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Involvement of IL-6 and IL-1 receptor antagonist on intellectual disability

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ABSTRACT

Imbalances in the regulation of pro-inflammatory cytokines have been increasingly correlated with several neurodevelopmental disorders and their role in neuronal development is being investigated. To assess the possible influence of cytokines on the onset of intellectual disability (ID), we studied the polymorphisms of thirteen proinflammatory cytokine genes in 81 patients and 61 healthy controls. We demonstrated a significant association of interleukin-6 (IL-6) single-nucleotide polymorphism (SNP) (−174 G/C and nt565 G/A), and interleukin-1 receptor antagonist (IL-1RA) (Mspa-I 11100) SNP with ID. Moreover, the IL-6 SNPs is an unfavorable genetic predisposition for females. The evaluation of circulating levels of IL-6 and IL-1RA showed that the serum concentrations of IL-6 were significantly higher in ID patients than in controls. These data suggest that functional cytokine gene polymorphisms may influence the development of ID.

1. Introduction

Intellectual disability (ID), also called mental retardation, is characterized by significantly impaired cognitive functions and includes a component relating to mental functioning and relating to an individuals' functional skill in their environment. People with intellectual disabilities can learn new skills, but more slowly compared with normal individuals. The World Health Organization describes ID as a disorder characterized by the presence of incomplete or arrested mental development, mainly owing to the deterioration of concrete functions at each stage of development that contribute to the overall level of intelligence, such as cognitive, language, motor and socialization functions. Many causal factors related to ID have been identified and can be classified as genetic, acquired (congenital and developmental), environmental and socio-cultural [1]. However, in 40% of cases the cause of ID is unknown [2].

ID has an estimated prevalence of 1–3% in developed countries and represents the result of several pathological processes that act on the central nervous system (CNS) [3]. Over the years, human genetic studies have identified many candidate genes that cause ID but the mechanisms by which these genes regulate cognitive function remain poorly understood [4]. Among others, molecules from the immune system, such as cytokines, have been reported as important and interactions between the immune system and CNS have long been under investigation. In particular, the discovery of multiple functions of cytokines in the CNS suggests that they play a central role in complex CNS functions such as cognition [5,6] and cytokine-mediated pathophysiological processes underling cognitive impairments [7,8]. Besides the detrimental effects on cognitive function, mainly linked to neuroinflammation and neuropathological processes, cytokines have important physiological roles in CNS functions such as learning, memory and cognition. Constitutive expression of cytokines in the CNS is primarily from astrocytes and microglia, although neuronal expression has been observed [7,9]. Specifically, evidence shows that pro-inflammatory cytokines interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF) are involved in the molecular and cellular mechanisms that mediate complex cognitive processes [10,11] and play a key role in synaptic plasticity, long-term potentiation (LTP), neurogenesis and memory consolidation [12]. In the CNS, cognition, as a complex of processes such as attention, executive function, learning and memory, consciousness and language, is collectively dependent upon hippocampal-dependent synaptic plasticity. Synaptic plasticity is regulated by specific LTP and long-term depression (LTD), which respectively strengthen or weaken synaptic connectivity following

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Table 1
Patient clinical features.

Sex	
Male, *n* [%]	49 [60.5]
Female, *n* [%]	32 [39.5]
Average age in years ± SD	14 ± 4.08
Intellectual disability grade	
Mild, *n* [%]	58 [71.6]
Moderate, *n* [%]	22 [27.2]
Severe or profound, *n* [%]	1 [1.20]

stimulation, and is the underlying cellular/molecular mechanism important for memory consolidation [13–15]. It was demonstrated that both the over-expression and absence of cytokines directly influenced hippocampal-dependent forms of memory and synaptic plasticity [10,16,17].

Moreover, it is now an accepted clinical idea that imbalances in the immune system, perhaps caused by interactions of multiple cytokines, cause or contribute to many neurodevelopmental disorders [18,19]. Elevated levels of pro-inflammatory cytokines have been associated with autism and schizophrenia, Down syndrome and epilepsy [20–22].

Cytokines production is under genetic control. Individual differences in levels of interleukins can be attributed to gene polymorphisms within the coding, intron, or promoter regions. Recently, the association between genetic variants of cytokines IL-1β, IL-6 and cognitive function [23] in healthy elders of the general population was reported [24–27]. Despite these findings, the involvement of inflammatory cytokines in cognitive disorders is not well understood. Because inherited genetic variation may have a key role in brain impairment, genetic evidence provides an important means of establishing the role of cytokines in cognitive and relational disorders.

To investigate an association among genetic polymorphic variations, plasma levels of proinflammatory cytokines and ID we examined single nucleotide polymorphisms (SNPs) of thirteen cytokine genes. Moreover, serum levels of significantly associated cytokines SNPs were evaluated. The results suggest that IL-6 and IL-1RA SNPs may have a role in ID and peripheral blood IL-6 concentrations are also associated with ID.

2. Materials and methods

2.1. Study participants

The study comprised 142 subjects: 81 ID patients and 61 unaffected controls, matched for age and sex. The group of patients, enrolled at the Department of Neuropsychiatric Disorders of the Civil Hospital of L'Aquila, Italy, included 49 males and 32 females aged between 23 and 5 years. Table 1 shows the demographic and diagnostic characteristics of our ID patients.

The control subjects underwent a routine medical checkup in the outpatient clinic of the Department of Pediatrics of the Civil Hospital of L'Aquila. A detailed clinical examination was performed in all cases and a diagnosis of intellectual disability was made according to the Child Development and Neuropsychiatric Clinics of the S. Salvatore Hospital, L'Aquila, Italy.

All study subjects were Italians and resided in the same geographic area in Italy. The study was performed with the approval of the ethics committee (Prot. n. 0102550 – 10/21/2011) and written informed consent was obtained from all the subjects.

2.2. Cognitive measures

Based on age, the Wechsler Primary and Preschool Scale of Intelligence (WPPSI-R) and Wechsler Intelligence Scale for children (WISC-III) were used to study intellectual functioning in verbal and performance cognitive domains and the child's general intellectual ability. Vineland Adaptive Behaviour Scales were used to measure personal and social skills required for everyday living. The Child Behavior CheckList (CBCL) was used to detect emotional and behavioral problems in children and adolescents and the Conners' Continuous Performance Test (C-CPT), which measures and evaluates a child's attention span and ability to maintain focus on a task, was used to support the conclusions.

Exclusion criteria for ID patients were genetic conditions (Down syndrome, fragile X syndrome, and phenylketonuria). Environmental conditions that interfere with the growth and development of the brain such as prenatal (severe maternal malnutrition, alcohol and drug abuse), during birth (hypoxia, extreme prematurity) and after birth (severe head injury, malnutrition of the child, severe emotional neglect or abuse) factors were included.

2.3. DNA extraction

Genomic DNA was extracted from whole blood according to the manufacturer's protocol (QIAamp DNA blood MiniKit, Qiagen, Courtaboeuf, France) and kept at −20 °C until use. DNA concentration and purity was determined using a spectrophotometer (Beckman Instruments, Inc. Fullerton, CA. 92834-3100).

2.4. Cytokine gene polymorphisms

Cytokine gene polymorphisms were investigated in patients and controls using the Pel-Freez Cytokine Genotyping Kit, a PCR-based method designed to detect simultaneously polymorphisms of thirteen cytokine genes (IL1α, IL1β, IL1R, IL1RA, IL4Ra, IL12, IFNγ, TGFβ, TNFα, IL2, IL4, IL6, and IL10) (Fig. 1). Each tray contained forty-eight formulations of specific lyophilized primer sets used to amplify genomic DNA. Briefly, PCR reactions were performed using a mixture composed of PCR Buffer, water and Taq DNA Polymerase. A volume of 50 μL of DNA sample (75–125 ng/μL) was added to the buffer mixture and 10 μL of the reaction mixture was dispensed into each well. The PCR products were then electrophoresed by 1.5% agarose gel containing 0.5 mg/ml ethidium bromide and documented by photography. The genotype specificity was determined by the presence or absence of a specific PCR product of known size.

2.5. Cytokine measurements

Serum samples from patients and healthy controls were separated from venous blood and stored at −80 °C until cytokine analysis. The determination of cytokine levels was performed by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Human IL-6 ELISA Kit EH2IL6, Thermo scientific, and Human IL-1RA ELISA Kit EK0782, Boster Biological Technology), performed in accordance with the manufacturer's protocol. The sensitivity of the assays was <1 pg/ml for IL-6 and <2 pg/ml for IL-1RA. Samples were run in triplicate and the mean value was used for further statistical analysis.

3. Statistical analysis

Pearson's chi-square test and Fisher's exact test were used to evaluate genotype and allele frequencies of all cytokines in both ID patients and controls and the odds ratios (OR) and 95% confidence intervals (CIs) were calculated to assess the relative risk conferred by a particular allele and genotype. Categorical variables were compared by χ^2 test. Statistical significance was assumed when $p < 0.05$. To compare the mean values of cytokines, the

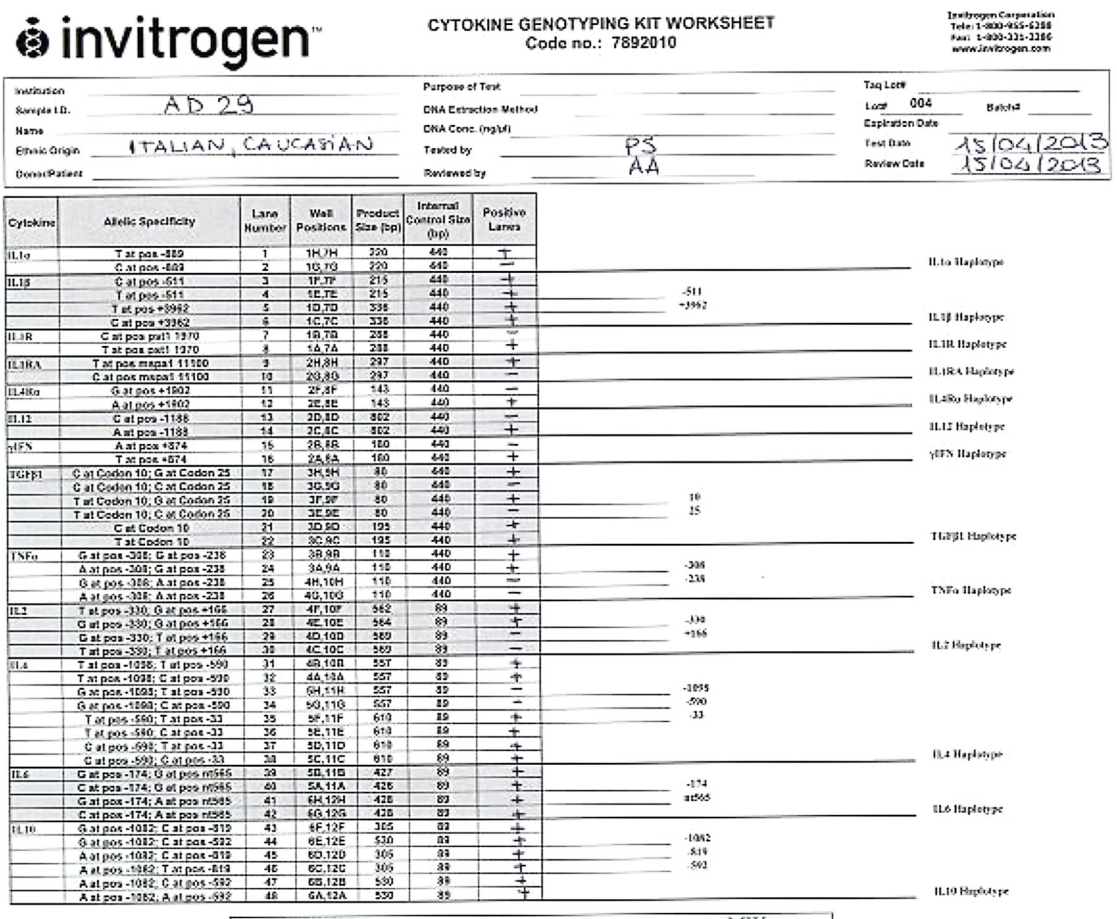

Fig. 1. Scan of a representative cytokine genotyping worksheet. Below the table, an image of a representative gel electrophoretic band patterns is inserted. MW indicates ΦX-174 HelII digest molecular weight marker.

Mann–Whitney *U*-test was used. Statistical analysis was performed using IBM SPSS Statistics 16.0 software (SPSS/IBM, Chicago, IL, USA).

4. Results

4.1. Cytokine gene polymorphisms

The cytokine polymorphisms analyzed are shown in Fig. 2. Our results demonstrated a statistically significant association between polymorphisms of IL-6 and IL-1RA and ID as shown in Fig. 3. The presence of an IL-6 (−174) G/C genotype detected in 42.1% of patients and in 22.2% of controls indicated an increased risk of developing ID ($p < 0.05$; OR: 2.60; 95% CI: from 1.188 to 5.716) whereas the IL-6 (−174) C/C (1.3% patients vs 16.3% controls, $p < 0.005$; OR: 0.07; 95% CI: from 0.008 to 0.556) and IL-6 (nt565) A/A (1.3% patients vs 14.5% controls, $p < 0.005$; OR: 0.089; 95% CI: from 0.009 to 0.646) genotypes were protective. Moreover, a positive association of IL-1RA Mspa-I 11100 T/T genotype ($p < 0.05$; OR 2.33; 95% CI: from 1.117 to 4.625) and a negative

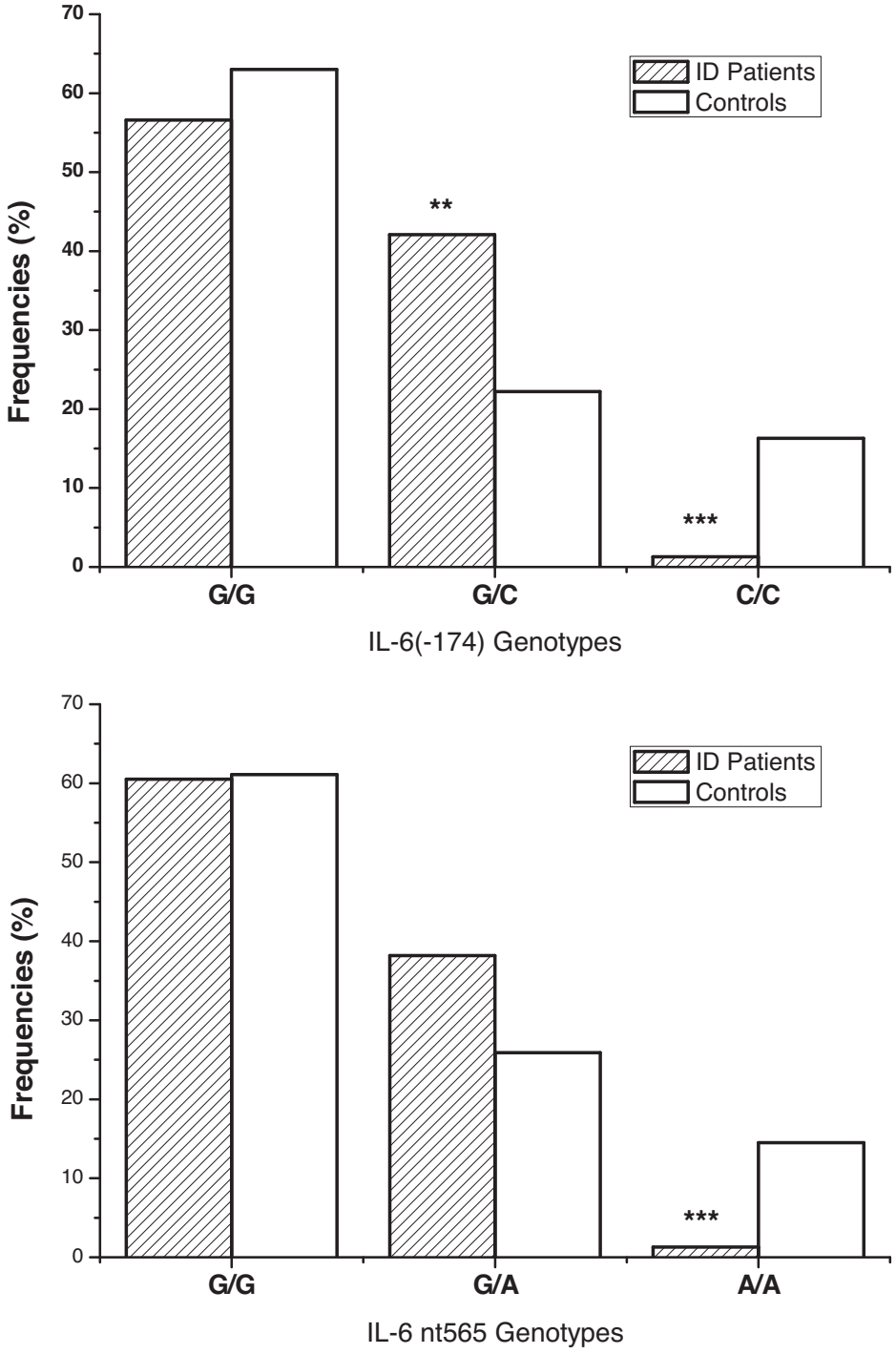

Fig. 2. Frequency distributions of IL-6 174 (top) and nt565 (bottom) genotypes in ID patients and controls. Values are given as percentage of each population. Person's chi-square test and Fisher's exact test were used to evaluate significant differences. ***$p < 0.001$, **$p < 0.005$ vs control.

association of IL-1RA Mspa-I 11100 C/T genotype with ID ($p < 0.005$; OR 0.375; CI: from 0.186 to 0.756) was also observed (Table 2). Gender differences in frequency distributions of genotypes were detected for IL-6 polymorphisms in ID patients. A significantly higher frequency in females than in males was observed for IL-6 (nt565) G/A SNP ($p < 0.05$; OR; 2.9011; CI: from 1.1080 to 7.5958). In contrast, IL-6 (nt565) G/G SNP was observed more frequently in males than females ($p < 0.05$; OR; 0.3828; CI: from 0.1475 to 0.9932) (Fig. 4). No differences between patients and controls were detected for the other cytokine polymorphisms examined.

4.2. Haplotype analysis of the IL-6 gene

To perform haplotype analysis of the IL6 gene, the genotype result of −174 was considered, for each individual, together with the genotype result of nt565 SNPs. All combinations of haplotypes that were present in patients and controls are shown in Fig. 5. From this analysis, we observed that the GG/CA combination was associated with a significantly increased risk of ID as compared with the controls (38.1% patients vs 20% controls, $p < 0.05$; OR: 2.468; 95% CI: from 1.102 to 5.529) and, in contrast, the CA/CA combination was associated with a minor risk for developing ID (1.3%

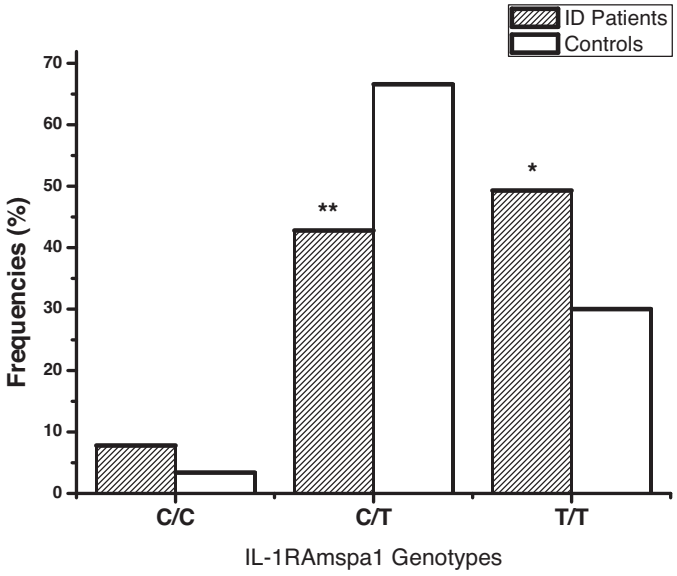

Fig. 3. Graphical representation of IL-1RA Mspa-I 11100 SNP in ID patients and controls. Values are given as percentage of each population. Person's chi-square test and Fisher's exact test were used to evaluate significant differences. $^{**}p < 0.005$; $^{*}p < 0.05$ vs control.

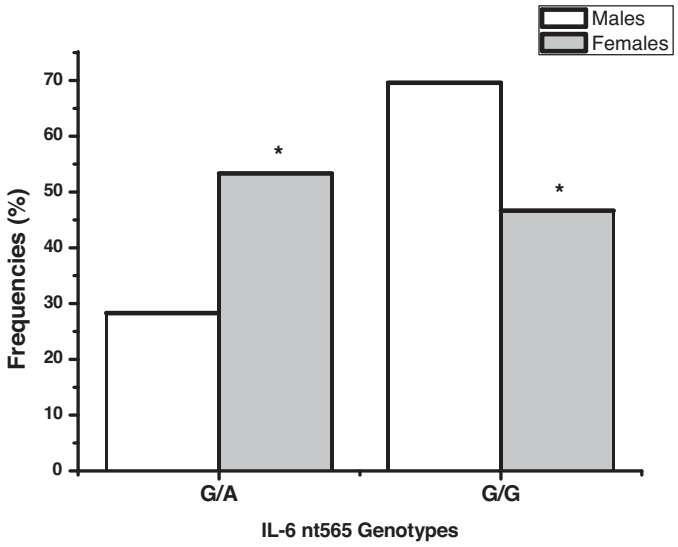

Fig. 4. Graph representing the percentage of frequency of IL-6 genotypes in males and females. Statistical significances were obtained with Person's chi-square test and Fisher's exact test. $^{*}p < 0.05$ vs males.

patients vs 14.5% controls, $p < 0.05$; OR: 0.078; 95% CI: from 0.009 to 0.646) (Table 3). Additionally, in studying the frequency distributions related to gender we detected a statistically significant difference in the percentage of females who carried the GG/CA combination with respect to males (55.2% females vs 44.8% males, $p < 0.05$; OR: 2.9; 95% CI: from 1.108 to 7.596) (Fig. 6).

Table 2
Genotype frequencies (%) of cytokines in ID patients and controls.

Polymorphism	ID patients $n = {}^{a}76$ [%]	Control subjects $n = {}^{a}55$ [%]	X^2	p value
IL-6 [−174]				
Genotypes				
GG	43 [56.6]	34 [63.0]	0.18	ns
GC	32 [42.1]	12 [22.2]	5.01	0.02
CC	1 [1.30]	9 [16.3]	10.25	0.0017
Alleles				
G	118 [77.6]	80 [72.7]	0.83	ns
C	34 [22.4]	30 [27.3]	0.83	ns
IL-6 nt565				
Genotypes				
GG	46 [60.5]	33 [61.1]	0.01	ns
GA	29 [38.2]	14 [25.9]	2.34	ns
AA	1 [1.3]	8 [14.5]	8.73	0.004
Alleles				
G	121 [79.6]	80 [72.7]	1.69	ns
A	31 [20.4]	30 [27.3]	1.69	ns
Polymorphism	ID patients $n = {}^{b}77$ [%]	Control subjects $n = {}^{b}60$ [%]	X^2	p value
IL-1RAmspa1 11100				
Genotypes				
CC	6 [7.8]	2 [3.4]	1.22	ns
CT	33 [42.8]	40 [66.6]	7.68	0.009
TT	38 [49.3]	18 [30.0]	5.22	0.02
Alleles				
C	45 [29.2%]	44 [36.6]	2.09	ns
T	109 [70.8]	76 [63.4]	1.70	ns

[a] IL-6 frequencies were not available for 5 patients and 6 controls.
[b] IL-1RA frequencies were not available for 4 patients and 1 control.

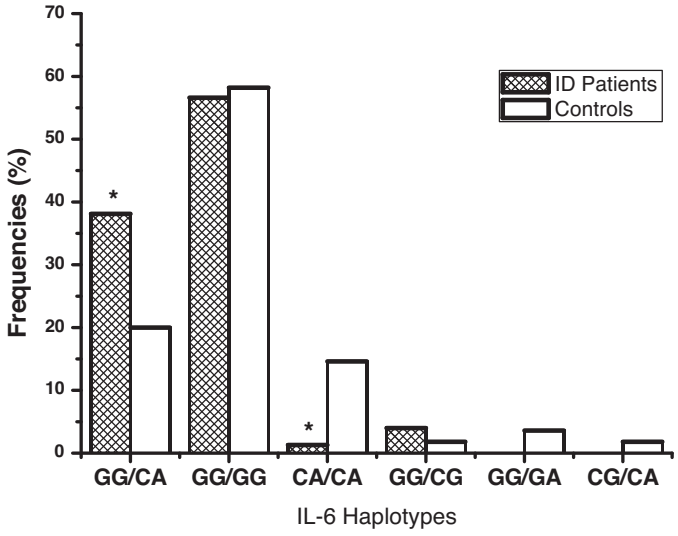

Fig. 5. Graphical representation of IL-6 174 haplotypes in ID patients and controls. Significances were calculated with Person's chi-square test and Fisher's exact test. $^{*}p < 0.05$ vs control.

4.3. Serum cytokine levels

To obtain combined information on serum concentrations of IL-6 and IL-1RA and their polymorphisms in ID patients to identify correlations with protein release, we measured serum cytokine levels. Significantly higher levels of IL-6 were observed in patients

Table 3
Combinations of IL-6 haplotypes in ID patients and controls.

IL-6 gene [−174/nt565]	ID patients $n = 76$ [%]	Controls $n = 55$ [%]	OR [95% CI]	p value
GG/CA	29 [38.1]	11 [20]	2.468 [1.102–5.529]	0.03
GG/GG	43 [56.6]	32 [58.2]	–	ns
CA/CA	1 [1.3]	8 [14.6]	0.078 [0.009–0.646]	0.01
GG/CG	3 [4]	1 [1.8]	–	ns
GG/GA	0 [0]	2 [3.6]	–	ns
CG/CA	0 [0]	1 [1.8]	–	ns

OR, odds ratios.

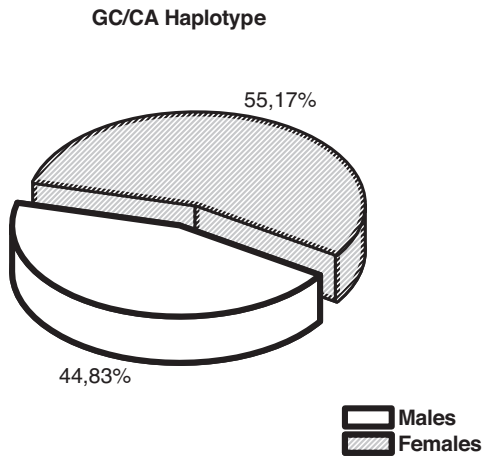

Fig. 6. Distribution of the percentages of haplotype frequencies for IL-6 SNPs in males and females. $^{*}p < 0.05$ vs males.

with the −174 GC genotype compared with control subjects (8.75 ± 7.5 pg/ml vs 5.69 ± 3.2 pg/ml, $p = 0.004$) (Fig. 7). However, no significant difference of IL-1RA serum concentrations was observed in ID patients compared with the controls (630.65 ± 92.37 pg/ml vs 639.48 ± 125.78 pg/ml).

5. Discussion

Intellectual disability, according to a definition by the American Association on Mental Retardation (AAMR), is characterized by significant limitations of intellectual functioning and adaptive behavior, considered as a set of adaptive skills conceptual, social and practical. This disability is a condition diagnosed before the age of 18. There are many causes of ID, however, specific causes are only identified in 25% of cases and the diagnosis is often not made until the child is in elementary school and has difficulty in mastering academic skills. The delay in diagnosis is a critical issue when planning care, because early intervention programs, including appropriate sensory, motor and cognitive activities, may give children with ID the best chance of success.

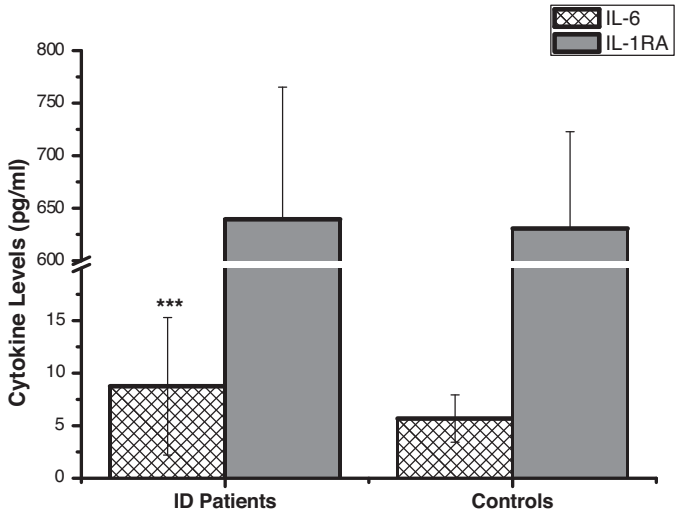

Fig. 7. Histogram of IL-6 and IL-1RA serum levels in ID patients and controls measured by ELISA. The mean values were compared by Mann–Whitney *U*-test. $^{***}p < 0.001$.

Excluding the syndromic intellectual disability, in which intellectual deficit associated with other medical and behavioral signs and symptoms are present, the diagnosis of non-syndromic intellectual disability, in which intellectual deficit is the sole clinical feature, is based only on specific clinical assessment tools. However, the distinction between syndromic and non-syndromic ID is often blurred. A better understanding of the molecular and genetic causes of ID might help clinicians to perform more accurate diagnoses when planning therapeutic interventions. Genetic causes of ID are thought to be present in 25–50% of cases and increasing numbers of genes are being identified that cause ID, although certain pathways and molecules are emerging as central contributors to normal cognition.

To the best of our knowledge, this is the first study to evaluate the involvement of cytokines in individuals with ID. We report a significant association between mild/moderate ID and SNPs of two main pro-inflammatory cytokines. Specifically, the genotype T/T of Mspa-I 11100 in IL-1RA and the genotype G/C of (−174) in IL-6 are associated with an increased risk of developing ID. Moreover, analysis of the possible combinations of haplotypes formed by the two SNP (−174 and nt565) of IL-6 has shown that the presence of the GGCA haplotype constitutes a risk factor for ID. We reported that the prevalence of mental retardation was higher among males than females, in accord with many other previous studies. Surprisingly, we observed a gender difference for IL-6-nt565 genotypes. This gender divergence remained evident when we analyzed the IL-6 haplotypes. The G/A 565nt IL-6 genotype was more frequent in females, but alone it was not associated with ID. However, the GGCA risk haplotype is an unfavorable predisposing genetic factor, particularly for females. Even if genetic variation among different populations accounted for the divergent results, these findings show that gender differences should be considered in association analysis, because many genes have been demonstrated to function differently in males and females. Furthermore, the genotypes C/C of (−174) and A/A of 565 in IL-6 and the genotype C/T of Mspa-I 11100 in IL-1RA may be protective toward ID. When serum levels of IL-6 and IL-1RA were analyzed, high levels of IL-6 were significantly associated with the presence of ID but no differences were detected for IL-1RA between ID and controls. The SNP-174 G/C of IL-6 was previously associated with increased IL-6 production [28] and shown to affect *in vitro* IL-6 transcription and IL-6 plasma levels [29,30]. Although there have been some divergences among published studies regarding the association between IL-6 SNPs and IL-6 serum levels [31], it is reasonable to hypothesize that SNP-174 G/C of IL-6 could be used as a marker for local or systemic IL-6 secretion.

Previous studies demonstrated that the IL-6 (-174) polymorphism was associated with ID in cystic periventricular leucomalacia in preterm infants [32] and that the expression of IL-6 increased in various neurological disorders such as Alzheimer's disease or meningitis [33–35]. IL-6 is a regulator of neurogenesis, which is important for cognitive function and hippocampal-dependent learning. The association of IL-6 and learning and memory has been widely reported [6,35–37]. Specifically, it seems to have a regulatory role on LTP in the hippocampus by limiting memory acquisition both *in vitro* and in free-moving rats and is dependent on NMDA receptor activation [38]. Wei H. et al. [39] demonstrated impaired cognitive abilities and deficits in learning in mice with elevated IL-6 in the brain. Moreover, transgenic mice not expressing IL-6 showed improved cognitive functioning [40]. However, IL-6 deficiency was also shown to impair recognition memory [41]. This discrepancy might be explained because the basal level of IL-6 may influence some forms of learning and memory but not others. Furthermore, it must be noted that cytokines interact in a network where the function of one cytokine can be modified, modulated or substituted by others [42]. It has been hypothesized that IL-6 has

an opposite role to IL-1 in synaptic plasticity and LTP maintenance, by inducing negative feedback and inhibiting the effects of IL-1 on LTP maintenance [6,38] and/or by curtailing synaptic improvement at neighboring synapses [43].

Here, we also reported an association of the genotype SNP MspaI 11100 T/T of IL-1RA with ID and a protective role for C/T SNP. The IL-1 family is a growing family of cytokines also comprising receptors and receptor antagonists IL-1RA. The IL-1 system is tightly regulated at multiple levels by diverse mechanisms including receptor antagonists. Currently, there is no clear evidence for a causal relationship between IL-1RA and cognitive function, but it was reported that IL-1RA acts strongly as an IL-1 antagonist by binding to IL-1R-1 with an affinity higher than that of IL-1 [44]. It was also demonstrated that IL-1, and particularly IL-1β, plays a dual role in hippocampal-dependent learning processes [5,45] and that increases in IL-1RA may prevent the adverse cognitive effects of IL-1β [46]. In contrast, intracerebral injection of IL-1RA worsened learning and memory in a variety of animal models [47,48] and, blocking of the IL-1ß receptor by IL-1RA during the LTP process in the hippocampus, impaired the maintenance of LTP [49,50]. Some studies have also explored the polymorphic variations of IL-1RA [51,52] in association with attention deficit hyperactivity disorder, but with conflicting results.

6. Conclusions

Our data support the hypothesis that IL-6 may play an important role in the development of ID. Thus, IL-6 gene SNPs might serve as novel genetic markers of susceptibility for ID in the Italian population. In addition, we demonstrated an association between ID patient IL-6 SNPs and IL-6 serum levels, where ID patients overproduced IL-6. Moreover, the polymorphism MSPA1 11100 T/T IL-1RA was associated with a greater risk of developing ID, but no changes of IL-1RA levels were detected in patients when compared to controls.

In conclusion, our findings suggest that the IL-6 and IL-1RA polymorphisms as well as the overexpression of IL-6 might have significant implications in the pathogenesis of ID. Cytokine screening of ID patients has the potential to become an important tool in clinical diagnostic settings. Specifically, the characterization of these polymorphisms will provide insight into ID onset supporting the use of cytokine genes in multi-gene panel analysis of ID patients. To identify and understand the genetic etiology of ID allows for the opportunity of prenatal diagnosis, guidance with disease management, acceptance of the disability, and connection with other parents and support groups.

Conflict of interest

No conflict of interest to declare.

Ethical statement

Ethical standards were maintained by following ethical guidelines. Consent was sought using a form approved by the local Ethics Committee. The experiments of the present study comply with the current Italian laws and are in accordance with the Declaration of Helsinki.

Acknowledgment

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