

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Munthe-Fog L, Hummelshøj T, Honoré C, Madsen HO, Permin H, Garred P. Immunodeficiency associated with *FCN3* mutation and ficolin-3 deficiency. *N Engl J Med* 2009;360:2637-44.

## Supplement

### Pyrosequencing

Initially, a fragment of the *FCN3* exon 5, covering the frame shift mutation at position *FCN3+1637delC*, was amplified by PCR using the following primers: 5'-CGCCCCCTCGGTGTCCA-3' and 5'-biotin-CCACCTTGAGCGGCTGGTA-3'.

PCR amplifications were carried out in 50 µl volumes containing: ~50 ng genomic DNA, 0.25 µM of each primer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris·HCl, pH 8.4, and 0.4 units of Platinum Taq DNA polymerase (Invitrogen).

The PCR reactions were performed at following cycle conditions; 2m94°C, 35x (30s94°C, 60s58°C, 60s72°C), 5m72°C. The amplicate (7 µl) were analyzed on a 2% agarose gel containing ethidium bromide. The remaining product was immobilized to Streptavidin conjugated sepharose beads (cat. nr. 17-5113-01, GE Healthcare) in binding buffer (10 mM Tris, 2 M NaCl<sub>2</sub>, 1 mM EDTA, 0.1% Tween 20), purified and denatured using NaOH 0.5 M, 70% EtOH and washing buffer (10 mM Tris-Acetate, pH 7.6) all performed with a PyroMark Vacuum Prep Workstation (Biotage). Biotinylated single strands were released into 40 µl of annealing buffer (0.43 g MgAc, 2.42 g Tris in 1000 ml Milli-Q) containing 40 µM of the sequencing primer (5'-CCATGTCACAAAAGACTG-3') and heated to 80°C for 2 min. Sequencing started with position -5 relative to the *FCN3+1637delC* position. The genotyping was performed using the PSQ 96MA SNP software version 2.02 (Biotage). Samples generating inconclusive sample reports were rerun by Pyrosequencing or sequenced by traditional Sanger method.

## **Polymerase chain reaction and DNA sequencing**

Direct sequencing of the promoter region of the *FCN3*, spanning from position -1 to -1092 bp, as well as all eight exons and intron-exon boundary sequences were performed on genomic DNA templates from all samples suspected for carrying the *FCN3+1637delC* frame shift mutation. Samples generating inconclusive Pyrosequencing reports were sequenced in exon 5. The promoter regions were amplified by PCR in one PCR fragment and the coding regions of each exons were amplified using primers designed from flanking intronic or untranslated sequences. Each fragment was amplified by using a single primer set (supplement table 1), where the forward primers contained a 5'-T7 sequence (5'-ttatacgactcacta-3') except for the *FCN3* promoter region, which were sequenced with internal sequencing primers. PCR amplifications were carried out in 20- $\mu$ l volumes containing: 50 ng genomic DNA, 0.25 $\mu$ M of each primer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris·HCl, pH 8.4, and 0.4 units of Platinum Taq DNA polymerase (Invitrogen). The PCR reactions were performed at the following cycling parameters: 2min94°C, 15 cycles (30sec94°C, 60sec62°C, 60sec72°C), 15 cycles (30sec94°C, 60sec58°C, 60sec72°C), 5min72°C and were sequenced in the forward direction using the ABI BigDye cycle sequencing terminator kit, V 3.1 (Applied Biosystems, Foster City, CA) according to the protocol using 5'-biotinylated sequence primers. Sequence reactions were purified on the PyroMark Vacuum Prep Workstation (Biotage) using streptavidin conjugated sepharose beads (GE Healthcare). Sequence analysis was performed on an ABI Prism 3100 Genetic Analyser (Applied

Biosystems). The resulting DNA sequences were aligned using BioEdit software, and DNA polymorphisms were confirmed visually from sequence electropherograms. All DNA variations, which were only observed in only one individual, were confirmed by new PCR followed by resequencing.

**Supplemental Table 1: FCN3 primers**

The forward primary PCR primers contain a 5' T7 sequence (taatacgactcacta) except for the FCN3 promoter #1 which is sequenced with intern sequencing primers listed below (Seq FCN3-A-D)

**FCN3 primary PCR primers**

	<u>Forward</u>	<u>Reverse</u>
Promoter #1	5'-gccagaatcactttctaagtgc-3'	5'-ccaagcaggagaagccac-3'
Exon 1	5'-ctgaaggaggaaatactcca-3'	5'-gcagagcccagattatgaaac-3'
Exon 2	5'-gttcataatctggctctgc-3'	5'-aaattgctactttcctgccttc-3'
Exon 3	5'-ctctggctccaagtctctg-3'	5'-ccaagcagagatcccacc-3'
Exon 4	5'-cggctccactggtgctc-3'	5'-tgtggggaggatcttgcc-3'
Exon 5	5'-ggccaagatcctcccaca-3'	5'-tctgggtggttctggctcc-3'
Exon 6	5'-caagggaatgtaggtcatag-3'	5'-caggatggcagacagtaacc-3'
Exon 7	5'-ggttactgtctgccatctg-3'	5'-acagaggagacaggattgcc-3'
Exon 8	5'-attatatctccaaagtgccag-3'	5'-ggacaggcaagcagaggtg-3'

**Biotinylated sequencing primers**

T7 primer	5'-taatacgactcacta-3'
Seq FCN3-A	5'-gatgttgactccatcctgg-3'
Seq FCN3-B	5'-ggagttcactctgttgccc-3'
Seq FCN3-C	5'-ggttcaccatgtggcc-3'
Seq FCN3-D	5'-ctatgaatagtgacaactgctacc-3'

**Ficolin-3 Serum Measurement**

Serum Ficolin-3 concentrations were measured in randomly selected patients with the wildtype genotype at position +1637 as well as in all the heterozygous

and mutant homozygous patients. Briefly, microtiter plates (Maxisorb, Nunc) were coated with 100µl monoclonal anti-Ficolin-3 antibody (FCN334, 2.5 µg/ml) in PBS buffer by incubating at 4°C overnight<sup>6</sup>. The wells were washed three times with PBS-T and sera were diluted 1:640 with PBS containing 0.05% Tween 20 (PBS-T) and added to the plate in triplicates. A standard 2-fold dilution series (1:160–1:20480) of pooled normal human serum in PBS-T was added to the plates in triplicates. A negative control without sample was included and the plate was incubated for 3 h at 37°C. After washing 100 µl of biotinylated FCN334 (2.5 µg/ml) in PBS-T was added to the wells and incubated at 4°C overnight. HRP-conjugated streptavidin diluted 1:5000 in PBS-T was added to the wells and incubated for 1 h at 37°C. After washing, OPD substrate solution was added and the OD was measured at 490 nm.

### **Western Blotting**

Serum from the family of the index case, the index case, a normal control individual and recombinant Ficolin-3 were investigated. Serum samples were diluted 1:42 in SDS-buffer with or without reducing agent. Samples were heated for 5 min at 90°C and applied to SDS-PAGE using 3-8% Tris-acetate (non-reducing conditions) or 10% Bis-Tris (reducing conditions) gels in tris-acetate or MOPS running buffer, respectively (all from Invitrogen, Taastrup, Denmark). The separated proteins were subsequently blotted onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, United Kingdom). The membranes were blocked using a 5% skim milk solution in PBS containing 0.05% tween (PBS-T)

for 1 hour at room temperature (RT) shaking. After a brief wash in PBS-T, the membranes were incubated for 1 hour with a biotinylated polyclonal anti-Ficolin-3 antibody (R&D systems, Abingdon, United Kingdom) at RT shaking. Following incubation, the membranes were washed thoroughly in PBS-T and subsequently incubated with horseradish peroxidase HRP-conjugated streptavidin (GE Healthcare, Buckinghamshire, United Kingdom). Membranes were developed onto ECL hyperfilm (GE Healthcare, Buckinghamshire, United Kingdom) with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, USA).

### **Ficolin-2 Depletion**

A total of 200 µl serum was diluted 1:1 in hepes-buffer (25mM HEPES, 155mM NaCl, 5mM CaCl<sub>2</sub>, pH = 7.4) and 50 µl GlcNAc-agarose beads (Sigma-Aldrich, Broendby, Denmark) was added to the serum and incubated overnight end-over-end at 4°C. As a control, buffer was added instead of the beads. Following incubation, the beads were removed by centrifugation. Depletion of Ficolin-2 was confirmed using both a Ficolin-2 specific ELISA and SDS-PAGE/Western blot (data not shown). No decrease in the Ficolin-3 concentration was observed following GlcNAc-agarose incubation (data not shown).

### **Ficolin-3 dependent complement activation assay**

A 10% bovine serum albumin (BSA) solution was diluted 1:1 in a 5.4M sodium acetate solution and incubated on ice for 1 hour. During the incubation, acetic anhydride (Sigma-Aldrich, Broendby, Denmark) was added to the solution at 12

min intervals to a final conc. of 0.5mM. Following incubation, the sample was desalted with PBS-buffer using amicon ultra-15 centrifugal filter devices (Millipore, Copenhagen, Denmark). Acetylated BSA (10µg/ml) or non-treated BSA was coated in 96-well maxisorp microtiter plates (Nunc, Roskilde, Denmark) overnight at 4°C in PBS-buffer. Wells were washed thrice in hepes-buffer (25mM HEPES, 155mM NaCl, 5mM CaCl<sub>2</sub>, pH = 7.4) containing 0.05% tween (hepes-T) and blocked for 1 hour with 200µl/well hepes-T. Following blocking, the wells were incubated with serial dilutions of sera diluted in hepes-T for 30 min at 37°C and subsequently washed in hepes-T. We used a normal serum pool, serum from the Ficolin-3 deficient patient, sera deficient of C1q<sup>13</sup> or MBL<sup>14</sup> and sera depleted for ficolin-2. To reconstitute for Ficolin-3 deficiency we used recombinant Ficolin-3 produced as described<sup>15</sup>. C4 deposition was detected using a rabbit polyclonal anti-human C4 antibody (DAKO, Glostrup, Denmark) and an HRP-conjugated anti-rabbit IgG (GE healthcare, Buckinghamshire, United Kingdom). The plates were developed with OPD substrate (DAKO, glostrup, Denmark) and H<sub>2</sub>O<sub>2</sub> for 15 min and the enzymatic reaction was terminated by adding 1M H<sub>2</sub>SO<sub>4</sub> to the wells. The optical density of the wells was measured at 490nm.