
MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

MIGUEL VERÍSSIMO CARVALHO LINCE DUARTE

# *Clinical Features and Genetical Analysis in Chronic Granulomatous Disease: a 25-Year Experience from a Regional Tertiary Care Center*

ARTIGO CIENTÍFICO ORIGINAL

ÁREA CIENTÍFICA DE PEDIATRIA

Trabalho realizado sob a orientação de:

DRª SÓNIA CRISTINA GASPAR DE LEMOS

PROFESSORA DOUTORA GUIOMAR GONÇALVES DE OLIVEIRA

MARÇO 2021

**Agradecimentos**

Este mestrado traduz uma longa caminhada que nunca foi feita só.

Foi um privilégio ter partilhado com os colegas de curso este caminho, nem sempre fácil, mas sem dúvida gratificante.

Um muito obrigado a todos os professores que cruzaram o meu percurso estudantil e que, de uma forma ou de outra, me marcaram pelo seu exemplo e ensinamento.

À Professora Doutora Guiomar Gonçalves de Oliveira, co-orientadora deste trabalho, pela partilha do seu muito conhecimento, pelo incentivo e pela imprescindível revisão do manuscrito.

À minha orientadora, Doutora Sónia Cristina Gaspar de Lemos, pela conceção deste projeto tão desafiante, pela disponibilidade, dedicação e empenho constantes e pelas críticas e conselhos na revisão do manuscrito. Aqui lhe exprimo a minha gratidão.

Por último, à minha família que estiveram e estão sempre comigo em todos os passos que dou. Vocês são a minha força e o meu farol.

# *Clinical Features and Genetical Analysis in Chronic Granulomatous Disease: a 25-Year Experience from a Regional Tertiary Care Center*

Miguel Veríssimo Carvalho Lince Duarte[1]

Guiomar Gonçalves de Oliveira[2,3]

Sónia Cristina Gaspar de Lemos[2,4]

1- Integrated Master's Degree, Faculty of Medicine, University of Coimbra, Portugal

2- University Clinic of Pediatrics, Universidade de Coimbra Faculdade de Medicina, Coimbra, Coimbra, Portugal

3- Child Developmental Center, Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Coimbra, Portugal

4- Primary Immunodeficiency Consult, Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Coimbra, Portugal

**Address for correspondence**: Pólo III – Pólo das Ciências da Saúde, Azinhaga de Santa Comba, Celas 3000-548 Coimbra

**Email**: miguelvclinceduarte@gmail.com

**Resumo:**

A doença granulomatosa crónica (DGC) é uma imunodeficiência primária rara, com infeções graves causadas por bactérias e fungos catalase-positivos, complicações inflamatórias e autoimunes. A prevenção de complicações infeciosas envolve profilaxia direcionada. O conhecimento dos padrões genéticos, clínicos e microbiológicos locais podem ter interesse na adequação da profilaxia e tratamento.

**Material e Métodos:** Realizou-se um estudo longitudinal retrospetivo de 25 anos, com análise dos dados clínicos e genéticos de doentes com DGC, confirmada geneticamente.

**Resultados:** A população incluiu 10 doentes (9 com DGC ligada ao cromossoma X (DGC-X) e 1 com DGC autossómica recessiva (DGC-AR). Verificou-se uma incidência de 1:53.221 nados-vivos. Nos doentes sem diagnóstico pré-natal (n=8) a mediana de idade de início clínico foi de 0,96 anos (0,25 – 4,08) e de diagnóstico de 4,38 anos (0,25-17,75) traduzindo-se num atraso de diagnóstico de 2,84 anos (0-16,25). Contabilizaram-se 110 infeções, 56 das quais graves: gânglios linfáticos (100% doentes), trato gastrointestinal (70%), fígado e baço (60%) foram os locais mais afetados e *Staphylococcus aureus* e *Salmonella* spp. os microrganismos mais frequentemente isolados. A profilaxia teve impacto na incidência de infeções (7,53 para 6,58 infeções/100 pacientes-mês) e na taxa de positividade das culturas realizadas (67,6% para 46,6%).

**Discussão:** A tipologia das infeções e dos microrganismos envolvidos diferiu do reportado noutras series. As infeções pulmonares foram pouco frequentes enquanto as infeções do fígado e baço tiveram elevada expressividade. Também se verificou ausência ou isolamento residual de microrganismos habitualmente associados à DGC como *Aspergillus e Burkholderia*. A profilaxia teve impacte nas infeções globais e ganglionares, mas não reduziu significativamente a taxa de infeções graves, e resultou num aumento de infeções do trato gastrointestinal.

**Conclusão:** Apesar de o prognóstico da DGC ter melhorado significativamente, o conhecimento dos padrões genéticos e microbiológicos locais pode ser importante para adequar esquemas terapêuticos e reduzir o *disease-burden*.

**Palavras-Chave**: Imunodeficiência Primária, Doença Granulomatosa Crónica; Infeção; Inflamação, Combinação Trimetoprima e Sulfametoxazol, Estudo Retrospetivo.

**Abstract**

**Introduction**: Chronic granulomatous disease (CGD) is a rare primary immunodeficiency characterized by recurrent, life-threatening bacterial and fungal infections and inflammatory and autoimmune events. Prevention of infectious complications involves targeted prophylaxis. Knowledge of local genetic, clinical and microbiological patterns may be of interest in the tailoring of prophylaxis and treatment.

**Material and Methods**: A 25-year retrospective longitudinal study was conducted with analysis of clinical and genetic data based on medical records of patients with genetically confirmed CGD.

**Results**: This population includes 10 patients, 9 with X-linked disease and 1 with autosomal recessive disease. An incidence rate of 1:1:53.221 live births was reported. In patients without prenatal diagnosis (n=8), the median age of clinical onset was 0.96 years (0.25 - 4.08) and the median age at diagnosis was 4.38 years (0.25 - 17.75), resulting in a diagnostic delay of 2.84 years (0 - 16.25). A total of 110 infections occurred, 56 of which were severe. Lymph nodes (100% patients), gastrointestinal tract (70%), liver and spleen (60%) were the most affected sites and *Staphylococcus aureus* and *Salmonella* spp. were the most frequently isolated microorganisms. Prophylaxis impacted the incidence of infections (7.53 to 6.58 infections/100 patients-month) and the yield of the cultures performed (67.6% to 46.6%).

**Discussion**: The sites of infection and microorganisms implied differed from the reported in other series. Lung infections were infrequent while liver and spleen infections were highly expressive. Also, there was an absence or residual isolation of microorganisms commonly associated with CGD such as *Aspergillus* and *Burkholderia*. Prophylaxis decreased overall infections and lymph node infections but did not significantly reduce the rate of severe infections and resulted in an increase in gastrointestinal tract infections.

**Conclusion**: Although the prognosis of CGD has improved significantly, knowledge of local genetic and microbiological patterns is important to adapt therapy regimens and reduce the disease-burden of chronic granulomatous disease.

**Keywords**: Primary Immunodeficiency Diseases; Granulomatous Disease, Chronic; Infections; Inflammation; Retrospective Studies; Trimethoprim, Sulfamethoxazole Drug Combination

## Introduction

Chronic Granulomatous Disease (CGD) is a rare primary immunodeficiency, with an incidence of 1/200.000 to 1/250.000 live births, caused by an inability of phagocytic leukocytes to kill a variety of microorganisms (1-3). It is characterized by susceptibility to infectious, inflammatory and autoimmune complications. (4)

It can manifest at any age; however, the first manifestations tend to occur in the first 2 years of life, with most of the patients being diagnosed before 5 years of age (2, 3, 5). Age of clinical onset is also impacted by the genotype. (6)

CGD is caused by defects in the oxidative burst in phagocytes (neutrophils, monocytes, macrophages, and eosinophils), as a result of alterations in the NADPH Oxidase complex. This complex is composed of two membrane-bound components (p22phox, p91phox), forming a heterodimer (cytochrome b558), and three cytosolic regulatory subunits (p47phox, p67phox, and p40phox) (5, 7). Mutations in any of the 5 genes encoding the subunits of this complex result in a decrease in the production of reactive oxygen species (ROS), compromising the phagocytosis of microorganisms. (5, 8)

The most common defect in European and American populations (approximately 70%) occurs in the *CYBB* gene (p91phox), located on the short arm of the X chromosome (Xp21.1-p11.4). Because it is inherited in a X-linked recessive manner, most patients are male. However, in females, extreme lionization phenomena may occur, leading to skewed X-chromosome inactivation (9, 10). Generally, X-linked variants show a more severe form of the disease, particularly the lower the production of ROS. (6, 11, 12)

Autosomal recessive forms are equally distributed in the female and male population (6, 7). They are mainly caused by mutations in the *p47phox* gene (about 20% of cases) and in the *p67phox* and *p22phox* genes (with 5% in each type) and more rarely in the p40phox gene (5, 6). They are more frequent in countries with high levels of consanguinity, where they may have higher incidence than the X-linked form. (4, 13, 14)

Definitive diagnosis is made by granulocyte function tests, with consequent genetic study for identification of the specific mutation, relevant in future counseling. (15, 16)

CGD is characterized by severe and recurrent infections of the lung, skin, bone, lymph nodes and liver, and is mainly caused by a restricted group of catalase-positive bacteria and fungi: *Staphylococcus aureus*, *Salmonella* spp., *Serratia marcescens*, *Nocardia*, *Aspergillus fumigatus* and *Aspergillus nidulans* (3, 17). Patients with CGD are also at high risk for infection with mycobacterium species, with the development of local or disseminated disease after Bacillus Calmette–Guérin (BCG) vaccination (18, 19). The relative prevalence of microbial agents involved has changed over the last decades. (1)

Additionally, there is some regional variability (6, 20), which should be considered. Several uncommon bacteria, *Chromcobacterium violaceum*, *Francisella philomiragia*, *Granulibacter bethesdensis* and *Burkholderia gladioli*, reported in recent decades, are virtually pathognomonic of CGD, and their infection should raise suspicion of this disease. (5, 8)

With the introduction of prophylaxis with sulfamethoxazole-trimethoprim (SMX-TMP) and itraconazole, a decrease in the incidence of infections was observed (17, 18). SMX-TMP reduced the incidence from 15.8 to 6.9 bacterial infections per 100 patient-months in patients with CGD-X, and from 7.1 to 2.4 per 100 patient-months in patients with CGD-AR (21). A randomized trial of 39 patients receiving placebo or itraconazole demonstrated that itraconazole prophylaxis decreased the incidence of fungal infections and is well tolerated. (22)

CGD is also characterized by dysregulated inflammatory response, which results in the formation of typical granulomatous lesions (8). These present with cutaneous, pulmonary, gastrointestinal or bladder location and can cause significant morbidity, through occlusion of adjacent viscera. (23)

Currently, hematopoietic stem-cell transplantation (HSCT) is the only curative treatment, being increasingly recommended at an earlier age (24). However, the scarce availability of bone-marrow donors and, until recently, the need for precise histocompatibility between donor and patient, to decrease the risk of Graft-versus-Host Disease (GvHD), greatly limited its use (15). Gene therapy, currently in clinical trials, may emerge as an alternative. Nonetheless promising results are yet to be delivered, due to low efficiency and low maintenance of the transcribed gene expression. (5, 25)

The prognosis of patients with CGD has dramatically improved (26), attributable to earlier diagnosis and better therapeutic management, with most patients reaching adulthood (1, 3, 17) and having median age of death of 30-40 years (5). Still, the cumulative effects of recurrent infections and inflammations lead to chronic complications that condition patients functionally, socially and educationally, with significant loss of quality of life (3). Thus, optimizing the management of infectious and inflammatory events is essential to reduce the disease burden.

The objective of this study is to characterize genetical, clinical and microbiological patterns in the region, to optimize the diagnosis and management of this patients, and verify the effectiveness of prophylaxis with SMX-TMP and itraconazole in each infection site.

## Materials and Methods

### Patients

The study data was obtained by a retrospective analysis of the electronic and paper-based clinical records of ten patients diagnosed with CGD over the past 25 years (1991 to the present) in a Portuguese Pediatric Tertiary Center - *Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra* (HP-CHUC). For confidentiality purposes, patient data was anonymized, and only authorized personnel had access to the original files. Some of these patients have been referred to in previous publications (10, 27). The disease was confirmed by functional and genetic studies. The functional test, used for the evaluation of the neutrophil oxidative burst, was the 123-dihydrorhodamine (123DHR). Study follow-up ended with adulthood at age 18 years (n=3) or with HSCT (n=3).

### Data Collection

All infectious events from birth to current age, adulthood or HSCT were analyzed in detail. Severe infection was defined as infection requiring intravenous treatment, hospitalization or resulting in death. Infections requiring months of intravenous therapy and multiple admissions were counted as one. For each infectious event, the age of occurrence, location, microbiology and severity were analyzed. For inflammatory and autoimmune events, the age of occurrence, location and type were analyzed. Family history was also considered, including consanguinity.

### Statistical analysis

Univariate analysis was performed by calculating frequencies, measures of central location and dispersion for each variable alone. The results are presented as medians and minimum/maximum values for continuous variables and as percentages for nominal variables. The incidence rate of infection was presented as infections/100 patients-month. The Wilcoxon test was used to compare infection incidence rates pre- and post-prophylaxis for total infections, severe infections, and infections per site, for the population and each subgroup (male XL-CGD, female XL-CGD and AR-CGD). The Chi-square test was used to assess the association between usage of prophylaxis and positivity of microbiological cultures. The delay of occurrence of infectious and inflammatory events was analyzed by the Kaplan-Meier non-parametric method and the log-rank test was performed for comparisons.

The threshold for statistical significance was set to $p<0.05$ in all analysis. Statistical analysis and graph construction were performed using SPSS program version 26.0 (IBM, Armonk, New York, USA).

## Results

Patients General Characteristics

This study included ten patients (nine male) from eight families. Nine had X-linked CGD (XL-CGD) and one had autosomal recessive CGD (AR-CGD) due to a mutation in the *CYBA* gene. The only female patient had XL-CGD with extreme lionization of the X chromosome (Table 1). Six had a positive family history, with manifestations compatible with CGD, and in one there was consanguinity (patient 6). In two patients the diagnosis was prenatal.

Of the three patients who underwent HSCT, one (patient 7) died of post-transplant lymphoproliferative disease (PTLD), associated with Epstein-Barr virus (EBV) infection, two years after transplantation. Nine patients remain alive. The median follow-up for all patients was 14.46 years (5.75-18) (Table 2).

***Table 1*** – Genetic and Demographic Data

| Patient | Sex | Family History | Mutation | | | | |
|---|---|---|---|---|---|---|---|
| | | | Heritage | Protein | Gene | cDNA | RefSeq |
| **1** | M | No | XL-CGD | p91-phox | *CYBB* | c.676C>T | NM_000397.3 |
| **2** | M | Yes | XL-CGD | p91-phox | *CYBB* | c.45 + 2delT | NM_000393.4 |
| **3** | M | Yes | XL-CGD | p91-phox | *CYBB* | c.252G>A | NM_000397.3 |
| **4** | M | Yes | XL-CGD | p91-phox | *CYBB* | c.252G>A | NM_000397.3 |
| **5** | M | Yes | XL-CGD | p91-phox | *CYBB* | c.2T>A | NM_000397.4 |
| **6** | M | No | AR-CGD | p22-phox | *CYBA* | c.287+1G>A | NM_000101.3 |
| **7** | M | No | XL-CGD | p91-phox | *CYBB* | c.58G>C | NM_000397.4 |
| **8** | M | Yes | XL-CGD | p91-phox | *CYBB* | c.1462-2A>G | NM_000397.4 |
| **9** | M | Yes | XL-CGD | p91-phox | *CYBB* | c.252G>A | NM_000397.3 |
| **10** | F | No | XL-CGD | p91-phox | *CYBB* | c.972_973delCA | NM_000397.4 |

CGD – Chronic Granulomatous Disease. XL-CGD – X-linked CGD. AR-CGD – autosomal recessive CGD. p91-phox – Membrane NADPH oxidase complex. p22-phox Membrane NADPH Complex. RefSeq - NCBI Reference Sequence Database

In patients without prenatal diagnosis, the median age of clinical onset was 0.96 years (0.25- 4.08) and the diagnosis age was 4.38 years (0.25-17.75), resulting in a diagnostic delay of 2.84 years (0-16.25). In the population, infection was the main mode of revelation of the disease (70%), with family screening contributed to the diagnosis (30%). The age of the first infectious event was at 1.05 years (0.25-4.08), of the first inflammatory event at 4.58 years (1.5-9.67). HSCT occurred at 14.58 years (4.58-14.67) (Table 2).

***Table 2*** – General Characteristics

| | | |
|---|---|---|
| **Patients without prenatal diagnosis (n=8)** | **Clinical onset age, years** | **0.96 (0.25 – 4,08)** |
| | XL-CDG (CYBB) | 1 (0.25 – 4.08) |
| | AR-CGD (CYBA) | 0.75 |
| | Diagnosis age, years | 4.38 (0.25-17.75) |
| | XL-CDG (CYBB) | 4.08 (0.25-17.75) |
| | AR-CGD (CYBA) | 11.92 |
| | Diagnostic delay, years | 2.84 (0-16.25) |
| **Population (n=10)** | Mode of revelation | |
| | Infection | 70% |
| | Family Screening | 30% |
| | Follow up, years | 14.46 (5.75-18) |
| | HSCT age, years | 14.58 (4.58-14,67) |

Results are expressed as median (range, minimum–maximum) for quantitative variables and in total number (%) for qualitative variables. CGD – Chronic Granulomatous Disease. XL-CGD – X-linked CGD. AR-CGD – autosomal recessive CGD. HSCT – hematopoietic stem-cell transplantation.

Estimated Incidence

The incidence in the region Centro (NUTS II) for the timeframe (1994-2020) is estimated to be 1:53.221. There was also an average of 19.700 births per year (Supplementary Table 1).

Initial manifestations attributable to CGD and manifestations leading to diagnosis

The clinical findings at onset of CGD were persistent or suppurative lymphadenitis in five patients, three of which related to BCG vaccine, pneumonia in two patients and osteomyelitis in one. The manifestations leading to diagnosis were hepatic or hepatosplenic abscesses in three patients, muscle abscess, osteomyelitis, meningitis and fever without focus in one patient each. In one case (patient 3), the diagnosis was suspected after re-evaluation of his infectious history due to a diagnosed sibling. In the two patients with prenatal diagnosis and under prophylaxis, the first manifestations of CGD were persistent or suppurative lymphadenitis (Supplementary Table 2).

Infectious Events

All patients started prophylactic treatment with SMX-TMP and itraconazole after diagnosis, and those of prenatal diagnosis, soon after birth. Patients 3 and 7 also received Interferon-Gamma (IFN-γ) as adjuvant therapy in some infectious complications.

A total of 110 infectious events occurred, 56 of which were severe. All patients had at least 1 severe infection. Infections of the lymph nodes, skin, gastrointestinal tract and liver/spleen were the most frequent. All patients had at least one lymph node infection and six had at least one liver/spleen infection. In infections of the gastrointestinal tract, gastroenteritis predominated, with three rectal and anal abscesses also occurring. Lung infections accounted for 5.40% of all infections (Table 3).

***Table 3*** - Sites and Characteristics of the 110 Infections

| Sites and Characteristics of Infections | Number of occurrences, n (%) | Number of Patients Involved, n |
|---|---|---|
| **Lymph Node** | 29 (26.3%) | 10 |
| **Skin** | 24 (21.8%) | 6 |
| **Ear** | 5 (4.5%) | 3 |
| **Gastrointestinal** | 23 (20.0%) | 7 |
| **Gastroenteritis** | 16 (14.7%) | 6 |
| **Rectal and Anal Abscess** | 3 (2.7%) | 3 |
| **Chronic Gastritis** | 2 (1.8%) | 2 |
| **Pseudomembranous Colitis** | 2 (1.8%) | 1 |
| **Liver and Spleen** | 12 (10.9%) | 6 |
| **Lung** | 6 (5.4%) | 4 |
| **Bone** | 2 (1.8%) | 2 |
| **Meninges** | 1 (0.9%) | 1 |
| **Blood** | 4 (3.6%) | 3 |
| **Others** | 4 (3.6%) | - |

Regarding the time of the first infection in each location, lymph node infections presented the earliest at 1.18 years (0.3-10.7). This was followed, in increasing age, by infections of bone 3.18 years (0.3-6.1); lung 4.06 years (1.5-6.9); gastrointestinal 4.22 years (1.1-9.7); liver and spleen 5.24 years (0.6-15.3); skin 6.31 years (1.6-10.3); ear 6.50 years (2.6-10.7); meninges 11.58 years and blood 14.5 years (Supplementary Figure 1).

Infectious Agents

61 infections (56%) had a positive culture with 64 microorganisms being isolated. Gram-negative bacteria predominated (53.10%) followed by gram-positive (32.81%). Mycobacteria were identified in 9.50%. Fungi and protozoa were rare (3.13% and 1.56% respectively). The bacteria most frequently isolated were *Staphylococcus aureus* (n=12), *Salmonella* spp. (n=10) and Serratia marcescens (n=7). The following gram-negative bacteria were isolated two times each *Escherichia coli*, *Helicobacter pylori*, *Klebsiella*

*pneumoniae*, *Pseudomonas* spp.. Furthermore, *Legionella* spp., *Bartonella* spp., *Campylobacter* spp., *Fusobacterium* spp., *Haemophilus influenzae*, *Morganella morganii*, *Proteus mirabilis*, *Shigella* spp. and *Yersinia* spp. were isolated one time each. Other gram-positive bacteria isolated two times were *Clostridium difficile*, *Staphylococcus epidermidis. Enterococcus avium*, *Nocardia* spp., *Peptostreptococcus* spp., *Streptococcus anginosus* and *Streptococcus pyogenes* were also isolated one time each. The isolated mycobacteria were one *Mycobacterium non tuberculosis* and five post-BCG. The following fungi were isolated one time each: *Aspergillus* spp. and *Candida albicans*. The isolated protozoan was one *Giardia lamblia* (Supplementary Table 3).

The culture yield in the various locations was: 51.72% lymph node, 63.63% skin, 20.00% ear, 96.00% gastrointestinal, 27.27% liver and spleen, 33.33% lung, 100.00% bone, 100.00% meninge, 33% blood.

*Staphylococcus aureus* was the main cause of infection in the lymph nodes (n=6) and skin (n=5). In the gastrointestinal system, *Salmonella* spp. is the most frequently identified species (n=8) (Figure 1).

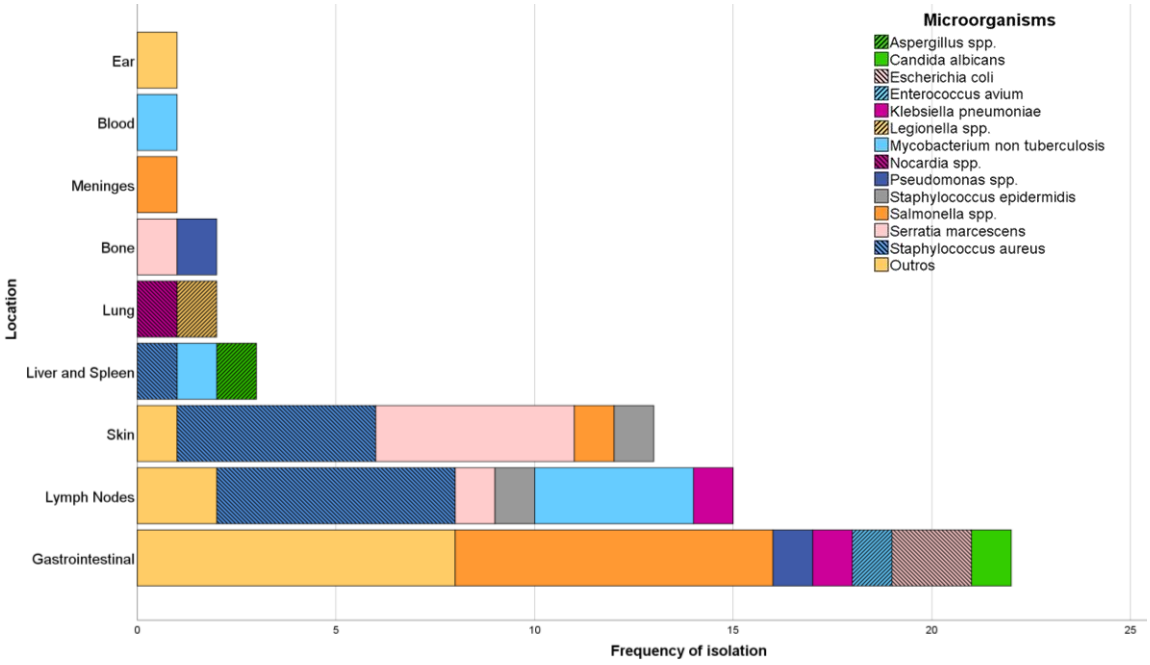



***Figure 1*** - Microorganisms isolated at the infection sites. The results are expressed as absolute number per infection site.

BCGitis data are included in the specific locations (Lymph Node and Liver/Splenic) but given their high prevalence it was decided to also treat them separately. Eight patients received vaccination against BCG and four of them had at least one manifestation of BCGitis. There was a case of disseminated BCGitis with hepatosplenic abscesses (Supplementary Table 4).

Effect of Prophylaxis

Total patient follow-up was 134.42 years (49.33 pre-prophylaxis and 85.08 post-prophylaxis). There was an overall reduction in the incidence of infection with the usage of prophylaxis from 7.53 to 6.58 infections/100 patients-month ($p=0.05$), but not on severity infection 3.43 to 2.53 infections/100 patients-month ($p=0.069$) (Figure 2). Due to differences in phenotype, the effect of prophylaxis is further described separately for patients with XL-CGD male, XL-CGD female and AR-CGD.

Regarding the incidence rate of infection, in male XL-CGD patients (eight patients) infection prior to prophylaxis usage it was 10.59 infections/100 patients-month and with prophylaxis 7.47 infections/100 patients-month ($p=0.116$). A similar trend was observed in severe infections with an incidence of 5.93 infections/100 patients-month prior to prophylaxis usage and 3.37 infections/100 patients-month with prophylaxis ($p=0.116$).

In male XL-CGD the effect of prophylaxis in each infection site was also verified. In the lymph nodes there was a decrease from 4.20 to 1.80 infections/100 patients-month ($p<0.05$) and in the gastrointestinal tract there was an increase from 0.4 to 2.1 infections/100 patients-month ($p<0.05$). Effect on skin, ear, liver and spleen, lung, bone and blood showed no statistically significant difference (Supplementary Table 5).

In the AR-CGD patient, the incidence rate of infection prior to prophylaxis was 3.50 infections/100 patients-month and for severe infection was 2.80 infections/100 patients-month; in the female patient (XL-GCD), the recorded incidence rate was 3.29 infections/100 patients-month and 2.34 infections/100 patients-month respectively for the same indicators. With prophylaxis, no infections were recorded in both patients, due to short follow-up time (3.42 and 0.25 years respectively).

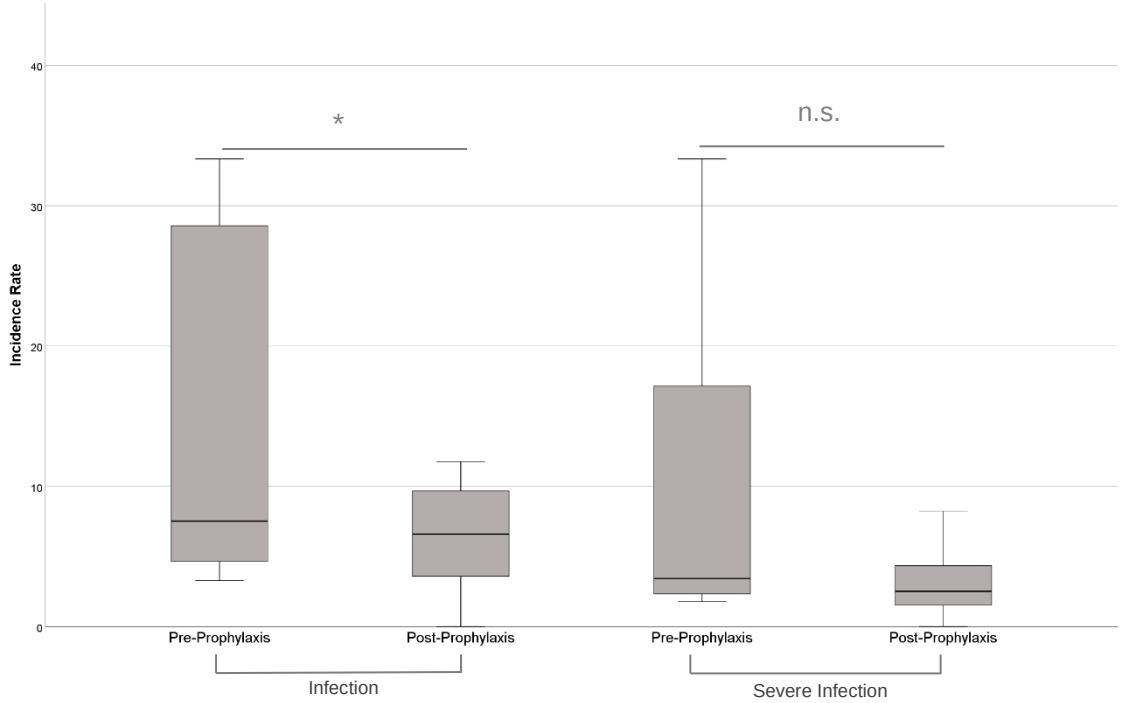



***Figure 2*** - Impact of prophylaxis on infection incidence rate and serious infection incidence rate. Data is expressed by median, quartiles, minimum and maximum of individual incidence rates in infection/100 patient-month. *: $p<0.05$; n.s.: not significant.

There was a decrease in the yield of cultures with the usage of prophylaxis. In pre-prophylaxis 67.6% were positive and post-prophylaxis 46.6% were positive ($p<0.05$). There was no significant difference in the incidence of classical microorganisms due to prophylaxis.

Inflammatory and Autoimune events

24 inflammatory and autoimmune events were documented. Six of the patients had an inflammatory event and two an autoimmune one (Supplementary Table 6).

The inflammatory events consisted of oral thrush/stomatitis/aphthous ulcers in four patients, granulomas in three patients, located in the skin and mesentery two times each and in the esophagus, mediastinum and infratemporal fossa one time each. Gastrointestinal manifestations also occurred with bowel stenosis and granulomatous colitis (two patients each), inflammatory cystitis and follicular hyperkeratosis (one patient each).

The autoimmune events consisted of autoimmune thrombocytopenia (20% of the patients) or celiac disease (10%). Additionally, five patients presented hypochromic microcytic anemia. Patient 6 had Leber's amaurosis and patients 1 and 10 had, respectively, a mother and a 3rd degree relative with Systemic Lupus Erythematosus.

Age at Infectious and Inflammatory Events

There was a clear predominance of infectious events (n=110) compared to inflammatory events (n=20). Kaplan-Meier test was conducted to compare the occurrence of the first infectious and inflammatory event of the ten patients. With the application of log-rank test it was confirmed that there was a statistically significant difference between them ($p<0.05$). It is also shown that the first infectious event occurs at a significantly younger age than the first inflammatory event, respectively 1.05 years (0.25-4.08) and 4.58 years (1.5-9.67). None of the patients had any inflammatory manifestation before an infectious one (Figure 3).

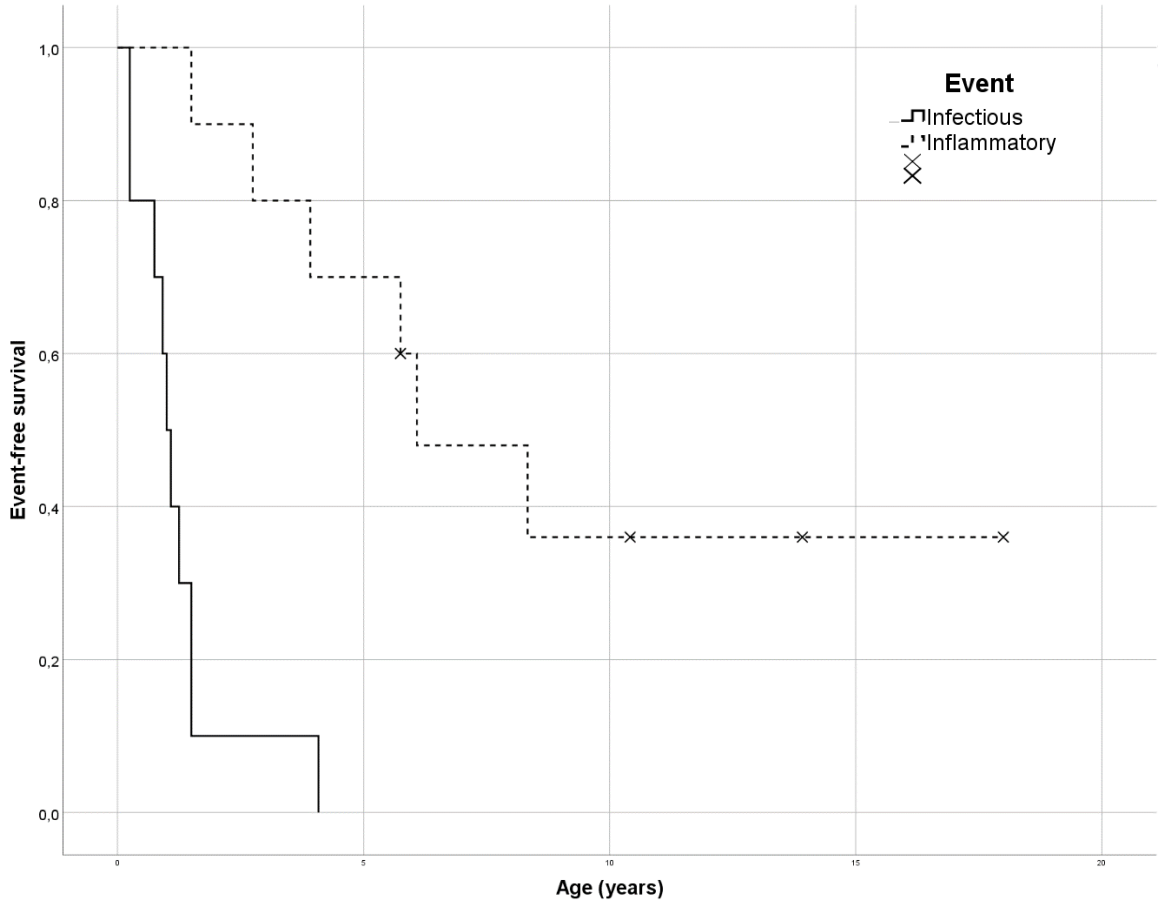



***Figure 3*** – Event-free survival curves for infections and inflammatory events. Log-rank test p=0.05

## Discussion

In this study we investigated the inheritance and clinical expression of CGD in ten patients diagnosed over the last 25 years in a third level Portuguese pediatric hospital. We characterized infectious, inflammatory and autoimmune events during follow-up. Microorganisms found were isolated and identified.

The incidence of CGD in our region is remarkably high, comparing with other studies (1, 6). This can be due to national asymmetries in the distribution of the disease or high clinic awareness.

Clinical expression and age of diagnosis were similar to those described worldwide; however, some important differences did appear. The clinical onset age (0.96 years) was lower than other European series (3, 17), probably due to universal vaccination with BCG in the National Vaccination Programme (PNV) until 2017. BCGitis was exceptionally common as a clinical onset manifestation, representing half of them. This finding is consistent with other countries that perform BCG vaccination (18, 28). There were significant delays in diagnosis which may be explained by: patients 3 and 10, less clinical exuberance (sparce pulmonary, cutaneous and lymph node infections); patient 6, erroneous values of oxidative burst (studied over 5 years until established diagnosis).

In our study the lymph nodes were the most prevalent site of infection. This was significantly higher than other European, American and Asian series (1, 3, 6, 18), but consistent with studies conducted in other Mediterranean countries, where high prevalence was also found (2, 17). These results suggest a regional trend. The prevalence of lung infections was low compared to most series worldwide (1, 3, 6, 29, 30). *Aspergillus* spp., *Burkholderia cepacia* or *Nocardia* spp. account for a significant proportion of pneumonias (1, 11), therefore the residual presence of these microorganisms in our population may explain this finding.

Liver and splenic infections had high frequency compared to the worldwide trend (1, 3, 6, 30). They accounted for about 10.90% of infections and six patients had at least one event, contributing to long hospital stays and had significant high morbidity.

The most frequently cultured micro-organisms were *Staphylococcus aureus*, *Salmonella* spp. and *Serratia* spp. The presence of Aspergillus spp. was remarkably residual, contrary to most studies (3). This trend was also seen in other recent Mediterranean studies (2, 17), which may be due to environmental factors or high sensitivity to itraconazole.

Atypical agents of infection were also isolated, such as *Helicobacter pylori*, *Fusobacterium* spp., *Peptostreptococcus* spp., *Morganella morganii* and *Yersinia* spp.

Despite being commonly associated with this disease, *Burkholderia* spp., was absent, and *Nocardia* spp. and *Klebsiella* pneumoniae were remarkably rare, consistent with recent European studies. (3, 17)

BCG infections were relatively frequent due to universal vaccination with BCG in all newborns. It is expected that the clinical reality will change in the future because of the BCG vaccination strategy, which changed from universal to individual. As of 2017, only children with individual or community risk factors for tuberculosis were vaccinated. The majority of our population had suspicious family history, so it is crucial that the pediatrician and family physician thoroughly verify family history of primary immune deficiency to prevent use of live vaccines, including BCG. (31)

Prophylaxis substantially reduced the incidence of infections, with a statistically significant effect on lymph nodes infections, consistent with what has been described in the literature (32). Despite an apparent reduced incidence of severe infection, there was no statistically significant effect. Severe and life-threatening events were still present with prophylaxis (29). The increased incidence of gastrointestinal infections with prophylaxis may be due to intestinal dysbiosis phenomena and increasing antibiotic resistance (33). Most patients came from hospitals in their areas of residence; therefore, the pre-diagnosis (and consequently pre-prophylaxis) manifestations may be underestimated, particularly non severe ones. Additionally, the number of post-prophylaxis infections may be overestimated due to regular and specialized medical follow-up and greater accuracy and accessibility of clinical records.

The culture yield rate was low, particularly after the introduction of prophylaxis. Definition of targeted therapy was therefore hampered and frequently required the administration of broad-spectrum empirical antimicrobial regimens to control severe infections in CGD. Molecular identification methods may improve this situation. Still, due to antifungal prophylaxis, non-culture methods such as Galactomannan currently have limited usage. (34)

Despite infection being largely predominant in our study, inflammation, especially of the intestinal tract and oral cavity, also occurred. Granulomas, stenosis and granulomatous colitis were predominant. A lower prevalence of granulomatous colitis was found comparing to other European and American series (3, 29, 35) and significantly lower than in Spain (17). Autoimmune thrombocytopenia and celiac disease were observed but discoid lupus erythematosus was absent contrary to other European series (3, 6). Nevertheless, given the rarity of these manifestations and given the size of our population it is difficult to draw any meaningful conclusions. An earlier median age of inflammation onset than described in Europe was observed (3), still it is expected to

increase due to ageing of our population and the absence of inflammatory manifestations in several patients.

## Conclusion

This is the first Portuguese retrospective longitudinal study on CGD. Infection sites and microorganisms involved in this group of patients differed from the reported in other series. Lung infections were unexpectedly infrequent, while liver and spleen infections were highly expressive. Furthermore, microorganisms usually associated with CGD, such as *Aspergillus* spp. and *Burkholderia* spp., were residually isolated or absent.

Prophylaxis had an impact on lymph node infections but did not significantly reduce the rate of severe infections and resulted in an increase in gastrointestinal tract infections.

Since curative options are still limited (HSCT depends on the existence of compatible donors and gene therapy is still restricted to clinical trials), knowledge of local microbiological patterns may optimize antimicrobial regimens decreasing the disease-burden.

## Supplementary Data

Supplementary materials are available after the references.

## Acknowledgments

The authors thank Dr. Ana Brett in the definition of severe, relapsing and recurrent infection and Dr. Ana Luísa Carvalho in the uniformization of genetic nomenclature.

**References**


1. Winkelstein JA, Marino MC, Johnston RBJ, Boyle J, Curnutte J, Gallin JI, et al. Chronic Granulomatous Disease: Report on a National Registry of 368 Patients. Medicine. 2000;79(3):155-69.
2. Beghin A, Comini M, Soresina A, Imberti L, Zucchi M, Plebani A, et al. Chronic Granulomatous Disease in children: a single center experience. Clinical Immunology. 2018;188:12-9.
3. Dunogué B, Pilmis B, Mahlaoui N, Elie C, Coignard-Biehler H, Amazzough K, et al. Chronic Granulomatous Disease in Patients Reaching Adulthood: A Nationwide Study in France. Clinical Infectious Diseases. 2017;64(6):767-75.
4. Kanariou M, Spanou K, Tantou S. Long-term observational studies of chronic granulomatous disease. Current Opinion in Hematology. 2018;25(1):7-12.
5. Arnold DE, Heimall JR. A Review of Chronic Granulomatous Disease. Advances in Therapy. 2017;34(12):2543-57.
6. van den Berg JM, van Koppen E, Ahlin A, Belohradsky BH, Bernatowska E, Corbeel L, et al. Chronic granulomatous disease: the European experience. PloS one. 2009;4(4):e5234-e.
7. Roos D. Chronic granulomatous disease. British medical bulletin. 2016;118(1):50-63.
8. Dinauer MC. Primary immune deficiencies with defects in neutrophil function. Hematology American Society of Hematology Education Program. 2016;2016(1):43-50.
9. Marciano BE, Zerbe CS, Falcone EL, Ding L, DeRavin SS, Daub J, et al. X-linked carriers of chronic granulomatous disease: Illness, lyonization, and stability. Journal of Allergy and Clinical Immunology. 2018;141(1):365-71.
10. Amaral J, Paiva A, Ramos F, Stasia M-J, Lemos S. X-linked chronic granulomatous disease in a female carrier with novel pathogenic mutation and skewed X-inactivation. Annals of Allergy, Asthma & Immunology. 2018;120:328-9.
11. Marciano BE, Spalding C, Fitzgerald A, Mann D, Brown T, Osgood S, et al. Common severe infections in chronic granulomatous disease. Clin Infect Dis. 2015;60(8):1176-83.
12. Kuhns DB, Alvord WG, Heller T, Feld JJ, Pike KM, Marciano BE, et al. Residual NADPH oxidase and survival in chronic granulomatous disease. N Engl J Med. 2010;363(27):2600-10.
13. Rawat A, Vignesh P, Sharma A, Shandilya JK, Sharma M, Suri D, et al. Infection Profile in Chronic Granulomatous Disease: a 23-Year Experience from a Tertiary Care Center in North India. J Clin Immunol. 2017;37(3):319-28.

14. Köker MY, Camcıoğlu Y, van Leeuwen K, Kılıç S, Barlan I, Yılmaz M, et al. Clinical, functional, and genetic characterization of chronic granulomatous disease in 89 Turkish patients. J Allergy Clin Immunol. 2013;132(5):1156-63.e5.
15. Lacerda-Pontes R, Gomes LN, Albuquerque RSd, Soeiro-Pereira PV, Condino-Neto A. The extended understanding of chronic granulomatous disease. Current Opinion in Pediatrics. 2019;31(6):869-73.
16. Di Matteo G, Giordani L, Finocchi A, Ventura A, Chiriaco M, Blancato J, et al. Molecular characterization of a large cohort of patients with Chronic Granulomatous Disease and identification of novel CYBB mutations: An Italian multicenter study. Molecular Immunology. 2009;46(10):1935-41.
17. Robles-Marhuenda A, Álvarez-Troncoso J, Rodríguez-Pena R, Busca-Arenzana C, López-Granados E, Arnalich-Fernández F. Chronic granulomatous disease: Single-center Spanish experience. Clinical Immunology. 2020;211:108323.
18. Zhou Q, Hui X, Ying W, Hou J, Wang W, Liu D, et al. A Cohort of 169 Chronic Granulomatous Disease Patients Exposed to BCG Vaccination: a Retrospective Study from a Single Center in Shanghai, China (2004–2017). Journal of Clinical Immunology. 2018;38(3):260-72.
19. Li T, Zhou X, Ling Y, Jiang N, Ai J, Wu J, et al. Genetic and Clinical Profiles of Disseminated Bacillus Calmette-Guérin Disease and Chronic Granulomatous Disease in China. Frontiers in Immunology. 2019;10(73).
20. Wu J, Wang W-F, Zhang Y-D, Chen T-X. Clinical Features and Genetic Analysis of 48 Patients with Chronic Granulomatous Disease in a Single Center Study from Shanghai, China (2005–2015): New Studies and a Literature Review. Journal of Immunology Research. 2017;2017:8745254.
21. Margolis DM, Melnick DA, Ailing DW, Gallin JI. Trimethoprim-Sulfamethoxazole Prophylaxis in the Management of Chronic Granulomatous Disease. The Journal of Infectious Diseases. 1990;162(3):723-6.
22. Gallin JI, Alling DW, Malech HL, Wesley R, Koziol D, Marciano B, et al. Itraconazole to Prevent Fungal Infections in Chronic Granulomatous Disease. New England Journal of Medicine. 2003;348(24):2416-22.
23. Marciano BE, Rosenzweig SD, Kleiner DE, Anderson VL, Darnell DN, Anaya-O'Brien S, et al. Gastrointestinal Involvement in Chronic Granulomatous Disease. Pediatrics. 2004;114(2):462-8.
24. Chiesa R, Wang J, Blok HJ, Hazelaar S, Neven B, Moshous D, et al. Hematopoietic cell transplantation in chronic granulomatous disease: a study of 712 children and adults. Blood. 2020;136(10):1201-11.
25. Roos D. Chronic Granulomatous Disease. Methods Mol Biol. 2019;1982:531-42.

26. Wolach B, Gavrieli R, de Boer M, Gottesman G, Ben-Ari J, Rottem M, et al. Chronic granulomatous disease in Israel: Clinical, functional and molecular studies of 38 patients. Clinical Immunology. 2008;129(1):103-14.
27. Campos M, Rocha G, Cordeiro A, Lemos S, Paiva A, Silva I, et al. Serratia osteomyelitis and chronic granulomatous disease. Acta médica portuguesa. 2011;24:449-52.
28. Blancas-Galicia L, Santos-Chávez E, Deswarte C, Mignac Q, Medina-Vera I, León-Lara X, et al. Genetic, Immunological, and Clinical Features of the First Mexican Cohort of Patients with Chronic Granulomatous Disease. Journal of Clinical Immunology. 2020;40(3):475-93.
29. Jones LB, McGrogan P, Flood TJ, Gennery AR, Morton L, Thrasher A, et al. Special article: chronic granulomatous disease in the United Kingdom and Ireland: a comprehensive national patient-based registry. Clin Exp Immunol. 2008;152(2):211-8.
30. Kobayashi S, Murayama S, Takanashi S, Takahashi K, Miyatsuka S, Fujita T, et al. Clinical features and prognoses of 23 patients with chronic granulomatous disease followed for 21 years by a single hospital in Japan. European Journal of Pediatrics. 2008;167(12):1389-94.
31. Shearer WT, Fleisher TA, Buckley RH, Ballas Z, Ballow M, Blaese RM, et al. Recommendations for live viral and bacterial vaccines in immunodeficient patients and their close contacts. J Allergy Clin Immunol. 2014;133(4):961-6.
32. Liese J, Kloos S, Jendrossek V, Petropoulou T, Wintergerst U, Notheis G, et al. Long-term follow-up and outcome of 39 patients with chronic granulomatous disease. J Pediatr. 2000;137(5):687-93.
33. Willmann M, Vehreschild MJGT, Biehl LM, Vogel W, Dörfel D, Hamprecht A, et al. Distinct impact of antibiotics on the gut microbiome and resistome: a longitudinal multicenter cohort study.
34. King J, Henriet SSV, Warris A. Aspergillosis in Chronic Granulomatous Disease. J Fungi (Basel). 2016;2(2).
35. Bortoletto P, Lyman K, Camacho A, Fricchione M, Khanolkar A, Katz BZ. Chronic Granulomatous Disease: A Large, Single-center US Experience. Pediatr Infect Dis J. 2015;34(10):1110-4.

## Supplementary Data

***Supplementary Table 1*** – Estimated incidence of CGD

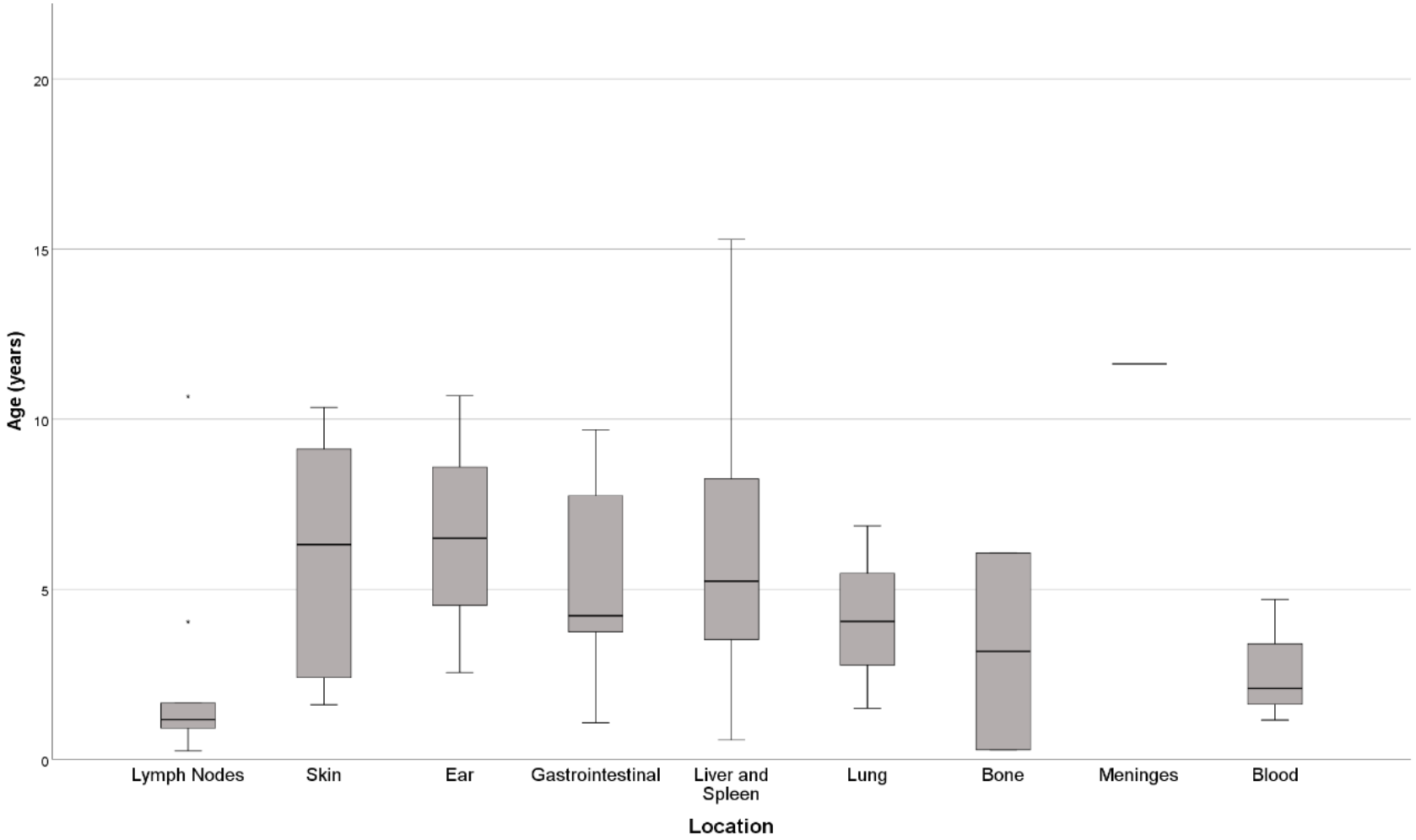
| Year | Live Births* | CGD Births |
|---|---|---|
| **1994** | 22.238 | 2 |
| **1995** | 21.804 | 1 |
| **1996** | 22.366 | |
| **1997** | 22.947 | |
| **1998** | 22.751 | 1 |
| **1999** | 23.441 | |
| **2000** | 23.973 | 1 |
| **2001** | 22.415 | |
| **2002** | 22.765 | |
| **2003** | 22.461 | |
| **2004** | 21.854 | |
| **2005** | 21.710 | |
| **2006** | 20.805 | |
| **2007** | 19.973 | 1 |
| **2008** | 20.156 | 1 |
| **2009** | 18.934 | |
| **2010** | 19.127 | 1 |
| **2011** | 18.342 | |
| **2012** | 17.195 | |
| **2013** | 15.733 | 1 |
| **2014** | 15.556 | |
| **2015** | 16.096 | 1 |
| **2016** | 16.252 | |
| **2017** | 15.926 | |
| **2018** | 16.064 | |
| **2019** | 15.871 | |
| **2020** | 15.452 | |

*Births on NUTS II Centro, Instituto Nacional de Estatística. 1994 and 2020 data were calculated considering the percentage of the national births in the region in adjacent years due to incomplete data.

***Supplementary Table 2*** – Onset and Diagnostic manifestations

| **Patient** | **Age at clinical onset** | **Onset Manifestation** | **Age at diagnosis** | **Suspicious Manifestation** | **HSCT** |
|---|---|---|---|---|---|
| **1** | 1 | BCGitis | 2,92 | Probable *Aspergillus* spp. hepatic abscess | No |
| **2** | 0,25 | BCGitis | 0,58 | Disseminated BCGitis with multiple hepatosplenic abscess | Yes |
| **3** | 4,08 | *Legionella* spp. pneumonia | 7,17 | ---------- | No |
| **4** | 0,25 | *S. marcescens* osteomyelitis | 0,25 | *S. marcescens* osteomyelitis | No |
| **5** | 1,08 | Inguinal lymphadenitis | Prenatal | ---------- | No |
| **6** | 0,75 | *S. aureus* lymphadenitis | 11,92 | *Salmonella* spp. meningitis | Yes |
| **7†** | 0,92 | Cervical lymphadenitis | 4,67 | Fever without focus | Yes |
| **8** | 1,5 | Cervical BCGitis | 4,08 | Hepatic Abscess | No |
| **9** | 1,25 | *S. aureus* cervical supurative lymphadenitis | Prenatal | ---------- | No |
| **10** | 1,5 | Pneumonia | 17,75 | *S. marcescens* deltoid muscle abscess | No |

Age at clinical onset and diagnosis are expressed in years. HSCT – Hematopoietic stem-cell transplantation. *S. marcescens* - *Serratia marcescens*. *S. aureus* - *Staphylococcus aureus*. **†** - Post-Transplant Death




***Supplementary Figure 1*** – Box Plot representing the association between age and first infectious manifestation at each location. Age is expressed by median, quartiles, minimum and maximum, in years.

***Supplementary Table 3*** – Isolated Microorganisms

| **Microorganisms** | **Cases, n (%)** | **Microorganisms** | **Cases, n (%)** |
|---|---|---|---|
| **Gram-negative Bacteria** | **34 (53,13%)** | **Gram-positive Bacteria** | **21 (32,81%)** |
| *Salmonella* spp. | 10 (15,63%) | *Staphylococcus aureus* | 12 (18,75%) |
| *Serratia marcescens* | 7 (10,94%) | *Clostridium difficile* | 2 (3,13%) |
| *Escherichia coli* | 2 (3,13%) | *Staphylococcus epidermidis* | 2 (3,13%) |
| *Helicobacter pylori* | 2 (3,13%) | *Nocardia* spp. | 1 (1,56%) |
| *Klebsiella pneumoniae* | 2 (3,13%) | *Enterococcus avium* | 1 (1,56%) |
| *Pseudomonas* spp. | 2 (3,13%) | *Peptostreptococcus* spp. | 1 (1,56%) |
| *Bartonella* spp. | 1 (1,56%) | *Streptococcus anginosus* | 1 (1,56%) |
| *Campylobacter* spp. | 1 (1,56%) | *Streptococcus pyogenes* | 1 (1,56%) |
| *Fusobacterium* spp. | 1 (1,56%) | **Mycobacteria** | **6 (9,38%)** |
| *Haemophilus influenzae* | 1 (1,56%) | *Mycobacterium non tuberculosis* | 1 (1,56%) |
| *Legionella* spp. | 1 (1,56%) | post-BCG | 5 (7,81%) |
| *Morganella morganii* | 1 (1,56%) | **Fungi** | **2 (3,13%)** |
| *Proteus mirabilis* | 1 (1,56%) | *Aspergillus* spp. | 1 (1,56%) |
| *Shigella* spp. | 1 (1,56%) | *Candida albicans* | 1 (1,56%) |
| *Yersinia* spp. | 1 (1,56%) | **Protozoa** | **1 (1,56%)** |
| | | *Giardia lamblia* | 1 (1,56%) |

Data is expressed in absolute numbers. Percentages are the number of episodes with a positive culture of a microorganism divided by the total number of isolated microorganisms.

***Supplementary Table 4*** – BCGitis event characteristics

| BCGitis | Number of events | Number of patients with ≥ 1 event | % of patients ≥ 1 event |
|---|---|---|---|
| **Local** | 3 | 3 | 37,5 |
| **Regional** | 1 | 1 | 12,5 |
| **Disseminated** | 1 | 1 | 12,5 |
| **Total** | 5 | 4 | 50 |

The total number of reported episodes in all patients is compared to the number of patients who has suffered from ≥1 episode in absolute numbers and percentage of the vaccinated patients (n=8).

***Supplementary Table 5*** – Incidence rate per location in male XL-CGD

| Location | Pre | Post |
|---|---|---|
| Lymph Nodes | 4,20 | 1,80 |
| Skin | 1,69 | 1,64 |
| Ear | 0,80 | 0,20 |
| Gastrointestinal | 0,40 | 2,10 |
| Liver and Spleen | 1,27 | 0,81 |
| Lung | 0,85 | 0,31 |
| Bone | 0,42 | 0,10 |
| Blood | 0,85 | 0,10 |

Incidence rate are expressed in infections/100 patients-month. Pre – Pre-prophylaxis. Post – Post-Prophylaxis. Statistically significant effect in lymph nodes (p=0.046) and gastrointestinal (p=0.043)

***Supplementary Table 6*** – Characteristics of the 24 inflammatory and autoimmune events

| Characteristics of Event | Episodes, n (%) | Patients involved, n |
|---|---|---|
| **Inflammatory** | 20 (83,33%) | 6 |
| **Granuloma** | 7 (29,17%) | 3 |
| Cutaneous | 2 (8,33%) | 1 |
| Esophageal | 1 (4,17%) | 1 |
| Mesenteric | 2 (8,33%) | 2 |
| Mediastinal | 1 (4,17%) | 1 |
| Infratemporal Fossa | 1 (4,17%) | 1 |
| **Gastrointestinal** | 7 (29,17%) | 4 |
| Stenosis | 4 (16,67%) | 2 |
| Granulomatous Colitis | 3 (12,5%) | 2 |
| **Urological** | 1 (4,17%) | 1 |
| Inflammatory Cystitis | 1 (4,17%) | 1 |
| **Oral** | 4 (16,67%) | 4 |
| **Dermatological** | 1 (4,17%) | 1 |
| **Autoimmune** | 4 (16,67%) | 2 |
| **Celiac Disease** | 1 (4,17%) | 1 |
| **Autoimmune thrombocytopenia** | 3 (12,5%) | 2 |

Results are expressed in number of occurrences (and % of the total number of episodes) and number of patients ≥ 1 event.