RESEARCH ARTICLE

Functional study of TNF-α promoter polymorphisms: literature review and meta-analysis

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Accepted for publication June 6, 2011

To cite this article: Mekinian A, Tamouza R, Pavy S, Gestermann N, Ittah M, Mariette X, Miceli-Richard C. Functional study of TNF-α promoter polymorphisms, literature review and meta-analysis. *Eur. Cytokine Netw.* 2011; 22(2): 88-102 doi:10.1684/ecn.2011.0285

ABSTRACT. The functional consequences of TNF-α promoter SNPs are still controversial and, to date, the functional consequences of TNF-α haplotype combinations in healthy subjects have not been assessed. In order to assess functional consequences of each TNF-α polymorphism and of their haplotype combination, TNF-α expression and secretion by LPS-stimulated monocytes from 50 healthy subjects were assessed. Monocytes were isolated and cultured for four hours, after 100 ng/mL LPS stimulation. mRNA expression was quantified using the real-time polymerase chain reaction, and TNF-α levels were measured by enzyme-linked immunosorbent assay. Each subject was genotyped for TNF-α -857 C/T, -238 G/A, -308 G/A polymorphisms. In order to confirm definitively the functional consequences of these TNF-α polymorphisms, we then performed a systematic review of the literature for TNF-α SNPs, and then a meta-analysis of the functional studies of the TNF-α -308 G/A SNP. No association between TNF-α mRNA or protein level expression, and TNF-α -238G/A, -308G/A, -857C/T polymorphisms, studied either independently or in haplotype combinations, was revealed. Using a meta-analysis for the TNF-α -308 G/A polymorphism, we confirmed the absence of any association between TNF-α mRNA and protein levels, and TNF-α -308 G/A genotypes. This study and meta-analysis of the literature confirmed the absence of any functional consequences of the TNF-α -308G/A promoter polymorphism, either alone, or in various haplotype combinations in healthy subjects.

Key words: TNF-**α**, single nucleotide polymorphisms, haplotypes, functional study, review

TNF-α antagonists have dramatically improved the outcome in rheumatoid arthritis (RA) [1]. Nevertheless, about 30% of patients fail to obtain any clinical improvement. Several studies have investigated the association between the *TNF*-α -308 G/A polymorphism and the response to TNF-α antagonists in RA, however, the results have been contradictory. Our group has suggested that the ancestral haplotype of the TNF-α promoter, *TNF*-α -238G/-308G/-857T, the most frequent among Caucasians, is associated with a lower response to adalimumab in homozygous carriers [2]. When analyzed independently, the *TNF*-α -308 G/A genotype was not associated with any specific response profile to adalimumab [2].

Some studies have investigated the functional consequences of TNF-α promoter polymorphisms. Most of these studies have focused on each SNP, and have mainly relied on transfection models or *ex vivo* cell culture, however, these too lead to controversial results [3]. Recent *in vivo* studies did not confirm any functional effect of the TNF-α SNPs when analyzed separately [4, 5]. The functional consequences of *TNF*-α promoter haplotype combinations have not been investigated to date in healthy subjects.

The first aim of this study was to determine whether the three *TNF*-α polymorphisms, as well as their haplotype combinations, were associated with varying levels of TNF-α production in healthy subjects. Moreover, in order to confirm the functional consequences of these TNF-α polymorphisms, we performed a systematic review of the literature and conducted a meta-analysis of previously published functional studies of *TNF*-α promoter SNPs.

PATIENTS AND METHODS

Patients

Fifty healthy subjects (mean age 47±17 years; 29 females) were included in the study. These subjects had no chronic illness, were not taking corticosteroids and were exhibiting no increase in acute phase reactants when included in the study.

All subjects gave their informed consent to participate to the study.

doi: 10.1684/ecn.2011.0285

Monocyte stimulation and RNA extraction

Firstly, PBMCs were isolated by density gradient centrifugation on Ficoll-Hypaque (Sigma Chemical Co., St. Louis MO, USA), immediately after blood collection. Monocytes were isolated using LS column positive selection (Miltenyi Biotec).

Purified monocytes were stimulated with 100 ng/mL of LPS (*E. coli* 055 ; Sigma Chemical Co.), and cultured for four hours at 37°C and 5% CO_2 (RPMI 1640, glutamax Gibco, 10% SVF Dutscher, penicillin 100 U/mL, streptomycin 100 μg/mL, HEPES buffer 10 mM, sodium pyruvate 1mM, and amino acids, Invitrogen). In addition, unstimulated, baseline monocytes were studied as a control.

Total mRNA was extracted using commercially available RNeasy Minikit (Qiagen), and cDNA synthesized with Omniscript^{TM} Reverse Transcriptase (Qiagen) and oligo(dT) primers (Promega).

TNF-α mRNA quantification

Real-time PCR was carried out to quantify TNF-α expression using a LightCycler^{TM} (Roche, Mannheim, Germany).

Primer probes used for TNF-α mRNA quantification were *For*: 5'-CAGAGGGAAGAGTTCCCCAG-3' and *Rev:* 5'-TGTAGCCATGTTGTAGCA -3', with a final product size of 108 bp.

In order to normalize any possible variation in mRNA extraction and cDNA synthesis, expression of β-actin, a housekeeping gene, was also used. Primer sets of β-actin were *For:* 5'-GCTGTGCTACGTCGCCCT- 3' and *Rev*: 5'-AAGGTAGTTTCGTGGATGCC-3', with a final product size of 200 bp.

TNF-α and β-actin PCR products were detected using the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics). To correct for variations in mRNA recovery and reverse transcription yield, the amount of TNF-α cDNA was normalized with β-actin. Results were expressed as increases in normalized values, over that observed with untreated cells. Quantitative PCR runs were considered only if amplification efficiencies were high (slopes ranging from -3.2 to -3.6). Each sample was processed in duplicate, with initial incubation at 96°C for 10 min, and thermal conditions followed 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. For each run, serially diluted cDNA of control PBMCs was used for quantitative standards. We determined the cell equivalence (CE) number of TNF-α and β-actin mRNA in each sample according to the standard curve generated from values obtained with PBMCs. The unit number showing the relative TNF-α mRNA level in each sample was determined as a value of TNF-α CE normalized with β-actin CE. Melting-curve analysis was performed to assess the specificity of the PCR product. Results were expressed in duplicate as the TNF-α/β-actin ratio at baseline, after stimulation, and a -fold stimulation/baseline ratio.

Soluble TNF-α quantification

TNF-α production in cell-free supernatants (sTNF-α) was determined in duplicate at baseline and after stimulation using Quantikine enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, USA).

TNF-α SNP genotyping

All subjects were genotyped for three TNF-α gene polymorphisms (-258G/A, -308G/A, and -857C/T). These polymorphisms were genotyped by allelic discrimination TaqMan PCR using a commercial assay kit C_7514879 (PE Applied Biosystems).

We used PHASE (version 2.1) software to perform haplotype reconstructions. This Bayesian algorithm provides the most likely pairs of haplotypes carried by each subject. The average probability of PHASE certainty in haplotype inference was 99% for *TNF*-α haplotypes.

Statistical analysis

All quantitative data are expressed as means (SD) or medians (IQ25-75) according to their distribution, and all qualitative data as frequencies (percentages). All genotyped SNPs were in Hardy-Weinberg equilibrium.

For each SNP, genotypes and haplotypes were analyzed for mRNA expression and TNF-α levels using the Mann-Whitney test.

Statistical analysis was performed on GraphPad (5.1 version; 2007), and significance was defined as $p<0.05$.

LITERATURE REVIEW AND META-ANALYSIS

Search strategy

The bibliographic search was performed using Medline (January 1990 to October 2009), by two investigators (AM and CMR) employing the following key words: gene polymorphism, single nucleotide polymorphism, TNF-alpha, TNF-alpha -308 G/A, TNF-alpha -238 G/A, TNF-alpha -376 G/A, TNF-alpha -863 C/A, TNF-alpha -1031 T/C, whole blood, PBMCs, monocytes, TNF-alpha mRNA, TNF-alpha plasma levels, healthy, functional study. Transfection studies in animals were excluded. All other articles were included in the literature review.

Study selection for the meta-analysis

In order to be included in the meta-analysis, the studies had to have to reported functional consequences of *TNF*-308 G/A SNP, as assessed by mRNA and/or TNF-alpha protein levels. Studies involving non-Caucasians were included if the reported GG genotype frequency was similar to that found in Caucasians. When different categories of subjects were analyzed in the same study, as for example patients with a disease condition and healthy controls, they were independently included in the meta-analysis. Studies involving transfection *in vitro* were excluded from the analysis. Inclusion and exclusion criteria for the meta-analysis were checked independently for each article by two reviewers (AM and CMR). In cases of disagreement, articles were re-examined and discussed until consensus was achieved.

Data collection

The main outcome measures of TNF-α -308 G/A functional consequence were TNF-α mRNA and protein levels, as expressed in means with SD or SE. Data collection and data extraction were performed by one reviewer (AM) using a predefined form.

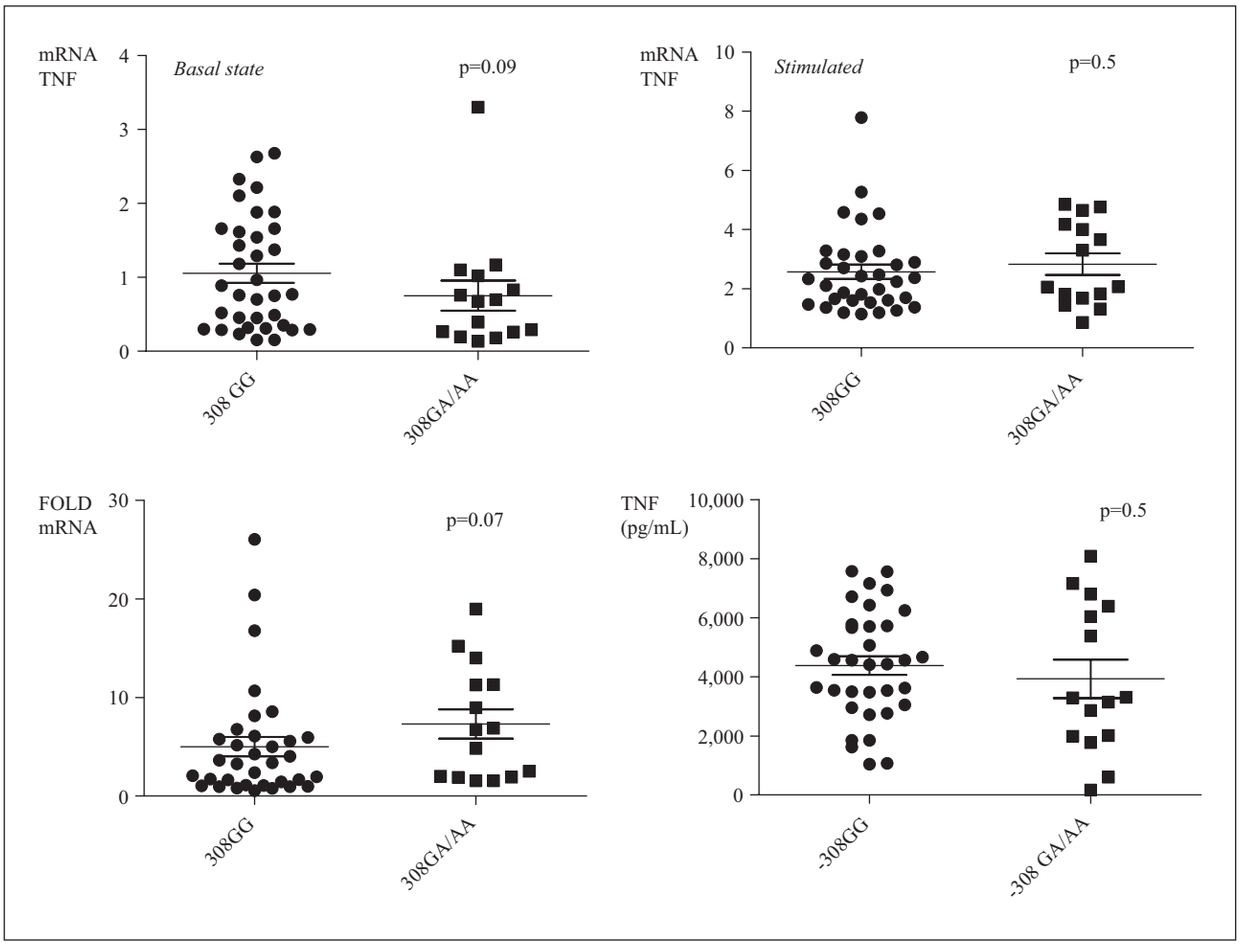

Figure 1
mRNA and TNF-α protein levels according to the *TNF*-α -308 G/A SNP.

Statistical analysis

The meta-analysis was performed according to the recommendations of the Cochrane Collaboration [6]. The standardized mean difference (SMD) was the effect measure of interest assessing the association between the *TNF*-α -308 G/A promoter polymorphism and TNF-α mRNA and protein levels. For the meta-analysis, summary SMDs were computed using a random effects models (for data demonstrating significant heterogeneity, $I^2 \geq 50\%$). To assess heterogeneity across studies, we used the I^2 statistic based on Cochran's heterogeneity statistic (Q). Firstly, all studies were included in the meta-analysis and the *TNF*-α -308 GG genotype was compared against the AG/AA combined group according to mRNA and protein levels. Because of the differences in populations and protocols between studies, a sub-analysis was performed according to the methods (*e.g.* circulating TNF-α levels or PBMCs under stimulation), and the subjects included in the studies (healthy subjects or with disease conditions). Then, a meta-analysis was conducted in similar way to compare the common GG genotype with the very rare, AA genotype. Meta-analyses were computed using RevMan analyses software (Review Manager (RevMan), In. Version 5.0 ed. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration; 2008).

RESULTS

TNF-α promoter polymorphism distribution

Genotype and haplotype distributions for 50 healthy subjects are shown in the *supplementary table 1*. Three subjects carried the rare *TNF*-α -308 AA genotype and one subject carried the rare *TNF*-α -857 TT genotype. Eighteen subjects (36%) were homozygous carriers of the *TNF*-α GGC/GGC ancestral haplotype.

TNF-α mRNA and TNF-α protein levels

The TNF-α/βactin mRNA ratios were 0.9±0.8 at baseline, 2.6±1.4 after LPS stimulation, and 5.7±5.8 when expressed as -fold after LPS stimulation relative to baseline expression. There was no detectable TNF-α protein level (sTNF-α) at baseline for all subjects, but was reached 4,240±2,050 pg/mL after LPS stimulation. The mRNA and sTNF-α levels were significantly different before and after LPS stimulation, either in the whole group, or after stratification according to various genotype and haplotype combinations ($p < 0.001$).

Association between TNF-α mRNA, sTNF-α and TNF-α genotypes

As the *TNF*-α -308 AA genotype was rare, we compared the *TNF*-α -308 GG genotype group to the combined *TNF*-α -308 AG and AA genotype group (*TNF*-α -308 AG/AA) *(figure 1)*. Likewise, the *TNF*-α -238 GG genotype was compared to the combined *TNF*-α -238 AG and AA genotypes; the *TNF*-α -857 CC genotype was compared to the combined *TNF*-α -857 CT and TT genotypes. We found no association between TNF-α mRNA or protein levels in culture supernatants, or any of these *TNF*-α genotypes *(supplementary figure 1 and supplementary table 2)*.

Association between TNF-α mRNA, sTNF-α and TNF-α haplotype combinations

TNF-α mRNA and sTNF-α levels were compared between *TNF*-α GGC/GGC and the other haplotype combinations. Levels of TNF-α mRNA and of sTNF-α for the *TNF*-α GGC homozygotous carriers were no different from the other haplotype combination carriers *(figure 2)*. Similar results were observed when analysis was performed for the TNF-α GGC/GAC and GGC/GGT haplotypes *(supplementary table 2)*. Other haplotype combinations could not be analyzed as they were underrepresented.

Literature review for TNF-α -308 G/A SNP

The literature search yielded 52 citations. There were seven studies involving *in vitro* transfection methods *(table 1)*. Five studies analysed TNF-α mRNA levels in the basal state or after stimulation *(table 2)*. Nineteen studies examined TNF-α protein levels, in the basal state or after stimulation, either in whole blood (seven studies), in peripheral blood mononuclear cells (PBMCs) (seven studies), in monocytes, in lymphocytes, in cerebrospinal fluids, in nasal secretions and in chorioamnion (n=1, respectively) *(table 2)*. Nineteen studies evaluated TNF-α circulating levels *(table 3)*.

Regarding studies of the *TNF*-α -308 G/A SNP selected for meta-analysis, the literature search yielded 45 citations. Twenty-four studies exploring the functional consequences of *TNF*-α -308 G/A were excluded, because data were expressed as medians with ranges or interquartiles, or were insufficient for analysis. Twenty one studies met the inclusion criteria for the meta-analysis. Among them, three studies examined TNF-α mRNA levels and 19 studies examined TNF-α protein levels. In seven studies, various conditions or patient categories were analyzed, and were included separately in the meta-analysis. Data from our study were also included in the meta-analysis.

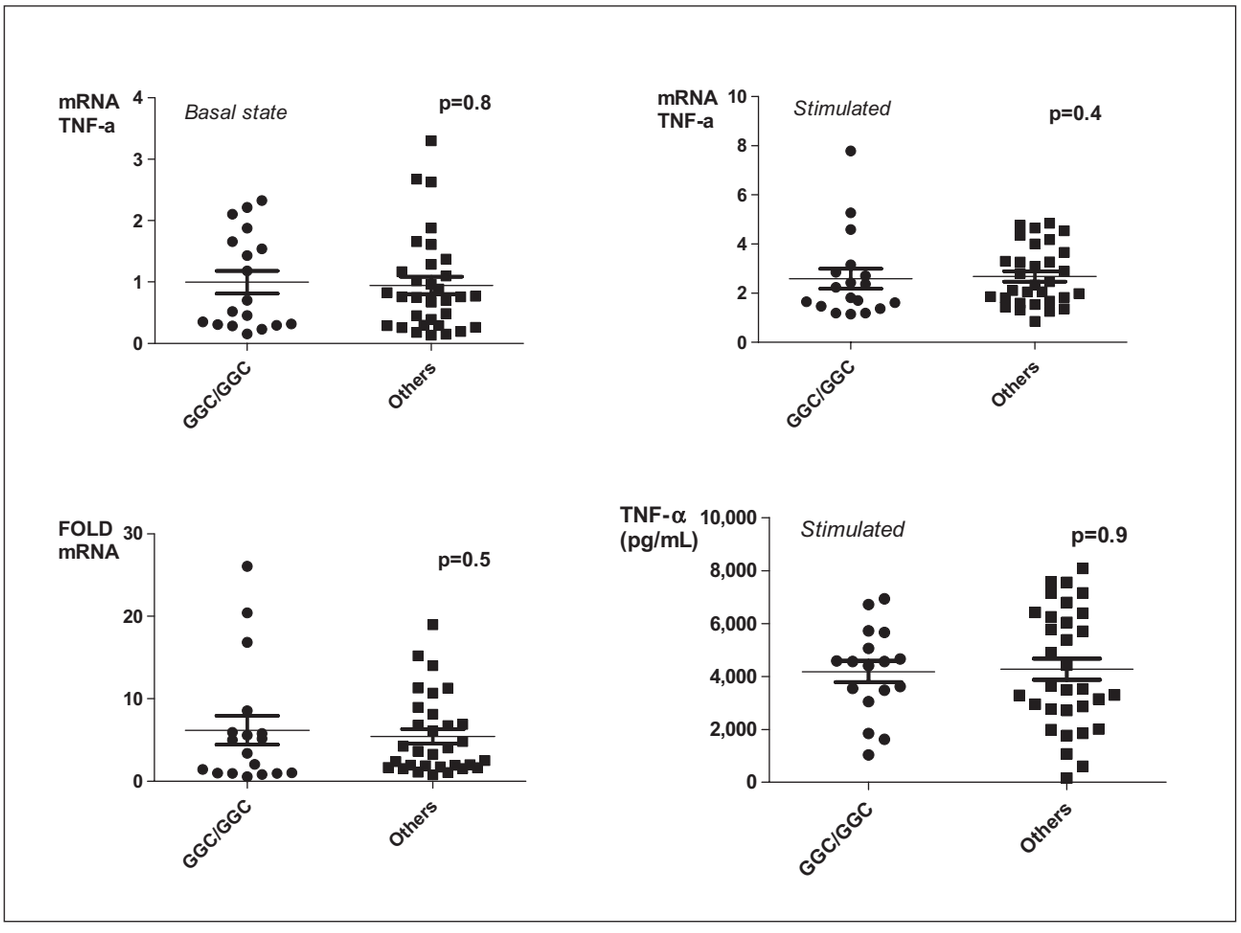

Figure 2
mRNA and TNF-α protein levels according to the *TNF*-α GGC/GGC haplotype combination.

Table 1
Studies *in vitro* using transfection to assess TNF-α promoter activity of the *TNF*-α -308G/A SNP.

Author Year	5' Segment	3' Segment	Cell lineage	TNF-2/TNF-1 (unstimulated)	Stimulation (ng/mL)	Time (hours)	TNF-2/TNF-1 (stimulated)
Stuber 1996 [13]	-1,173 to +130	-	MonoMac6	-	LPS (100)	20	none
Kroeger 1997 [14]	-993 to +110	-	U937	1	PMA (20)	24	1.3
Bailey 2001 [15]	-1,073 to +130	-	U937	1.3	LPS+PMA (1,000/10)	18	1.1
Kroeger 2000 [16]	-993 to +110	+1,957 to +2,792	U937	-	PMA (20)	18	**2.2***
Kroeger 2000 [16]	-993 to +110	+1,957 to +2,792	U937	1.4	TNF-(5) IFN-g (100 u/mL) LPS (5,000)	24	1.1 1.1 **2.5***
Kroeger 1997 [14]	-993 to +110	+1,957 to +2,792	U937	1	PMA (20)	24	**2***
Kroeger 2000 [16]	-993 to +110	+1,957 to +2,792	THP1	-	PMA (20)	18	1
Brinkman 1996 [17]	-619 to +108	-	Jurkat	1	PMA (10)	8/24	1.1 /1.1
Bailey 2001 [15]	-1,073 to +130	-	Jurkat	1	CD23/CD8/PMA(10)	8	0.8
Karimi 2009 [18]	-992 to +110	-	Jurkat	**1.53* (5 μg)** **1.97*(3 μg)** **2.05*(1 μg)**	-	-	-
Kroeger 1997 [14]	-993 to +110	-	Jurkat	0.64	PMA (20)	24	1.5
Stuber 1996 [13]	-1,173 to +130	-	Jurkat	-	PMA	20	1.2
Brinkman 1996 [17]	-619 to +108	+108 to 1951	Jurkat	1	PMA (10)	8/24	1 /1.1
Kroeger 1997 [14]	-993 to +110	+1,957 to +2,792	Jurkat	1.1	PMA (20)	24	**1.7***
Kroeger 2000 [16]	-993 to +110	+1,957 to +2,792	Jurkat	-	PMA (20)	18	**3***
Karimi 2009 [18]	-992 to +110	+1,957 to +2,792	jurkat	1	-	-	
Wilson 1997 [19]	-585 to +106	-	Raji	**5.5***	PMA (50)	48	**8.4***
Brinkman 1996 [17]	-619 to +108	-	Raji	0.8	PMA (50)	24/48	1.1/1
Bailey 2001 [15]	-1073 to +130	-	Raji	1	PMA (50)	24	1.1
Kroeger 2000 [16]	-993 to +110	+1,957 to +2,792	Raji	-	PMA (20)	18	1.3
Brinkman 1996 [17]	-619 to +108	+108 to 1,951	Raji	1	PMA (50)	24/48	1/0.9
Kroeger 2000 [16]	-993 to +110	+1,957 to +2,792	Hela	-	PMA (20)	18	1
Kroeger 2000 [16]	-993 to +110	+1,957 to +2,792	HepG2	-	PMA (20)	18	1.4

* $p<0.05$ TNF-2 *vs* TNF-1.

Literature review for TNF-α -238 G/A, TNF-α-376 G/A, TNF-α -863 C/A, TNF-α -1031 C/T SNPs

The literature search yielded 12 citations. Ten studies involved the *TNF*-α -238 G/A SNP, two studies *TNF*-α -376 C/T, four studies *TNF*-α -863 C/A and one study *TNF*-α -1031 T/C *(supplementary table 4)*.

Meta-analysis of the TNF-308G/A SNP

As the *TNF*-α -308 AA genotype is rare, we first compared the *TNF*-α -308 GG group to the combined *TNF*-α -308 AG/AA group. We found no difference between the TNF-α protein levels of the combined *TNF*-α -308 AG/AA group and those of the *TNF*-α -308 GG genotype group: the standardized mean difference (SMD) was -0.19, with a 95% confidence interval [-0.48, +0.11] (*figure 3A*). The heterogeneity across studies was large with I^2=90%. Similarly, no difference could be seen when analysis was performed by subgroup involving circulating levels of TNF-α or TNF-α levels in PBMCs after stimulation, in healthy subjects, or in different disease conditions *(supplementary figures 1-4)*. Furthermore, no difference was observed in TNF-α mRNA levels: the SMD was -0.97 (95% confidence interval [-2.67, 0.73]) *(figure 3B)*. When the rare AA genotype was compared to the GG genotype, no difference was shown concerning TNF-α protein levels: the SMD was -1.01 (95% confidence interval [-2.24, 0.22]) *(figure 4)*. The number of studies available was insufficient to evaluate the mRNA levels between the TNF-α GG and AA genotypes.

DISCUSSION

We have analyzed for the first time the functional consequences of *TNF*-α SNPs and haplotype combinations in healthy subjects, at both transcriptional and post-translational levels. This study was performed at the basal state, and after stimulation in monocytes, the cells that are mainly responsible for TNF-α production. We found no significant association between TNF-α production and any of the SNP genotypes or haplotype combinations. Meta-analysis of the literature confirmed this absence of association between TNF-α production and SNP genotypes.

Identifying predictive factors of the response to TNF-α antagonists in patients with inflammatory diseases remains a challenging goal. Two types of biomarkers have been suggested to be associated with this response: a high level of plasma TNF-α before treatment [7], and the presence of the rare *TNF*-α -308 A allele. It was tempting to suggest that an association between these biomarkers and the hypothesis that a functional consequence of these TNF-α promoter polymorphisms could be a variation in the levels of TNF-α production. Many studies have examined the functional consequence of the *TNF*-α -308 G/A SNP on TNF-α production, but the results remain controversial *(tables 1-3)* [7-51]. The low number of patients included in each study or the fact that the rare A genotype (TNF-2) was underrepresented, may explain these discrepancies. These studies also differed as regards culture duration, concentration of LPS used for cell stimulation, and stimuli.

Table 2
Functional relevance of -308 G/A SNP *ex vivo* (literature studies).

Author / Year	Total (n)	GG (n)	Ethnic origin	Condition	Methods	TNF-2/TNF-1 protein (unstimulated)	Stimulation (dose; time)	TNF-2/TNF-1 protein (stimulated)	TNF-2/TNF-1 mRNA (unstimulated)	TNF-2/TNF-1 mRNA (stimulated)
Huizinga 1997 [35]	179	106	C	Healthy	Whole blood	-	LPS 10 ng/mL (6 h)	1.2	-	-
Huizinga 1997 [35]	179	106	C	Healthy		-	LPS 1,000 ng/mL (6 h)	0.8	-	-
Turner 1995[36]	22	14	C	Heart transplants		-	LPS (4 h)	1.1	-	-
Ba de Jong 2000 [37]	129	-	C	Healthy		-	LPS 1,000 ng/ml (4 h)	1	-	-
Westendorp 1997[38]	188	-	C	Meningitis		No difference	LPS 10 and 1,000 ng/mL (6 h)	No difference	-	-
Baseggio 2001 [10]	21	17	C	Lymphoma		No difference	LPS 1 μg/mL (0.5-24 h)	No difference	-	***2.1 (0.5h)****
Louis 1998 [39]	57	41	C	Healthy		-	LPS 100 ng/mL (3 h)	1.2	-	-
Louis 1998 [39]	57	41	C	Healthy		-	LPS 1 ng/mL (4, 24 h)	***1.8* (24 h)***	**-**	**-**
Heesen 2003 [40]	61	39	C	Healthy		-	LPS 100 ng/mL (4 h)	***1.3****	**-**	**-**
Pachman 2000 [41]	29	24	C	Healthy	PBMCs	0.7	-	-	-	-
Somoskovi 1999 [42]	44	26	C	Sarcoidosis		0.5	LPS 1 μg/mL (24 h)	0.6	-	-
He 1995 [9]	47	35	C	MS		-	MBP	-	0.6	0.6
Mycko 1995 [43]	53	39	C	MS		0.6	LPS 10 μg/mL (4 h)	0.9	-	-
Mycko 1995 [43]	81	68	C	Healthy		0.5	LPS 10 μg/mL (4 h)	0.9	-	-
O'Dwyer 2008 [8]	50	35	C	Septic shock (day 1)		-	-	-	1.2	-
O'Dwyer 2008 [8]	50	35	C	Septic shock (day 7)		-	-	-	No difference	-
Oregon 2008 [12]	100	41	NC	Healthy		-	-	-	1	-
Oregon 2008 [12]	50	41	NC	RA		-	-	-	***0.3****	-
Pachman 2000 [41]	37	19	C	DM		***2.8****	-	-	-	-
Fernandez 2002 [44]	62	44	C	Healthy		-	Conca A 10 μg/mL (48 h)	No difference	-	-
Huang 1999 [45]	33	13	C	Myasthenia		-	AntiCD3 (72 h)	***1.8****	**-**	**-**
Huang 1999 [45]	53	22	C	Healthy		-	AntiCD3 (72 h)	***1.8****	**-**	**-**
Fernandez 2002 [44]	53	40	C	Liver transplants		-	Conca A 10 μg/mL (48 h)	***1.8****	**-**	**-**
Bouma 1996 [46]	42	26	C	IBD		-	AntiCD3/CD28 (48 h)	***1.3****	**-**	**-**
Pociot1995 [47]	78	68	C	Healthy	Monocytes	-	LPS 250 pg/mL (18 h)	1.3	-	-
Our study	50	35	C	Healthy		0	LPS 100 pg/mL (4 h)	1	0.9	1.1
Lu 2008 [11]	67	33	NC	AS		-	LPS 2 μg/mL (0-8 h)	-	1	***3.7 *(2h)***
Hoffmann 2001 [48]	32	24	C	Healthy	Lymphocytes	-	AntiCD3/CD28 (72 h)	0.9	-	-
Billolikar 2005 [49]	50	17	C	Asthma	Nasal secretions	***0.3****	-	-	-	-
Tarkowski 2000 [50]	50	32	C	AD	CSF	1	-	-	-	-
Hernandez 2003 [51]	18	13	NC	Chorioamnion	-	1	LPS 1/5/10 ng/mL (24 h)	0.8/1/1.1	-	-
Hernandez 2003 [51]	18	13	NC	Chorioamnion	-	1	LPS 50 ng/mL (24 h)	***2.1****	**-**	**-**

* $p<0.05$ TNF-2 *vs* TNF-1.
** $p<0.05$ TNF-1 *vs* TNF-2.
C: Caucasians; NC: non-Caucasians; MS: multiple sclerosis; AS: ankylosing spondylitis; DM: dermatomyositis; IBD: inflammatory bowel disease; AD: Alzheimer's disease; CSF: cerebrospinal fluid; PBMCs: peripheral blood mononuclear cells; Conca: concavalin; A/G: TNF-2/TNF-1; "-": not available; no difference: data not shown.

Table 3
TNF-α plasma levels according to -308 G/A SNP (literature studies).

Author Year	Total (n)	GG (n)	Ethnic origin	Condition	TNF-2/TNF-1
Skoog 1999 [20]	156	99	C	Healthy	1.1
Oregon 2008 [12]	100	93	NC	Healthy	1
Wennberg 2002 [21]	84	-	C	Healthy	No difference
Kubota 1998 [22]	211	164	C	Heart failure	0.8
Ito 1999 [23]	48	35	NC	Idiopathic cardiomyopathy	1.1
Mira 1999 [24]	89	54	C	Septic shock	1.7
Baseggio 2001 [10]	21	17	C	Lymphoma	No difference
Cuchacovich 2004 [25]	20	10	NC	RA	1.4
Marotte 2008 [7]	39	27	C	RA	5.4
Fijen 2001 [26]	12	6	C	Healthy (after IV LPS)	1
Tang 2000 [27]	112	86	NC	Postoperative (before sepsis)	0.9
Sharma 2007 [28]	112	92	NC	Healthy	
Sharma 2007 [28]	83	74	NC	Sarcoïdosis	
Kovar 2007 [29]	87	59	C	Healthy (after IV LPS)	1
O'Dwyer 2008 [8]	14	-	C	Septic shock	No difference
Gordon 2004 [30]	213	135	C	Septic shock	No difference
Tang 2000 [27]	42	29	NC	Postoperative (sepsis)	***1.6****
Appoloni 2001 [31]	33	25	C	Septic shock	***1.8****
Gonzalez 2003 [32]	50	41	C	IBD	***7.3****
Bhushan 2008 [33]	104	74	NC	Obesity with obstructive sleep apnoea	***1.3****
Haddy 2005 [34]	752	607	C	Healthy families	***0.8 (p=0.06)***
Oregon 2008 [12]	50	41	NC	RA	***0.6****

* p<0.05 TNF-2 *vs* TNF-1.
** p<0.05 TNF-1 *vs* TNF-2.
C: Caucasians; NC: non-Caucasians; RA: rheumatoid arthritis, IBD: inflammatory bowel diseases; IV: intravenous; A/G: TNF-2/TNF-1; no difference: data not shown.

Few studies analyzed *ex vivo* the effect of *TNF*-α SNPs at the transcriptional level *(table 1)*. Using a transfection protocol, some groups investigated the transcriptional promoter activity of each allele *in vitro (table 2*) [13-19]. One group found nearly twice as much transcription of *TNF*-α -308 AG/AA (TNF-2), but the selective importance of 3'UTR [14, 16] was not confirmed in their last study [18]. Another group found similar results, with a very large *TNF*-α -308 AG/AA to *TNF*-α -308 GG (TNF-2/TNF-1) ratio, but this was mainly due to an unusual resistance of TNF-1 to PMA stimulation [19]. A few *in vivo* studies, using allele-specific transcript quantification (ASTQ) and haploCHIP, could not confirm any difference concerning -308 G/A SNP [4, 5, 17]. Studies that analyzed TNF-α protein levels either *ex vivo* or after stimulation are also contradictory, although they mainly reported no effect *(tables 1*, *3)* [7, 8, 10, 12, 20-51].

Some of the previous studies were conducted in different pathological situations, such as lymphoma, septic shock, RA, and not in healthy subjects. In order to avoid interference from uncontrolled factors that could affect TNF-α production (*e.g.* treatments, infection *versus* inflammation), we chose to study healthy subjects to better ascertain the consequences of genetic background on TNF-α expression. Regarding the 17 studies in the literature that involved healthy subjects, there was no agreement as to the functional consequences of TNF-1 or TNF-2. Using the meta-analysis, which included the most of these studies, we were able to identify the absence of any functional relevance of the *TNF*-α -308 G/A SNP on transcriptional as well as post-translational levels, and thus confirm our own experimental results. More interestingly, using the meta-analysis, we were able to bring together the largest number of patients carrying the rare *TNF*-α -308 AA genotype, but we found no difference between this and the *TNF*-α -308 GG genotype. In light of our results and of the literature review, the absence of any significant effect of *TNF*-α -308 G/A SNP on TNF-α production is very clear, in particular in healthy subjects.

Only a few studies have analyzed other *TNF*-α SNPs *(supplementary table 3)*. As regards the *TNF*-α -238 G/A SNP, our results confirm other studies in healthy subjects; however, the *TNF*-α -238 AG/AA combined genotype was underrepresented. Huizinga *et al*. found more important levels of TNF-α in the *TNF*-α -238 GG group, but they could not confirm these results under different LPS concentrations [35]. In pathological situations, two studies revealed contradictory results [12, 28]. As regards other SNPs, available data are too scarce to allow any conclusions to be drawn [8, 20, 21, 35, 37, 52].

Taking into account the TNF-α involvement in the pathogenesis of RA, some studies investigated the association of TNF-α promoter SNPs with response to TNF-α antagonists. Again, similarly to the functional studies for TNF-α production, a number of these studies remain controversial. In 86 patients with RA, the presence of the *TNF*-α -308 G/G genotype correlated with a better response to

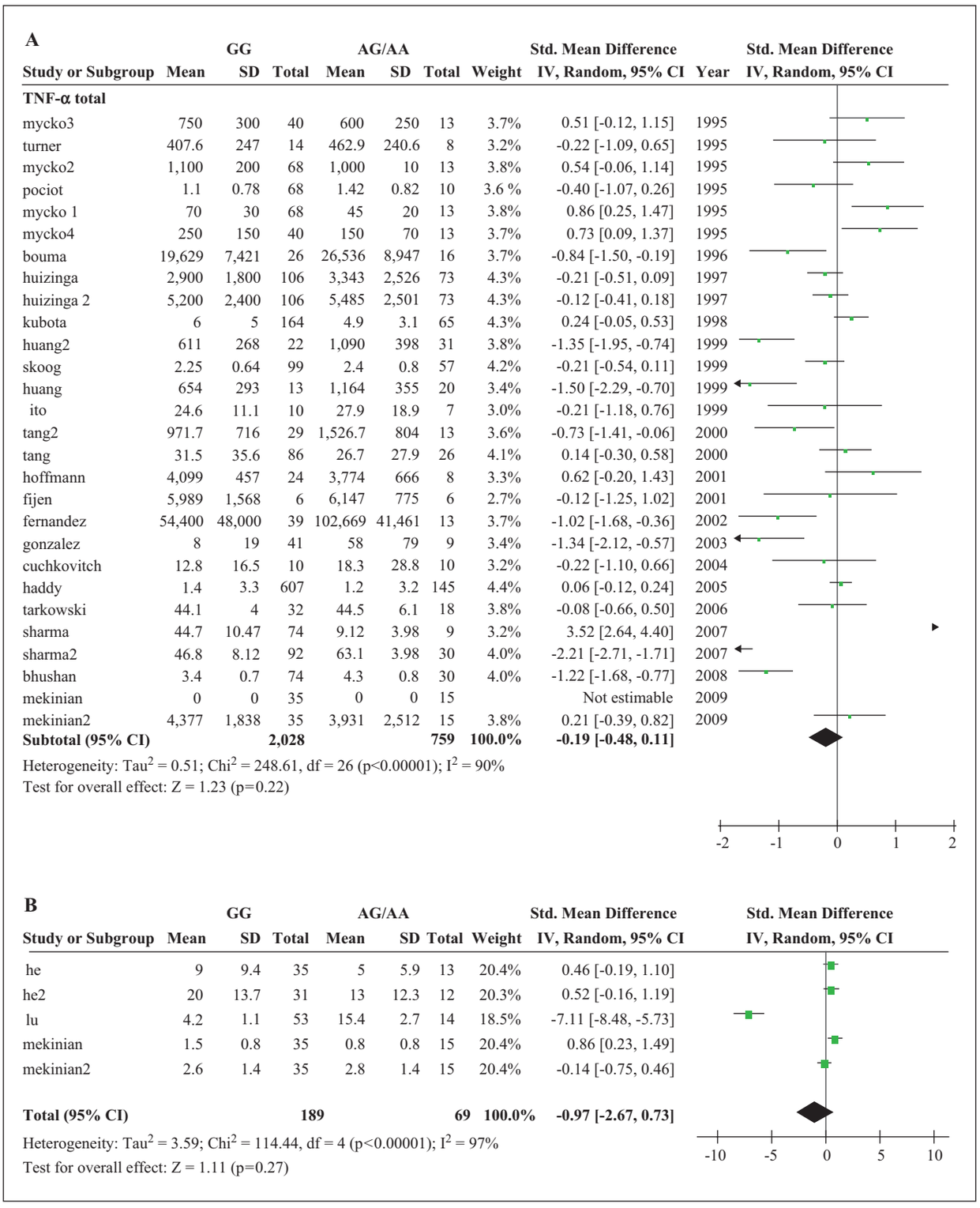

Figure 3
A) Meta-analysis of TNF-α protein levels [TNF-1 (GG) *vs* TNF-2 genotypes (AG/AA)].
B) Meta-analysis of mRNA levels (TNF-1 *vs* combined TNF-2 genotypes).

etanercept than that seen for the *TNF*-α -308 A/G genotype [53]. Another study showed that patients carrying the rare *TNF*-α -308A/A genotype responded poorly to etanercept, whereas the genotype for this SNP was not associated with response to infliximab [54]. A recent meta-analysis including all studies published until 2010, showed that the *TNF*-α -308A/G SNP, the most frequently investigated, was not associated with any clinical response in RA [55].

Recently, our group found that the ancestral TNF-α haplotype combination, and not any particular SNP alone, was associated with response to TNF-α antagonists in RA [2]. Nevertheless, our results show, for the first time, that

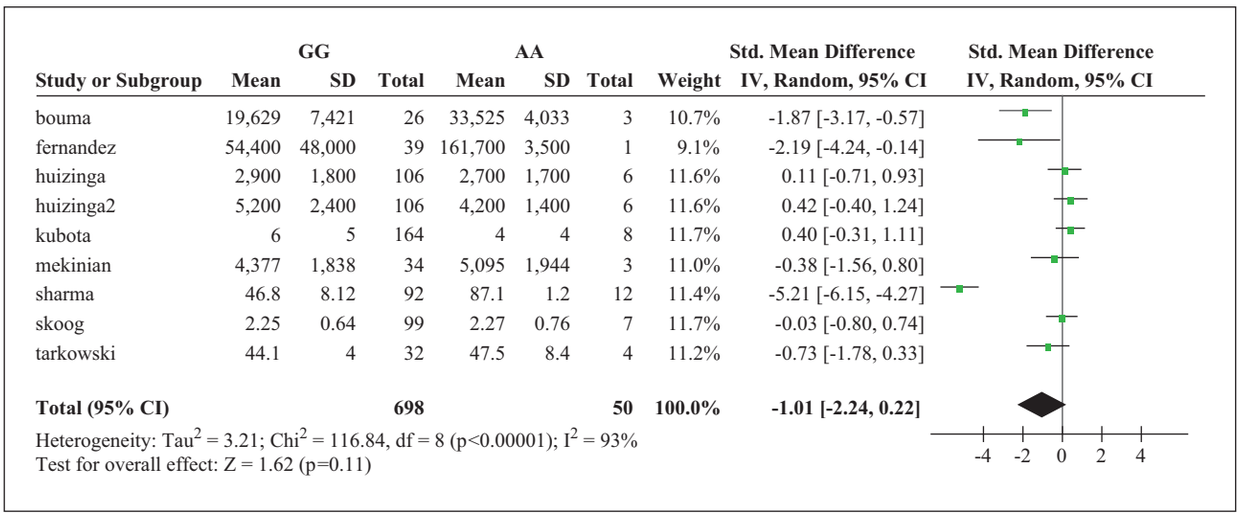

Figure 4

Meta-analysis of TNF-α protein levels (GG *vs* AA genotype).

haplotype combinations of TNF-α promoter polymorphisms do not lead to significant differences in TNF-α production in healthy subjects. In particular, while the *TNF*-α GGC/GGC haplotype has been shown to be associated with response to adalimumab in RA, this haplotype combination does not lead to different TNF-α mRNA or protein levels. Nevertheless, other mechanisms could explain the different responses of the haplotype combinations to TNF-α antagonists, and in particular their different binding to TNF-α. *TNF*-α promoter polymorphisms could also affect the production of other cytokines, as higher IFNα levels have been shown to be associated with TNF-2 [56]. However, we failed to demonstrate any difference in IFNα production associated with *TNF*-α genotypes and different haplotype combinations (data not shown). Linkage disequilibrium between lymphotoxin alpha (LTA) and *TNF*-α polymorphisms should also be taken into account, as etanercept is able to bind and inhibit both TNF-α and LTA, and therefore limit the effect of these cytokines on cellular responses. *TNF*-α haplotype combinations with *LTA* polymorphisms have been associated with various levels of LTA and TNF-α [5, 28, 52]. In conclusion, this study shows the absence of any significant functional association between TNF-α production and SNP genotypes or haplotype combinations of the *TNF*-α gene promoter.

Disclosure. *None of the authors has any conflict of interest or financial support to disclose.*

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SUPPLEMENTARY DATA

Table 1
Genotype and haplotype combination frequencies.

TNF-α polymorphism	Genotypes/haplotypes	n (%)
-238G/A	GG	45 (90)
	GA	5 (10)
	AA	0
	G allele	95 (95)
	A allele	5 (5)
-308G/A	GG	35 (70)
	GA	12 (24)
	AA	3 (6)
	G allele	82 (82)
	A allele	18 (18)
-857C/T	CC/CC	37 (74)
	CT/TT	12 (24)
	TT/TT	1 (2)
	C allele	86 (86)
	T allele	14 (14)
Haplotypes	GGC/GGC	18 (36)
	GGC/GAC	11 (22)
	GGC/GGT	11 (22)
	GGC/AGC	5 (10)
	GAC/GAC	3 (6)
	GGC/GAT	1 (2)
	GGT/GGT	1 (2)

TNF-α haplotypes are written in order -238G/A, -308G/A, -857C/T.

Table 2
Absence of association between TNF-α mRNA and protein levels, TNF-α SNP genotypes, and haplotype combinations.

TNF-α Genotypes/haplotypes (n)	mRNA TNF-α (unstimulated)	mRNA TNF-α (stimulated)	mRNA TNF-α (fold)	Protein TNF-α (pg/mL)
-238 GG (45)	0.8 [0.13-3.3]	1.6 [0.9-7.8]	3.8 [0.6-26]	4,023 [160-8,080]
-238 GA/AA (5)	1.4 [0.3-1.8]	1.4 [1.3-4.5]	2.4 [1.7-6.8]	4,428 [2,712-6,248]
-308 GG (35)	0.8 [0.15-2.7]	2.3 [1.1-7.8]	3.3 [0.6-26]	4,494 [1,041-7,571]
-308 GA/AA (15)	0.7 [0.13-3.3]	2.1 [0.9-4.9]	6.7 [1.5-18]	3,290 [160-8,080]
-857 CC (37)	0.7 [0.13-3.3]	2.2 [0.9-7.8]	4.4 [0.6-26]	4,417 [160-8,080]
-857 CT/TT (13)	0.8 [0.15-2.7]	2.2 [1.4-4.4]	3.5 [0.8-11]	3,640 [1,070-7,571]
GGC/ GGT (11)	0.9 [0.15-2.7]	2.3 [1.4-4.4]	2.5 [0.8-10.7]	4,889 [1,070-7,571]
Others (39)	0.7 [0.13-3.3]	2.2 [0.9-7.8]	4.3 [0.6-26]	4,023 [160-8,080]
GGC/ GAC (11)	0.4 [0.13-3.3]	3.3 [0.9-4.8]	6.7 [1.5-19]	3,290 [160-8,080]
Others (39)	0.15 [0.8-2.7]	2.2 [0.1-7.8]	3.3 [0.6-26]	4,494 [1,041-7,571]

Values are medians with ranges. Haplotype combinations are written in order -238G/A, -308G/A, -857C/T.
$p>0.05$ between different genotypes and haplotype combinations.

Table 3
Studies concerning other SNPs of the TNF-α promoter.

Author Year	Total (n)	Condition	Method	Polymorphism	mRNA ratio (unstimulated)	TNF- protein ratio (unstimulated)	Stimulation Concentration	mRNA ratio (stimulated)	TNF-α protein ratio (stimulated)
Pociot 1995 [47]	78	Healthy	Monocytes	-238G/A	-	-	LPS 250 pg/mL (18 h)	-	1.1
Westendorp 1997 [38]	188	Meningitis	Whole blood		-	No difference	LPS 10/1,000 ng/mL (6 h)	-	No difference
Ba de Jong 2002 [37]	129	Healthy	Whole blood		-	-	LPS 1,000 ng/mL (4 h)	-	1
Haddy 2005 [34]	752	Healthy	Plasma levels		-	No difference	-	-	-
Oregon 2008 [12]	100	Healthy	Plasma levels PBMC (mRNA)		1	1	-	-	-
Gordon 200 [30]	53	Septic shock	Plasma levels		-	No difference	-	-	-
Lu 2008 [11]	67	AS	Monocytes		1	-	LPS 2 μg/mL (0-8 h)	1.1	-
Huizinga 1997 [35]	179	Healthy	Whole blood		-	-	LPS 10/1,000 ng/mL (6 h)	-	1.5*/ 1.2
Oregon 2008 [12]	50	RA	PBMC (mRNA) Plasma levels		**2***	**1.3***	-	-	-
O'Dwyer 2008 [8]	62	Septic shock (day 1 and 7)	PBMC (mRNA) Plasma levels		No difference	No difference	-	-	-
Sharma 2007 [28]	112	Healthy	Plasma levels		-	1.1	-	-	-
Sharma 2007 [28]	83	Sarcoidosis	Plasma levels		-	**0.5***	-	-	-
Our study	50	Healthy	Monocytes		0.6	0	LPS 100 ng/mL (4 h)	0.9	1.1
Ba de Jong 2002 [37]	129	Healthy	Whole blood	-376G/A	-	-	LPS 1,000 ng/mL (4 h)	-	1
Huizinga 1997 [35]	179	Healthy	Whole blood		-	-	LPS 10/1,000 ng/mL (6 h)	-	0.8/ 0.8
Skoog 1999 [19]	156	Healthy	Plasma levels	-863C/A	-	**0.9***	-	-	-
Wennberg 2002 [20]	84	Healthy	Plasma levels		-	1	-	-	-
Sharma 2006 [52]	113	Asthma	Plasma levels		-	**0.8***	-	-	-
O'Dwyer 2008 [8]	62	Septic shock (day 1 and 7)	PBMC(mRNA) Plasma levels		**1.5*** (day 1) No difference (day 7)	No difference (days 1 and 7)	-	-	-
Sharma 2006 [52]	113	Asthma	Plasma levels	-1031T/C	-	**0.8***	-	-	-

* $p<0.05$ -238G *vs* -238A, -376A *vs* -376G, -863A *vs* -863C, or -1031 C *vs* -1031T.
RA: rheumatoid arthritis; AS: ankylosing spondylitis; no difference: data not available.

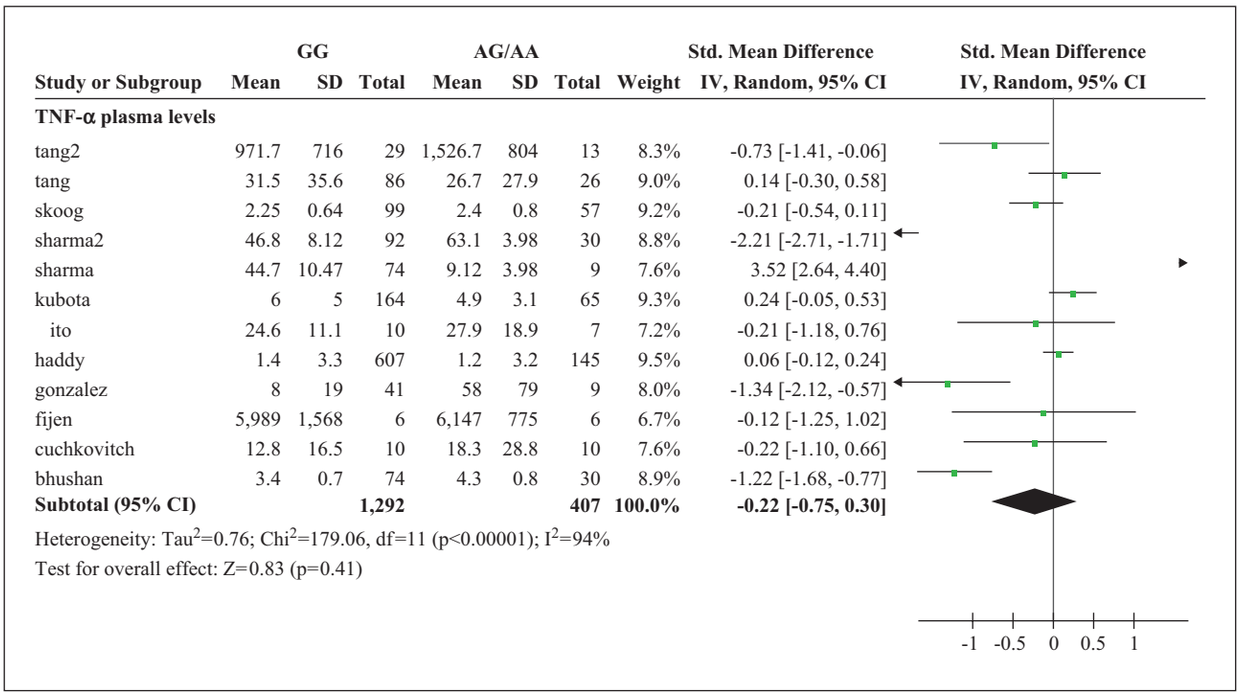

Figure 1
TNF-α protein levels according to SNP -308G/A genotypes (studies of TNF-α serum levels).

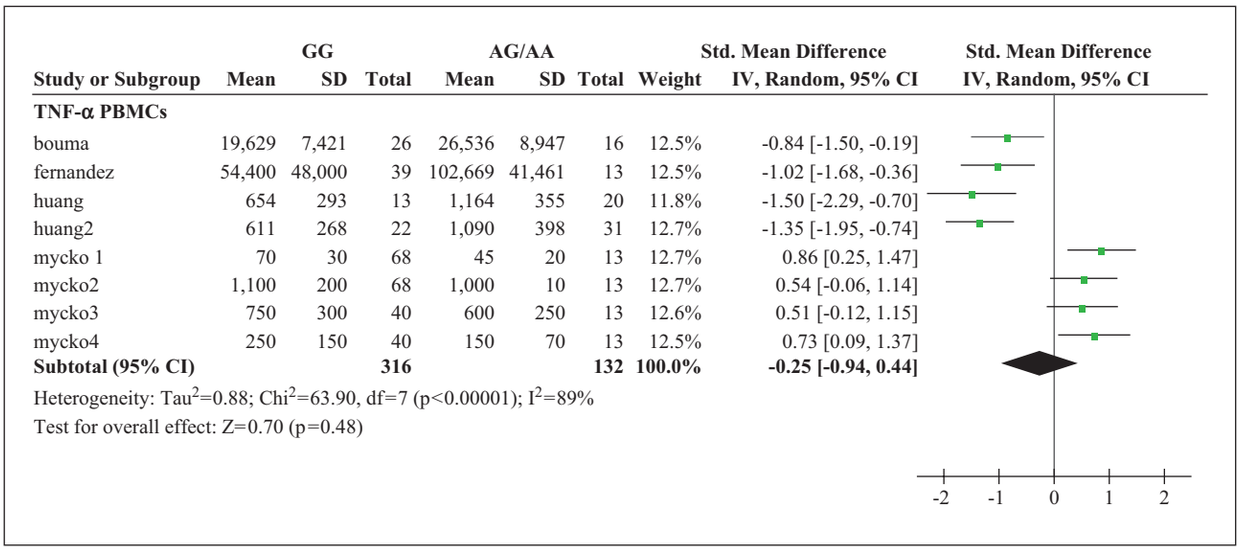

Figure 2
TNF-α protein levels according to SNP -308G/A genotypes (PBMCs studies).

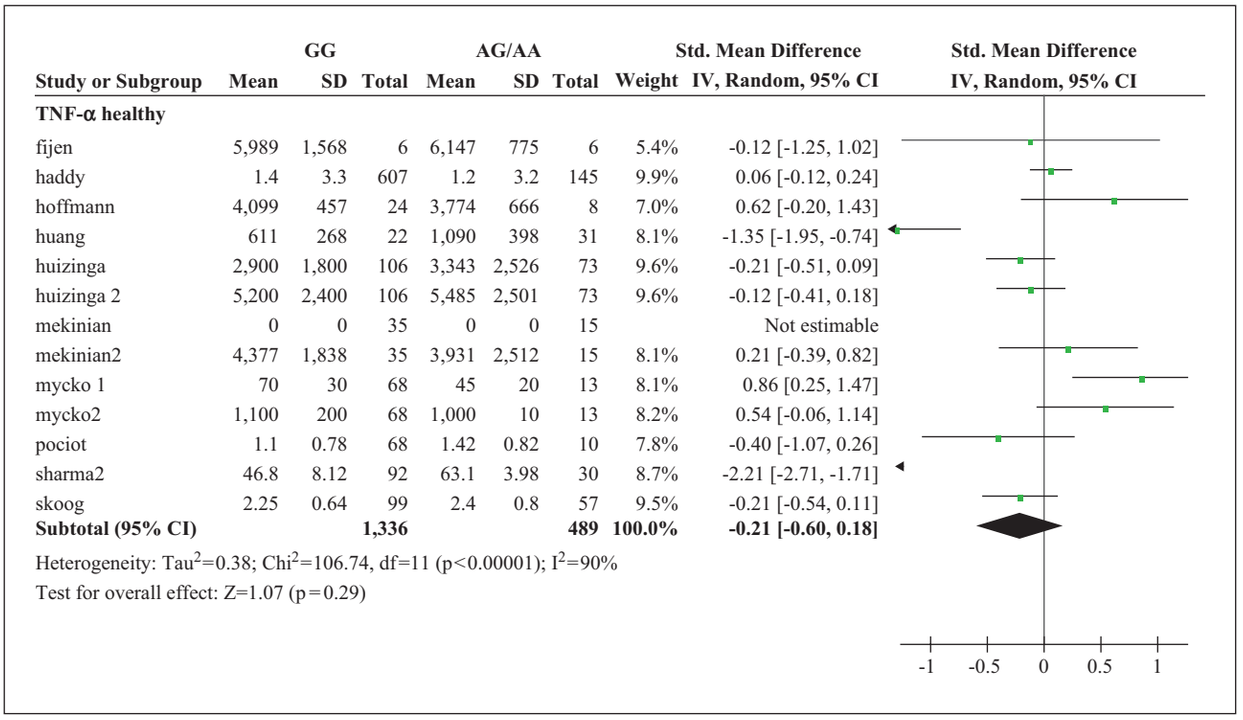

Figure 3
TNF-α protein levels according to SNP -308G/A genotypes (studies in healthy subjects).

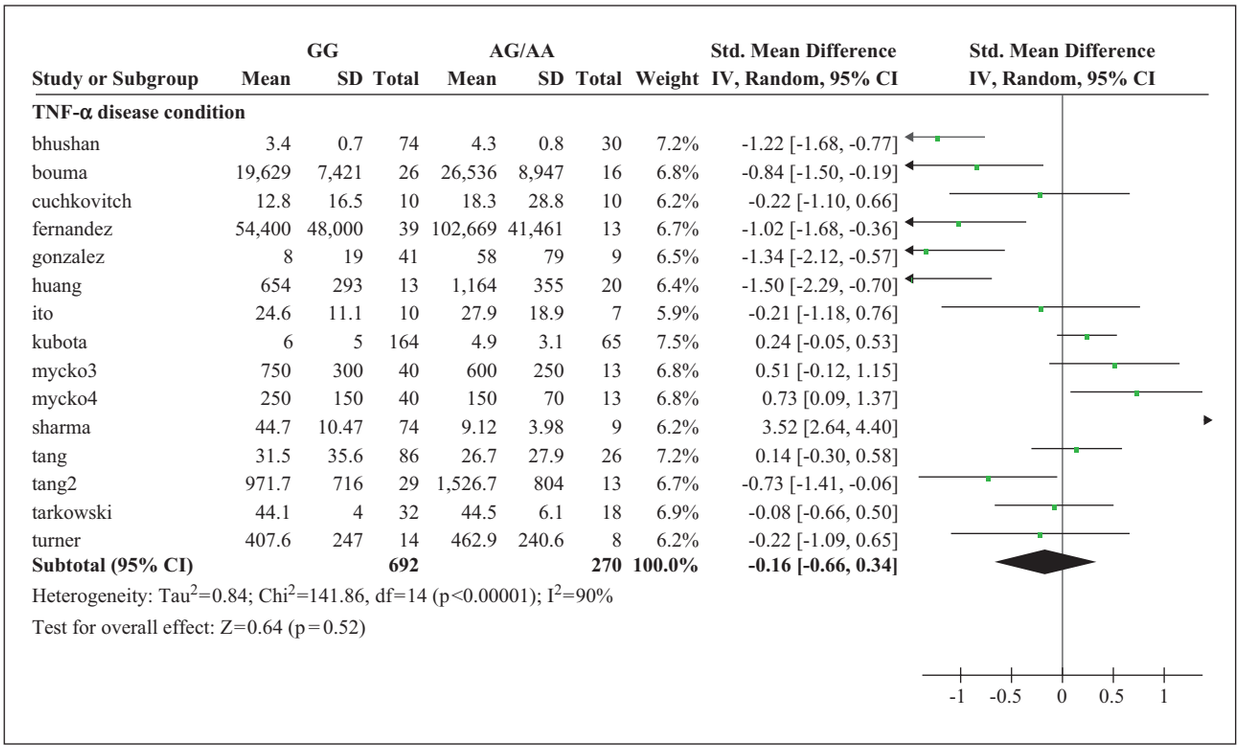

Figure 4
TNF-α protein levels according to SNP -308G/A genotypes (studies of patients with various diseases).