non-standard coefficient, standard error, *Z*-test values, *p*-values of significance, and standard coefficient. Based on the results of the covariance analysis, the covariance relationship between Professionalism and Emotional Care was found to be significant. The standardization coefficient of 1.642 indicates a strong association between these two latent variables. Therefore, it was suggested to add the path relationship for the analysis, as it would help to provide a more accurate representation of the relationships between these variables in the SEM model. Adding the path relationship would also help to improve the goodness of fit of the model and enhance the overall accuracy and reliability of the results. Hence, it is essential to analyze the path relationship between Professionalism and Emotional Care to derive meaningful insights from the data and improve the understanding of their interrelationship.

Discussion

Nurses have a crucial role to play in infertility treatment and are instrumental in offering care and support (*18*). They can provide educational material on fertility treatment, help with treatment manipulation, and offer emotional support throughout the process (*28*). Nurses often collaborate with a multidisciplinary team, which may include reproductive endocrinologists and psychologists, to develop a personalized care plan for each patient.

For the SEM method to be applied, the selected variables were assumed to follow the normal distribution (*29*). Therefore, the basic descriptive characteristics were computed to assess the normality assumption of the data. The z-score values were then calculated based on the standard deviation, skewness, and kurtosis values, which were found to be in the normal range of ± 1.5 and

Table 3. The model regression coefficient

Factor (Latent variable)	Analyzed variables (manifest variables)	Non-standardized coefficients	Standardization coefficient	Standard error	*Z*	*p*
Professionalism	Satisfaction	0.047	0.144	0.021	2.243	0.025*
Emotional Care	Satisfaction	0.047	0.144	0.021	2.250	0.024*
Satisfaction	Medical adherence	4.104	1.000	1.519	2.702	0.007**

*$p < 0.05$, **$p < 0.01$.

Table 4. Model fit indices

χ^2	df	*p*	Chi-square degrees of freedom ratio	GFI	RMSEA	RMR	CFI	NFI	NNFI
-	-	> 0.05	< 3	> 0.9	< 0.10	< 0.05	> 0.9	> 0.9	> 0.9
2247.169	40.000	0.000****	56.179	0.450	0.525	8.211	0.453	0.450	0.248

Abbreviations: df, degree of freedom; GFI, goodness-of-fit index; RMSEA, root mean square error of approximation; RMR, root mean square residual; CFI, comparative fit index; NFI, normed fit index; NNFI, non-normed fit index. ****$p < 0.0001$.

Table 5. Table of path-node covariance relationships

Factor A	Factor B	Non-standard estimated coefficient	Standard estimate coefficient	Standard error	*Z*	*p*
Professionalism	Emotional Care	0.320	1.642	0.040	7.944	0.000****

****$p < 0.0001$.

± 3, respectively (*30*). The GFI represents the amount of variance and covariance accounted for by the model, and a score of 0.90 or higher is generally considered an acceptable fit. The CMIN/DF is the ratio of the Chi-Square statistic to the degrees of freedom, and a value of 2 or lower indicates a good model fit. Therefore, if the data depicts a pattern of normality, and the GFI and CMIN/DF values are within the acceptable range, then the SEM model can be considered a suitable method for estimating and verifying the relationships between the variables (*31*). The significance level was set at 5% ($\alpha = 0.05$). A good model fit is indicated by high values for GFI, CFI, and NFI, as well as low values for CMIN/DF and RMSEA (*32,33*). By examining these fit test summaries, it was possible to verify the suitability of the SEM model and ensure that it accurately represented the relationships between the variables.

According to the table of Factor loading coefficient, it can be concluded that in the final SEM model, the seven variables: Professional knowledge, Execution quality, Human care, Service attitude, Attitude, Medical adherence, and Recommendation can effectively explain the potential factors they represent. This means that they can be considered as different manifestations of the same underlying factor: different variables on the same factor. This also indicates a high degree of internal consistency and reliability of these variables across the model. This is important for assessing the quality and improving the delivery of healthcare.

The table of model path coefficients, it can be seen that in the final SEM model, Professionalism and Emotional Care have a more significant positive effect on satisfaction, which means that if the level of Professionalism and Emotional Care of medical staff was higher. This means that patients are more satisfied with their care if their Professionalism and Emotional Care are higher. In addition, satisfaction also has a significant positive effect on medical adherence, the more satisfied the patient is with the healthcare service, the more medical adherence the patient is on the healthcare service.

According to the results of the analysis of covariance, there is a significant covariance between the variables Professionalism and Emotional Care with a standardized coefficient of 1.642, indicating a strong correlation between these two variables. It is recommended that the relationship between these two variables be added to the SEM model for further analysis. This will allow for a more comprehensive assessment of the quality of healthcare services and patient satisfaction, and provide guidance for the improvement of the healthcare delivery system.

This study's findings shows that high-quality care and positive attitudes among nursing staff can notably enhance the compliance of individuals undergoing infertility treatment. Furthermore, improvements made to the quality of care and service attitudes can enhance the outcome and overall satisfaction of infertility patients to a considerable extent. Consequently, hospital management should prioritize rigorous training and assessments of nursing staff to augment their professional skills and service quality, thereby boosting patient compliance and treatment effectiveness. The limitations of this study were that the questions listed in the questionnaire were not sensitive enough to identify the level of care and secondly, as the patients had many different nurses caring for them, they could not be evaluated uniformly. These contents need to be collected with more information for more comprehensive analysis.

Conclusions

Patient satisfaction and nursing care have long been focal points in the field of healthcare. The professional and research communities have consistently directed their attention towards assessing patients' contentment with medical services. This trend has driven the creation of methodological platforms and the initiation of new research endeavors, aiming to unveil novel determinants influencing the ultimate evaluation of patient satisfaction (*34*). As the pressure to enhance healthcare efficiency and sustainability intensifies, the standards for nursing quality continue to escalate. This trend is poised to significantly impact patients' loyalty to healthcare facilities and exert a direct influence on nursing practices and service delivery. As a result, research efforts are dedicated to a comprehensive exploration and clarification of these dynamic changes, with the goal of providing improved responses to the ongoing evolution within the healthcare domain.

Currently, we are in the process of establishing various mechanisms and formulating strategies to enhance healthcare quality. While a standardized platform and unified assessment parameters are yet to be established, optimal parameters for healthcare quality can be defined, potentially initiating a process of continuous improvement. The in-depth investigation into the causes of dissatisfaction and exploration of methods to address discontent have led to issues related to patient satisfaction progressively becoming a focal point across multiple disciplines. The purpose of this study was to analyze and assess the determinants influencing the overall satisfaction of infertility patients seeking treatment at our hospital. The study sample comprises 1200 patients who received outpatient or inpatient treatment. In this context, both Professional knowledge and Execution quality exhibit standardized loading coefficients exceeding 0.4, indicating their substantial variance-explaining capacity for latent variables within the model. Standardized loading coefficients measure the strength of relationships between observed variables and latent variables, with values above 0.4 generally considered relatively strong associations. The insights provided by the model's path coefficient table reveal significant relationships among different variables and suggest their

potential co-expression on the same factor. This aids in a deeper understanding of the constituents of satisfaction and its related factors in the study. The effectiveness of three pathways in the model, namely Professionalism > Satisfaction, Emotional Care > Satisfaction, and Satisfaction > Medical adherence, has been confirmed through the regression coefficient table. These pathways are not only statistically significant but also reject the null hypothesis, further supporting their significance in the relationships within the model. This provides crucial information for a comprehensive comprehension of the relationships between satisfaction and medical adherence, along with associated influencing factors. The covariance analysis emphasizes the covariance relationship between Professionalism and Emotional Care. The significant result indicates a covariant association between Professionalism and Emotional Care. With a standardized coefficient of 1.642, a robust correlation between these variables is suggested. The results of the SEM indicate that Professional knowledge, Execution quality, Human Care, Service attitude, accommodation satisfaction, and medical staff satisfaction have a positive impact on nursing satisfaction.

The limitations of this study include the uneven distribution of the research sample in specific regions. The methods of comparative analysis and the size of the research sample remain crucial factors in the evaluation process. Nevertheless, our research results furnish valuable information for healthcare personnel.

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Development of aids to relieve vulvodynia during the postpartum period

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Abstract: Postpartum women live with a low quality of life due to pain caused by episiotomy and perineal laceration. In particular, they endure pain when sitting for long periods of time to breastfeed. The purpose of this study is to develop a sitting aid to alleviate postpartum vulvodynia. This study was conducted in the following four phases from July 2017 to May 2019. They are: material selection and molding, cleaning and disinfection testing, pressure distribution measurement testing, and trial testing by postpartum women. The main material was a 100% polypropylene object with a three-dimensional reticular fiber spring structure and fiber density of 3.8 kg/m^2. As a result, a sitting aid that withstands washing and disinfection well in the medical field and is breathable. It had moderate resilience and elasticity and reduced pressure on the seating surface for women weighing approximately 45 kg and 55 kg, but we were skeptical about its use for women weighing more than that. The completed sitting aid is noninvasively effective in improving the quality of life of many postpartum women, but the density and thickness of the main material should be reexamined to meet the needs of women in a wider weight range. In addition, a self-administered questionnaire survey of trial users revealed that some women did not experience relief from vulvodynia even after using the sitting aid. Such women also had physical problems such as discomfort in the lower back, difficulty breastfeeding, and difficulty standing up. For women with multiple physical problems, individual causes should be addressed.

***Keywords*:** postpartum women, episiotomy, perineal laceration, vulvodynia

Introduction

Lacerations of the birth canal often occur during the parturition period (*1*). Lacerations may occur spontaneously at the perineum, cervix, vagina, or vulva, or by extension of a perineotomy wound (*1,2*). To prevent severe perineal lacerations, perineal massage during pregnancy and parturition (*3,4*), and Pilates exercises during pregnancy (*5,6*), However, none of these reports have yet been confirmed to be effective, nor have superior interventions been identified (*7*). In many countries, perineotomy may be used to prevent severe perineal lacerations (*8*). The use of elective perineotomy remains useful and should be performed based on clinical judgment and maternal or fetal indications. Even today, perineotomy is routinely performed in some countries (*9*). There have been reports of the application of herbal creams or herbal oils to perineal incision wounds (*10-12*), Far-infrared irradiation of perineotomy wounds for pain relief (*13*), low-frequency therapy (*14*), and low-level laser therapy (*15*) have been used, however, their efficacy has not been proven (*1,8*). There is also a report that auricular acupuncture was effective in relieving vulvodynia in postpartum women, but the number of patients involved was 29 and the mechanism of action was not reported (*16*). Thus, perineal lacerations and perineal injuries caused by perineotomy are not only painful but also have significant physical and psychological effects on the lives of many postpartum women (*17,18*).

Circular seats made of rubber or urethane or postpartum chairs made of urethane covered with synthetic leather have traditionally been used for vulvodynia pain during the sitting position in the postpartum period. However, these have the problem that prolonged use causes vulvar congestion and traction of the skin around the wound, which adversely affects wound healing and wound pain (*19*). Therefore, it is important to develop an aid that does not increase wound pain and does not interfere with wound healing even after prolonged use in the sitting position during repeated postpartum breastfeeding (*20*). It is also important that the assistive device be breathable, washable, and disinfectable to maintain hygiene. Effective aids to relieve postpartum vulvodynia have not yet been developed.

The purpose of this study was to develop an assistive device to prevent vulvar pain augmentation that occurs in

the sitting position.

Materials and Methods

This study was conducted in the following four phases. The four phases were conducted from July 2017 to May 2019.

Phase 1. Material selection, molding (July 2017 to December 2017): In this phase, we selected the appropriate materials and developed the initial molds for the sitting aid.

Phase 2. Washing and disinfection tests (January 2018 to November 2018): During this phase, the washability and disinfectability of the materials used in the sitting aid were thoroughly tested to ensure hygiene and safety.

Phase 3. Body pressure distribution measurement test (December 2018 to February 2019): This phase involved testing the pressure distribution characteristics of the sitting aid, using body pressure measurement techniques to assess its effectiveness in reducing vulvar pain.

Phase 4. Trial test by postpartum women (March 2019 to May 2019): In the final phase, the developed sitting aid was trialed by postpartum women to evaluate its practical effectiveness and gather user feedback.

Material

In this study, two materials were selected to develop the sitting aid, focusing on their suitability for the postpartum period. This period is characterized by lochia discharge, making it essential to use materials that are easy to clean and disinfect. Additionally, postpartum women often use sanitary pads, increasing the need for materials that offer breathability to prevent discomfort and skin issues. The selected materials also needed to provide an appropriate level of firmness for comfort and support.

CALFIBER®: *i*) Material: 100% polypropylene, *ii*) Structure: Three-dimensional reticular fiber spring structure, *iii*) Fiber density: 3.8 kg/m^2, *iv*) Characteristics: High elasticity and resilience, breathability, heat retention, light weight, heat resistance, easy to keep clean.

V-lap®: *i*) Material: 100% polyester, *ii*) Structure: Vertical non-woven fabric structure, *iii*) Characteristics: Breathable, lightweight, heat resistant, disposable.

Both materials satisfy the specific requirements of postpartum care, offering comfort, hygiene, and practicality, essential for products intended for use during the postpartum period.

Molding

Circular seats can cause the vulva to become congested when sitting on them because of the indentation in the middle. Therefore, we devised a shape that does not press on the vulva when sitting and does not cause congestion. The sitting aid is made by punching the above material into a U-shape in order to support weight on the entire thigh. The shape and size of the sitting aids produced are shown in Figure 1.

Washing and disinfection tests

The washing and disinfection tests were conducted under the general medical instrument washing methods outlined (*21*). The following three types of washing and disinfection tests were performed using CALFIBER® punched into a U-shape (hereinafter referred to as "U-shaped CALFIBER®"). For each washing or disinfection of the U-shaped CALFIBER®, the size of the nine points shown in Figure 2 was measured with a ruler. In addition, the presence of deformation was visually determined. V-lap® was excluded from the washing and disinfection tests because it is a disposable product.

Test 1: Washing five times in a fully automatic household washing machine on the "standard course" using a laundry net for clothes.

Test 2: Repeat 5 times with a 15-minute soak in a 0.5% sodium hypochlorite solution.

Test 3: Washing with Washer Disinfector (Stihlco DS1000G manufactured by MS Corporation): Washing with tap water for 1 minute (room temperature) ⇒ Drain ⇒ Alkaline detergent 0.5% washing (warm water 90°C for 1 minute) ⇒ Drain ⇒ Neutralize with acid detergent (warm water 50°C for 1 minute) ⇒ Drain ⇒ Rinse (warm water 50°C for 1 minute) Drain ⇒ Final rinse (hot water 80°C for 1 minute) ⇒ Drain ⇒ Dry (50°C to 60°C for 2 minutes) 5 times. The time for each process starts when the water in the tank reaches the set temperature.

Body pressure distribution measurement test

For the body pressure distribution measurement test, we utilized FSA sensors (Vista Medical Ltd; Winnipeg, Manitoba, Canada) to compare the body pressure distribution during seating of three types of seating assistive devices: a rubber circular seat ("rubber seat"), a urethane foam circular seat ("urethane seat"), and a U-shaped seat with a fabric cover over the V-lap® on CALFIBER® (hereinafter referred to as "U-shaped seat"). The body pressure distribution of subjects sitting on the seat was compared. The body pressure distribution of the subject sitting on the seat was approximately 45% of body weight. To evaluate the suitability and effectiveness of the sitting aids specifically for postpartum women, the participants in this study were postpartum women, one for each specified weight category, approximately weighing 45 kg, 55 kg, and 65 kg, respectively. The research procedure consisted of measuring the subjects' weight, setting up the sitting aids, setting up the body pressure distribution measurement test device, having the subjects sit on the seats, and measuring the body pressure distribution.

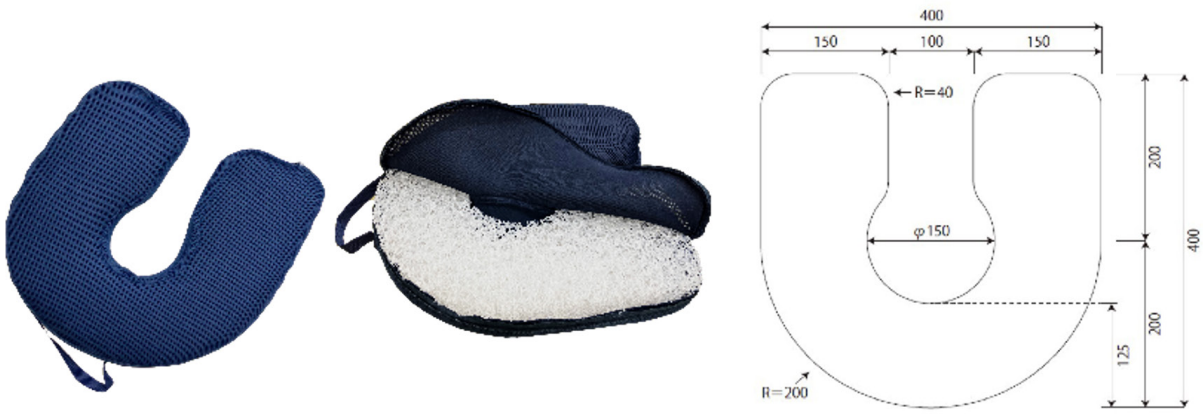

Figure 1. The shape and size of the sitting aids produced.

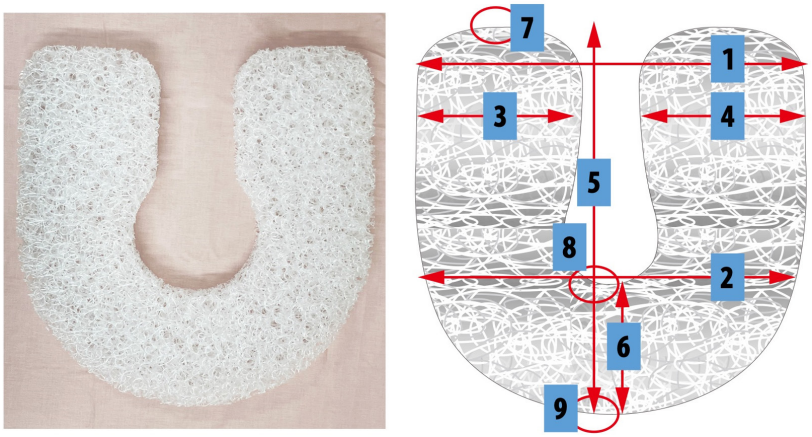

Figure 2. U-shaped CALFIBER®, the size of the nine points.

The test procedure was as follows: *i*) The height of the seat and the floor was adjusted to match the length of the lower leg of the subjects. Subjects were seated without shoes or slippers, ensuring that the soles of both feet were fully in contact with the floor. *ii*) Subjects were instructed to sit deeply, leaning slightly forward with their hands on their knees. After about one minute of position adjustment to find a comfortable posture, data on body pressure distribution were collected. The data collected included images showing the complete shape of the buttocks to ensure comprehensive pressure mapping.

Trial test by postpartum women

Two hundred postpartum women (number and rate of collection: 189, 94.5%) hospitalized at a maternity hospital in Tokyo after vaginal delivery were asked to try the U-shaped seat, compare it with a urethane seat, and answer a self-administered questionnaire. The self-administered questionnaire included age, postpartum weight, newborn weight, number of previous deliveries, method of delivery, presence of episiotomy and perineal laceration, and comfort with the U-shaped seat compared to a urethane seat.

Basic statistics were calculated for the response results. Spearman rank correlation analysis was performed on the relationship between vulvar pain during seating and other factors. SPSS Statistics Ver. 24 (IBM, USA) was used for the analysis.

Ethical consideration

This study was conducted after obtaining approval from the research ethics review of the National Center for Global Health and Medicine (Approval No. NCGM-G-002376-00).

Participants in the sitting aid trial and the self-administered questionnaire survey were informed of the purpose of the study in writing and orally, and asked to participate in the study on a voluntary basis. Consent was confirmed by submission of a self-administered, unsigned questionnaire.

Results

Evaluation after washing or disinfection

After cleaning and disinfection, no gross deformation was observed in any of the methods and times. The measurements are shown in Table 1. There was virtually no change in size at any point in any cleaning or

Table 1. Measurements after cleaning or disinfection of U-shaped CALFIBERR

Washing or disinfection method	Measuring point	Before (cm)	1st (cm)	2nd (cm)	3rd (cm)	4th (cm)	5th (cm)
Household Washer	1	40	40	40	40	40	40
	2	39	39	39	39	39	39
	3	15	15	15	15	15	15
	4	15	15	15	15	15	15
	5	40	40	40	40	40	40
	6	12.5	12.5	12.5	12.5	12.5	12.5
	7	5.8	5.8	5.8	5.8	5.8	5.8
	8	5.9	5.9	5.8	5.8	5.8	5.8
	9	5.7	5.7	5.7	5.7	5.7	5.7
Washer Disinfector	1	40.0	40.0	40.0	40.0	40.0	40.0
	2	37.0	37.0	37.0	37.0	37.0	37.0
	3	15.0	15.0	15.0	15.0	15.0	15.0
	4	15.0	15.0	15.0	15.0	15.0	15.0
	5	40.0	40.0	40.0	40.0	40.0	40.0
	6	12.5	12.5	12.5	12.5	12.5	12.5
	7	5.8	6.0	6.0	6.0	6.0	6.0
	8	5.9	5.9	6.0	6.0	6.0	5.9
	9	5.7	6.0	6.0	6.0	6.0	6.0
Immersion in 0.5% sodium hypochlorite solution	1	39	39	39	39	39	39
	2	37	37	37	37	37	37
	3	15	15	15	15	15	15
	4	15	15	15	15	15	15
	5	37	37	37	37	37	37
	6	12.5	12.5	12.5	12.5	12.5	12.5
	7	6	6	6	6	6	6
	8	6	6	6	6	6	6
	9	6	6	6	6	6	6

Table 2. Results of body pressure distribution measurement test

Female Weight	Material	Minimum pressure (kPa)	Maximum pressure (kPa)	Average pressure (kPa)	Standard deviation (kPa)
45kg	U-Shaped seat	0.00	15.40	3.50	4.11
	Urethane seat	0.00	26.52	4.17	5.77
	Rubber seat	0.00	21.33	4.25	5.89
55kg	U- Shaped seat	0.00	16.57	4.26	4.52
	Urethane seat	0.00	20.88	4.45	5.38
	Rubber seat	0.00	25.38	4.59	5.94
65kg	U- Shaped seat	0.00	26.66	5.49	5.69
	Urethane seat	0.00	23.64	5.37	6.13
	Rubber seat	0.00	26.66	6.92	5.69

disinfection method or frequency. Additionally, there was no change in tactile feel or color.

Evaluation of Body Pressure Distribution Measurement

The body pressure distribution measurement test was conducted to compare the pressure distribution of U-shaped seats, rubber, and urethane seats, and the results are shown in Table 2.

The test results indicated distinct differences in pressure distribution among the different seat types. Notably, for participants weighing 45 kg and 55 kg, the U-shaped seat demonstrated the lowest body pressure, in terms of both maximum and average pressure. This suggests that the U-shaped seat provided better pressure relief for individuals in these weight categories. Conversely, for participants weighing 65 kg, the urethane seat showed the lowest body pressure for both maximum and average measurements. This indicates that the urethane seat may be more effective in distributing body pressure for individuals in this higher weight range.

Responses to self-administered questionnaire by trial users

The mean age of the subjects was 32.5 ± 4.1 years, the mean postpartum weight was 59.1 ± 7.6 kg, and the mean birth neonatal weight was 3,059 ± 354.5 g. Ninety-six (50.8%) were primiparas and 93 (49.2%) were multiparas. The method of delivery was spontaneous vaginal delivery in 161 (85.6%), suction in 25 (13.3%), and forceps in 2 (1.1%). 93 (54.4%) had episiotomy and 78 (45.6%) did not.122 (74.4%) had perineal laceration and 42 (25.6%) did not.

Table 3 shows the results of U-shaped seat compared to urethane seat. When asked to compare the urethane seat with the U-shaped seat, about 88% of the respondents reported no or less vulvar pain when seated in the U-shaped seat, and about 76% reported no or less Discomfort due to steaminess. Table 4 shows the association between the presence of vulvodynia and other factors when seated. Sense of back strain, difficulty getting up, difficulty breastfeeding, and discomfort due to steam were significantly associated with the presence of vulvodynia.

Discussion

Production of a sitting aid to relieve postpartum vulvodynia

This study has been instrumental in developing a new sitting aid designed to alleviate vulvodynia in postpartum women, particularly addressing issues arising from prolonged sitting during breastfeeding. For many years, prolonged pressure on the vulva when postpartum women assume the sitting position for breastfeeding has increased the pain of the episiotomy wound (*19*). In this study, a new sitting aid was developed to solve this problem (*2*). Although no detailed studies have been conducted, many conventional sitting aids pull on the skin around the episiotomy wound, causing the wound to become congested (*19*). In addition, many of them were difficult to wash or caused steaminess after prolonged sitting. This research has allowed us to develop a sit-to-stand device using selected materials and designs that can solve many of these problems. In the field of obstetrics and midwifery, there have been many years of research on delivery assistance methods that prevent the occurrence of serious perineal laceration (*22*) and episiotomy methods that prevent subsequent perineal laceration (*23-25*). However, these methods cannot completely eliminate the occurrence of perineal laceration or episiotomy-related discomfort (*7,8*). Therefore, during the episiotomy procedure and when perineal laceration occurs, it is necessary to be aware of the postpartum woman's quality of life and methods to alleviate vulvar pain. There have been reports on suture methods that reduce postpartum vulvar pain (*26*). The limitation in the use of drugs during the postpartum period, particularly due to breastfeeding, necessitates non-pharmacological approaches for pain management (*27*). The development of this new sitting aid is a significant advancement in this regard. It represents a noninvasive, drug-free approach for alleviating vulvar pain in postpartum women, aligning with the need for safe and effective pain relief methods during lactation.

Table 3. Results of U-shaped seat compared to urethane seat

respondent	Vulvar pain when seated *n* (%)	Discomfort due to steaminess *n* (%)
None	119 (63.0)	63 (33.3)
Mild	48 (25.4)	82 (43.4)
Moderate	19 (10.1)	43 (22.8)
Severe	3 (1.6)	1 (0.5)

Ease of cleaning and disinfection

A critical aspect of postpartum care involves maintaining hygiene, particularly with tools and aids. The "U-shaped CALFIBER®", developed in this study, underwent rigorous cleaning and disinfection tests, proving its resilience and suitability for clinical settings. These tests have demonstrated that the "U-shaped CALFIBER®" can be easily sterilized and used as an aid for postpartum bleeding. Because of the excretion of lochia after childbirth, sitting aids often become soiled. It is important that they can be easily washed and sterilized to maintain a hygienic environment, and also reduce the workload for clinical staff, making the "U-shaped CALFIBER®" a beneficial addition to postpartum care practices.

Influence of less pressure on the seat surface on pain relief of the episiotomy wound

The results of the pressure distribution measurement test showed that pressure on the seat surface was reduced for women weighing 45 kg and 55 kg. Based on these results, we believe that the product will be less effective for women weighing more than this. Overall, the results highlights that the effectiveness of the seat types in terms of pressure distribution varies depending on the body weight of the user, with the U-shaped seat being more beneficial for lighter individuals and the urethane seat performing better for heavier individuals. Therefore, it is necessary to reconsider the density of the material

Table 4. Association between vulvodynia and other factors during seating

Variable	Sense of strain felt in the lower back	Difficulty breastfeeding	Difficulty stand up	Discomfort due to steaminess
Vulvodynia when seated				
Rs	0.308**	0.364**	0.426**	0.266**
p	< 0.001	< 0.001	< 0.001	< 0.001

Spearman rank correlation analysis. **Correlation is significant at the 0.01 level (2-tailed).

(CALFIBER®) and the thickness of the SEAT in order to meet the needs of postpartum women of various weights in the future.

Postpartum women's needs for sitting aids as indicated by the opinions of trial users

The subjects who used the seating aids developed in this study and participated in the self-administered questionnaire survey can be considered to be an average population of the present day in Japan, based on their backgrounds and an overview of their deliveries. Compared to conventional rubber or urethane seats, the U-shaped seat caused less vulvar pain and discomfort due to steam when seated, and many respondents found it easier to change positions from a sitting to standing position. These results suggest that the moderate elasticity, resilience, and air permeability of CALFIBER® had a positive effect on the sitting posture of postpartum women.

Many of the women who reported having vulvodynia even after using the U-shaped seat also had other problems (back strain, difficulty breastfeeding, difficulty standing up) at the same time. For women with many physical problems, sitting aids alone may not be sufficient to provide vulvodynia relief. For such women, the use of medications that have less impact on lactation and postpartum care of the pelvic floor muscles may be helpful (*28*-*30*). In addition to midwives and obstetricians, it may be more effective to involve multiple professions with such individuals.

Limitations and future study

There are two limitations to this study: first, we were unable to confirm the durability of the product with continuous use, and second, we cannot completely rule out reporting bias using the self-administered questionnaire method. Now, the authors would like to make some recommendations for future research. To address these two limitations, the researchers should conduct observations of the productions over time (and also conduct a seated repetitive tapping test) and interview the users.

Conclusion

Through the four phases of this study, a sitting aid that relieves vulvodynia in postpartum women was created using 3D reticulated polypropylene fiber as the primary material. Sitting aids made of materials with moderate elasticity, stretchability, and breathability are effective in relieving postpartum vulvodynia. However, there is room for future improvement in the density and thickness of the material. In addition, women with multiple physical problems, including vulvodynia, need to be treated individually.

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Crisis management for the future: Building a platform to provide information on emerging and re-emerging infectious diseases from normal times in Japan

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Abstract: At the beginning of the mpox (disease caused by monkey pox) epidemic, there was no platform in Japan to provide appropriate information on emerging and re-emerging infectious diseases (EIDs), and the number of accesses to bioterrorism-related information sites increased rapidly. Even though the interest in mpox was much smaller than in coronavirus infectious disease, emerged in late 2019 (COVID-19), the increase in the number of views were much greater than during the COVID-19 epidemic. This may not be because mpox is bioterrorism-related as an analog of smallpox, but rather because there were no other websites providing information on mpox. For future crisis management, there should be a platform to provide information on possible epidemics of EIDs from normal times in Japan.

***Keywords*:** mpox, COVID-19, social media platforms, EIDs

Introduction

In the recent emerging and re-emerging infectious diseases (EIDs), such as coronavirus infectious disease, emerged in late 2019 (COVID-19) and mpox (disease caused by the monkeypox virus), once an epidemic started, many information web sites have been created. These include a variety of qualities from official government websites to personal blogs; there was a significant problem with social networking sites and websites that provide false information about COVID-19 and other health-related issues like mpox (*1*). The spread of misinformation and disinformation on these platforms can have serious consequences for public health and safety (*2*). On the other hand, however, unless it is a re-emerging infection caused by an already well-known disease, there are often no websites that provide correct information in normal times. This report takes an example of a bioterrorism-related information website and examined the need to provide the information on EIDs in normal times.

Bioterrorism Information Website in Japan

"baiotero-taio-homupeji" (a website providing information on bio-terrorism in Japanese), which was developed in 2008 to provide information on clinical diagnosis and testing procedures for bioterrorism-related diseases for medical institutions (*3*), and was opened to the public in 2016 in anticipation of international situations and mass gathering events in Japan (*3,4*). The database lists diseases that are expected to be used for bioterrorism, and related diseases that require identification and differentiation, and provides details of the diseases, definitive diagnostic methods, and treatment. The major bioterrorism-related diseases include anthrax, which has been used in bioterrorism, smallpox, which has a high fatality rate when used, viral hemorrhagic fever, rabies, and other 11 important diseases. And this list also includes 23 related diseases such as dengue fever, typhus rash, and relapsing fever. Most of the diseases listed are currently no cases of infection in Japan and difficult to diagnose or to differentiate from other fatal diseases.

Number of visits to bioterrorism information websites

The number of accesses to our website from 2019 to July 2023 in Figure 1. We found a rapid increase in the number of accesses after January 2020, when a case of

COVID-19 was confirmed in Japan. We did not provide information on COVID-19 on our website because it had already been more than a month since the case in Wuhan, China, and COVID-19 is not a bioterrorism-related disease. However, since information was provided on SARS as a disease that must be differentiated from bioterrorism, it can be assumed that the keywords used in the search led to visit to this website. The number of accesses to this site in May 2022, when mpox was prevalent in Europe and the United States, shows a sharp increase in the number of accesses to this site, which is much higher than the number of accesses at the start of the COVID-19 epidemic. Figure 2 shows the number of daily accesses in May 2022; the number of accesses increased rapidly on May 19, reaching the largest number of daily accesses ever. This was the day that Web news sites and TV news programs reported the confirmation of several domestic cases of mpox in the United States and the United Kingdom, with returnees from Nigeria as the index case. Despite the fact that mpox had a much smaller risk population and less interest in the general population than COVID-19, these results were achieved not only because of the increased sensitivity of the general population to information about media coverage of EIDs since the COVID-19 outbreak, but also there were no other informational websites about mpox. In fact, the website visits quickly declined and did not increase after 2023, when domestic cases began to increase in Japan (Figure 1). This is not only because social interest became smaller, but also because

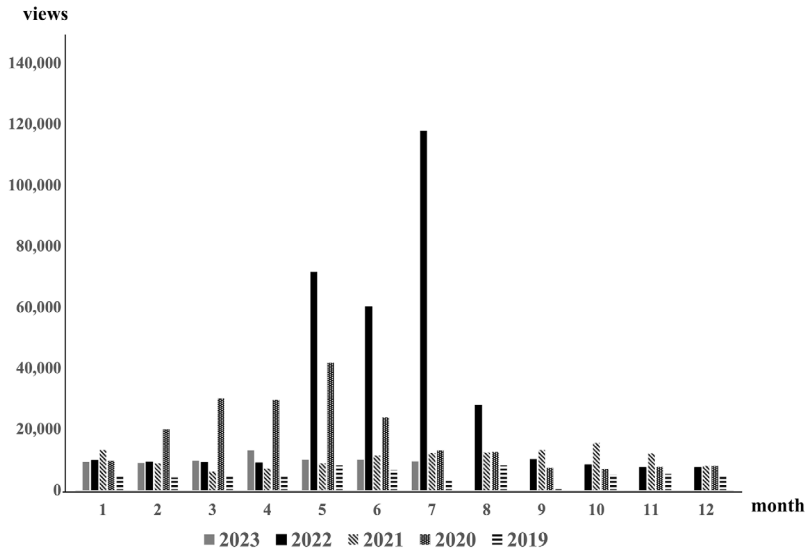

Figure 1. Bioterrorism-related information websites views from 2019 to July 2023. Data compiled through July 2023, no data after August, 2023.

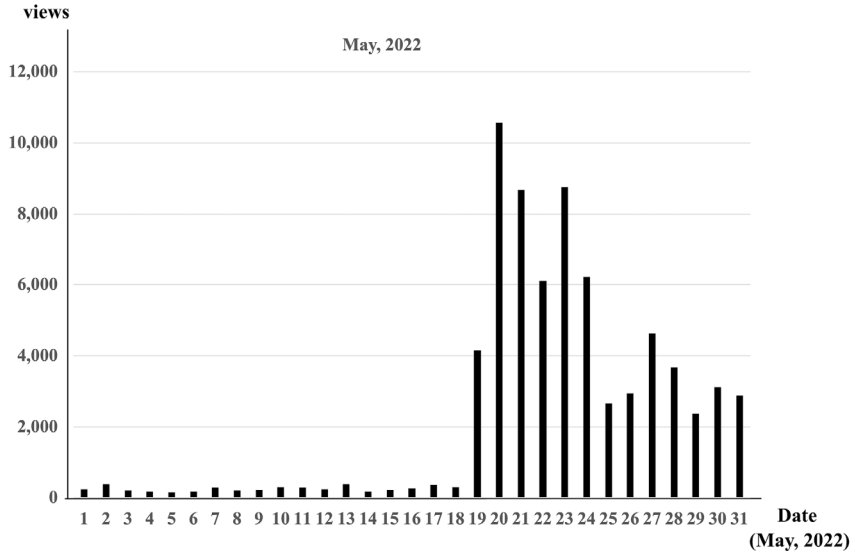

Figure 2. Number of views to bioterrorism-related informational websites per day in May 2022.

other websites were available around this time to convey information about mpox (*5,6*).

Is information on emerging and reemerging infectious diseases necessary even in normal times?

Having a dedicated information site that provides up-to-date and accurate information on EIDs helps to enhance preparedness efforts. When public health authorities and the general public are well-informed about potential threats, they can take proactive measures to prevent or mitigate the spread of diseases. EIDs may not always be at the forefront of public attention during normal times. Our website is only intended to provide information on bioterrorism and not to provide information on EIDs. It was not intended to be commercial in any way, nor was it intended to increase the number of views, so we were able to continue to provide information even in normal times. Under such circumstances, an actual and unexpected infectious disease outbreak occurred, making us aware of the need for a place to provide information in normal times.

An information site serves as a platform to raise awareness about EIDs can educate people about the signs and symptoms of diseases, modes of transmission, and prevention strategies. It becomes a dependable resource for healthcare professionals, researchers, policymakers, and the public. An information site can facilitate collaboration and coordination among different stakeholders, including healthcare organizations, government agencies, non-governmental organizations, and international partners. A dedicated information site can help combat misinformation and rumors, preventing the spread of panic and fear during infectious disease outbreaks. The site can serve as a hub for collecting and analyzing data related to infectious diseases. This data can be crucial for understanding trends, identifying risk factors, and designing evidence-based interventions. An information site can also showcase ongoing research and innovations in the field of infectious diseases. It can encourage researchers to share their findings, leading to advancements in diagnostics, treatments, and vaccines. Overall, an information site for emerging and re-emerging infectious diseases is a valuable tool for public health preparedness, response, and education, not only during crises but also during more stable periods. It contributes to building resilient healthcare systems and reducing the impact of future outbreaks.

The information sites for EIDs are necessary not only during times of crisis but also in normal times. Recently, Social media platforms like Twitter (named "X" from July 2023) have provided important insight into the public's perceptions of global outbreaks like mpox (*7*). Social media platforms also have many minority users such as LGBTQ+ (an abreviation for lesbian, gay, bisexual, transgender, queer, and/or questioning), and communities have formed. Therefore, like mpox, it can be an excellent tool with respect to EIDs that are prevalent in limited populations such as men who have sex with men (MSM) in each region, rather than being prevalent in different geographic parts of the world (*8*). However, it is well known that misinformation and disinformation about mpox is often spread on Twitter (named "X" from July 2023), and there are many unresolved issues.

Our bioterrorism information website may have played a certain role in providing information immediately after the mpox epidemic this time. However, this is not the original purpose of our website. In 2023, cases of presumed domestic infection began to occur, although new findings on mpox have also been reported from domestic cases *(9-12)*, it is difficult to continuously update necessary information other than bioterrorism after an epidemic. For future crisis management, there should be a platform to provide information on possible epidemics of EIDs from normal times in Japan.

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Proposal to apply a "Positive Emotion, Engagement, Relationships, Meaning, and Accomplishment (PERMA)" based approach to manage the COVID-19-related mental health problems in the era of long COVID

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Abstract: Long COVID (LC)-related health problems are highly concerned. Many patients seem to have "recovered" from an acute SARS-CoV-2 infection, however, they might experience various symptoms, almost involving all organs and systems. Of those, neuropsychiatric symptoms like depression, anxiety, and post-traumatic stress disorder (PTSD) are not rare. These problems significantly impact the quality of life (QOL) of patients, family, and caregivers, even lead a tragic suicide outcome. Other than the conventional psychological and medical approaches, here, we proposal a positive emotion, engagement, relationships, meaning, and accomplishment (PERMA)-based approach to fight against these COVID-19-related mental health problems (CRMHPs). This approach is characterized by positive psychological interventions and self-achievements, which has been proved to be a powerful tool against mood disorders in common people. Nowadays, abolishment of certain prophylactic measures (such as isolation, lockdown, compulsorily wearing a mask and maintaining social distance, measures to avoid crowding) enables us to have more opportunities to contact patients and implement the PERMA-based approach to the patients with CRMHPs. We believe that application of PERMA-based approach is conducive to alleviate the influence of the CRMHPs and improve their QOL.

Keywords: positive psychological intervention, PERMA model, long COVID, COVID-19-related mental health problems

At present, long COVID (LC)-related health problems are highly concerned since it has been a marked clinical problem in the routine clinical practice. Although the definition of LC remains in vague, it is commonly used to include the post-acute COVID-19-related sequelae (*1*). Ballering *et al*. estimated that approximately 65 million people worldwide are suffering from LC (*2*). Many patients seem to have "recovered" from an acute SARS-CoV-2 infection, however, they might experience various symptoms, almost involving all organs and systems of the whole body, certainly the neurological system is involved (*1*), of those, neuropsychiatric symptoms including depression, anxiety, and post-traumatic stress disorder (PTSD) are not rare (*1*). The causes of COVID-19-related mental health problems (CRMHPs) are quite complex. Overall, they can be classified into biological factors (brain injuries due to direct viral infection, related immunological/inflammatory reactions, and the systemic damage of the other organs, *etc*.) and social factors (worries due to illness *per se*, prophylactic measures like isolation or lockdown, unemployment, deterioration of the economic, *etc*.). Although the acute COVID-related respiratory/systemic symptoms were controlled, patents are commonly suffering from long-term mood disorders. Herrman *et al*. reported that patients infected with SARS-CoV-2 have double risk of development of mood disorders (*3*), about 30–40% of patients are estimated to have CRMHPs (*4*), *vs.* only 10–35% of those with non-COVID diseases (*5,6*).

CRMHPs may influence the daily life of patients. Severe depression and/or anxiety state may markedly reduce the quality of life (QOL) of patients *per se*, family members, even the caregivers. If leave CRMHPs untreated, the situation might become worse, even induce the final tragic outcome, namely suicide. A study reported that the suicide rates were increased in 2021, which were

believed to be associated with the COVID-19 pandemic. Accordantly, intervention and control of CRMHPs have been regarded as important tasks, for both the medical and social workers.

There are no specific therapies for treating the mood disorders. Globally, a battery of strategies were proposed for intervention of CRMHOs in the context of LC, for example: health at home campaign recommended by world health organization, computerized cognitive behavioral therapy (*7*) and mindfulness-based online intervention (*8*) in China, online multimedia psychophysiological educational interventions in Iran (*9*), and Bhramari pranayama intervention (*10*) and Yoga (*11*) in India, of those, psychological counseling services play a crucial role. However, such services have limitations. Main limitations lie in most of interventions are focusing on recording and measuring frequency of CRMHPs as well as the changes of scores in the behavioral assessments. Ignorance of the patients' actual psychological condition as well as lack of interaction/communication with the patients might be a stumbling block for such services reaching a satisfactory efficacy.

Based on these contexts, here, we proposal a positive emotion, engagement, relationships, meaning, and accomplishment (PERMA) approach for intervention of CRMHPs, which is implementing in our institutes. As shown as in Figure 1, when a patient potentially having CRMHPs steps into a hospital, a medical staff will evaluate his/her mental state with several mental assessment tools. When the CRMHPs are confirmed, the positive psychological intervention (PPI) will be initiated. The PPI including five domains (please read the following statements), and the medical staff will evaluate the improvement of the mental state termly (Figure 1). Although the appropriate parameters and follow-up timing are still under investigation, in light of the reported ameliorations in other diseases (*12-17*), the efficacy of this PERMA approach on CRMHPs is therefore highly anticipated.

A PERMA model falls into the field of positive psychology (*12*) developed by Martin Seligman (*18*). According to Seligman's theory, flourishes of human well-being should include these five domains, namely positive emotion, better engagement, relationships, meaning, and accomplishment (*19*). This model focuses not only the medical issues, but also the interpersonal relationship, interaction and communications, supports from the surroundings, and personal value and achievement. It is in line with the spirits of bio-psycho-social medical model. As a PPI (*vs*. conventional symptom-based treatments), this PERMA model may continuously and actively "give" good emotion to the patients. It attempts to help the patients to construct a good interpersonal relationship. Moreover, this approach is also good for finding/correcting their negative emotions. Hence, it is not surprising that the PERMA-based PPI has good efficacy in terms of treating the mood disorders. Indeed, numerous previous studies have documented that application of the PERMA approach achieved improvement of the psychological state as well as QOL in patients with various diseases, such as depression (*13*), stroke (*14*), acute liver failure (*12*), lung cancer chemotherapy (*15*), breast cancer (*16*), and in patients undergoing hemodialysis (*17*). In terms of the COVID-19 pandemic, Sánchez-Hernández *et al*. documented the importance of PPI and self-care to maintain good psychological state in the context of COVID-19 pandemic (*20*). Other than the patients, a Korean report confirmed the efficacy of the PERMA-based PPI in improvement of the well-being and sleep quality in the South Korean emergency workers who were struggling with depression and sleep quality

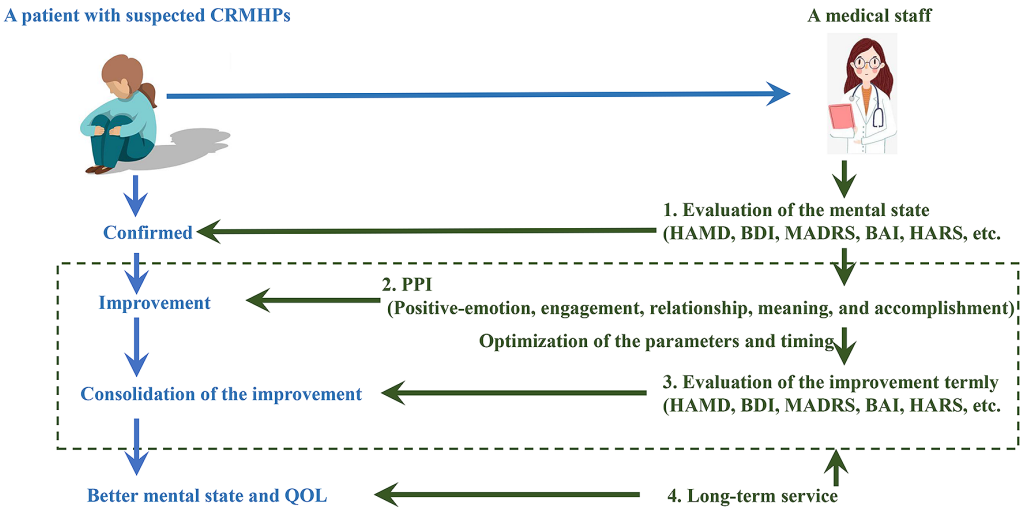

Figure 1. A diagram of the processes of the PERMA-based approach. BAI: Beck anxiety inventory; BDI: Beck depression inventory; CRMHPS: COVID-19-related mental health problems; HARS: Hamilton anxiety rating scale; HAMD: Hamilton depression scale (HAMD); MADRS: Montgomery-Asberg depression rating scale; PPI: positive psychological intervention; QOL: quality of life.

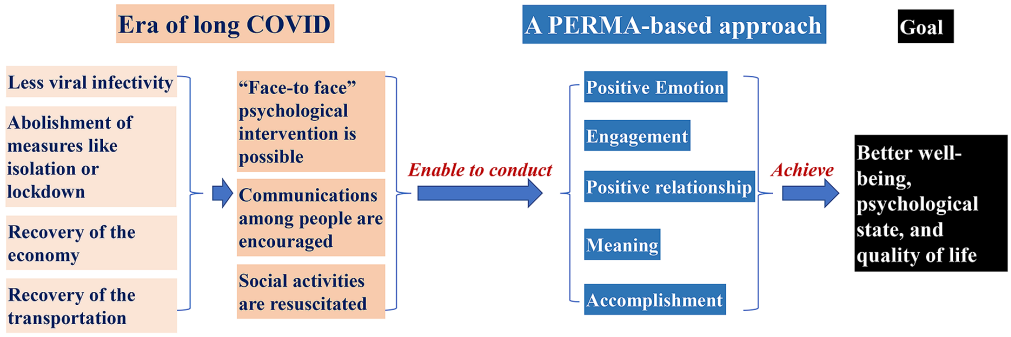

Figure 2. Application of the PERMA-based approach to manage the COVID-19-related mental health problems in the era of long COVID. PERMA: Positive Emotion, Engagement, Relationships, Meaning, and Accomplishment.

during the COVID-19 pandemic (*21*). In addition, the PERMA model was used to evaluate/ intervention the well-being in various populations affected by the COVID-19 pandemic, such as language teachers (*22*), mid-career musicians (*23*), university students (*24*), patients with acquired brain injury (*25*). However, due to the communicable nature of COVID-19, investigations directly involving COVID-19 patients were limited. Alternatively, the uncertain, long-term, less-communicable (might be) natures of LC, enable the clinicians using this PERMA model to conduct investigation/intervention of the psychological state of patients with LC.

In terms of application the PERMA model in LC, these five elements should be seriously implemented: *i*) Positive Emotion: As a fundamental intervention tool in the PERMA model, actively online multimedia psychoeducational interventions had been proved to be useful in relief of the stress of COVID-19 patients (*9*). For LC patients, performing a face-to-face intervention might have a better efficacy. Moreover, observing the psychological state of patients outside of clinic, and performing a timely intervention became possible. *ii*) Engagement: Government should be aroused to emphasize the CRMHPs in the context of LC. More facilities for psychological service should be set up and more public participations should be proposed. Patients with CRMHPs are encouraged to attend more social activities, and set up a certain specific of life goal. *iii*) Positive relationship: Healthy education should be implemented to the public to establishment of the proper knowledge, attitude, and practice (KAP) to LC. Family members and the public should be educated to eliminate discrimination and prejudice to LC patients. Meanwhile, the patients are also encouraged to attempt to make good relationship with all related people. *iv*) Meaning: As per previous studies, the theme of "find meaning" was conducive to the patients to alleviation of stress (*9*) and improvement of psychological state (*26*). Thus, helping the patients to find proper life meaning, and correct the "biased" life meaning should be a fundamental work of the psychological workers. *v*) Accomplishment: Indeed, we should help the patients to setup a serial of "accomplishments" during the daily life. Any tiny progress in terms of fighting against the illness, along with the other achievements should be inspired and praised. Finding their self-value and reconstructing the self-confidence might be the soul of "accomplishment".

Taken together, the CRMHPs remain remarkable complaints in patients with LC. Rather than the situation in COVID-19 pandemic, measures like isolation, lockdown, compulsorily wearing a mask and maintaining social distance, and avoid crowding are abolished, making face-to-face counsel and intervention became available. The medical staffs have more opportunities to directly contact patients. Meanwhile, patients have more chances to communicate with their doctors, caregivers, family members, psychological works, *etc*. This is helpful for the patients suffering from CRMHPs to obtain PPI, such as the PERMA-based approach, to construct better interpersonal relationship, to find his/her life meaning, and to achieve the self-value, and ultimately achieve better well-being, psychological state and QOL. All of these must be helpful to ameliorate the CRMHPs in LC (Figure 2). In this regard, the PERMA-based approach is highly recommended in the era of LC.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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