Original Article

Diagnostic Modalities Based on Flow Cytometry for Chronic Granulomatous Disease: A Multicenter Study in a Well-Defined Cohort

Hatice Ezgi Baris, MD[a], Ismail Ogulur, PhD[b,c], Bengu Akcam, MSc[b,c], Ayca Kiykim, MD[d], Dilek Karagoz, MD[e], Berkay Saraymen, PhD[f], Gamze Akgun, BS[b,c], Sevgi Bilgic Eltan, MD[b,c], Sezin Aydemir, MD[d], Zeynep Akidagi, MSc[g,h], Esma Bentli, MSc[i], Ercan Nain, MD[b,c], Nurhan Kasap, MD[b,c], Dilek Baser, MSc[b,c], Derya Ufuk Altintas, MD[e], Yildiz Camcioglu, MD[d], Gözde Yesil, MD[j], Ahmet Ozen, MD[b,c], Mustafa Yavuz Koker, PhD[g], Elif Karakoc-Aydiner, MD[b,c,*], and Safa Baris, MD[b,c,*] *Istanbul, Adana, Kayseri, and Nevsehir, Turkey*

What is already known about this topic? Chronic granulomatous disease is caused by mutations affecting subunits of the nicotinamide adenine dinucleotide phosphate oxidase complex. It is characterized by susceptibility to severe infections such as pneumonia, cutaneous and deep tissue abscesses, lymphadenitis, osteomyelitis, and septicemia in addition to inflammatory manifestations. Assessment of the nicotinamide adenine dinucleotide phosphate oxidase function by the dihydrorhodamine test and screening for subunit mutations by genetic analysis help establish the diagnosis.

What does this article add to our knowledge? A flow cytometry–based algorithm facilitates the diagnosis of chronic granulomatous disease by rapidly identifying the defective nicotinamide adenine dinucleotide phosphate oxidase subunits. The same method also detects the carrier status by offering a range of expression values in comparison with healthy controls.

How does this study impact current management guidelines? The results of this study will be useful for the early diagnosis and treatment of chronic granulomatous disease by providing flow cytometry–based algorithm for targeted gene sequencing.

BACKGROUND: Chronic granulomatous disease (CGD) is characterized by defective microbial killing due to mutations affecting subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. Definitive genetic identification of disease subtypes may be delayed or not readily available.

OBJECTIVE: Sought to investigate the role of intracellular staining of NADPH oxidase enzyme subunits in predicting the respective genetic defects in patients with CGD and carriers.

METHODS: Thirty-four patients with genetically inherited CGD, including 12 patients with X-linked CGD (gp91$^{\text{phagocyte oxidase (phox)}}$ deficiency due to cytochrome b-245, beta polypeptide [*CYBB*] mutations) and 22 patients with autosomal-recessive CGD (p22$^{\text{phox}}$, p47$^{\text{phox}}$, and p67$^{\text{phox}}$ deficiency due to cytochrome b-245, alpha polypeptide [*CYBA*], neutrophil cytosolic factor 1 [*NCF1*] and *NCF2* mutations, respectively) were recruited from different immunology centers and followed up prospectively. Dihydrorhodamine testing and NADPH oxidase subunit expression in white blood cells were determined by flow cytometry.

RESULTS: gp91$^{\text{phox}}$ and p22$^{\text{phox}}$ defects, which result in simultaneous loss of both proteins due to their complex formation, were differentiated only by comparative analysis of

[a]Faculty of Medicine, Department of Pediatrics, Marmara University, Istanbul, Turkey
[b]Faculty of Medicine, Pediatric Allergy-Immunology, Marmara University, Istanbul, Turkey
[c]Istanbul Jeffrey Modell Diagnostic and Research Center for Primary Immunodeficiencies, Istanbul, Turkey
[d]Faculty of Medicine, Division of Pediatric Allergy-Immunology, Istanbul-Cerrahpasa University, Istanbul, Turkey
[e]Faculty of Medicine, Division of Pediatric Allergy-Immunology, Çukurova University, Adana, Turkey
[f]ERNAM-Nanotechnology Research and Application Center, Erciyes University, Kayseri, Turkey
[g]Faculty of Medicine, Department of Immunology, Erciyes University, Kayseri, Turkey
[h]Vocational College, Kapadokya University, Nevsehir, Turkey
[i]Genome and Stem Cell Center, Erciyes University, Kayseri, Turkey
[j]Faculty of Medicine, Department of Genetics, Bezmialem Vakif Gureba University, Istanbul, Turkey

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Corresponding author: Safa Baris, MD, Division of Pediatric Allergy/Immunology, Marmara University, Fevzi Çakmak Mah. No. 41, Pendik/Istanbul/Turkey. E-mail: safabaris@hotmail.com.
* These authors contributed equally to this work.
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Abbreviations used
AR-CGD- autosomal-recessive CGD
$A22^R$- autosomal, A22 CGD with residual oxidase activity
$A47^0$- autosomal, A47 CGD with residual oxidase activity
$A67^R$- autosomal, A67 CGD with residual oxidase activity
CGD- chronic granulomatous disease
CYBA- cytochrome b-245, alpha polypeptide
CYBB- cytochrome b-245, beta polypeptide
DHR- dihydrorhodamine-1,2,3
MFI- mean fluorescence intensity
NADPH- nicotinamide adenine dinucleotide phosphate
NCF- neutrophil cytosolic factor
OS- overall survival
phox- phagocyte oxidase
SI- stimulation index
$X91^0$- X-linked, X91 CGD with null oxidase activity

patients' and mothers' intracellular staining. $p47^{phox}$ and $p67^{phox}$ protein expression was almost undetectable in patients compared with carrier mothers and healthy controls. The expression values of the respective subunits were found to be significantly higher in all controls as compared with carrier mothers, which in turn were higher than those of patients.

CONCLUSIONS: Analysis of NADPH oxidase enzyme subunits by flow cytometry in patients and carriers is useful in the rapid prediction of the genetic defect of patients with CGD, thus guiding targeted sequencing and aiding in their early diagnosis.

Key words: *Chronic granulomatous disease; NADPH oxidase; flow cytometry; Dihydrorhodamine; gp91; p22; p47; p67*

INTRODUCTION

Chronic granulomatous disease (CGD) is a primary immune deficiency disorder characterized by recurrent bacterial and fungal infections due to defective nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which is responsible for the production of reactive oxygen species in the phagocytes.[1-3] The nicotinamide adenine dinucleotide phosphate oxidase enzyme complex is composed of 5 subunits: 2 transmembrane glycoproteins $gp91^{phagocyte\ oxidase\ (phox)}$ and $p22^{phox}$ that form the heterodimeric cytochrome b_{558}, and 3 cytosolic subunits $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ that form a heterotrimer. Upon microbial binding into the phagocyte cell membrane, the association of the cytosolic heterotrimer with the transmembrane cytochrome b_{558} results in a number of enzymatic reactions that convert molecular oxygen to reactive oxygen species, which are required for effective killing of pathogenic bacteria and fungi in the phagocytic leukocytes. Each of these subunits is encoded by a different gene: the X-linked cytochrome b-245, beta polypeptide (*CYBB*) for $gp91^{phox}$ and the autosomal cytochrome b-245, alpha polypeptide (*CYBA*) for $p22^{phox}$, neutrophil cytosolic factor-1 (*NCF1*) for $p47^{phox}$, *NCF2* for $p67^{phox}$, and *NCF4* for $p40^{phox}$. Mutations in any one of the respective genes can give rise to CGD.[3,4] Recently, a new genetic defect in cytochrome b-245 chaperone 1 (*CYBC1*) was described that leads to decreased $gp91^{phox}$ expression and consequently results in CGD.[5,6] As a result of the aforementioned genetic defects, patients suffer from severe infections such as pneumonia, cutaneous and deep tissue abscesses, lymphadenitis, osteomyelitis, and septicemia in addition to inflammatory manifestations.[1,7-9]

There are currently 2 tests widely used in the diagnosis of CGD, which are based on the determination of the NADPH oxidase activity in stimulated blood phagocytes: nitro-blue tetrazolium slide test and dihydrorhodamine-1,2,3 (DHR) test.[10,11] Both tests are capable of detecting female X-CGD carriers and thus predict the pattern of this form of inheritance.[12-14] In addition, the DHR flow patterns generally correlate with the clinical presentation of patients.[7,15] However, abnormal results with DHR assay can be also observed in patients with myeloperoxidase deficiency who usually exhibit normal nitro-blue tetrazolium test result.[16] Therefore, the results of DHR and nitro-blue tetrazolium should be evaluated carefully in each suspected patient during the diagnosis.

Because of the chronicity of the disease with intractable symptoms that are often difficult to treat, early diagnosis is important in the management.[1,17] Definitive molecular diagnosis is possible with gene sequencing, aided by immunoblotting, techniques that are not routinely available and usually take a longer time to be achieved. In addition, mutation analysis is not only time consuming but also an expensive method of diagnosis in low-resource settings. Flow cytometric assessment of the NADPH oxidase components following intracellular staining could be an easier and faster method to identify the defective subunit and at a lower cost compared with mutational analysis. This assay was previously applied in some CGD forms with successful results.[18-21] However, there is no detailed study that investigates the role of flow cytometry in CGD diagnosis, and provides reference values for protein expressions of NADPH oxidase subunits and detection of the carrier status. We present here a flow cytometry—based algorithm that can facilitate the identification of the defective protein in patients and confirm the carrier status in family members by offering a range of expression values comparing to healthy controls.

METHODS

Patients and study design

This multicenter study involved 34 patients followed with a diagnosis of CGD. Clinical and demographic features of the patients were solicited from their medical records. These records included clinical history, laboratory test results, and mutation analysis of each patient. Patients and mothers were evaluated by the DHR test and by intracellular staining of NADPH oxidase subunits. The study was approved by the Marmara University, School of Medicine Ethics Committee (09.2017.230), and a written informed consent was obtained from all parents. Because of the young age of some patients, a simple oral description of the study was presented to participating children in the presence of their parent(s) and a verbal assent was requested.

Dihydrorhodamine-1,2,3 test

The DHR assay was performed as described previously.[13,22] Briefly, isolated neutrophils were incubated with DHR, stimulated with phorbol 12-myristate 13-acetate, and analyzed by flow cytometry. The results were presented as the stimulation index (SI; the ratio of the mean fluorescence intensity [MFI] of the stimulated to the unstimulated cells). The SIs of neutrophils with 1.5 or less

TABLE I. The demographic, clinical, and mutation data of patients with CGD

Patient no. (family no.)	Current age (y)/sex	AOO (mo)	AOD (mo)	Follow-up (y)	Consanguinity	CGD subtype	DHR SI	Residual activity*	Outcome	Mutation	Zygosity	Reported mutation
P1 (F1)	12.7/F	42	84	5.7	Yes	A22	6	$A22^R$	Alive	c.70G>A, p.Gly24Arg	Homozygous	27
P2 (F2)	16/F	10	12	15	No	A22	1.1	$A22^0$	Alive	c.288-15C>G, p.Leu97ArgfsX94	Homozygous	27
P3 (F3)	6.8/M	2	4	6.5	No	A22	1	$A22^0$	Alive	c.27G>A/c.251_263del13bp, p.Trp9Ter	Compound heterozygous	27
P4 (F4)	17.3/F	7	24	15.3	Yes	A22	1	$A22^0$	Alive	c.58+2T>G, p.?	Homozygous	7
P5 (F5)	15.3/M	6	60	10.3	Yes	A22	2.7	$A22^R$	Alive	c.70G>C, p.Gly24Arg	Homozygous	Unreported
P6 (F6)	4.2/M	7	14	3.1	No	X91	1	$X91^0$	Died, after HSCT	c.897G>T, p.Lys299Asn	Hemizygous	7
P7 (F7)	5.8/M	1	8	5.2	No	X91	1	$X91^0$	Alive, transplanted and well at 18 mo after HSCT	c. 469C>T, p.Arg157X	Hemizygous	2
P8 (F8)	12.2/M	24	27	10	No	X91	1	$X91^0$	Alive	c.469C>T, p.Arg157X	Hemizygous	2
P9 (F9)	11.6/M	5	20	10	No	X91	1	$X91^0$	Alive	c.674+2T>G, p.?	Hemizygous	28
P10 (F10)	8/M	1.5	35	5.1	No	X91	1.1	$X91^0$	Alive	Gross deletion 4-6 exons	Hemizygous	2
P11 (F11)	3.2/M	1	15	2	No	X91	1	$X91^0$	Died, after HSCT	c.742dupA, p.Ile248AsnfsX36	Hemizygous	7
P12 (F12)	1.3/M	4	4	1	No	X91	1	$X91^0$	Alive	c.676C>T, p.Arg226X	Hemizygous	7
P13 (F13)	3.3/M	4	4	3	No	X91	1	$X91^0$	Alive, transplanted and well at 12 mo after HSCT	c.1094T>G, p.Leu365Arg	Hemizygous	Unreported
P14 (F14)	4.8/M	3.5	24	2.8	No	X91	1	$X91^0$	Alive	c.388C>T, p.Arg130X	Hemizygous	2
P15 (F14)	6.2/M	1	39	3	No	X91	1	$X91^0$	Alive	c.388C>T, p.Arg130X	Hemizygous	2
P16 (F15)	2.2/M	1	3	2	Yes	X91	1	$X91^0$	Died, Aspergillus infections, sepsis	c.217C>T, p.Arg73X	Hemizygous	2
P17 (F16)	3/M	4	8	2.3	Yes	X91	1	$X91^0$	Alive	c.675-2A>G, p.?	Hemizygous	Unreported
P18 (F17)	21/F	72	154	7.4	Yes	A47	2.1	$A47^0$	Alive	c.579G>A, p.Trp193X	Homozygous	7
P19 (F17)	32/M	192	290	7.2	Yes	A47	2	$A47^0$	Alive	c.579G>A, p.Trp193X	Homozygous	7
P20 (F18)	30/M	12	300	4	Yes	A47	2.2	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27
P21 (F19)	24/M	216	216	6.1	Yes	A47	2.6	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27
P22 (F20)	16/M	108	124	5.6	Yes	A47	1.9	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27
P23 (F20)	27.5/M	96	192	11.5	Yes	A47	2	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27
P24 (F21)	7.4/M	21	24	5.2	Yes	A47	1.9	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27
P25 (F22)	12/F	72	80	5.6	Yes	A47	5.6	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27
P26 (F23)	18.1/M	48	192	2.1	Yes	A47	2.7	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27
P27 (F24)	35.7/M	60	175	21.2	Yes	A47	3.8	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27
P28 (F25)	13/F	48	48	9	Yes	A47	1.6	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27
P29 (F25)	23/F	24	168	8	Yes	A47	2.6	$A47^0$	Alive	c.75_76delGT, p.Tyr26HisfsX26	Homozygous	27

(continued)

TABLE I. *(Continued)*

Patient no. (family no.)	Current age (y)/sex	AOO (mo)	AOD (mo)	Follow-up (y)	Consanguinity	CGD subtype	DHR SI	Residual activity*	Outcome	Mutation	Zygosity	Reported mutation
P30 (F26)	17/M	36	171	2	Yes	A67	1	$A67^0$	Alive	c.279C>G+c.366+1G>C, p. Asp93Glu+del exon 4	Homozygous	27
P31 (F27)	11.7/F	72	127	1.2	No	A67	3.9	$A67^R$	Alive	c.767_768insA, p.Glu257LysfsX15	Homozygous	27
P32 (F28)	1.3/F	2	4	1	No	A67	1	$A67^0$	Alive	c.229C>T, p.Arg77X	Homozygous	27
P33 (F29)	23/M	2	60	18.1	Yes	A67	1	$A67^0$	Alive	c.229C>T, p.Arg77X	Homozygous	27
P34 (F30)	13.6/M	3	8	3	Yes	A67	1	$A67^0$	Alive, transplanted and well at 8 mo after HSCT	c.229C>T, p.Arg77X	Homozygous	27

AOD, Age of diagnosis; *AOO*, age of onset; *HSCT*, hematopoietic stem cell transplantation.
*According to the DHR test.

were accepted as oxidase-null, whereas SI values greater than 1.5 were grouped as oxidase-residual activity.[13]

Intracellular staining of NADPH enzyme components

Whole-blood staining and flow cytometry was used to determine the enzyme components. Fifty microliter of whole blood was fixed and permeabilized with Beckman Coulter IntraPrepTM kit (A07803, Indianapolis, Ind). For each tube, 5 μL of the respective mAbs was added as follows: for p22, mouse monoclonal (MCA4686A488, clone: CS9, Bio-Rad, Hercules, Calif); for gp91, mouse monoclonal (MCA4685A488, clone: NL7, Bio-Rad); for p47, rabbit monoclonal (ab179457, Abcam, Cambridge, UK); and for p67, rabbit monoclonal (ab109523, Abcam). The cells were incubated at 4°C in the dark for 1 hour. Cells were washed and centrifuged for 5 minutes. For p47 and p67 staining, secondary antibody Alexa Fluor 647−conjugated goat antirabbit polyclonal antibody (ab181474, Abcam) diluted at ratio 1:200 was added for 15 minutes. Stained cells were analyzed by BD FACSCalibur (BD Biosciences, San Jose, Calif) by gating on neutrophils. Protein expression was calculated as the difference in MFI (ΔMFI) between cells stained with the respective specific antibodies and their isotype controls; the results were analyzed as a percentage of ΔMFI of healthy control assayed on the same day.[23,24]

Molecular genetic analysis

Genomic DNA was isolated from total blood leukocytes and analyzed for mutations in exons and exon/intron boundaries of *CYBB*, *CYBA*, *NCF1*, and *NCF2* by means of PCR amplification, followed by sequence analysis. Because the *NCF1* gene has 2 pseudogenes that share more than 99% of homology with the real gene, a gene-scan analysis was performed for hotspot 2 GT deletion in exon 2 by determining the pseudogene/gene ratio. In healthy individuals, the ratio is usually 2:1 and in carriers of exon 2 GT deletion 5:1, due to unequal crossing-over between *NCF1* and one of its pseudogenes, causing a deletion of *NCF1* on one allele and gain of one pseudogene on the other allele. The ratio in patients born from two 5:1 carriers will be 0, due to loss of *NCF1* on both alleles (see Figure E1 in this article's Online Repository at www.jaci-inpractice.org).[25,26]

Statistical analysis

Continuous variables were expressed as median (range), and categorical variables were described in frequencies and percentages. Nonparametric continuous variables were compared by Mann-Whitney *U* test, whereas comparison of categorical variables was performed by Fisher exact test. Overall survival (OS) was calculated from the initiation of treatment to death or the last follow-up examination. Analysis of OS was done using the Kaplan-Meier method. *P* values less than .05 were considered statistically significant. Statistical analysis was done using SPSS 20 (SPSS Inc, Chicago, Ill) and GraphPad Prism 8 (GraphPad Software Inc, San Diego, Calif).

RESULTS

Clinical and demographic data

Thirty-four patients from 3 medical centers in Turkey were enrolled in this study. The mean current age of the cohort was 13.5 ± 9.23 years. There were 4 families with more than 1 affected member. Demographic data and clinical outcomes of the study cohort are summarized in Table I. A high consanguinity rate was observed in our cohort (n = 20 [58.8%]). Most patients

(n = 19 [55.9%]) were younger than age 1 year at the onset of disease symptoms. However, only 8 patients (23.5%) were diagnosed with CGD before age 1 year. Patients with X-CGD presented (3.7 [1-4.5] months vs 39 [7-72] months; $P < .001$) and diagnosed (14.5 [6.0-25.5] months vs 104.1 [24-175] months; $P = .001$] at a younger age compared with the patients with autosomal-recessive CGD (AR-CGD). The delay in diagnosis was higher in patients with AR-CGD (5.1 ± 3.2 vs 1.1 ± 0.2 years; $P = .002$).

Abscesses were the most common disease manifestation at first presentation (n = 22 [64.7%]). Other presenting features at the onset of the disease were pneumonia (n = 8 [23.5%]), prolonged fever (n = 5 [14.7%]), lymphadenitis (n = 2 [5.9%]), and bacteremia (n = 1 [2.9%]). One female patient with AR-CGD had no symptoms and was diagnosed by family screening by the DHR test after her older brother was diagnosed with CGD.

Infectious complications are presented in Table II. The most common site for abscesses was the skin (n = 15 [44.1%]). Other locations were the perianal region (n = 6 [17.6%]), liver (n = 2 [5.9%]), bone (n = 2 [5.9%]), teeth (n = 1 [2.9%]), and lungs (n = 1 [2.9%]).

Granulomas were found in 11 (32.3%) patients and were most common in lymph nodes (n = 8 [23.5%]). Other tissues in which granulomas developed were the liver (n = 2 [5.9%]), skin (n = 1 [2.9%]), lung (n = 1 [2.9%]), bone (n = 1 [2.9%]), and the brain (n = 1 [2.9%]). Autoimmune manifestations were uncommon in our cohort and presented in the form of colitis in only 2 siblings with X-CGD.

All patients received antimicrobial prophylaxis with trimethoprim sulfamethoxazole and itraconazole. Twenty patients (58.8%) were also treated with IFN-γ. Five patients had a transplantation (14.7%) and 2 died soon after transplantation because of graft failure and infections. The other 3 patients (P7, P13, P34) are now at 18, 12, and 8 months posttransplantation, respectively, without complications. One patient (P16) died after severe pulmonary aspergillosis at age 2 years. The mean duration of follow-up and OS were higher in those with AR-CGD compared with those with X-CGD (7.7 ± 5.5 vs 4.1 ± 2.9 years; $P = .03$) and (100% vs 65.5% ± 17.3%; $P = .01$). The OS of the subtypes of the diseases are summarized in Figure E2 in this article's Online Repository at www.jaci-inpractice.org.

TABLE II. The infectious complications and species of patients with CGD

Infectious complications	No. of patients (%)
Recurrent otitis	5 (14.7)
Recurrent sinusitis	2 (5.9)
Pneumonia	24 (70.6)
Skin abscesses	14 (44.1)
Recurrent deep tissue and organ abscesses	12 (35.3)
Recurrent oral thrush and fungal skin infections	9 (26.5)
Deep situated infection or sepsis	17 (50.0)
Osteomyelitis	3 (8.8)
Infectious species	
MSSA	8 (23.5)
Serratia	3 (8.8)
Escherichia coli	1 (2.9)
Citrobacter	1 (2.9)
Klebsiella	2 (5.8)
Aspergillus	6 (17.6)

MSSA, Methicillin-sensitive *Staphylococcus aureus*.

Genetic mutations, DHR test results, and disease activity

The patients were analyzed for the gene defects related to the CGD. The mutations are presented in Table I. There were 12 patients with *CYBB*, 12 patients with *NCF1,* 5 patients with *CYBA*, and 5 patients with *NCF2* mutations. Two patients with *CYBB* (c.1094T>G, p.Leu365Arg and c.675-2A>G) and 1 patient with *CYBA* (c.70G>C, p.Gly24Arg) had novel mutations; the other mutations were described previously (Table I).[2,27,28] There were no *de novo* mutations in our cohort. In line with literature,[27,28] the GT deletion in *NCF1* was most common among $p47^{phox}$-deficient patients. The DHR was performed in all patients and their mothers, and according to the DHR results residual NADPH oxidase activity was evaluated. As expected in general,[7] all our patients with $gp91^{phox}$ and most patients with $p22^{phox}$ and $p67^{phox}$ defects were found to have very low DHR SI (≤1.5; median, 1.00; 1.00-1.10), showing absent reactive oxygen intermediates in those patients (X-linked, X91 CGD with null oxidase activity [$X91^0$]; autosomal, A22 CGD with null oxidase activity [$A22^0$]; and autosomal, A67 CGD with null oxidase activity [$A67^0$]). In contrast, all patients with $p47^{phox}$ defects demonstrated residual oxidase activity (autosomal, A47 CGD with residual oxidase activity [$A47^0$]) (>1.5; median, 2.17; 1.96-2.66), indicating that the DHR SI can differentiate these patients from patients with other defects ($P = .001$). Interestingly, 3 patients (2 with missense *CYBA* and 1 with insertion *NCF2* mutations) showed residual activity more than 1.5 (autosomal, A22 CGD with residual oxidase activity [$A22^R$] and autosomal, A67 with residual oxidase activity [$A67^R$], median SI, 3.90; 2.70-6.00). To determine the clinical phenotypes and association with reactive oxygen intermediate activity, we compared both groups (null vs residual). Nineteen patients (55.8%) with SI less than 1.5 ($X91^0$, $A22^0$, $A67^0$) presented significantly earlier (2.5 ± 3.0 years) when compared with the patients (n = 14 [44.2%], 12.5 ± 7.1 years, $P = .001$) with SI more than 1.5 ($A47^0$, $A22^R$, and $A67^R$). The rate of documented infections was higher in patients with null oxidase activity (98% vs 42%; $P = .010$).

Intracellular staining of NADPH oxidase enzyme complex in patients and carrier mothers

Patients with CGD can have any 1 of 6 different gene defects: most of the patients have X-CGD, whereas the others have AR forms.[8,24,29] We used flow cytometry to screen 27 patients and their carrier mothers for the different components of the NADPH oxidase enzyme complex to determine whether intracellular staining of the NADPH oxidase enzyme complex can predict the gene defect (Figure 1). In general, the quantified differences in MFI values between specific antibody and isotype control staining (ΔMFI) were statistically lower in patients and higher in healthy controls, whereas the related protein showed an intermediate staining in carrier mothers (Figures 2 and 3; Table III). Because the 2 transmembrane glycoproteins $gp91^{phox}$ and $p22^{phox}$ are bound together to form cytochrome b_{558}, a deficiency of either protein results in the loss of both complexed proteins. Consequently, in our cohort, all $gp91^{phox}$- and $p22^{phox}$-mutated patients demonstrated the same flow cytometric staining

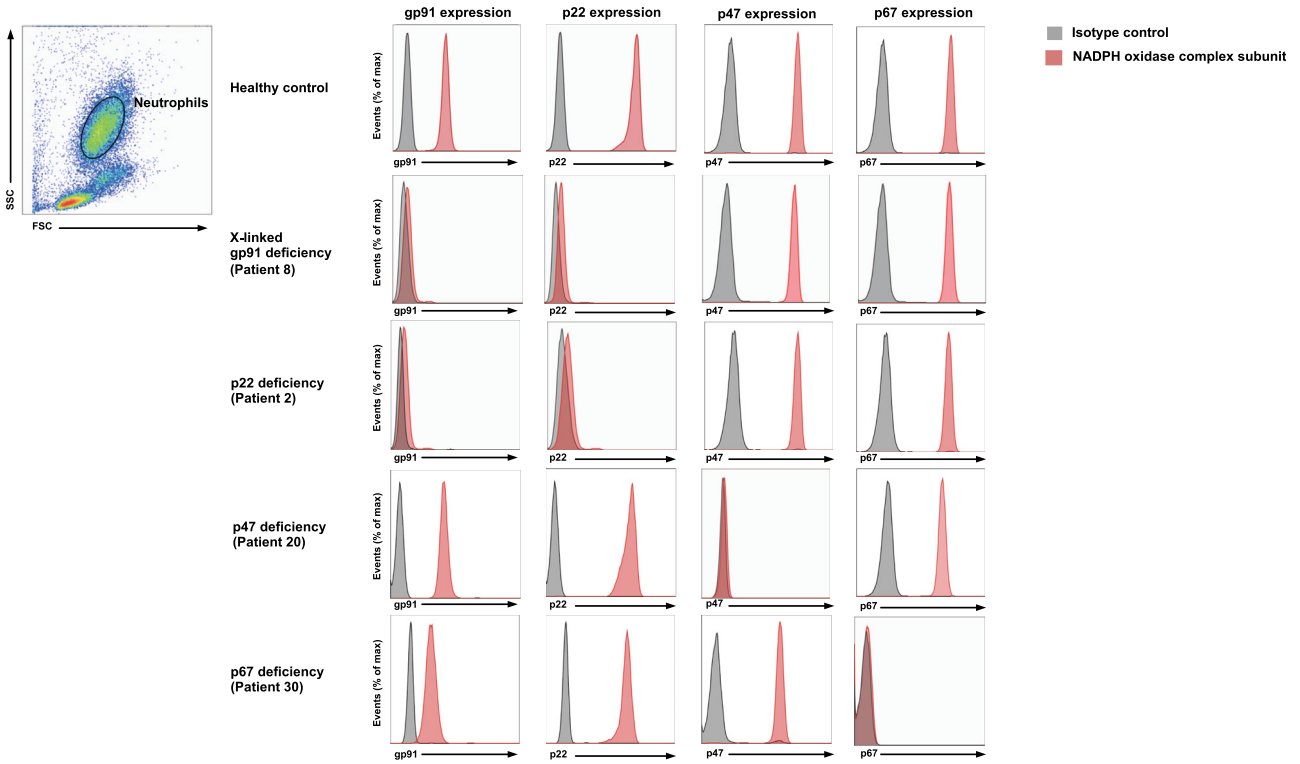

FIGURE 1. Neutrophil gating histograms of NADPH oxidase enzyme subunits analyzed by flow cytometry in patients with CGD and healthy controls. *FSC*, Forward scatter; *max*, maximum; *SSC*, side scatter.

pattern of very low expression of both proteins, which could not on its own differentiate the 2 subtypes of the disease. However, comparison of patient and carrier mother intracellular staining results was helpful in differentiating the 2 defects (Figure 2, *A* and *B*). Because of X chromosome lyonization, mothers with X-linked CGD have 2 neutrophil populations, one harboring the normal *CYBB* allele and the other the mutant one, that respectively exhibit normal and deficient NADPH oxidase enzyme activity.[10] Therefore, neutrophils of *CYBB* mutation carrier mothers displayed bimodal distribution for the $p22^{phox}$ protein staining, mirroring the bimodal DHR staining (Figure 2, *A*). Of note, normal maternal DHR and protein expression of $gp91^{phox}$ is expected in the *de novo CYBB* mutations in male patients, which were not detected in our cohort. However, the neutrophils of *CYBA* mutation carrier mothers exhibited normal DHR staining and monomodal distribution for $p22^{phox}$ protein with normal or intermediate expression pattern compared with healthy controls (Figure 2, *B*).

In the case of $p47^{phox}$ and $p67^{phox}$ deficiencies, the protein expression of the respective subunit was virtually undetectable in patients when compared with that of carrier mothers showing normal DHR and healthy controls (Figure 3, *A* and *B*). When calculated as a percentage of the expression of the respectively related protein in controls assessed on the same day, the neutrophil median protein expression in the patients was as follows: $gp91^{phox}$: 57.4% (range, 35.7%-65.7%), $p22^{phox}$: 11.2% (range, 9.2%-21.7%), $p47^{phox}$: 1.9% (range, 0.0%-3.8%), and $p67^{phox}$: 0.9% (range, 0.4%-2.6%). For comparison, the median protein expression in obligate carriers was approximately half of the controls, although there was an overlap in the range of carriers and healthy controls in $gp91^{phox}$ and $p22^{phox}$ expressions (Table III). When analyzed in aggregate, the quantified ΔMFI values of all controls were significantly higher compared with those of patients and carriers (Figure 2, *C* and *D*; Figure 3, *C* and *D*). In addition, the carrier mothers displayed higher ΔMFI values in comparison to patients for the $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ proteins (Figure 2, *D*; Figure 3, *C* and *D*).

We also compared the residual intracellular subunit protein expression between patients with null and residual oxidase activity. In our cohort, there were 2 $p22^{phox}$-deficient patients with missense mutations (P1 and P5), which demonstrated residual oxidase activity with the DHR test (SI; P1: 6, P5: 2.7) and both of them exhibited higher protein expression when compared with the rest of $p22^{phox}$-deficient patients (20% ± 2% vs 9.6% ± 0.6%; $P = .01$).

Collectively, our results showed that intracellular staining was successful in predicting the CGD subtype in patients and carriers. On the basis of our results, we provide a flowchart for the diagnosis of patients with CGD for potential NADPH oxidase subunit defects by combining intracellular subunit protein staining in patients and carriers (Figure 4).

DISCUSSION

In this report, we demonstrate and validate the utility of using flow cytometry for the rapid determination of the target NADPH oxidase subunit in patients with CGD without *de novo* mutations. By using a carefully vetted cohort of 34 genetically inherited patients and their parents from different immunology centers in Turkey, we show that flow cytometric screening of NADPH oxidase subunits provides a fast, highly sensitive, and

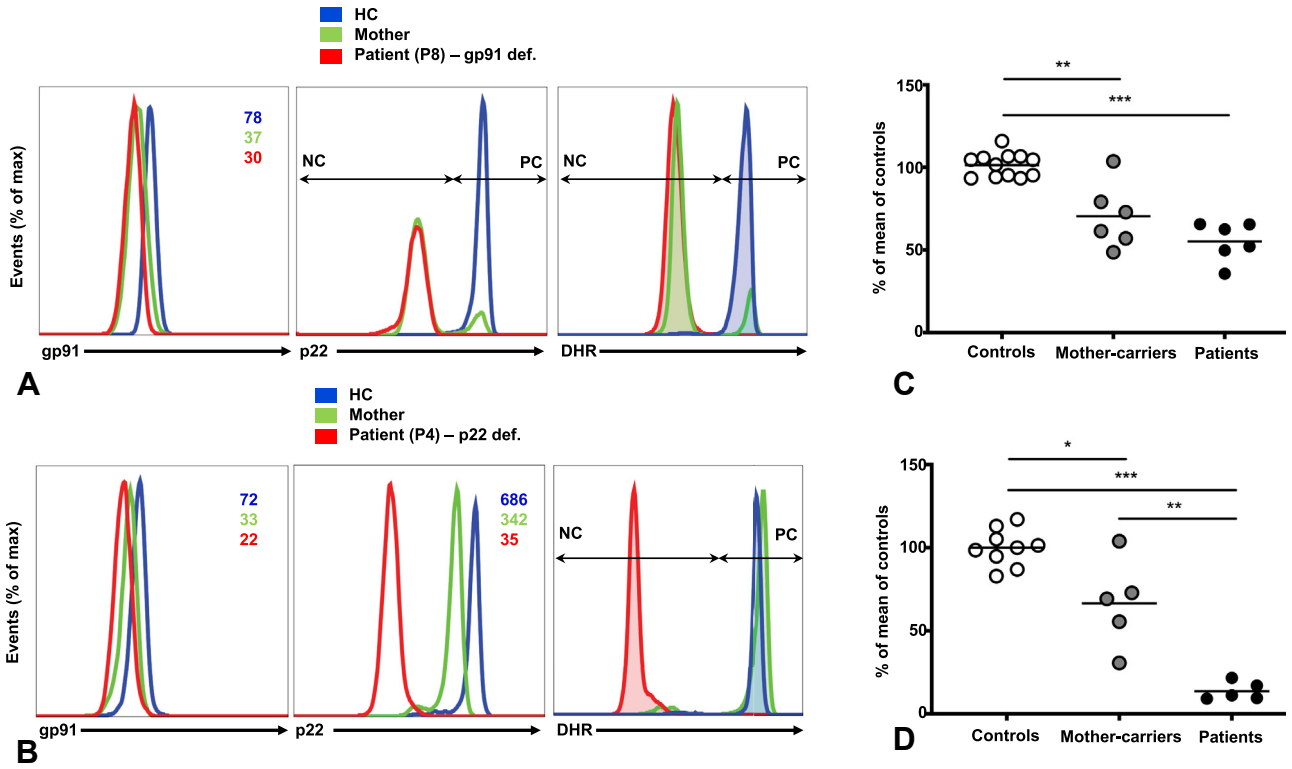

FIGURE 2. gp91^{phox} and p22^{phox} protein expression profiles differentiate patients from HCs and carriers. (**A** and **B**), Flow cytometric analysis of gp91^{phox} and p22^{phox} expression and respiratory burst of *CYBB* (patient 8) and *CYBA* (patient 4)-mutated patients, carrier mothers, and HCs' neutrophils. MFI values are indicated with the related colors. (**C** and **D**), Quantitative expression of gp91^{phox} and p22^{phox} in neutrophils of patients, carrier mothers, and HCs. *HC*, Healthy control; *max*, maximum; *NC*, negative cell; *PC*, positive cell. *$P < .05$, **$P < .01$, ***$P < .001$, 1-way ANOVA test with Bonferroni posttest analysis.

accurate determination of the lesioned subunit. Targeted detection of the genetic defect in CGD based on flow cytometric assessment of protein expression of the enzyme components can thus guide the genetic diagnosis and provide an alternative cost-effective diagnostic method in low-resource settings. The utility of this flow cytometric approach extended beyond the provision of a diagnostic tool to shed light on the relationship between the respective genetic lesions, the level of subunit protein expression, DHR test activity, and clinical outcomes. Thus, it may also aid together with the other investigations in formulating a "prognostic matrix" relevant to disease course and outcome.

Flow cytometric analysis demonstrated that in our cohort mutations decreased or eradicated NADPH oxidase enzyme complex subunits' expression in all patients with CGD. Previously, Roos et al[2] described the protein levels on the basis of immunoblot analysis as absent (°), diminished (⁻), or normal (⁺) for all the CGD-related proteins. The proteins can be nonfunctional or have residual activity. Most *CYBB* mutations in our cohort were described previously as null (°) protein with no residual activity.[2] However, depending on the nature of the mutation, it is possible that some patients with X-linked CGD with missense mutations can also exhibit residual activity resembling that in patients with p47^{phox} AR-CGD.[30] All our patients with X-CGD or p22^{phox} deficiency had very low expression of both gp91^{phox} and p22^{phox}, as expected, because the 2 proteins are bound together in the membrane to form cytochrome b_{558} and loss of one of them affects the expression of the other protein.[18] However, the expression level of the proteins is related to the mutation types, such that those mutations in *CYBB* leading to the complete loss of gp91^{phox} result in undetectable p22^{phox}, whereas some missense mutations displayed variable expression of gp91^{phox} with proportionally detectable p22^{phox} expression as described by Kuhns et al.[3] Because of the same staining pattern observed in gp91^{phox} and p22^{phox} deficiencies, differentiation between the 2 subtypes necessitates additional evaluations. Thus, concurrent analysis of patients and their mother carriers discriminates gp91^{phox} from p22^{phox} subtype in patients with different mutations, except for gp91^{phox}-deficient patients with *de novo* mutations, in which mothers will show normal DHR and subunit staining.

Comprehensive evaluation of the clinical, immunologic, and genetic characteristics of our cohort revealed several features relevant to this investigation. Because of the high rate of consanguinity, AR-CGD was observed more frequently in our cohort compared with X-linked CGD. DHR test residual activity was associated with later disease onset, and patients with X-CGD presented clinically earlier than patients with AR-CGD who overall had better survival. Our results provided a reference data set by comparing patients' and mothers' MFI values to those of healthy controls for gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} deficiencies. The patients showed the lowest, whereas the carrier mothers usually displayed intermediate values in comparison to the healthy controls.

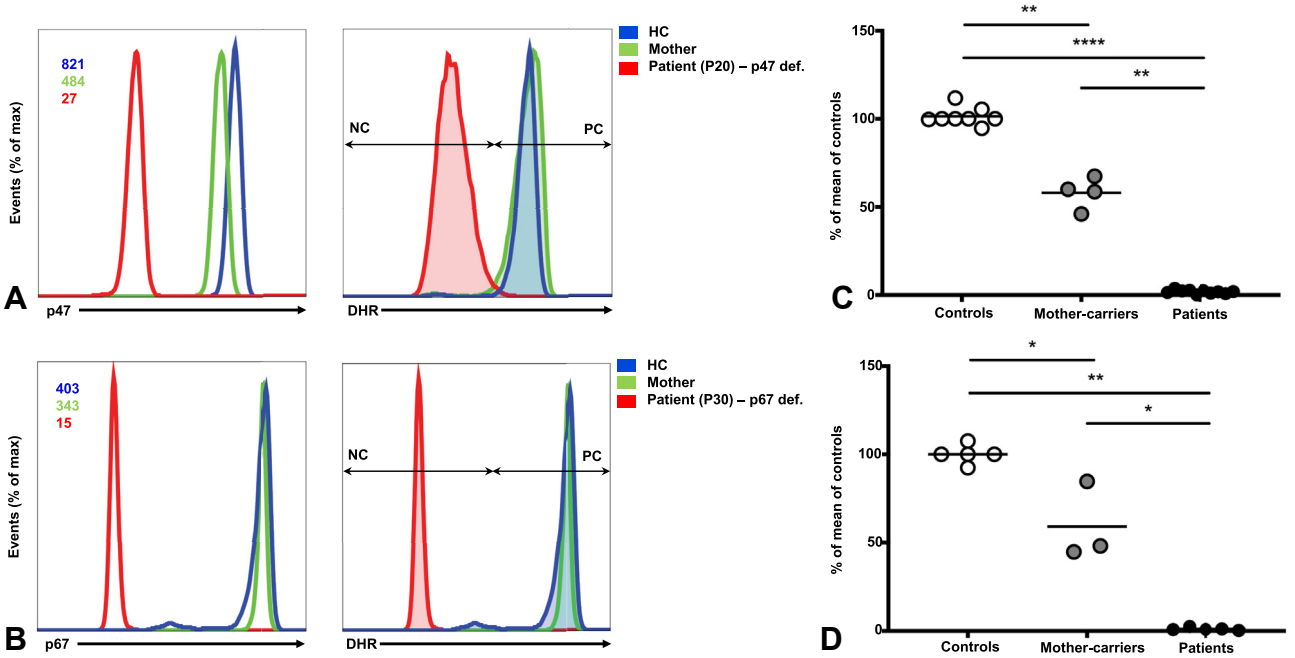

FIGURE 3. The pattern of p47^{phox} and p67^{phox} protein expression differentiates patients from HCs and carriers. (**A** and **B**) Flow cytometric analysis of p47^{phox} and p67^{phox} expression and respiratory burst in *NCF1* (patient 20) and *NCF2* (patient 30)-mutated patients, carrier mothers, and HCs' neutrophils. MFI values are indicated with the related colors. (**C** and **D**) Quantitative expression of p47^{phox} and p67^{phox} in neutrophils of patients, carrier mothers, and HCs. *HC*, Healthy control; *Max*, maximum; *NC*, negative cell; *PC*, positive cell. $*P < .05$, $**P < .01$, $****P < .0001$, 1-way ANOVA test with Bonferroni posttest analysis.

TABLE III. The range of expression of NADPH oxidase enzyme subunits in healthy individuals, carriers, and affected patients shown as percent of mean ΔMFI

	ΔMFI percentage			
Group	**gp91^{phox}**	**p22^{phox}**	**p47^{phox}**	**p67^{phox}**
Healthy	103.2 (93.85-115.9)	100.1 (82.9-117.1)	100.0 (94.7-111.8)	100.0 (92.4-107.6)
Carriers (mother)	67.2 (48.7-103.6)	69.3 (30.7-103.9)	59.4 (46.0-67.5)	48.1 (44.9-84.7)
Patients	57.4 (35.7-65.7)	11.2 (9.2-21.7)	1.9 (0.0-3.8)	0.9 (0.4-2.6)

Mutations in patients with X-linked CGD are heterogeneous. As described previously, 65% of *CYBB* gene mutations are single nucleotide mutations that can cause missense, nonsense, or either splice side mutations.[2] Our genetic results showed similarity with those of previously described patients with X-CGD, while also identifying 2 novel mutations with complete loss of residual oxidase activity (c.1094T>G and c.675-2A>G). Interestingly, the c.469C>T mutation was observed in 2 unrelated families, raising the possibility of a common founder mutant allele in our population.[7]

Clearly, the mutation in *NCF1*, which causes a dinucleotide GT deletion in a GTGT tandem repeat at the start of exon 2, is a mutational hotspot that is the most common mutation observed in all patients with AR-CGD with p47^{phox} defect.[27] The reason for the predominance of this mutation is related to the *NCF1* pseudogenes, which have more than 99% homology with the native gene but lack a GT sequence at the start of exon 2 that causes a premature stop codon. Recombination of these pseudogenes with real *NCF1* gene leads to insertion of GT deletion into *NCF1*, which results in CGD.[27] However, mutations in *CYBA* and *NCF2* genes generally lead to either total loss of protein (p22°, p67°) or diminished protein expression (p22^{-}, p67^{-}). However, in some cases, mutations can be associated with normal protein expression.[27] In our study, there were 2 patients with missense mutations in *CYBA* that were associated with residual protein expression and oxidase activity, as opposed to the other mutations, which resulted in the total loss of protein expression and consequently absent oxidase activity. Our results demonstrate that evaluation of the DHR test results in light of the NADPH oxidase subunit intracellular staining pattern will help predict the locus of the culprit mutation in patients with CGD.

Koker et al[19] defined 12 *CYBA*-mutated patients with null oxidase activity in most of the patients, whereas 3 patients were detected to have residual activity with SI more than 1.5. Two of these patients with residual oxidase activity have missense mutation as p.Gly24Arg, which was also detected in our 2 *CYBA*-mutated patients. Interestingly, although they did not find any

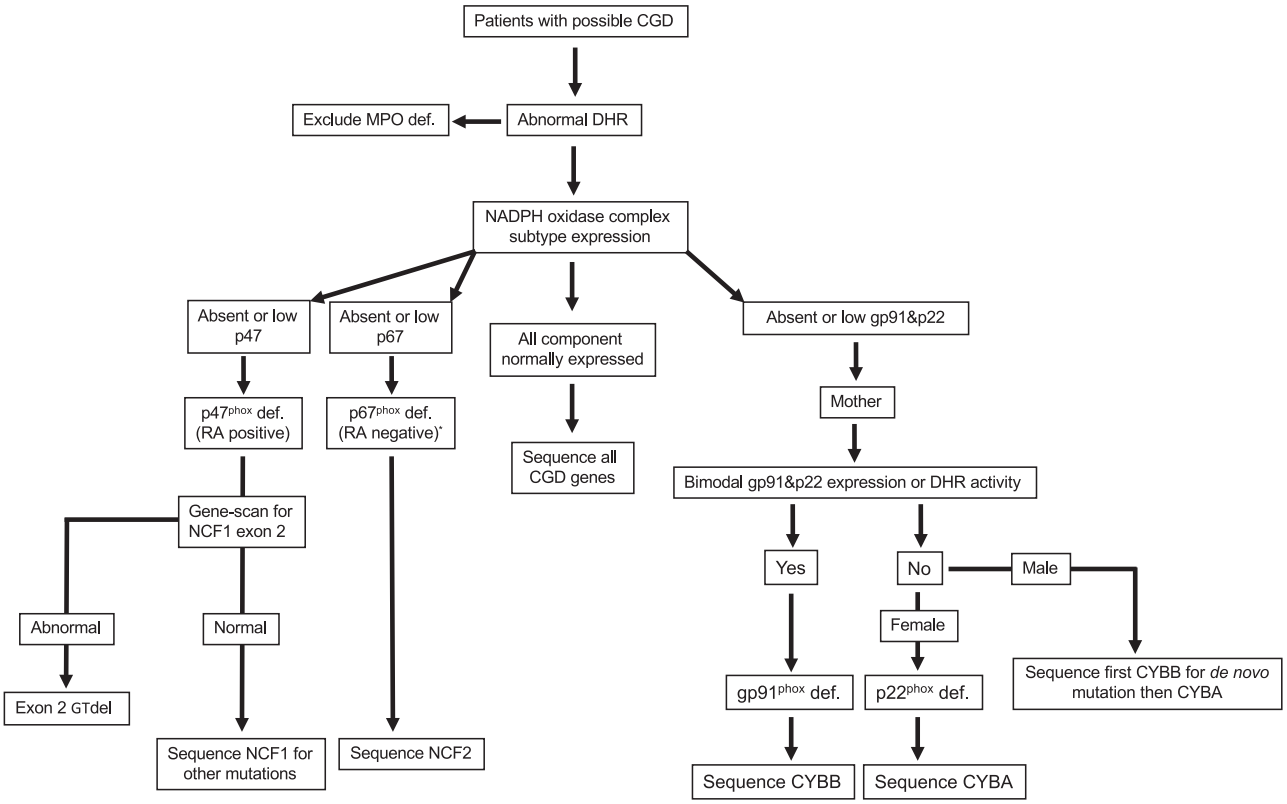

FIGURE 4. Flowchart for diagnosis of CGD by flow cytometry assay. *MPO*, Myeloperoxidase; *RA*, residual oxidase activity. *Rarely, some patients with *NCF2* mutation may present with residual oxidase activity.

$p22^{phox}$ and $gp91^{phox}$ expression in their patients with p.Gly24Arg mutation, we observed a residual $p22^{phox}$ expression as 20% ± 2% of normal controls, which can explain the residual oxidase activity observed in those patients. The differences in protein expression even for the same mutations can be related with the different antibodies used for staining in both studies.

In our cohort, the intracellular staining for $p47^{phox}$ and $p67^{phox}$ protein expression was very low or undetectable in all $p47^{phox}$- and $p67^{phox}$-deficient patients. These results were compatible with the results of previous studies.[20,27] Interestingly, our patients with delGT showed residual oxidase activity with SI more than 1.5, which was lower than the described value as 3 or more in the Koker et al[7] study. These results may relate to the technical variations across different laboratories, necessitating that every center should set its own reference values.

A limitation of our study was related to the mAb (NL7) used for the $gp91^{phox}$ protein staining, which detects only the cytoplasmic part of the protein. In general, the staining yielded weak and close signals among patients, healthy donors, and carrier mothers, but was still useful to differentiate patients from others. This limitation was also described previously in other studies using polyclonal antibodies for the $gp91^{phox}$ component.[20] The mAb 7D5, which binds to an extracellular epitope of $gp91^{phox}$ protein, demonstrated a more reliable signal for the protein.[18,31] Therefore, more specific mAbs are needed for more effective staining. It is worth noting that up to one-third of all X-linked defects may present with *de novo* mutations. Those patients will display decreased or abolished $gp91^{phox}$ protein expression, whereas their mothers will have normal expression.[18] Furthermore, it cannot be ruled out that rare mutations in *CYBB*, *CYBA*, *NCF1*, and *NCF2* genes may on occasion result in normal protein expression. Therefore, in all suspected patients, mutation analysis should be offered for definitive diagnosis.

CONCLUSIONS

We here demonstrated the diagnostic utility of flow cytometric analysis of intracellular NADPH oxidase enzyme subunits in a large multicenter CGD cohort and proposed a step-by-step diagnostic algorithm for the evaluation of patients with suspected CGD. Concomitant flow cytometric analysis of NADPH oxidase enzyme subunits in patients and carrier mothers is useful in predicting the defective gene and can thus allow targeted genetic sequencing to provide rapid and cost-effective diagnosis of CGD.

Acknowledgment

This article is dedicated to the memory of Professor Mustafa Yilmaz who passed away in 2019.

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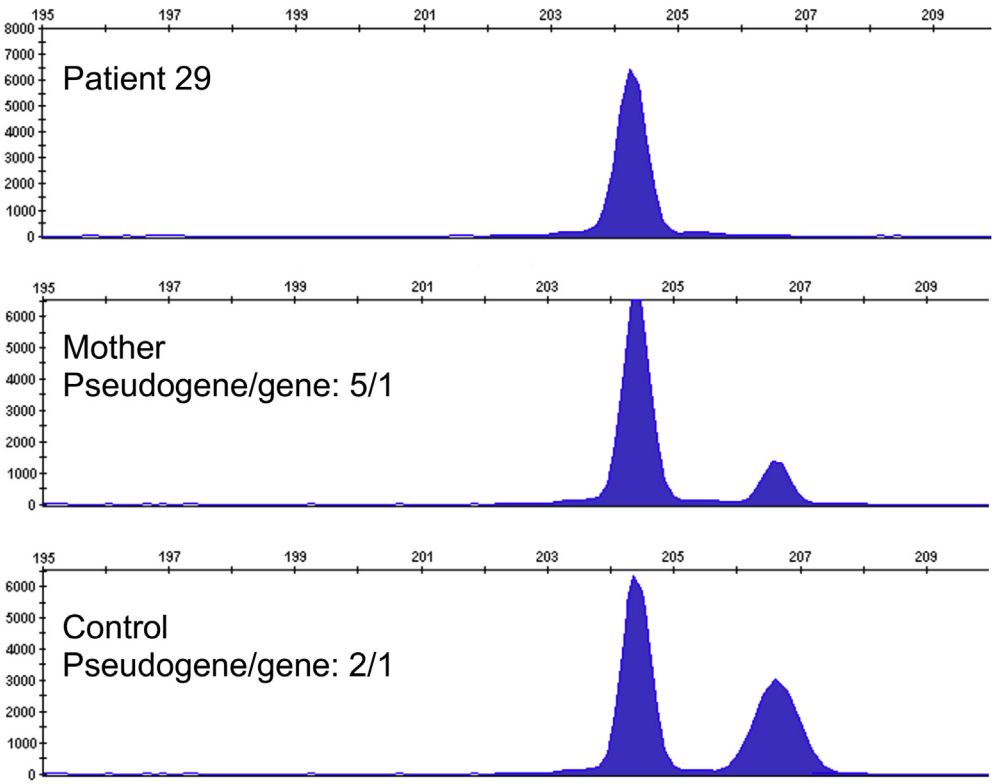

FIGURE E1. A gene-scan analysis result of P29. The patient has lost wild-type *NCF1* genes and includes only *NCF1* pseudogenes (with GT deletion in exon 2). The ratio of pseudogene/gene is 5/1 in carrier mother and 2/1 in healthy control.

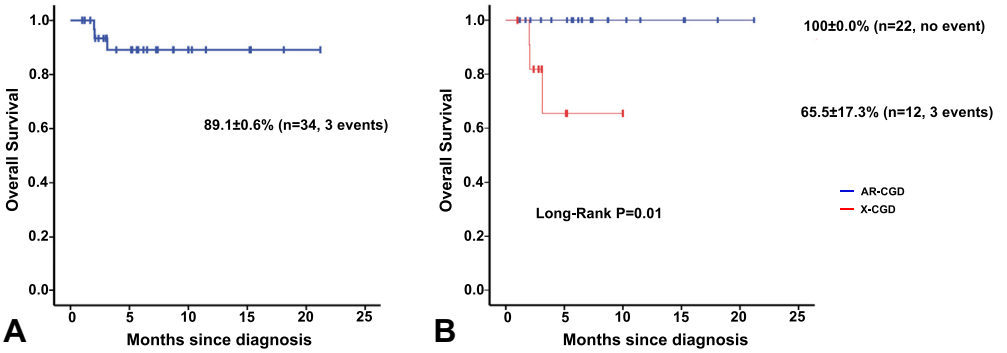

FIGURE E2. The OS rate is higher in patients with AR-CGD than in patients with X-CGD. (**A**) The Kaplan-Meier analysis shows the OS of the patients with CGD. (**B**) The comparison of OS between patients with AR-CGD and patients with X-CGD.