Current Topics in Genetics
Vol. 4, 2010

Review

# Molecular diagnosis of patients with chronic granulomatous disease

**Marianne A. Jakobsen* and Torben Barington**

Department of Clinical Immunology, Odense University Hospital, DK-5000 Odense C, Denmark

## ABSTRACT

Chronic granulomatous disease (CGD) is a rare inherited disorder of the innate immune system characterized by severe recurrent bacterial and fungal infections. The syndrome is caused by mutation in any of the five genes coding for the components of the NADPH oxidase complex. The most common form of CGD (~69 % of cases) is caused by mutations in the X-linked *CYBB* gene and is often more severe than the autosomal recessive forms that are caused by mutations in *CYBA*, *NCF-1*, *NCF-2* or *NCF-4*. The mutations detected in *CYBB*, *CYBA* and *NCF-2* are heterogeneous and often family-specific. In contrast, in more than 94 % of patients with *NCF-1* deficiency a single mutation, a GT deletion (ΔGT) of a GT-GT repeat in the start of exon 2, has been identified. This predominance is caused by recombination events involving *NCF-1* and highly homologous pseudogenes that co-localize to the same chromosomal region. Here, we give a review of the genetic background of chronic granulomatous disease, describe an algorithm and suitable methods for the molecular diagnosis and discuss the problems one may encounter in the analysis.

**KEYWORDS**: CGD, *CYBA*, *CYBB*, *NCF-1*, *NCF-2*, *NCF-4*

## INTRODUCTION

Chronic granulomatous disease (CGD) is a rare inherited disorder of the innate immune system caused by a defect of the NADPH oxidase activity in phagocytic cells, e.g. neutrophils, monocytes, macrophages and eosinophils. Microorganisms are phagocytosed normally but persist inside the cells because the activity of the NADPH oxidase is markedly diminished or completely absent resulting in very low or no production of superoxide and thereby its toxic derivatives which are important for the killing of the invading microorganisms [1, 2]. Therefore, CGD patients are susceptible to severe recurrent infections at the body surfaces e.g. the skin, the airways, the gut as well as the lymph nodes by a broad spectrum of catalase-positive bacteria and fungi [3]. The major clinical manifestations of CGD are pyoderma, pneumonia, inflammation of the gastrointestinal tract, lymphadenitis, liver abscess and osteomyelitis [4, 3, 5]. The incidence of CGD is in between 1/200 000 and 1/250 000 live-births [4].

The NADPH oxidase is a multicomponent system composed of the transmembrane heterodimeric flavocytochrome b558 composed of gp91phox and p22phox and a cytosolic complex involving p47phox, p67phox and p40phox [6, 7, 8, 9, 10, 11, 12, 13]. The activity of the complex is also dependent on the participation of either of the low molecular weight GTP-binding proteins rac 1 or rac 2 [14, 13]. The activity of the complex is spatially regulated as the cytosolic complex only translocates to the membrane upon activation. The active NADPH oxidase accepts electrons from NADPH on the cytosolic side and donates them to oxygen in the phagosome or extracellularily thereby producing superoxide [12]. Superoxide rapidly dismutates and forms hydrogen peroxide

*Corresponding author
marianne.jakobsen@ouh.regionsyddanmark.dk

which again gives rise to other reactive oxygen species (ROS). The components are encoded by five genes: gp91phox by X-linked *CYBB*, p22phox by *CYBA*, p47phox by *NCF-1*, p67phox by *NCF-2* and p40phox by *NCF-4* [9]. Mutations in any of these genes result in CGD with two thirds of cases caused by mutation in *CYBB* and one third by mutations in any of the other four genes [15, 16, 17, 18]. Similarly, a single patient with a related immunodeficiency caused by a defect in *rac-2* has been described [1]. However, the onset of symptoms, the age at diagnosis and the severity of infections are very dependent on the nature of the mutations and the resulting effect on the function of the proteins. Patients with mutation in the X-bound *CYBB* appear to have an earlier onset and a more severe clinical phenotype than patients with mutation in any of the autosomal genes [4]. Furthermore, it has been shown that the risk of immune-mediated complications may also depend on mutations in host-defense molecules like myeloperoxidase and mannan binding lectin [19].

### *CYBB*

An X-linked inheritance of CGD was proposed already in 1967 due to the high relative incidence of male patients [20]. In 1986, the gene for *CYBB* was cloned and localised to Xp21.1 [21, 22] and later, the protein product was shown to be the 90 kd gp91phox component of the flavocytochrome b558 [8]. The *CYBB* gene is around 33.5 kb and contains 13 exons [23, 24]. Mutations in *CYBB* lead to reduced or no expression of gp91phox, and thereby flavocytochrome b558. More than 358 different mutations causing CGD have been described for CYBB [1, 25] and these mutations often tend to be family-specific. The mutations include 1) nucleotide changes in the promoter region affecting the transcription of *CYBB* 2) missense and nonsense mutations causing amino acid changes and introduction of premature STOP codons, respectively, 3) mutations in the splice sites leading to the deletion of one or more exons in the mRNA 4) small deletions or insertions causing frame shifts and finally 5) large deletions comprising one or more exons from the *CYBB* gene [25, 16, 4; //bioinf.uta.fi/CYBBbase].

### *CYBA*

The expression of flavocytochrome b558 is missing in X-linked CGD as described above but also in some patients with autosomal recessive CGD. In 1990, the gene for *CYBA* was cloned and localized to chromosome 16q24 [26]. The gene spans 8.5 kb and contains 6 exons that encode a ~600 bp open reading frame. Dinauer *et al*. (1990) showed that the missing expression of flavocytochrome b558 in 3 unrelated patients with autosomal recessive CGD was caused by mutations in *CYBA*. Since, 55 pathogenic allelic mutations have been described for *CYBA* as well as 12 clinically insignificant polymorphisms [15, 27, 17]. These mutations seem to be heterogeneous and tend to be family specific like the mutations identified in *CYBB*. Approximately, 6 % of all reported CGD cases are accounted for by mutations in *CYBA* [15]. However, the genetics of CGD varies in different ethnic groups as the number of mutations in *CYBA* is roughly equally to mutations in *CYBB* in patients from Iran [28].

### *NCF-1*

In 1989, Volpp *et al*. [29] isolated and determined the sequence of the *NCF-1* mRNA. Later, the gene was localized to chromosome 7q11.23 [30]. The *NCF-1* wildtype gene is around 16 kb from ATG start codon to the polyadenylation signal and contains 11 exons. A high proportion of the gene consists of repetitive elements like SINE (short interspersed elements) and ALU repeats [31]. Exon 6 and 7 code for $SH3_A$ and exon 8 and 9 for $SH3_B$, the regions interacting with p22phox and p67phox, respectively [17]. Two pseudogenes (*ψNCF-1*) with over 98 % identity to wildtype *NCF-1* co-localize to chromosome 7q11.23 and carry a reading frame shifting deletion of GT (ΔGT) in a GT-GT repeat present in the start of exon 2 in the functional gene. Besides this, only 5 exonic SNPs and 31 intronic differences separate the pseudogenes from the functional gene [31, 32]. The most common form of autosomal recessive CGD is caused by mutation in *NCF-1* and accounts for around 20 % of all reported CGD cases [15]. In the group of patients with p47-phox deficiency, the ΔGT deletion in *NCF-1* has been identified in more than 94 % of patients [1, 33] and probably arises by recombination events

between *NCF-1* and ψ*NCF-1* [34, 35]. The existence of these highly homologous genes and the fact that transcripts of both *NCF-1* and ψ*NCF-1* are present in normal individuals [31] make the identification of other mutations than ΔGT in *NCF-1* very difficult. This is further complicated by data showing the existence of ψ*NCF-1* without the usual signature of ΔGT [36]. However, mutations besides ΔGT have been described in *NCF-1* [37, 38, 39, 40].

### *NCF-2*

The large component, p67phox, of the cytosolic part of the NADPH oxidase interacts with p47phox, p40phox and rac-2 and is encoded by *NCF-2*. In 1990, the cDNA for *NCF-2* was cloned [41] and the gene was localized to chromosome 1q25 [30]. The *NCF2* gene spans around 40 kb and contains 16 exons [42]. Exons 2 to 4 encode four 34-mer peptide repeats that form a scaffold interacting with rac-2, whereas exons 13 to 14 and exons 15 to 16 encode the regions interacting with p40phox and p47phox, respectively [17]. Around 54 different disease causing mutations and 12 clinically insignificant polymorphisms have been described for *NCF-2* [17]. The mutations are heterogeneous and often family-specific like the mutations identified in *CYBA* and *CYBB*. Mutation of *NCF-2* is the rarest form of autosomal recessive CGD and accounts for approximately 5 % of all cases [15]. However, the prevalence of *NCF-2* mutant families is higher in studies from Turkey, which is likely explained by the high incidence of consanguineous marriages [43].

### *NCF-4*

In 1993, p40phox was identified as the third SH3-containing protein in the activating cytosolic part of the NADPH oxidase complex and a 1054 bp cDNA was cloned [9]. The *NCF-4* gene is localised on chromosome 22q13.1. The gene is around 17 kb and includes 8 exons that encode a protein of 339 amino acids [23]. The C-terminal part of *NCF-4* codes for the region binding to p67phox [17]. A splice variant encoding a protein of 348 amino acids has been identified where the C-terminal residues 245-348 are different from the known p40phox [44]. The function of the isoform of p40-phox is unknown. The isoform does not interact with p67-phox and it is only weakly expressed in myeloid cells [44]. Only recently, the first patient with a mutation in *NCF-4* causing a defect in the NADPH oxidase activity was identified [18]. The patient is compound heterozygous for a frame shift mutation causing a premature stop codon on one allele and a missense mutation (R105Q) on the other allele [18].

## Molecular diagnosis of chronic granulomatous disease

The molecular diagnosis of patients suspected for CGD should start with a functional test for NADPH oxidase activity in neutrophils and monocytes. The nitroblue tetrazolium test (NBT) is now gradually being replaced by the more sensitive dihydrorhodamine (DHR) test in which cells after uptake of dihydrorhodamine and stimulation by phorbol myristate acetate (PMA) oxidize the compound to the fluorochrome rhodamine which may be quantified by flow cytometry [45]. Oxidation requires hydrogen peroxide produced by the NADPH oxidase, but also peroxidases are needed and in case of a negative result deficiency of myeloperoxidase should be excluded [46]. Almost all CGD patients will have no formation of rhodamine compared with an unstimulated control, but a few patients with autosomal recessive defects involving missense mutations may have some oxidation, but less than cells from normal controls [47]. Female heterozygous carriers of a CYBB mutation will show two populations - one with normal oxidation and one without oxidation - due to lyonisation of one of the X chromosomes. Usually the two populations are about equal in size, but lyonisation may be skewed and in rare situations CGD may be precipitated in heterozygous females with a very small fraction of normal phagocytes. Patients with an abnormal DHR test should be tested genetically to establish the diagnosis and to allow for genetic counselling of the family. The algorithm should consider the a priory likelihood of mutations in the five genes as well as the technical challenges associated with the detection of them, especially the detection of non-ΔGT mutations in *NCF-1*.

In the following, we present our current algorithm for the genetic workout. This consists of a fragment analysis, PCR and sequencing of the relevant genes. The fragment analysis has previously been described [40]. PCR and sequencing primers are listed in Tables 1-7 with the corresponding PCR setup. As six genes need to be examined, a flow taking into account the distribution of mutations in the different genes has been set up (Figure 1). Firstly, male patients and female patients with a broad spectrum or two tops indicative of skewed lyonisation in the DHR test are analysed for mutation in the X-bound *CYBB*. If no mutation is found or if the patient is a female with a single clearly abnormal top in the DHR test, the patients are analysed with a fragment analysis for the common ΔGT deletion in *NCF-1*. An abnormal distribution (5:1 (compound heterozygous) or 6:0 (homozygous)) of ΔGT results in a further analysis of NCF-1 mRNA and DNA, whereas an analysis showing a normal 2:1 distribution of ΔGT is followed by PCR and sequencing for *CYBA*, *NCF-2*, *NCF-1* cDNA, *NCF-1* DNA, in that order, until a genetic explanation for the immunodeficiency is found. Rarely, *NCF-4* and *rac-2* must be sequenced.

**Table 1.** Primers for *CYBB* DNA sequencing.

| | |
|---|---|
| CYBB1f | 5'-GTGATCAAATAGCTGGTTAG |
| CYBB1r | 5'-ATGGACTCAGTCTGTTTACA |
| CYBB2f | 5'-GTGTAGTGACACTGTTGAGG |
| CYBB2r | 5'-AAGTGACTGTTGCCCAGGAA |
| CYBB3f | 5'-TCTGAAGGACCTTCCTGTAC |
| CYBB3r | 5'-GGCTCATGAGTATTCTACAG |
| CYBB4f | 5'-TGCATCTCTCTGAACCTCAG |
| CYBB4r | 5'-GTGAGGACACTAGGATTGAC |
| CYBB5f | 5'-GTTCTGGAGACCCAGTTCTT |
| CYBB5r | 5'-TTGGCAAAGGAGAGGTCTTC |
| CYBB6f | 5'-GTCTGTGAGGGATGATTAGG |
| CYBB6r | 5'-ACCTCTGCTATCCTCTCACA |
| CYBB7f | 5'-GGGAGCAATAAGCTATCGCT |
| CYBB7r | 5'-GACACATGTTTTAGCTGTTCC |
| CYBB8f | 5'-GTTCCCCATTGTGACTAAGG |
| CYBB8r | 5'-GTAGGTCTTCTCTCTGTTGT |
| CYBB9f | 5'-GAACTCCTGTGTTGTCCCTA |
| CYBB9r | 5'-CCTGTGTGTTTTGTTGGAGAC |
| CYBB10f | 5'-GGAAGCACCCAATAGATACA |
| CYBB10r | 5'-TGGTCTCTGCCAACCTTAGC |
| CYBB11f | 5'-CTGTGAAATGTCCAGAGCCT |
| CYBB11r | 5'-AATGAGAGTGAATGGACCGG |
| CYBB12f | 5'-GCTTGAGAGAGATCCAGTTT |
| CYBB12r | 5'-GAATCTTCTGTTGGGCATGA |
| CYBB13f | 5'-ACAAACAGGCTGAACCACAC |
| CYBB13r | 5'-GCTCTCAAAACCATAGAGCTC |

PCR was run in 1X PCR buffer I (Applied Biosystems), 0.25 mM dNTP mix, 0.5 pmol/μl of the appropriate forward and reverse primer in a total volume of 20 μl using 10 ng/μl template and 0.025 U/μl HotStarTaq DNA polymerase (Qiagen; (Copenhagen, Denmark)). An initial denaturation at 95°C for 15 min, was followed by 35 cycles of 94°C for 60 sec, 59°C for 60 sec and 72°C for 60 sec with a final 10-min extension at 72°C. PCR primers were also used for sequencing.

**Table 2.** Primers for *CYBB* mRNA.

| | |
|---|---|
| CYBBm1f | 5'-GTCCGAGAAAGGCAAACACA |
| CYBBm1r | 5'-ACCTCTCACAGAGATACAGAA |
| CYBBm1fseq | 5'-AGTAGCACTCTCTGAACTTG |
| CYBBm1rseq | 5'-CAAGTTCAGAGAGTGCTACT |
| CYBBm2f | 5'-CAGAGAGTTTGGCTGTGCAT |
| CYBBm2r | 5'-TGAGCATTTGGCAGCACAAC |
| CYBBm2fseq | 5'-CACTGCCAGTGAAGATGTGT |
| CYBBm2rseq | 5'-ACACATCTTCACTGGCAGTG |

Synthesis of cDNA was performed in 1X PCR buffer II, 5 mM $MgCl_2$, 1 U/μl RNnase inhibitor, 0.1 mM dNTP mix, 2.5 μM random hexamer primer, 2.5 U/μl MulV reverse transcriptase (all from Applied Biosystems) and 51 μl of mRNA in a total volume of 100 μl. The reaction mixtures were incubated at 42°C for 30 min, and at 94°C for 5 min. PCR was run with PCR primers (not denoted seq) in 1X PCR buffer I (Applied Biosystems), 0.2 mM dNTP mix and 10 μl of cDNA in a total volume of 25 μl. The PCR was amplified with an initial denaturation at 94°C for 12 min, 40 cycles at 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 60 sec, with a final 7-min extension at 72°C**.** Both PCR primers and sequencing primers were used for sequencing.

### Difficulties met in the molecular analysis

*CYBB* is a large gene with 13 exons, but still it is relatively easy to sequence the exons and the surrounding splice sites. However, because

**Table 3.** Primers for *CYBA* DNA.

| | |
|---|---|
| CYBA1f | 5'-AATAGACGCCTGGTTGACAC |
| CYBA1r | 5'-ACTTCCCCACCCTGTAAGTA |
| CYBA2f | 5'-CTCTGCTCTTCACCAGTGCA |
| CYBA2r | 5'-GAGGCAAACAGCTCACTGTG |
| CYBA3f | 5'-TGAGGGTCTCACTATGTTGCC |
| CYBA3r | 5'-CAAGCACCAAAGGGTTGGTT |
| CYBA4f | 5'-TTGGTGCTTGTGGGTAAACC |
| CYBA4r | 5'-AATCCTGCACACTAGACAGC |
| CYBA5f | 5'-GCCTTGTCCTGAGACTTTGT |
| CYBA5r | 5'-AGCCTCCGCATCTTTGTCTG |
| CYBA6f | 5'-CTGAGTGGCAGTCACACCAT |
| CYBA6r | 5'-GGAAGGCGATGCTGATGTTA |

Exon 2 to 5 of *CYBA* was amplified in 1X PCR buffer I, 0.2 mM dNTP mix, 0.25 pmol/µl of the appropriate forward and reverse primers in a total volume of 50 µl using 2 ng/µl template and 0.05 U/µl AmpliTaqGold (Applied Biosystems). An initial denaturation at 94°C for 12 min, 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec with a final 7-min extension at 72°C was used.

Exon 1 was amplified in 1X PCR buffer I (Applied Biosystems), 0.5 µl DMSO, 0.25 mM dNTP mix, 0.25 pmol/µl forward and reverse primer in a total volume of 20 µl using 2.5 ng/µl template and 0.05 U/µl HotStar Taq DNA polymerase (Qiagen). An initial denaturation at 95°C for 15 min, 40 cycles of 95°C for 60 sec, 52°C for 60 sec and 72°C for 1 min 50 sec with a final 10-min extension at 72°C was used.

Similar, the GC rich exon 6 was amplified in 1X PCR buffer I (Applied Biosystems), 0.5 µl DMSO, 0.25 mM dNTP mix, 0.25 pmol/µl forward and reverse primer in a total volume of 20 µl using 2.5 ng/µl template and 0.05 U/µl HotStar Taq DNA polymerase (Qiagen). An initial denaturation at 95°C for 15 min, 40 cycles of 95°C for 60 sec, 56°C for 60 sec and 72°C for 3 min with a final 10-min extension at 72°C was used.

several mutations in the promoter region and intron exist affecting transcription and splicing, respectively, and because several large deletions of more than 1 exon have been described [16 http://bioinf.uta.fi/CYBBbase/], *CYBB* mRNA should also be analysed to make sure that these mutations will also be discovered in female patients.

*CYBA* is a small gene with only 6 exons. However, exon 1 and 6 are very GC rich and

**Table 4.** Primers for *NCF-1* DNA.

| | |
|---|---|
| NCF1_1f | 5'-TTTAAGGCGCAGCCTGGAAGT |
| NCF1_1r | 5'-GTAGATCCAGGCTCAAAGAC |
| 2LB2 | 5'-GTGCACACAGCAAAGCCTCT* |
| 2RB2 | 5'-CTAAGGTCCTTCCCAAAGGGT* |
| NCF1_3f | 5'-ACGTTTGTGCCCTTTCTGCA |
| NCF1_3r | 5'-ACATGAGGTGTTCAGAGTGG |
| 3L | 5'-CCAATCTCGTGCTTTTCCAA* |
| 3R | 5'-GCCAATGACCCCCTGACA* |
| 4L | 5'-CCCTCTCGGGCTTGACCT* |
| 4R3 | 5'-GCAAAACACAGAAAGTCCCA* |
| NCF1_5f | 5'-TTGGGAAGTTCTTCTGCAGG |
| NCF1_5r | 5'-CCAATTCTTCAGCAGGGTAG |
| NCF1_6f | 5'-AGCAGAGACTCAAGATGCCA |
| NCF1_6r | 5'-CATTTAGGGCCCCTAACAGA |
| NCF1_7f | 5'-GAGCATTGGACATCAGTGTG |
| NCF1_7r | 5'-GACTCCAAAGCAGTCACTCA |
| NCF1_8f | 5'-AGTGCCTGGAGGAAGGCCA |
| NCF1_8r | 5'-TCCCTGCTACTGAGATGGAA |
| NCF1_9f | 5'-TTCAGAGAGAGCCCTGAAAC |
| NCF1_9r | 5'-CAAGCTATGGCCTAGACAGA |
| NCF1_10f | 5'-GTGGAAAACCGCCCAGGCT |
| NCF1_10r | 5'-CTCCCTCTGATTCCGCCCT |
| NCF1_11f | 5'-GAGCAGGAGTTGGAGAACG |
| NCF1_11r | 5'-AGACGCCAGGCTCTATAGAA |

*Same set of primers are used for PCR and sequencing. PCR and sequencing are performed as described in [40].

**Table 5.** Primers for functional *NCF-1* mRNA.

| | |
|---|---|
| NCFm131f | 5'-AGCCAGCACTATGTGTACAT |
| NCFm1325r | 5'-AGACGCCAGGCTCTATAGAAC |
| NCFm481fseq | 5'-GACATACTTGATGCCCAAAG |
| NCFm500rseq | 5'-CTTTGGGCATCAAGTATGTC |
| NCFm911fseq | 5'-CAAAAGTCAGGGCAAGACGT |
| NCF930rseq | 5'-ACGTCTTGCCCTGACTTTTG |

The two first primers are used for both PCR and sequencing. Primers denoted seq. are only used for sequencing.
PCR and sequencing are performed as described in [40].

**Table 6.** Primers for *NCF-2* DNA.

| | |
|---|---|
| NCF2_1f | 5'-CTACTCATCTGGCCCAGAAA |
| NCF2_1r | 5'-TCCGAAATGCAATGGGGTTG |
| NCF2_2f | 5'-GAATGCACACCATTGTCCCA |
| NCF2_2r | 5'-CTTCTGCCTCACCTGCTGAT |
| NCF2_3f | 5'-TACTTGGACTCAGGGTTGCT |
| NCF2_3r | 5'-AACTGTCAGCCATCCATCCA |
| NCF2_4f | 5'-CCAGGCAACATTCTCAGAGT |
| NCF2_4r | 5'-GTGTTACCCAGACTCACAGA |
| NCF2_5f | 5'-CCAAGGGTTCCTACCTTAAC |
| NCF2_5r | 5'-CTTTCAGCCAAACCTCTGTC |
| NCF2_6f | 5'-GCTGAAATCCTAATCCAGAGG |
| NCF2_6r | 5'-TCAGGCAACTCAGCACACAT |
| NCF2_7f | 5'-GACAGTGTTCTCATCAGTCAG |
| NCF2_7r | 5'-GAAGAAGCCTGACATTCCAG |
| NCF2_8f | 5'-ATCTCGGCAGTTGCAGTTAG |
| NCF2_8r | 5'-AAGAAGGCAGCAGATACTGC |
| NCF2_9f | 5'-TGCAGACCAGCCAAGTTCTT |
| NCF2_9r | 5'-TAGCTGGTGCCATCTCAAGG |
| NCF2_10f | 5'-GACGTTATCTGCATGTGGCT |
| NCF2_10r | 5'-GCTGCGTGGTCATTAGCTGA |
| NCF2_11f | 5'-GCTCCCTAGAAGCAGAGACT |
| NCF2_11r | 5'-GTAAGGTAGCAACTGGTTCG |
| NCF2_12f | 5'-GAGAGAAAGCCTCAGACACT |
| NCF2_12r | 5'-TGTTGAGGAAGTGGCTCAGT |
| NCF2_13f | 5'-CTACACAAGGCACTGATCAC |
| NCF2_13r | 5'-TTCTTCTCTCTGCCCTTGAC |
| NCF2_14f | 5'-TCTGTGTGGAATAGCCAGAC |
| NCF2_14r | 5'-GTCTCAGTACAGTATACAGCA |

PCR was run in 1X PCR buffer I, 0.2 mM dNTP mix, 0.25 pmol/µl of the appropriate forward and reverse primers in a total volume of 50 µl using 2 ng/µl template and 0.05 U/µl AmpliTaqGold (Applied Biosystems). An initial denaturation at 94°C for 12 min, 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec with a final 7-min extension at 72°C was used. PCR primers were also used for sequencing.

**Table 7.** Primers for *NCF-4* DNA.

| | |
|---|---|
| NCF4_121f | 5'-GAAGTGGATTCCTGCAAACC |
| NCF4_540r | 5'-TGTTGATCAGGGACTGCAGA |
| NCF4_3171f | 5'-GAGAGACGAATGTTGGCTTC |
| NCF4_3507r | 5'-GTCCTGTTGAGAACCACTGA |
| NCF4_4041f | 5'-ACCCTTCTGCATCCTTATCC |
| NCF4_4370r | 5'-TGGAAGGAAGGAGAGTCAGC |
| NCF4_6460f | 5'-GATGAAAGTTGGATGGTGCC |
| NCF4_6790r | 5'-AAGCCATACCCAGGGATGGT |
| NCF4_9551f | 5'-TAAAGAGAGGAGGGCTGATG |
| NCF4_9957r | 5'-CTCCAAGCCTGAACATGACT |
| NCF4_10752f | 5'-AGGAGCAATTGCAGCCACAT |
| NCF4_11100r | 5'-TGAGGCTGTAACCCCTTCCT |
| NCF4_11461f | 5'-TGTAAGGCACTTCTATAGGA |
| NCF4_11750r | 5'-TCCACCCTCACCTCTATCTT |
| NCF4_14727f | 5'-TTAGAAGTGACCAGCTCAGC |
| NCF4_15425r | 5'-CAGCACCTTAATACTAGTGC |
| NCF4-exon89rseq | 5'-TGGAGTAAGGAGGCATCAG |
| NCF4-exon89fseq | 5'-GGCTGCGTTGCTACTACTA |
| NCF4_exon10fny | 5'-AGATTACTTGGATGACACG |
| NCF4_17330r | 5'-TAAATGACGCCCTGTGGATG |

PCR was run in 1X PCR buffer I, 0.2 mM dNTP mix, 0.25 pmol/µl of the appropriate forward and reverse primers in a total volume of 25 µl using 2 ng/µl template and 0.05 U/µl AmpliTaqGold (Applied Biosystems). An initial denaturation at 96°C for 12 min, 40 cycles of 96°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec with a final 7-min extension at 72°C was used. PCR primers were also used for sequencing.

can be difficult to sequence. By adding DMSO in both the PCR and sequencing reactions, fine sequencing results are obtained.

As already mentioned, the analysis of mutations in *NCF-1* is complicated by the presence of two pseudogenes (*ψNCF-1*) almost 98 % identical to the normal gene with a reading frame shifting ΔGT deletion in the start of exon 2 as the most distinct difference [31]. Noack *et al*, 2001 [39] described an approach using long range PCR of genomic DNA with primers sensitive to ΔGT in exon 2 in order to avoid amplification of the pseudogenes. This approach, however, may misinterpret mutations in pseudogenes without the common ΔGT signature as CGD causing mutations [36]. Therefore, we have chosen to combine a fragment analysis previously described by Dekker *et al*, 2001 [48] with a PCR targeting *NCF-1* mRNA [40]. In the fragment analysis both NCF-1 and pseudogene genomic DNA are amplified, but may be separated due to the length

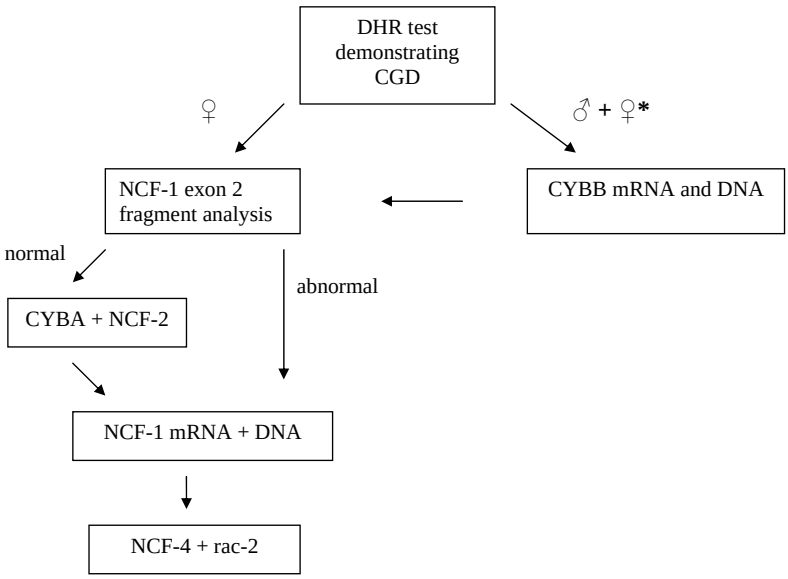


**Figure 1. The flow for molecular diagnosis of CGD**. Only patients with abnormal DHR test are subjected to genetic analysis. Male patients are analysed for mutation in the X-bound *CYBB* while only female patients with a fraction of cells with normal oxidation in the DHR test go on to this analysis. The next step is to analyse for the common ΔGT deletion in *NCF-1*. If an abnormal ratio of ΔGT-containing to non-ΔGT-containing loci is detected, *NCF-1* mRNA and DNA are examined. In contrast, an analysis showing a normal 2:1 distribution of ΔGT is followed by PCR and sequencing of first *CYBA* and *NCF-2* and then by *NCF-1* cDNA and DNA sequencing. If no mutations have been detected in these four genes, *NCF-4* and *rac-2* must be sequenced. In any case, the algorithm stops when mutations that explain the clinical condition are found.
*Female patients with two tops indicative of skewed lyonisation

difference caused by the deletion. The fragment analysis will thus give the ratio of genes containing the GTGT repeat contra genes with ΔGT, thereby ensuring that the presence of pseudogenes without the ΔGT signature will be discovered from the start of the analysis. Furthermore, patients compound heterozygous for the ΔGT mutation are easily detected. However, some mutations in *NCF-1* may cause nonsense mediated decay of the mRNA making it difficult to amplify the full length *NCF-1* mRNA in the following reverse transcription PCR. This problem may by overcome by cloning the PCR amplicon followed by sequencing of 8-16 clones [40].

## CONCLUSION

Chronic granulomatous disease is a rare inherited immunodeficiency cause by mutation in any of the five genes encoding the components of the NADPH oxidase complex making the molecular diagnosis complex. Moreover, the possibility for uneven lyonisation of the X chromosomes and the presence of pseudogenes further complicate the diagnostic unravelling of *CYBB* and *NCF-1* mutations, respectively. The algorithm presented here constitutes a rational way of solving these problems analysing the genes according to the prevalence of mutations and identifying disease causing mutations with a minimum of PCR amplification and sequencing.

## REFERENCES

1. Heyworth, P. G., Cross, A. R., and Curnutte, J. T. 2003, Curr. Opin. Immunol., 15, 578.
2. Jurkowska, M., Bernatowska, E., and Bal, J. 2004, Arch. Immunol. Ther. Exp. (Warsz.)., 52, 113.
3. Seger, R. A. 2008, Br. J. Haematol., 140, 255.

4. Winkelstein, J. A., Marino, M. C., Johnston, R. B. Jr., Boyle, J., Curnutte, J., Gallin, J. I., Malech, H. L., Holland, S. M., Ochs, H., Quie, P., Buckley, R. H., Foster, C. B., Chanock, S. J., and Dickler, H. 2000, Medicine (Baltimore), 79, 155.
5. Marciano, B. E., Rosenzweig, S. D., Kleiner, D. E., Anderson, V. L., Darnell, D. N., Anaya-O'Brien, S., Hilligoss, D. M., Malech, H. L., Gallin, J. I., and Holland, S. M. 2004, Pediatrics, 114, 462.
6. Segal, A. W. 1987, Nature, 326, 88.
7. Nunoi, H., Rotrosen, D., Gallin, J. I., and Malech, H. L. 1988, Science, 242, 1298.
8. Dinauer, M. C., Orkin, S. H., Brown, R., Jesaitis, A. J., and Parkos, C. A. 1987, Nature, 327, 717.
9. Wientjes, F. B., Hsuan, J. J., Totty, N. F., and Segal, A. W. 1993, Biochem. J., 296 (Pt 3), 557.
10. Lomax, K. J., Leto, T. L., Nunoi, H., Gallin, J. I., and Malech, H. L. 1989, Science, 245, 409.
11. Nauseef, W. M. 2004, Histochem. Cell Biol., 122, 277.
12. Roos, D. 1996, Immunol. Today., 17, 517.
13. Heyworth, P. G., Bohl, B. P., Bokoch, G. M., and Curnutte, J. T. 1994, J. Biol. Chem., 269, 30749.
14. Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G., and Segal, A. W. 1991, Nature, 353, 668.
15. Cross, A. R., Noack, D., Rae, J., Curnutte, J. T., and Heyworth, P. G. 2000, Blood Cells Mol. Dis., 26, 561.
16. Heyworth, P. G., Curnutte, J. T., Rae, J., Noack, D., Roos, D., van, K. E., and Cross, A. R. 2001, Blood Cells Mol. Dis., 27, 16.
17. Roos, D., Kuhns, D. B., Maddalena, A., Bustamante, J., Kannengiesser, C., de, B. M., van, L. K., Koker, M. Y., Wolach, B., Roesler, J., Malech, H. L., Holland, S. M., Gallin, J. I., and Stasia, M. J. 2010, Blood Cells Mol. Dis., 45, 246.
18. Matute, J. D., Arias, A. A., Wright, N. A., Wrobel, I., Waterhouse, C. C., Li, X. J., Marchal, C. C., Stull, N. D., Lewis, D. B., Steele, M., Kellner, J. D., Yu, W., Meroueh, S. O., Nauseef, W. M., and Dinauer, M. C. 2009, Blood, 114, 3309.
19. Foster, C. B., Lehrnbecher, T., Mol, F., Steinberg, S. M., Venzon, D. J., Walsh, T. J., Noack, D., Rae, J., Winkelstein, J. A., Curnutte, J. T., and Chanock, S. J. 1998, J. Clin. Invest., 102, 2146.
20. Windhorst, D. B., Holmes, B., and Good, R. A. 1967, Lancet., 1, 737.
21. Baehner, R. L., Kunkel, L. M., Monaco, A. P., Haines, J. L., Conneally, P. M., Palmer, C., Heerema, N., and Orkin, S. H. 1986, Proc. Natl. Acad. Sci. USA., 83, 3398.
22. Royer-Pokora, B., Kunkel, L. M., Monaco, A. P., Goff, S. C., Newburger, P. E., Baehner, R. L., Cole, F. S., Curnutte, J. T., and Orkin, S. H. 1986, Nature, 322, 32.
23. 2004, Nature, 431, 931.
24. Ross, M. T., Grafham, D. V., Coffey, A. J., Scherer, S., McLay, K., Muzny, D., Platzer, M., Howell, G. R., Burrows, C., Bird, C. P., Frankish, A., Lovell, F. L., Howe, K. L., Ashurst, J. L., Fulton, R. S., Sudbrak, R., Wen, G., Jones, M. C., Hurles, M. E., Andrews, T. D., Scott, C. E., Searle, S., Ramser, J., Whittaker, A., Deadman, R., Carter, N. P., Hunt, S. E., Chen, R., Cree, A., Gunaratne, P., Havlak, P., Hodgson, A., Metzker, M. L., Richards, S., Scott, G., Steffen, D., Sodergren, E., Wheeler, D. A., Worley, K. C., Ainscough, R., Ambrose, K. D., nsari-Lari, M. A., Aradhya, S., Ashwell, R. I., Babbage, A. K., Bagguley, C. L., Ballabio, A., Banerjee, R., Barker, G. E., Barlow, K. F., Barrett, I. P., Bates, K. N., Beare, D. M., Beasley, H., Beasley, O., Beck, A., Bethel, G., Blechschmidt, K., Brady, N., Bray-Allen, S., Bridgeman, A. M., Brown, A. J., Brown, M. J., Bonnin, D., Bruford, E. A., Buhay, C., Burch, P., Burford, D., Burgess, J., Burrill, W., Burton, J., Bye, J. M., Carder, C., Carrel, L., Chako, J., Chapman, J. C., Chavez, D., Chen, E., Chen, G., Chen, Y., Chen, Z., Chinault, C., Ciccodicola, A., Clark, S. Y., Clarke, G., Clee, C. M., Clegg, S., Clerc-Blankenburg, K., Clifford, K., Cobley, V., Cole, C. G., Conquer, J. S., Corby, N., Connor, R. E., David, R., Davies, J., Davis, C., Davis, J., Delgado, O., Deshazo, D., Dhami, P., Ding, Y., Dinh, H., Dodsworth, S., Draper, H., Dugan-Rocha, S., Dunham, A., Dunn, M.,

Durbin, K. J., Dutta, I., Eades, T., Ellwood, M., Emery-Cohen, A., Errington, H., Evans, K. L., Faulkner, L., Francis, F., Frankland, J., Fraser, A. E., Galgoczy, P., Gilbert, J., Gill, R., Glockner, G., Gregory, S. G., Gribble, S., Griffiths, C., Grocock, R., Gu, Y., Gwilliam, R., Hamilton, C., Hart, E. A., Hawes, A., Heath, P. D., Heitmann, K., Hennig, S., Hernandez, J., Hinzmann, B., Ho, S., Hoffs, M., Howden, P. J., Huckle, E. J., Hume, J., Hunt, P. J., Hunt, A. R., Isherwood, J., Jacob, L., Johnson, D., Jones, S., de Jong, P. J., Joseph, S. S., Keenan, S., Kelly, S., Kershaw, J. K., Khan, Z., Kioschis, P., Klages, S., Knights, A. J., Kosiura, A., Kovar-Smith, C., Laird, G. K., Langford, C., Lawlor, S., Leversha, M., Lewis, L., Liu, W., Lloyd, C., Lloyd, D. M., Loulseged, H., Loveland, J. E., Lovell, J. D., Lozado, R., Lu, J., Lyne, R., Ma, J., Maheshwari, M., Matthews, L. H., McDowall, J., McLaren, S., McMurray, A., Meidl, P., Meitinger, T., Milne, S., Miner, G., Mistry, S. L., Morgan, M., Morris, S., Muller, I., Mullikin, J. C., Nguyen, N., Nordsiek, G., Nyakatura, G., O'Dell, C. N., Okwuonu, G., Palmer, S., Pandian, R., Parker, D., Parrish, J., Pasternak, S., Patel, D., Pearce, A. V., Pearson, D. M., Pelan, S. E., Perez, L., Porter, K. M., Ramsey, Y., Reichwald, K., Rhodes, S., Ridler, K. A., Schlessinger, D., Schueler, M. G., Sehra, H. K., Shaw-Smith, C., Shen, H., Sheridan, E. M., Shownkeen, R., Skuce, C. D., Smith, M. L., Sotheran, E. C., Steingruber, H. E., Steward, C. A., Storey, R., Swann, R. M., Swarbreck, D., Tabor, P. E., Taudien, S., Taylor, T., Teague, B., Thomas, K., Thorpe, A., Timms, K., Tracey, A., Trevanion, S., Tromans, A. C., d'Urso, M., Verduzco, D., Villasana, D., Waldron, L., Wall, M., Wang, Q., Warren, J., Warry, G. L., Wei, X., West, A., Whitehead, S. L., Whiteley, M. N., Wilkinson, J. E., Willey, D. L., Williams, G., Williams, L., Williamson, A., Williamson, H., Wilming, L., Woodmansey, R. L., Wray, P. W., and Yen, J. 2005, Nature, 434, 325.

25. Kannengiesser, C., Gerard, B., El, B. J., Henri, D., Kroviarski, Y., Chollet-Martin, S., Gougerot-Pocidalo, M. A., Elbim, C., and Grandchamp, B. 2008, Hum. Mutat., 29, E132.
26. Dinauer, M. C., Pierce, E. A., Bruns, G. A., Curnutte, J. T., and Orkin, S. H. 1990, J. Clin. Invest., 86, 1729.
27. Rae, J., Noack, D., Heyworth, P. G., Ellis, B. A., Curnutte, J. T., and Cross, A. R. 2000, Blood., 96, 1106.
28. Teimourian, S., de, B. M., and Roos, D. 2010, J. Clin. Immunol., 30, 587.
29. Volpp, B. D., Nauseef, W. M., Donelson, J. E., Moser, D. R., and Clark, R. A. 1989, Proc. Natl. Acad. Sci. USA, 86, 7195.
30. Francke, U., Hsieh, C. L., Foellmer, B. E., Lomax, K. J., Malech, H. L., and Leto, T. L. 1990, Am. J. Hum. Genet., 47, 483.
31. Chanock, S. J., Roesler, J., Zhan, S., Hopkins, P., Lee, P., Barrett, D. T., Christensen, B. L., Curnutte, J. T., and Gorlach, A. 2000, Blood Cells Mol. Dis., 26, 37.
32. Gorlach, A., Lee, P. L., Roesler, J., Hopkins, P. J., Christensen, B., Green, E. D., Chanock, S. J., and Curnutte, J. T. 1997, J. Clin. Invest., 100, 1907.
33. Casimir, C. M., Bu-Ghanim, H. N., Rodaway, A. R., Bentley, D. L., Rowe, P., and Segal, A. W. 1991, Proc. Natl. Acad. Sci. USA., 88, 2753.
34. Roesler, J., Curnutte, J. T., Rae, J., Barrett, D., Patino, P., Chanock, S. J., and Goerlach, A. 2000, Blood, 95, 2150.
35. Vazquez, N., Lehrnbecher, T., Chen, R., Christensen, B. L., Gallin, J. I., Malech, H., Holland, S., Zhu, S., and Chanock, S. J. 2001, Exp. Hematol., 29, 234.
36. Heyworth, P. G., Noack, D., and Cross, A. R. 2002, Blood, 100, 1845.
37. de, B. M., Singh, V., Dekker, J., Di, R. M., Goldblatt, D., and Roos, D. 2002, Prenat. Diagn., 22, 235.
38. Roos, D., de, B. M., Koker, M. Y., Dekker, J., Singh-Gupta, V., Ahlin, A., Palmblad, J., Sanal, O., Kurenko-Dept, Jolles, S., and Wolach, B. 2006, Hum. Mutat., 27, 1218.
39. Noack, D., Rae, J., Cross, A. R., Ellis, B. A., Newburger, P. E., Curnutte, J. T., and Heyworth, P. G. 2001, Blood, 97, 305.
40. Jakobsen, M. A., Pedersen, S. S., and Barington, T. 2009, Genet. Test Mol. Biomarkers, 13, 505.

41. Leto, T. L., Lomax, K. J., Volpp, B. D., Nunoi, H., Sechler, J. M., Nauseef, W. M., Clark, R. A., Gallin, J. I., and Malech, H. L. 1990, Science, 248, 727.
42. Kenney, R. T., Malech, H. L., Epstein, N. D., Roberts, R. L., and Leto, T. L. 1993, Blood, 82, 3739.
43. Koker, M. Y., Sanal, O., van, L. K., de, B. M., Metin, A., Patiroglu, T., Ozgur, T. T., Tezcan, I., and Roos, D. 2009, Eur. J. Clin. Invest., 39, 942.
44. Hasebe, T., Someya, A., and Nagaoka, I. 1999, FEBS Lett., 455, 257.
45. O' Gorman, M. R. and Corrochano, V. 1995, Clin. Diagn. Lab Immunol., 2, 227.
46. Mauch, L., Lun, A., O' Gorman, M. R., Harris, J. S., Schulze, I., Zychlinsky, A., Fuchs, T., Oelschlagel, U., Brenner, S., Kutter, D., Rosen-Wolff, A., and Roesler, J. 2007, Clin. Chem., 53, 890.
47. Yu, G., Hong, D. K., Dionis, K. Y., Rae, J., Heyworth, P. G., Curnutte, J. T., and Lewis, D. B. 2008, Clin. Immunol., 128, 117.
48. Dekker, J., de, B. M., and Roos, D. 2001, Exp. Hematol., 29, 1319.