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ARTICLE

# Low prevalence of *APP* duplications in Swedish and Finnish patients with early-onset Alzheimer's disease

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**Familial early-onset Alzheimer's disease with cerebral amyloid angiopathy (EOAD/CAA) was recently associated with duplications of the gene for the amyloid-$\beta$ precursor protein (*APP*). In this study, we have screened for duplications of *APP* in patients with EOAD from Sweden and Finland. Seventy-five individuals from families with EOAD and 66 individuals with EOAD without known familial inheritance were screened by quantitative PCR. On the basis of the initial results, a portion of the samples was also investigated with quantitative multiplex PCR. No duplications of *APP* were identified, whereby we conclude that this is not a common cause of EOAD in the Swedish and Finnish populations, at least not in our collection of families and cases.**
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## Introduction

Genetics is one of the major risk factors for developing Alzheimer's disease (AD). Four genes are known to be involved in AD and mutations in three of these cause autosomal dominant early-onset Alzheimer's disease (EOAD): the amyloid-$\beta$ precursor protein (*APP*) on chromosome 21,[1] presenilin 1 (*PSEN1*) on chromosome 14[2] and presenilin 2 (*PSEN2*) on chromosome 1.[3] In addition, the $\varepsilon 4$ allele of apolipoprotein E (*APOE*) on chromosome 19 increases the risk of developing late-onset sporadic AD and decreases the age at onset in a dose-dependent manner.[4] Most of these genetic variants are believed to result in increased production of the amyloid-$\beta$ (A$\beta$) peptide or a shift in the A$\beta_{40}$/A$\beta_{42}$ ratio to increase the amount of the more amyloidogenic peptide A$\beta_{42}$. Amyloid-$\beta$ is cleaved from APP by $\gamma$-secretase whose active constituent is one of the presenilin proteins[5] and *APOE* $\varepsilon 4$ has been associated with increased A$\beta$ deposition.[6]

Variability in the expression of *APP* can also cause AD. Individuals with Down's syndrome (DS, trisomy 21) usually develop AD pathology in early middle age due to an extra copy of the *APP* gene.[7] Also, two research groups have recently reported that duplications of *APP*, in the absence of trisomy 21, can cause familial EOAD with cerebral amyloid angiopathy (EOAD/CAA).[8,9]

In this study, we screened for *APP* duplications in affected subjects from Swedish and Finnish families and cases with EOAD in an effort to estimate the frequency of *APP* duplications in EOAD patients in these populations.

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## Materials and methods

### Samples

The Swedish sample set consisted of 85 patients from the Memory Clinics of Uppsala Academic Hospital and Karolinska University Hospital, Huddinge. After a quality

**Table 1** Samples analyzed for *APP* gene dose

| Cohort | Total samples | Familial EOAD | Sporadic EOAD | Age at onset ± SD |
|---|---|---|---|---|
| Swedish | 77 | 45 | 32 | 56.9 ± 6.1 |
| Finnish | 64 | 30 | 34 | 59.7 ± 5.3 |
| Total | 141 | 75 | 66 | 58.2 ± 5.9 |

control, eight samples were removed. The remaining 77 samples with age at disease onset ≤65 years (41–65 years, mean ± SD = 56.9 ± 6.1 years; 57% women) were included in a screen for *APP* duplications. All patients were clinically diagnosed as possible or probable EOAD according to NINCDS-ADRDA.[10] Autopsy was performed on 17 of these patients, 11 of whom had congophilic vessels. Forty-five patients had familial EOAD with at least one known first-degree relative affected by AD, and 32 were cases without known familial inheritance (Table 1). The Swedish sample collection included the two families with the Swedish (K670N/M671L)[11] and Arctic (E693G)[12] *APP* mutations.

The Finnish sample set consisted of 64 patients from eastern Finland, all examined at the Kuopio University Hospital, with age at disease onset ≤65 years (45–65 years, mean ± SD = 59.7 ± 5.3; 72% women). All patients fulfilled clinical criteria for probable AD according to NINCDS-ADRDA.[10] Thirty patients had familial EOAD where at least two affected first-degree relatives in two generations had been documented.[13] The remaining 34 patients had sporadic EOAD (Table 1). All samples had been screened for mutations in *APP*, *PSEN1* and *PSEN2* without positive findings.

This study was approved by the Ethics Committees of Uppsala University and Kuopio University Hospital.

### Allele quantification

Primers for *APP*1i (intron after exon 1), *APP*2ei (exon/intron border of exon 2), *APP*9ei (exon/intron border of exon 9), *APP*16i (intron after exon 16), glycogenin 2 (*GYG2*) on chromosome X and deleted in azoospermia 4 (*DAZ4*) on chromosome Y were designed using the PrimerExpress software (Applied Biosystems, Foster City, CA, USA); sequences are available upon request. Primers from within the *APP* promoter, *APP*5e (exon 5) and *APP*18i (the intron after exon 18) were used in Sleegers *et al*.[9] Primers for the control genes hemoglobin beta (*HBB*) on chromosome 11, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) on chromosome 12 and ubiquitin C (*UBC*) on chromosome 12, have previously been used in Johnson *et al*,[14] West *et al*[15] and in Sleegers *et al*,[9] respectively.

The Swedish samples were screened for gene load by quantitative PCR (qPCR) using the SYBR green chemistry on the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Richmond, CA, USA). The samples were amplified in a volume of 25 μl, containing 10 ng of genomic DNA, 2 × Power SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of each primer, using the universal thermal cycling parameters (Applied Biosystems). Each plate included triplicate DNA samples from EOAD patients, a healthy control as calibrator sample and two DS patients as positive controls. Wells without template were included as negative controls. SD were calculated for the triplicates. If the SD for a triplicate was more than 0.3, the sample was rerun. The number of gene copies was determined relative to *HBB* and normalized to the calibrator sample using the $2^{-\Delta\Delta C_t}$ method (Applied Biosystems).

The Finnish samples were screened for the presence of *APP* duplications using both qPCR and a quantitative multiplex PCR (qmPCR) assay. For qPCR, 25 ng of genomic DNA was amplified in a volume of 25 μl together with 2 × Power SYBR Green PCR Master Mix (Applied Biosystems) and 600 nM of each primer (*APP*5e or *APP*16i). Each plate included DNA samples from EOAD patients, four DS patients and four healthy controls, all analyzed in duplicates. The gene dose of *APP* was calculated using the standard curve method (Applied Biosystems), compared to *HBB* and normalized to the mean of the healthy controls. The qmPCR was performed in a multiplex PCR using *APP*5e, *APP*16i and *HBB* where all forward primers were labeled with the fluorescent dye FAM. The reaction mix contained 25 ng of DNA, primers for *APP*5e (200 nM), *APP*16i (100 nM) and *HBB* (200 nM), 10 × buffer, 10 mM dNTP and 2.5 U HotStarTaq polymerase enzyme (QIAGEN, Hilden, Germany). The number of cycles needed to reach the exponential phase was determined experimentally. Subsequently, 1 μl fluorophore-labeled PCR product was mixed with 10 μl HiDi Formamide and 0.75 μl GeneScan 600 LIZ Size Standard (Applied Biosystems). The PCR product was denatured at 95°C for 3 min and cooled on ice for 2 min. Samples were analyzed through capillary electrophoresis using an ABI 3100 (Applied Biosystems) and the results were viewed using GeneMapper v.3.0 (Applied Biosystems). The peak height was used to determine gene dose of *APP*5e and *APP*16i compared to *HBB*, and all samples were normalized to the mean of the healthy controls.

### SNP arrays

To verify a potential duplication of the *APP* gene, one sample from the Swedish cohort was run on GeneChip Human Mapping 250K Nsp Arrays (Affymetrix, Santa Clara, CA, USA) according to protocol. The Nsp array has 45 single nucleotide polymorphisms (SNPs) within *APP*. Results were analyzed with the GTYPE software (Affymetrix).

## Results

As the qPCR method is dependent on high-quality DNA, the Swedish samples were evaluated by screening with *GYG2* on chromosome X. Gene dose data were thereafter related to gender (Figure 1a). Eight samples did not

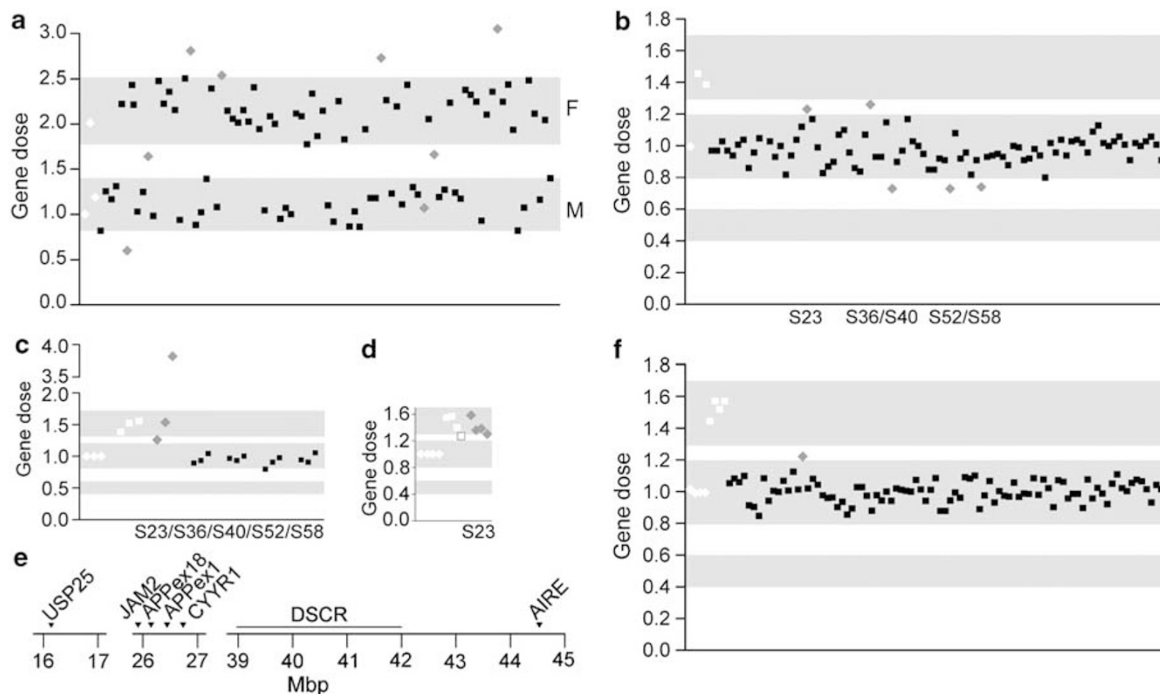


**Figure 1** Gene dose analyses. (**a**) Quality control of the Swedish samples using *GYG2* on chromosome X for correlation to gender, a value of 0.8–1.4 represents men and 1.8–2.5 represents women; (**b**) Analysis of *APP* gene dose in the Swedish cohort using primers for intron 16 of *APP*. Values between 0.4 and 0.6 correspond to a deletion, 0.8–1.2 is normal gene dose and 1.3–1.7 denotes a duplication; (**c**) follow-up analysis of five Swedish samples; primers for the promotor, intron 9 and intron 18 of *APP* were used; (**d**) one Swedish sample in a follow-up analysis of *USP25*, *JAM2*, *CYYR1* and *AIRE*; (**e**) location of the analyzed genes on chromosome 21. Down's syndrome critical region (DSCR) is located in the region of 39–42 Mbp;[16] (**f**) analysis of *APP* gene dose in the Finnish samples using primers for intron 16 of *APP*. *APP* gene dose in the Swedish cohort was determined through qPCR, compared to *HBB* and normalized to the calibrator sample using the $2^{-\Delta\Delta C_t}$ method. All samples were run in triplicates and SD were calculated. If the SD for a triplicate was more than 0.3, the sample was rerun. Number of gene copies in the Finnish cohort was determined through qmPCR, compared to *HBB* and normalized to the mean of four healthy control samples. White diamonds represent healthy controls, white squares represent Down's syndrome, gray diamonds represent samples outside the specified range and black squares are samples with values within the set gene dose range.

show the expected gene dose on chromosome X and were therefore considered unreliable. They were excluded from further analyses after confirmation of gender with *DAZ4* on chromosome Y. The remaining 77 Swedish samples were screened for *APP* gene dose using qPCR with primers for *APP*16i (Figure 1b) and *APP*1i or *APP*2ei. Initially, five samples with values outside the normal gene dose were detected in the Swedish cohort. Four of the samples were considered normal based on further analysis with additional primers within the *APP* gene (the promotor, *APP*9ei and *APP*18i) (Figure 1c). One sample still appeared duplicated and the extent of the increased gene dose region was analyzed with two genes flanking *APP*, *JAM2* and *CYYR1*. Two genes in more distant regions of chromosome 21, *USP25* and *AIRE*, were also analyzed. These genes were compared to *HBB* (Figure 1d), indicating duplication of one or several regions spanning >28 Mbp on chromosome 21 (Figure 1e). This region also spanned the Down's syndrome critical region (DSCR), the smallest duplicated region of chromosome 21 identified in patients with partial trisomy 21,[16] but there were no indications that this patient had DS. To investigate whether this ambiguous duplication could simply be due to a deletion of the control gene, all four genes and *APP*16i were compared to *GAPDH* and *UBC*. This did not alter the results. However, the sample demonstrated no duplications of the *APP* locus when run on a GeneChip 250K SNP array or analyzed using qmPCR.

The Finnish samples were screened for *APP* duplications using *APP*16i and *APP*5e in both qPCR and qmPCR. Seven samples demonstrating increased gene dose in qPCR were re-extracted from peripheral blood lymphocytes and qPCR analysis was repeated. After this, all samples demonstrated normal values. One sample demonstrated a slightly elevated gene dose in qmPCR for both *APP*16i (Figure 1f) and *APP*5e (1.20/1.22), but gene dose was normal (1.02/1.11) in qPCR analysis.

## Discussion

This screen for *APP* duplication included a total of 141 EOAD samples from Sweden and Finland. However, no

duplication of the *APP* locus could be identified. In two recent studies using French[8] and Dutch[9] samples, the frequencies of *APP* duplication were calculated to 8 and 2.7% of familial EOAD, respectively. If these frequencies were valid also for the Swedish and Finnish populations, we would have expected to find between two and six cases of *APP* duplication in our familiar cases. However, the occurrence of *APP* duplication seems to be more infrequent in the Swedish and Finnish populations.

One sample was concluded to be a false positive finding, as the putative duplication was very large and spanned DSCR, which is not consistent with a non-DS diagnosis.[16] There is a hypothetical possibility that this is not an uninterrupted duplication on one chromosome, but rather two independent duplications on separate chromosomes where DSCR is not included. However, this sample did not show any signs of duplication when tested with two other methods, qmPCR and GeneChip SNP arrays.

Neither qPCR nor qmPCR analyses demonstrated evidence of *APP* gene duplication among the Finnish EOAD patients. Although qmPCR was less variable than qPCR, there were no indications of false negative results using either method, as DS samples consistently demonstrated duplication of chromosome 21. We therefore consider both methods reliable for gene dose screening.

French[8] and Dutch[9] studies have demonstrated *APP* duplication ranging from minimally 0.29 up to 6.37 Mb, and containing either only the *APP* gene[9] or up to 12 genes.[8] All these duplications were presented with EOAD/CAA. Also, a report on an *APP* duplication in a Finnish family[17] was associated with progressive cognitive decline with coexisting intracerebral hemorrhages, but where the affected family members displayed a cognitive profile somewhat different from that typically seen in AD. Moreover, DS subjects with three copies of chromosome 21, and thereby of *APP*, usually develop AD although intracerebral hemorrhages have only occasionally been reported.[18] These differences in disease presentation could be related to which and how many additional genes are within the duplicated region. Nonetheless, the central role of *APP* duplications for AD development is emphasized further by a rare case of DS caused by partial trisomy 21 distal to the *APP* gene. This individual did not show any signs of AD, neither neuropsychological nor neuropathological, at her death at age 78 years.[19]

In conclusion, we screened a collection of patients from Sweden and Finland with AD and onset at or before 65 years of age, but found no cases of *APP* duplications. We therefore conclude that duplication of the *APP* gene is not a common cause of EOAD in Swedish and Finnish populations.

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