Analysis of polymorphisms in the promoter region and protein levels of interleukin-6 gene among gout patients

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Abstract

Objective

To explore the associations between the polymorphisms and protein levels of interleukin-6 (IL-6) gene and gout disease.

Methods

A total of 120 male gout patients and 184 healthy controls were enrolled. Each patient was matched with 1-2 gout-free controls by age within three years. Four polymorphisms in the promoter of IL-6 gene, including -597G/A, -572C/G, -373A(m)T(n), and -174G/C, and the IL-6 levels were analyzed. The clinical characteristics and biochemical markers in plasma were measured, including age of gout onset, duration of gout history, tophus number, gout attack frequency, uric acid, total cholesterol, triglycerides and creatinine.

Results

The mean IL-6 level for gout patients was 9.80 (±11.76 pg/ml) which showed no significant difference from the controls (7.06±7.58 pg/ml, p=0.230). When the IL-6 levels were dichotomized according to the median value (5 pg/ml), there were significantly higher proportions of the gout patients (59.66%) than controls (44%) with high IL-6 levels (OR=1.88, 95% CI=1.17-3.02, p=0.008). Unique genotype was found at polymorphisms -174G/C and -597G/A. Neither the polymorphisms -572C/G nor -373A(m)T(n) in the genotype or allele distributions showed a significant association related to clinical characteristics, biochemical markers, IL-6 levels or gout disease (all p>0.05).

Conclusions

Those with gout disease have greater proportions of high IL-6 levels in plasma than controls, and there is no significant association between the four polymorphisms in the promoter region of IL-6 gene and gout disease.

Key words

Interleukin-6, gout, polymorphism promoter.

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Introduction

Gout is characterized by peripheral arthritis resulting from the deposition of monosodium urate (MSU) crystals in joint or periarticular tissue. There are four phases in gout disease including asymptomatic hyperuricemia, acute arthritis, intercritical and chronic tophaceous phases. During the acute arthritis phase, the monocytes and immature macrophages secrete cytokines including tumor necrosis factor-alpha (TNF-α), interleukin- 1beta (IL-1β), IL-6, IL-8 and interferon-γ, to enhance endothelial activation, recruitment and activation of leukocytes (1, 2). The secretion of IL-6, together with TNF-α and IL-1, is required for the induction of acute phase reactions (3). Furthermore, high levels of IL-6 in synovial fluid were also observed in those patients with chronic arthritis (4, 5), and because of the broad spectrum of activities, increased secretion of IL-6 may be responsible for local and systemic signs and symptoms in patients with chronic arthritis, and it has been suggested that IL-6 may contribute to the pathophysiology of inflammatory arthritis.

IL-6 gene was mapped at chromosome 7. Four polymorphisms were found in the promoter region in the IL-6 gene including -597G/A, -572C/G, -373A(m)T(n), and -174G/C (7). Different genotypes at these polymorphisms were demonstrated to associate with different IL-6 levels, furthermore, the IL-6 levels also were demonstrated to be associated with many diseases, such as chronic periodontitis, systemic-onset juvenile chronic arthritis, primary Sjögren's syndrome and rheumatoid arthritis (8-10). However, the genotypes at these four polymorphisms and their protein levels were seldom revealed to show a relationship with gout disease, even though the gout disease also has the chronic arthritis syndrome. Some studies have reported the factors associated with gout disease were hyperuricemia, abnormal creatinine, higher TG, GPT and alcohol consumption, as well as the polymorphisms -863C/A in TNF-α gene (11, 12), and lack associations between polymorphisms -308G/A in TNF-α gene or in IL-4 gene (13). Here, except for the aforementioned factors associated with gout disease, we intend to explore the associations in the genotypes at polymorphisms -597G/A, -572C/G, -373A(m)T(n) and -174G/C in the IL-6 gene and the protein levels between those with gout disease and those free of gout.

Materials and methods

This study was designed as a cross-sectional study in which all the participants were male. We matched one gout patient with 1-2 gout free controls by age within a range of three years. Blood sample in fasting was drawn and informed consent was obtained from all participants during 2004 and 2005. A total of 120 gout patients were enrolled from Kaohsiung Chang-Gung Memorial Hospital and 184 healthy controls were enrolled from a community clinic. All the gout patients were diagnosed by a clinical rheumatic physician, and all the healthy controls were also diagnosed to be free from gout.

We measured all the participants for biochemical markers, including creatinine, uric acid, total cholesterol (TC) and triglycerides (TG) in the plasma by an automated multichannel chemistry analyzer (Toshiba 200). The gout patients' clinical characteristics, including tophus, age of gout onset, duration of gout history and gout attack frequency during the last year were also measured.

Plasma IL-6 level was measured in duplicate using an ELISA kit according to the manufacturer's instructions (R&D Systems, MN, USA). Sample was added to a monoclonal anti-IL-6 antibody which was pre-coated onto a microplate. After washing away any unbound substances, an enzyme-linked polyclonal anti-IL-6 antibody was added, and then a substrate solution was added to develop color. The resultant color reaction was read using a Microplate Reader (Opsys MR, DYNEX Technologies) at 450 nm wavelength.

Four polymorphisms including -597G/A, -572C/G, -373A(m)T(n), and -174G/C, in the promoter region of IL-6 gene were identified by a polymerase chain reaction (PCR) and DNA direct sequencing method (7). The primers used in the PCR procedure were 5'-AGT GGG

CTG AAG CAG GTG AAG AAA for forward, and 5'-CTG ATT GGA AAC CTT ATT AAG ATT GT for reverse. The temperature in PCR procedure for initial denaturation was 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 40 sec; and a final extension at 72°C for 7 min and then the samples were maintained at a final 4°C (ABI 9700). The fragment length was 578 bp after PCR amplification. A total of 30µl of PCR product was used for the DNA direct sequencing (ABI 310) to reveal the genotypes of the four polymorphisms.

Statistics

Student t-test was used to test for a significant difference in the mean age and IL-6 levels between gout patients and controls. The gout patients' age, IL-6 levels, clinical characteristics and biochemical markers, including duration of gout disease, age of gout onset, uric acid, TC, TG and creatinine, were tested by one-way analysis of variance (ANOVA) among different genotypes at polymorphisms -572C/G and -373A(m)T(n). The *p*-value estimated from IL-6 levels was obtained after log transformation of original IL-6 levels. The differences in the mean tophus number and attack frequency during the last years were analyzed by the Kruskal-Wallis test among different genotypes at the above two polymorphisms. The chi-square test was used to detect the Hardy-Weinberg (HW) equilibrium for polymorphisms -572C/G and -373A(m)T(n) among the control group.

The odds ratios (OR) and 95% confidence intervals (95% CI) were used to assess the strength of relationship in the inherited models, genotype and allele distributions of polymorphisms -572C/G and -373A(m)T(n) between the patient groups and controls, and the respondent *p*-value was estimated by chi-square test. When the expected value was less than 5, the *p*-value was estimated by Fisher's exact test. If the *p*-value was less than 0.05 or the range of 95% CI did not include unity, the difference was considered to be statistically significant. SAS software (V9.13) was used for the statistical analysis.

Table I. The OR and 95% CI of genotypes, allele and inherited models in the polymorphisms -174G/C, -373A(m)T(n), -572C/G and -597G/A in interleukin-6 gene between gout patients and controls.

	Gout patients (n=120; %)	Controls (n=184; %)	OR	95% CI	*p*-values
Age (years)	54.89 ± 14.92	55.88 ± 15.89	–	–	0.588
Interleukin -6 levels (pg/ml)	9.80 ± 11.76	7.06 ± 7.58	–	–	0.230
Polymorphisms -572C/G					
Genotypes					
GG	6 (5.00)	7 (3.80)	1.20	0.39-3.70	0.756
CG	38 (31.67)	71 (38.59)	0.75	0.46-1.22	0.244
CC	76 (63.33)	106 (57.61)	1.0		
Allele frequency					
G	50 (20.83)	85 (23.10)	0.88	0.59-1.30	0.511
C	190 (79.17)	283 (73.90)	1.0		
Dominant Model					
CG/GG	44 (36.67)	78 (42.39)	0.79	0.49-1.26	0.320
CC	76 (63.33)	106 (57.61)	1.0		
Recessive model					
GG	6 (5.00)	7 (3.80)	1.33	0.44-4.06	0.615
CC/CG	114 (95.00)	177 (96.20)	1.0		
Polymorphism -373A(m)T(n)					
Genotypes					
A10T10/A10T10	78 (65.00)	113 (61.41)	1.0		
A10T10/A10T11	30 (25.00)	51 (27.72)	0.85	0.50-1.46	0.558
A10T10/A9T11	7 (5.83)	15 (8.15)	0.68	0.26-1.73	0.413
A10T11/A10T11	2 (4.17)	3 (2.72)	1.45	0.41-5.17	0.566
A10T11/A9T11	2	1			
A10T9/A10T10	1	0			
A8T12/A10T10	0	1			
Allele frequency					
A10T10	194 (80.83)	293 (79.62)	1.0		
A10T11	36 (15.00)	58 (15.76)	0.94	0.60-1.48	0.780
A9T11	9 (3.75)	16 (4.35)	0.85	0.37-1.96	0.702
A10T9	1 (0.42)	0 (0.00)	–	–	–
A8T12	0 (0.00)	1 (0.27)	–	–	–
Polymorphism -174G/C					
Genotypes					
GG	120 (100)	184 (100)	–	–	–
GC	0 (0)	0 (0)	–	–	–
CC	0 (0)	0 (0)	–	–	–
Polymorphism -597G/A					
Genotypes					
GG	120 (100)	184 (100)	–	–	–
GA	0 (0)	0 (0)	–	–	–
AA	0 (0)	0 (0)	–	–	–

–: the method was not applied; OR: odds ratio: 95% CI: 95% confidence intervals.

Results

A total of 304 male subjects participated in this study; the mean age was 55.49±15.50 years, and there was no significant difference between patients and controls (*p*=0.588; Table I). The genotypes and allele frequency at polymorphisms -597G/A, -572C/G, -373A(m)T(n) and -174G/C between patients and controls are displayed in

Table II. The associations between clinical characteristics and genotypes at polymorphisms -572C/G and -373A(m)T(n) in IL-6 gene among gout patients.

Clinic characteristics (mean±SD)	-572C/G			-373 A(m)T(n)§			
	CC (n=76)	CG (n=38)	GG (n=6)	A10T10/A10T10 (n=78)	A10T10/A10T11 (n=30)	A10T10/A9T11 (n=7)	Total (n=120)
Current age (years)	55.82 ± 14.19	52.29 ± 15.22	59.67 ± 21.72	55.72 ± 14.18	52.87 ± 14.92	49.86 ± 16.93	54.62 ± 14.51
Age of gout onset (years)	46.76 ± 13.61	41.94 ± 12.83	52.20 ± 23.22	46.57 ± 13.80	42.96 ± 12.37	42.71 ± 15.70	45.40 ± 13.56
Duration of gout history (years)	8.80 ± 5.76	9.74 ± 6.70	4.60 ± 3.36	8.83 ± 5.48	9.48 ± 7.15	7.14 ± 6.52	8.89 ± 5.97
No. of tophus†	1.78 ± 4.08	1.55 ± 3.16	0.17 ± 0.41	1.76 ± 4.04	1.87 ± 3.48	0.29 ± 0.76	1.70 ± 3.77
Median (min, max)	0 (0, 23)	0 (0, 15)	0 (0, 1)	0 (0, 23)	0 (0, 15)	0 (0, 2)	0 (0, 23)
Attacks /last year†	1.71 ± 2.69	1.61 ± 2.74	2.83 ± 3.97	1.77 ± 2.83	1.53 ± 2.49	1.71 ± 2.29	1.70 ± 2.70
Median (min, max)	1 (0, 10)	1 (0, 10)	1 (0, 10)	1 (0, 10)	1 (0, 10)	1 (0, 5)	1 (0, 10)
Uric acid (mg/dl)	7.70 ± 2.14	8.47 ± 2.16	7.18 ± 1.50	7.76 ± 2.11	8.55 ± 2.29	7.97 ± 1.70	7.98 ± 2.15
Total Cholesterol (mg/dl)	199.42 ± 42.48	205.05 ± 61.51	221.67 ± 40.63	199.49 ± 42.41	200.00 ± 46.67	234.57 ± 106.25	201.82 ± 49.51
Triglycerides (mg/dl)	185.67 ± 104.96	185.51 ± 111.89	191.17 ± 103.18	187.44 ± 107.20	182.41 ± 102.65	208.86 ± 143.97	187.48 ± 107.61
Creatinine (mg/dl)	1.40 ± 0.36	1.37 ± 0.41	1.42 ± 0.22	1.40 ± 0.37	1.37 ± 0.44	1.34 ± 0.20	1.39 ± 0.38

†The *p*-value was estimated by Kruskal-Wallis test.
§The genotypes A10T11/A10T11, A10T11/A9T11, A10T9/A10T10 and A8T12/A10T10 were not included in the table due to small sample size.
All the $p>0.05$ between the clinical characteristics and polymorphisms -572C/G and -373 A(m)T(n).

Table I. The results showed that none of the participants in this study had allele C at polymorphism -174G/C and allele A at polymorphism -597G/A; *i.e.*, both polymorphisms -174G/C and -597G/A were homozygous and unique in the genotype distributions among male Taiwanese in our study. Regarding the HW equilibrium in polymorphisms -572C/G and -373A(m)T(n) in the control group, these showed the genetic distributions were in HW equilibrium (p=0.242 for -572C/G; p=0.282 for -373A(m)T(n)). In the polymorphism -572C/G, the participants have the genotype CC mostly (59.87%; 182/304), and it was taken to be the baseline. When the genotypes CG or GG were compared to genotype CC, they did not show a significant difference between patients and controls (Table I). In addition, neither the dominant model nor the recessive model showed a significant association in statistics between gout patients and controls (p>0.05).

There were five alleles composed of the polymorphism -373A(m)T(n), and these resulted in seven genotypes in our study (Table I). Most of the participants have genotype A10T10/A10T10 (61.84%; 191/304) and was taken as baseline. The results showed that those carrying genotypes A10T10/A10T11 and A10T10/A9T11 did not have a significant difference compared to genotype A10T10/A10T10 between the patients and the controls (p>0.05). We combined the other four genotypes – A10T11/A10T11, A10T11/A9T11, A10T9/A10T10 and A8T12/A10T10 – as one group, due to the small sample size, with the result that the genotypes also show no significant association with gout (p=0.566). The allele frequencies of the polymorphism -373A(m)T(n) are also listed in Table I, and none showed a significant association with gout disease (p>0.05).

The associations in clinical characteristics, biochemical markers and current age between different genotypes at polymorphisms -572C/G and -373A(m)T(n) among gout patients are listed in Table II. The clinical characteristics including age of gout onset, duration of gout history, tophus number, gout attack frequency during the last year, and the biochemical markers, including uric acid, total cholesterol, triglycerides and creatinine in plasma were measured. Those carrying genotype GG at polymorphism -572C/G have lower values of gout history and tophus number (4.60±3.36 years, 0.17±0.41, respectively), but they did not show a significant difference in statistics when compared to the genotypes CC or CG (all p>0.05). In the meantime, of all the aforementioned clinical characteristics and biochemical variables, none showed a significant association with the genotypes at polymorphisms -572C/G and -373A(m)T(n) (all p>0.05).

The IL-6 levels were measured among 119 gout patients and 175 controls, and they were dichotomized into 2 categories according to the median value (5 pg/ml) of all the participants. The associations between IL-6 levels and genotypes and allele frequency at polymorphisms -572C/G and -373A(m)T(n) among all the participants are listed in Table III. We chose the genotype CC at polymorphism -572C/G as the baseline genotype, and compared this to the genotypes CG and GG; the results did not show a significant association between the IL-6 levels and these genotypes (p>0.05). The same result was also found between the allele frequency at polymorphism -572C/G and IL-6 levels (p=0.626). Regarding the polymorphism -373A(m)T(n), neither the genotypes nor the allele frequency shows a significant association with IL-6 levels (all p>0.05, Table III).

The associations between gout disease and IL-6 levels among those carrying different genotypes in polymorphisms -572C/G and -373A(m)T(n) are listed in Table IV. In the gout patients, a total of 59.17% (71/119) of the IL-6 levels

Table III. The OR and 95% CI of interleukin-6 levels in those carrying different alleles or genotypes at polymorphisms -572C/G and -373A(m)T(n) among all participants.

	Interleukin -6 levels (pg/ml) ≥5	<5	OR (95% CI)	*p*-values
Polymorphism -572C/G				
Genotypes				
CC	91 (61.49)	87 (59.59)	1.0	
CG	52 (35.14)	52 (35.62)	0.96 (0.59 - 1.55)	0.856
GG	5 (3.38)	7 (4.79)	0.68 (0.21 - 2.23)	0.526
Allele frequency				
C	234 (79.05)	226 (77.40)	1.0	
G	62 (20.95)	66 (22.60)	0.91 (0.61 - 1.34)	0.626
Polymorphism -373A(m)T(n)				
Genotypes				
A10T10/A10T10	97 (65.94)	91 (62.33)		
A10T10/A10T11	39 (26.35)	37 (25.34)	0.99 (0.58 - 1.69)	0.967
A10T10/A9T11	9 (6.08)	12 (8.22)	0.70 (0.28 - 1.75)	0.447
A10T11/A10T11	1 (0.68)	4 (2.74)	–	–
A10T11/A9T11	2 (1.35)	0 (0.00)	–	–
A10T9/A10T10	0 (0.00)	1 (0.68)	–	–
A8T12/A10T10	0 (0.00)	1 (0.68)	–	–
Allele frequency				
A10T10	242 (81.76)	233 (74.20)	1.0	
A10T11	43 (14.53)	45 (14.33)	0.92 (0.58 - 1.45)	0.720
A9T11	11 (3.72)	12 (3.82)	0.88 (0.38 - 2.04)	0.770
A10T9	0 (0.00)	1 (0.32)	–	–
A8T12	0 (0.00)	1 (0.32)	–	–

OR: odds ratio; 95% CI: 95% confidence intervals; – : the method was not applied.

Table IV. The OR and 95% CI of interleukin -6 levels in those carrying different alleles or genotypes at polymorphisms -572C/G and -373A(m)T(n) between gout patients and controls.

	Gout patients ≥5/< 5 (pg/)	Controls ≥5/<5 (pg/)	OR (95% CI)	*p*-values	p_c-values
Total	71/48 (59.66)	77/98 (44.00)	1.88 (1.17-3.02)	0.008	0.008
Polymorphism -572C/G					
Genotypes					
CC	42/34 (55.26)	49/53 (48.04)	1.34 (0.74-2.43)	0.340	1.000
CG	27/11 (71.05)	25/41 (37.88)	4.03 (1.70-9.51)	0.001	0.003
GG	2/3 (40.00)	3/4 (42.86)	0.89 (0.09-9.16)	0.921§	1.000
Polymorphism -373A(m)T(n)					
Genotypes†					
A10T10/A10T10	45/33 (57.69)	52/58 (47.27)	1.52 (0.85-2.73)	0.159	0.477
A10T10/A10T11	20/10 (66.67)	19/27 (41.30)	2.84 (1.09-7.42)	0.031	0.093
A10T10/A9T11	5/2 (71.43)	4/10 (28.57)	6.25 (0.84-46.57)	0.159§	0.477

†The genotypes A10T11/A10T11, A10T11/A9T11, A10T9/A10T10 and A8T12/A10T10, and alleles A10T9 and A8T12 were omitted from the table due to small sample size.
p_c: the *p*-value was corrected by multiplied with the genotype number for multiple comparisons; §the *p*-value was estimated by Fisher's exact test.
OR: odds ratio; 95% CI: 95% confidence intervals.

were greater than or equal to the median (5 pg/ml), showing a significant difference compared to the controls (44.00%; OR=1.88, 95% CI=1.17-3.02, *p*=0.008). Among those carrying heterozygous genotype CG at polymorphism -572C/G, the gout patients have 71.05% (27/38) with high IL-6 levels (≥5 pg/ml), which also showed a significant difference compared to the controls (37.88%; OR=4.03, 95% CI=1.70-9.51, p_c=0.003). However, among those carrying genotypes CC or GG at polymorphism -572C/G, or among those carrying each genotype at polymorphism -373A(m)T(n), no similar significant association existed between the gout disease and IL-6 levels (all *p*>0.05, Table IV).

Discussion

Gout is a common and complex multifactorial disease in which both genetic and environmental factors were demonstrated to contribute to severity and occurrence. Environmental factors associated with gout disease have been revealed including obesity, alcohol consumption, hyperuricemia, higher creatinine and triglycerides levels (11, 12), and the genetic factors, including TNF-α, chromosome 4q25, 1q21 and urate transporter 1 (URAT1), were proposed to have an important role in the development of gout disease (12, 14-16). Another aspect contributing to the development of gout disease is the proinflammatory cytokine, such as IL-6, which is produced at the site of inflammation and is an important mediator in the acute gout phase. Some studies have demonstrated that the MSU crystals can induce IL-6 production in synoviocytes and monocytes (1, 17), and the IL-6 is also known to be an endogenous pyrogen (18) and the gene is the major cytokine inducer gene during the acute gout phase (19). Desgeorges *et al*. also revealed that IL-6 levels were significantly lower in the sera of healthy controls than those of gout patients (20). Our study also demonstrates the gout patients have significantly greater proportions of high IL-6 levels in serum than the controls, especially for those carrying genotype CG at polymorphism -572C/G. Regarding the IL-6 protein levels in those carrying different polymorphisms in the IL-6 gene, some studies showed that those carry genotype GG at polymorphism -174G/C had higher IL-6 protein levels than the genotype CC carriers (9); those carrying the genotype CC or CG at polymorphism -572C/G had a lower IL-6 protein levels than genotype GG (21); and for the polymorphism -373A(m)T(n), allele A9T11 was associated with lower serum IL-6 levels (8).

Even though our data did not show the significant difference in the IL-6 levels among those carrying different genotype at polymorphism -572C/G, nevertheless we showed that while those carrying genotype CG the gout patients had higher IL-6 levels than the controls. This finding can further support the role of IL-6 levels in the inflammatory gouty arthritis.

During the acute inflammation phase, the leukocyte infiltrate initially is mostly neutrophilic, and then monocytes predominate (22-24). In contrast, in chronic inflammation, most of the associated cells were mononuclear cells, such as macrophages and lymphocytes (22, 23). The mechanisms that control the transition from neutrophil to monocyte recruitment during the transformation from acute to chronic inflammation are poorly understood. It is possible that the IL-6/soluble IL-6 receptor α (sIL-6Rα) complex plays an important role in this transition, and it has been shown to recruit of leukocytes directly at inflammatory sites (25). In addition, IL-6/sIL-6Rα was shown to stimulate synoviocyte proliferation (26) and osteoclast maturation (27), and the sIL-6R is as an important consideration in IL-6-mediated effects within the joint (28). However, our study did not show a significant contribution to gout disease in the genetic components at the polymorphisms -572C/G and -373A(m)T(n) in IL-6 gene. We hypothesized that under the dysfunction of IL-6/sIL-6Rα complex, the transition from neutrophil to monocyte recruitment will not be complete, and the inflammatory syndrome of the gout patients will persist. Therefore, we suggest a molecular analysis between gout disease and IL-6 gene should be performed in combination with both IL-6 and sIL-6Rα genes.

Regarding the polymorphism -174G/C in IL-6 gene, even though this polymorphism was revealed to be associated with systemic juvenile chronic arthritis (9), and high proportions of heterozygosity were found in western countries (9, 29), but most of those living in eastern populations do not have the allele C variant, including Taiwanese (29), Japanese (30) and Koreans (31). In our study, the allele C at polymorphism -174G/C was not found among any of the participants either, *i.e.*, all of the subjects showed a homozygosis distribution of genotype GG. In a similar homozygosis genotype distribution at polymorphism -597G/A the allele A was also absent from our study. It may be considered that a high linkage disequilibrium existed among the four polymorphisms in the IL-6 gene, and we suggest that the polymorphism -572C/G can be representative of the four polymorphisms among male Taiwanese.

In summary, a total of 120 male gout patients and 184 healthy controls were included to explore the associations between gout disease and IL-6 levels and the polymorphisms in IL-6 gene. We demonstrate that the gout patients have greater proportions of high IL-6 levels in the plasma than the controls, especially in those carrying the genotype CG at polymorphism -572C/G. Nevertheless, none of the four polymorphisms in IL-6 gene, -597G/A, -572C/G, -373A(m)T(n) and -174G/C, was revealed to have a significant association with both gout disease and IL-6 levels.

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