

Human leukocyte antigen - B27 and antibodies to *Klebsiella pneumoniae* in Ankylosing Spondylitis: associations and clinical outcome

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Abstract

Ankylosing spondylitis (AS) is a chronic disabling rheumatic disease with indefinite etiology. Human leukocyte antigen-B27 (HLA-B27) carriage and *Klebsiella pneumoniae* (*K. pneumoniae*) infections may contribute to the etiopathogenesis of AS. The objective of this study was to determine the association of HLA-B27 carriage, serum immunoglobulin G (IgG) to *K. pneumoniae* with AS, and its clinical outcome. In a case-control study, HLA-B27 carriage was detected by polymerase chain reaction, serum IgG to *K. pneumoniae* was measured by ELISA, and *K. pneumoniae* was isolated from the stool of 40 AS patients who were compared to age and sex-matched 40 normal individuals. Clinical findings, disease activity, and functional ability were evaluated for all AS patients. HLA-B27, serum IgG to *K. pneumoniae*, and fecal carriage of *Klebsiella* were significantly higher in AS patients when compared to controls ($p < 0.001$ for all). Disease activity and functional score categories were significantly higher in HLA-B27 positive AS patients with an elevated titer of *K. pneumoniae* IgG than in HLA-B27 negative patients with low titer of *K. pneumoniae* IgG ($p < 0.012$ and $p < 0.001$, respectively). In conclusion, HLA-B27 carriage and *K. pneumoniae* infections could play a significant role in the development and clinical outcome of AS patients.

Keywords: HLA-B27 and *K. pneumoniae* in Ankylosing Spondylitis.

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Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disorder that affects the axial skeleton, peripheral joints, and extra-articular structures, with a male-to-female prevalence of approximately 3:1.¹

The occurrence of AS might be linked to some factors, including genetic, immunologic, microbial infection, and endocrinal abnormality as its definite etiology is still uncertain.² So, the etiopathogenesis of AS has been a matter of extensive molecular, immunological, and microbiological research. *Klebsiella pneumoniae*

(*K. pneumoniae*), as a microbial factor, has been reported as a triggering or perpetuating factor in AS, especially in patients carrying human leukocyte antigen-B27 (HLA-B27) antigen.³

In this respect, a molecular mimicry theory has been suggested because of similarities in amino acid sequences between HLA-B27 and *K. pneumoniae* antigens. This was evidenced by the specific cross-reaction of monoclonal anti-HLA-B27 antibodies with *K. pneumoniae*, as well as the more avid binding of anti-HLA-B27 allogeneic human tissue-typing sera with *K. pneumoniae* antigens compared to other tissue-typing sera.⁴ Accordingly, antibodies produced after *K. pneumoniae* infection can bind to HLA-B27 molecules on lymphocytes, fibroblasts, and chondrocytes and stimulate an inflammatory process due to cytokines, chemokines, complements, and proteinases release.⁵

Therefore, a multifactorial pathogenesis is considered in AS especially HLA-B27 carriage. This molecule may misfold in the endoplasmic reticulum triggering dendritic cells expression of interleukin 23 and intracellular peptides presentation to T cells leading to cross-reactivity and tissue inflammation. Moreover, intestinal flora dysregulation associated with AS may have an important role in the development and progression of AS.³

To elucidate these issues among AS patients in our locality, this study was conducted to investigate the association of HLA-B27 and *K. pneumoniae* with the pathogenesis and clinical outcome of AS patients recruited from the Rheumatology Department, Zagazig University Hospitals.

Subjects and Methods

This case-control study was conducted at the Departments of Medical Microbiology and Immunology and Rheumatology, Rehabilitation, and Physical Medicine, Faculty of Medicine, Zagazig University. During the period between January 2021 and June 2022, a total of 40 AS patients were consecutively recruited from inpatient and follow-up units of the Rheumatology Department. Patients were diagnosed according to the Assessment of SpondyloArthritis International Society (ASAS) criteria.⁶ Patients with other coexisting

rheumatic disease or any form of spondyloarthropathy, overt infection, on treatment with anti-microbial agents were excluded. An equal number (n=40) of normal volunteers were randomly selected as a control group, and they were matched regarding age and sex. All controls were >18 years old, without any associated autoimmune disorder, active infection, or history of spondyloarthritis in a first-degree relative nor a history of current treatment with any anti-microbial agents.

Methods

Data collection

A detailed history was obtained from all study participants. A complete physical examination including provocative tests of sacroiliitis was performed for 40 AS patients. Patients were evaluated for associated enthesitis and dactylitis. Extra-articular involvement (uveitis, prostatitis, cardiovascular, gastrointestinal, pulmonary, and renal) was evaluated by a specialist consultation. Finally, the body mass index was calculated by using weight in kilograms (kg) divided by the square of height in meters (m²).

Clinical and Radiological Assessment

- The Bath Ankylosing Spondylitis Metrology Index (BASMI) was used to assess spinal mobility.⁷ In all patients, the BASMI composite index was calculated as the sum (0-10) of each score (0, 1 or 2) for five parameters representing a range of motion assessment in the spinal and lumbar regions. Modified Schober's test, lateral flexion, intermalleolar distance, occipital wall distance, tragus-to-wall distance, and cervical rotation were all evaluated.

- Disease activity was assessed by the validated Arabic version of Bath AS Disease Activity Index (BASDAI) calculation^{8,9} with subsequent calculation of the Ankylosing Spondylitis Disease Activity Score (ASDAS) with C-reactive protein (ASDAS-CRP).¹⁰ Functional ability was assessed using the validated Arabic version Bath AS Functional Index (BASFI).^{9,11} (BASDAI and BASFI were scored on a 0 to 10 visual analogue scale (VAS), with higher values indicating a worse status. BASDAI value of ≥ 4 represents an active

disease, whereas a value < 4 indicates an inactive disease. ASDAS less than 1.3 reflects an inactive disease. An ASDAS value ≥ 2.1 indicates high disease activity.^{12, 13}

- To confirm the presence of sacroiliitis, all patients underwent conventional radiography (Anteroposterior view) and magnetic resonance imaging (MRI) of the sacroiliac joints (SIJs), and scoring was done using the modified New York criteria for AS.^{6, 14}

Sampling

For all participants, venous blood samples (10 ml) were obtained. Of these 5 ml were collected on EDTA-containing tubes and used to determine the erythrocyte sedimentation rate (ESR) by the westergren method and for the polymerase chain reaction (PCR). Serum samples were obtained from the other 5 ml and divided into two parts. The first part was used to measure the C reactive protein (CRP) level

and the second was stored at -20°C until used in the ELISA reaction.

Detection of HLA-B27 by polymerase chain reaction

This was done for all participants. Total DNA was isolated from anticoagulated blood samples using commercial kits (Cat no. 51304, QIAamp DNA Blood Mini Kit, Qiagen Inc., Valencia, CA, USA), according to manufacturer's instructions. PCR reactions were carried out according to Kaur et al., 2019¹ in a total volume of 25 μl which contained 2.5 μl of 10X reaction buffer with 2.5 mM MgCl_2 , 2.5 μl of 2.5 mM dNTPs, 1U HotStart *Taq* DNA polymerase, 5 μl of DNA extract, 1pmol/ μl of each primer (HLA-B27 specific primers and human growth hormone (HGH) primers, as internal controls). Primer sequences, PCR conditions, and the size of amplified products are listed in Tables 1 & 2. Amplified products measuring 141 bp were considered positive for HLA-B27.¹

Table1. Primer's sequence and amplicons size.

Gene	Primer sequence	Size of amplicon
B27ex294F	F 5'- CTACGTGGACGACACGCT-3';	141 bp
B27ex2199RC	R 5'- AGTCTGTGCCTTGG CCTTGC-3'	
HGHI	5'-CAGTGCCTTCCCAACCATTCCCTTA-3'	437 bp
HGH2	5'ATCCACTCACGGATTCTGTGTGTTTC-3'	

Table2. Conditions of PCR reactions.

Cycle	Temperature	Time	Number of cycles
Initial denaturation	96 $^{\circ}\text{C}$	4 min	1
Denaturation	96 $^{\circ}\text{C}$	30 sec	40
Annealing	58 $^{\circ}\text{C}$	30 sec	40
Extension	72 $^{\circ}\text{C}$	30 sec	40
Final extension	72 $^{\circ}\text{C}$	5 min	1

Isolation of *K. pneumoniae* from stool specimens

Stool samples were inoculated into trypticase soy broth as an enrichment broth and incubated for 24 hours at 37°C . Subcultures from trypticase soy broth were streaked on MacConkey media and incubated for 24 hours at 37°C . The isolates were identified based on colony morphology, Gram stain, and conventional biochemical methods and species level was confirmed by an automated mass spectrometry microbial identification system

(Vietec MS MaldiToF Mass Spectroscopy, (bioMérieux. Inc. Durham. USA). Isolates of *K. pneumoniae* were kept at 4°C on brain heart infusion agar (BHI) slants for further processing.

Detection of *K. pneumoniae* antibody (IgG) by ELISA

This was carried out according to the method described by Raj Kumar HRV et al., 2016¹⁵

- Preparation of *K. pneumoniae* antigen: this was performed according to the method of AL Kady et al., 2019¹⁶. Previously stored cultures of

K. pneumoniae were subcultured on BHI broth for 24 hours. Then, they were inoculated onto BHI agar plates for further 24 hours and then 4 to 5 colonies were harvested from plates, suspended, and mixed in 3 ml of sterile normal saline. The suspension was heated to 60°C for 30 minutes before centrifugation at 1509 xg for 15 minutes. The supernatant was removed, and the pellet was washed three times with 400 µl of phosphate-buffered saline with Tween 20 (PBS-T). Finally, carbonate-bicarbonate buffer was gradually added to the pellet, and the turbidity was adjusted to 4 McFarland standard tubes to achieve an approximate concentration of 12×10^8 cfu/ml.

- Coating ELISA plates with *K. pneumoniae* antigens: for sensitization, each well of a 96-microwell plastic plate (Greiner Bio-One, Germany) was loaded with 100 µl of the prepared antigen suspension, and the plate covered and incubated for 3 hours at 37°C and kept at 4°C overnight. Each well was aspirated and washed three times using 400 µl of PBS –T ensuring complete removal of the liquid at each step. Then for each well, 150 µl of 1% bovine serum albumin was added as a blocking solution and kept at 37°C for 2 hours then washed 3 times as described above.

- ELISA steps: Test serum samples were diluted 1:10 using PBS then added to the corresponding wells in duplicate and incubated at 37°C for 90 minutes. The wells were washed with PBS-T fluid 3 times. Then in each well, 100 µl of the Horse Radish Peroxidase conjugated with anti-human IgG (Diasorin S.P.A.UK) was added. The wells were incubated at 37°C for 90 minutes

then washed 3 times. After that, 100 µl of chromogenic substrate was added to each well and the plate was incubated for 20 min in dark at 37°C. Finally, 50 µl of the stop solution was added to each well. The optical density (OD) of each well was immediately determined using an ELISA reader (Stat fax 303 plus, USA) set to 492 nm and the mean OD of each duplicate was calculated.

Statistical analysis

Statistical packages (EPI-info Version 6.04 and SPSS Version 20 inc. Chicago, USA) were used to analyze collected data. Quantitative data were represented as the mean, standard deviation, and range. The Chi-square test (χ^2) was used to compare proportions as appropriate. The independent t- test was used for quantitative analysis of normally distributed data to detect difference between two different groups. A p value of < 0.05 was considered statistically significant at 95% confidence interval.

Results

This case-control study included a total of 80 participants, divided into 2 groups matched in age and sex. The 40 AS patients had a mean age of 31.7 ± 5.2 and male/female distribution was 28/12. The mean age of the 40 normal controls was 31.32 ± 8.54 and male/female distribution of 24/16. Disease onset, duration, comorbidities, articular, extraarticular manifestation, activity scoring, and treatment were reported for all patients in Table 3.

Table 3. Demographic, clinical, and radiological characteristics of AS patients and controls.

Variables	AS patients (n =40)	Control (n =40)
Age (years) mean $\pm$ SD	31.7 ± 5.2	31.32 ± 8.54
Age at disease onset(years) mean $\pm$ SD	23.8 ± 3.6	-
Disease duration (years) mean $\pm$ SD	8.1 ± 4.9	-
Gender, n (%)		
Male	28(70%)	24(60%)
Female	12(30%)	16(40%)
Body Mass Index, kg/m ² mean $\pm$ SD	26.92 ± 3.1	26.2 ± 2.9
Smoking status, n (%)	15(37.5%)	12(30%)

Table 3. Continued.

Variables	AS patients (n =40)	Control (n =40)
Current comorbidities, n (%)	17(42.5%)	
Hypertension	8(15%)	
Dyslipidemia	5(12.5%)	-
Ischemic heart disease	2(5%)	
Diabetes mellitus	2(5%)	
Peripheral involvement n (%)	10(25%)	-
Inflammatory back pain n (%)	40(100%)	
Cervical pain n (%)	24(60%)	
Clinical Sacroiliitis n (%)	21(52.5%)	-
Enthesitis, n (%)	14(35%)	-
Dactylitis, n (%)	4(10%)	-
Extra-articular manifestations, n (%)		
Anterior uveitis	8(15%)	
GIT	1(2.5%)	
Pulmonary	11(27.5%)	
CVS	8(20%)	
Renal	0(0%)	-
Prostatitis	0(0%)	
Presence of deformities, n (%)		
Kyphosis	10(25%)	-
Flexion deformity	8(20%)	
BASDAI score (range: 0–10) mean $\pm$ SD	4.22 $\pm$ 1.83	-
ASDAS-CRP score mean $\pm$ SD	2.1 $\pm$ 1.08	-
BASFI score (range: 0–10) mean $\pm$ SD	4.8 $\pm$ 1.86	2.5 $\pm$ 1.03
BASMI score (range: 0–10) mean $\pm$ SD	4 $\pm$ 1.5	2.1 $\pm$ 1.01
Sacroiliitis grade1, n (%)	4(10%)	
Sacroiliitis grade2, n (%)	14(35%)	-
Sacroiliitis grade 3, n (%)	10(25%)	
Sacroiliitis grade 4, n (%)	12(30%)	-
MRI inflammatory lesions of the SIJ n (%)	19(47.5%)	-
MRI structural lesions of the SIJ n(%)	26(65%)	
Current medications, n (%)		
NSAIDs	27(67.5%)	
Corticosteroids	2(5%)	
c- DMARDs		
MTX	25(62.5%)	
SSZ	6(15%)	
Biological treatment		
Anti-TNF	7(17.5%)	-
Anti-IL 17	23(57.5%)	

GIT; gastrointestinal, CVS; cardiovascular, NSAD; non-steroidal anti-inflammatory drugs, c-DMARDs; Disease modifying anti-rheumatic drugs, MTX; methotrexate, SSZ; sulfasalazine, TNF; Tumor necrosis factor, IL-17; interleukin 17.

Laboratory characteristics of AS patients and control groups are shown in Table 4. The ESR, CRP, HLA-B27, serum anti-*Klebsiella*

pneumoniae IgG, and fecal carriage of *Klebsiella* were more significantly higher in AS patients' group compared to the control group.

Table 4. Laboratory characteristics of AS patients and controls.

Variable	AS patients (n=40)	Control (n=40)	*p value
ESR, mm/h (mean $\pm$ SD)	32.2 $\pm$ 10.3	18.92 $\pm$ 5.23	<0.001
CRP, mg/ dL (mean $\pm$ SD)	7.9 $\pm$ 6.8	1.58 $\pm$ 0.57	<0.001
HLA-B27, n (%)	28 (70 %)	6 (15%)	<0.001
Anti- <i>Klebsiella pneumoniae</i> antibodies (OD)			
Range	0.633-1.77	0.231-0.522	<0.001
Mean OD $\pm$ SD	1.52 $\pm$ 0.18	0.42 $\pm$ 0.9	
Fecal carriage of <i>Klebsiella</i> n (%)	21 (52.5%)	4 (10.0%)	<0.001

t= Student t test. χ^2 = Chi-Square test. *P $\leq$ 0.05 is significant.

Additionally, the mean serum level of IgG to *K. pneumoniae* was significantly higher in HLA-B27

positive AS patients in comparison to HLA-B27 negative AS ones (p <0.001) (Table 5).

Table 5. Frequency of HLA -B27 antigen and IgG to *K. pneumoniae* among the 40 AS patients.

Item	<i>K. pneumoniae</i> IgG Mean OD $\pm$ SD	*p value
Patients with HLA-B27 positive (n=28)	1.58 $\pm$ 0.12	<0.001
Patients with HLA-B27 negative (n=12)	0.99 $\pm$ 0.28	

*P $\leq$ 0.05 is significant. t= Student t test.

To assess the relation between HLA-B27 and *K. pneumoniae* IgG with the clinical outcome of AS, the disease activity and the functional score categories (BASDAI, BASFI and ASDAS-CRP) were compared. There were significantly higher

scores recorded in AS patients with HLA-B27 positive and elevated titer of IgG to *K. pneumoniae* compared to AS patients with HLA-B27 negative and low titer of IgG to *K. pneumoniae* (Table 6).

Table 6. Relation of disease activity and functional score categories to HLA-B27 carriage and level of anti-*Klebsiella pneumoniae* IgG of the 40 AS patients.

	BASDAI, n (%)		<i>p</i> value	BASFI, n (%)		<i>p</i> value	ASDASCRP, n (%)		<i>p</i> value
	< 4 (n=18)	≥4 (n=22)		< 4.6 (n=18)	≥4.6 (n=22)		< 1.3 (n=15)	≥1.3 (n=25)	
HLA-B27									
Positive	9 (50.0)	19(86.4)	0.013	8(44.4)	20(90.9)	0.001	7(46.7)	21(84.0)	0.012
Negative	9(50.0)	3(13.6)		10(55.6)	2(9.1)		8(53.3)	4(16.0)	
Anti- <i>Klebsiella pneumoniae</i> antibodies (OD)									
Mean ± SD	0.7±0.1	1.4±0.56	<0.001	0.75±0.11	1.2±0.44	0.001	0.68±0.1	1.55±0.33	<0.001
Range	0.63-0.85	0.9-1.77		0.71-0.91	1.1-1.75		0.66-0.98	0.99-1.66.	

t= Student t test. χ^2 = Chi-Square test. *P $\leq$ 0.05 is significant.

Discussion

This study investigated the association of HLA-B27 and *K. pneumoniae* with the pathogenesis and clinical outcome of AS patients attended Zagazig University Hospitals.

In this study, HLA-B27 was detected in 70 % of AS patients, significantly higher than in the controls (15%). This comes consistent with findings of a previous study, reported that 88.9% of the examined AS patients were HLA-B27 positive in comparison to 7.7 % of the controls ($p < 0.001$).¹⁷ In addition, another study reported that the carriage of the HLA-B27 molecule was associated with the initiation of inflammation in articular and extra-articular sites of AS patients which agreed with the current study finding.¹⁸ Moreover, *Moshrif et al.*, 2018 found a similar positive rate of HLA-B27 carriage where 74.5% of their AS patients were HLA B27 positive.¹⁹ On the other hand, there was no difference in HLA-B27 carriage between the patient and control groups ($p > 0.05$) in another previous similar study.²⁰

Additionally, other studies reported lack of association between two HLA-B27 subtypes, HLA-B*27:06 and HLA-B*27:09, with AS disease and they attributed the development of AS to genes located outside of the major histocompatibility complex locus such as loci of the IL-23/IL-17 axis, tyrosine kinase 2, signal transducer and activator of transcription (STAT) 3, IL6R, IL27, as well as several aminopeptidase genes.^{8, 21, 22} These demonstrate the complex immunopathogenesis of the disease where multiple immunologic mechanisms contribute together to disease production.^{3, 23}

The possible role of the microbiome in AS etiology might be attributed to several elements such as changes in intestinal permeability, triggering of immune response and molecular similarity.²⁴ The results of the current study demonstrated significantly higher fecal carriage of *Klebsiella* among AS patients compared to the controls. Similar findings were previously reported, particularly with active AS patients.^{25, 26} Moreover, in support to the current finding, a previous meta-analysis study reported a significantly higher fecal carriage rate of *K. pneumoniae* to have a probable important role

in the occurrence of AS.⁴ This could be explained by molecular mimicry theory where there was structural similarity between some components of *K. pneumoniae* with specific genetic or somatic sequences in human.³ However, low fecal carriage of *K. pneumoniae* (12%) has been reported in spondyloarthritis patients in a different study.¹⁸ This inconsistency might be attributed to differences in the collection and culture methods for fecal samples and the disease activity or stage at the time-point of sampling.²⁷

The current study demonstrated that AS patients had significantly higher levels of serum anti-*Klebsiella pneumoniae* IgG than controls. Similar results were previously reported where the IgM, IgG, and IgA anti-*Klebsiella* antibody levels were found to be significantly higher in AS patients when compared to control individuals.^{4,8,29} Furthermore, previous studies from different countries with different geographical locations demonstrated significantly elevated levels of *Klebsiella* antibodies in AS patients, along with elevated levels of anti-collagen antibodies, pointing to the shared molecular and immunological cross-reactivity features that exist between *Klebsiella* antigens and different types of collagens.^{25, 30} However, contradictory to the current study findings, a previous study in Canada, did not find any difference in anti-*Klebsiella* antibodies between the AS patients and control subjects.³¹ Moreover, previous studies reported that normal controls had a significantly higher prevalence of anti-*Klebsiella* antibodies compared to patients with spondyloarthritis and AS, respectively.^{17, 32} The analysis at different timing, whether at the initiation stage or later, and whether during disease activity or remission might explain these controversial results.

The current study revealed that among the studied AS patients, there was a significantly higher mean serum level of IgG to *K. pneumoniae* in HLA-B27 positive patients compared to HLA-B27 negative ($p < 0.001$). This comes consistent with findings of a previous study where IgG antibody levels to the *Klebsiella*, *Yersinia*, and *Salmonella* heat shock proteins were significantly higher in HLA-B27

positive patients and normal individuals than HLA-B27 negative controls.³³ Furthermore, antibodies directed against an epitope of a *K. pneumoniae*-derived protein were detected in 190 AS patients where 189/190 patients were HLA-B27 positive, implying an association between the HLA-B27 carriage and the ability to stimulate an immune response against *K. pneumoniae*-derived antigens that cross-react with autoantigens.³⁴ However, Hjelholt et al., 2013 reported no significant differences between both groups.³⁵

The current study demonstrated a significant impact of HLA-B27 and *K. pneumoniae* IgG on the clinical outcome of AS. As the disease activity and functional score categories (BASDAI, BASFI and ASDAS-CRP) were significantly increased in AS patients with HLA-B27 positive and elevated titer of IgG to *K. pneumoniae* than in AS patients with HLA-B27 negative and low titer of IgG to *K. pneumoniae*. This comes in line with findings of a previous study.¹⁷ However; inconsistent results were reported elsewhere. Some reports declared a significant impact of HLA-B27 on disease activity assessed by ASDAS-CRP ($p < 0.001$) with no statistical differences in BASDAI or BASFI scores.³⁶ Another report found that a median of BASDAI was five times higher in HLA-B27 positive patients than HLA-B27 negative patients ($p = 0.033$) while no differences regarding BASFI ($p = 0.086$) or ASDAS-CRP ($p = 0.058$) were detected.³⁷ Interestingly, while no significant difference in BASDAI and BASFI scores was found between HLA-B27 antigen positive and negative cases in some studies,^{35,38,39,40} other studies reported that HLA-B27-negative AS patients had significantly higher BASDAI and BASFI scores as well as higher prevalence of arthritis, dactylitis, and extra-articular manifestations than HLA-B27 positive AS patients.⁴¹ These differences might be attributed to the patient status, treatment modalities, and method of measurement of damage at the time of the study.

In conclusion, this study demonstrated that HLA-B27 and *K. pneumoniae* showed significant relationship with AS development and clinical severity. Anti-*Klebsiella* measures could be combined with current medical treatment in the management of AS patients.

Author Contributions

HME, MAG contributed to the study conception and design. HME, MAG, WKM, HAE contributed to methodology, analysis, and interpretation of data. WKM, HAE provided clinical support. HME wrote the manuscript draft. HME, MAG, WKM provided review and editing. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

the study protocol was reviewed and approved by the ethics Institutional Review Board of the Faculty of Medicine, Zagazig University (ZU-IRB; #6459/11.2020).

Informed consent

Each subject provided written informed consent to participate.

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