

Clinical, functional, and genetic characterization of chronic granulomatous disease in 89 Turkish patients

Mustafa Yavuz Köker, MD, PhD,^a Yıldız Camcıoğlu, MD,^b Karin van Leeuwen, BSc,^c Sara Şebnem Kılıç, MD,^d Işıl Barlan, MD,^e Mustafa Yılmaz, MD,^f Ayşe Metin, MD, PhD,^g Martin de Boer, BSc,^c Hüseyin Avcılar, MD, PhD,^h Türkan Patiroğlu, MD,ⁱ Alişan Yıldırım, MD,^j Olcay Yeğin, MD,^k İlhan Tezcan, MD, PhD,^l Özden Sanal, MD,^l and Dirk Roos, PhD^c Kayseri, Istanbul, Bursa, Adana, Ankara, Samsun, and Antalya, Turkey, and Amsterdam, The Netherlands

Background: Chronic granulomatous disease (CGD) is a rare primary immunodeficiency disorder of phagocytes resulting in impaired killing of bacteria and fungi. A mutation in one of the 4 genes encoding the components p22^{phox}, p47^{phox}, p67^{phox}, and p40^{phox} of the leukocyte nicotinamide dinucleotide phosphate reduced (NADPH) oxidase leads to autosomal recessive (AR) CGD. A mutation in the *CYBB* gene encoding gp91^{phox} leads to X-linked recessive CGD.

Objective: The aim of this study is to show the correlation between clinical, functional, and genetic data of patients with CGD from Turkey.

Methods: We report here the results of 89 patients with CGD from 73 Turkish families in a multicenter study.

Results: Most of the families (55%) have an AR genotype, and 38% have an X-linked genotype; patients from 5 families with a suspected AR genotype (7%) were not fully characterized. We compared patients with CGD according to the severity of NADPH oxidase deficiency of neutrophils. Patients with A22⁰, A67⁰ or X91⁰ phenotypes with a stimulation index of 1.5 or less have early clinical presentation and younger age at diagnosis (mean, 3.2 years). However, in p47^{phox}-deficient cases and in 5 other AR cases with high residual oxidase activity (stimulation index ≥ 3), later and less severe clinical presentation and older

age at diagnosis (mean, 7.1 years) were found. Pulmonary involvement was the most common clinical feature, followed by lymphadenitis and abscesses.

Conclusion: Later and less severe clinical presentation and older age at diagnosis are related to the residual NADPH oxidase activity of neutrophils and not to the mode of inheritance. CGD caused by A22⁰ and A67⁰ subtypes manifests as severe as the X91⁰ subtype. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Chronic granulomatous disease, dihydrorhodamine-1,2,3 assay, *CYBB*, *CYBA*, *NCF1*, *NCF2*, nicotinamide dinucleotide phosphate reduced oxidase, mean fluorescence intensity, stimulation index

Chronic granulomatous disease (CGD; OMIM no. 306400) is a rare (approximately 1 case in 200,000-250,000 newborns) genetic disorder of the innate immune system. The disease is characterized by a severe susceptibility to bacterial and fungal infections caused by the failure of phagocytic leukocytes to produce microbicidal reactive oxygen intermediates (ROIs). The source of these radicals is superoxide generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multicomponent enzyme complex expressed in phagocytic leukocytes (neutrophils, monocytes, eosinophils, and macrophages).¹⁻³

The disease is classified by mutations in specific subunits of the NADPH oxidase enzyme. A defect in membrane gp91^{phox} (phagocyte oxidase [phox]), which is encoded by the *CYBB* gene on the X chromosome, leads to X-linked recessive chronic granulomatous disease (X-CGD; approximately 70% of patients worldwide).¹⁻³ A defect in any one of the other 4 components of the NADPH oxidase (ie, p22^{phox}, p47^{phox}, p67^{phox}, and p40^{phox} encoded by the autosomal *CYBA*, *NCF1*, *NCF2*, and *NCF4* genes, respectively) leads to autosomal recessive (AR) CGD (AR-CGD).^{1,4,5} The majority of patients with X-linked recessive CGD (X-CGD) are given a diagnosis before the age of 2 years, whereas patients with p47^{phox} deficiency might have their condition undiagnosed until adulthood.^{1,3,4}

X-linked CGD is caused by mutations in the *CYBB* gene, which encodes gp91^{phox}, a key membrane protein in the phagocyte NADPH oxidase system.² This protein is the large subunit of the membrane-bound flavocytochrome *b*₅₅₈ complex; the small subunit is p22^{phox}. Heterodimer formation between gp91^{phox} and p22^{phox} is important for stable expression of each subunit in phagocytes. Immunoblotting or flow cytometric analysis with specific antibodies for individual components of NADPH oxidase helps to identify the deficient protein in the majority of cases.^{6,7}

Both the positive family history for CGD and granulocyte function tests in parents and siblings are critical for differentiation

From ^athe Immunology Department and GenKök Laboratory of Immunology, Faculty of Medicine, University of Erciyes, Kayseri; ^bPediatric Infectious Diseases, Clinical Immunology and Allergy Division, Cerrahpaşa Medical School, University of Istanbul; ^cSanquin Research and the Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam; ^dthe Pediatric Immunology Division, Faculty of Medicine, University of Uludağ, Bursa; ^ethe Pediatric Allergy and Immunology Division, Faculty of Medicine, University of Marmara, Istanbul; ^fthe Pediatric Allergy and Immunology Division, Faculty of Medicine, University of Çukurova, Adana; ^gthe Pediatric Immunology Division, Ankara Hematology-Oncology Hospital of Children, Ankara; ^hKök Biotek Company, Technocity of University of Erciyes, Kayseri; ⁱthe Pediatric Hematology and Immunology Division, Faculty of Medicine, University of Erciyes, Kayseri; ^jthe Pediatric Allergy and Immunology Division, Faculty of Medicine, University of 19 Mayıs, Samsun; ^kthe Pediatric Immunology Division, Faculty of Medicine, University of Akdeniz, Antalya; and ^lthe Pediatric Immunology Division, Faculty of Medicine, University of Hacettepe, Ankara.

Supported by TÜBİTAK (The Scientific and Technological Research Council of Turkey), project no. 110S252, and part of the E-Rare program of the European Union.

Disclosure of potential conflict of interest: M. Y. Köker has received grants, support for travel to meetings for the study or other purposes, and travel/accommodation/meeting expenses from TÜBİTAK. Y. Camcıoğlu and A. Metin have received grants from TÜBİTAK. D. Roos has received a grant from the E-Rare program of the European Union. The rest of the authors declare that they have no relevant conflicts of interest. Received for publication November 29, 2012; revised May 10, 2013; accepted for publication May 23, 2013.

Corresponding author: Mustafa Yavuz Köker, MD, PhD, Department of Immunology, Faculty of Medicine, University of Erciyes, Kayseri, Turkey. E-mail: mykoker@erciyes.edu.tr and kokyavuz@hotmail.com.

0091-6749/\$36.00

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http://dx.doi.org/10.1016/j.jaci.2013.05.039

Abbreviations used

AR:	Autosomal recessive
A22 ^R :	A22 CGD with residual oxidase activity
CGD:	Chronic granulomatous disease
DHR:	Dihydrorhodamine-1,2,3
MFI:	Mean fluorescence intensity
NADPH:	Nicotinamide dinucleotide phosphate reduced
NBT:	Nitroblue tetrazolium
phox:	Phagocyte oxidase
PMA:	Phorbol 12-myristate 13-acetate
ROI:	Reactive oxygen intermediate
SI:	Stimulation index
X-CGD:	X-linked recessive chronic granulomatous disease

of the mode of inheritance (X-CGD or AR-CGD).³ The nitroblue tetrazolium (NBT) microscope test is a simple test for diagnosis of CGD but has been largely replaced by flow cytometry. The dihydrorhodamine-1,2,3 (DHR) assay, in addition to being a rapid and sensitive assay for the diagnosis of CGD, can also easily detect carriers of X-linked disease by using flow cytometry.^{6,8} A mosaic of oxidase-positive and oxidase-negative neutrophils in a female member of a family with CGD strongly suggests X-CGD carrier status caused by random inactivation of the paternal or maternal X chromosome in each female cell.^{6,7,9} Carriers in families with autosomal CGD can only be detected by means of mutation analysis.^{7,10} Consanguineous marriage is a risk factor for AR-CGD.⁹⁻¹¹

In the present study we have applied the DHR assay for the diagnosis of CGD and flow cytometric and Western blot analysis with specific antibodies for subgroup detection. On this basis, the *CYBB*, *CYBA*, *NCF1*, and *NCF2* genes were sequenced, and the clinical and molecular characterization of 89 patients with CGD from 73 Turkish families are reported. We compared patients with CGD with regard to the NADPH oxidase activity of the patients' neutrophils and also evaluated the functional, clinical, and molecular spectrum of the disease.

METHODS**Patients**

Between 2002 and 2012, heparinized venous blood samples from 89 patients with CGD and 310 relatives and from 100 healthy control subjects were sent by their physicians from different university hospitals throughout Turkey to the Immunology Laboratory of the University of Erciyes, following their procedures and appropriate informed consent protocols. The study protocol was approved by the Ethical Committee of the University of Erciyes and financially supported by TÜBİTAK (the Scientific and Technological Research Council of Turkey) within Euro-CGD project number 110S252.

Functional analysis of neutrophils

Total leukocytes were isolated from 100 to 200 μ L of blood from healthy control subjects, patients, and relatives by means of lysis of the erythrocytes in the pellet fraction with a nonfixing lysis solution of 155 mmol/L NH_4Cl , 10 mmol/L NaHCO_3 , and 0.1 mmol/L EDTA. Neutrophils were purified from white cells by using standard procedures.¹² The capacity of the neutrophils to generate ROIs was tested both with the NBT slide test and the DHR assay.⁷

The DHR assay was performed (with slight modifications) with total leukocytes, as described by Köker et al.⁶⁻⁸ In this test isolated neutrophils were incubated with DHR, stimulated with phorbol 12-myristate 13-acetate (PMA), and analyzed by means of flow cytometry. The results are shown as the stimulation index (SI; ie, the ratio of the mean fluorescence intensity [MFI] of the

stimulated cells and that of the unstimulated cells). For more details, see the **Methods** section in this article's Online Repository at www.jacionline.org. In cases in which 2 distinct fluorescent populations were observed, the SI for each neutrophil population was calculated.^{6,9}

Subgroup analysis of patients with CGD by using flow cytometry and Western blot analysis

Expression of NADPH oxidase components was analyzed by means of flow cytometry in the Immunology Laboratory of the University of Erciyes, and the results were compared with those after Western blot analysis carried out in the Sanquin Laboratory (Amsterdam, The Netherlands). The tests were performed by using previously defined methods with small modifications of antibodies.^{6,9} We used antibodies specific for gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} from Santa Cruz Biotechnology (Santa Cruz, Calif) for subgroup detection of samples in flow cytometry.

DNA analysis

Genomic DNA was isolated from total blood leukocytes by using standard procedures and analyzed for mutations in exons and exon/intron boundaries of *CYBB*, *CYBA*, *NCF1*, and *NCF2* by means of PCR amplification of each exon with its intronic boundaries, followed by sequence analysis.^{11,13}

Statistical analysis

Statistical analysis was accomplished with the SPSS statistical software package, version 16 (SPSS, Chicago, Ill). Both χ^2 and Fisher exact tests were used to compare differences in percentages for categorical variables. *P* values of less than .05 were considered statistically significant. The Student *t* test (or Mann-Whitney *U* test in nonparametric conditions) was used to compare means for continuous variables. Kaplan-Meier graphs were used to show survival rates.

RESULTS

Eighty-nine patients with clinically suspected CGD (25 female and 64 male patients; median age, 10 years) were enrolled in the study (Table I). The diagnosis of CGD was confirmed by using the NBT test, the DHR assay, or both. Thirty-four (38.2%) patients from 28 families were given a diagnosis of X-CGD because the DHR assay performed in their mothers showed nonfunctional and functional PMN subpopulations indicative of the X-linked recessive carrier state. The remaining 55 (61.8%) patients from 45 families were suspected to have AR-CGD.

In all patients with X-CGD and 20 of the patients with suspected AR-CGD, no expression of either gp91^{phox} or p22^{phox} protein in the leukocytes was detected. Subsequent genetic analysis revealed mutations in *CYBB* in 31 of the 34 patients suspected of having X-CGD (the remaining 3 are still under investigation). Mutations in *CYBA* were found in all 20 patients with suspected AR-CGD without gp91^{phox}/p22^{phox} expression. In 17 of the patients with suspected AR-CGD, there was no p47^{phox} expression (confirmed by means of mutation analysis of *NCF1*). In 13 patients from the suspected AR-CGD group, we found a lack of p67^{phox} expression (confirmed by means of mutation analysis of *NCF2*). Five patients with suspected AR-CGD are still under investigation.

Altogether, in our cohort 31 (34.8%) patients (from 25 families) had X-CGD (gp91^{phox} deficiency), 20 (22.5%) patients (18 families) had p22^{phox} deficiency, 17 (19.1%) patients (9 families) had p47^{phox} deficiency, and 13 (14.6%) patients (13 families) had p67^{phox} deficiency (Table I). Eight (9.0%) patients (8 families) are still under investigation for complete characterization.

The 81 fully characterized patients with CGD belong to 65 families. In 39 of 40 families with proved AR, the parents were

TABLE I. Characteristics of the patients and families classified by CGD subtype

CGD subtypes	Patients, no. (%)	Families, no. (%)	Sex	Age (y)* at diagnosis† (median age)	Deceased (no.)
X-CGD (gp91 ^{phox} defective)	34 (38.2%)	28 (38.4%)	34 M	2.72 ± 0.37 (2)	3 M
AR-CGD	50 (56.2%)	40 (54.7%)	25 M, 25 F	5.16 ± 0.68 (3)	6 (2 M, 4 F)
p22 ^{phox} defective	20 (22.5%)	18 (24.6%)	10 M, 10 F	4.50 ± 0.83 (3.5)	2 (1 M, 1 F)
p47 ^{phox} defective	17 (19.1%)	9 (12.3%)	10 M, 7 F	7.32 ± 1.64 (4)	1 F
p67 ^{phox} defective	13 (14.6%)	13 (17.8%)	5 M, 8 F	3.42 ± 1.21 (2)	3 (1M, 2 F)
Unknown subtypes‡	5 (5.6%)	5 (6.9%)	5 M	5.00 ± 0.56 (4)	—
All CGD cases	89 (100%)	73 (100%)	64 M, 25 F	4.24 ± 0.45 (3)	9 (4 F, 5 M)

F, Female; M, male.

*Data are presented as means ± SEMs.

†For age less than 1 year, a median age of 0.5 years was used for mean calculations in all patients.

‡Suspected AR-CGD with consanguineous families and without a carrier mother in the DHR assay.

related to each other (28 first-degree cousins and 11 more distantly related). In 3 of 28 families with the X-linked mutations, the parents were first-degree cousins. Indeed, homozygous mutations in *CYBA*, *NCF1*, or *NCF2* were found among their children with AR-CGD. There was a significant association between consanguineous marriage and CGD form ($P < .001$); the number of consanguineous marriages among the parents of patients with fully characterized AR-CGD was much higher than in parents of patients with X-CGD (39/40 [97.5%] vs 3/25 [12.0%] families). Eight families have 2 and 4 families have 3 affected members.

At the time of the last assessment or death, the patients ranged in age from 1.5 to 37 years (median age, 10 years); 9 had died. At the time of diagnosis, the median age was higher (4 years) in p47^{phox}-deficient patients and lower (2 years) in the gp91^{phox}-deficient patients when compared with the median age of all patients with CGD (3 years, Table I).

In the X-CGD group neutrophil function was defective in all patients, as determined by using the DHR assay, with a mean SI of 1.07 ± 0.17 (Fig 1 and see Table E1 in this article's Online Repository at www.jacionline.org). Table E1 records the MFIs and SIs in the patient and control groups. The female carriers ($n = 41$) had dual neutrophil populations. Other members ($n = 70$) of families with X-CGD had normal neutrophil DHR assay results. At the time of the last assessment or death, patients with X-CGD ranged in age from 1.5 to 32 years (median age, 8.5 years); 3 had died (see Table E2 in this article's Online Repository at www.jacionline.org). Table E2 records characteristics and mutations in patients with X-CGD.

In the AR-CGD group the mean SI of most of the p22^{phox}- and p67^{phox}-deficient patients was 1.05 ± 0.13 (see Table E1), except in 5 cases: P5, P6, P14, and P15 in the p22^{phox}-deficient and P12 in the p67^{phox}-deficient groups (Fig 1 and see Table E3 in this article's Online Repository at www.jacionline.org). Table E3 records characteristics and mutations in patients with AR-CGD. All patients with a p47^{phox} defect and 5 cases (A22 CGD with residual oxidase activity [A22^R] and A67⁺) had residual NADPH oxidase activity, with an average SI of 5.59 ± 3.06 in the DHR assay (see Table E1). At the time of the last assessment or death, patients with AR-CGD ranged in age from 2.5 to 37 years (median age, 12 years); 6 had died (see Table E3).

Sequence analysis in patients with CGD and carriers

Table E2 shows the mutations found in *CYBB* in the patients with X-CGD, and Table E3 shows the mutations found in *CYBA*, *NCF1*, and *NCF2* in the patients with AR-CGD. All

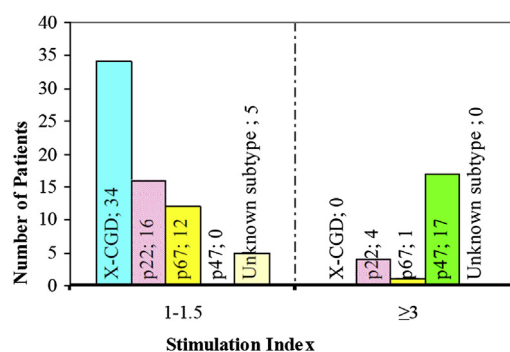


FIG 1. DHR stimulation indices of neutrophils with PMA in CGD subgroups. Patients with SIs of 1.5 or less ($n = 67$) and patients with SIs of 3 or greater ($n = 22$) are shown. The oxidase activity in p22^{phox} and p67^{phox} deficiency is as low as in gp91^{phox} deficiency, except in patients with residual oxidase activity, which have missense mutations in p22^{phox}, p.(Gly24Arg), p.(Ala124Val), p.(Ala125Thr) and in p67^{phox}, p.(Ala202Val) (Table E3).

mothers of patients with X-CGD and 15 other female relatives of patients with X-CGD were found to be heterozygous carriers for the mutations in their related patients. These results fully correlated with their carrier status in the DHR assay (see Table E2). All investigated parents of patients with AR-CGD were heterozygous carriers of the mutations found in their children.

Residual NADPH oxidase activity and patients

The mean DHR SI indicating the residual ROI production capacity of the neutrophils of p47^{phox}-deficient patients was 5.6 (range, 3-12; see Table E3), which is approximately 5 times higher than the mean SI of patients with the X91⁰, A22⁰, and A67⁰ phenotypes (SI ≈ 1.06 ; see Table E3). We also found a higher residual ROI production in 5 cases with missense mutations (4 patients with A22^R phenotype, p.[Gly24Arg], p.[Ala124Val], p.[Ala125Thr] and 1 patient with A67⁺ phenotype, p.[Ala202Val]; Fig 1 and see Table E3). The SIs of neutrophils were grouped into 1.5 or less ($n = 67$, oxidase-null) and 3 or greater ($n = 22$, oxidase-residual). The mean age at diagnosis of patients with SIs of 1.5 or less was 3.3 years, whereas the mean age of patients with SIs of 3 or greater was 7.1 years ($P < .001$, Fig 2 and Table II).

Medical history of patients

The age at onset of clinical symptoms in most of the patients with X-CGD ($n = 28$ [82.4%]) and in 50% of the patients with

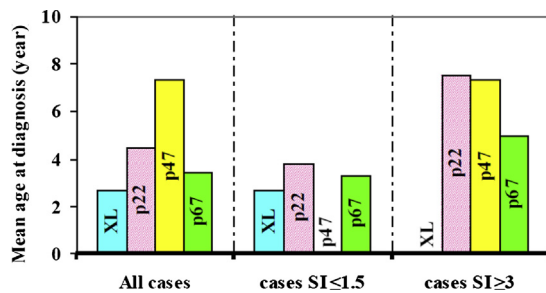


FIG 2. Mean age at diagnosis in CGD subtypes in relation to SIs. Cases with SIs of 1.5 or less (oxidase-null) have a lower mean age at laboratory diagnosis (3.3 years) than cases with SIs of 3 or greater (oxidase-residual; 7.1 years, $P < .001$, Table II). XL, X linked.

TABLE II. Mean age at laboratory diagnosis in CGD subtypes in relation to residual oxidase activity

Mean age at laboratory diagnosis (y)	All cases	X-CGD	P value	AR-CGD	A22	A47	A67
All cases	4.2	2.7	<.001	5.2	4.5	7.3	3.4
SI ≤ 1.5 cases*	3.3	2.7	.01	3.6	3.8	—	3.3
SI ≥ 3.0 cases†	7.1	—	—	7.2	7.5	7.3	5

*Including oxidase-null cases (X91⁰, A22⁰, and A67⁰ phenotypes).

†Including oxidase-residual cases (A47⁰, A22^R, and A67⁺ phenotypes).

AR-CGD was in the first year of life. The age of laboratory diagnosis was grouped into 1 or less, 1 to 5 or less, 5 to 10 or less, and greater than 10 years (Fig 3). Most of the oxidase-null patients (55/67 [82%]) were given a diagnosis at 5 years of age or earlier. In oxidase-residual patients this proportion with early diagnosis (12/22 [55%]) was significantly lower ($P < .001$, Fig 3). The mean age at laboratory diagnosis in patients with the X91 phenotype (2.7 years) was similar to those of patients with the A22 (3.8 years) and A67 (3.3 years) phenotypes with an SI of 1.5 or less (Table II) and were significantly lower than the mean age of patients with the A47 phenotype (7.3 years; $P < .001$; Fig 2 and Table II).

BCG complications. In the second month of life, all patients received BCG vaccine containing a live attenuated form of *Mycobacterium bovis*, with the exception of 8 patients with an affected sibling in their families. BCG-itis, a severe lymphadenopathy after tuberculosis vaccination, occurred in 22.5% of our patients; 13 of them (14.6%; 4 with X-CGD and 9 with AR-CGD [2 with A22⁰, 1 with A22^R, 3 with A47⁰, and 3 with A67⁰ phenotypes]) had clinical symptoms resembling tuberculosis and were given antituberculosis therapy. There was no difference in the occurrence of BCG-itis with regard to sex or oxidase activity or between the AR-CGD and X-CGD groups.

Clinical presentation. The most common presenting finding was pneumonia (the main causative agents were fungi, especially *Aspergillus* species), which was detected in 90% of the patients, followed by suppurative lymphadenitis, which was detected in 76% of patients; deep-seated abscesses in the lung, liver, kidney, and intra-abdominal tissues (the main responsible agents were *Aspergillus* and *Staphylococcus* species) were detected in 72% of the oxidase-null patients (Table III).¹⁴ The incidence of these severe infections was significantly less in oxidase-residual patients: 64% ($P = .02$), 32% ($P < .001$), and 33% ($P < .001$), respectively. However, cutaneous/subcutaneous

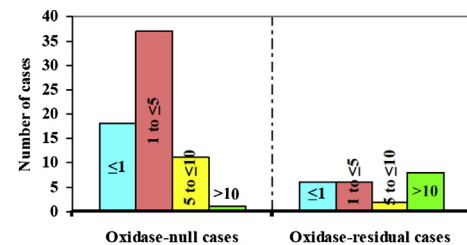


FIG 3. Number of patients with CGD and the age at laboratory diagnosis in relation to residual oxidase activity. The age at laboratory diagnosis was grouped into 1 or less, 1 to 5 or less, 5 to 10 or less, and greater than 10 years. The percentage of oxidase-null patients ($n = 55$ [82%]) given a diagnosis at 5 years of age or earlier was higher than that of oxidase-residual patients ($n = 12$ [55%]).

TABLE III. Incidence of severe* infections in patients with CGD

Infections	Oxidase-null patients (n = 67)	Oxidase-residual patients (n = 22)	P value	Hazard ratio (95% CI)
Pneumonia	90%	64%	.02	0.05-0.47
Lymphadenitis	76%	32%	<.001	0.22-0.66
Liver/deep-seated abscess	72%	33%	<.001	0.18-0.62
Cutaneous/subcutaneous infections	39%	63%	.04	0.48-0.02
Osteomyelitis	25%	18%	.46	0.12-0.26
Sepsis	31%	27%	.71	0.18-0.26
Enteric infections	25%	14%	.20	0.06-0.30
Urinary tract infections	25%	13%	.20	0.06-0.29
Otitis media	21%	27%	.56	0.27-0.15
Perianal abscess	22%	14%	.33	0.09-0.26
Brain abscess	18%	14%	.62	0.13-0.21

Results as a percentage of patients with at least 1 severe infection.

*An infection was considered severe when hospital admission and intravenous antimicrobial treatment, surgical intervention, or both was required.¹⁴

infections were more prevalent in oxidase-residual patients (Table III, $P = .04$).

Ongoing disease with CGD. Two patients with the X91⁰ phenotype and 2 patients with the A22⁰ phenotype (age >7 years) had a history of selective IgA deficiency, with levels of less than 6.67 mg/dL. Two cases with the A22⁰ and A22^R phenotypes had hypogammaglobulinemia. The case with A22^R phenotype was accompanied by sensorineural deafness; this patient has a deaf sister. One patient had X-CGD with growth hormone deficiency, 1 patient with the A22⁰ and 1 patient with the A67⁰ phenotypes had chronic idiopathic thrombocytopenic purpura, and 1 patient with the A67⁰ had Behçet disease, with no other family members with these diseases. Additionally, 1 patient with the A67⁰ phenotype had cor pulmonale, and granuloma formation of the stomach occurred in 2 patients with the X91⁰ phenotype and 1 patient with the A22⁰ phenotype. Seborrheic dermatitis in patients with the A67⁰ and A67⁺ phenotypes was also encountered.

Chronic manifestations of CGD. The most common chronic manifestation in patients with CGD was anemia caused

TABLE IV. Chronic manifestations of patients with CGD

Symptoms*	Our cohort results				Wolach et al (n = 38) ¹⁷	United States (n = 550) ¹⁵	Jones et al (n = 94) ¹⁶
	Oxidase-null patients (n = 67)	Oxidase-residual patients (n = 22)	P value	Hazard ratio (95% CI)			
Anemia	63%	64%	.94	0.24-0.22	74%	NM	NM
Lymphadenopathy	67%	72%	.62	0.27-0.16	68%	98%	40%
Failure to thrive	58%	27%	.01	0.09-0.52	50%	50%	75%
Chronic lung disease	58%	18%	<.001	0.20-0.60	29%	NM	77%
Hepatomegaly	39%	23%	.13	0.05-0.34	32%	50%-90%	NM
Splenomegaly	36%	18%	.08	0.02-0.37	29%	60%-80%	NM
Stomatitis/gingivitis	49%	23%	.14	0.05-0.48	29%	15%-50%	24%

NM, Not mentioned.

*Results are percentages of patients with chronic manifestation.

by chronic infection (63% in oxidase-null and 64% in oxidase-residual patients). Other manifestations were lymphadenopathy (67%) and stomatitis (49%) in oxidase-null patients and lymphadenopathy (72%) and stomatitis (23%) in oxidase-residual cases. Failure to thrive was noticed significantly more (58%) in oxidase-null patients than in oxidase-residual patients (27%; $P = .01$, Table IV).¹⁵⁻¹⁷ Chronic lung disease was also significantly more frequent (58%) in oxidase-null patients than in oxidase-residual patients (18%; $P < .001$, Table IV).

Autoimmune phenomena. Autoimmune/autoinflammatory complications were found in nearly half of the patients. Among them, 4 patients with the X91⁰ phenotype and 1 patient with the A22⁰ phenotype had inflammatory bowel disease (IBD). Twenty patients with X-CGD and 18 patients with AR-CGD (4 with p47⁰, 5 with p22⁰, 1 with p22^R, and 8 with A67⁰ phenotypes) had stomatitis/gingivitis, and 3 patients with oxidase-null AR-CGD had reactive arthritis. Familial Mediterranean fever symptoms and uveitis occurred in 1 patient with the A67⁺ phenotype. Idiopathic thrombocytopenia (1 patient with the A22⁰ and 1 patient with the A67⁰ phenotypes), pericardial effusions without infective etiology (1 patient with the X91⁰ and 1 patient with the A67⁰ phenotypes) and autoimmune hepatitis (2 patients with the X91⁰ phenotype) were the other autoimmune disorders that occurred in our cohort.

Infecting microorganisms and therapies. Among microorganisms that were isolated in 63 patients with severe infections, fungal infections were the most common and the leading cause of death (78%). *Aspergillus fumigatus* was the most common cause of pulmonary invasive aspergillosis (24 cases; 1 patient with the A22⁰ phenotype with *Aspergillus flavus* [leading to paraplegia caused by vertebral abscesses] and 1 patient with the X91⁰ phenotype with *Aspergillus nidulans* [with vertebral abscesses]) and of brain abscesses (6 oxidase-null and 1 oxidase-residual cases, of which 5 required surgical removal). *Candida albicans* was detected in 5 patients with pneumonia.

In particular, among the bacterial infections, *Mycobacterium tuberculosis* caused tuberculosis in 4 patients with X-CGD and 5 patients with AR-CGD. *Staphylococcus aureus* was the most commonly isolated organism from patients with suppurative adenitis and deep skin infections (18 cases). *S. aureus* was also isolated from a liver abscess (6 cases) and pneumonia (3 cases). Other microorganisms involved were *Escherichia coli* (urine, 3 cases), *Salmonella* species (liver and bone, 5 cases), *Serratia marcescens* (osteomyelitis and sepsis in 2 patients with the X91⁰ phenotype), and *Klebsiella* species (1 patient with the A67⁰ phenotype with furuncle and pneumonia). Another uncommon pathogen was

Echinococcus granulosus in a patient with the A22^R phenotype with a liver hydatid cyst.

Treatment. After diagnosis, all patients were started on prophylaxis with trimethoprim-sulfamethoxazole and itraconazole. In severe cases IFN- γ was added to the treatment protocol. Before the initiation of prophylaxis, most of the patients (90%) had at least 1 admission history caused by infections before 3 years of age (median diagnostic age) in oxidase-null and 4 years of age in oxidase-residual patients. Six patients (3 with X91⁰, 2 with A22⁰, and 1 with A67⁰ phenotypes) underwent successful stem cell transplantation from fully matched related donors and are doing well now. No patients in this cohort have been treated with gene therapy.

Cause of death and survival rate

Nine patients with CGD, including 6 patients with AR-CGD (3 with A67⁰, 2 with A22⁰, and 1 with A47⁰ phenotypes) and 3 patients with the X91⁰ phenotype (Table I), died during follow-up because of sepsis caused by *Aspergillus* infections in the major organs (brain and lung). The mean age at diagnosis in deceased patients was 2.0 ± 1.5 years, and the mean age at death 10.8 ± 6.7 years. The age range of all deceased patients was 3 to 24 years. Residual oxidase activity was absent, except from 1 patient with the A47⁰ phenotype with an SI of 4 from a low socioeconomic region; she died from *Aspergillus* species-induced pneumonia at the age of 12 years. Overall survival probability in our cohort was 0.63 in a 20-year interval. The survival probability was 0.93 in the oxidase-residual group and 0.38 in the oxidase-null group ($P = .026$, see the left lower survival graphs of Fig 4). Retrospectively, in our cohort most of the deceased family members were male, and death history in members of families with oxidase-null patients was 23 of 59 families and 3 of 14 families in oxidase-residual patients ($P = .17$).

DISCUSSION

In the present study we investigated the inheritance mode and clinical expression of CGD in Turkey. In contrast to previous reports from the United States, Europe, and Japan, where X-CGD is more prevalent,^{14-16,18-20} AR inheritance is the predominant form in Turkey, which might be explained by the high rate of consanguinity.

In the current CGD cohort, with an additional 45 new patients since the previous report,^{7,10,13} the ratio of patients with X-CGD to patients with AR-CGD has increased because 20 patients from the large city of Istanbul have now been included in the study, and

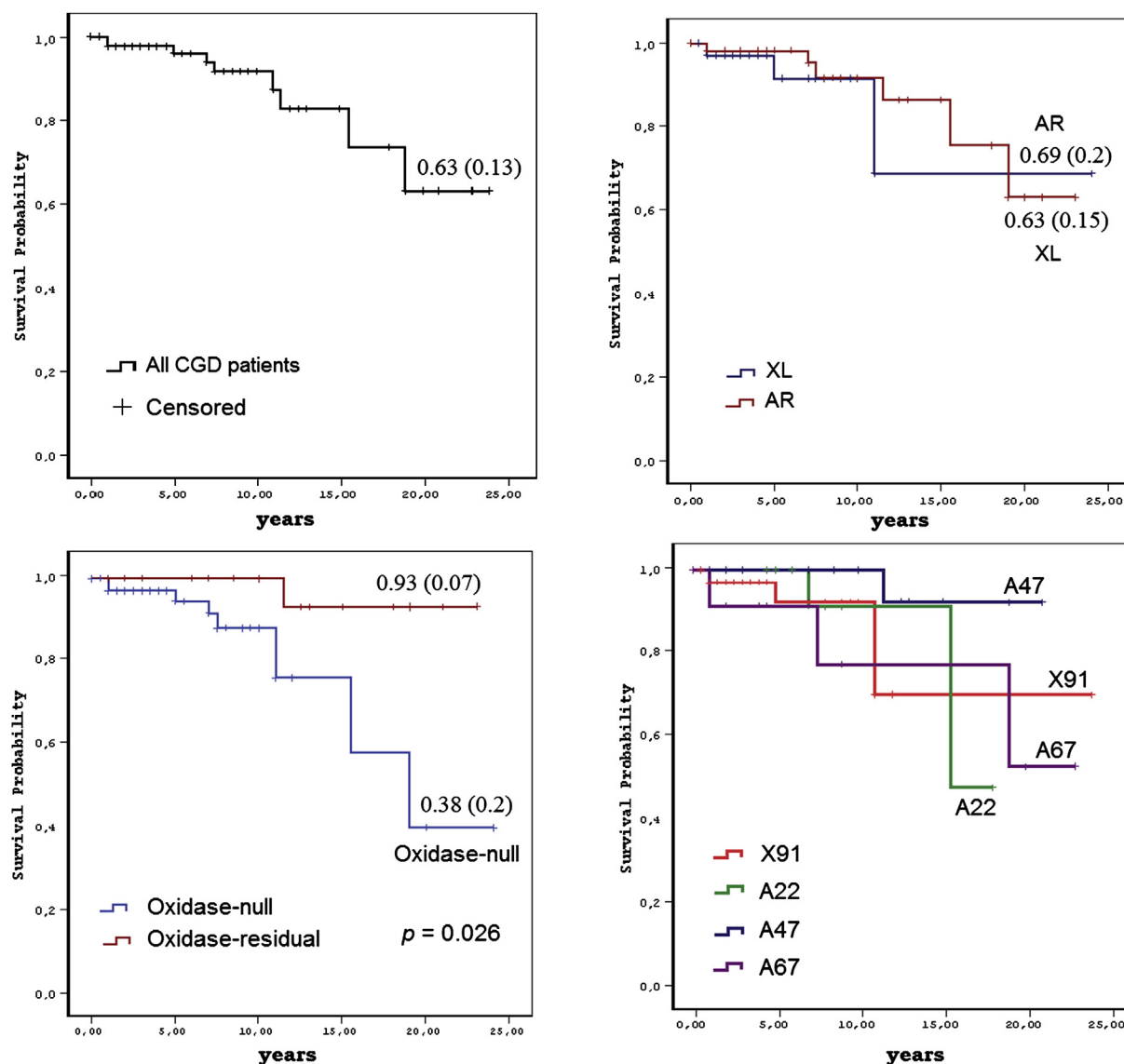


FIG 4. Kaplan-Meier graphs showing the survival rate of patients with CGD. Survival curves after diagnosis of CGD (from upper left to lower right) for the entire cohort of patients, for patients with X-linked and AR disease, for patients with or without residual NADPH oxidase activity, and for all CGD subtypes are shown. There was a difference between groups with or without residual NADPH oxidase activity ($P = .026$).

half of them had the X-CGD subtype. This high percentage is probably due to early diagnostic facilities and the availability of modern health services to newborns in that city. In our first study with a small number of families, the percentage of X-CGD was approximately 33%⁷; now it is 38.2% (Table I). A second factor is the lower percentage of consanguineous marriages in large cities compared with rural areas, which might also have a decreasing effect on the AR-CGD prevalence.

The onset of the disease was earlier in patients with X-CGD compared with those with AR-CGD. All of our patients with X-CGD manifested their first infections very early in life, usually during the first year. Likewise, the mean age of diagnosis was lower in the patients with X-CGD compared with those with AR-CGD (2.7 vs 5.2 years). These findings reflect a delay in clinical presentation of the AR form of the disease. A similar result was also found in the US registry, where the mean age at

diagnosis was 3 years in patients with X-linked disease compared with 7.8 years in those with the AR form.¹⁵ Likewise, in a large European study the mean age at diagnosis was lower in patients with X-CGD versus AR-CGD (4.9 vs 8.8 years).⁴ Similar findings were also reported in other studies.^{14-19,21} The basic difference in our results from those reported is the age at diagnosis of patients with AR-CGD (5.2 years, Table I), which was lower than in the European and US registries, probably because of the higher percentage of patients with the A22 (22.5%) and A67 (14.6%) phenotypes with no residual NADPH oxidase activity and the lower percentage of patients with CGD with residual oxidase activity (p47^{phox} deficient, 19.1%) in our cohort in comparison with 30% of p47^{phox}-deficient CGD cases in other studies. Our cohort showed that the mean ages at laboratory diagnosis in patients with the X91^{phox} phenotype (2.7 years) were similar to those of oxidase-null patients with AR-CGD (3.6 years) and

were significantly lower than the mean age of patients with the A47^{phox} phenotype (7.3 years) and that of all oxidase-residual cases (7.2 years, $P < .001$, Table II). With the availability of better CGD diagnostic services, p47^{phox}-deficient, mild clinical cases might be diagnosed earlier. The percentage of p22^{phox}-deficient patients in Turkey (22.5%) is greater than the worldwide rate of 5% of all CGD cases (Table I).^{5,15} The reason for this difference is not clear; a founder effect, as in Korea,²² is not likely because the Turkish patients show a heterogeneous genetic background (see Table E3).

Iranian investigators⁹ reported that p22^{phox} deficiency causes more severe clinical symptoms than other autosomal forms of CGD, possibly because of the interaction of this membrane subunit with the catalytic gp91^{phox} subunit of the NADPH oxidase. However, we demonstrate that the severity of the disease is related to residual ROI production capacity, which is similar in patients with the X91⁰, A22⁰, and A67⁰ phenotypes (Table III). Indeed, when we compare all patients with CGD with respect to the residual ROI production of neutrophils, there is a significant correlation ($P < .001$) between null oxidase activity ($SI \leq 1.5$) and early clinical presentation (Fig 3) and younger age at laboratory diagnosis (Fig 2). The SI in the DHR assay was about 5-fold higher in our oxidase-residual patients than in the oxidase-null patients with A91⁰, A22⁰ or A67⁰ phenotypes (see Table E1). Therefore residual ROI production is an important parameter affecting clinical course, in line with the study of Kuhns et al,³ although these authors only scored survival and gastrointestinal symptoms. We confirmed the relation between residual NADPH oxidase activity and survival (Fig 4).³ In our cohort severe infections, such as pneumonia, suppurative lymphadenitis, and deep-seated abscesses, were detected significantly more frequently in the oxidase-null patients than in the oxidase-residual patients ($P = .02$, $P < .001$, and $P < .001$; Table III). Furthermore, chronic manifestations, such as chronic lung disease and failure to thrive, were also seen significantly more in oxidase-null patients than in oxidase-residual patients (Table IV), which might be due to repeated infection histories.

The most common clinical manifestation of CGD in our patients was pulmonary system involvement, which is in accordance with previous reports.^{4,9,14,15,17,21} In our study the lymphoreticular system was the second most common site of infection, which is in line with some,¹⁴ but not all,¹⁶⁻¹⁸ previous reports, followed by skin involvement in oxidase-residual patients. In oxidase-null patients the lymphoreticular system involvements were followed in frequency by deep-seated/liver abscess. BCG-itis, a severe lymphadenopathy caused by vaccination of tuberculosis, occurred in 20% of our patients. Although all newborns in Turkey are routinely immunized with BCG at birth in accordance with the national vaccination program, the rate of BCG-itis in our cohort is lower than the Iranian rate (56%)⁹; perhaps the differences are related to the nature of tuberculosis (BCG strain) vaccine or other factors, such as dosage of vaccine and time of vaccination.

In conclusion, our experience with 89 patients with CGD demonstrates that later clinical presentation and older age at diagnosis are related to the residual NADPH oxidase activity of the neutrophils. Oxidase-null patients with the X91⁰, A22⁰, and A67⁰ phenotypes are more likely to have severe infections than those with residual oxidase activity (A47⁰, A22^R, and A67⁺ phenotypes). Our findings provide insight into the differences between CGD forms with respect to the pattern and severity of the disease. In this way our findings might help to predict

outcomes and thereby increase awareness and help optimize treatment in these patients. These findings can be also useful in the genetic counseling of families with an affected child.

We thank Dr Demet Hafizoğlu (University of Uludağ) and Dr Safa Barış (University of Marmara) for organization of patients and Berkay Saraymen (University of Erciyes) for kindly helping us with the laboratory experiments of the samples. We thank Dr Ferhan Elmalı (University of Erciyes) for statistical analysis.

Key messages

- Oxidase-null patients with CGD of the X91⁰, A22⁰, or A67⁰ phenotypes are more likely to have severe infections than those with the A47⁰ phenotype.
- Later and less severe clinical presentation and older age at diagnosis of CGD are related to the residual NADPH oxidase activity of neutrophils.

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METHODS

DHR assay

In this test isolated neutrophils were incubated for 15 minutes with DHR and stimulated with PMA. This process was analyzed by means of flow cytometry in which the neutrophil population was identified by its typical pattern of forward and side scatter and selected by gating, without including dead or apoptotic cells. Analysis of control cells was always included. The dihydrorhodamine fluorescence is shown as the SI (ie, the ratio of the [geometric] mean [channel] fluorescence intensity of the stimulated cells and the MFI of the unstimulated cells). At least 10,000 cells were analyzed for each test. MFI was measured as arbitrary units. Samples were analyzed in the FITC channel of the BD FACSCanto II (Becton Dickinson Biosciences, San Diego, Calif) flow cytometric device. FACSDiva software 6.1.2 was used for evaluation. The mean MFI was 163 ± 13.7 in unstimulated control samples. After PMA stimulation, at least 50-fold increases are expected in normal neutrophil oxidase activity.

The dihydrorhodamine fluorescence and SI data were experimentally (empirically) separated into 2 distinct subpopulations on the basis of fluorescence intensity or SI; these subpopulations were called oxidase-null patients (MFI <250 AU or SI ≤ 1.5) and oxidase-residual patients (MFI ≥ 500 AU or SI ≥ 3).

We prefer using SIs for defining the functional capacity of neutrophils. Our laboratory experience is that the samples collected from different patients with CGD could not be analyzed on the same day. Therefore the MFI of a sample might be affected in each experiment by using flow cytometric device calibrations and compensation adjustment. Furthermore, other factors, such as gating strategy of neutrophils, the amount of PMA added for the stimulation, time interval for incubation and analysis of fresh 1- or 2-day-old blood samples might affect the MFI. In contrast, the SI is a ratio, and therefore unwanted fluorescence effects on the MFI are mostly eliminated. The variation

in baseline fluorescence (in the absence of PMA) might affect this ratio (SI), but we use catalase for inhibiting carryover of hydrogen peroxide from one cell to another, thus decreasing the baseline activity in each experiment. Our flow cytometric gating strategy mostly eliminates all nonresponding dead or apoptotic cells. Thus we are confident that the SI is a reliable way of presenting our data. A comparison between the MFI at rest and after PMA stimulation with SIs for all patients is shown in Table E1.

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TABLE E1. MFIs and SIs in the patient and control groups

Sample groups	Mean MFI $\pm$ SD unstimulated samples (AU)	Mean MFI $\pm$ SD PMA-stimulated samples (AU)	*Mean SI $\pm$ SD PMA-stimulated/unstimulated
Control (n = 100)	163 $\pm$ 13.7	11,228 $\pm$ 2,453	66 $\pm$ 10
Patients with X-CGD (n = 34)	152 $\pm$ 12.6	164 $\pm$ 30.8	1.07 $\pm$ 0.17
AR-CGD without residual activity (n = 33)	156 $\pm$ 11.7	165 $\pm$ 27.0	1.05 $\pm$ 0.13
AR-CGD with residual activity (n = 22)	157 $\pm$ 10.2	885 $\pm$ 485	5.59 $\pm$ 3.06
CGD without residual activity (n = 67)	154 $\pm$ 12.0	164 $\pm$ 30.0	1.06 $\pm$ 0.15

AU, Arbitrary unit; MFI, mean fluorescence intensity.

*The SI value is calculated according to MFI data from each patient.

TABLE E2. Characteristics and mutations in patients with X-CGD

Patient no. (family no.)	Age at diagnosis	f-Age	DHR SI	Subtype	Mutation	Nucleotide change in CYBB gene	Exon	Amino acid change in gp91 ^{phox}	Carrier*	Carrier NN (%)	Reference
P1 (F1)	<1	5	1	X91 ^O	Nonsense	c.83G>A	2	p.[Trp28X]	CM	53%	E1
P2 (F2)	1.5	5	1	X91 ^O	Splice site	c.141+2T>C	2	p.[Leu16_Gly47del]	CM	28%	Unpublished
P3 (F3)	3.5	9	1	X91 ^O	Nonsense	c.217C>T	3	p.[Arg73X]	CM	35%	E1
P4 (F4)	8	32	1	X91 ^O	Nonsense	c.271C>T	4	p.[Arg91X]	CM	50%	E1
P5 (F5)	8	9	1	X91 ^O	Splice site	c.338-1G>A	del5	p. [Ala113GlufsX3]	CM	80%	Unpublished
P6 (F6)	2	3 (deceased)	1	X91 ^O	Deletion	c.450_451delAA	5	p.[Ser151fsX13]	CM, CS	67%, 50%	E1
P7 (F7)	2	5	1	X91 ^O	Nonsense	c.469C>T	5	p.[Arg157X]	CM	60%	E1
P8 (F8)	2	4	1	X91 ^O	Nonsense	c.469C>T	5	p.[Arg157X]	CM	17%	Unpublished
P9 (F9)	1	9	1	X91 ^O	Nonsense	c.676C>T	7	p.[Arg226X]	CM	60%	E1
P10 (F10)	2	4	1	X91 ^O	Nonsense	c.676C>T	7	p.[Arg226X]	CM	40%	Unpublished
P11 (F10)	<1	3	1	X91 ^O	Nonsense	c.676C>T	7	p.[Arg226X]	CM	40%	Unpublished
P12 (F11)	3	11	1	X91 ^O	Nonsense	c.700G>T	7	p.[Glu234X]	CM, CS, CG	53%, 82%, 65%	E2
P13 (F12a)	2	7 (deceased)	1	X91 ^O	Nonsense	c.736C>T	7	p.[Gln246X]	CM, CG, CGG	80%, 66%, 38%	E2
P14 (F12b)	3	15	1	X91 ^O	Nonsense	c.736C>T	7	p.[Gln246X]	CM, CG, CMA	50%, 38%, 66%	E2
P15 (F12b)	1	12	1	X91 ^O	Nonsense	c.736C>T	7	p.[Gln246X]	CM, CG, CMA	50%, 38%, 66%	E2
P16 (F13)	1	1.5	1	X91 ^O	Insertion	c.742dupA	7	p.[Ile248AsnfsX36]	CM	37%	Unpublished
P17 (F14)	3	10	1	X91 ^O	Nonsense	c.868C>T	8	p.[Arg290X]	CM, CS	28%, 50%	Unpublished
P18 (F15)	6	10	1	X91 ^O	Missense	c.897G>T	8	p.[Lys299Asn]	CM	65%	Unpublished
P19 (F16)	<1	8	1	X91 ^O	Missense	c.1012C>G	9	p.[His338Asp]	CM	25%	Unpublished Novel
P20 (F17)	3	13	1	X91 ^O	Missense	c.1016C>A	9	p.[Pro339His]	CM	16%	E1
P21 (F17)	1	10	1	X91 ^O	Missense	c.1016C>A	9	p.[Pro339His]	CM	16%	E1
P22 (F18)	<1	3	1	X91 ^O	Missense	c.1031C>T	9	p.[Ser344Phe]	CM	64%	Unpublished
P23 (F19)	7	16	1	X91 ^O	Deletion	c.1038delT	9	p.[Glu347ArgfsX39]	CM	10%	E1
P24 (F20)	<1	5	1.5	X91 ^O	Missense	c.1235G>A	10	p.[Gly412Glu]	CM, CS	30%, 70%	Unpublished
P25 (F20)	2	11	1.5	X91 ^O	Missense	c.1235G>A	10	p.[Gly412Glu]	CM, CS	30%, 70%	Unpublished
P26 (F21)	3	14 (deceased)	1.4	X91 ^O	Splice site	c.1315-1G>T	del11	p.[Ile439_Gln487del]	CM	70%	E1
P27 (F21)	<1	10	1.4	X91 ^O	Splice site	c.1315-1G>T	del11	p.[Ile439_Gln487del]	CM	70%	E1
P28 (F22)	1.5	7	1	X91 ^O	Deletion	c.1528_1529delTT	12	p.[Leu510ValfsX8]	CM	40%	E1
P29 (F23)	<1	2.5	1	X91 ^O	Deletion	CYBB del	1-13	NR	CM, CMA	66%, 58%	Unpublished
P30 (F24)	1.5	3	1	X91 ^O	Deletion	CYBB del	1-13	NR	CM, CS	50%, 32%	Unpublished
P31 (F25)	2	2.5	1	X91 ^O	Deletion	CYBB del	1-13	NR		30%	Unpublished
P32 (F26)	4	5	1	X91 ^O	—	?	—	—	CM	58%	Unpublished
P33 (F27)	7	15	1.5	X91 ^O	—	?	—	—	CM, CS	57%, 51%	Unpublished
P34 (F28)	8	16	1	X91 ^O	—	?	—	—	CM	60%	Unpublished
Control subjects	10-40		56-76	—	—	Wild-type	—	Wild-type	—	—	—

CM, Carrier mother; CS, carrier sister; CG, carrier grandmother; CGG, carrier great grandmother; CMA, carrier maternal aunt; f-Age, age at final follow-up or death; NN, percentage of normal neutrophils; NR, not relevant; P, patient.

*Genetically confirmed.

TABLE E3. Characteristics and mutations in patients with AR-CGD

Patient no. (family no.)	Sex	Age at diagnosis	f-Age	DHR SI	Subtype	Mutation	Nucleotide change in <i>CYBA</i> gene	Exon	Amino acid change in p22 ^{phox}	Reference
A22 group										
P1 (F1)	F	2	10	1	A22 ^O	Splice site	c.58+2T>G	int1	ND	Unpublished Novel
P2 (F2)	F	3	11	1	A22 ^O	Deletion	c.58+4_+7delAGTG	int1	p.[Ile20SerfsX77]	E3
P3 (F3)	F	3	12	1	A22 ^O	Deletion	c.58+4_+7delAGTG	int1	p.[Ile20SerfsX77]	E3
P4 (F4)	M	1	3	1	A22 ^O	Deletion	c.58+4_+7delAGTG	int1	p.[Ile20SerfsX77]	Unpublished
P5 (F5)	F	3	21	4	A22 ^R	Missense	c.70G>A	2	p.[Gly24Arg]	E3
P6 (F6)	F	4	10	4	A22 ^R	Missense	c.70G>A	2	p.[Gly24Arg]	E3
P7 (F7)	M	3	6	1	A22 ^O	Missense	c.74G>A	2	p.[Gly25Asp]	Unpublished Novel
P8 (F8)	M	<1	16 (deceased)	1	A22 ^O	Insertion	c.166dupC	3	p.[Arg56ProfsX157]	E3
P9 (F9)	M	3	10	1	A22 ^O	Insertion	c.166dupC	3	p.[Arg56ProfsX157]	E3
P10 (F10)	F	3	10 (deceased)	1	A22 ^O	Insertion	c.166dupC	3	p.[Arg56ProfsX157]	E3
P11 (F10)	M	<1	5	1	A22 ^O	Insertion	c.166dupC	3	p.[Arg56ProfsX157]	E3
P12 (F11)	F	10	15	1	A22 ^O	Splice site	c.369+1G>A	6	p.[Leu97ArgfsX68]	E4
P13 (F12)	F	6	15	1.5	A22 ^O	Nonsense	c.339C>A	5	p.[Cys113X]	E3
P14 (F13)	F	18	31	5	A22 ^R	Missense	c.371C>T	6	p.[Ala124Val]	E4
P15 (F14)	M	6	12	3	A22 ^R	Missense	c.373G>A	6	p.[Ala125Thr]	Unpublished
P16 (F15)	M	4	12	1	A22 ^O	Deletion	c.408delC	6	p.[Lys137SerfsX54]	E3
P17 (F16)	M	5	12	1	A22 ^O	Deletion	c.408delC	6	p.[Lys137SerfsX54]	E3
P18 (F17)	F	6	8	1	A22 ^O	Deletion	Exon 1 del	—	ND	Unpublished
P19 (F17)	M	4	6	1	A22 ^O	Deletion	Exon 1 del	—	ND	Unpublished
P20 (F18)	M	6	11	1	A22 ^O	Deletion	Large deletion	3-6	ND	E3
A47 group										
						<i>NCF1</i> gene	p47 ^{phox}			
P1(F1)	M	4	17	3	A47 ^O	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	E2
P2(F1)	M	2	9	3	A47 ^O	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	E2
P3(F2)	M	15	25	3	A47 ^O	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	Unpublished
P4(F3)	M	16	19	3	A47 ^O	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	Unpublished
P5 (F3)	F	12	14	3	A47 ^O	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	Unpublished
P6 (F4)	F	16	17	5	A47 ^O	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	Unpublished
P7 (F5a)	M	1	16	6	A47 ^O	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	Unpublished
P8 (F5b)	M	16	37tw	11	A47 ^O	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	Unpublished
P9 (F5b)	M	16	37tw	12	A47 ^O	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	Unpublished
P10 (F6)	M	1	20	5	A47 ^O	Splice site	c.574G>A	6	p.[Asp151_Alal227del]	E5
P11 (F7)	F	2	15	4	A47 ^O	Splice site	c.574G>A	6	p.[Asp151_Alal227del]	E5
P12 (F7)	F	<1	12 (deceased)	4	A47 ^O	Splice site	c.574G>A	6	p.[Asp151_Alal227del]	E5
P13 (F7)	F	<1	9	4	A47 ^O	Splice site	c.574G>A	6	p.[Asp151_Alal227del]	E5
P14 (F8)	M	10	25	5	A47 ^O	Nonsense	c.579G>A	7	p.[Trp193X]	Unpublished
P15 (F8)	F	<1	13	5	A47 ^O	Nonsense	c.579G>A	7	p.[Trp193X]	Unpublished
P16 (F8)	F	11	26	9	A47 ^O	Nonsense	c.579G>A	7	p.[Trp193X]	Unpublished
P17 (F9)	M	1	16	10	A47 ^O	Nonsense	c.579G>A	7	p.[Trp193X]	E2
					Comp. het	Deletion	c.75_76delGT	2	p.[Tyr26HisfsX26]	—
A67 group										
						<i>NCF2</i> gene	p67 ^{phox}			
P1 (F1)	M	<1	5	1	A67 ^O	Nonsense	c.229C>T	3	p.[Arg77X]	E6
P2 (F2)	F	2	3 (deceased)	1	A67 ^O	Nonsense	c.229C>T	3	p.[Arg77X]	Unpublished
P3 (F3)	F	4	24	1	A67 ^O	Missense	c.279C>G + intron4+1G>C	4	p.[Asp93Glu]	E6
P4 (F4)	F	2	6	1	A67 ^O	Missense	c.279C>G + intron4+1G>C	4	p.[Asp93Glu]	E6
P5 (F5)	F	3	7	1	A67 ^O	Missense	c.279C>G + intron4+1G>C	4	p.[Asp93Glu]	E6
P6 (F6)	M	<1	8 (deceased)	1	A67 ^O	Missense	c.279C>G + intron4+1G>C	4	p.[Asp93Glu]	Unpublished
P7 (F7)	F	17	17	1	A67 ^O	Missense	c.279C>G + intron4+1G>C	4	p.[Asp93Glu]	Unpublished
P8 (F8)	F	5	24 (deceased)	1	A67 ^O	Nonsense	c.304C>T	4	p.[Arg102X]	E4
P9 (F9)	F	2	11	1.2	A67 ^O	Missense	c.409T>A	5	p.[Trp137Arg]	E4
P10 (F10)	M	5	12	1.3	A67 ^O	Nonsense	c.410G>A	5	p.[Trp137X]	Unpublished Novel
P11 (F11)	M	<1	2.5	1	A67 ^O	Nonsense	c.410G>A	5	p.[Trp137X]	Unpublished Novel
P12 (F12)	F	2	25	12	A67 ⁺	Missense	c.605C>T	6	p.[Ala202Val]	E6
P13 (F13)	M	1	11	1.2	A67 ^O	Insertion	c.767_768dupAA	9	p.[Glu257LysfsX15]	Unpublished

(Continued)

TABLE E3. (*Continued*)

Unknown group									
P1 (F1)	M	4	5	1		—		—	
P2 (F2)	M	10	15	1		—		—	
P3 (F3)	M	3	10	1		—		—	
P4 (F4)	M	4	8	1		—		—	
P5 (F5)	M	4	5	1.4		—			
Control subjects	M/F	10-40	56-76	—	—	Wild-type	—	Wild-type	

Comp. het, Compound heterozygote; *F*, female; *f-Age*, age at final follow-up or death; *M*, male; *ND*, not determined; *P*, patient; *tw*, twin.