

Molecular basis of immunoglobulin heavy constant G4 gene (*IGHG4*)-related low serum IgG4 subclasses in Down syndrome

Mohammed A. Jeraiby, MD.

ABSTRACT

الأهداف: دراسة الآلية الجزيئية لمصل الدم المنخفض IgG4 على مستوى الجين G4 الثابت الثقيل للجلوبيولين المناعي (*IGHG4*). المرضى الذين يعانون من متلازمة داون أكثر عرضة للإصابة بتشوهات مناعية تجعلهم عرضة للعدوى. من بين الحالات الشاذة الأخرى، قام الأفراد المصابون بالـ (DS) بتغيير تركيزات المصل لبعض الفئات الفرعية من الغلوبولين المناعي (IgG)، ولا سيما الفئات الفرعية IgG4.

المنهجية: في هذه الدراسة المستقبلية، أجرينا تفاعل البوليميراز المتسلسل الكمي في الوقت الفعلي (qPCR) خلال الفترة من ديسمبر 2017م إلى يونيو 2019م في مستشفى جامعة سانت إتيان، سانت إتيان، فرنسا لقياس عدد نسخ *IGHG4* ومقارنة تلك النتائج مع مرجع الجين (*36B4*). اعتبرت نسبة *IGHG4/36B4* طبيعية عندما تتراوح بين 0.8 و 1.2. أربعة وأربعون مريضاً في DS، من بينهم 23 مريضاً في DS يحملون مصل الدم المنخفض IgG4 و 21 مريضاً DS مع مصل طبيعي IgG4 (المستوى أكثر من 0.1 جم/لتر). قمنا بمقارنة مجموعة المرضى مع 38 متبرعاً سليماً (مجموعة السيطرة) بدون DS.

النتائج: عثرنا على الحذف المتغاير لجين السلسلة الثقيلة *IGHG4* في 16 (69.57%) من مرضى DS مع انخفاض مصل IgG4 مقابل 2 (9.52%) DS مع مصل طبيعي ($p=0.0001$). في المجموعة الضابطة، وجد الحذف في 5.26% (2/38) من العينة.

الخلاصة: يرتبط جين *IGHG4* المفرد ذو السلسلة الثقيلة ارتباطاً وثيقاً بانخفاض IgG4 في المصل في مجتمعنا مع DS، ولكن يجب تقييم العوامل الأخرى ذات الصلة في العمل المستقبلي.

Objectives: To investigate the molecular mechanism of low serum IgG4 at the level of the immunoglobulin heavy constant G4 gene (*IGHG4*). Patients with Down syndrome (DS) are more likely to exhibit immunological abnormalities that predispose them to infection. Among other anomalies, individuals with DS have altered serum concentrations of some subclasses of immunoglobulin G (IgG), particularly the IgG4 subclasses.

Methods: In this prospective study, quantitative real-time polymerase chain reaction (qPCR) was carried out from December 2017 to June 2019 in the University Hospital of Saint-Etienne, Saint-Etienne, France to measure the number of *IGHG4* copies and

to compare those outcomes with a reference gene (*36B4*). An *IGHG4/36B4* ratio was considered normal when between 0.8 and 1.2. Forty-four DS patients, comprising 23 DS patients carrying severe low serum IgG4 and 21 DS patients with normal serum IgG4 (level >0.1 g/L). The patient group was compared with 38 healthy donors (controls) without DS.

Results: The heavy chain gene *IGHG4* heterozygous deletion was found in 16 (69.57%) DS patients with low serum IgG4 versus in 2 (9.52%) DS with normal serum IgG4 ($p=0.0001$). In the control group, deletion was found in 5.26% (2/38) of the sample.

Conclusion: The heavy chain gene *IGHG4* haploinsufficiency is highly correlated with low serum IgG4 in our population with DS, but other relevant factors must be assessed in future work.

Keywords: Down syndrome, low serum immunoglobulin G4, immunoglobulin heavy chain G4 gene, qPCR

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From the Department of Biochemistry, Faculty of Medicine, Jazan University, Jazan, Kingdom of Saudi Arabia.

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Address correspondence and reprint request to: Dr. Mohammed A. Jeraiby, Biochemistry Department, Faculty of Medicine, Jazan University, Jazan, Kingdom of Saudi Arabia. E-mail: mojer2011@hotmail.com
ORCID ID: <https://orcid.org/0000-0002-5428-9167>

Patients with Down syndrome (DS) are more likely to exhibit immunological abnormalities that predispose them to infection.¹⁻³ Among other anomalies, individuals with DS have altered serum concentrations of some subclasses of immunoglobulin G (IgG), particularly the IgG4 subclasses.⁴ In line with this, Anneren et al,⁴ found low serum IgG4 in 68% of DS patients. This is critical, as Loh et al,⁵ found that 90%

of DS patients with severe respiratory infections had a low serum IgG4 subclass, while only 25% of healthy DS patients showed such a deficiency. However, the role of low serum IgG4 has not been fully established, as many affected persons in the general population are asymptomatic. For example, one study found that up to 15% of typically developing children and 10% of adults may have a low serum IgG4.⁶ In the context of DS, low serum IgG4 may be an important factor in recurrent infections, the development of autoimmune diseases, and even stroke.^{7,8} Despite being the lowest of all IgG subclasses, comprising approximately 4% of total IgG, IgG4 may play an important role in the body's mucosal defense because of its relatively higher concentration in external secretions.⁹ Low levels in one or more IgG subclasses can be caused by T-cell dysfunction, deletions of the constant region of the heavy chain genes, or abnormalities of isotype switching.¹⁰ Furthermore, *in vitro* lymphocyte T and lymphocyte B function abnormalities are present in individuals with low IgG levels, suggesting that low IgG subclass levels may be indicative of an underlying lymphocyte B defect, a possible lymphocyte T defect, and significant immunodepression.^{11,12} The current literature has not provided a clear consensus on the pathogenic link between low IgG4 and the immunoglobulin heavy constant G4 gene (*IGHG4*) in DS.

The aim of the present study was to investigate the molecular mechanism of low serum IgG4 at the level of the heavy chain gene (*IGHG4*) in DS.

Methods. This prospective cross-sectional study was performed at the University Hospital of Saint-Etienne, St. Etienne, France, from December 2017 to June 2019. The inclusion criteria was all cases with DS and immunoglobulin investigation. Those who did not do an immunoglobulin investigation and did not have DS were excluded from this study.

This study included 44 patients with DS, 23 women (52.3%) and 21 men (47.7%) divided into 2 groups. All patients were over 7 years of age (range: 8-57 years), the age at which IgG4 reaches adult levels.¹³ The first group comprised patients with DS carrying severely low serum levels of IgG4 (<0.02 g/L) and DS subjects having normal serum levels of IgG4 (level >0.1 g/L), (normal range: 0.04-0.86). The patients were compared

to 38 healthy donors (controls) who did not have DS.

Ethical considerations were followed in agreement with the Declaration of Helsinki throughout this study, which was approved by the Research Committee of Jazan University (REC42/1/119). Consent was obtained from the patients' parents.

The IgG4 levels were analyzed in serum using Optilite (The Binding Site Group Ltd., 8 Calthorpe Road, Edgbaston, Birmingham, B15 1QT, UK) according to the manufacturer's instructions and measured in the immunology laboratory at the University Hospital of Saint-Etienne, France. A quantitative real-time polymerase chain reaction (qPCR) was carried out using the Step One Plus system (APPLIED Biosystems, Carlsbad, CA, version 2.3) to measure the number of *IGHG4* copies present and compare that number to a reference gene (*36B4*). The process was completed in 14.5 µL mixtures containing Taq polymerase, buffer solution, SYBR Green (QuantiTect SYBR® Green PCR Kits, QIAGEN), and 5 µL of extracted DNA 2 ng/µl from whole blood samples with specific primers (*IGHG4*-FOR, 5'-aatcttctctctgcagagtccaaatg-3' and *IGHG4* REV, 5'gttgcttacctgggcatga-3'), which were designed to amplify *IGHG4* gene exon 2. Because the structure of *IGHG4* is homologous with immunoglobulin heavy constant G2 (*IGHG2*), we included this gene in this study using primers *IGHG2*-FOR, 5'-caaatgtgtgtcagtgccca-3' and *IGHG2*-REV, 5'-aagactgacgtctctgccac-3' and tested other different structures in immunoglobulin-like immunoglobulin heavy constant E using primers (*IGHE*) *IGHE*-FOR, 5'-gctgcaaaaacatccctcca-3' and *IGHE* REV, 5'gtggtggctggraaggtcata-3' as well.¹⁴ The reaction was performed in 2 steps, programmed for 45 cycles with primer hybridization at 60°C. The first step consisted of using primers of a reference gene, for which the number of copies is known. The second step consisted of using the primers of the gene of interest, *IGHG4*, for which the number of copies is desired. An *IGHG4/36B4* ratio was considered normal (2 copies of *IGHG4*) when the resulting values were between 0.8 and 1.2. To maintain the precision of the technique, electrical pipettes (Xplorer Eppendorf) were used, all DNA assays were carried out on the same day, and the same DNA dilutions were used for the 2 plates (*36B4* and *IGHG4*).

The data was analyzed using the Statistical Package for Social Sciences (SPSS) version 22 (IBM Corp., Armonk, NY, USA). Means and standard deviations were used to express continuous variables. Categorical variables were expressed as percentages. A set of t-tests was used for continuous variables that exhibited normal

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distributions. Mann-Whitney U tests were used for non-parametric variables. The Shapiro-Wilk test was used to assess the normality of the data. A general linear model was used to assess the association between *IGHG4* and B cell function, IgG, IgG1, IgG2, IgG3, IgA, and IgM. A *p*-value of <0.05 was considered significant.

Results. Among a total of 446 DS subjects, immunoglobulin was evaluated in 53 (11.9%) patients with recurrent infection. Twenty-three patients with DS (11 males and 12 females) carried severe low serum IgG4 (<0.02 g/L), with 5 having an IgG4 level of 0 g/L and 21 (10 males and 11 females) having normal serum levels of IgG4 (level >0.1 g/L).

Among 23 patients with DS with low/undetected IgG4, 3 had congenital heart disease (13%), 2 had otitis media (8.7%), one had pulmonary airway malformation, 4 had hypothyroidism (17.4%), and one had type 1 diabetes. All patients had experienced recurrent infections, mostly lower respiratory tract and ear-nose-throat infections. In the group with normal IgG4 levels, 2 suffered from hypothyroidism, one with celiac disease, and one with congenital heart malformation.

Regarding the immunoglobulin levels in the group with low/undetected IgG4 and the group with normal IgG4 serum levels, almost all patients had a normal mean ratio for the levels of IgA, IgM, and IgG. Normal IgG subclass distribution, except that low IgG2 serum levels (IgG2 normal range: 2.4-7 g/l) were noted in the group with low/undetected IgG4 and normal IgG4 serum levels with mean ratios of 2.20 ± 2.20 and 2.24 ± 0.85 ($p < 0.900$) (Table 1).

In total, 82 individuals participated in screening for *IGHG4* gene deletion (44 DS with and without low IgG4 levels versus 38 healthy controls). The mean ratio

of *IGHG4* in 23 DS patients with a low IgG4 level was 0.70 ± 0.31 , which is significantly lower than the mean ratio of *IGHG4* in the 21 DS patients with normal IgG4 levels at 0.97 ± 0.16 , ($p = 0.008$). The low serum IgG4 patients also differed from the control group (healthy subjects without DS), who had a mean ratio of 0.99 ± 0.16 , $p < 0.001$.

In the 23 (52.3%) DS patients with low serum IgG4, 16 (69.6%) had heterozygous gene deletion, while 7 (30.4%) did not. The lowest value was 0.20 and the highest was 1.23. Among those 21 (47.7%) DS patients with normal IgG4 levels, 19 (90.5%) had no deletion, while only 2 had it (9.5%; $p < 0.001$). The minimum ratio for patients with normal IgG4 was 0.52 and the maximum was 1.16. In the control group of 38 healthy donors, only 2 (5.26%) had the deletion, whereas 36 (94.7%, $p < 0.001$) had no detectable *IGHG4* deletion (Figure 1). In examining *IGHG2* and *IGHE* among patients with *IGHG4* deletion, only one patient (9.1%) presented with both an *IGHG2* and an *IGHE* deletion (Table 2).

Table 3 shows the association between *IGHG4* and B cell, IgA, and IgM total IgG and IgG sub-classes. It is clear that there is a non-significant direct relationship between *IGHG4* and B cell function, IgG, IgA, and IgM. There was a non-significant inverse relationship between *IGHG4* and IgG1, IgG2, and IgG3.

Discussion. To evaluate the *IGHG4* gene in DS patients with low serum IgG4, a molecular diagnosis of the *IGHG4* gene revealed a deletion rate of 69.57% (16/23) among DS patients with low IgG4 levels. In contrast, Pan et al,¹⁵ reported heterozygous deletion rates for the *IGHG4* gene of 1.5% in the Caucasian population and 4% in the Black population. This

Table 1 - Mean and standard deviation for B cell functions deficiency for patients with low IgG4.

Variables	With low/undetected IgG4 level	With normal IgG4 level	P-value
	Mean±SD		
IGHG4	0.53±0.17	1.10±0.10	<0.001*
B cell (cell/uL)	163.25±91.65	199.71±67.44	0.357
IgG	13.41±2.63	12.41±3.72	0.471
IgG1	9.07±1.89	8.36±2.72	0.475
IgG2	2.20±0.69	2.24±0.85	0.900
IgG3	1.83±0.95	1.20±0.84	0.144
IgA	2.88±1.31	2.24±1.32	0.292
IgM	0.69±0.29	0.67±0.36	0.875

IGHG4: immunoglobulin heavy constant G4 gene, IgG: immunoglobulin G,
SD: standard deviation

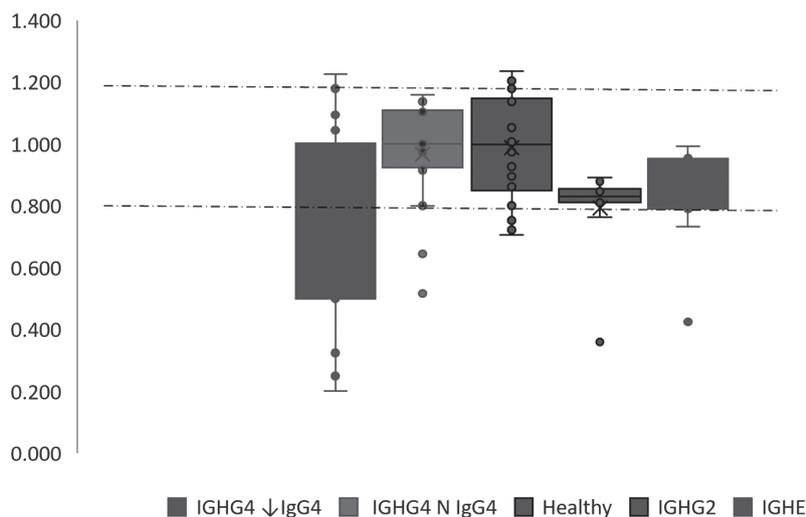


Figure 1 - Comparison of immunoglobulin heavy constant G4 gene (*IGHG4*)/*36B4* ratios: Down syndrome (DS) with low IgG4 level, DS with normal IgG4 level (N IgG4), healthy control and *IGHG2/36B4* ratios, *IGHE/36B4* ratios

Table 2 - Comparison of *IGHG4/36b4*, *IGHG2/36b4*, and *IGHE/36b4* for DS with Low IgG4 level.

Comparison Group	N	Min.	Max.	Median	Mean	SD	P-value
Ratio of <i>IGHG4/36b4</i> for DS with ↓IgG4	23	0.20	1.23	0.64	0.70	0.31	
Ratio of <i>IGHG2/36b4</i> for DS with ↓IgG4	11	0.36	0.89	0.83	0.79	0.15	0.167
Ratio of <i>IGHE/36b4</i> for DS with ↓IgG4	11	0.42	0.99	0.91	0.85	0.16	0.149

IGHG4: immunoglobulin heavy constant gamma 4

Table 3 - General linear model for the association between immunoglobulin heavy constant G4 gene (*IGHG4*) with B cell functions.

Parameter	B	95% confidence interval		P-value
		Lower bound	Upper bound	
Intercept	0.177	-0.869	1.223	0.723
B cell (cell/uL)	0.001	-0.001	0.003	0.294
IgG	0.127	-0.354	0.609	0.581
IgG1	-0.103	-0.694	0.487	0.714
IgG2	-0.104	-0.643	0.436	0.687
IgG3	-0.261	-0.569	0.047	0.091
IgA	0.036	-0.089	0.161	0.547
IgM	0.203	-0.321	0.728	0.421

IgG: immunoglobulin G

discrepancy between the present study and prior work can be attributed to differences in the techniques used by Pan et al.¹⁵ The authors used a pulsed field gel electrophoresis technique (PFGE), which provides a resolution that can identify only large deletions. In this study, I used a qPCR technique, which identifies smaller deletions. To further probe the high frequency of this deletion in this study, I studied the number of copies of the *IGHG4* gene in a control group of 38 healthy people without DS, finding the deletion rate to be 5.26%. This suggests that the higher rate of deletion in our study resulted from the qPCR technique.

Nevertheless, I have studied only the deletions at the level of exon 2. To specify the size of this deletion, it would be interesting to extend qPCR to other exons. Another possible technique for identification would be array comparative genomic hybridization (array CGH), which has very good resolution. According to Pan et al,¹⁵ deletions are quite rare; thus, defects in the expression of IgG mainly result from poor regulation of the expression of the heavy chain gene of the constant region. However, Olsson et al,¹⁶ have identified deletions at the level of the DNA sequence of IgG1, and Zhao et al,¹⁷ have shown that there were mutations at the level of the DNA sequence IgG2. Moreover, additional analysis of genetic polymorphisms in individuals with low serum IgG4 levels revealed an association between decreased IgG4 levels and a minor structural defect in the *IGHG4* gene.^{18,19} On the other hand, some data from chromosomal microarrays have also defined the potential clinical significance of the *IGHG4* locus. Heterozygous deletion of *IGHG4* is likely benign, according to ClinVar.²⁰

Study limitations. There are some short comings in this study. Due to a lack of other technique, for example CGH array in our laboratory, the *IGHG4* deletion in DS, need further investigation for confirmation. Therefore, I hope to improve the above mentioned issue to provide a basis for molecular investigation in DS, particularly among those with severe low/undetected serum IgG4 and who have had repeated infections.

In conclusion, patients with recurrent infection had severely low/undetected serum IgG4 levels, as well as a severely low *IGHG4/36B4* ratio. Because DS is a polygenic disease, the causes of low/undetected serum IgG4 in patients with DS are complex and difficult to study. These deficits could be caused by extrinsic factors linked to DS-related disturbances, which are especially important in the presence of heterozygous DS. Critically, these findings show that *IGHG4* deletion is common in DS patients with low IgG4 levels. Despite

the study's limitations, this finding found a genetic explanation for low/undetected IgG4 in DS patients with repeated infections. This finding may influence future recommendations for *IGHG4* genetic testing in DS patients with low IgG4 levels.

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