

## Association of Helicobacter Pylori Infection with Oxidative DNA Damage and Atherosclerosis in Rheumatoid Arthritis Patients

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### Abstract

**Objectives:** to evaluate the associations between Helicobacter pylori (HP) with oxidative DNA damage and subclinical atherosclerosis in rheumatoid arthritis (RA) patients.

**Methods:** Eighty RA patients were divided into two groups according to the presence of anti-HP antibodies. In addition, to forty healthy volunteers. All patients were subjected to DAS-28, ESR, hsCRP, RF, Anti-CCP, Lipid profiles, serum anti-HP antibodies, 8-hydroxydeoxyguanosine, Oxidized LDL, IL-6, carotid intima media thickness (cIMT) and flow mediated dilatation of the brachial artery (FMD).

**Results:** HP positive RA patients revealed significantly higher disease activity, RF, Anti-CCP, dyslipidemia, 8-OHdG, ox-LDL, cIMT and lower FMD% compared to HP negative patients. There was positive correlation between anti-HP antibodies with disease activity parameters, ox-LDL, 8-OHdG and cIMT as well as negative correlation with FMD%. In multiple regression analysis, IgG antibodies against H pylori were associated with DAS-28 ( $p < 0.001$ ), hsCRP ( $p < 0.01$ ), 8-OHdG ( $p < 0.01$ ), cIMT ( $p < 0.001$ ) and FMD% ( $p < 0.001$ ).

**Conclusions:** Chronic infection with HP in RA patients is significantly associated with oxidative stress and DNA damage. Detection and eradication of HP infection in RA patients may reduce the burden of atherosclerosis and its associated morbidity and mortality.

**Keywords:** Helicobacter pylori; Oxidative DNA damage; Atherosclerosis; Rheumatoid arthritis

### Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disorder primarily characterized by symmetric destructive polyarthritis affecting small, medium, and large joints. A number of genetic and environmental factors contribute to disease onset and severity [1]. In addition, a number of viral and bacterial pathogens such as Helicobacter pylori (HP) may have a role in disease pathogenesis as well [2].

HP is a Gram-negative flagellated bacterium is widely prevalent, with approximately 50% of the western world and over 80% of those living in developing countries infected with the bacterium [3]. The association of HP infection in the pathogenesis of RA is controversial. Chronic infection with HP serves as a source of persistent antigenic stimulation triggering autoimmunity [3].

Recent studies have investigated the role of oxLDL in increasing oxidative stress, excess generation of reactive oxygen species (ROS)

and subsequent lipid peroxidation (LPO) derived DNA damage as critical events in the formation of atherosclerotic lesions [4].

The aim of our study was to evaluate the associations between HP with oxidative DNA damage and subclinical atherosclerosis in RA patients.

### Material and Methods

This study was carried out on eighty rheumatoid arthritis patients selected from the outpatient clinics of Physical Medicine, Rheumatology & Rehabilitation department, Tanta University Hospitals. The patients fulfilled the American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) 2010 criteria for diagnosis of rheumatoid arthritis [5]. RA patients were divided into two equal groups according to the presence or absent of Immunoglobulin G (IgG) antibodies to Helicobacter pylori (anti-HP antibodies). In addition, to forty healthy HP negative volunteers matched in age and sex were involved in this study as controls. Informed consents were taken from each patient and controls. The study was approved by Local Research Ethics Committee of Faculty of Medicine (Approval code: 31079), Tanta University.

We exclude patients with previously treated for HP infection ,who had received antibiotics; proton pump inhibitors or bismuth compounds in the preceding 4 weeks, known history of previous cardiovascular disease, smoking, pregnancy , conditions that affect lipid profile as (diabetes mellitus, hypothyroidism, liver or kidney disease, Cushing syndrome, obesity). As well as patients receiving medications affecting lipid metabolism.

## All Patients were Subjected to the Following Assessment

### Clinical assessment

Disease activity was assessed by measuring the disease activity for 28 joint indices score (DAS-28). Components of DAS-28 are erythrocyte sedimentation rate, patient-assessed global score (0-100), swollen and tender joint counts (0-28) [6].

### Laboratory investigations

Overnight fasting-state 7 ml of venous blood samples were taken from the controls and RA Patients.

1.6 ml was transferred into tube containing 0.4 ml sodium citrate for ESR assessment, 1 ml was placed in EDTA for complete blood count. The rest of blood was delivered slowly into a dry sterile centrifuge tube, and then centrifuged as soon as possible at 2000 rpm for 10 minutes. Lipid profile and RF were determined immediately and aliquots of serum were immediately stored at -80°C until the time of analysis.

**A-Routine laboratory investigations including:** ESR (mm/h) was determined by Westergren method. hsCRP concentrations were measured using the Diamed Eurogen CRP ELISA kit. Lipid profiles including serum triglyceride (TG) level, serum total cholesterol (TC) level, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were analyzed using kits supplied by BioMerieux Vitek Inc. (Durham, NC,USA). RF was determined by nephelometry (Behring, Marburg, Germany). Cutoff value was 15.0 U/ml . Serum anti-CCP was measured by ELISA (QuantaLite CCP version 3.1 for IgG/IgA; Inova Diagnostics, San Diego, CA, USA). Cutoff value was 20 arbitrary units, according to the manufacturer's instructions.

**B-Specific laboratory investigations including:** 1-H pylori and CagA serology:-Immunoglobulin G (IgG) antibodies to H pylori (anti-HP antibodies) were measured by a commercial enzyme-linked immunosorbent assay (Pyloriset® EIA-G; Orion Diagnostica, Espoo, Finland). The range of serum levels of anti-HP antibodies was between 100 U and 12,800 U.H pylori infection was defined as IgG anti-HP antibodies >300 U.

2-8-hydroxydeoxyguanosine (8-OHdG): Serum levels of 8-OHdG were measured ELISA kits (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan) [7]. 8-OHdG Check is a

competitive enzyme-linked immunosorbent assay (ELISA) utilizing monoclonal antibody (clone N45.1) which is highly specific for DNA damage, not cross react with RNA oxidation products such as 8-hydroxy-guanine and 8-hydroxy-guanosine.

3-Oxidized LDL (oxLDL): Serum Ox-LDL levels were measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Sweden) [8].

4- IL-6 concentrations were determined by ELISA (Roche Diagnostics GmbH, Mannheim, Germany).

### Radiological assessment

**Assessment of carotid atherosclerosis by intima media wall thickness (cIMT):** Common carotid arteries were assessed using SAMSUNG MEDISON (UGEO H60) with a linear transducer (midfrequency, 10 MHz). The radiologist for this study was blinded to other data for RA patients and controls. Measurement of cIMT was taken at three points on each side: common carotid artery (10 mm before the bulb), bulb (5-10 mm cranially to the start of the bulb), and internal carotid artery (10 mm after the flow divider). The mean of IMT among the six segments studied were assessed. A cut off value of IMT was 0.72 mm, patients who had IMT>0.72 mm were considered to have atherosclerosis [9].

**Assessment of flow mediated dilatation (FMD) of the brachial artery:** FMD was assessed on the brachial artery with the same machine used for the assessment of carotid IMT. The test was performed according to the International Brachial Artery Reactive Task Force guidelines. FMD was expressed as the relative increase in brachial artery diameter during hyperemia, and defined as: post hyperemia diameter - basal diameter/basal diameter × 100 [10].

### Statistical Analysis

All data were analyzed using SPSS software (version11; SPSS Inc., Chicago, IL, USA). Baseline characteristics are presented as mean ± standard deviation and as frequency (percentage) for discrete variables. Comparisons between groups and association between characteristics of patients and ultrasonography (US) grading were conducted using analysis of variance (ANOVA) and Fisher LSD test. Correlation between variables was examined using Pearson's correlation coefficient. Multiple linear regression analysis was performed after adjustment for age and disease duration to assess independent associations between HP, clinical evaluation, laboratory and radiological measurement. P value <0.05 was considered statistically significant.

### Results

Baseline demographics, biochemical and ultrasonography characteristics of subjects with rheumatoid arthritis and controls were summarized in Table 1. There were no significant difference between the three groups as regard age and sex

Characteristic	RA, HP Seropositive (n=40)	RA, HP Seronegative (n=40)	Controls (n=40)
Age (years)	42.73 ± 9.8	42.51 ± 8.7	41.40 ± 10.61
Sex (male/female)	(3/37)	(2/38)	(4/36)

Disease duration /y	10.01 ± 6.71	9.76 ± 8.12	-----
ESR (mm/h)	30.32 ± 21.76 <sup>*,**</sup>	20.76 ± 15.51 <sup>*</sup>	8.33 ± 3.12
hs-CRP (mg/dl)	5.49 ± 3.9 <sup>*,**</sup>	3.5 ± 2.23 <sup>*</sup>	2.1 ± 1.84
DAS28	5.99 ± 0.72 <sup>**</sup>	4.01 ± 0.51	-----
RF, IU/ml	210 ± 190.3 <sup>*,**</sup>	178 ± 162.2 <sup>*</sup>	14.57 ± 6.43
Anti-CCP, U/ml	357.3 ± 323.6 <sup>*,**</sup>	305.7 ± 287.9 <sup>*</sup>	14.31 ± 8.25
TC (mg/dl)	228.13 ± 12.75 <sup>*,**</sup>	155.76 ± 15.12 <sup>*</sup>	147.10 ± 16.08
TG (mg/dl)	159.93 ± 31.97 <sup>*,**</sup>	121.33 ± 27.31 <sup>*</sup>	100.30 ± 29.80
LDL-C (mg/dl)	154.66 ± 28.33 <sup>*,**</sup>	132.33 ± 14.41 <sup>*</sup>	122 ± 16.46
HDL-C	43.06 ± 10.12 <sup>*,**</sup>	49.60 ± 11.85 <sup>*</sup>	54 ± 11.45
TC/HDL-C	5.15 ± 1.34 <sup>*,**</sup>	3.71 ± 1.58 <sup>*</sup>	3.2 ± 0.27
LDL-C/HDL-C	3.76 ± 0.82 <sup>*,**</sup>	2.90 ± 1.43 <sup>*</sup>	2.49 ± 0.81
8-OHdG (ng/mL)	1.39 ± 1.16 <sup>*,**</sup>	0.82 ± 0.56 <sup>*</sup>	0.25 ± 0.15
ox-LDL (U/L)	56.0 ± 20.9 <sup>*,**</sup>	46.2 ± 14.7 <sup>*</sup>	38.5 ± 9.2
IL-6(pg/ml)	26.64 ± 17.75 <sup>*</sup>	25.98 ± 19.95 <sup>*</sup>	5.3 ± 3.15
FMD%	4.72 ± 2.03 <sup>*,**</sup>	5.93 ± 1.69 <sup>*</sup>	7.93 ± 2.61
cIMT, mm	0.84 ± 0.27 <sup>*,**</sup>	0.73 ± 0.18 <sup>*</sup>	0.51 ± 0.11

Values represent the mean ± standard deviation. Significance was determined using one-way analysis of variance for independent samples (ANOVA) and Fisher LSD test. <sup>\*</sup>p<0.001 compared to controls. <sup>\*\*</sup>P<0.001 compared with HP seronegative RA. RF, Rheumatoid factor; anti-CCP: anti-cyclic citrullinated peptide; ESR, erythrocyte sedimentation rate; hsCRP, highly sensitive C-reactive protein; DAS-28, disease activity for 28 joint indices score; TC, total cholesterol; TG: triglycerides; LDL-C: low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; 8-OHdG, 8-Hydroxy-2-deoxyguanosine; oxLDL, Oxidized low-density lipoprotein; IL-6, interleukin-6; FMD, flow-mediated dilation; cIMT, carotid intima media thickness.

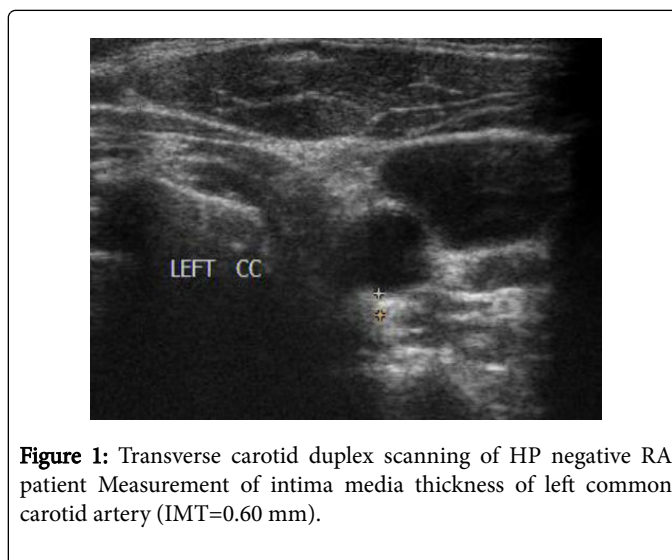
**Table 1:** Baseline demographics, biochemical and radiologic characteristics of subjects with rheumatoid arthritis and controls.

As regard disease activity and serological tests in our RA patients, DAS-28, ESR, hsCRP, RF and anti-CCP levels were significantly higher in RA patients compared to controls. In addition, there were significantly higher levels of disease activity parameters (DAS28, ESR and hsCRP), RF and anti-CCP in HP positive RA patients compared to HP negative patients.

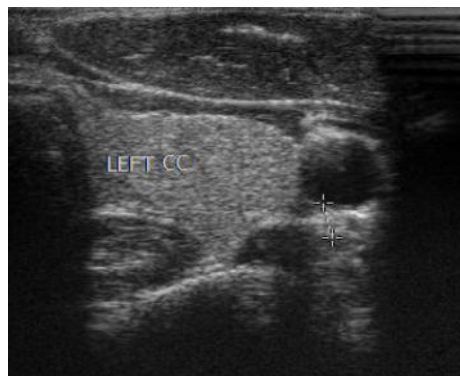
All RA patients exhibited significantly higher levels of TC, LDL-C and TG compared to controls. In addition, HDL-C levels were significantly lower compared to controls. As a consequence, the atherogenic ratio of TC/HDL-C as well as LDL-C/HDL-C was significantly higher in RA patients compared to controls. Moreover, HP positive RA patients revealed significantly higher lipid profile and dyslipidemia compared to HP negative patients.

In our study, 8-OHdG, ox-LDL and IL-6 levels were significantly higher in RA patients compared to controls. Moreover, HP positive RA patients showed significantly higher levels of serum 8-OHdG and ox-LDL compared to HP negative RA patients.

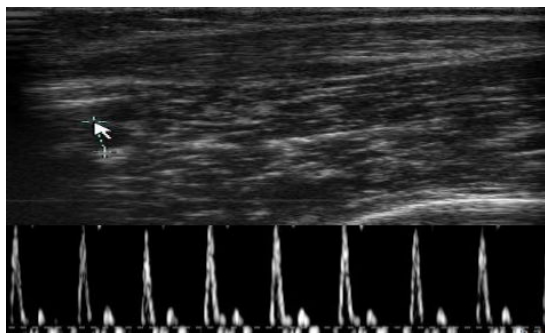
RA patients showed significantly higher cIMT and lower FMD percentage compared to controls (Figures 1-4). In addition, RA patients who were seropositive to HP had significantly higher cIMT and lower FMD compared to seronegative patients.



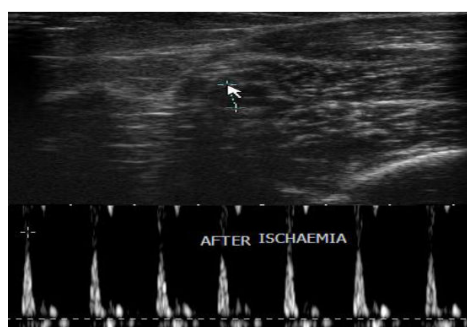
**Figure 1:** Transverse carotid duplex scanning of HP negative RA patient Measurement of intima media thickness of left common carotid artery (IMT=0.60 mm).



**Figure 2:** Transverse carotid duplex scanning of HP positive RA patient Measurement of intima media thickness of left common carotid artery (IMT=1.3 mm).



**Figure 3:** Ultrasound image of the brachial artery at base line of HP positive RA patient. The arrow is pointed to the measured diameter of the brachial artery (4.21 mm).



**Figure 4:** Ultrasound image of the brachial artery one minute after hyperemic stimulus. The diameter of the brachial artery was 4.32 mm. There was Impairment of flow mediated dilatation of the brachial artery (FMD=2.6%) in the same patient.

The mean value of IgG antibodies against H pylori in seropositive RA patients was  $1316 \pm 176$  U. There was positive correlation between IgG antibodies levels with disease activity parameters (DAS-28, ESR

and hsCRP), ox-LDL, 8-OHdG and cIMT. In addition, their levels had a negative correlation with FMD% (Table 2 ).

Characteristic	r	P value
ESR (mm/ h)	0.589	p<0.001
hs-CRP (mg/dl)	0.43	p<0.001
DAS28	0.565	p<0.001
TC (mg/dl)	0.019	NS
RF, IU/ml	0.009	NS
Anti-CCP, U/ml	0.043	NS
TG (mg/dl)	0.119	NS
LDL-C (mg/dl)	0.047	NS
HDL-C	0.037	NS
TC/HDL-C	0.019	NS
LDL-C/HDL-C	0.046	NS
8-OHdG (ng/mL)	0.72	p<0.001
ox-LDL (U/L)	0.635	p<0.001
IL-6(pg/ml)	0.089	NS
FMD%	-0.645	p<0.001
cIMT, mm	0.696	p<0.001

Pearson's correlation coefficient. P value <0.05 was considered statistically significant; NS: nonsignificant.

**Table 2:** Correlation of HP antibodies in seropositive RA patients with laboratory and radiological data.

The results of multivariate linear regression analysis after adjustment for age and disease duration, the variables associated with an increase in IgG antibodies against H pylori were DAS-28 ( $\beta=0.37$ ;  $p<0.001$ ), hsCRP ( $\beta=0.16$ ;  $p<0.01$ ), 8-OHdG ( $\beta=0.15$ ;  $p<0.01$ ), cIMT ( $\beta=0.39$ ;  $p<0.001$ ) and FMD% ( $\beta=-0.49$ ;  $p<0.001$ ).

## Discussion

Rheumatoid arthritis is a complex, chronic autoimmune disease that affects approximately 0.5%–1% of the population worldwide. In recent years, it became an evidence that the excess mortality was mainly due to the cardiovascular disease [11]. Many studies demonstrated a relationship between HP infection and the extra digestive tract diseases [12]. The cardiovascular disease was suggested to be related to the infection which may be due to its effect on lipid metabolism [13].

In the present study, there were significant differences in the parameters of disease activity (DAS28, ESR and hsCRP) and serological tests (RF, anti-CCP) of RA between HP positive patients and HP negative patients. In addition, there was positive correlation between HP IgG antibodies with the disease activity parameters.

Hasni reported that the presence of HP in gastric mucosa resulted in chronic immune system activation with current cytokine signaling, infiltration of gastric mucosa by neutrophils, macrophages,

lymphocytes with subsequently generation of antibodies and effector T cells [14]. *H. pylori* may cause a loss of self-tolerance through molecular mimicry, polyclonal activation and epitope spread. In addition, reported studies on mice, B lymphocytes stimulated by HP urease antigen revealed production of several autoantibodies such as: IgM-type rheumatoid factor (RF IgM), anti-single stranded DNA antibody, and antiphosphatidylcholine (anti-PC) antibody [15]. However, Ishikawa et al. concluded that HP infection was not associated with disease activity in RA patient [16]. Moreover, Zentilin et al. reported that the improvement of morning stiffness and arthropathy in the HP positive RA patients after 4 months of HP eradication was due to the possibility that the medications may had a therapeutic effect on arthropathy in those patients [17].

Our HP positive RA patients revealed significantly higher dyslipidemia compared to HP negative patients. Several studies have suggested a relationship between HP infection and coronary heart disease; some of the underlying mechanisms still need to be discovered. It has been reported that chronic HP infection resulted in decreased HDL-C levels. These lipid alterations could contribute to the initiation and development of atherosclerosis [18]. HDL-C played a key role in the reverse cholesterol transport, protecting LDL against oxidation. Also, it was found that HDL-C reduced lipoprotein associated peroxides [19].

Buzás et al. reported that the increase of cholesterol, LDL-C and the decrease of HDL-C levels of infected people creates an atherogenic lipid profile which could promote atherosclerosis with its complications [20]. Moreover, Feingold et al. documented that serum triglyceride and HDL-C levels can change during the acute phase of bacterial infection [21]. These alterations promoted atherogenesis, which have been attributed to the action of bacterial lipopolysaccharide (LPS). In addition, Volanen, et al. expressed that the administration of endotoxin (LPS) induced the production of several cytokines [22]. The cytokines had the ability to increase serum triglyceride level in animals. They also suggested that changes in lipid profile appeared to be related to the production of inflammatory cytokines by the cells which were chronically infected with gram-negative bacteria.

In our study, 8-OHdG, ox-LDL and IL-6 levels were significantly higher in HP positive RA patients when compared to HP negative patients. In previous studies, it was obvious that neutrophils were infiltrated in the synovial fluid. This infiltration was the main reason for the oxidative stress which resulted in oxidative products that were elevated in the lipids and proteins of RA patients [23]. DNA damage was demonstrated through assessments of 8-oxo-dG in urine, synovial fluids, and primary blood lymphocytes of RA patients [24].

Enhanced reactive oxygen species (ROS) production, lipid oxidation, protein oxidation and DNA damage lead to the failure of antioxidant defense system. This failure contributed to tissue damage of cell membrane and mitochondria or sarcoplasmic reticulum in cardiac myocytes [25,26]. HP is capable of making high levels of ROS production from the neutrophil. Also, HP infection leads to the de novo synthesis of ROS in epithelial cells after exposure to infection. These high concentrations of ROS were linked to various forms of DNA damage, including oxidative DNA damage, DNA deamination and alkylation [27].

Previous study on atherosclerotic patients documented the presence of various DNA abnormalities, such as chromosomal aberrations, loss of heterozygosity and microsatellite instability, micronucleus, and DNA strand breaks. Moreover, 8-OHdG might be not only just a

biomarker of oxidative stress, but also may take part in the pathogenesis of atherosclerosis. 8-OHdG has a mutagenic effect which could lead to G-T transition mutations and A-C transition mutations [28].

HP strains have been shown to induce higher levels of proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-8 in gastric epithelial cells. The proinflammatory cytokines lead to increased oxidative stress. Consequently, the infected cells and gastric tissues exhibit increased oxidative DNA damage which was a critical event in the formation of atherosclerotic lesions [29].

In this study, RA patients who were seropositive to HP had significantly higher cIMT and lower FMD% compared to seronegative patients. Furthermore, there was positive correlation between HP IgG antibodies with hs-CRP, ox-LDL, 8-OHdG and cIMT. On the other hand there was a negative correlation of HP IgG antibodies and FMD%. These results suggested a relationship between HP infection with subclinical atherosclerosis and endothelial dysfunction in RA patients.

Longo-Mbenza et al. demonstrated that the severity of HP infection was significantly associated with risk factors for CVD, such as diabetes mellitus, arterial hypertension, high levels of serum fibrinogen, increased total cholesterol and low HDL-cholesterol [30]. They explained that chronic HP infection with exacerbation of inflammation and dyslipidemia may contributed to early onset of atherosclerosis.

Oshima et al. demonstrated that HP seropositive subjects revealed high levels of serum CRP and attenuated FMD [31]. They explained different mechanism underlying the causal role of HP infection in endothelial dysfunction. HP may have the direct effect on the structure and function of vascular endothelial cells. HP may induce a disturbance of proliferation and apoptosis and to decrease viability of cultured vascular endothelial cells. Moreover, an infection from HP may cause malabsorption of folate, vitamin B6, and vitamin B12. The infection leads to the failure of methylation by 5-methyl-tetrahydrofolic acid and hyperhomocysteinemia which was toxic to endothelial cells.

## Conclusion

Chronic infection with HP in RA patients was significantly associated with oxidative stress and DNA damage. Clinical and public health implications for the detection and eradication of *H. pylori* infection in RA patients may reduce the burden of atherosclerosis and its associated morbidity and mortality.

## Conflict of Interest

None

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