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Interferon-Gamma-Dependent Immunity in *Bacillus Calmette-Guérin* Vaccine Osteitis Survivors

Laura Pöyhönen, MD¹, Liisa Kröger, MD, PhD², Heini Huhtala, MSc³, Johanna Mäkinen, PhD⁴, Jussi Mertsola, MD, PhD⁵, Ruben Martinez-Barricarte, PhD⁶, Jean-Laurent Casanova, MD, PhD^{6,7,8,10,11}, Jacinta Bustamante, MD, PhD^{7,8,9}, Qiushui He, MD, PhD^{4,12}, and Matti Korppi, MD, PhD¹

¹Center for Child Health Research, University of Tampere and University Hospital, Tampere, Finland ²Department of Pediatrics, University Hospital, Kuopio, Finland ³School of Health Sciences, University of Tampere, Tampere, Finland ⁴Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare, Turku, Finland ⁵Department of Pediatrics, University of Turku and University Hospital, Turku, Finland ⁶St. Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller Branch, The Rockefeller University, New York, NY, USA ⁷Laboratory of Human Genetics of Infectious Diseases, Necker Branch, INSERM U1163, Necker Hospital for Sick Children, Paris, France, EU ⁸Paris Descartes University, Imagine Institute, Paris, France, EU ⁹Howard Hughes Medical Institute, New York, USA ¹⁰Pediatric Hematology-Immunology Unit, Necker Hospital for Sick Children, Paris, France, EU ¹¹Center for the Study of Primary Immunodeficiencies, Paris AP-HP, Necker-Enfants Malades Hospital, Paris, France, EU ¹²Department of Medical Microbiology and Immunology, University of Turku, Turku, Finland

Abstract

Background—Inborn errors of interferon-gamma (IFN- γ) –mediated immunity underlie disseminated disease caused by *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) live vaccines. We hypothesized that some patients with osteitis after BCG vaccination may have an impaired IFN- γ immunity. Our aim was to investigate IL-12 and IFN- γ *ex-vivo* production stimulated with BCG and BCG+IFN- γ or BCG+IL-12, respectively, in BCG osteitis survivors.

Methods—Fresh blood samples were collected from 132 former BCG osteitis Finnish patients now aged 21-49 years, and IL-12 and IFN- γ were measured in cell cultures with and without stimulation with BCG and with BCG+IFN- γ or BCG+IL-12, respectively. As a pilot study, known disease-causing genes controlling IFN- γ immunity (*IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO* and *CYBB*) were investigated in 20 selected patients by whole-exome -sequencing.

Results—By the limit of <5th percentile, *ex-vivo* IL-12 concentration and increase in concentration was low in five, and *ex-vivo* IFN- γ concentration and increase in concentration was low in six patients (including two samples with both IL-12 and IFN- γ findings). By the limit of

<10th percentile, an additional six and four patients were respectively detected (including two samples with both findings). With two exceptions, low concentrations and low increases in concentrations picked-up the same cases. Mutations in known disease-causing IFN- γ -related genes were not found in any of these patients.

Conclusion—These findings call for searching of mutations in new genes governing IFN- γ -dependent immunity to live BCG vaccine.

Keywords

Interleukin-12; Interferon-gamma; Bacillus Calmette-Guérin; Osteitis; Vaccination

Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis*. Approximately one third of the world's population is infected with *M. tuberculosis*, but only 10% of infected hosts develop an active disease.¹ Host immunity and variation especially in innate immunity influence the risk to get TB and other mycobacterial diseases.^{2,3} After phagocytosis of mycobacteria, macrophages produce different mediators like interleukin-1 (IL-1) which enhance the maturation of dendritic cells, and IL-12 which enhance the maturation of T helper type-1 (Th1) cells, further promoting the production of interferon-gamma (IFN- γ).⁴

Bacillus Calmette-Guérin (BCG) vaccine has been globally used as a vaccine against TB.⁵ Currently, most western countries have stopped universal BCG vaccinations due to a risk of vaccination complications and low TB prevalence. However, severe complications like BCG osteitis have been rare in most populations, for example in Japan only 0.2 cases per 100,000 vaccinations.⁶

BCG vaccination of newborns belonged to the national vaccination program in Finland from 1941 to 2006, and the coverage of the vaccinations was nearly 100%.^{7,8} The National Public Health Institute registered systematically invasive complications such as BCG osteitis until 1988.⁷⁻⁹ According to the national registers, 222 children developed BCG osteitis confirmed by culture of BCG strain, by typical histology and age, or by both during the years 1960-1988.^{7,8} The mean age when the first signs of osteitis appeared was 1.5 years (range 0.3-5.7 years), and 96% of the children were vaccinated within the first days after birth and the remaining 4% during the first year of life.^{7,8} Between 1971 and 1978, when the Copenhagen BCG strain was in use, the annual incidence of BCG osteitis increased to 36.9 (mean) per 100,000 vaccinated newborns, and decreased after the change to the Glaxo BCG vaccine to 6.4 (mean) per 100,000 vaccinated newborns^{7,8}, which still was higher than that reported from other countries.⁶

Human IFN- γ plays an important role in the immune response to BCG vaccine and in the defense against mycobacteria. The understanding of the pathogenesis of mycobacterial diseases has been improved by studies of Mendelian susceptibility to mycobacterial disease (MSMD). It is a rare genetic condition that makes an individual susceptible to weakly virulent mycobacteria, such as BCG¹⁰⁻¹⁴ and atypical mycobacteria. The patients are also

vulnerable to the more virulent *M. tuberculosis*^{3,13,15} and half have suffered from typhoidal or non-typhoidal salmonellosis.¹²⁻¹⁴ MSMD patients are usually otherwise healthy, and their immunity is intact except for a limited defect in innate immunity affecting IFN- γ . Since the first observations in 1996^{12,16}, nine MSMD causing genes have been discovered.¹³ Seven of these genes are autosomal (*IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15* and *IRF8*), and two are X-linked (*NEMO* and *CYBB*). By 2015, 18 different disorders have been defined due to the high level of allelic heterogeneity, and these disorders are linked to the function of the IFN- γ pathway, impairing either the production of IFN- γ or the response to IFN- γ .¹³

The hypothesis of the study was that the patients with severe complications like osteitis after BCG vaccination have a compromised function of the IFN- γ pathway. The aim of the study was to investigate, by using blood cell cultures obtained from the former BCG osteitis patients, IL-12 and IFN- γ *ex-vivo* production stimulated with BCG and BCG+IFN- γ or BCG +IL-12, respectively. Fresh whole blood samples were available from 132 of those 222 adults who had suffered from BCG osteitis in infancy.^{8,9} The nine known genes of MSMD were investigated by whole exome-sequencing (WES) in 20 study subjects as a pilot study.

Materials and Methods

The diagnostics of invasive complications caused by BCG vaccinations was centralized in one national laboratory (National Public Health Institute, Helsinki, Finland) from 1960 to 1988, and during these 29 years, altogether 222 BCG osteitis cases were diagnosed in Finland.^{8,9} In 2007-2008, a questionnaire and invitation to the study was sent to 203 (91.4%) former BCG osteitis patients with current address available, and 160 (78.8%) replied. The questionnaire included questions on chronic illnesses, long-term use of medicines, repeated or chronic infections, and mycobacterial infections. Nineteen subjects were not contacted; six had moved to overseas, and no residence information was available from 13 subjects with BCG osteitis in early childhood.

Fresh whole blood samples were obtained from 132 of the 160 former BCG osteitis patients who agreed to attend the study. Blood samples were collected into heparinized tubes at laboratories around the country and were immediately (within 12 hours) transported to the Tuberculosis Reference Laboratory, National Institute for Health and Welfare, Turku, Finland.

At the laboratory, the blood samples were diluted 1:2 in RPM 1640 medium (GibcoBRL) supplemented with penicillin at 10000 U/ml (GibcoBRL). The detailed protocol of whole blood culture and stimulation was described previously.¹⁷ In brief, 6 ml of the diluted blood sample was dispensed into four wells (1.5 ml/ well) of a 24-well plate (Nunc). They were cultured and stimulated with BCG (4 $\times$ 10⁷ cells/ 0.1ml), BCG and recombinant IFN- γ (50 μ l, 165000U/ml), or BCG and recombinant IL-12 (50 μ l, 700 ng/ml), respectively. For each sample, whole blood without stimulation was used as a negative control. After 18 hours of incubation, 450 μ l supernatant was collected from each well for determination of IL-12 and after 48 hours of incubation, all supernatants left in each well were collected for determination of IFN- γ . The cytokine concentrations were determined by using ELISA kits

(Quantikine, R&D systems) according to the manufacture's protocol. The detection limit of the test was 0.7 pg/ml for IL-12 and 17.6 pg/ml for IFN- γ .

The differentials of white blood cells were counted in 119/ 132 (90%) study subjects before the samples were transported, and the IL-12 and IFN- γ concentrations in the cell cultures are expressed per peripheral blood mononuclear cells (PBMCs), including lymphocytes and monocytes. The IL-12 and IFN- γ concentrations (in pg/ml) per PBMCs (1.0×10^9 /l) were used in the analyses.

Data on PBMCs counts were available in 119, data on stimulated IL-12 concentrations in 125 and data on stimulated IFN- γ concentrations in 131 cases. Calculated concentrations per PBMCs were present in 115 (IL-12) and 118 (IFN- γ) cases, respectively.

Given the implication of IFN- γ pathway in mycobacterial infections, the nine known genes of MSMD (18 known gene defects) were studied in 20 study subjects as a pilot study. The samples were picked-up to include cases with low and high IL-12 concentrations after BCG +IFN- γ stimulation, and low and high increases in stimulated concentration, and those with low and high IFN- γ concentrations after BCG+IL-12 stimulation, and low and high increases in stimulated concentration, respectively. WES was performed by the New York Genome Center using an Illumina HiSeq 2500 machine, with an Agilent 71 Mb SureSelect exome kit. The reads were aligned to the human reference genome with a BWA aligner, and then re-calibrated and annotated with GATK¹⁸, PICARD (<http://picard.sourceforge.net/>) and ANNOVAR.¹⁹ The variations were further filtered and investigated with our in-house on-line server. The known gene defects of *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO* and *CYBB* were examined as described earlier.¹³

Ethics

The study was accepted by the Ethics Committee of the Tampere University Hospital District. A written consent was obtained from the study subjects, including permission to perform genetic studies concerning susceptibility to mycobacterial infections.

Statistical Analysis

The SPSS 19.0 package (IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp) was used for statistical analyses. Exploratory data analyses revealed that the IL-12 and IFN- γ concentrations and increases in concentrations were non-normally distributed, and therefore, the results are expressed as medians, 5th, 10th, 25th, 75th, 90th and 95th percentiles and ranges. The PBMC counts (cells $\times 10^9$ /L) are expressed as means, standard deviations (SD) and ranges.

Results

Study Subjects and Peripheral Blood Mononuclear Cells

The median age of the 132 former BCG osteitis patients was 33.0 years (range 21-49 years), and 72 (54.5 %) were females. None of the 160 study subjects, including those 132 with blood samples for cell cultures available, reported TB, or any other mycobacteriosis than infant BCG osteitis.

Among the 132 patients with blood samples available, 8% reported allergy, 7% asthma, 3% hypothyreosis, 3% psoriasis, 2% rheumatoid arthritis, 2% epilepsy, 2% multiple sclerosis, and less than 1% diabetes, spondylarthritis, stroke, breast cancer or migraine. Seven patients (5%) reported an earlier *Salmonella* infection.

The mean PBMC count (SD, range) was $2.80 \times 10^9/l$ (0.89, 0.60-5.49). The figures for lymphocytes were 2.35 (0.81, 0.54-4.80) and for monocytes 0.45 (0.18, 0.06-1.10).

IL-12 and IFN- γ Concentrations With and Without Stimulations

Before BCG stimulation, IL-12 was measurable (>0.7 pg/ml) in 17 (13%) and IFN- γ was measurable (>17.6 pg/ml) in 15 (11%) samples. The respective figures were 84 (64%) and 131 (99%) after stimulation with BCG. Thus, IL-12 concentration was below the detection limit in 48 (36%) cases even after BCG stimulation.

The median IL-12 concentration per PBMCs was 38.1 pg/ml after BCG+IFN- γ stimulation. The median IFN- γ concentration per PBMCs was 16708.7 pg/ml after BCG+IL-12 stimulation (Table 1).

The median increase of the IL-12 concentration per PBMCs was 36.5 pg/ml between BCG alone and BCG+IFN- γ stimulations. The median increase of the IFN- γ concentration per PBMC was 12516.0 pg/ml between BCG alone and BCG+IL-12 stimulations (Table 2).

By the limit of $<5^{\text{th}}$ percentile, IL-12 *ex-vivo* concentration and increase in concentration was low in five and *ex-vivo* IFN- γ concentration and increase in concentration in six cases, two cases with low concentrations and low increases in both IL-12 and IFN- γ as included (Table 3). By the limit of $<10^{\text{th}}$ percentile, *ex-vivo* IL-12 concentration and increase in concentration was low in additional six and *ex-vivo* IFN- γ concentration and increase in concentration in additional four cases, two cases with low concentrations and low increases in both IL-12 and IFN- γ as included (Table 3). With two exceptions, low *ex-vivo* concentrations and low increases in concentrations picked-up the same cases. None presented with a low ($<10^{\text{th}}$ percentile) stimulated IFN- γ and a high ($>90^{\text{th}}$ percentile) stimulated IL-12 concentration, or with a low ($<10^{\text{th}}$ percentile) stimulated IL-12 and a high ($>90^{\text{th}}$ percentile) stimulated IFN- γ value in cell cultures (Data not shown).

The reported current or earlier diseases in low ($<5^{\text{th}}$ or 5- 10^{th} percentiles) IL-12 or IFN- γ producers are presented in Table 3. There were four subjects with low production of both IL-12 and IFN- γ , and one of them reported asthma, one diabetes, one stroke and one reported no disease (Table 3).

Gene Defects of the IFN- γ Pathway

Known genes of MSMD including *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO* and *CYBB* were studied in 20 study subjects by WES. No mutations were found in coding regions of these genes. Seven of them belonged to the groups of low ($<10^{\text{th}}$ percentile) stimulated *ex-vivo* IL-12 concentration and low increase in concentration (N=4), or low ($<10^{\text{th}}$ percentile) stimulated *ex-vivo* IFN- γ concentration and low increase in concentration (N=3).

Register for causes of death

No residence information was available from 13 subjects with BCG osteitis in early childhood, but their dates of birth were recorded in the study documents. The register for causes of death (Statistics Finland) charted at a group level the causes of death of all deceased persons who had been born at the same day as the 13 former BCG osteitis patients without any residence information available. No person had died of tuberculosis or any other infection.

Discussion

The IFN- γ pathway is crucial for immunity against mycobacteria, including BCG, and to date, 18 disorders of the IFN- γ pathway have been identified.¹³ In order to reveal potential cases for defects of the IFN- γ pathway predisposing to complications of BCG vaccination, we analyzed the *ex-vivo* levels of IL-12 and IFN- γ in blood cell cultures stimulated with BCG alone and BCG+IFN- γ or BCG+IL-12, respectively, in 132 former BCG osteitis patients. Since reference values are not available, we classified the concentrations and increases in concentrations as low by the limit of 5th percentile or 10th percentile. Low stimulated *ex-vivo* IL-12 concentrations and low increases in concentrations revealed the same cases, with only one exception, and low stimulated *ex-vivo* IFN- γ concentrations and low increases in concentrations also revealed the same cases, again with only two exceptions. There were four cases (20% of all those with low concentrations or low increases in either IL-12 or IFN- γ) with low stimulated concentrations and low increases in concentrations of both IL-12 and IFN- γ . In the previous study¹⁷, which was done by the same methodology as our study, all 50 healthy BCG-vaccinated controls responded to the stimulation with BCG with increased IFN- γ production and all but two with increased IL-12 production in cell cultures. In line, IFN- γ was measurable in 11% of cases before BCG stimulation but in 99% after BCG stimulation in the present study. The respective figures for IL-12 were 13% and 64%. IL-12 was measurable in all cases after stimulation with BCG + IFN- γ . On average, the stimulated IFN- γ and IL-12 levels were lower in the present study than in the historical controls¹⁷, but as is well-known, the comparisons with historical controls studied in different laboratories are not reliable. Anyway, our results suggest some weakness of the IFN- γ pathway, although we could not reveal any increased infection morbidity or mortality after BCG osteitis was recovered.

An important result of the present study was that mutations in known genes affecting IFN- γ pathway were not found in those 20 cases which were studied, though seven of them presented with low stimulated *ex-vivo* IL-12 or IFN- γ concentrations or low increases in concentrations, and two presented with low stimulated *ex-vivo* concentrations and low increases of both IL-12 and IFN- γ .

Among 11 Iranian children who presented with invasive complications like cervical lymphadenitis or osteitis after BCG vaccination, 10 (91%) had an impaired *ex-vivo* IFN- γ response to BCG+IL-12 stimulation.²⁰ Evidently, these patients had MSMD, a genetic defect either in IL-12 receptor function or in IFN- γ production, but gene tests were not done. Instead, no significant differences were found in stimulated IL-12p40 and IFN- γ concentrations in cell cultures between 11 patients with non-TB mycobacterial cervical

lymphadenitis and healthy controls from Israel.²¹ Despite the negative result, the authors considered MSMD possible. In the present study, only 64% of the former BCG osteitis patients had measurable IL-12 concentration after BCG stimulation in cell cultures, but after BCG+IFN- γ stimulation, IL-12 was measurable in all cases. Instead, IFN- γ was measurable in all cell cultures after BCG stimulation. However, the study did not allow an evaluation of the magnitude of these responses due to the lack of appropriate healthy controls or reference values. Despite this, our results suggest that stimulation tests of blood cells may help to select patients for genetic studies, when thus far uncovered gene defects are searched. The currently known 18 gene defects of the IFN- γ pathway in *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO* and *CYBB* genes account for only half the known MSMD cases.¹³

The most common MSMD is complete recessive IL-12R β 1 deficiency, for which BCG disease is the most common infection.^{13,22} The clinical phenotype ranges from fatal infection in infancy to asymptomatic course throughout adulthood.²² Interestingly, BCG vaccination or BCG disease protects against subsequent environmental mycobacteriosis.²² Multifocal osteitis occurs frequently in partial dominant IFNGR1 and STAT1 diseases.^{13,23,24} However, BCG osteitis and multifocal osteitis are separate clinical entities, and all our BCG osteitis patients presented with only one focus.⁸ The cellular phenotype of partial IFNGR1 disease is characterized by a reduced *ex-vivo* response to IFN- γ .²² Likewise, the defect of the cellular IFN- γ response is partial in STAT1 disease, and the outcome of infections including BCG disease has mainly been good.¹³ In MSMD patients, BCG infections like other mycobacterioses have diverse manifestations ranging from localized to disseminated cases. Thus, the beneficial prognosis of early-childhood BCG osteitis in the patients of the present cohort does not rule out MSMD.

Evidently, the high BCG osteitis incidence in Finland reflects a selective genetic susceptibility to the disease, and the lack of severe or exceptional infections in later life rules out primary immune deficiencies. Recently, we have documented differences in polymorphisms of genes regulating mannose binding lectin production (*MBL2* gene) and toll-like receptor-2 subfamily function between the subjects of the present early-life BCG osteitis cohort and Finnish population-based controls.^{25,26} In this cohort, three patients had the homozygous variant *MBL2* genotype (O/O), and in one of them, IFN- γ concentration was at the 5th percentile level after stimulation with BCG and IL-12. No other evidence of low IFN- γ or IL-12 production was found.

The strength of this study is the large number of BCG osteitis patients from a period of 29 years in a country with high BCG osteitis incidence during the study years.⁷⁻⁹ At that time, the microbiological diagnostics of BCG complications was centralized, and complications were systematically recorded in one national register. In addition, the homogeneity of the study patients who are all of Finnish origin is an advantage in genetic studies. We were able to document patients with low *ex-vivo* stimulated IL-12 and/or IFN- γ production; this finding is commonly linked with susceptibility to mycobacterial disease. MSMD genetics were available for two of four BCG osteitis survivors who had low *ex vivo* production of both IL-12 and IFN- γ . No mutations were identified.

There are some weaknesses of this study. First, we were able to collect fresh blood samples from only 60% of the former BCG osteitis patients. This is mainly because of long distances within the country, and therefore, a selection bias is unlikely. Since references in healthy individuals are not available, the significances of the stimulated IL-12 and IFN- γ responses could not be assessed. The lack of a control group is a clear weakness of the study. On the other hand, to be representative of the population, the control group should be large covering different age groups and different parts of the country. Since the distances in our country are long and the transfer of some samples within 24 hours to the reference laboratory was challenging, we had 25 transfer control samples from healthy persons, but this material is too small and also non-representative to serve as a study control.

In conclusion, known gene defects of the IFN- γ pathway were not found in a sample of 20 adults from Finland with a history of BCG osteitis in infancy. On the other hand, there were marked differences in *ex-vivo* IL-12 and IFN- γ concentrations after stimulations, when the whole cohort of 118 subjects were examined. This suggests that there may be defects in the production of IL-12 and/ or IFN- γ , causing decreased immunity and increased susceptibility to BCG. Based on current results, further genetic studies even to find novel genetic defects are indicated, and they could first be focused on those with low *ex-vivo* IL-12 or IFN- γ concentrations or low increases in concentration.²⁷ The final goal of the studies on the association between genetic factors and BCG infections is to identify risk groups for severe complications of BCG vaccination.

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Table 1
IL-12 and IFN- γ concentrations in cell cultures per PBMC in 115 and 118 adults with BCG osteitis in infancy

	IL-12 concentration (pg/ml) after BCG+IFN- γ stimulation (N=115)	IFN- γ concentration (pg/ml) after BCG+IL-12 stimulation (N=118)
5 th percentile	3.5	2229.4
10 th percentile	5.2	4474.2
25 th percentile	12.4	8597.8
Median	38.1	16708.7
75 th percentile	66.0	31330.9
90 th percentile	109.6	45801.5
95 th percentile	152.4	59741.3
Range	2.9-274.7	543.0-81402.3

Table 2
Increases of IL-12 and IFN- γ concentrations per PBMC after BCG+IFN- γ or BCG+IL-12 stimulations, respectively, compared to concentrations after BCG stimulation alone. The detection limit was used for calculations if the concentration after BCG stimulation was non-measurable

	Increase in IL-12 concentration (pg/ml) (N=114)	Increase in IFN- γ concentration (pg/ml) (N=110)
5 th percentile	3.5	2236.9
10 th percentile	5.1	4390.3
25 th percentile	11.7	7035.0
Median	36.5	12516.0
75 th percentile	61.2	22393.9
90 th percentile	108.9	32620.3
95 th percentile	147.7	43708.4
Range	-3.0-260.5	990.3-66182.5

Table 3
Twenty low IL-12 or IFN- γ producers identified with four criteria based on results after BCG+IFN- γ or BCG+IL-12 stimulations of cell cultures, respectively (the *ex-vivo* concentration or increase in concentration of IL-12 or IFN- γ <5th percentile or <10th percentile, respectively)

No	Male (m), female (f), age (years)	IL-12 concentration low	IL-12 increase low	IFN- γ concentration low	IFN- γ increase low
<5th percentile					
25	f, 45			x	x
64	f, 32	x	x	x	x
65	m, 36*			x	x
66	f, 34			x	x
67	m, 34*	x	x		
72 ¹	f, 22	x	x	x	x
74	f, 47			x	x
82	m, 28			x	
128 ²	f, 41*	x	x		
132 ³	f, 34*	x	x		
5th-10th percentile					
26	m, 33			x	x
59	f, 38*	x	x		
62	m, 35				x
110	f, 28	x	x		
115 ⁴	f, 37	x	x		
130 ⁵	f, 35*	x	x	x	x
137 ⁶	f, 48*	x	x	x	x
141 ^{1,3}	f, 46		x		
142	f, 49			x	
155	f, 21	x	x		x

* *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO* and *CYBB* genes were studied, in seven cases, and all tests gave a negative result.

Reported diseases:

- ¹ Asthma;
- ² Allergy;
- ³ Salmonella infection;
- ⁴ Multiple sclerosis;
- ⁵ Diabetes;
- ⁶ Stroke

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