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The simultaneous presence of IL-1B and TNFA two-positions risk haplotypes enhances the susceptibility for celiac disease

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Abstract

To assess the joint contribution of interleukin 1 beta (IL-1B) and tumor necrosis factor alpha (TNFα) to the genetic risk of developing celiac disease (CD), we analyzed four biallelic polymorphisms of TNFA and IL-1B genes in 228 patients and 244 healthy controls. The individual contribution of TNFA −308A and IL-1B −511C alleles was weak (OR 1.47 and 1.66, respectively) and was null for TNFA −238 A/G and IL-1B +3953 C/T single nucleotide polymorphisms (SNPs). Due to the potential linkage disequilibrium between TNFA, human leukocyte antigen (HLA) -DQA1 and HLA-DQB1 genes, only individuals carrying DQ2 antigen (DQ2-positive) were considered to perform haplotype analyses. Two-position risk haplotypes were first defined by the combined presence of −511C and +3953T alleles for IL-1B (OR 9.402) or −308A and −238A alleles for TNFA (OR 15.389). The TNFA/IL-1B combined haplotype-stratified association analysis showed that the simultaneous presence of TNFA risk and IL-1B non-risk haplotypes (OR 13.32) but not TNFA non-risk and IL-1B risk haplotypes (OR 0.71) is associated with CD. Interestingly, our data suggest that the coexistence of both risk haplotypes seems to work synergistically (OR 29.59), which enhances the risk of developing CD.
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1. Introduction

Celiac disease (CD)[1] is a chronic multisystem disorder induced by gluten in genetically susceptible individuals. Immunologic mechanisms play a major role in disease development, which is characterized by early induction of an inflammatory response preceding an antigen-specific T-cell response [1–3]. The exposure of the small intestinal mucosa to gluten peptides up-regulates the expression of pro-inflammatory cytokines typically related to the innate immune response, such as interleukin 1 (IL-1) [4], tumor necrosis factor alpha (TNFα) [5], and IL-18 [6] among others. The involvement of IL-1B and TNFα in the early stages of CD might be related to their regulatory role in

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[1] *Abbreviations:* TNFA, tumor necrosis factor A gene; TNFα, tumor necrosis factor alpha cytokine; IL-1B, interleukin 1 beta; CD, celiac disease; IEL, intraepithelial lymphocyte; HLA, human leukocyte antigen; DQ2-positive, individuals carrying DQ2 antigen (official name for the HLA antigen assigned by the World Health Organization Nomenclature Committee for Factors of the HLA System); IgA, immunoglobulin A; IgG, immunoglobulin G; EmA, antiendomysial antibodies; tTG, anti-tissue transglutaminase antibodies; SNPs, single nucleotide polymorphisms.

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the transcription of early inflammatory molecules, such as intercellular adhesion molecule, cyclooxigenase type 2 and inducible nitric oxide synthase [7–10]. Tumor necrosis factor alpha has been associated with a broad range of immunomodulatory roles in CD; for example, it activates the expression of matrix metalloproteinases [11], recruits cells to the lamina propria, enhances intraepithelial lymphocyte (IEL) proliferation and induces chemokinesis by IEL [12].

Susceptibility to CD is strongly associated with alleles HLA-DQA1*0501 and HLA-DQB1*02 encoded by HLA-DQA1 and HLA-DQB1genes, carried by over 95% of individuals with CD [13,14]. The role of the HLA-DQA1 and HLA-DQB1 genes is well understood in terms of both genetic and functional effects, however it partially accounts for disease heritability. Thus, the frequency of carriers of DQ2 antigen in the general population (20–30%) suggests that DQ2 is necessary but not sufficient for development of CD and that additional genes might be required to confer risk for CD.

Regarding the role of tumor necrosis factor A (TNFA) gene in CD susceptibility, controversial results have been obtained. Although much evidence supports the role of the TNFA −308 A/G SNP as a genuine disease susceptibility factor [15–19], it is also considered that TNFA polymorphisms could be part of an extended haplotype including HLA-DQA1*0501 and HLA-DQB1*02 alleles and constitute a passive component carried at the HLA class III region of CD haplotypes [20–22]. For these reasons we focused our analysis on DQ2-positive individuals.

The IL-1 gene cluster encoding IL-1A, IL-1B and IL-1 receptor antagonist (IL-1RA), a naturally occurring competitive inhibitor of IL-1-induced pro-inflammatory activity, is located in the chromosomal region 2q12–22. At least three SNPs in the IL-1B gene have been reported, all of them consisting of a C-to-T base transition at positions −511 and −31 in the promoter and at +3953 in exon 5 [23,24]. The influence of IL-1B polymorphisms has been studied in patients with several autoimmune disorders [25–29]. When a familial approach was used to study individuals of Spanish Caucasian origin, no statistically significant differences were found in the transmission patterns of either IL-1B −511 C/T or IL-1RN variable number of tandem repeats in patients with CD [30].

As there is increasing evidence that SNPs on different genes could act synergistically on disease susceptibility [31–33], the present study evaluated the influence of IL-1B, TNFA, and the combined effect of IL-1B and TNFA alleles in the genetic risk for developing CD taking into account the potential linkage between TNFA and HLA class II genes. We exclude the analysis of IL-1RN gene because investigation of the relationship between IL-1RA genotypes and concentrations of IL-1β and IL-1RA protein produced, a critical aspect in regulation of inflammation [34], is out of our study's scope.

2. Materials and methods

2.1. Patients and control subjects

We assessed 228 consecutive Latin American Caucasian patients diagnosed with CD and a group of 244 unrelated healthy volunteers as controls. All patients and control subjects attending the Small Intestinal Section of the "Dr. C.B. Udaondo" Gastroenterology Hospital in Buenos Aires were informed of the aim of the study and gave their written, informed consent to be included. The study was approved by the local Ethics Committee of the Gastroenterology Hospital, informed of internationally endorsed standards for the application of the Helsinki Declaration.

Patients and controls belonged to the same ethnic group, which is primarily European Caucasian with much lower native American and West African genetic contribution [35]. Our study test and control populations show individual admixture [36]. Compared with controls, chromosomal segments harboring either TNFA and/or IL-1B in CD patients might show an excess of ancestry from a hypothetical putative high-risk European or Native American parental origin. Though, a potentially hidden stratification given the ethnic admix of our study population is not controlled, the presence of such stratification would not invalidate our findings.

Diagnosis of CD was based on the presence of clinical features, including characteristic celiac enteropathy [37], the presence of a positive CD-related serology [antibodies] and the response to a strictly monitored gluten-free diet. Autoimmune enteropathies were excluded by the absence of anti-enterocyte autoantibodies determined by an indirect immunofluorescence method using human small intestinal substrate [38]. Intestinal biopsy samples were fixed in 10% formalin for conventional histological assessment. At the time of diagnosis of CD, antiendomysial antibodies (EmA) were determined by indirect immunofluorescence on monkey esophagus substrate [38], and IgA or IgG subtypes of anti-tTG were performed by ELISA using a commercial kit (INOVA Diagnostics Inc., San Diego, CA, USA) [39]. Demographic and clinical information for all subjects is shown in Table 1.

2.2. HLA-DQA1 and HLA-DQB1 typing

HLA-DQA1*0501 and HLA-DQB1*02 genotyping was performed using sequence-specific primers (PCR-SSP) as previously described [40]. Primers (Operon Technologies, Inc., Alameda, CA) were used at 0.25 μM and Taq DNA polymerase at 25 units. The PCR mixture was run for 25 cycles at 94, 55 and 72 °C during 20, 50 and 20 s, respectively.

2.3. Cytokine genotyping

Genomic DNA was extracted from 10 ml of EDTA-anticoagulated blood using the standard salting out technique. Genotyping for the −511 C/T and the +3953 C/T

Table 1
Epidemiological and clinical characteristics of patients with celiac disease (CD) and healthy volunteer controls

Characteristic	CD	Controls
Total number	228	244
Mean age at study, yr (range)	39 (18–71)	38 (16–70)
Age at diagnosis of CD, yr (range)	36 (1–71)	31 (1–70)
Gender (female/male)	192/36	206/38
Clinical characterization at diagnosis of CD, *n*		
Classical	164	—
Subclinical	56	—
Silent	8	—
Marsh histological classification type, *n*		
I	6	—
III a	6	—
III b	10	—
III a + III b	10	—
III c	150	—
III c + III b	44	—
III a + III b + III c	2	—
EmA tests at diagnosis[a]		
Positive	220	—
Negative	8	244
Anti-tTG tests at diagnosis[b]		
Positive	218	—
Negative	10	244

CD, celiac disease; yr, years; IgA, immunoglobulin A; IgG, immunoglobulin G; EmA, antiendomisial antibodies; anti-tTG, anti-tissue transglutaminase antibodies.
[a] IgA and IgG subtypes of EmA were determined by indirect immunofluorescence on monkey esophagus substrate [38].
[b] IgA and IgG subtypes of anti-tTG were determined by ELISA using a commercial kit [39].

SNPs was performed on a Bio-Rad thermal cycler (“iCycler”; Bio-Rad, Hertfordshire, UK) by a PCR-RFLP technique as previously described [28]. The TNFA −308 G/A and −238 G/A polymorphisms were also analyzed using PCR-RFLP analysis [41,42]. Positive and negative controls were included with each batch of samples.

2.4. Statistical analysis

Data were managed and stored using the SPSS program (V9.05 for Windows, Chicago, IL).

Allelic frequencies in the patient and control groups were calculated by direct counting. Allele distribution was compared between groups by Fisher’s exact tests. Crude odds ratios (OR) and 95% confidence intervals (95% CI) were calculated, and values of $p < 0.05$ were considered statistically significant. When detailed, an adjustment for potential multiple testing errors was carried out by Bonferroni correction. Hardy–Weinberg equilibrium testing was calculated using Arlequin® software (University of Geneva, Geneva, Switzerland) as described in detail elsewhere [43]. Since the gametic phase was unknown, for the haplotype analyses, pairwise linkage disequilibrium measures were investigated, and haplotypes were constructed using the expectation-maximization algorithm implemented in Arlequin® software.

3. Results

Table 1 shows the demographic features and clinical information for CD patients and healthy controls. No statistical differences were found for gender and age between groups.

The −511 IL-1B, +3953 IL-1B, −308 TNFA and −238 TNFA SNPs were examined in patients and controls regardless of the carriage of DQ2. Table 2 states the number of subjects successfully genotyped for −511 or +3953 IL-1B, −308 or −238 TNFA SNPs. The proportion of these alleles was consistent with the Hardy–Weinberg equilibrium in the controls.

The −511C allele is more frequent in CD patients than controls (63% vs. 53%, OR 1.659, 95% CI: 1.261–2.184, $p = 0.00003$), whereas there are no significant differences at IL-1B +3953 position. In accordance with previous associations of TNFA in CD, the TNFA −308A allele is a weak risk factor in our population (OR 1.470, 95% CI: 1.130–1.912, $p = 0.0042$) whereas there are no significant differences at TNFA −238 position between experimental groups (Table 2).

3.1. HLA-DQA1 and HLA-DQB1genes

The presence of HLA-DQA1*0501 and HLA-DQB1*02 alleles has not been previously assessed in our population. We identified the presence of both HLA-DQA1*0501 and HLA-DQB1*02 alleles in 63/244 healthy control popula-

Table 2
Distribution of IL-1B and TNFA alleles in patients with celiac disease (CD) and healthy controls

Allele	CD	Controls
	$n = 210$[#]	$n = 224$[##]
−511 IL-1B		
C, (%) n^a	(0.63) 266[*]	(0.53) 236
T, (%) n^a	(0.37) 154	(0.47) 212
	$n = 212$[#]	$n = 216$[##]
+3953 IL-1B		
C, (%) n^a	(0.71) 300	(0.72) 312
T, (%) n^a	(0.29) 124	(0.28) 120
	$n = 220$[#]	$n = 242$[##]
−308 TNFA		
A, (%) n^a	(0.62) 274[**]	(0.53) 256
G, (%) n^a	(0.38) 166	(0.47) 228
	$n = 191$[#]	$n = 214$[##]
−238 TNFA		
A, (%) n^a	(0.41) 156	(0.38) 163
G, (%) n^a	(0.59) 226	(0.62) 265

CD, celiac disease. Due to diverse technical failures some patients were not successfully genotyped: n = number of patients ([#]from a total of 228 CD patients or [##]from a total of 244 controls). n^a = number of alleles. Allele distribution was compared between groups by the two-tailed Fisher’s exact test.
[*] OR 1.659, 95% CI: 1.261–2.184, $p = 0.00003$.
[**] OR 1.470, 95% CI: 1.130–1.912, $p = 0.0042$.

tion (25.8%) and in 212/228 CD patients (93%). Only DQ2-positive individuals were included in the analysis of TNFA and IL-1B haplotypes, and in the TNFA/IL-1B combined haplotype-stratified association analysis described bellow.

3.2. Definition of IL-1B and TNFA risk haplotypes

The likelihood-ratio test showed linkage disequilibrium among positions −511 and +3953 for IL-1B ($p < 0.00001$ in CD patients, and 0.0097 in controls) and among positions −308 and −238 for TNFA ($p = 0.049$ in CD patients and $p < 0.0001$ in controls) and also the presence of the four predictable IL-1B and TNFA haplotypes in both groups.

To reveal the causes of linkage disequilibrium within IL-1B and TNFA loci, respectively, the presence of allelic interactions within two-positions haplotypes for IL-1B (−511 and +3953) and TNFA (−308 and −238) was separately assessed in DQ2-positive individuals.

One hundred ninety-eight (−511) and 200 (+3953) samples from a total of 212 DQ2-positive CD patients were genotyped for IL-1B SNPs and similarly, 58 (−511) and 59 (+3953) samples from a total of 63 DQ2-positive controls were respectively genotyped for those IL-1B SNPs. Only 186 samples from DQ2-positive CD patients and 54 samples from DQ2-positive controls were successfully genotyped for both −511 and +3953 IL-1B SNPs. Consequently, the analysis of interaction at IL-1B gene (Table 3) included 372 haplotypes carried by 186 CD patients and 108 haplotypes carried by 54 controls. This analysis demonstrated a positive interaction between IL-1B −511C and +3953T alleles in CD patients. Hence, a two-position IL-1B risk haplotype was defined by the presence of −511C and +3953T alleles (haplotype CT, OR 9.402, Table 3).

Two hundred and twelve (−308) and 175 (−238) samples from a total of 212 DQ2-positive CD patients were genotyped for TNFA SNPs and similarly, 63 (−308) and 55 (−238) samples from a total of 63 DQ2-positive controls were, respectively genotyped for those TNFA SNPs. Only 167 samples from DQ2-positive CD patients and 55 samples from DQ2-positive controls were successfully genotyped for both −308 and −238 TNFA SNPs. Consequently, the analysis of interaction at TNFA (Table 4) included 334 haplotypes carried by 167 CD patients and 110 haplotypes carried by 55 controls. The analysis demonstrated a positive interaction between TNFA −308A and −238A alleles in CD patients. Hence, a two-position TNFA risk haplotype was defined by the presence of −308A and −238A alleles (haplotype AA, OR 15.389, Table 4).

Table 3
Distribution of haplotype frequencies at IL-1B locus and analysis of interaction between −511 and +3953 alleles in DQ2-positive patients and controls

	allele −511C			allele −511T	
	CD	Controls		CD	Controls
allele +3953T	88*	13	allele +3953T	152	33
allele +3953C	18	25	allele +3953C	114	37

Haplotypes CT, CC, TT and TC were defined at IL-1B locus as the combination of alleles −511 C/T and +3953 C/T. Haplotype frequency data were estimated by the maximum likelihood method. One hundred and eighty-six CD patients and 54 controls were included in the analysis. Two ORs were calculated in parallel by the two-tailed Fisher's exact test for CD ($n = 372$) and control haplotypes ($n = 108$).
p_c: value obtained by Bonferroni correction for multiple comparisons as the product of p value by the number of comparisons performed.
* OR 9.402, 95% CI: 4.056–21.790, $p < 0.0001$, $p_c < 0.0002$; haplotype CT vs CC.

Table 4
Distribution of haplotype frequencies at TNFA locus and analysis of interaction between −238 A/G and −308 A/G alleles in DQ2-positive patients and controls

	allele −308A			allele −308G	
	CD	Controls		CD	Controls
allele −238 A	95*	3	allele −238 A	38**	38
allele −238 G	107	52	allele −238 G	94	17

Haplotypes AA, AG, GA and GG were defined at TNFA locus as the combination of alleles at positions −308 and −238. Haplotype frequency data were estimated by the maximum likelihood method. One hundred and sixty-seven CD patients and 55 controls were included in the analysis. Two ORs were calculated in parallel by the two-tailed Fisher's exact test for CD (n=334) and control (n=110) haplotypes.
p_c: value obtained by Bonferroni correction for multiple comparisons as the product of p value by the number of comparisons performed.
* OR 15.389, 95% CI: 4.652–50.912, $p < 0.0001$, $p_c < 0.0002$; haplotype AA vs AG.
** OR 0.181, 95% CI: 0.091–0.3588, $p < 0.0001$, $pc < 0.0002$; haplotype GA vs GG.

3.3. TNFA/IL-1B combined haplotype-stratified association analysis

To investigate whether SNPs in different genes work synergistically as a susceptibility factor for CD, a combined TNFA/IL-1B haplotype-stratified association analysis was performed based on the study of Botto et al. [44]. The IL-1B and TNFA haplotypes were considered as two individual dichotomous factors (i.e., R: risk and NR: non-risk). The results described in 3.2 allowed us to define CT as the "IL-1B R" haplotype, the sum of CC, TC and TT haplotypes as "IL-1B NR" haplotypes, AA as the "TNFA R" haplotype and the sum of AG, GA and GG haplotypes as "TNFA NR" haplotypes. The genetic inheritance of both TNFA and IL-1B haplotypes inherently has four groups ("TNFA R /IL-1B R", "TNFA NR /IL-1B R", "TNFA R/IL-1B NR" and "TNFA NR/IL-1B NR"), which were considered four different strata for the analysis. The group of patients with the "TNFA NR /IL-1B NR" haplotype was used as a reference group to perform three comparisons and compute three ORs, those for each R haplotype alone and those for the combination of TNFA and IL-1B R haplotypes (Table 5). Twenty-six percent CD and 24.60% control haplotypes were "non determined", and

consequently excluded from the initial number of CD ($n = 424$) and control ($n = 126$) haplotypes (Table 5). The risk associated with the presence of the TNFA R haplotype alone (OR 15.389, Table 4) persisted rather unmodified despite of the coexistence of the IL-1B NR haplotype (OR 13.32, Table 5). Conversely, the loss of association between TNFA NR/IL-1B R combined haplotype and CD (OR 0.75, Table 5) indicates that the IL-1B R haplotype remains associated with CD only when the TNFA R haplotype is present. The positive association between the IL-1B R haplotype and CD, shown in Table 3, might have occurred due to underestimation of the presence of TNFA R haplotypes in carriers of IL-1B R haplotype. The simultaneous presence of both R haplotypes confers a twofold greater susceptibility (OR 29.59, Table 5) compared to the presence of the TNFA R haplotype alone, shown in Table 4 (OR 15.389).

4. Discussion

There is convincing evidence that innate immune responses occur in the initial phases of CD. Given these processes, since it was demonstrated that cytokine polymorphisms could affect the clinical manifestations of CD [45] and also due to the important role of these molecules in inflammatory responses, we investigated the possible association of polymorphisms in IL-1B and TNFA with CD. The analysis of CD patients and healthy controls irrespectively of the carriage of DQ2 revealed a positive association between the IL-1B −511C and TNFA −308A alleles and the risk of developing CD. Haplotype analysis of DQ2-positive CD patients and controls showed that, of the two-positions IL-1B haplotypes defined, uniquely CT conferred susceptibility for CD, and the same was true for the haplotype TNFA AA.

Table 5
Distribution of TNFA/IL-1B combined haplotype frequencies and haplotype-stratified association analysis in DQ2-positive patients with celiac disease (CD) and healthy controls

TNFA −308 −238 haplotype	IL-1B −511 +3953 haplotype	CD $n^h = 424$	Controls $n^h = 126$	OR, p, and p_c values vs reference	95% CI
R	R				
AA	CT	36	0		
Total R/R combined haplotypes		36(8.57%)	0(0.00%)	29.59, $p < 0.0001$, $p_c < 0.0003$	1.789–489.46
NR	R				
AG	CT	0	8		
GC	CT	22	17		
GG	CT	24	1		
Total NR/R combined		46(11.10%)	26(20.63%)	0.71, $p = $ ns	0.408–1.248
R	NR				
AA	CC	29	0		
AA	TC	24	2		
AA	TT	13	0		
Total R/NR combined haplotypes		66(15.71%)	2(1.58%)	13.32, $p < 0.0001$, $p_c < 0.0003$	3.170–55.960
NR	NR				
AG	CC	31	18		
AG	TC	59	13		
AG	TT	4	7		
GA	CC	0	6		
GA	TC	13	14		
GA	TT	0	0		
GG	CC	46	1		
GG	TC	0	2		
GG	TT	13	6		
Total reference NR/NR combined haplotypes		166(39.52%)	67(53.17%)	—	—
ND		110(25.94%)	31(24.60%)		

CI, confidence interval; ^{h}, number of haplotypes; R, risk; NR, non risk; ND, no determined haplotypes.
The heading of the table indicates the total number of haplotypes from CD patients (424) and controls (126) respectively. The distribution of combined TNFA/IL-1B haplotypes from 212 CD patients and 63 controls was inferred using an expectation-maximization algorithm for multilocus genotypic data. The number of ND haplotypes excluded from the analysis is stated in the last row. For the haplotype-stratified association analysis, each of three subgroups of combined TNFA/IL1B haplotypes (R/R, NR/R and R/NR) was contrasted with the reference combined haplotype (NR/NR) and separately analyzed by a two-tailed Fischer's exact test. Results are presented in a two-by-four table. p_c: value obtained by Bonferroni correction for multiple comparisons as the product of p value by the number of comparisons performed.

Subsequently we investigated whether interactions between risk haplotypes might strengthen this primary association. TNFA/IL-1B combined haplotype-stratified association analysis revealed that an even stronger association was apparent with the combination of both risk haplotypes. Our data suggest that this combination of alleles might be a valuable genetic marker to identify individuals at greater risk of developing CD. Since the present study was performed in DQ2-positive CD patients and controls we can exclude the possibility that the associations observed might result from linkage disequilibrium between TNFA, HLA-DQA1 and HLA-DQB1 genes, but not with other true mutation(s) in nearby genes that remain still undefined.

The mechanism underlying the interactions between risk haplotypes might be a shift of the balance toward increased pro-inflammatory cytokine expression, which could exacerbate the immune response towards the ingested gluten. The likely synergistic effects of IL-1B and TNFα could start at the level of their regulatory roles in the transcription of early inflammatory molecules; the functional implications of that deserve further investigation. A recent article assessed the relevance of several variants of genes related to innate immunity in CD, such as those encoding pro-inflammatory cytokines. The authors concluded that IL-1B does not seem to play a major role in the genetic predisposition for CD; however, the study failed to consider interactions between SNPs located in different genes [30]. Accordingly, our results suggest that the variable influences of IL-1B polymorphisms on cytokine levels previously reported [28] might be correlated with the presence of additional modifier genes and with the reciprocal effects between IL-1B and interacting alleles in different genetic contexts.

In conclusion, the present study evaluated polymorphisms associated with two pro-inflammatory cytokine genes in a series of DQ2-positive Argentinian patients with CD and control population. We found a significant association between coexistence of IL-1B and TNFA two-loci risk haplotypes and CD. The present results add novel insights to the hypothesis that the interaction among SNPs in different genes makes up the genetic background that constitutes the risk of developing CD.

The presence of undetected population stratification, the requirement for an independent population confirmation, and the modification by genetic or environmental cofactors that differ among study populations have been proposed among the causes for the irreproducibility in gene association studies. In our study we have considered the probable linkage disequilibrium between TNFA, HLA-DQA1 and HLA-DQB1 genes, and discussed the effects of a potentially hidden stratification given by ethnic admix. Reproducibility of the statistical association between CD and the coexistence of both risk haplotypes herein described remains to be demonstrated by simple replication in an independent population sample. Our results highlight the need to perform simultaneous analysis of multiple modifier genes rather than to study a single locus in complex diseases.

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