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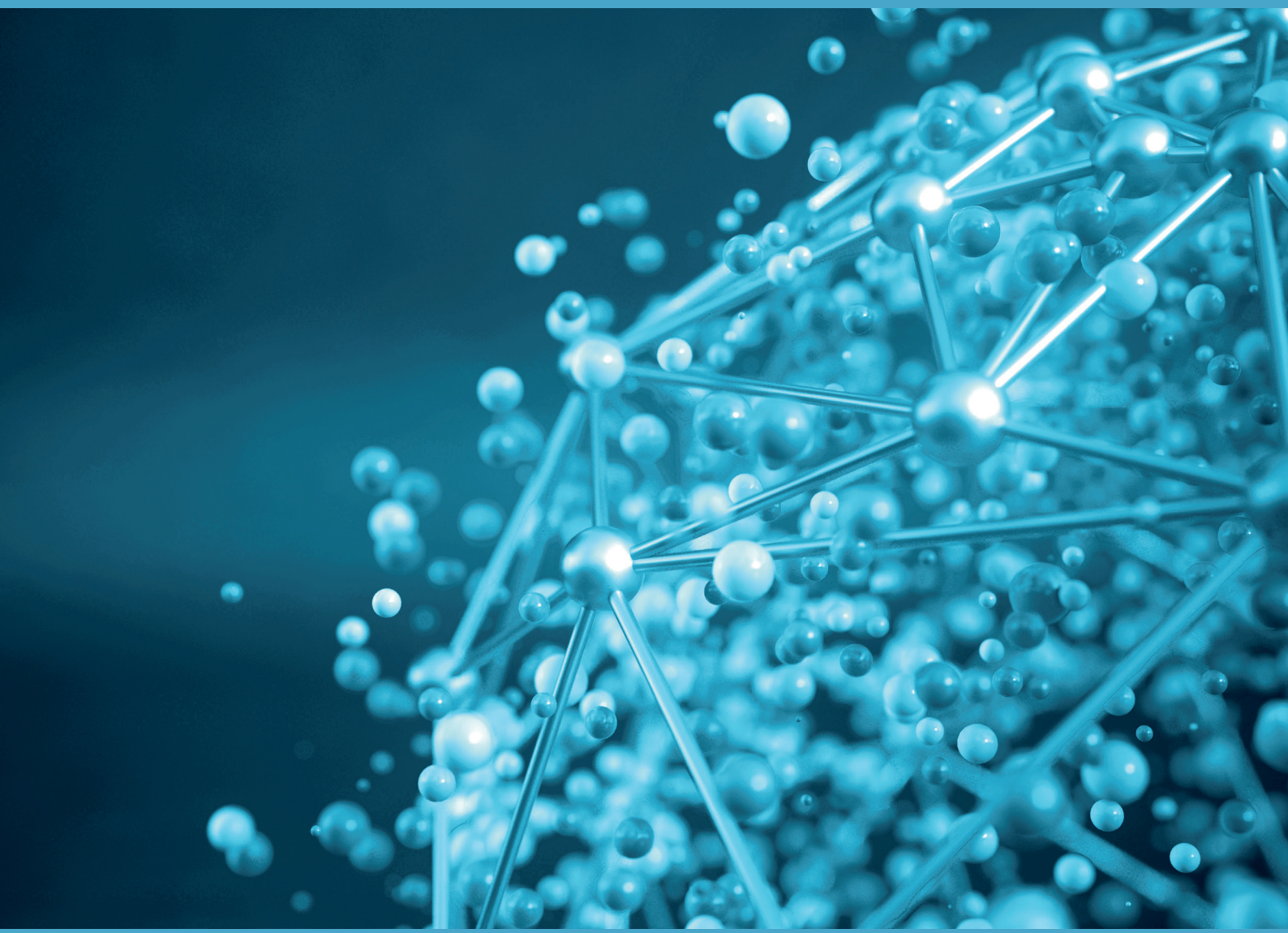

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JCB | GENERAL INFORMATION

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ORIGINAL RESEARCH ARTICLE

Impact of clinico-biochemical variations on the etiopathogenesis of cataract: a case-control study

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ABSTRACT

Purpose: Cataract is a major cause of blindness worldwide with a greater prevalence in developing countries like India. Owing to speculations about the relationship of various biochemical markers and cataract formation this case-control study was designed with the aim to know the impact of serum blood sugar, serum electrolytes and serum calcium on the etiopathogenesis of cataract in Kashmiri population.

Methods: A total of 300 cases diagnosed with cataract and 360 healthy controls were taken for the study. Serum of all the cases and controls was analyzed for blood sugar and calcium using spectrometric techniques. Sodium and potassium were analyzed using Ion-Selective Electrode technology. All the investigations were done on *ABBOTT c4000* fully automatic clinical chemistry analyzer.

Results: Most of the patients in our study were ≥50 years of age having posterior subcapsular cataract. The mean levels of serum fasting blood sugar (mg/dL), serum sodium (mmol/L), serum potassium (mmol/L) and serum calcium (mg/dL) were 99.4 ± 7.7; 140.4 ± 2.5; 4.2 ± 0.5; and 8.9 ± 0.5, respectively, in cases compared to 107.7 ± 12.3; 142.9 ± 5.0; 3.8 ± 0.5; and 8.3 ± 1.7, respectively, in healthy controls. A significantly higher number of cataract cases had elevated serum glucose and sodium levels, low serum potassium and calcium levels compared to healthy controls.

Conclusions: Hyperglycemia, hypernatremia, hypokalemia and hypocalcemia can independently increase the patients' risk to cataracts. Corrections in these biochemical parameters may reduce cataract incidence.

Keywords: Blood pressure, Blood sugar, Calcium, Cataract, Intraocular pressure, Potassium, Sodium

Introduction

The International Agency for the Prevention of Blindness has defined cataract as the clouding or opacification of the normally clear lens of the eye or its capsule that obscures the passage of light through the lens to the retina (1,2). This disease, which can significantly reduce patient's quality of life, is still one of the main ophthalmic public health conditions in developed and developing countries (3). Cataracts are mainly divided into nuclear cataract (NC), cortical cataract (CC), posterior sub capsular (PSC) cataract, acquired cataract and congenital cataract (4). Senile cataract is one of the common types of acquired cataract which occurs as a consequence of the aging process. It is characterized by initial opacity in the lens with subsequent swelling of lens and final shrinkage with complete loss of transparency (5). Cataract is detected by an eye examination that includes a visual activity test, slit lamp exam (SLE) and dilated eye exam (6).

Worldwide, cataract has caused >50% vision loss including 33.4% blind people and 18.4% people with moderate to severe visual impairment. Globally 10.8 million people were blind and 35.1 million people were visually impaired from cataract in 2010 (7). Additionally, data from the World Health Organization (WHO) has estimated that this number will increase to 40 million in 2025 due to the aging populations with greater life expectancies (8). Up to 50 million people in

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the world suffer from senile cataract (9,10), and its prevalence in developing countries is much more than in developed ones (11). In India, the prevalence of blindness due to cataract was reported to be 8% in the age group of >50 years, as per the National blindness survey (12). An estimated 20 lakh new cases of cataract are being added to the burden every year in this country, showing a steep rise ranging from 0.5% above 30 years to 94.5% above 70 years (13). Cataract accounts for 62.6% of all blindness, affecting 9-12 million bilaterally blind persons (14). The WHO/NPCB (National Programme for Control of Blindness) survey has shown that there are over 22 million blind in India and 80.1% of these are blind due to cataract (15).

Cataract is caused by degeneration and opacification of the lens fibers already formed. Any factor that disturbs the critical intra- and extracellular equilibrium of water and electrolytes or deranges the colloid system within the fibers tends to bring about opacification (16). Several studies have been carried out to elucidate risk factors which are responsible for development of cataract. Extensive research has established age, ion imbalance, altered calcium levels, diabetes and UV light exposure as causative risk factors for cataract, while recent studies have identified other potential risk factors like exogenous estrogen, nutrition, dietary fat and genetics which might play a role in the development of cataract (17). Although cataract affects all age groups its incidence increases with advancing age (18). Senile cataract affects equally persons of either gender, usually above the age of 50 years (19). The association between diabetes and cataract formation has been shown in clinical, epidemiological and basic research studies. Due to increasing numbers of type 1 and type 2 diabetics worldwide, the incidence of diabetic cataracts steadily rises (20). Several clinical studies have shown that cataract development occurs more frequently and at an earlier age in diabetic compared to nondiabetic patients (20). Many studies have shown that serum electrolyte (potassium and sodium) concentration directly affects the concentration of electrolytes in aqueous humor and thereby induces cataract formation. Concentration of sodium in lens is less compared to serum concentrations whereas it is vice versa in case of potassium concentrations, and this cationic balance is maintained by the osmotic pressure and thus water balance by the action of enzyme Na^+/K^+ ATPase. Any imbalance in between the electrolytes leads to cataract formation (21). Calcium is of particular concern in cataract. This cation is essential for various lens fiber cell metabolism processes (22). It has been shown that lens calcium content correlates with opacity in cataractous human lenses (23) and subsequent changes in serum calcium concentration might be an important factor in the development of cataract (24).

Demographic and clinico-biochemical biomarkers have been previously linked with the development of cataract from other parts of the world, but so far only few studies have been reported from the Indian subcontinent regarding the interrelationship of cataract (25,26). Keeping in view the ethnicity and relatively conserved genetic pool of Kashmiri population, this case-control study was designed to elucidate the role of demographic and clinico-biochemical parameters in the etiopathogenesis and severity of cataract in Kashmiri population.

Materials and methods

Ethics

This study was performed in line with the principles of the Declaration of Helsinki. Ethical clearance for the study was sought from Institutional Review Board, Government of Medical College Srinagar vide No. 2022T/ETH/GMC. All the patients included in the study were informed about the study and informed consent both in vernacular as well as in English language was taken before eliciting history and sample collection from study subjects. Standard questionnaire or patient proforma was properly recorded and drafted as per socio-demographic and clinico-biochemical parameters. The authors affirm that human research participants provided informed consent for publication of their details.

Study design

This was a case-control study conducted by the Department of Biochemistry in collaboration with the Department of Ophthalmology, Government Medical College Srinagar and associated SMHS hospital Srinagar, J&K, from March 2020 to March 2022.

Study subjects and sample size

The study was conducted in ethnic population of Kashmir; no restrictions were made among patients with respect to gender and dwelling. A total of 300 patients diagnosed with cataract and 360 healthy controls were enrolled for the study. Keeping the power of study as 80% and allocation ratio of 1.2 and effect size of 0.4, the sample size was calculated by G power 23.0.1 version.

Inclusion and exclusion criteria

"Cases" included all those individuals who were >18 years of age and diagnosed with cataract. "Cases" excluded all those individuals with any other eye ailment and symptomatic disease (liver, kidney, heart or other), trauma, infection, inflammation of eye, cancer or any other genetic abnormality. "Controls" included healthy individuals >18 years of age.

Ophthalmic examination

Each patient was subjected to various ophthalmic measurements. Uncorrected visual acuity was measured with Snellen chart. Refraction was done and the best corrected visual acuity was noted. Intraocular pressure (IOP) measurement was done with non-contact tonometer. Corrected IOP was calculated after measuring central corneal thickness by ultrasonic pachymetry. Detailed anterior segment examination using slit lamp was done to rule glaucoma and associated ocular pathology. Detailed fundus examination under full mydriasis obtained by 0.8% tropicamide and 5% phenylephrine was done with direct ophthalmoscopy, 78D and indirect ophthalmoscopy.

Sample collection

A total of 04 mL of venous blood was collected from each cataract patient and healthy control in a coagulation activating red top vial. Blood was collected from each individual after 8-10 hours of fasting. Blood was immediately centrifuged for separation of serum. Serum was properly stored at –20°C till biochemical analysis was done in the Biochemistry diagnostic laboratory, SMHS hospital Srinagar.

Biochemical analysis for electrolytes

Serum electrolyte levels were estimated on *ABBOTT ARCHITECT c4000* fully automated clinical chemistry analyzer using Ion-Selective Electrode. The reference ranges of serum sodium levels were taken between 135 and 145 mmol/L and serum potassium levels as 3.5-4.5 mmol/L.

Biochemical analysis for calcium

Serum calcium levels were estimated spectrophotometrically on *ABBOTT ARCHITECT c4000* fully automated clinical chemistry analyzer using Arsenazo III dye. The reference ranges of serum calcium were taken as: 8.5-10.2 mg/dL.

Biochemical analysis of glucose

Serum glucose levels were measured spectrophotometrically on ABBOTT ARCHITECT c4000 fully automated clinical chemistry analyzer. Glucose is phosphorylated by hexokinase (HK) in the presence of adenosine triphosphate (ATP) and magnesium ions to produce glucose-6-phosphate (G6P) and adenosine diphosphate (ADP). Glucose-6-phosphate dehydrogenase (G6PDH) specifically oxidizes G6P to 6-phosphogluconate with the concurrent reduction of nicotinamide adenine dinucleotide (NAD) and readily to nicotinamide adenine dinucleotide reduced (NADH). One micromole of NADH is produced for each micromole of glucose consumed. The NADH produced absorbs light at 340 nm and can be detected spectrophotometrically as an increased absorbance. The reference ranges of serum glucose were taken as: 99-100 mg/dL (normal) and >100 mg/dL (impaired).

Statistical analysis

The data was analyzed using IBM Statistical Package for the Social Sciences (SPSS) software v. 25.0. Descriptive statistics was performed and data was presented as frequency (N) and percentage (%). Continuous data was presented as mean and standard deviation. Chi-square test was used to compare proportions between groups as deemed proper by the statistical expert. P value <0.05 was considered statistically significant.

Results

Table I contains the socio-demographic and clinicopathological characteristics of cataract cases and healthy controls. Cases and controls were matched with respect to age and gender; 17.5% (63 of 360) of the controls were <50 years of age as compared to 21% (63 of 300) of cases who were <50 years of age. With respect to gender, 47.5% (171 of 360) controls were men as compared to 52.0% (156 of 300) of men having cataract. Accordingly, 90.0% (324 of 360) of the controls were inhabitants of rural areas as compared to 80% (240 of 300) of cases who belonged to rural areas. The cataract patients were evaluated for various clinical parameters. Hypertension was present in 13.0% (39 of 300) of cataract patients. The family history of cataract was present in 4.0% (12 of 300) of cases. The history of any other eye disorder was present in 23.0% (69 of 300) of cataract cases. Further the IOP of all the patients was measured wherein 91.0% (273 of 300) had normal IOP while 9.0% (27 of 300) had higher IOP.

TABLE I - Socio-demographic and clinicopathological characteristics of cases having cataract and controls included in the study

–	Controls N = 360 (%)	Cases N = 300 (%)	χ^2	p value
Age group				
<50 years	63 (17.5)	63 (21.0)	1.2	0.15
≥50 years	297 (82.5)	237 (79.0)		
Gender				
Men	171 (47.5)	156 (52.0)	1.3	0.15
Women	189 (52.5)	144 (48.0)		
Dwelling				
Rural	324 (90.0)	240 (80.0)	13.1	<0.0001
Urban	36 (10.0)	60 (20.0)		
Hypertension				
No	–	261 (87.0)	–	–
Yes		39 (13.0)		
Family history of cataract				
No	–	288 (96.0)	–	–
Yes		12 (4.0)		
H/o any other eye disorder				
No	–	231 (77.0)	–	–
Yes		69 (23.0)		
Eyes affected				
One	–	177 (59.0)	–	–
Both		123 (41.0)		
Type of cataract				
Nuclear		00 (0.0)		
Cortical	–	75 (25.0)	–	–
Posterior subcapsular		225 (75.0)		
Grade				
I and II	–	165 (55.0)	–	–
III and IV		135 (45.0)		
IOP				
Normal	–	273 (91.0)	–	–
High		27 (9.0)		

H/o = history of; IOP = intraocular pressure.

Table II depicts the biochemical parameters of cataract cases and healthy controls; 69.0% (207 of 300) of cases had impaired blood sugar level as compared to 22.5% (81 of 360) of controls having impaired blood sugar, and the association was found to be statistically significant (p<0.0001); 36.3% (109 of 300) of cases had hypernatremia as compared to only 3.0% (11 of 360) of controls with hypernatremia (p<0.0001). Cataract cases had significantly low potassium levels compared to controls (17.5% vs. 6.1%; p<0.0001). In addition, 38.0% (114 of 300) of cases had hypocalcemia as compared to only 12.5% (45 of 360) of controls with hypocalcemia and the difference was statistically significant (p<0.0001).

TABLE II - Biochemical parameters of cataract cases and healthy controls

Parameters	Controls N = 360 (%)	Cases N = 300 (%)	OR (95% CI)	p value
Fasting blood sugar				
Normal	279 (77.5)	93 (31.0)	7.6 (5.4-10.8)	<0.0001
Impaired	81 (22.5)	207 (69.0)		
Sodium levels				
Normal	349 (97.0)	191 (63.7)	18.1 (9.5-34.4)	<0.0001
High	11 (3.0)	109 (36.3)		
Potassium levels				
Normal	338 (93.9)	249 (83.0)	3.1 (1.9-5.3)	<0.0001
Low	22 (6.1)	51 (17.0)		
Calcium levels				
Normal	315 (87.5)	186 (62.0)	4.2 (2.9-6.3)	<0.0001
Low	45 (12.5)	114 (38.0)		

CI = confidence interval; OR = odds ratio.

Table III shows the mean levels of various biochemical parameters in cases and healthy controls. A statistically significant difference was observed between cases and controls with respect to mean fasting blood sugar levels in mg/dL (107.7 ± 12.3 vs. 99.4 ± 7.7); mean sodium levels in mmol/L (142.9 ± 5.0 vs. 140.4 ± 2.5); mean potassium level in mmol/L (3.8 ± 0.5 vs. 4.2 ± 0.5); mean calcium level in mg/dL (8.3 ± 1.7 vs. 8.9 ± 0.5).

TABLE III - Levels (mean ± SD) of various biochemical parameters in cataract cases and healthy controls

Parameters	Controls (mean ± SD)	Cases (mean ± SD)	p value
Fasting blood sugar (mg/dL)	99.4 ± 7.7	107.7 ± 12.3	<0.0001
Sodium levels (mmol/L)	140.4 ± 2.5	142.9 ± 5.0	<0.0001
Potassium levels (mmol/L)	4.2 ± 0.5	3.8 ± 0.5	<0.0001
Calcium levels (mg/dL)	8.9 ± 0.5	8.3 ± 1.7	<0.0001

SD = standard deviation.

Table IV shows the association of fasting blood sugar levels with various socio-demographic, clinico-pathological and biochemical parameters of cataract patients and controls. In each subgroup of age, gender, dwelling, sodium, potassium and calcium levels, a statistically significant difference in serum blood levels was noted between cases and controls (p<0.0001). Among patients with PSC cataract, 72.0% (162 of 225) had impaired blood sugar level as compared to CC wherein only 60% (45 of 75) had impaired blood sugar, and the difference was statistically significant (p = 0.05). No association was found between blood sugar levels and any other parameter of cases and controls.

Table V describes the association of serum sodium levels with various socio-demographic, biochemical and clinicopathological parameters of cataract cases and controls. A statistically significant difference in serum sodium levels was noted between cases and controls in each subgroup of age, gender, dwelling, potassium and calcium levels (p<0.0001). Among cataract patients with grade I and II disease, 41.2% (68 of 165) had high sodium levels while among cataract patients with grade III and IV disease, only 30.4% (41 of 135) had high sodium levels and the difference was statistically significant (p = 0.05). We did not observe any other parameter influencing the sodium levels in cataract patients.

Table VI displays the association of serum potassium levels with various socio-demographic, clinicopathological and biochemical parameters of cataract patients and controls. Among cases, 20% (48 of 240) of rural inhabitants had low potassium levels as compared to 6.5% (21 of 324) of rural control subjects having low potassium levels (p<0.0001). With respect to calcium level, 31.6% (36 of 114) of hypocalcemia cases had hypokalemia as compared to only 2.2% (01 of 45) of hypocalcemia controls having hypokalemia, and the difference was statistically significant (p<0.0001). Among hypertensive cases, 15.4% (18 of 39) had hypokalemia but among normotensive cases only 12.6% (21 of 261) had hypokalemia, and the difference was statistically significant (p<0.0001). Importantly, 33.3% (09 of 27) cases having high IOP were hypokalemic but among cases having normal IOP, only 15.4% (42 of 273) had hypokalemia, and the difference was statistically significant (p<0.0001). No association was found between serum potassium levels and any other parameter of cases and controls.

Table VII depicts the association of serum calcium levels with various socio-demographic, clinicopathological and biochemical parameters of cataract cases and controls. A statistically significant difference in calcium levels was noted between cases and controls in each subgroup of age, gender and dwelling (p<0.0001). We did not observe any other parameter influencing the calcium levels in cataract patients.

Discussion

We evaluated cataract patients with respect to various socio-demographic, clinicopathological and biochemical characteristics. As aging itself is a major risk factor for the development of cataract in both women and men (27), most of the cataract patients were from the age group of ≥50 years. It has been suggested that with aging the alteration

TABLE IV - Association of fasting serum glucose levels with various socio-demographic, biochemical and clinicopathological parameters of cataract cases and controls

Socio-demographic/ clinicopathological/ biochemical parameters	Fasting serum glucose levels in controls			Fasting serum glucose levels in cases			OR (95% CI)	p value
	N = 360 (%)	Normal 279 (77.5)	Impaired 81 (22.5)	N = 300 (%)	Normal 93 (31.0)	Impaired 207 (69.0)	7.6 (5.4-10.8)	<0.0001
Age group								
<50 years	63 (17.5)	57 (90.5)	06 (9.5)	63 (21.0)	17 (27.0)	46 (73.0)	25.7 (9.3-70.4)	<0.0001
≥50 years	297 (82.5)	222 (74.7)	75 (25.3)	237 (79.0)	76 (32.1)	161 (67.9)	6.2 (4.2-9.1)	<0.0001
Gender								
Men	171 (47.5)	141 (82.5)	30 (17.5)	156 (52.0)	48 (30.8)	108 (69.2)	10.5 (6.2-17.7)	<0.0001
Women	189 (52.5)	138 (73.0)	51 (27.0)	144 (48.0)	45 (31.3)	99 (68.8)	5.9 (3.6-9.5)	<0.0001
Dwelling								
Rural	324 (90.0)	255 (78.7)	69 (21.3)	240 (80.0)	75 (31.3)	165 (68.8)	8.1 (5.5-11.9)	<0.0001
Urban	36 (10.0)	24 (66.7)	12 (33.3)	60 (20.0)	18 (30.0)	45 (70.0)	4.6 (1.9-11.3)	<0.001
Sodium levels								
Normal	349 (97.0)	270 (77.4)	79 (22.6)	191 (63.7)	58 (30.4)	133 (69.6)	7.8 (5.2-11.6)	<0.0001
Elevated	11 (3.0)	09 (81.8)	02 (18.2)	109 (36.3)	35 932.1)	74 (67.9)	9.5 (1.9-46.3)	0.002
Potassium levels								
Normal	338 (93.9)	261 (77.2)	77 (22.8)	249 (83.0)	84 (33.7)	165 (66.3)	6.6 (4.6-9.5)	<0.0001
Low	22 (6.1)	18 (81.8)	04 (18.2)	51 (17.0)	09 (17.6)	42 (82.4)	21 (5.7-77.1)	<0.0001
Calcium levels								
Normal	315 (87.5)	246 (78.1)	69 (21.9)	186 (62.0)	60 (32.3)	126 (67.7)	7.4 (4.9-11.2)	<0.0001
Low	45 (12.5)	33 (73.3)	12 (26.7)	114 (38.0)	33 (28.9)	81 (71.1)	6.7 (3.1-14.6)	<0.0001
Hypertension								
No	–	–	–	261 (87.0)	78 (29.9)	183 (70.1)		0.3
Yes				39 (13.0)	15 (38.5)	24 (61.5)	0.6 (0.3-1.3)	
Family history of cataract								
No	–	–	–	288 (96.0)	84 (29.2)	204 (70.8)		0.002
Yes				12 (4.0)	009 (75.0)	03 (25.0)	0.13 (0.03-0.5)	
H/o any other eye disorder								
No	–	–	–	231 (77.0)	72 (31.2)	159 (68.8)		1.000
Yes				69 (23.0)	21 (30.4)	48 (69.6)	1.03 (0.5-1.8)	
Eyes affected								
One	–	–	–	177 (59.0)	57 (32.2)	120 (67.8)		0.6
Both				123 (41.0)	36 (29.3)	87 (70.7)	1.1 (0.6-1.8)	
Type of cataract								
Cortical	–	–	–	75 (25.0)	30 (40.0)	45 (60.0)		0.05
Posterior subcapsular				225 (75.0)	63 (28.0)	162 (72.0)	1.7 (0.9-2.9)	
Grade								
I and II	–	–	–	165 (55.0)	45 (27.3)	120 (72.7)		0.1
III and IV				135 (45.0)	48 (35.6)	87 (64.4)	0.6 (0.4-1.1)	
IOP								
Normal	–	–	–	273 (91.0)	81 (29.7)	192 (70.3)		0.12
High				27 (9.0)	12 (44.4)	15 (55.6)	0.5 (0.2-1.1)	

CI = confidence interval; H/o = history of; IOP = intraocular pressure; OR = odds ratio.

TABLE V - Association of serum sodium levels with various socio-demographic, biochemical and clinicopathological parameters of cataract cases and controls

Socio-demographic/ clinicopathological/ biochemical parameters	Serum sodium levels in controls			Serum sodium levels in cases			OR (95% CI)	p value
	N = 360 (%)	Normal 349 (97.0)	High 11 (3.0)	N = 300 (%)	Normal 191 (63.7)	High 109 (36.3)	18.1 (9.5 -34.4)	<0.0001
Age group								
<50 years	63 (17.5)	63 (100.0)	0 (0.0)	63 (21.0)	25 (39.7)	38 (60.3)	–	<0.0001
≥50 years	297 (82.5)	286 (96.3)	11 (3.7)	237 (79.0)	166 (70.0)	71 (30.0)	11.1 (5.7-21.5)	<0.0001
Gender								
Men	171 (47.5)	162 (94.7)	09 (5.3)	156 (52.0)	99 (63.5)	57 (36.5)	10.3 (4.9-21.8)	<0.0001
Women	189 (52.5)	187 (98.9)	02 (1.1)	144 (48.0)	92 (63.9)	52 (36.1)	52.8 (12.5-221.7)	<0.0001
Dwelling								
Rural	324 (90.0)	314 (96.9)	10 (3.1)	240 (80.0)	150 (62.5)	90 (37.5)	18.8 (9.5-37.2)	<0.0001
Urban	36 (10.0)	35 (97.2)	01 (2.8)	60 (20.0)	41 (68.3)	19 (31.7)	16.2 (2.0-127.3)	<0.0001
Potassium levels								
Normal	338 (93.9)	329 (97.3)	09 (2.7)	249 (83.0)	162 (65.1)	87 (34.9)	19.6 (9.6-39.9)	<0.0001
Low	22 (6.1)	20 (90.9)	02 (9.1)	51 (17.0)	29 (56.9)	22 (43.1)	7.1 (3.8-13.3)	<0.0001
Calcium levels								
Normal	315 (87.5)	304 (96.5)	11 (3.5)	186 (62.0)	121 (61.5)	65 (34.9)	14.8 (7.5-29.0)	<0.0001
Low	45 (12.5)	45 (100.0)	00 (0.0)	114 (38.0)	70 (61.4)	44 (38.6)	–	<0.0001
Hypertension								
No	–	–	–	261 (87.0)	165 (63.2)	96 (36.8)		0.7
Yes				39 (13.0)	26 (66.7)	13 (33.3)	0.9 (0.4-1.7)	
Family history of cataract								
No	–	–	–	288 (96.0)	184 (63.9)	104 (36.1)		0.7
Yes				12 (4.0)	07 (58.3)	05 (41.7)	1.2 (0.3-4.0)	
H/o any other eye disorder								
No	–	–	–	231 (77.0)	153 (66.2)	78 (33.8)		0.11
Yes				69 (23.0)	38 (55.1)	31 (44.9)	1.6 (0.9-2.7)	
Eyes affected								
One	–	–	–	177 (59.0)	119 (67.2)	58 (32.8)		0.14
Both				123 (41.0)	72 (58.5)	51 (41.5)	1.4 (0.9-2.3)	
Type of cataract								
Cortical	–	–	–	75 (25.0)	53 (70.6)	22 (29.3)		0.16
Posterior subcapsular				225 (75.0)	138 (61.3)	87 (38.6)	1.5 (0.8-2.6)	
Grade								
I and II	–	–	–	165 (55.0)	97 (58.8)	68 (41.2)		0.052
III and IV				135 (45.0)	94 (69.6)	41 (30.4)	0.6 (0.3-1.0)	
IOP								
Normal	–	–	–	273 (91.0)	175 (64.1)	98 (35.9)		0.6
High				27 (9.0)	16 (59.3)	11 (40.7)	1.2 (0.5-2.7)	

CI = confidence interval; H/o = history of; IOP = intraocular pressure; OR = odds ratio.

TABLE VI - Association of serum potassium levels with various socio-demographic, biochemical and clinicopathological parameters of cataract cases and controls

Socio-demographic/ clinicopathological/ biochemical parameters	Serum potassium levels in controls			Serum potassium levels in cases			OR (95% CI)	p value
	N = 360 (%)	Normal 338 (93.9)	Low 22 (6.1)	N = 300 (%)	Normal 249 (83.0)	Low 51 (17.0)	3.1 (1.9-5.3)	<0.0001
Age group								
<50 years	63 (17.5)	62 (98.4)	01 (1.6)	63 (21.0)	48 (76.2)	15 (23.8)	19.3 (2.4-151.8)	<0.0001
≥50 years	297 (82.5)	276 (92.9)	21 (7.1)	237 (79.0)	201 (84.8)	36 (15.2)	2.3 (1.3-4.1)	<0.0003
Gender								
Men	171 (47.5)	163 (95.3)	08 (4.7)	156 (52.0)	132 (84.6)	24 (15.4)	3.7 (1.6-8.5)	<0.0001
Women	189 (52.5)	175 (92.6)	14 (7.4)	144 (48.0)	117 (7.4)	27 (18.8)	2.8 (1.4-5.7)	<0.0002
Dwelling								
Rural	324 (90.0)	303 (93.5)	21 (6.5)	240 (80.0)	192 (80.0)	48 (20.0)	3.6 (2.0-6.2)	<0.0001
Urban	36 (10.0)	35 (97.2)	01 (2.8)	60 (20.0)	57 (95.0)	03 (5.0)	1.8 (0.1-18.4)	1.000
Calcium levels								
Normal	315 (87.5)	294 (93.3)	21 (6.7)	186 (62.0)	171 (91.9)	15 (8.1)	1.2 (0.6-2.4)	0.6
Low	45 (12.5)	44 (97.8)	01 (2.2)	114 (38.0)	78 (68.4)	36 (31.6)	20.3 (2.6-153.2)	<0.0001
Hypertension								
No	–	–	–	261 (87.0)	228 (87.4)	21 (12.6)		<0.0001
Yes				39 (13.0)	33 (84.6)	18 (15.4)	5.9 (2.8-12.2)	
Family history of cataract								
No	–	–	–	288 (96.0)	240 (83.3)	48 (16.7)		0.4
Yes				12 (4.0)	09 (75.0)	03 (25.0)	1.6 (0.4-6.3)	
H/o any other eye disorder								
No	–	–	–	231 (77.0)	195 (84.4)	36 (15.6)		0.27
Yes				69 (23.0)	54 (78.3)	15 (21.7)	1.5 (0.7-2.9)	
Eyes affected								
One	–	–	–	177 (59.0)	150 (84.7)	27 (15.3)		0.35
Both				123 (41.0)	99 (80.5)	24 (19.5)	1.3 (0.7-2.4)	
Type of cataract								
Nuclear								
Cortical	–	–	–	75 (25.0)	57 (76.0)	18 (24.0)		0.07
Posterior subcapsular				225 (75.0)	192 (85.3)	33 (14.7)	0.5 (0.2-1.0)	
Grade								
I and II	–	–	–	165 (55.0)	132 (80.0)	33 (20.0)		0.16
III and IV				135 (45.0)	117 (86.7)	18 (13.3)	0.6 (0.3-1.1)	
IOP								
Normal	–	–	–	273 (91.0)	231 (84.6)	42 (15.4)		0.051
High				27 (9.0)	18 (66.7)	09 (33.3)	2.7 (1.1-6.5)	

CI = confidence interval; H/o = history of; IOP = intraocular pressure; OR = odds ratio.

TABLE VII - Association of serum calcium levels with various socio-demographic, clinicopathological and biochemical parameters of cataract patients and controls

Socio-demographic/ clinicopathological/ biochemical parameters	Serum calcium levels in controls			Serum calcium levels in cases			OR (95% CI)	p value
	N = 360 (%)	Normal	Low	N = 300 (%)	Normal	Low		
		315 (87.5)	45 (12.5)		186 (62.0)	114 (38.0)	4.2 (2.9-6.3)	<0.0001
Age group								
<50 years	63 (17.5)	54 (85.7)	09 (14.3)	63 (21.0)	40 (63.5)	23 (36.5)	3.4 (1.4-8.2)	0.007
≥50 years	297 (82.5)	261 (81.7)	36 (12.1)	237 (79.0)	146 (61.6)	91 (38.4)	4.5 (2.9-6.9)	<0.0001
Gender								
Men	171 (47.5)	147 (86.0)	24 (14.0)	156 (52.0)	96 (61.5)	60 (38.5)	3.8 (2.2-6.5)	<0.0001
Women	189 (52.5)	168 (88.9)	21 (11.1)	144 (48.0)	90 (62.5)	54 (37.5)	4.8 (2.7-8.4)	<0.0001
Dwelling								
Rural	324 (90.0)	282 (87.0)	42 (13.0)	240 (80.0)	144 (60.0)	96 (40.0)	4.4 (2.9-6.7)	<0.0001
Urban	36 (10.0)	33 (91.7)	03 (8.3)	60 (20.0)	42 (70.0)	18 (30.0)	4.7 (1.2-17.3)	0.02
Hypertension								
No	–	–	–	261 (87.0)	168 (64.4)	93 (35.6)		0.3
Yes				39 (13.0)	18 (46.2)	21 (53.8)	2.1 (1.0-4.1)	
Family history of cataract								
No	–	–	–	288 (96.0)	180 (62.5)	108 (37.5)		0.3
Yes				12 (4.0)	06 (50.0)	06 (50.0)	1.6 (0.5-5.2)	
H/o any other eye disorder								
No	–	–	–	231 (77.0)	147 (63.6)	84 (36.4)		0.3
Yes				69 (23.0)	39 (56.5)	30 (43.5)	1.3 (0.7-2.3)	
Eyes affected								
One	–	–	–	177 (59.0)	114 (64.4)	63 (35.6)		0.3
Both				123 (41.0)	72 (58.5)	51 (41.5)	1.2 (0.7-2.0)	
Type of cataract								
Nuclear								
Cortical	–	–	–	75 (25.0)	51 (68.0)	24 (32.0)		0.2
Posterior subcapsular				225 (75.0)	135 (60.0)	90 (40.0)	1.4 (0.8-2.4)	
Grade								
I and II	–	–	–	165 (55.0)	99 (60.0)	66 (40.0)		0.4
III and IV				135 (45.0)	87 (64.4)	48 (35.6)	0.8 (0.5-1.3)	
IOP								
Normal	–	–	–	273 (91.0)	171 (62.6)	102 (37.4)		0.5
High				27 (9.0)	15 (55.6)	12 (44.4)	1.3 (0.6-2.9)	

CI = confidence interval; H/o = history of; IOP = intraocular pressure; OR = odds ratio.

in membrane permeability of the lens epithelium coupled with the changes in sodium and potassium ion levels in aqueous humor may accentuate ionic imbalance within the lens and lead to the development of cataract (28). In our study, a slightly higher percentage of men population was affected compared to women. According to different studies, women are more prone to getting most types of cataract than men. This is most likely due to lower estrogen levels after menopause in women (29). In our study, proportion of rural participants diagnosed with cataract was much higher compared to urban participants, which may be due to the differential exposure to contextual factors (30). Furthermore, we speculate that rural areas lack quality healthcare and hygiene, hence putting inhabitants at more risk of developing cataract and its related complications. Our results are in line with the observations from other parts of the country reporting significantly higher incidence of cataract in rural areas (31). We observed only 13.0% of the cataract cases to be hypertensive, but according to Lee et al hypertension could induce conformational changes to proteins in lens capsules,

thereby exacerbating the cataract formation (32). Per previous reports, the risk of cataract increases with long-standing hypertension (33). Furthermore, some studies suggest that antihypertensive medications (such as diuretics and beta-blockers) are related to cataract (34). In our study, only few cataract patients had a family history of cataract. Our findings are consistent with epidemiological studies demonstrating more prevalent occurrence of age-related cataracts in close relatives of cataract patients than in the general population (35). In addition, genetic studies have shown the effect of specific genes in the development of cataractous lenses (35). Only one eye was affected in most of the cataract patients, which supports the previous findings that cataract may be present in one or both eyes but cannot spread from one eye to another (36). Most of the enrolled patients were having PSC cataract, which is the most common morphological form of cataract (37) and commonly studied across the country (38). PSC cataract produces more and faster vision deterioration than other forms of cataract, and patients report earlier for cataract surgery, which may be a potential explanation for its elevated prevalence (39).

Various studies are going on all over the world to clarify the relationship between biochemical elements and cataract formation (40), and most of the studies from different populations have not found a remarkable difference in blood biochemical elements between cataract patients and healthy controls (41-43). To further explore the biochemical intricacies of cataract we evaluated the cases as well as controls for various biochemical markers and deduced the relationship, if any, between the disease development and biochemical markers. As evident from the dataset, most of the cataract patients in our study were having significantly impaired fasting blood sugar levels compared to controls. A previous study has found a significant relationship between hyperglycemia and incidence of cataracts (44). *In vivo* or *in vitro* studies showed evidence that diabetes mellitus is the cause of cataract (45,46). Cataracts have multiple etiologies, one of which is chronic hyperglycemia which has been related to many systemic and ocular complications such as loss of vision. It has been suggested that the polyol via which the enzyme aldose reductase catalyzes the reduction of glucose into sorbitol is a central part of the mechanism of cataract development. The increased intracellular accumulation of sorbitol leads to a hyperosmotic effect, resulting in hydropic lens fibers that degenerate and form cataract (47,48). In our study population, the frequency of cataract patients having hypernatremia was significantly higher compared to controls, which is in line with the study conducted by Mathur and Pai who reported significant hypernatremia in cataract patients compared to the controls (49). Since the lens metabolism is associated with aqueous humor, which itself is produced from blood secretions, serum electrolyte concentration directly affects electrolytes of aqueous humor and in turn lens metabolism (26). Studies have upheld that, in case of increased concentrations of sodium in aqueous humor, it is difficult for the sodium pumps to maintain a low intracellular sodium ion level. In turn, a higher sodium ion concentration of the aqueous humor, coupled with an altered membrane permeability of lens, increases the intracellular sodium ion concentration leading to hydration of the lens, thereby resulting in loss of its transparency and development of cataract (50). In the current study, we observed hypokalemia in significantly higher number of cataract patients compared to controls. Several studies revealed strong association of low serum potassium levels with CCs (51,52). According to Duncan and Bushell, cortical and mature cataracts had very low serum potassium levels (53). The frequency of hypocalcemia in cataract cases was significantly higher compared to controls. Low serum calcium levels in cataract patients were seen in other populations, clearly supporting our results (54). Cataract is the most common ocular symptom of hypocalcemia (55). Seemingly, because of deposition of calcium in soft tissues producing reduced vision/cataract or calcification of basal ganglia, calcium gets depleted in human serum (56).

In the further part of the study we deciphered the effect of various factors on the relationship between biochemical parameters and cataract. Surprisingly none of the factors significantly affected the relationship of serum glucose, serum sodium and serum calcium with cataract. In addition, we have also identified various factors modifying the association between hypokalemia and cataract. The frequency of hypokalemic rural cataract patients was significantly higher compared to rural hypokalemic controls. Difference in mean serum potassium concentrations among Kashmiri cataract cases and healthy controls residing in rural locations might be due to difference in the quality of diet among the two groups (56,57). Interestingly we observed a significantly higher proportion of hypokalemic cataract patients coexistent with hypocalcemia. Although hypokalemia and hypocalcemia exhibit multiple interrelated acid-base and electrolyte abnormalities such as hypophosphatemia, respiratory/metabolic alkalosis, mixed acid-base disorders (58), the coexistence of these in cataract has not yet been reported and debated upon. Further, hypokalemia was significantly associated with hypertension in cataract patients. Even though the association of hypokalemia and hypertension has been previously reported (33,34), the common causes of hypertension with hypokalemia have been well established, which include essential hypertension with diuretic use, primary aldosteronism, Cushing's syndrome, pheochromocytoma, renal vascular disease and malignant hypertension (59). Therefore, patients with coexistent hypertension and hypokalemia need to be evaluated further to establish the reason for the development of cataract.

Conclusion

In conclusion, serum glucose, sodium, potassium and calcium levels were identified as the potentially modifiable parameters deranged in cataract. In future, large-scale studies to enhance the battery of biochemical markers and to establish cause and effect relationship between biochemical markers and cataract need to be done.

Limitation of the study

The sample size of the study is relatively modest.

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Conflict of interest: The authors disclose that there are no financial or non-financial interests that are directly or indirectly related to the work submitted for publication.
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ORIGINAL RESEARCH ARTICLE
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PIVKA-II or AFP has better diagnostic properties for hepatocellular carcinoma diagnosis in high-risk patients

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is a lethal cancer. Two biomarkers were used for HCC diagnosis including alpha-fetoprotein (AFP) and protein induced by vitamin K absence-II or antagonist (PIVKA-II). However, data on biomarkers and HCC diagnosis are not consistent. This study aimed to evaluate if PIVKA-II, AFP, or a combination of both biomarkers had the best diagnostic properties for HCC.

Methods: This was a prospective study and enrolled patients 18 years or over with a high risk for HCC. AFP and PIVKA-II levels were calculated for HCC diagnosis. Diagnostic properties of both biomarkers were reported with sensitivity, specificity, and a receiver operating characteristic (ROC) curve.

Results: There were 260 patients with high risk for HCC in this cohort. Of those, 219 patients were diagnosed with HCC: confirmed by biopsy in 7 patients (2.69%) and by imaging in the others. Median values of AFP and PIVKA-II were 56 ng/mL and 348 mAU/mL, respectively. PIVKA-II level of 40 mAU/mL had sensitivity of 80.80%, while AFP of 10 ng/mL had sensitivity of 75.80%. A combination of PIVKA-II at 100 mAU/mL or over and AFP of 11 ng/mL gave sensitivity of 60.30%. The ROC curve of PIVKA-II plus AFP was significantly higher than the AFP alone (0.855 vs. 0.796; p = 0.027), but not significantly different from the PIVKA-II alone (0.855 vs. 0.832; p = 0.130).

Conclusion: PIVKA-II may have more diagnostic yield for HCC compared with AFP. It can be used alone without a combination with AFP.

Keywords: Hepatitis B virus, Hepatitis C virus, Sensitivity, Specificity

Introduction

Hepatocellular carcinoma (HCC) is a common cancer. It is the sixth most common cancer and the second highest in cancer-related death globally. In 2015, there were 854,000 new cases and 810,000 deaths in the world (1). There were three common causes of HCC: hepatitis B virus (HBV), alcohol, and hepatitis C virus (HCV). These causes related to HCC deaths in 33%, 30%, and 21%, respectively (1). High-risk patients for HCC were patients with cirrhosis, chronic HBV infection, or lesions identified on a surveillance ultrasound in patients with cirrhosis, chronic HBV infection, or prior HCC (2).

The main two markers for HCC are alpha-fetoprotein (AFP) and new biomarker: protein induced by vitamin K absence-II or antagonist (PIVKA-II). These markers play an important role in diagnosis, differential diagnosis, and prognosis. A nomogram of both AFP and PIVKA-II can be used to differentiate HCC and intrahepatic cholangiocarcinoma with an area under the receiver operating characteristic (ROC) curve of 95.1% (3). Both biomarkers can be used as a prognostic factor after several treatments such as radiofrequency, surgical resection, embolization, or radiotherapy (4-12). PIVKA-II level lower than 25 mAU/mL or equal after radiotherapy had long progression-free survival (p = 0.004) (4). Additionally, both biomarkers can indicate tumor size, tumor differentiation, vascular invasion, and those treated with hepatitis B/C antiviral agents (13-16).

As HCC is a lethal cancer with 5-year survival rate of under 15%, abdominal ultrasonography is used for HCC surveillance (17,18). However, its sensitivity was low at 63% (19). Biomarkers were studied and used as an alternative

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diagnostic method particularly for HCC including AFP and PIVKA-II (20-22). The advantage of AFP is widely available, while PIVKA-II is highly specific to HCC. Data on biomarkers and HCC diagnosis are not consistent. A report from the USA found that AFP was more sensitive than PIVKA-II (70% vs. 66%) in patients with HCC compared with patients with cirrhosis (23). On the other hand, a study from Nigeria found that PIVKA-II was more sensitive than AFP (96.8% vs. 62.9%) in patients with HCC vs. control with benign liver disease (24). A study from Korea recommends to use PIVKA-II combined with AFP to diagnose HCC in patients with HBV infection (25). These results showed that data on biomarkers and HCC diagnosis are not consistent and varied among countries, in which more data on this issue are required. This study aimed to evaluate if PIVKA-II, AFP, or a combination of both biomarkers had the best diagnostic properties for HCC.

Materials and methods

This was a prospective study conducted at University Hospital, Khon Kaen University, Thailand. Data were collected from the HCC project. The inclusion criteria were patients 18 years or over with a high risk for HCC. The high risk for HCC was defined by the American Association for the Study of Liver Diseases (AASLD) guidelines for HCC management (17): cirrhosis or presence of liver nodule(s) of 1 cm or over in size. Those with pregnancy, obstructive jaundice, vitamin K, or warfarin administration and presence of extrahepatic malignancy were excluded. The study protocol was approved by the ethics committee in human research, Khon Kaen University, Thailand (HE621134). This study was a part of HCC project of Khon Kaen University, Thailand.

Eligible patients provided a written informed consent prior to study participation. Data were collected as follows: baseline characteristics, laboratory results, and radiographic findings. Baseline characteristics included age, sex, etiology of cirrhosis, comorbid diseases, and the Child-Pugh score for cirrhosis. Laboratory tests in the study were platelet count, serum creatinine, prothrombin time, liver function test, AFP, and PIVKA-II. All samples were tested for AFP and PIVKA-II by using a test kit (μTASWako i30; FUJIFILM Wako Pure Chemical Corporation). Radiographic findings were numbers of liver mass, largest mass size (cm), and portal vein invasion. HCC was diagnosed by either confirmation by pathological findings or radiographic findings of arterial hypervascularity followed by venous and/or delayed phase or washout of contrast (17).

Statistical analyses

Descriptive statistics were used to calculate mean (1st-3rd interquartile ranges) or number (percentage) of the study population. AFP and PIVKA-II levels were calculated for HCC diagnosis by logistic regression analysis. Results were shown as various cutoff points with their diagnostic properties including sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, a ROC curve, and an area under the ROC curve with 95% confidence interval (CI). All statistical analyses were performed using STATA software version 10.1.

Results

There were 260 patients with a high risk for HCC in this cohort. Of those, 219 patients were diagnosed with HCC: confirmed by biopsy in 7 patients (2.69%) and by imaging in the others. The other 41 patients had a diagnosis of dysplastic nodule (25 patients; 60.98%), regenerative nodule (8 patients; 19.51%), hemangioma (5 patients; 12.20%), liver cyst (1 patient; 2.44%), fibronodular hyperplasia (1 patient; 2.44%), and hepatic adenoma (1 patient; 2.44%). Baseline characteristics and laboratory results are shown in Tables I and II. The median age was 58 years with male predominance (81.15%). The most common cause of HCC was HCV (43.85%) with a proportion of cirrhosis of 98.46%: mostly Child-Pugh score class A (81.15%). The median largest size of liver mass was 3.5 cm. The median values of AFP and PIVKA-II were 56 ng/mL and 348 mAU/mL, respectively.

The PIVKA-II level of 40 mAU/mL had sensitivity of 80.80% with 75.60% specificity, while AFP of 10 ng/mL had sensitivity of 75.80% with 65.90% specificity (Tabs. III and IV). A combination of PIVKA-II at 100 mAU/mL or over and AFP of 11 ng/mL gave sensitivity of 60.30% with 92.70% specificity. The ROC curve of PIVKA-II plus AFP was significantly higher than the AFP alone (0.855 vs. 0.796; p value = 0.027), but not significantly different from PIVKA-II alone (0.855 vs. 0.832; p value = 0.130), as shown in Figure 1.

TABLE I - Baseline characters of patients with a high risk for hepatocellular carcinoma (n = 260)

Factors	Median (1st-3rd quartile) or number (percentage)
Age, years	58 (54-63)
Male sex, n (%)	211 (81.15)
Etiology	
HBV	98 (37.69)
HCV	114 (43.85)
HBV plus HCV	6 (2.31)
NAFLD	14 (5.38)
ALD	27 (10.38)
AIH	1 (0.38)
Comorbid diseases	
None	193 (74.23)
Diabetes	38 (14.62)
Hypertension	12 (4.62)
Cirrhosis	256 (98.46)
Child-Pugh score A	211 (81.15)
Child-Pugh score B	36 (13.85)
Child-Pugh score C	9 (3.46)

AIH = autoimmune hepatitis; ALD = *alcoholic* liver disease; HBV = hepatitis B virus; HCV = hepatitis C virus; NAFLD = nonalcoholic fatty liver disease.

TABLE II - Laboratory results of patients with a high risk for hepatocellular carcinoma (n = 260)

Factors	Median (1st-3rd quartile) or number (percentage)
Platelet $\times 10^3/mm^3$	145 (102-220)
Creatinine (mg/dL)	0.94 (0.80-1.15)
Prothrombin time (sec)	12.3 (11.5-13.4)
Albumin (g/dL)	3.9 (3.4-4.4)
Bilirubin (mg/dL)	1.0 (0.6-1.6)
Alanine aminotransferase (U/L)	51 (29-86)
Aspartate transaminase (U/L)	74 (40-133)
Alkaline phosphatase (U/L)	135 (88-196)
Alpha-fetoprotein (ng/mL)	59 (6-1,598)
PIVKA-II (mA/mL)	348 (31-10,442)
Radiographic findings	
Number of liver nodules, n (%)	
1	156 (90.00)
2	46 (17.69)
3	12 (4.62)
≥4	46 (17.69)
Largest size (cm)	3.5 (1.8-7.5)
Portal vein invasion, n (%)	
No invasion	181 (69.62)
Main portal vein invasion	56 (21.54)
Non-main portal vein invasion	23 (8.85)

PIVKA-II = protein induced by vitamin K absence-II or antagonist.

TABLE III - Performance characteristics of protein induced by PIVKA-II (mAU/mL) for diagnosing HCC in patients with a high risk for HCC

Cutoff	Sensitivity	Specificity	LR+	LR-
≥40	80.80 (75.00-85.80)	75.60 (59.70-87.60)	3.31 (1.93-5.70)	0.25 (0.18-0.35)
≥60	78.50 (72.50-83.80)	80.50 (65.10-91.20)	4.03 (2.15-7.52)	0.27 (0.20-0.36)
≥80	75.30 (69.10-80.90)	82.90 (67.90-92.80)	4.41 (2.24-8.70)	0.30 (0.23-0.39)
≥100	72.10 (65.70-78.00)	87.80 (73.80-95.90)	5.92 (2.59-13.50)	0.32 (0.25-0.40)
≥120	69.40 (62.80-75.40)	87.80 (73.80-95.90)	5.69 (2.49-13.0)	0.35 (0.28-0.44)

HCC = hepatocellular carcinoma; LR+ = positive likelihood ratio; LR- = negative likelihood ratio; PIVKA-II = vitamin K absence-II or antagonist.

TABLE IV - Performance characteristics of AFP (ng/mL) for diagnosing HCC in patients with a high risk for HCC

Cutoff	Sensitivity	Specificity	LR+	LR-
≥10	75.80 (69.60-81.30)	65.90 (49.40-79.90)	2.22 (1.44-3.42)	0.37 (0.27-0.51)
≥12	73.10 (66.70-78.80)	70.70 (54.50-83.90)	2.50 (1.54-4.04)	0.38 (0.28-0.51)
≥14	69.40 (62.80-75.40)	73.20 (57.10-85.80)	2.59 (1.55-4.32)	0.42 (0.32-0.55)
≥20	65.30 (58.60-71.60)	78.00 (62.40-89.40)	2.97 (1.66-5.34)	0.45 (0.35-0.57)
≥200	44.70 (38.00-51.60)	90.20 (76.90-97.30)	4.59 (1.79-11.80)	0.61 (0.52-0.72)

AFP = alpha-fetoprotein; HCC = hepatocellular carcinoma; LR+ = positive likelihood ratio; LR- = negative likelihood ratio.

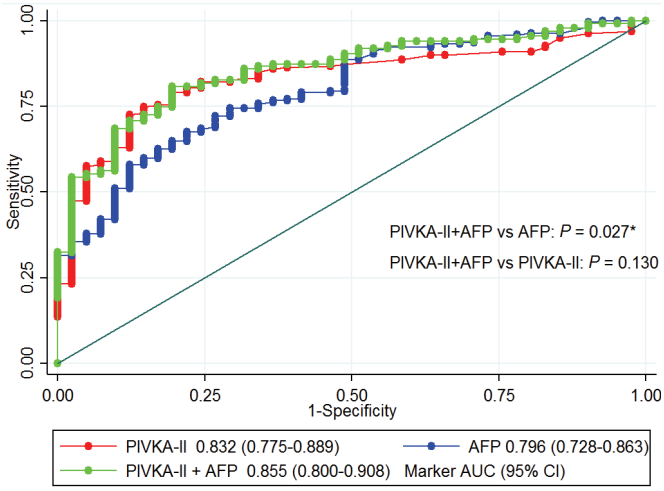

Fig. 1 - Receiver operating characteristic (ROC) curves and their area under the ROC curves (95% confidence interval) of protein induced by vitamin K absence-II or antagonist (PIVKA-II), alpha-fetoprotein (AFP), and the combination of both biomarkers to predict hepatocellular carcinoma (HCC) in patients with a high risk for HCC.

Discussion

This cohort found that PIVKA-II was more sensitive for HCC diagnosis than AFP and can be used alone without a combination with AFP.

The results of this study were comparable with two previous studies to detect HCC in cirrhosis patients with liver nodules of 1 cm or over (26-28). Note that this study had larger study population than the other two studies (260 vs. 128 vs. 90). With larger sample size in this study, PIVKA-II was more sensitive to detect HCC than AFP (80.80% vs. 75.80%) by the cutoff point of 40 mAU/L and 10 ng/mL, respectively. Compared with the study from France, the sensitivity of PIVKA-II was comparable (80.80% vs. 77%) as well as the cutoff point (40 vs. 42 mAU/L). However, this study had different results compared with the US study (23), in which AFP had

better sensitivity than PIVKA-II in HCC with a sample size of 208 patients. AFP at 10.9 ng/mL had higher sensitivity at 66% compared with 56% sensitivity of PIVKA-II at 221.5 mAU/mL. These differences may be due to different control. In the previous study, patients with cirrhosis without liver mass served as controls, while non-HCC patients were cirrhotic patients with liver mass in this study. Another possible explanation is the property of PIVKA-II, which is an indicator of microvascular invasion (26). High PIVKA-II of over 90 mAU/mL had higher risk of microvascular invasion by 3.5 times (95% CI 1.08, 11.8; p value 0.043). In this study, 30.38% of patients had portal vein invasion, which may be an indicator of microvascular invasion (Tab. II).

In this study, we found that PIVKA-II can be used for HCC diagnosis without a need to combine with AFP. These results were different from the study from Korea (25). The areas under the ROC curve of these combinations were significantly different from PIVKA-II alone (0.912 vs. 0.870) or AFP alone (0.902 vs. 0.812) for those with liver cirrhosis in the previous study. Once again, these differences may be due to different study population. The previous study enrolled patients with HBV infection and categorized into three groups: non-cirrhotic HBV infection, cirrhosis without HCC, and HCC group (no data whether cirrhosis or not). Additionally, the cirrhosis group in the previous study may or may not have liver nodules like in this study. Note that HBV infection was accounted in only 37.69% in this study. Another explanation is different cutoff points for PIVKA-II and AFP. The cutoff points for these two markers in the previous study were 40 mAU/mL and 25 ng/mL, while the cutoff points in this study were 100 mAU/mL and 11 ng/mL. Note that a combination of these two biomarkers had lower sensitivity but higher specificity.

Even though both AFP and PIVKA-II are useful diagnostic markers for HCC, a previous report found that they may not be a good marker for small HCC nodules less than 2 cm as they have sensitivity of approximately 50% (29). However, they may be used for HCC detection particularly in hepatitis virus-related HCC (22,30). A study from China found similar findings as this study but different cutoff for both AFP and PIVKA-II (31). A combination of AFP and PIVKA-II model is better than AFP alone but comparable with PIVKA-II alone. Therefore, PIVKA-II may be used alone without a combination with AFP to diagnose HCC. Compared with benign liver disease, this study had PIVKA-II and AFP cutoff points at 40 mAU/mL and 10 ng/mL while the Chinese study had cutoff points of 43.47 mAU/mL and 21.47 ng/mL for PIVKA-II and AFP. The different cutoff points may be from different study population and sample size. This study had larger sample size and most patients (80%) had HBV or HCV as a cause, while the Chinese study did not show causes of HCC. Another study conducted with liver cirrhosis as a control group also found that similar findings of PIVKA-II alone were comparable with a combination of PIVKA-II and AFP for HCC diagnosis (32).

There are some limitations in this study. First, etiologies of cirrhosis in this study are varied; HCV was the most common cause (43.85%) followed by HBV infection (37.69%). The results of this study may not be applicable for other countries with different causes of cirrhosis or HCC. Second, we used different cutoff points for a combination of PIVKA-II and AFP as discussed earlier. Third, some associated factors with hepatitis virus or fatty liver such as sleep apnea were not studied (33-38). No predictor for HCC was studied as well as systematic review (39-42). Finally, note that control group in this study were those with high risk for HCC: presence of liver mass of 1 cm or more in size. As this study enrolled high-risk patients for HCC with various causes, various Child-Pugh score classification, various liver nodule sizes, these may lead to possible selection biases. Further studies are necessary before considering these biomarkers even for a general evaluation of the HCC diagnosis.

In conclusion, PIVKA-II may have more diagnostic yield for HCC compared with AFP. It can be used alone without a combination with AFP.

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REVIEW

Laboratory biomarkers of delayed cerebral ischemia following subarachnoid hemorrhage: A systematic review

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ABSTRACT

Delayed cerebral ischemia (DCI) substantially contributes to disability and death in subarachnoid hemorrhage (SAH) patients; however, its pathophysiology is incompletely understood and diagnostic and therapeutic strategies are lacking. Biomarkers may help to elucidate the pathophysiology, optimize early diagnosis, or provide treatment targets. We systematically searched PubMed and Embase on October 13, 2021, for studies that evaluated at least one laboratory biomarker in patients with DCI, using the most up-to-date definition of DCI as proposed by a panel of experts in 2010. Quality of studies was assessed using the Newcastle-Ottawa Scale or Cochrane Collaboration's risk of bias assessment tool. Biomarkers of clinical and radiological DCI were analyzed separately. Results were meta-analyzed if possible, otherwise narratively reviewed. Biomarkers were classified as significant, inconclusive, or nonsignificant. We defined validated biomarkers as those with significant results in meta-analyses, or in at least two studies using similar methodologies within the same time interval after SAH. The search yielded 209 articles with 724 different biomarkers; 166 studies evaluated 646 biomarkers of clinical DCI, of which 141 were significant and 7 were validated biomarkers (haptoglobulin 2-1 and 2-2, ADAMTS13, vWF, NLR, P-selectin, F2-isoprostane); 78 studies evaluated 165 biomarkers of radiological DCI, of which 63 were significant and 1 was a validated biomarker (LPR). Hence, this review provides a selection of seven biomarkers of clinical DCI and one biomarker of radiological DCI as most promising biomarkers of DCI. Future research should focus on determining the exact predictive, diagnostic, and therapeutic potentials of these biomarkers.

Keywords: Biomarker, Delayed cerebral ischemia, Subarachnoid hemorrhage

Introduction

Delayed cerebral ischemia (DCI) is an important and potentially preventable contributor to disability and death in subarachnoid hemorrhage (SAH) patients in whom the causative aneurysm has been obliterated (1,2). The pathophysiology of DCI is complex and multifactorial; however, it is not yet fully understood. As a result, an accurate diagnostic test and effective treatment are both lacking.

Biomarkers may not only help to further elucidate the pathophysiology of DCI, but could also aid in optimizing early diagnosis and prompt treatment. Even though the present literature is brimmed with biomarker studies of DCI, accurate biomarkers of DCI have not yet been established. In 2018, an extensive systematic review proposed a panel analysis of 6 selected genetic and 15 protein biomarker candidates for further prospective validation of DCI prediction (3). However, this review used a very broad definition of DCI, including also DCI defined solely as angiographic vasospasm on digital subtraction angiography or increased flow velocities on transcranial Doppler (TCD) ultrasonography. Historically, angiographic vasospasm was considered the sole cause of DCI; however, in the past decades research has

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shown that DCI and vasospasms are two different entities (4). Therefore, the combined use of both vasospasm and DCI in the 2018 review may have misguided the selected panel of biomarkers.

In 2010, a panel of experts proposed a strict definition of DCI, divided into a clinical and radiological definition, to be used as outcome measure and to enable more reliable meta-analyses of future clinical trials and observational studies (4). We used these definitions of DCI to systematically review the current literature on laboratory biomarkers of DCI and selected a new panel of the most promising biomarkers. The selected biomarkers may be useful for the prediction or diagnosis of DCI, either individually or combined.

Methods

The Preferred Reported Items for Systematic Review and Meta-analysis (PRISMA) 2020 guidelines were followed. This review was not registered and a review protocol was not prepared.

Search strategy and selection criteria

PubMed and Embase were searched for relevant studies from January 1, 1985, to October 13, 2021. The search strategy can be found in the supplemental data (Supplementary material, page 2-3). Two authors (MAT and HL) independently selected eligible studies. Titles and abstracts were screened for potential eligibility when covering primary studies on biomarkers of DCI in SAH patients either written in Dutch or English. Of the selected studies, full texts were screened and studies were considered eligible if they met the following criteria: (1) patients with SAH of whom (2) at least 95% were diagnosed with a causative aneurysm, (3) measurement of at least one laboratory biomarker (4) during hospital admission, (5) using either the radiological or clinical definition of DCI as proposed in 2010 (criteria listed in Tab. I), and (6) the studies had to perform, or allow for, statistical analysis on the biomarker in question and DCI. Exclusion criteria were: (1) studies with mixed clinical and radiological DCI cohorts, in which patients with either clinical or radiological DCI could not be distinguished from the mixed cohort, (2) biomarker levels in patients with and without DCI were inconsistent (e.g., different numbers in tables/figures and text), (3) no level of statistical significance was given, nor could be calculated, (4) studies in children only, (5) the same patient population (similar years and hospital of patient inclusion) was used by more than one study and, within those studies, the same biomarker was measured and compared similarly between DCI and no DCI patients. In this case, the study with the smallest sample size, and if sample sizes were equal the most outdated study, was excluded. Lastly, we checked studies on nimodipine administration. In 1988 and 1989, two randomized controlled trials showed efficacy of nimodipine in preventing DCI, resulting in a shift in SAH management (5,6). Therefore, we excluded studies with SAH patients before 1985. SAH cohorts between 1985 and 1990 were only included if nimodipine administration was explicitly mentioned. All papers after 1990 were included, unless nimodipine was described explicitly as being not part of the treatment protocol.

TABLE I - Criteria of the clinical and radiological definition of DCI

Clinical DCI	
Defined as:	*Excluded if:*
• A decrease in level of consciousness (described by a decrease of the Glasgow Coma Scale score or an increase of the National Institutes of Health Stroke Scale score) ***or***	• Vague definitions of DCI, e.g., "clinical deterioration," "neurological deterioration," "worsening of neurological condition," without specifically mentioning either decreased level of consciousness or focal neurological impairment

• Focal neurological impairment (e.g., aphasia, motor impairment, sensory deficits, neglect)	• The DCI cohort also includes patients with solely signs of vasospasm without clinical symptoms of DCI. For example, patients with:
	  • Arterial narrowing on CT-A or DSA
	  • Increased flow velocities on TCD

Radiological DCI	
Defined as:	*Excluded if:*
• New ischemia/infarction/stroke or	• Defined as "presence of ischemia/infarction/stroke" without defining "new," "secondary," or "due to DCI/associated terms of DCI"
• Secondary ischemia/infarction/stroke or	• Presence of ischemia/infarction/stroke determined by an imaging modality other than noncontrast CT or MRI, e.g., CT perfusion, CT-A)
• Ischemia/infarction/stroke due to DCI (or associated DCI terms, including delayed ischemic/neurological deficit, [clinical] vasospasm, secondary ischemia/infarction)	• Other nonimaging methods used to assess ischemia/infarction/stroke, e.g., brain tissue oxygenation monitoring and microdialysate biomarkers

CT = computed tomography; CT-A = CT-angiography; DCI = delayed cerebral ischemia; DSA = digital subtraction angiography; MRI = magnetic resonance imaging; TCD = transcranial Doppler.

Data extraction

The same authors (MAT and HL) independently extracted baseline SAH characteristics (age, gender, World Federation of Neurological Societies [WFNS] grade and Fisher grade), the study's definition of DCI, the number of patients with and without DCI, and biomarker-related data.

If studies addressed both clinical and purely radiological DCI separately, biomarker-related data were extracted for both definitions. The biomarker-related data concerned laboratory biomarkers only (clinical and radiological biomarkers [heart rate, blood pressure, temperature, TCD velocities, computed tomography (CT) perfusion variables etc.] were left out of consideration). Biomarker data had to concern levels of individual biomarkers or ratios of two biomarkers, that is, combinations of three or more biomarkers, such as decision tree analysis or a self-made calculated score based on multiple biomarkers, were left out of consideration. Data included the biomarker medium (blood, cerebrospinal fluid [CSF], microdialysate [MD], urine, or other), timing of measurement(s), and all statistical analyses regarding the biomarker and the occurrence of DCI. If the biomarker medium was not mentioned, it was assumed to be blood. A biomarker measured in multiple media, for example, in blood and CSF, was considered as two separate biomarkers.

Nongenetic biomarkers were stratified into biomarker groups, which were based on the multifactorial pathophysiology of DCI as described by a previously published systematic review (7). Of biomarkers with multiple functions, we aimed to select the function which the authors of the study considered as DCI-related and subsequently categorized the biomarker into that specific biomarker group. Nongenetic biomarkers which could not be stratified to any biomarker group related to the pathophysiology of DCI were categorized as "other." Within the "other" group, we aimed to cluster biomarkers with similar functions. Genetic biomarkers were categorized as a separate group. The extracted data were compared and discrepancies were discussed with a third author (DV) and solved.

Quality assessment

Two authors (MAT and DV) independently assessed the quality of included studies using the Newcastle-Ottawa Scale (NOS) for case control and cohort studies and the Cochrane Collaboration's risk of bias assessment tool for randomized trials. Studies could obtain a maximum of eight or nine points, depending on the use of a nonexposed cohort. The Cochrane Collaboration's risk of bias assessment tool comprises seven criteria, which can be scored as high, low, or unclear risk of bias. Discrepancies were discussed with a third opinion (WPV) and solved.

Data interpretation and analysis

Biomarkers of clinical DCI (primary outcome) were analyzed separately from biomarkers of radiological DCI (secondary outcome). We categorized biomarkers into significant and nonsignificant biomarkers. Biomarkers, which were studied by one study only, were considered significant if at least one measurement was significantly associated with DCI ($p < 0.05$) and nonsignificant if all measurements yielded nonsignificant results. If a biomarker was investigated by more than one study, the classification into significant and nonsignificant was either based on a biomarker-specific meta-analysis, if methodologically possible (similar type of analyses and timing of measurement), or by descriptive comparison. Meta-analyses were performed only if all relevant studies, which investigated the biomarker in question on a specific point in time after SAH, could be included, using Review Manager 5.4. For continuous data we used the inverse variance method with random effects model to calculate weighted mean differences with 95% confidence intervals. For dichotomous data we used the Mantel-Haenszel method with random effects model to calculate estimated odds ratio with 95% confidence intervals. A pooled effect size was estimated for each specific timing of biomarker measurement. Descriptive comparison of the studies was done by summarizing the results of all relevant studies in a biomarker-specific figure, which was then used to classify the biomarkers into significant, inconclusive, and nonsignificant. The biomarker-specific figures display the author, year, number of patients with and without DCI, the type of analyses, and the results divided into categories of sampling timing (day 0-3, 4-8, 9-13, and >14 after SAH). The method used for classification of biomarkers into significant, inconclusive, and nonsignificant based on the biomarker-specific figures is explained in the supplemental data (Supplementary material, page 4).

In order to select the most promising biomarkers of DCI, we evaluated whether significant biomarkers were validated by a second study. A validated biomarker was defined as a biomarker with significant results in at least two studies using similar methodologies within the same time interval after SAH, without any other study showing contradictory results. Biomarkers of which the meta-analysis of all relevant studies yielded a significant association were considered validated, too. Additionally, biomarkers that had only been investigated by one study were differentiated from those biomarkers that were investigated by a high-quality study. We defined high quality as a maximum (eight or nine) or maximum minus one point (seven out of eight or eight out of nine) quality assessment score.

Data have been reported according to the PRISMA reporting guidelines for systematic reviews and meta-analyses.

Results

Included studies

After removing duplicates, the search yielded a total of 5,820 studies. We excluded 3,900 studies during abstract screening and 1,711 studies during full-text screening, after which 209 articles (204 cohort and 4 case-control studies, 1 randomized controlled trial) remained (Fig. 1). Baseline characteristics of all included studies are listed in Table S1 of the supplemental data (Supplementary material, page 5-12). Altogether, the 209 studies measured 724 different biomarkers in 25,933 SAH patients (not corrected for overlapping

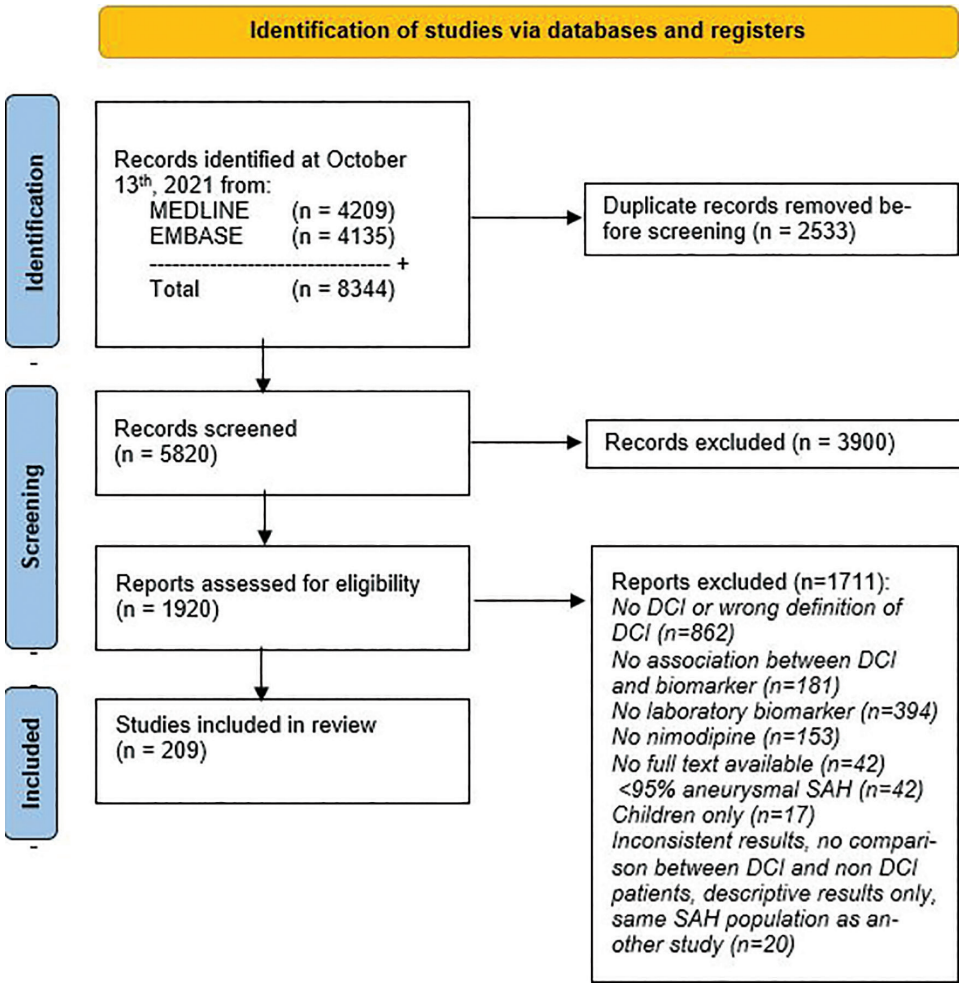

Fig. 1 - PRISMA 2020 flow diagram for systematic reviews.

patient cohorts). A summary of the NOS quality scores is listed in Table II, and the quality scores of individual studies are displayed in the baseline characteristics table (Table S1, page 5-12). The four case-control studies scored three, four (n = 2) and seven of nine points. The randomized controlled trial scored low risk on three of seven items.

TABLE II - Summary of the Newcastle-Ottawa Scale quality assessment scores of 204 cohort studies

Quality score	134 studies without control group (maximum 8 points)	70 studies with control group (maximum 9 points)
1 point	None	None
2 points	7 studies	2 studies
3 points	24 studies	8 studies
4 points	20 studies	16 studies
5 points	35 studies	20 studies
6 points	34 studies	14 studies
7 points	10 studies	7 studies
8 points	4 studies	3 studies
9 points	–	None

Clinical DCI

Biomarkers of clinical DCI were studied by 166 studies comprising 646 different biomarkers (Fig. 2) in 20,983 SAH patients of whom 5,810 (28%) developed DCI. Most biomarkers (588 of 646, 91%) were investigated by one study and 58 (9%) by more than one study (range 2 to 14, median 3). The distribution of the biomarkers among the biomarker groups is shown in Table III.

In total, 141 biomarkers (n = 88 nongenetic, n = 53 genetic) were classified as significantly associated with clinical DCI (Table S2, page 13-34). A summary of studied and significant biomarkers and specific trends observed within each biomarker group are displayed in Table III. Of the significant biomarkers, 121 were investigated by one study and 20 by more than one study. Meta-analyses were possible for five biomarkers (haptoglobin 1-1, 2-1, and 2-2, ADAM metallopeptidase with thrombospondin type 1 motif 13 [ADAMTS13] and von Willebrand factor [vWF]). The meta-analyses showed that SAH patients with haptoglobin polymorphism 2-1 were less likely to develop DCI and patients with haptoglobin polymorphism 2-2 were more likely to develop DCI (8-10). DCI patients had lower ADAMTS13 levels on day 1 and higher levels of vWF on day 4 after SAH, compared to patients without

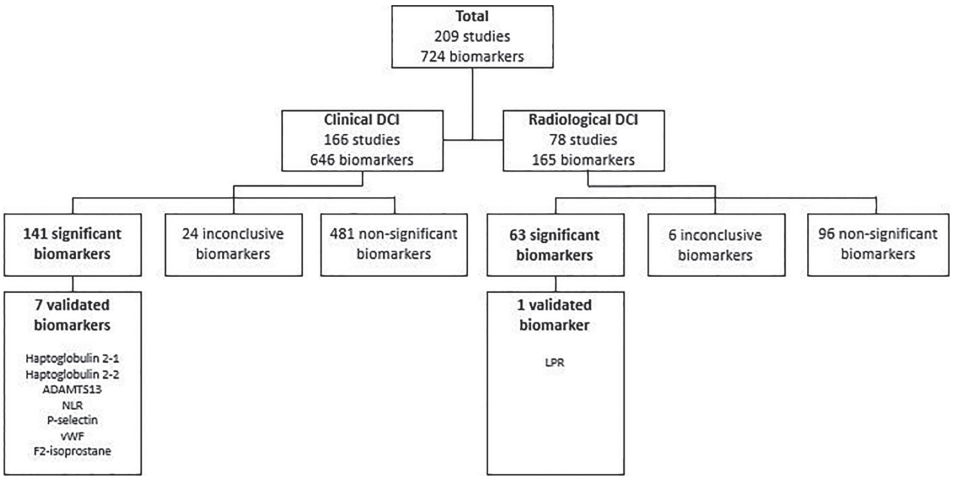

Fig. 2 - Flowchart of all biomarkers, divided into clinical and radiological DCI. ADAMTS13 = ADAM metallopeptidase with thrombospondin type 1 motif 13; LPR = lactate/pyruvate ratio; NLR = neutrophil/lymphocyte ratio; vWF = von Willebrand factor.

TABLE III - Number of studied and significant biomarkers and specific trends observed within each biomarker group of clinical DCI

Biomarker group	Biomarkers of clinical DCI		Specific trends observed among significant biomarkers within biomarker group
	Studied	Significant	
Nongenetic biomarkers	**225**	**88**	
Brain injury	16	8	All indicated more brain injury in patients with DCI. No specific trend in time.
Coagulation cascade	33	15	All indicated increased platelet activity, hypercoagulability, or hyperfibrinolysis. No specific trend in time.
Cortical spreading depressions	2	1	Excitotoxicity in first 48 h after SAH.
Endothelial injury	3	2	Both indicated more endothelial injury in DCI.
Inflammation and immune system	83	29	25 of 29 biomarkers indicated enhanced inflammation or immune response. Most frequently observed in first couple of days following SAH.
Oxidative stress	18	7	5 of 7 biomarkers indicated increased oxidative stress in patients with DCI. No specific trend in time.
Vascular tone	22	9	6 of 9 biomarkers indicated vasoconstriction, 2 indicated vasodilation, 1 was significant with unknown point estimate.
Volemic status	10	2	Both increased and decreased diuresis and natriuresis were observed in DCI.
Other	38	14	Subcategories were: cerebral metabolism, gender-specific growth factors, lipid metabolism, oxygen-carrying capacity, and stress response.
Genetic biomarkers	**421**	**53**	
Genomic	5	1	Function often unclear, some were involved in processes of oxidative stress, vascular tone, volemic status, stress response, inflammation and coagulation, among others.
MicroRNA	51	0	
Methylation	44	36	
Polymorphism	13	3	
RNA	15	6	
Single nucleotide polymorphism	293	7	

DCI = delayed cerebral ischemia; RNA = ribonucleic acid; SAH = subarachnoid hemorrhage.

DCI, in two studies (11,12). The results of the remaining 53 significant biomarkers that were investigated by multiple studies that could not be meta-analyzed were summarized in biomarker-specific figures. Based on the biomarker-specific figures, three other biomarkers (neutrophil/lymphocyte ratio [NLR] and P-selectin in blood and F2-isoprostane in urine) had significant results in two or more studies using similar methodologies and timing of measurement, and were considered validated biomarkers. Four studies on NLR showed a proinflammatory state on admission and preoperatively in patients with DCI compared to patients without DCI (13-16). P-selectin, a marker of endothelial activation, was found to have a significantly larger increase during DCI onset, compared to paired samples in the non-DCI group in two studies

TABLE IV - Quality of relevant studies of validated biomarkers

Validated biomarker	Studies	Quality of studies
Clinical DCI		
ADAMTS13	Li, 2017 (11)	3/8
	Tang, 2015 (12)	4/9
F2-isoprostane	Wiśniewski, 2020 (19)	6/9
	Wiśniewski, 2017 (20)	6/9
Haptoglobulin 2-1	Kim, 2018 (8)	3/8
	Leclerc, 2015 (9)	5/8
	Ohnishi, 2014 (10)	6/8
Haptoglobulin 2-2	Kim, 2018 (8)	3/8
	Leclerc, 2015 (9)	5/8
	Ohnishi, 2014 (10)	6/8
NLR	Liu, 2020 (13)	6/8
	Al-Mufti, 2019 (14)	6/8
	Wu, 2019 (16)	7/8
	Tao, 2017 (15)	5/8
P-selectin	Wang, 2011 (17)	7/9
	Frijns, 2006 (36)	5/8
vWF	Li, 2017 (11)	3/8
	Tang, 2015 (12)	4/9
Radiological DCI		
LPR	Kofler, 2020 (26)	4/8
	Torné, 2020 (27)	2/8

ADAMTS13 = ADAM metallopeptidase with thrombospondin type 1 motif 13; DCI = delayed cerebral ischemia; LPR = lactate/pyruvate ratio; NLR = neutrophil/lymphocyte ratio; vWF = von Willebrand factor.

(17,18). F2-isoprostane in urine on day 3 after SAH was significantly associated with the occurrence of DCI in two studies (19,20). The quality of studies from which we found the seven validated biomarkers is listed in Table IV. Both meta-analyses and biomarker-specific figures can be found in the supplemental data (Supplementary material, Figure S1-S58, page 35-45).

Among the 121 significant biomarkers investigated by one study only, 5 (n = 4 nongenetic, n = 1 genetic) biomarkers were investigated by high-quality studies: brain natriuretic peptide (21), osteopontin (22), tenascin C (23), and neutrophil/albumin ratio (24) in blood and endothelial nitric oxide synthase (SNP rs2070744 T-786C) using buccal swabs (25).

Radiological DCI

Biomarkers of radiological DCI were studied by 78 studies comprising 165 different biomarkers (Fig. 2) in 10,282 SAH patients of whom 2,254 (22%) developed DCI. Most biomarkers (146 of 165, 88%) were investigated by one study and 19 (12%) by more than one study (range 2 to 8, median 2). The distribution of the biomarkers among the biomarker groups is shown in Table V.

In total, 63 biomarkers (n = 39 nongenetic, n = 24 genetic) were classified as significantly associated with radiological DCI (Supplementary material, Table S2, page 13-34). A summary of the studied and significant biomarkers and specific trends observed within each biomarker group is given in Table V. Of the significant biomarkers, 56 were investigated by one study and 7 by multiple studies. Meta-analyses were not possible. Based on the biomarker-specific figures, one biomarker, lactate/pyruvate ratio (LPR) in blood, was considered validated as two independent studies showed that an LPR higher than 40 was associated with DCI, measured during the first weeks following SAH (26,27). The quality of the two studies on LPR was 4/8 and 2/8 (Table 4). The biomarker-specific figures can be found in the supplemental data (Supplementary material, Figure S59-S77, page 46-48).

Among the 56 significant biomarkers investigated by one study, 7 nongenetic blood biomarkers were investigated by a high-quality study: monocyte LFA-1, monocyte Mac-1, monocyte PSGL-1, neutrophil LFA-1, neutrophil Mac-1, neutrophil PSGL-1, and osteopontin (22,28).

Clinical vs. Radiological DCI

Of all biomarkers, 89 were studied for both clinical and radiological DCI (Supplementary material, Table S2, page 13-34). Eight blood biomarkers were significantly associated with both clinical and radiological DCI (d-dimer, platelet activating factor, interleukin-17, lectin complement pathway activity, osteopontin, brain natriuretic peptide, urea/creatinine ratio and serine proteinase inhibitor family E member 1 SNP rs2227631 + rs1799889 + rs6092 + rs6090 + rs2227684 + rs7242). Forty-five biomarkers were classified as nonsignificant for both clinical and radiological DCI. Of the remaining 36 biomarkers, the classification into significant, inconclusive, or nonsignificant differed between clinical and radiological DCI.

Discussion

This review has selected seven biomarkers of clinical DCI (haptoglobin polymorphisms 2-1 and 2-2, ADAMTS13, NLR, P-selectin, and vWF in blood and F2-isoprostane in urine) and one biomarker of radiological DCI (LPR in MD) as most promising biomarkers of DCI. Of note, the majority of significant biomarkers (121/141 of clinical DCI and 56/63 of radiological DCI) were only evaluated by one study, and although being potential biomarkers, especially the 12 biomarkers from high-quality studies, are in need of validation.

In 2018, a review on biomarkers of DCI yielded a panel of 21 predictive biomarkers (3); however, this review had three substantial limitations, which may have misguided the selected panel of biomarkers: (1) patients with solely angiographic vasospasm and increased flow velocities on TCD ultrasound, as indication of DCI, were also included, (2) the results of biomarkers measured in different media (CSF and blood) were combined, and (3) no differentiation between predictive and diagnostic biomarkers was made. In our present review, the most up-to-date definition of DCI was strictly applied during the selection of eligible articles and a biological substance measured in multiple media was considered a separate biomarker for each medium. As a result, our review yielded less studies (209 vs. 270). Although we found more biomarkers (724 vs. 257), our review yielded

TABLE V - Number of studied and significant biomarkers and specific trends observed within each biomarker group of radiological DCI.

Biomarker group	Biomarkers of radiological DCI		Specific trends observed among significant biomarkers within biomarker group
	Studied	Significant	
Nongenetic biomarkers	**100**	**39**	
Brain injury	6	2	All indicated more brain injury in patients with DCI. No specific trend in time.
Coagulation cascade	16	9	All four biomarkers of primary hemostasis indicated increased platelet activation/aggregation, three of four secondary hemostasis biomarkers showed hypercoagulability, one biomarker showed hyperfibrinolysis in patients with DCI. No specific trend in time.
Cortical spreading depressions	–	–	–
Endothelial injury	4	2	Both biomarkers indicated increased endothelial injury in DCI.
Inflammation and immune system	33	15	12 of 15 inflammation and immune system markers indicated increased inflammation and elevated immune response in patients with DCI. No specific trend in time.
Oxidative stress	4	2	Both biomarkers indicated increased oxidative stress in patients with DCI.
Vascular tone	8	1	Vasoconstriction between days 0 and 14 after SAH in DCI.
Volemic status	2	2	More diuresis and natriuresis were observed in DCI. More DCI in hyponatremia vs. no hyponatremia.
Other	27	6	Subcategories were: angiogenesis, cerebral metabolism, lipid metabolism, stress response, and protein metabolism.
Genetic biomarkers	**63**	**24**	
Genomic	3	0	Function often unclear; however, four biomarkers were involved in processes of coagulation, inflammation, and vascular tone.
MicroRNA	28	19	
Methylation	–	–	
Polymorphism	5	1	
RNA	–	–	
Single nucleotide polymorphism	27	4	

DCI = delayed cerebral ischemia; RNA = ribonucleic acid; SAH = subarachnoid hemorrhage.

only eight validated biomarkers, as opposed to 21 by Jabbarli et al., of which two (ADAMTS13 and NLR) were in agreement (3). The discrepancy between the panels of biomarkers selected by both reviews emphasizes the importance of a uniform definition of DCI, especially in the absence of solid diagnostic criteria.

The eight validated biomarkers of DCI are all related to known pathways of the multifactorial pathophysiology of DCI. In clinical DCI, increased platelet aggregability, thereby potentially leading to the formation of microthrombi, was observed by decreased admission levels of ADAMTS13 and increased levels of vWF on day 4 after SAH. As DCI usually occurs between days 4 and 10 after SAH (4), the early onset of significantly differing levels makes these biomarkers potentially predictive of DCI. Notwithstanding, the quality of the studies was rather low (NOS scores of 3/8 and 4/9), therefore a high-quality study is necessary to further elucidate matters.

Most inflammation and immune system biomarkers seem to be significantly elevated early in the course of SAH. Accordingly, elevated NLR (a validated biomarker) in the first couple of days following SAH was found to be significantly associated with clinical DCI in four independent studies. But, the exact predictive ability, including determination of an optimal cut-off value with accompanying accuracy, specificity, and sensitivity, remains to be determined. Whether NLR could also serve as a preventive treatment target is debatable, as NLR is a highly nonspecific biomarker and nonspecific anti-inflammatory therapies have thus far proven disappointing for the prevention of DCI (29). P-selectin was found to significantly increase during clinical DCI onset in two studies, which suggests that P-selectin may be a potential candidate for diagnosis or therapeutic treatment, for example, P-selectin antagonism. Thus far, therapies targeting P-selectin have only been performed in experimental settings of ischemic stroke, showing a reduction in infarct volume and better clinical outcome (30), and myocardial infarction, showing significantly reduced myocardial damage after percutaneous coronary intervention (31). P-selectin antagonism has not been studied in SAH, yet.

Three validated biomarkers of oxidative stress were found for clinical DCI in our review: F2-isoprostane in urine and genetic biomarkers haptoglobin polymorphism 2-1 and 2-2. Genetic markers often require extensive processing time and

are therefore unsuitable for acute diagnosis; however, early determination of genetic profiles, for example, on admission, may aid in stratification of patients at high risk of clinical DCI. As such, the haptoglobin polymorphisms 2-1 and 2-2 may be useful for patient stratification, and could perhaps provide a potential preventive treatment target, for example, the administration of exogenous haptoglobulin (32). For F2-isoprostane, whether the observed increased urine levels have preceded the onset of clinical DCI in all patients is unknown; therefore, we cannot distinguish whether these biomarkers are predictive or diagnostic. Further research should be performed to explore the temporal relationship of these markers in regard to clinical DCI development.

The remaining validated biomarker, LPR in MD, allows for continuous neuromonitoring providing direct metabolic information, which can be used to detect an ischemic state, that is, radiological DCI. Since DCI currently is a clinical diagnosis per exclusion, LPR may be a helpful additive in comatose patients, as it may detect metabolic distress in an early phase already, before hypodensities are visible on noncontrast head CT and when ischemic brain injury may still be preventable. However, this invasive technique yields metabolic information only locally, resulting in a detection failure of at least 20% (27).

The difficulty of DCI is in the incomplete understanding of its pathophysiology and lack of hard diagnostic criteria. Currently, two definitions are used to identify patients with DCI: clinical and radiological DCI (4). Even though adherence to one definition yields a different selection of patients than the other, both are strongly related to poor clinical outcome at follow-up, therefore both seem to be relevant (33-35). Differences in the two definitions are clearly observed in this review, too, as 36 of 89 biomarkers studied for both definitions showed dissimilar results. The clinical definition seems to be the most relevant, given that early detection and correction of altered biomarkers may prevent the onset of clinical deficits. However, not all patients are neurologically assessable, therefore in comatose patients we currently rely on the radiological definition for DCI diagnosis. Considering that radiological appearance of ischemia or infarction is not immediately visible, once radiological DCI is diagnosed the treatment window for ischemic disease has often elapsed. Diagnosing radiological DCI based on biomarkers may lead to earlier detection of radiological DCI, perhaps even within the treatment window.

Limitations of this review are the heterogeneity in definitions used for DCI in literature, which complicates the inclusion of relevant studies, the methodological heterogeneity among included studies, which has resulted in a largely narrative review with a suboptimal method used for classification of biomarkers, and a high proportion of poor quality studies. The quality of studies was not implemented in our method used for classification. As a result, even though the results of studies of validated biomarkers have been reproduced by a second group, some of the selected validated markers rely on studies with relatively poor quality (e.g., ADAMTS13, vWF, and LPR). Strengths of this review are the strict use of the most up-to-date definition of DCI and the inclusion of all types of laboratory markers in blood as well as CSF.

Conclusion

In this review, seven biomarkers of clinical DCI (haptoglobin polymorphisms 2-1 and 2-2, ADAMTS13, NLR, P-selectin, and vWF in blood and F2-isoprostane in urine) and one biomarker of radiological DCI (LPR in MD) were selected as the most promising biomarkers of DCI. Future research should focus on determining the exact predictive or diagnostic abilities of these biomarkers, and exploring whether these biomarkers could serve as preventive or therapeutic treatment targets for DCI.

Disclosures

CRediT author statement: Maud A. Tjerkstra conceptualization, methodology, formal analysis, investigation, writing—original draft **Homeyra Labib** conceptualization, methodology, formal analysis, investigation, writing—review and editing **Bert A. Coert** writing—review and editing **René Spijker** conceptualization, methodology, writing—review and editing **Jonathan M. Coutinho** writing—review and editing **Dagmar Verbaan** conceptualization, methodology, writing—review and editing, supervision **W. Peter Vandertop** conceptualization, methodology, writing—review and editing, supervision.

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ORIGINAL RESEARCH ARTICLE
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Analytical performance of the FDA-cleared Parsortix® PC1 system

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ABSTRACT

Introduction: The Parsortix® PC1 system, Food and Drug Administration (FDA) cleared for use in metastatic breast cancer (MBC) patients, is an epitope-independent microfluidic device for the capture and harvest of circulating tumor cells from whole blood based on cell size and deformability. This report details the analytical characterization of linearity, detection limit, precision, and reproducibility for this device.

Methods: System performance was determined using K_2-EDTA blood samples collected from self-declared healthy female volunteers (HVs) and MBC patients spiked with prelabeled cultured breast cancer cell lines (SKBR3, MCF7, or Hs578T). Samples were processed on Parsortix® PC1 systems and captured cells were harvested and enumerated.

Results: The system captured and harvested live SKBR3, MCF7, and Hs578T cells and fixed SKBR3 cells linearly between 2 and ~100 cells, with average harvest rates of 69%, 73%, 79%, and 90%, respectively. To harvest ≥1 cell ≥95% of the time, the system required 3, 5 or 4 live SKBR3, MCF7 or Hs578T cells, respectively. Average harvest rates from precision studies using 5, 10, and ~50 live cells spiked into blood for each cell line ranged from 63.5% to 76.2%, with repeatability and reproducibility percent coefficient of variation (%CV) estimates ranging from 12.3% to 32.4% and 13.3% to 34.1%, respectively. Average harvest rates using ~20 fixed SKBR3 cells spiked into HV and MBC patient blood samples were 75.0% ± 16.1% (%CV = 22.3%) and 68.4% ± 14.3% (%CV = 21.1%), respectively.

Conclusions: These evaluations demonstrate the Parsortix® PC1 system linearly and reproducibly harvests tumor cells from blood over a range of 1 to ~100 cells.

Keywords: Blood, Breast cancer, Circulating tumor cells, Liquid biopsy, Microfluidic devices, Neoplastic cells

Introduction

There is increasing interest in using liquid biopsy in the clinical setting as a tool for personalized medicine through the detection and characterization of cells that could seed metastasis, for longitudinal monitoring of cancer patients in real time, identifying treatment regimens or response and/or minimal residual disease (MRD). A liquid biopsy is ideal for these purposes due to the minimally invasive and repeatable nature of a blood draw, in contrast to traditional tissue biopsies (1-3).

Circulating tumor cells (CTCs) are cancer cells that have intravasated into the bloodstream or lymphatic system from primary and/or metastatic tumors (4,5). CTCs released from primary tumors into the bloodstream can travel to distant sites and form secondary tumors, an integral part of the metastatic process. Epithelial-mesenchymal transition (EMT) is a crucial process enabling CTC detachment, intravasation *into* the circulation, extravasation *from* the circulation, and, ultimately, metastases formation (1,5-7). Cancer cells circulate both as single CTCs and CTC clusters, with CTC clusters showing an increased metastatic potential (5,8). Isolating and

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characterizing the cells that have the potential to metastasize (i.e., CTCs) is vitally important, considering that ~90% of cancer deaths are due to metastasis (9).

Isolation and interrogation of CTCs has been challenging due to the rarity of these cells in the bloodstream, with approximately one cell in 10^5–10^6 peripheral blood mononuclear cells (PBMCs) (4,5,10). Yet it has been documented that the detection of CTCs is a clinically relevant predictor of disease progression and overall survival in patients with metastatic breast cancer (MBC) (11-16). A recent study using the Parsortix® system to obtain CTCs from blood reported a high concordance in predictive biomarker expression between the CTCs and tissue from the metastases, demonstrating that analysis of CTCs may serve as a surrogate sample for evaluation of biomarkers for breast cancer metastases (17).

Many technologies have been developed for CTC isolation, including biological and physical-based methods (7,18,19). Biological-based methods isolate CTCs based on phenotype and rely on antibodies to select cells of interest. Many of these, including the CellSearch® System, isolate CTCs based on expression of the epithelial cell adhesion molecule (EpCAM), which is expressed by a subset of CTCs. CTCs released into the bloodstream are often either undergoing or have undergone EMT, which involves upregulation of mesenchymal gene expression and downregulation of epithelial gene expression (20). This results in failure of EpCAM-based methods to efficiently isolate mesenchymal and/or EMTing CTCs, the CTC phenotype that may be more representative of cells capable of forming distant metastases (19). In addition, the use of antibodies for capture could impact downstream analysis (18,21) of the captured CTCs. These limitations identify a critical need for physical-based, or epitope-independent, CTC isolation methods enabling the capture and subsequent characterization of broader CTC populations.

The Parsortix® PC1 system is a semi-automated, epitope-independent microfluidic device that captures and subsequently harvests rare cells (including CTCs) from whole blood based on their size and deformability (22-29). This physical-based method of CTC isolation is not reliant on specific antigen expression for capture and enables enrichment of both epithelial and mesenchymal cells (22,24,25,29). The system also harvests the captured cells from the microfluidic device into a small volume of buffer (~210 µL) for subsequent user-defined evaluation.

The studies reported here were designed to evaluate the analytical performance of the Parsortix® PC1 system for the capture and harvest of breast cancer cells, including assessment of linearity, limit of detection (LoD), precision, and reproducibility, using whole blood samples spiked with live or fixed cultured breast cancer cell lines (SKBR3, MCF7, or Hs578T). The breast cancer cell lines chosen for spiking are representative of the heterogeneity observed in breast cancer and breast cancer CTCs (30-33). The analytical performance data generated herein plus the previously published clinical performance data (34) were included in the de novo request for the classification of the Parsortix® PC1 system (DEN200062) as a Class II medical device, which the Food and Drug Administration (FDA) granted in May 2022 (Online).

Methods

Blood sample collection

Blood was collected via venipuncture from self-declared healthy female volunteers (HVs) and via either a venipuncture or an existing port from MBC patients into 10 mL K_2-ethylenediaminetetraacetic acid (EDTA) tubes (Becton Dickinson). A minimum of 7.5 mL of blood per tube, up to a maximum of 80 mL of blood per donation, was obtained from each HV. A minimum of 5 mL of blood per tube was obtained from each MBC patient. The HVs were self-declared healthy, nonpregnant women aged ≥18 years with no known current illness or other serious health condition. MBC patients were women with documented evidence of MBC (as determined by any means). All women (HVs and MBC patients) gave written informed consent prior to collection of the blood samples under protocols that were reviewed and approved by the London-Surrey Borders Research Ethics Committee or the University of Rochester Research Study Review Board.

Cell lines

The breast cancer cell lines SKBR3, MCF7, and Hs578T were obtained from the American Type Culture Collection (ATCC) and were chosen because they are representative of CTCs that would be found in the blood of patients with MBC based on their immunotype, phenotype, morphology, and size (Tab. I) (30-33,35-38). Cell line maintenance is detailed in the Supplementary Materials.

TABLE I - Comparison of phenotype, immunophenotype, morphology, and size for SKBR3, MCF7, Hs578T, and CTCs

	SKBR3	MCF7	Hs578T	MBC patient-derived CTCs
Breast cancer subtype	HER2 enriched	Luminal A	Basal/triple negative	All subtypes
Phenotype	Epithelial	Epithelial	Mesenchymal	Epithelial, EMT, and mesenchymal
Receptor status	ER/PR- and HER2+	ER/PR+ and HER2-	ER/PR- and HER2-	ER/PR+/- and HER2-/+
Morphology	Grape-like	Mass	Stellate	Highly polymorphic
Size	15-17 µm (microscope and counter pipette) (Coumans et al (35))	16.5 µm (counter pipette) (Coumans et al (35))	No data	13.1 µm (Coumans et al (35)) 11.79 (4.51-33.11) µm (Zhao et al (36)) 13.1 (12-25) µm (Hao et al (37)) 11 (6-16) µm^2 (Coumans et al (38))

CTC = circulating tumor cell; EMT = epithelial-mesenchymal transition; MBC = metastatic breast cancer.

For each study, using the abovementioned cell lines, live or fixed cells (fixed using 4% formaldehyde [Sigma Aldrich]) were fluorescently labeled with CellTracker™ Green 5-chloromethylfluorescein diacetate (CMFDA; Life Technologies) as per the manufacturer's instructions. The labeled cell lines were spiked into freshly collected blood samples as detailed below.

Blood sample preparation

For linearity, LoD, and live cell precision studies, spike levels of ≤100 cells were directly spiked, as a single droplet, onto the side of empty 12 mL polystyrene tubes. The number of cells in the droplet was counted by two independent operators using a Leica fluorescence microscope. When an accepted cell count within the droplet (see Tab. SI) was confirmed by two independent operators, 7.5 mL of blood was added to the tube, ensuring the blood flowed over the droplet containing the spiked cells. Spike levels of >100 cells were spiked directly into blood samples from cell dilutions prepared in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; w/v) and 2 mM EDTA (BEP) (total volume of spike ≤5 µL). The numbers of cells spiked were calculated by spiking the same volume of cell dilution into six wells of a 96-well plate, each well containing 200 µL of 1× PBS (control plate), and either counting manually or using the Leica fluorescent microscope software and taking the mean of the six control spikes.

For precision and reproducibility studies using ~20 fixed, prelabeled SKBR3 cells, microfuge tubes containing known numbers of fixed, prelabeled SKBR3 cells (range between 17 and 25 cells) in ~180 µL of BEP were prepared ("Precision Tubes"). For spiking, the contents of a Precision Tube were pipetted into either 2.5 mL of PBS or a whole blood sample (study dependent), the tube was rinsed with 80 µL of additional PBS, and the 80 µL of additional PBS was pipetted into the PBS or whole blood sample.

ANGLE R&D Laboratory located in Guildford, UK, prepared and processed the samples unless stated otherwise.

Blood sample processing

All blood samples were processed within 8 hours of collection using Parsortix® PC1 systems and GEN3 6.5 µm cell separation cassettes at a constant pressure of 99 mbar (22). Captured cells were harvested from the cassettes directly into single wells on 96-well plates or onto polytetrafluoroethylene ("PTFE") printed slides with 21 hydrophobic droplet spots for subsequent enumeration using a fluorescence microscope. The number of cells in each harvest was counted by a minimum of two independent operators.

Linearity evaluation

Linearity was determined for each cell line individually by preparing HV blood samples spiked with 2 to ~100 live or fixed (SKBR3 only), prelabeled cells (Tab. SI). Data were obtained from a minimum of 10 HVs per cell line and eight different spike levels, with each HV providing data for all spike levels. Additional blood samples were prepared by spiking ~125, ~250, ~500, and ~1000 live, prelabeled SKBR3 cells into 7.5 mL aliquots of blood. Data for the higher cell spike levels were obtained from a minimum of 10 HVs for each spike level, with each HV providing data for all spike levels. The linearity studies were designed in accordance with the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guideline *EP06-A: Evaluation of Linearity of Quantitative Measurement Procedures: A Statistical Approach.*

Limit of blank and LoD evaluation

Limit of blank (LoB) of the system was determined using unspiked HV blood samples. LoD was determined for each cell line individually by preparing HV blood samples directly spiked with 1, 2, 3, 4, or 5 live, prelabeled cells. Data were obtained from a minimum of 60 samples for each spike level and cell line, performed in accordance with the CLSI guideline *EP17-A2: Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline—Second Edition.*

Precision evaluation

The precision studies were conducted in accordance with the CLSI guideline *EP05-A3: Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline–Third Edition*. Imprecision estimates for the Parsortix® PC1 system were evaluated for the different cell lines and spike levels using all three cell lines directly spiked into 7.5 mL aliquots of HV blood. For each cell line (SKBR3, MCF7, and Hs578T), 7.5 mL aliquots of blood from HVs were spiked with 5, 10, or ~50 live, prelabeled cells. For each spike level and cell line, data were obtained from a total of 100 samples, processed on 10 Parsortix® PC1 systems, two runs per system per day over a total of 5 nonconsecutive days. Additional precision studies evaluating fixed versus live cells, different cassette lots, and different laboratories were also conducted, and a description of these studies and their results can be found in the Supplementary Materials.

Reproducibility evaluation

A separate reproducibility study was conducted at the University of Rochester Medical Center (URMC). For this study, ≥5 mL of blood was collected from HVs and MBC patients into 10 mL K_2-EDTA tubes. The blood tubes were spiked with ~20 fixed SKBR3 cells using Precision Tubes. Data were collected from a total of 150 spiked samples (76 HVs and 74 MBC patients) processed on four different Parsortix® PC1 systems.

Data analysis

A detailed description of the data analysis methods used for each study can be found in the Supplementary Materials.

Results

Linearity

The harvesting of live, prelabeled SKBR3, MCF7, and Hs578T and fixed SKBR3 cells by the Parsortix® PC1 system

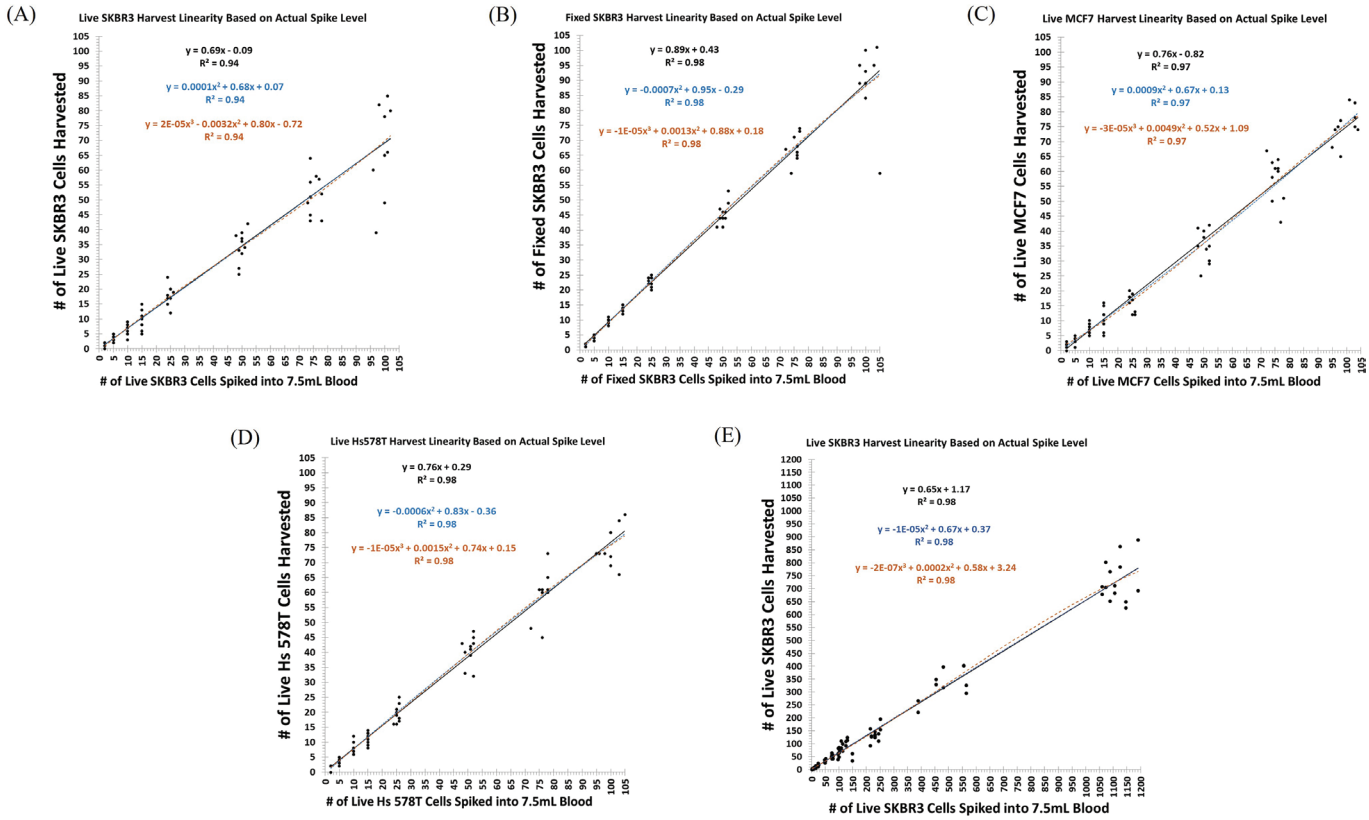

Fig. 1 - Parsortix® PC1 system observed linearity when processing blood samples spiked with a range of cell numbers for live SKBR3, MCF7, and Hs578T cells and fixed SKBR3 cells. Linear (first-order polynomial) regression analysis indicated in black, second-order polynomial regression analysis indicated in blue, and third-order polynomial regression analysis indicated in orange. (A) Live SKBR3 harvest linearity for between 2 and ~100 cells spiked into 7.5 mL of blood, with first-, second-, and third-order polynomial regression analyses shown (34). (B) Fixed SKBR3 harvest linearity for between 2 and ~100 cells spiked into 7.5 mL of blood, with first-, second-, and third-order polynomial regression analyses shown. (C) Live MCF7 harvest linearity for between 2 and ~100 cells spiked into 7.5 mL of blood, with first-, second-, and third-order polynomial regression analyses shown (34). (D) Live Hs578T harvest linearity for between 2 and ~100 cells spiked into 7.5 mL of blood, with first-, second-, and third-order polynomial regression analyses shown (34). (E) Live SKBR3 harvest linearity for between 2 and ~1,000 cells spiked into 7.5 mL of blood, with first-, second-, and third-order polynomial regression analyses shown.

was demonstrated to be linear over the range of 2 to ~100 cells spiked into 7.5 mL of blood (Fig. 1A-D). Additionally, linearity of harvest was shown to extend to at least 1,000 live SKBR3 cells spiked into 7.5 mL of blood (Fig. 1E). The best fit model for all cell lines was the first-order linear regression model (the solid black line).

Over the range of 2 to ~100 *live* SKBR3 cells, the linear regression model had a slope of 0.69, indicating an average harvest rate of ~69% (95% confidence interval [CI] = 65%-73%, R^2 = 0.94) (Fig. 1A) (34), whereas over the same range, *fixed* SKBR3 cells resulted in a slope of 0.89, indicating an average harvest rate of ~89% (95% CI = 85%-92%, R^2 = 0.98) (Fig. 1B). The data show both an increased variability for the recovery of live SKBR3 cells and better recovery of the fixed SKBR3 cells. This was not unexpected due to the effects of fixation on cultured cells, resulting in reduced deformability of the fixed cells compared to live cells. Over the range of 2 to ~100 live MCF7 cells, the linear regression model had a slope of 0.76, indicating an average harvest rate of ~76% (95% CI = 73%-79%, R^2 = 0.97) (Fig. 1C) (34) and over the same range, live Hs578T cells showed similar results with a slope of 0.76, indicating an average harvest rate of ~76% (95% CI = 74%-79%, R^2 = 0.98) (Fig. 1D) (34). Average harvest rates for each cell spike level, along with the standard deviation (SD) and percent coefficient of variation (%CV) for each cell line can be found in Tables SII–SV.

Detection limit (LoB, LoD)

Using a total of 63 unspiked blood samples, the LoB of the Parsortix® PC1 system was determined to be zero cells, as expected. Only one (1.6%) of the 63 unspiked blood samples was found to have a single prelabeled cell harvested. Considering the results from a separate cell carryover study where no carryover of cells was observed after the spiking of >2,000 cultured cells, the observation of a single cell in an unspiked sample is likely the result of a user error (e.g., improper labeling of the sample as a control when it was actually a spiked sample).

The LoD was determined for each cell line separately using a type II error level (β = false negative rate) of <5%, which equated to the spike level at which ≥95% of the replicate

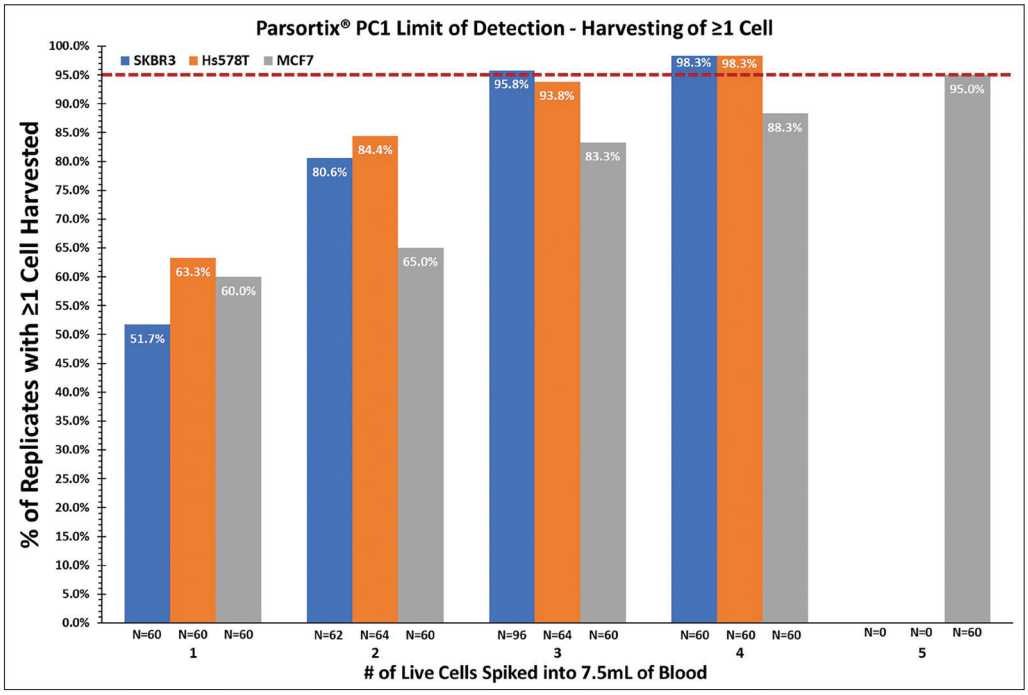

Fig. 2 - Parsortix® PC1 system limit of detection. Percent of replicates with ≥1 cell harvested when blood was spiked with 1, 2, 3, 4, or 5 live SKBR3, MCF7, or Hs578T cells. *Note*: For the SKBR3 and Hs578T cell lines, the 5-cell spike level was not evaluated as both of these cell lines showed recoveries of >1 cell at lower cell spike levels.

samples for a given cell line had ≥1 cell observed in the harvests (Fig. 2). For the SKBR3 and Hs578T cell lines, spiking of 5 live cells into blood was not performed as the detection limit, as per the specified definition, had already been reached at a cell spike level of fewer than 5 cells.

For live SKBR3 cells, the LoD was 3 cells (95.8% of the replicates spiked with 3 cells had ≥1 cell observed in the harvest); for live MCF7 cells, the LoD was 5 cells (95.0% of the replicates spiked with 5 cells had ≥1 cell observed in the harvest); and for live Hs578T cells, the LoD was 4 cells (98.3% of the replicates spiked with 4 cells had ≥1 cell observed in the harvest).

Precision

Precision studies were performed to assess the ability of the Parsortix® PC1 system to harvest multiple live breast cancer cell lines that are representative of the heterogeneity observed in breast cancer and breast cancer CTCs (30-33) at different spike levels. For each cell line (SKBR3, MCF7, and Hs578T) and spike level, 100 contrived samples consisting of live, prelabeled cells spiked into blood were processed using 10 different Parsortix® PC1 systems over 5 nonconsecutive days in each study. Imprecision estimates in these 5-day precision studies were determined for each cell line at three different spike levels (5, 10, and ~50 cells), with the cells being spiked into 7.5 mL of HV blood. The overall average harvest rates for the SKBR3, MCF7, and Hs578T cell lines were 72.0%, 70.9%, and 66.7%, respectively. The average harvest rates for each of the cell lines and cell spike levels ranged from 63.5% to 76.2%, the repeatability (within-run) %CV estimates ranged from 12.3% to 32.4%, and the reproducibility (within-laboratory) %CV estimates ranged from 13.3% to 34.1% (Tab. II). The repeatability and reproducibility %CV estimates for all of the cell line and cell spike level results combined were 26.3% (95% CI = 25.0%-27.6%) and 26.3% (95% CI = 25.2%-27.8%), respectively, with between-cell-type and between-spike-level %CV estimates of 6.6% (95% CI = 4.0%-10.6%) and 4.6% (95% CI = 1.6%-8.3%), respectively.

Additional precision studies were performed to evaluate the precision of the Parsortix® PC1 system for the capture and harvesting of tumor cells, taking into consideration changes in different variables including live versus fixed cells, separation cassette lots, and laboratory sites. A graphical representation of results from these additional precision studies evaluating live versus fixed cells, different cassette lots, and different laboratories can be seen in Figure 3, with further details provided in the Supplementary Materials.

Reproducibility

The overall combined average percentage of the ~20 prelabeled, fixed SKBR3 cells spiked into the blood samples from the 76 evaluable HV subjects and 74 evaluable MBC patients that were harvested was 70.0% ± 15.4% (95% CI = 62.3%-76.7%, median = 70.7%). The average percentage of spiked SKBR3 cells harvested in the HV subjects was 72.1% ± 16.1% (95% CI = 61.1%-80.9%, median = 75.0%) compared to 67.9% ± 14.3% (95% CI = 56.7%-77.4%, median = 68.4%) in the MBC patients (t-test p value = 0.098). Figure 4 shows a scatterplot of the percent harvest results in the HV subjects and MBC patients.

Although the average percent harvest results were not statistically significantly different between the two groups, the average percent harvest results in the MBC patients appeared to be slightly lower. As this was not expected, a possible reason for this was investigated, the results of which are described in the Supplementary Materials

TABLE II - Average harvest rates and imprecision estimates for the Parsortix® PC1 system when harvesting live SKBR3, MCF7, and Hs578T cells spiked into 7.5 mL of blood (2-factor ANOVA models using day and run as the factors)

Cell line	# of cells spiked	N	Mean % harvest	Within-run (Repeatability)		Between run		Between day		Within laboratory	
				SD	CV%	SD	%CV	SD	%CV	SD	%CV
Live SKBR3	5	100	73.6	22.8%	31.0	6.7%	9.1	0.0%	0.0	23.7%	32.2
	10	100	70.4	18.5%	26.3	0.0%	0.0	4.4%	6.3	19.1%	27.1
	~50 (48-52)	100	72.1	13.8%	19.1	0.0%	0.0	0.0%	0.0	13.8%	19.1
	5-~50	300	72.0	18.6%	25.8	3.5%	4.9	0.0%	0.0	18.9%	26.3
Live MCF7	5	100	68.0	22.0%	32.4	7.3%	10.7	1.3%	1.9	23.2%	34.1
	10	100	68.4	18.4%	26.9	0.0%	0.0	6.3%	9.2	19.5%	28.5
	~50 (48-52)	100	76.2	11.3%	14.8	0.0%	0.0	3.4%	4.5	11.8%	15.5
	5-~50	300	70.9	18.5%	26.1	2.1%	3.0	1.6%	2.3	18.7%	26.4
Live Hs578T	5	100	66.6	19.9%	29.9	5.9%	8.9	3.6%	5.4	21.0%	31.5
	10	100	63.5	17.8%	28.0	0.0%	0.0	7.5%	11.8	19.3%	30.4
	~50 (48-52)	100	70.0	8.6%	12.3	0.0%	0.0	3.4%	4.9	9.3%	13.3
	5-~50	300	66.7	16.8%	25.2	2.3%	3.4	3.8%	5.7	17.3%	25.9
Live SKBR3, MCF7, and Hs578T	5	300	69.4	22.6%	32.6	3.6%	5.2	0.0%	0.0	22.8%	32.9
	10	300	67.4	18.6%	27.6	0.0%	0.0	3.8%	5.6	19.0%	28.2
	~50 (48-52)	300	72.8	11.6%	15.9	0.0%	0.0	2.6%	3.6	11.9%	16.3
	5-~50	900	69.9	18.4%	26.3	1.4%	2.0	0.0%	0.0	18.4%	26.3

ANOVA = analysis of variance; %CV = percent coefficient of variation; SD = standard deviation.

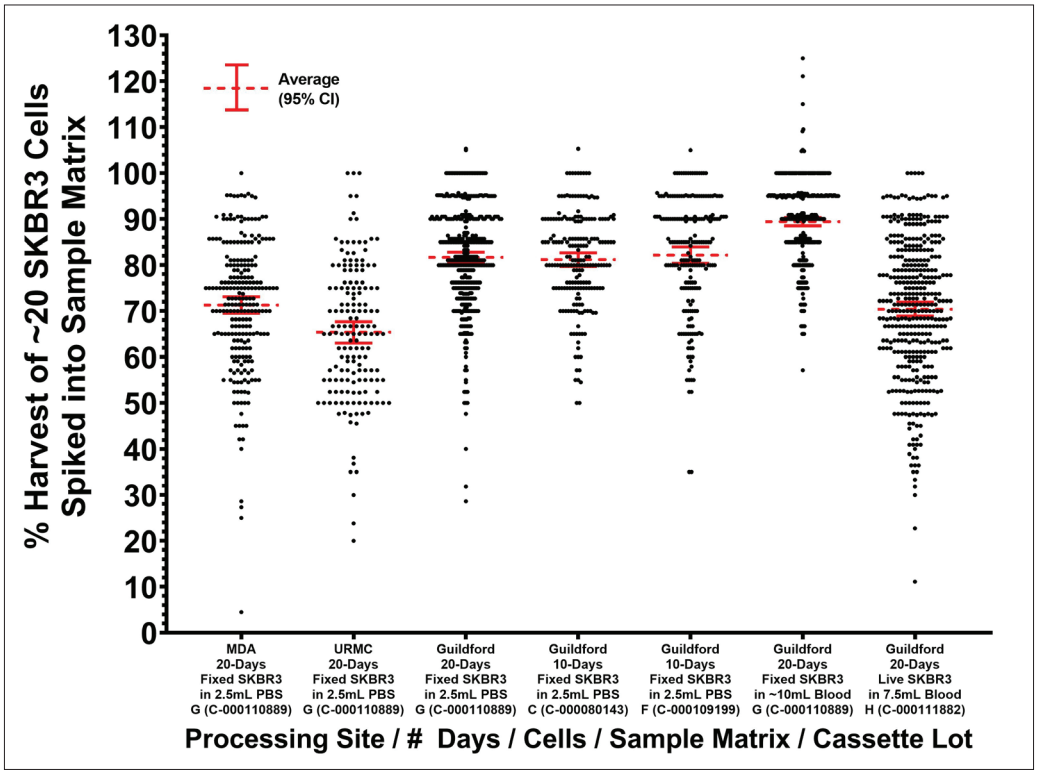

Fig. 3 - Precision of the Parsortix® PC1 system. Percent harvest (average and 95% CI shown) when spiking ~20 fixed SKBR3 cells into 2.5 mL PBS at different laboratory sites (MDA, URMC, ANGLE R&D) and using different separation cassette lots (Lot G, Lot C, and Lot F) or spiking ~20 fixed SKBR3 into 10 mL of blood, or ~20 live SKBR3 cells into 7.5 mL of blood. CI = confidence interval; PBS = phosphate-buffered saline.

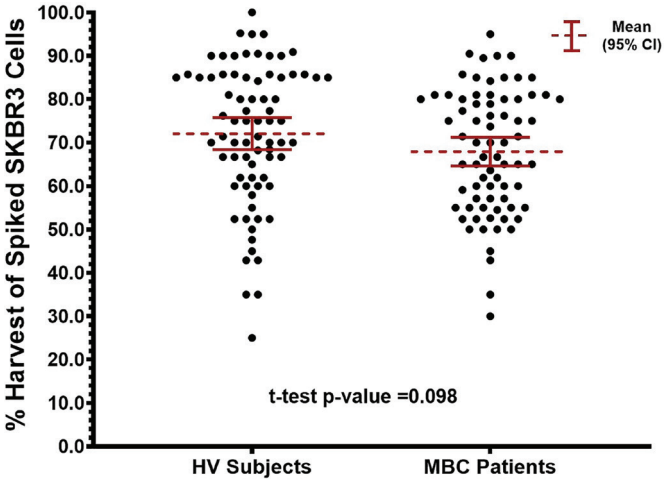

Fig. 4 - Reproducibility of the Parsortix® PC1 system. Percent harvest results from HV subject and MBC patient blood samples (≥5 mL of blood from a 10 mL K_2-EDTA tube) spiked with ~20 fixed, prelabeled SKBR3 cells. EDTA = ethylenediaminetetraacetic acid; HV = healthy female volunteer; MBC = metastatic breast cancer.

(Supplementary Figure 1A-B). This investigation showed significant differences in the average percent harvest results between different batches of fixed SKBR3 cells, providing evidence that the model system used in the analytical studies (i.e., cultured cell lines) contributed to the observed variabilities. This suggests that the actual imprecision of the Parsortix® PC1 system for the capture and harvest of CTCs may be lower than what was observed in these studies.

Conclusions

The analytical performance data show that the Parsortix® PC1 system harvested cultured breast cancer cells in a reproducible and linear fashion, with a minimum of 3 to 5 cells per 7.5 mL of blood required to ensure at least one cell will be harvested most of the time. The average repeatability and reproducibility %CV estimates for all of the live cell lines, chosen because they are representative of the heterogeneity expected to be seen in breast cancer CTCs, and all spike levels (5, 10, and ~50 cells) were both 26.3%, with the imprecision estimates decreasing as the number of cells spiked increased. Comparison of live and fixed SKBR3 cells showed that imprecision in the percentage of cells harvested by the system was higher when using live breast cancer cells, which was expected due to the increased deformability of live cells compared to more rigid fixed cells. This increased variability in harvest rate was also observed in the linearity data when comparing live and fixed breast cancer cells.

The analytical performance data reported here plus the previously published clinical performance data (34) were included in the submission for the de novo request for classification of the Parsortix® PC1 system (DEN200062) as a Class II medical device, which was granted by the FDA in May 2022 (Online). The Parsortix® PC1 system is the first FDA-cleared medical device for the capture and harvest of CTCs from the blood of MBC patients for subsequent, user-validated analysis.

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ORIGINAL RESEARCH ARTICLE
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Impact of clinico-biochemical and inflammatory biomarkers on the immunogenicity and efficacy of SARS-CoV-2 adenoviral vaccine: a longitudinal study

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ABSTRACT

Purpose: Due to a lack of effective antiviral treatment, several vaccines have been put forth to curb SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infection and to reduce the mortality and morbidity rate by eliciting a protective immune response, primarily through virus-neutralizing antibodies specific for SARS-CoV-2 spike protein. This longitudinal study was designed to evaluate the vaccine effectiveness and immune response following the administration of adenoviral vaccine, *COVISHIELD*, in Indian population who were previously uninfected with SARS-CoV-2 and to reveal the effect of various sociodemographic, inflammatory and biochemical factors on antibody response.

Methods: Briefly, the total immunoglobulin G (IgG) against SARS-CoV-2 spike and nucleocapsid protein along with the immunological markers were estimated by chemiluminescent microparticle immunoassay (CMIA) technology. Biochemical parameters were estimated by spectrometry.

Results: A total of 348 subjects received two doses of *COVISHIELD* (224 males, 124 females). The mean age of the study subjects was 42.03 ± 13.54 years. Although both the doses of COVISHIELD against SARS-CoV-2 spike protein induced a robust immune response that lasted for months in all the subjects, the total IgG titer against SARS-CoV-2 spike protein was found significantly higher in subjects ≥50 years of age, and those with obesity, elevated triglycerides and elevated lactate dehydrogenase levels.

Conclusions: There is a definite effect of age and biochemical factors on the immunogenicity of COVISHIELD. An understanding of these factors could not only impact the design of vaccines and help improve vaccine immunogenicity and efficacy but also assist in decisions on vaccination schedules, in order to combat this deadly pandemic.

Keywords: COVID-19, CRP, IgG, LDH, SARS-CoV-2, Spike protein, Total antibodies, Vaccines

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused panic, with the World Health Organization (WHO) declaring it as a global health emergency on January 30, 2020 (1,2). Since the start of the pandemic, SARS-CoV-2 has affected more than 50,59,75,895 people, causing more than 62,03,293 deaths worldwide by March 2022 (3). As of April 20, 2022, the total number of cases in Jammu and Kashmir was 4,53,955 including 4,49,140 cures and 4,751 deaths (4). Total vaccine doses administered in the union territory of Jammu and Kashmir were 2,24,75,070 (5). Although several treatment strategies have been developed, vaccination is still the most important means to combat SARS-CoV-2 infection or to curb the coronavirus disease 2019 (COVID-19) pandemic (6).

The Indian national vaccination program started from January 16, 2021, after the approval of two candidate vaccines namely *COVISHIELD* (ChAdOx1-nCOV-19 or AZD1222, acquired from Oxford University and AstraZeneca, manufactured by Serum Institute of India, Pune—a chimpanzee

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adenovirus-vectored vaccine expressing the SARS-CoV-2 spike protein) and COVAXIN (BBV-152, manufactured by Bharat Biotech, Hyderabad, in collaboration with Indian Council of Medical Research—an inactivated whole-virus vaccine) (7,8). *COVISHIELD* is one of the most commonly used vaccines in India, eliciting the antibody response against SARS-CoV-2 spike protein. *COVAXIN* is yet to get WHO approval, thus the popularity of this vaccine among common Indian masses is relatively less.

All vaccines follow the same basic principle, pre-exposing the immune system of a person to either killed or weakened pathogens, or some of the pathogen's structural parts, which leads the body to elicit an immune response (9). Similar to infection, vaccines result in early production of serum immunoglobulin (Ig)A, IgM and IgG antibodies, and also induce long-lasting memory B- and T-cell responses (10-12). *Adenoviral vaccines* are those in which viral gene delivery systems rely on a host viral genome (e.g., adenovirus) that typically lacks the genetic components necessary to produce new virions and that encodes antigenic components of the virus of interest to elicit a protective immune response, primarily through virus-neutralizing antibodies specific for spike protein of SARS-CoV-2. Such antibodies usually block the interaction of the virus with its cellular receptor or prevent conformational changes required for fusion of the virus with the host cell membrane and prevent the infection (13-15).

Although not very well established, various sociodemographic and clinico-biochemical factors affect the antibody production in response to vaccination. Male gender and higher age have been demonstrated to hamper SARS-CoV-2 vaccine immunogenicity (16). Similarly, different ethnic groups demonstrated varied antibody responses indicating a genetic influence on vaccine immunogenicity (17). In chronic obstructive pulmonary disease (COPD) and other lung infections, not only the initial response to pathogens and vaccines but also the strength with which the adaptive immune system responds to such challenges is impaired, leading to recurrent infections (18). Hypertension has been strongly associated with lower antibody titers in response to COVID-19 messenger ribonucleic acid (mRNA) vaccine (19). Hyperglycemia has been found to inhibit interleukin-6 (IL-6), thereby suppressing the antibody production (20). It was reported that obesity increased the likelihood of a poor vaccine-induced immune response (21). Obesity, dyslipidemia and excess adipose tissue could block the supply of nutrients to immune cells, leading to a constant low-grade inflammation thereby disrupting some immune responses, including those launched by T cells which can reduce the subsequent antibody production (22,23). Several studies showed that in severe SARS-CoV-2 infection, there is an increased serum level of inflammatory markers, such as C-reactive protein (CRP), lactate dehydrogenase (LDH), ferritin, D-dimer, and IL-6, all of which may result in cytokine storm (24) and lower levels of lymphocyte and platelets (25). IL-6 can reduce the production of fibronectin, albumin, and transferrin as well as the promotion of CD4+ T helper cells, which initiates the linking of innate and acquired immunity (26). Moreover, IL-6 is synthesized in the initial stages of inflammation and induces a number of acute-phase proteins, including CRP (27). High CRP has been shown to suppress T-cell function in autoimmune mouse models and to suppress dendritic cell (DC) function in normal people (28). More recent data suggest that hyperferritinemia may suppress immune responses by its ability to induce production of the anti-inflammatory cytokine IL-10 in lymphocytes (29).

Safety and immunogenicity are the major concerns regarding vaccines as their immunogenicity varies between individuals. Identifying the factors that affect the variability of antibody response to vaccines will not only contribute to studies on vaccines but also help to draft the strategies to combat this deadly pandemic as multiple variants of concern (VOCs) have the potential to jeopardize the effectiveness of vaccines. Overall no suitable study has been done till date that depicts the effect of various inflammatory and biochemical markers on antibody titer in response to vaccines against SARS-CoV-2 infection and vice versa. Therefore, the aim of this longitudinal study was to evaluate the vaccine effectiveness and immune response following the administration of *COVISHIELD* vaccine in Indian population who were previously uninfected with SARS-CoV-2 and to reveal the effect of various sociodemographic, inflammatory and biochemical factors that affect antibody response.

Materials and methods

Ethics statement

This study was performed in line with the principles of the Declaration of Helsinki. Ethical clearance for the study was sought from the Institutional Review Board, Government of Medical College Srinagar vide No. 1003/ETH/GMC. Informed consent both in vernacular as well as in English language was taken from the participants. Standard questionnaire or patient proforma was properly recorded and drafted as per sociodemographic and clinico-biochemical parameters. Vaccination data, including the name of the vaccine, number of doses received, dates, and place of administration, and clinical manifestations of COVID-19 were also recorded.

Study design

This was a longitudinal study undertaken in the Department of Biochemistry in collaboration with the Department of Social and Preventive Medicine, Government of Medical College Srinagar, from August 2021 to March 2022. Previously uninfected individuals receiving *COVISHIELD* vaccine were taken for the study. The study subjects were evaluated for various serum-based biomarkers at two different points of time. Figure 1 contains flow diagram depicting the elaborated study design.

Inclusion and exclusion criteria

All those individuals >18 years of age who received both doses of *COVISHIELD* and were not previously infected with SARS-COV-2 were included in the study. Individuals having any sort of genetic abnormality or malignancy were excluded from the study.

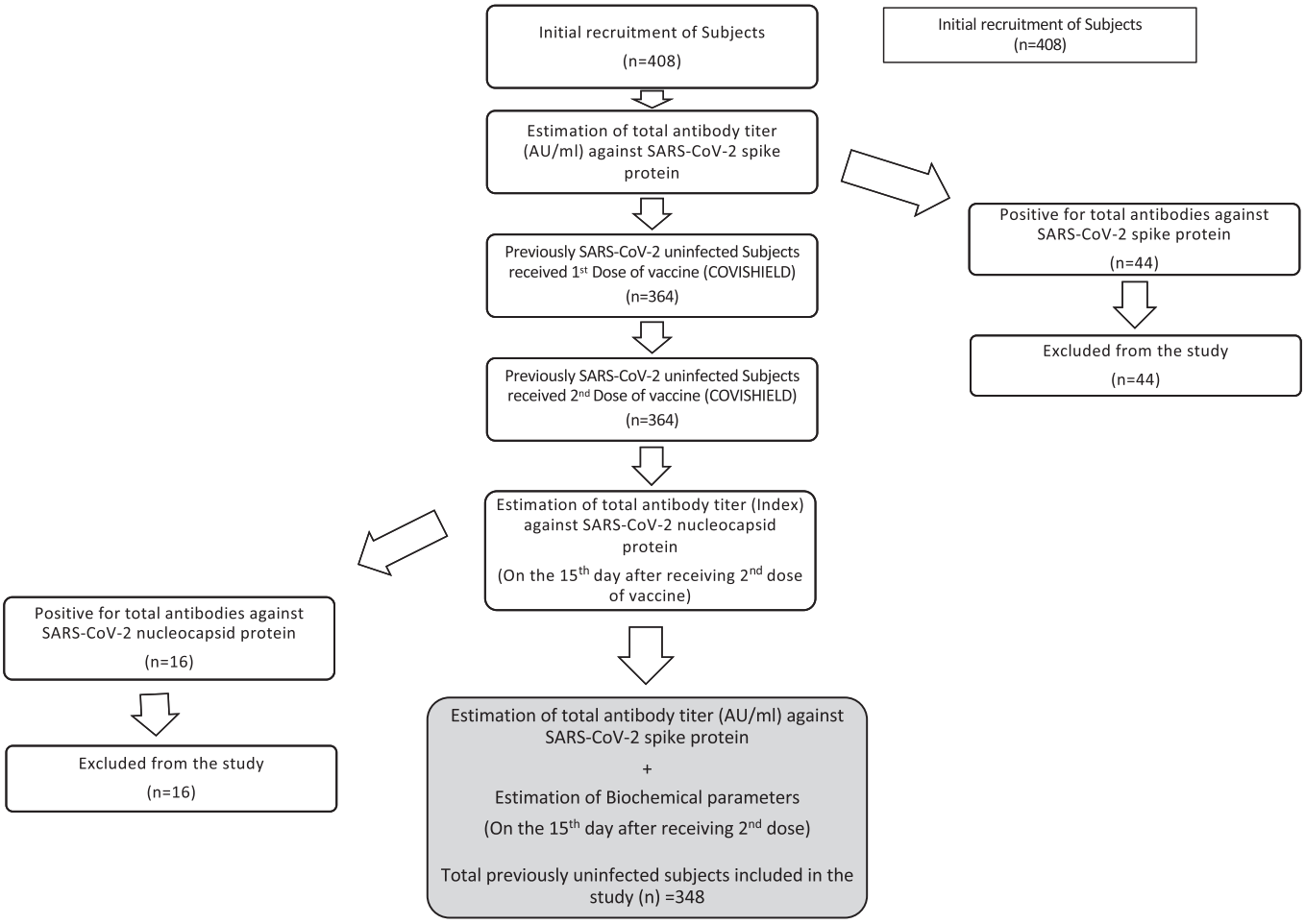

Fig. 1 - Schematic representation of study design.

Study subjects and sample size

A total of 348 participants who were previously uninfected with SARS-CoV-2 were taken for the final study. We estimated previous seroprevalence from the available data to be 40%. We expected that about 10% of the recruited subjects may have been naturally infected before, during or after the vaccination. We calculated sample size on the basis of above criteria with 5% random error and 80% power of study using G power version 20.1.0. The sample size came out to be 369. Adding 10% of the subjects (which may fall under the naturally infected category) the total sample size calculated was 406 (369 + 37). Ultimately, a total of 408 subjects were recruited for the study.

Sample collection

Four milliliters of venous blood sample was obtained from each study subject before vaccination and on the 15th day after receiving the second dose of vaccine. Blood samples were collected in red top (clot activator) vials and thereafter centrifuged at 4,500 rpm for 2 min at normal temperature for separation of serum. Serum samples were immediately stored at −20°C till further analysis.

Quantitative estimation of total antibodies (IgG) against SARS-CoV-2 spike protein

Before vaccination, a total of 408 individuals underwent quantitative estimation of total IgG against SARS-CoV-2 spike protein to rule out any previous natural infection from SARS-CoV-2. Subsequently, all those individuals who were negative for IgG against SARS-CoV-2 nucleocapsid protein (n = 348) were taken for further estimation of total IgG against SARS-CoV-2 spike protein on the 15th day in response to the second dose of *COVISHIELD.* The estimation was done on Abbott ARCHITECT i2000SR fully automated immunoassay analyzer using SARS-COV-2 IgGII Quant Reagent Kit (Abbott, USA). This procedure uses chemiluminescent microparticle immunoassay (CMIA) technology for the estimation of antibodies. The positive levels of SARS-CoV-2 total IgG were taken as ≥50.0 AU/mL.

Semi-quantitative estimation (index) of total antibodies (IgG) against SARS-CoV-2 nucleocapsid protein

On the 15th day after receiving second dose of *COVISHIELD*, all the previously uninfected study subjects (n = 364) were taken for the estimation of antibodies (Index) against SARS-CoV-2 nucleocapsid protein to rule out any natural infection

from SARS-CoV-2 after first or second dose of *COVISHIELD.* The estimation was done on Abbott ARCHITECT i2000SR fully automated immunoassay analyzer using SARS-CoV-2 IgG Reagent Kit (Abbott, USA). This procedure uses CMIA technology for the estimation of antibodies.

Determination of BMI

All the enrolled individuals (n = 348) underwent anthropometric assessment that included measurements of weight and height. Weight was measured without shoes using digital scales in an upright position and recorded to the nearest of 0.1 kg. Height was measured again without shoes in a standing position using a stadiometer to the nearest 0.1 cm. Body mass index (BMI) was calculated as per the formula: BMI (kg/m^2) = Weight (kg)/Height (m^2). BMI is an index of adiposity: individuals with BMI <18.5 kg/m^2 were considered underweight; 18.5-24.99 kg/m^2 as normal; 25-29.99 kg/m^2 as preobese; 30-34.99 kg/m^2 as obese class I and 35-39.99 kg/m^2 as obese class II.

Quantitative estimation of biochemical parameters

All the previously uninfected individuals who received *COVISHIELD* (n = 348) underwent estimation of fasting blood glucose, total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) on the 15th day after receiving the second dose of vaccine. Glucose was estimated by hexokinase/glucose-6-phosphate dehydrogenase assay using Glucose Reagent Kit (Abbott, USA). TC was estimated by cholesterol esterase method using Cholesterol Reagent Kit (Abbott, USA). TGs were estimated enzymatically using Triglyceride Reagent Kit (Abbott, USA). LDL-C was estimated by liquid selective detergent using MULTIGENT Direct LDL Reagent Kit (Abbott, USA). HDL-C was estimated by accelerator selective detergent using Ultra HDL Reagent Kit (Abbott, USA). These biochemical parameters were estimated on *Abbott ARCHITECT c4000SR* fully automated clinical chemistry analyzer. The reference ranges of analytes were taken as: fasting blood glucose: 70-126 mg/dL; TC: 50-200 mg/dL; TG: 50-200 mg/dL; LDL: ≤120 mg/dL (normal); HDL: >40 mg/dL in males (normal), >45 mg/dL in females (normal).

Quantitative estimation of Thyroid-Stimulating Hormone

All the previously uninfected individuals who received *COVISHIELD* (n = 348) were taken for the estimation of thyroid-stimulating hormone (TSH) on the 15th day after receiving the second dose of vaccine. This parameter was estimated on Abbott *ARCHITECT i2000SR* fully automated immunoassay analyzer using *TSH Reagent Kit* and employing the technology of CMIA. The reference range of TSH was taken as 0.35-5.2 uIU/mL.

Quantitative estimation of ferritin and IL-6

All the previously uninfected individuals who received *COVISHIELD* (n = 348) were taken for the estimation of ferritin and IL-6 on the 15th day after receiving the second dose of vaccine. These parameters were estimated on *Siemens ADVIA Centaur XP* fully automated immunoassay analyzer using *ADVIA Centaur FER Reagent Kit* and *ADVIA Centaur IL6 Reagent Kit* (Siemens, USA) and employing the technology of CMIA. The reference range was taken as: ferritin: 22-274 ng/mL in males, 05-204 ng/mL in females; IL-6: 0-4.4 pg/mL.

Quantitative estimation of LDH and CRP

All the previously uninfected individuals who received *COVISHIELD* (n = 348) were taken for the estimation of LDH and CRP on the 15th day after receiving the second dose of vaccine. These parameters were estimated on *Siemens Dimension RXL Max* fully automated clinical chemistry analyzer using *LDH Flex® reagent cartridge* and *RCRP Flex® reagent cartridge* (Siemens, USA) and employing the technology of spectrophotometry. The reference range was taken as: LDH: 125-220 U/L; CRP: 0-0.35 mg/dL.

Statistical analysis

Descriptive statistics was used to represent levels in terms of means ± standard deviation (SD). The total antibody (IgG) titer was converted to log scale for linearization. Linear regression analysis was performed to assess the relationship between antibody response and various sociodemographic, clinico-biochemical and inflammatory markers. The data of various categorical variables were skewed, so Mann-Whitney U-test was performed as there are different participants in each group, with no participant being in more than one group. The distribution of scores for subgroups of the independent variables had different shape, so the Mann-Whitney U-test was actually used to compare mean ranks. Statistical analysis was performed using Statistical Package for the Social Sciences v. 23.0 statistical software.

Results

A total of 408 subjects were taken for quantitative estimation of total antibodies (IgG) against SARS-CoV-2 spike protein before receiving any dose of vaccine. Out of 408 subjects only 44 tested positive for total IgG against SARS-CoV-2 spike protein and were excluded from the study. Consequently, a total of 364 previously uninfected subjects received two doses of *COVISHIELD* 84 days apart. On the 15th day after receiving the second dose of vaccine, semi-quantitative estimation of antibodies against SARS-CoV-2 nucleocapsid protein was done in all 364 subjects. Out of 364 subjects, only 16 tested positive for total IgG against nucleocapsid protein and were excluded from the study. Hence, a total of 348 subjects who were previously uninfected with SARS-CoV-2 and received two doses of *COVISHIELD* were taken for the estimation of clinico-biochemical, inflammatory parameters and total antibodies (IgG) against SARS-CoV-2 spike protein.

Characteristics of study subjects

Table I contains sociodemographic and clinico-biochemical characteristics of previously uninfected individuals. The sociodemographic factors including age, gender and ethnicity were studied to find out the distribution of these factors in the subjects. In this study, subjects were categorized

TABLE I - Sociodemographic and clinico-biochemical characteristics of enrolled individuals previously uninfected with SARS-CoV-2

Parameters	Study subjects N = 348 (%)
Age group	
<50 years	236 (67.8)
≥50 years	112 (32.2)
Gender	
Male	224 (64.4)
Female	124 (35.6)
Ethnicity	
Nonlocal	292 (83.9)
Local	56 (16.1)
H/o lung disease	
No	336 (96.6)
Yes	12 (3.4)
Hypertension	
No	292 (83.9)
Yes	56 (16.1)
T2DM	
No	296 (85.1)
Yes	52 (14.9)
BMI (kg/m²)	
Normal	152 (43.7)
Underweight	32 (9.2)
Preobese	116 (33.3)
Obese class I	44 (12.6)
Obese class II	04 (1.1)
Total cholesterol	
Normal	268 (77.0)
Elevated	80 (23.8)
TG	
Normal	208 (59.8)
Elevated	140 (40.2)
LDL-C	
Normal	228 (65.5)
Elevated	120 (34.5)
HDL-C	
Normal	144 (41.4)
Low	204 (58.6)
TSH	
Normal	264 (75.9)
Elevated	84 (24.1)

BMI = body mass index; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; T2DM = type 2 diabetes mellitus; TG = triglycerides; TSH = thyroid-stimulating hormone.

into two age groups: <50 and ≥50 years. Regarding the age, 67.8% (236 of 348) subjects fall in the age group of <50 years and 32.2% (112 of 348) were ≥50 years of age. With respect to gender, the ratio of males (224 of 348; 64.4%) was more than females (124 of 348; 35.6%). Regarding ethnicity, nonlocal residents were 83.9% (292 of 348) as compared to locals (56 of 348; 16.1%). Additionally, the clinico-biochemical parameters including history of lung disease, hypertension, type 2 diabetes mellitus (T2DM), TC, TG, LDL-C, HDL-C, TSH and anthropometric parameters including BMI were also determined on the 15th day after receiving the second dose of COVISHIELD. It was observed that 3.4% (12 of 348) subjects had a history of lung disease, 16.1% (56 of 348) had hypertension and 14.9% (52 of 348) had T2DM. With respect to BMI, 43.7% (152 of 348) were normal, 9.2% (32 of 348) were underweight, 33.3% (116 of 348) were preobese, 12.6% (44 of 348) were obese class I and 1.1% (04 of 348) were obese class II. As far as biochemical parameters are concerned, 23.8% (80 of 348) subjects had elevated cholesterol levels, 40.2% (140 of 348) had elevated TG levels, 34.5% (120 of 348) had elevated LDL-C levels, 58.6% (204 of 348) had low HDL-C levels, 24.1% (84 of 348) had elevated levels of TSH. Table II contains parameters of COVID test panel in study subjects. We observed that 24.1% (84 of 348) had elevated levels of LDH, 14.9% (52 of 348) had elevated levels of ferritin, 24.1% (84 of 348) had elevated levels of CRP and 32.2% (112 of 348) had elevated levels of IL-6 after receiving two doses of vaccine. Mean age of study subjects in years was 42.03 ± 13.54, mean BMI in kg/m² was 24.8 ± 4.50, mean fasting blood sugar levels in mg/dL were 106.75 ± 48.35. Regarding the various inflammatory parameters, mean LDH levels in U/L were 189.93 ± 53.43, mean ferritin levels in ng/mL were 134.6 ± 115.09, mean CRP levels in mg/dL were 0.26 ± 0.33, mean IL-6 levels in pg/mL were 5.4 ± 9.01. In addition, mean total IgG titer was 8952.8 ± 9587.5 AU/mL and mean (log) total IgG was 8.60 ± 1.01 AU/mL. Table III portrays the levels (mean ± SD) of various biochemical and inflammatory parameters in the study subjects.

TABLE II - Biochemical and inflammatory markers (COVID-19 test panel) in study subjects

Parameters	Study subjects
LDH levels	
Normal	264 (75.9)
High	84 (24.1)
Ferritin levels	
Normal	296 (85.1)
High	52 (14.9)
CRP levels	
Normal	264 (75.9)
High	84 (24.1)
IL-6 levels	
Normal	236 (67.8)
High	112 (32.2)

COVID-19 = coronavirus disease 2019; CRP = C-reactive protein; IL-6 = interleukin 6; LDH = lactate dehydrogenase.

TABLE III - Levels (mean ± SD) of various biochemical and inflammatory parameters in study subjects

Parameters	Study subjects Mean ± SD (Range)
Age (years)	42.03 ± 13.54 (19.0-75.0)
BMI (kg/m²)	24.8 ± 4.50 (16.4-34.1)
Fasting blood sugar (mg/dL)	106.75 ± 48.35 (70.0-349.0)
Total cholesterol (mg/dL)	172.10 ± 42.7 (84.0-317.0)

(*Continued*)

TABLE III - (*Continued*)

Parameters	Study subjects Mean ± SD (Range)
TG levels (mg/dL)	192.06 ± 99.64 (55.0-172.0)
LDL-C levels (mg/dL)	108.64 ± 36.12 (42.0-225.0)
HDL-C levels (mg/dL)	41.94 ± 10.44 (23.0-88.0)
TSH levels (µIU/mL)	4.34 ± 3.13 (0.3-21.5)
LDH levels (U/L)	189.93 ± 53.43 (125.0-503.0)
Ferritin levels (ng/mL)	134.6 ± 115.09 (3.6-554.1)
CRP levels (mg/dL)	0.26 ± 0.33 (0.002-2.3)
IL-6 levels (pg/mL)	5.4 ± 9.01 (0.0-57.1)
IgG (AU/mL)	8952.8 ± 9587.5 (314.5-40000.0)
(Log) IgG	8.60 ± 1.01 (5.7-10.6)

BMI = body mass index; CRP = C-reactive protein; HDL-C = high-density lipoprotein cholesterol; IgG = immunoglobulin G; IL-6 = interleukin 6; LDH = lactate dehydrogenase; LDL-C = low-density lipoprotein cholesterol; SD = standard deviation; TG = triglycerides; TSH = thyroid-stimulating hormone.

Association of total IgG levels with sociodemographic; clinico-biochemical characteristics and parameters of COVID-19 test panel

Table IV depicts the association between total IgG antibody titer against SARS-CoV-2 spike protein (determined on the 15th day after receiving the second dose of *COVISHIELD*) and various sociodemographic and clinico-biochemical characteristics of the study subjects using linear regression. On regression analysis, we observed that locals had significantly more total IgG titer against SARS-CoV-2 compared to nonlocals ((Regression Coefficient) RC = 0.109, p = 0.04). Underweight subjects and individuals having class I obesity had significantly more antibody titer when compared to subjects with normal BMI (RC = 0.23, p = 0.006; RC = 0.29, p<0.0001). Those with elevated levels of TG had more IgG titer compared to those having normal TG levels (RC = 0.0180) and this association between TG levels and total IgG titer was statistically significant (p = 0.001). We did not find significant association of any other sociodemographic and clinico-biochemical parameter with the generation of total IgG against SARS-CoV-2 spike protein in response to vaccination.

Table V illustrates the association between total IgG antibody titer (determined on the 15th day after receiving the second dose of *COVISHIELD*) and various parameters of COVID-19 test panel in study subjects using linear regression. We observed that subjects having high LDH levels produced significantly higher total IgG antibodies compared to those having normal LDH levels, and this association was statistically significant (RC = 0.15, p = 0.006). Subjects having normal levels of CRP had significantly higher antibody titer when compared to those having high CRP levels (−00122, p = 0.023).

TABLE IV - Association between total IgG antibody titer (on log scale) and various sociodemographic and clinico-biochemical characteristics of study subjects using linear regression

Parameters	Study group N = 348 (%)	Regression coefficient (Beta)	95% CI	p-Value
Age group				
<50 years	236 (67.8)	0.078	(−0.06-0.4)	0.15
≥50 years	112 (32.2)			
Gender				
Male	224 (64.4)	0.029	(−0.16-0.28)	0.59
Female	124 (35.6)			
Ethnicity				
Nonlocal	292 (83.9)	0.109	(0.01-0.59)	**0.04**
Local	56 (16.1)			
H/o lung disease	336 (96.6)	−0.10	(−1.11-0.05)	0.07
No	12 (3.4)			
Yes				
Hypertension				
No	292 (83.9)	−0.07	(−0.30-0.27)	0.92
Yes	56 (16.1)			
T2DM				
No	296 (85.1)	−0.04	(−0.49-0.19)	0.47
Yes	52 (14.9)			
BMI (kg/m²)				
Normal	152 (43.7)			
Underweight	32 (9.2)	0.23	(0.15-0.9)	**0.006**
Preobese	116 (33.3)	0.11	(−0.007-0.23)	0.06
Obese class I	44 (12.6)	0.29	(0.12-0.33)	**<0.0001**
Obese class II	04 (1.1)	−0.09	(−0.36-0.09)	0.2
Total cholesterol				
Normal	268 (77.0)	−0.05	(−0.37-0.14)	0.37
Elevated	80 (23.8)			
TG				
Normal	208 (59.8)	0.180	(0.16-0.59)	**0.001**
Elevated	140 (40.2)			
LDL-C				
Normal	228 (65.5)	−0.073	(−0.38-0.07)	0.17
Elevated	120 (34.5)			
HDL-C				
Normal	144 (41.4)	0.014	(−0.19-0.25)	0.79
Low	204 (58.6)			
TSH				
Normal	264 (75.9)	−0.005	(−0.26-0.23)	0.92
Elevated	84 (24.1)			

BMI = body mass index; CI = confidence interval; HDL-C = high-density lipoprotein cholesterol; IgG = immunoglobulin G; LDL-C = low-density lipoprotein cholesterol; T2DM = type 2 diabetes mellitus; TG = triglycerides; TSH = thyroid-stimulating hormone.
Bold represents statistically significant association.

As the frequency was skewed among subgroups of various independent variables, Mann-Whitney U-test was performed. Table VI depicts the association between total IgG antibody titer against SARS-CoV-2 spike protein and various sociodemographic and clinico-biochemical characteristics of

TABLE V - Association between total IgG antibody titer (on log scale) and various COVID-19 markers in study subjects using linear regression

Parameters	Study group N = 348 (%)	Regression coefficient (Beta)	95% CI	p-Value
LDH levels				
Normal	264 (75.9)	0.15	(0.1-0.6)	**0.006**
High	84 (24.1)			
Ferritin levels	296 (85.1)	0.09	(−0.05-0.5)	0.096
Normal	52 (14.9)			
High				
CRP levels				
Normal	264 (75.9)	−0.122	(−0.54-0.04)	**0.023**
High	84 (24.1)			
IL-6 levels				
Normal	236 (67.8)	0.055	(−0.10-0.35)	0.302
High	112 (32.2)			

CI = confidence interval; COVID-19 = coronavirus disease 2019; CRP = C-reactive protein; IgG = immunoglobulin G; IL-6 = interleukin-6; LDH = lactate dehydrogenase.
Bold represents statistically significant P value of <0.05.

study subjects using Mann-Whitney U-test. The results were identical to the results of linear regression with some notable differences. Surprisingly, subjects ≥50 years of age developed significantly more antibodies against SARS-CoV-2 spike proteins (U = 11368.00, p = 0.035). On performing Mann-Whitney U-test, there was no statistically significant difference in total IgG titer against SARS-CoV-2 between locals and nonlocals (p = 0.083). Differently, preobese subjects but not underweight individuals were having significantly more antibody titer when compared to subjects having normal BMI (U = 7440, p = 0.029).

TABLE VI - Association between total IgG antibody titer (on log scale) and various sociodemographic and clinico-biochemical characteristics of study subjects using Mann-Whitney U-test*

Parameters	Study group N = 348 (%)	Mean rank	U value	p-Value
Age group				
<50 years	236 (67.8)	166.67	11368.00	**0.035**
≥50 years	112 (32.2)	191.00		
Gender				
Male	224 (64.4)	171.13	13132.00	0.4
Female	124 (35.6)	180.60		
Ethnicity				
Nonlocal	292 (83.9)	170.40	6980.00	0.083
Local	56 (16.1)	195.86		
H/o lung disease				
No	336 (96.6)	174.79	1920.00	0.77
Yes	12 (3.4)	166.50		
Hypertension				
No	292 (83.9)	172.84	7692.00	0.48
Yes	56 (16.1)	183.14		
T2DM				
No	296 (85.1)	174.38	7660.00	0.95
Yes	52 (14.9)	175.19		
BMI (kg/m²)				
Normal	152 (43.7)	88.97	1896.00	0.50
Underweight	32 (9.2)	109.25		
Normal	152 (43.7)	125.45	7440.00	**0.029**
Preobese	116 (33.3)	146.36		
Normal	152 (43.7)	89.97	2048.00	**<0.001**
Obese class I	44 (12.6)	127.95		
Normal	152 (43.7)	79.13	208.00	0.28
Obese class II	04 (1.1)	54.50		
Total cholesterol	268 (77.0)			
	80 (23.8)			
Normal		177.60	9888.0	0.29
Elevated		164.10		
TG	208 (59.8)			
Normal	140 (40.2)	162.23	12008.00	**0.006**
Elevated		192.73		
LDL-C				
Normal	228 (65.5)	181.01	12196.00	0.096
Elevated	120 (34.5)	162.13		
HDL-C				
Normal	144 (41.4)	172.86	14452.00	0.79
Low	204 (58.6)	175.66		
TSH				
Normal	264 (75.9)	175.95	10704.00	0.63
Elevated	84 (24.1)	169.93		

BMI = body mass index; HDL-C = high-density lipoprotein cholesterol; IgG = immunoglobulin G; LDL-C = low-density lipoprotein cholesterol; T2DM = type 2 diabetes mellitus; TG = triglycerides; TSH = thyroid-stimulating hormone.
*Test was adopted as the distribution of scores for both groups of few independent variables was skewed.
Bold represents statistically significant P value of <0.05.

Table VII illustrates association between total IgG antibody titer and various parameters of COVID-19 test panel in study subjects using Mann-Whitney U-test. In contrast to the results of linear regression, we did not observe any statistical significance between total IgG antibody titer and CRP levels (p = 0.33).

Discussion

In this study based on 348 vaccinated participants, we showed variation in anti-spike total antibody (IgG) titer based on various sociodemographic and clinico-biochemical parameters. To the best of our knowledge, our study is the first to compare a short-term assessment of antibody response to vaccination with low-grade inflammation.

TABLE VII - Association between total IgG antibody titer (on log scale) and various COVID-19 markers in study subjects using Mann-Whitney U-test*

Parameters	Study group N = 348 (%)	Mean rank	U value	p-Value
LDH levels				
Normal	264 (75.9)	165.92	8824.00	**0.005**
High	84 (24.1)	201.45		
Ferritin levels				
Normal	296 (85.1)	170.64	6552.00	0.87
High	52 (14.9)	196.50		
CRP levels				
Normal	264 (75.9)	177.44	10312.00	0.33
High	84 (24.1)	165.26		
IL-6 levels				
Normal	236 (67.8)	170.86	12356.00	0.32
High	112 (32.2)	182.18		

COVID-19 = coronavirus disease 2019; CRP = C-reactive protein; IgG = immunoglobulin G; IL-6 = interleukin 6; LDH = lactate dehydrogenase.
*Test was adopted as the distribution of scores for both groups of few independent variables was skewed.
Bold represents statistically significant P value of <0.05.

A negative correlation between age and antibody titer in response to different vaccinations such as hepatitis B, seasonal influenza, tetanus and pneumococcal vaccine is well known (30,31). "Immunosenescence" combined with "Inflammaging" are the factors responsible for poor antibody production after vaccination in elderly individuals (32). Contrary to majority of findings, our study observed a significant upsurge of antibodies against SARS-CoV-2 spike protein in subjects ≥50 years of age. Although with age adaptive immunity diminishes, but to associate immunosenescence with the sequential process of aging can be deceptive. As per varied studies individuals of the same chronological age can have a widely variable "immunological age" (33,34). Interestingly, even if the loss of antibody production in the elderly has been noticed by various studies no age-related differences in the avidity and functional activity of antibodies induced by vaccination has been brought to the fore by major studies (35,36).

Sociodemographic factors such as race and ethnicity are known to influence susceptibility to infectious diseases and have recently been shown to be associated with immune response variations and adverse events following vaccination (37,38). Although not statistically significant, we observed generation of slightly higher total IgG in locals (Kashmiri individuals) than nonlocals after vaccination. The difference in the antibody response might be due to the fact that Kashmiri population is ethnic due to peculiar traditional and religious beliefs than the rest of the country due to which their genetic pool remains conserved and it is known that genetic determinants play an important role in regulating immune responses after infection or vaccination (39,40).

In our study, preobese and obese class I subjects produced significantly more antibodies after vaccination compared to subjects having normal BMI. There is a lot of evidence seconding the raised antibody titer in response to SARS-CoV-2 vaccination in obese individuals. Obesity is connected with substantial modification and profusion of immune cells in the adipose tissues with a noticeable decrease in Th2 cells, Treg cells, and M-2 macrophages (41,42). Adipose tissue of the obese subject expresses increased amounts of proinflammatory proteins such as tumor necrosis factor alpha (TNF-α), IL-6, transforming growth factor-beta 1 (TGF-β1), CRP, and monocyte chemotactic protein-1 (MCP-1) compared to adipose tissue of lean subjects, which subsequently upsurges immune response and antibody production (43,44). Obese and swollen adipose tissue encompasses >40% M-1 macrophages, which act as spring for a range of proinflammatory cytokines leading to a local as well as systemic inflammation that triggers immune cell activation (45). Callahan et al testified that obese adults had significantly higher antibody titer than adults with normal weight after first dose of vaccine (46). Studies have upheld that among obese subjects, the initial antibody response to H1N1 vaccine was robust (47).

In our study, it was also observed that individuals having high TG levels produced significantly more antibodies after vaccination. Numerous studies have shown that patients with isolated hypertriglyceridemia had augmented CRP and IL-6 concentration (48), thereby increasing CD4+ T helper cells and antibody production (49). Our observation was also supported by previous reports linking enhanced expression of B cells with dyslipidemia (50). Xu et al described that T and B lymphocytes were having markedly increased expression in patients with dyslipidemia compared to healthy controls (51). During lipolysis, TG release free fatty acids (FFA) in the presence of lipoprotein lipase (LPL), thereby inducing endothelial proinflammatory changes, tissue inflammation and vigorous immune response (52).

In this study, we also observed that those having high levels of LDH produced significantly increased IgG against SARS-CoV-2 after vaccination. LDH is a general indicator of acute or chronic tissue damage and is considered to be an inflammatory marker and predictor of increased immune response (53). During lung damage and other interstitial lung infections, LDH levels get increased (54). Therefore, more the concentration of natural infectious agent or artificial antigen, more is the inflammation and LDH level, hence more is the antibody response. Conventional or myeloid DCs are highly specialized antigen-presenting cells with a unique ability to prime naive T cells inducing the activation of the adaptive immune response (55). Increased lactate from infection and tissue injury due to increased LDH leads to extracellular acidosis, which in turn activates DCs inducing the activation of the adaptive immune response (56).

Limitation of the study

The data of subgroups of various independent variables are slightly skewed, which could affect the outcome of the study. Data skewness has been taken care of by converting antibody levels to log scale and applying Mann-Whitney U-test. The sample size of the study is relatively modest and further large-scale studies are needed to consolidate the inferences made in our study.

Conclusion

This study summarized that age, BMI, TG and LDH levels strongly influence immunogenicity of adenoviral vaccine against SARS-CoV-2. Our study gives an opportunity to understand the impact of biochemical and immunological factors affecting vaccine effectiveness. Therefore, our inferences could be utilized in designing vaccine studies and could help in designing personalized vaccination schedules that would ultimately lead to improvement in vaccine response and efficacy, especially at times of pandemics. In future, this study will assist to identify individuals at risk for low immunogenicity to adenoviral vaccines against SARS-CoV-2 and to subsequently intervene upon these risk factors.

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