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# Age at natural menopause is not linked with the follicle-stimulating hormone receptor region: a sib-pair study

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**Objective:** Studies have shown that age at natural menopause is heritable. Mutations in the FSH-receptor have been identified in women with premature ovarian failure (POF) and the FSH-receptor gene may, therefore, be considered a candidate gene for (early) menopausal age. This study investigates whether there is linkage between genetic markers in the FSH-receptor region and (early) age at menopause using a sib-pair design.

**Design:** Sib-pair based linkage analysis.

**Setting:** Sister pairs and their first-degree family members from The Netherlands.

**Patient(s):** The inclusion criteria for a family were natural menopause in upper or lower tail of the distribution of menopausal age in at least two sisters. A total of 126 families with at least one sib-pair were included in this study. Six polymorphic markers encompassing the FSH-receptor gene were genotyped.

**Intervention(s):** None.

**Main Outcome Measure(s):** Single point and multipoint logarithm of the odds (LOD) scores.

**Result(s):** None of the markers showed evidence in favor of linkage with overall age at natural menopause or early age at natural menopause.

**Conclusion(s):** Possibly, age at natural menopause in the more or less normal range is not part of the spectrum of phenotypes determined by mutations in the FSH-receptor gene. Alternatively, our results might be explained by genetic heterogeneity in the left tail of the distribution of menopausal age. This can limit the chance of finding a genetic locus, especially if this factor has a modest contribution to the phenotype. (Fertil Steril® 2004;81:611–6. ©2004 by American Society for Reproductive Medicine.)

**Key Words:** FSH-receptor, age at menopause, early menopause, sib-pair study, linkage

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Menopause marks the obligatory end of female reproductive life. It is claimed that menopause reflects the depletion of the follicle pool below a threshold value (1). The mean age at menopause is about 50 years in all populations and the range is roughly between 40 and 60 years. In approximately 1% of the women menopause is reached before age 40; this is defined as premature ovarian failure (POF) (2, 3). Premature ovarian failure is a collective term for which proposed causes include autoimmune disease, radiation, chemotherapy, and certain genetic criteria. The latter encompasses deletions of the X-chromosome (4, 5), fragile-X carriership (6), FSH-receptor mutations (7–11), and mutations in the FOXL2 gene (12, 13). Cessation of menses between age 40 and 45 years has previously been defined as early menopause (14–16).

It has been shown that variation in menopausal age is heritable to a large extent. Two independent twin studies provided heritability estimates of up to 0.53 (17) and 0.63 (95% confidence interval [CI] 0.53–0.71) (18) and heritability of age at menopause in siblings was demonstrated to be 0.85 (95% CI 0.61–0.92) (19). Known environmental and lifestyle factors account for only 3% of the variance in menopausal age (20).

Genes involved in idiopathic POF (i.e., POF without an established cause) might also be associated with early menopause or even affect the entire distribution of natural menopausal age due to co-segregation. This is supported by

**TABLE 1**

Inactivating mutations in the FSH-receptor gene.

| Author | Phenotype | Exon | Mutation | Functional studies |
|---|---|---|---|---|
| Aittomäki et al. (22) | Hypergonadotropic ovarian dysgenesis | 7 | Ala189Val | Failure of signal transduction |
| Beau et al. (8) | Secondary amenorrhea | 6 | Ile160Thr | Impaired cell surface expression |
| | | 10 | Arg573Cys | Altered signal transduction |
| Touraine et al. (11) | Primary amenorrhea | 9 | Asp224Val | FSH binding to receptor barely detectable |
| | | 10 | Leu601Val | Impairment of receptor signaling |
| Doherty et al. (9) | Primary amenorrhea | 7 | Ala189Val | Failure of signal transduction |
| | | 10 | Ala419Thr | Impairment of receptor signaling |

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the observation that women with early menopause and POF are found within the same families, suggesting a genetic relationship between early menopause and POF (15, 21).

In Finnish families with an extreme form of POF associated with hypergonadotropic ovarian dysgenesis, linkage was found with the region of the FSH-receptor gene on chromosome 2p. Subsequent functional studies demonstrated a mutation in the FSH-receptor gene causing failure of signal transduction of the receptor (22). Other groups searched for FSH-receptor mutations in patients with ovarian dysgenesis, but were unsuccessful (23, 24). However, in French women with normal sized ovaries, but primary or secondary amenorrhea at young age, partial loss of function mutations in the FSH-receptor gene were found (8, 11). Recently, a new mutation in the FSH-receptor gene was identified in another Finnish patient with primary amenorrhea and hypergonadotropic ovarian failure (9). From these studies it seemed that the locations of the mutations in the FSH-receptor gene determined residual receptor activity (Table 1). This suggests a continuum of clinical phenotypes, resulting from varying levels of residual receptor activity caused by the FSH-receptor mutations, of which only the most severe forms have been identified so far. A less severe phenotype, such as an early age at menopause, might also be associated with alterations in the FSH-receptor gene.

Although the FSH-receptor gene seems a good candidate gene for determining variations in age at menopause, currently no sensible prediction can be made about the location and type of mutation. A linkage approach permits studying such an effect without the need to specifically define individual genetic variants within the gene. In the present study, we aim to investigate linkage between anonymous genetic markers in the FSH-receptor region and (early) variation in age at natural menopause in a sib-pair study.

## MATERIALS AND METHODS

### Phenotype and Inclusion Criteria

The phenotype, or trait, of interest is age at natural menopause. Natural menopause is defined as proposed by the World Health Organization (25) as at least 12 consecutive months of amenorrhea not due to surgery or other obvious cause. Accordingly, women who were premenopausal at the time of either hysterectomy or bilateral oophorectomy are not eligible as probands. Women having had a unilateral oophorectomy are also considered ineligible because this is known to be associated with an earlier age at menopause (26, 27). For women who started using oral contraceptives (OC) or hormonal therapy (HT) within 1 year after their last period, age at menopause cannot be determined with certainty and are thus ineligible.

In quantitative trait locus analysis, power can be increased considerably by sampling sib-pairs with phenotypic values ( i.e., menopausal ages) in the extremities (28). However, extreme sib-pairs are rare in the population limiting their practical use. Applying less restrictive thresholds can allow for an increase in sample size that may outweigh the effort needed to identify and ascertain the extreme sib-pairs. Therefore, we aimed to ascertain women with menopausal ages of 45 years or earlier or 54 years or later (corresponding to 12.5 percentiles). In two specific situations menopausal ages were assigned as follows: [1] women who stopped using OCs before or at age 45 years and had no periods after that, are assigned a menopausal age of 1 year earlier, and [2] women who had a hysterectomy or bilateral oophorectomy after or at age 54 years and were still premenopausal at that time, are assigned a menopausal age of 1 year later. Where possible the parents or additional sisters or brothers are included to reconstruct the missing parental genotypes for phase determination.

### Family Ascertainment

We used several strategies to ascertain families. Two large-scale population-based cohorts, the diagnostisch onderzoek mammacarcinoom (DOM) and Prospect cohorts were used to identify possible probands. Information on menopausal age and whether or not menopause had occurred naturally was available for approximately 40,000 women in the region of Utrecht, The Netherlands.

The DOM cohort (29) consists of 20,555 women born between 1911 and 1925 and residing in the city of Utrecht, The Netherlands, who were invited for an experimental program for breast cancer screening between 1974 and 1980. All participants filled out questionnaires, underwent brief medical examinations, and donated an overnight urine sample. The Prospect-EPIC (European Prospective Investigation into Cancer and Nutrition) cohort consists of 17,357 women aged 50–69 years at enrollment in the study between 1993 and 1997. Design and sampling strategy have been described elsewhere (30). All participants filled out detailed questionnaires, underwent a brief medical examination, and a blood sample was drawn.

Probands fulfilling the inclusion criteria were approached and inquiries were made on the existence of sisters and if alive, on their menopausal status. Probands were sent pedigree forms to provide information on family structure and menopausal status of sisters. In the case of inconsistencies, physicians were requested to clarify data with a telephone call. If at least two sisters complied with the inclusion criteria, the family was asked to participate. To increase the level of participation blood samples (20 mL of EDTA–blood) were taken at their homes. Female participants were also asked to fill out a questionnaire on reproductive history. A total of 106 families were included using these cohorts.

Through press releases in newspaper and women's magazines, additional sib-pairs were sought. During a phone call, a first assessment of the family was made and if applicable, a pedigree form was sent. Further proceedings were followed as described previously. A total of 27 families were included in this way.

The Elderly Association of a small region in northwest Holland with approximately 200 male and female members aged 55 years or older, were willing to send their female members an information leaflet about this study with a reply coupon. This approach provided us with 4 families.

The Dutch Twin Registry is a volunteer registry founded in 1987 at the Free University in Amsterdam for scientific research purposes. According to their database, four dizygous twin pairs could be approached who experienced natural menopause in one of the extremes of the distribution. Two families agreed to participate.

Finally, three families were ascertained through outpatient clinics of the University Medical Center Utrecht and of the University Medical Center St. Radboud in Nijmegen, The Netherlands.

Table 2 gives an overview of the recruitment. In total, information and DNA of 428 individuals from 142 families was collected.

The Institutional Review Board of the University Medical Center Utrecht, The Netherlands, approved the study protocol. Written informed consent was obtained from all participants.

**TABLE 2**

Ascertainment of sib-pairs.

| Medium | No. of positive responses | No. of families included |
|---|---|---|
| Prospect cohort | 552 | 74 |
| DOM cohort | 282 | 32 |
| Newspaper | 200 phone calls | 8 |
| Elderly association | 40 written responses | 4 |
| Women's magazine | 125 phone calls | 19 |
| Dutch Twin Registry | 4 eligible DZ twins | 2 |
| Outpatient clinic Fertility UMCU/UMC St. Radboud | NA | 3 |
| Total | | 142 |

*Note:* NA = not applicable.

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## Genotyping

DNA was extracted from lymphocytes by high salt extraction (31). The 428 DNA samples were divided into six 96-well microtiter plates. Every DNA plate contained up to 86 unique DNA samples, six blind duplicate samples, three Centre d'Etudes du Polymorphisme Humain (CEPH) controls, and one negative control. Six microsatellite markers spanning a region of 16.3 cM encompassing the FSH-receptor gene were genotyped: D2S2240 (69.7 cM), D2S1352 (73.1 cM), D2S2153 (76.9 cM), D2S337 (80.7 cM), D2S380 (83.9 cM), and D2S441 (86 cM). Order and position of the markers are based on the Marshfield Genetic Map. Based on physical mapping, the FSH-receptor gene is located between markers D2S2240 and D2S1352.

Reverse primers were labeled with either 6-FAM or HEX fluorescent dyes (Isogen Bioscience, Maarssen, The Netherlands) at the 5′-end. The polymerase chain reaction (PCR) reactions were carried out in a 10-$\mu$L volume containing 1 $\times$ PCR Gold-buffer, 200 $\mu$mol/L of deoxy-NTPs, 2.5 mmol/L $MgCl_2$, 25 ng of each primer, 0.4 U AmpliTaq Gold (Applied Biosystems, Foster City, CA) and 25 ng of genomic DNA. Cycling conditions were 7 minutes at 94°C followed by 32 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, followed by a final extension at 72°C for 30 minutes. The PCR products were pooled and separated on an ABI 3700 sequencer (Applied Biosystems) and analyzed using GeneScan version 3.1 (Applied Biosystems). Allele sizes of the individual markers were determined using Genotyper version 2.1 software. The CEPH reference samples (1331-01, 1331-02, and 1347-02) were included to determine the appropriate size of the alleles. The 36 duplicate samples were included to estimate the proportion of mistyping of genotypes. All samples were double-checked by two independent investigators, who did not know the origin. The blind genotypes of the duplicate samples were compared to the original samples by a technician.

Genotyping failures resulted in exclusion of 16 families because the minimum requirement of two sisters with a phenotypic value was no longer reached. The final study population thus consisted of 126 families.

## Data Analysis

Genotype data were stored and handled using a database management program for PC compatible computers, written in FoxPro by L. A. Sandkuijl. Data verification included range checking for allele sizes and identification of individuals/families who show an unusually high number of uncommon alleles. Input files for Mapmaker/Sibs were prepared automatically from the database management system.

Given the previous reports on the FSH-receptor gene it may seem plausible that an effect of this gene on menopausal age, if any, would be most pronounced in sibs with an early age at menopause. Therefore, two analyses were conducted. The first contained all sib-pairs, that is, concordant early, concordant late, and discordant ages, at menopause. The second contained a subgroup of the former analysis, namely only the concordant early and discordant sib-pairs.

For both analyses, the quantitative trait locus mapping was done using the program Mapmaker/Sibs (32). Single-point and multipoint LOD (log of the odds that loci are linked) scores were computed through Haseman-Elston regression (33). A LOD score represents the plausibility of linkage between menopausal age and a marker. Haseman-Elston regression is a nonparametric procedure in which the squared difference in menopausal age of the pairs of siblings is regressed on the estimated proportion of alleles shared identical by descent. The sibships were analyzed using all possible distinct pairs (= n(n-1)/2) of phenotyped sibs.

# RESULTS

Of the total number of 382 individuals left in the study, 327 were assigned a phenotypic value. The remaining 55 individuals consisted of parents, brothers, and sisters without age at natural menopause who were included for phase determination. Although extreme sampling was applied, it sometimes occurred that women who in first instance were collected as additional siblings for phase determination, also experienced natural menopause, but at a less extreme age. The ages at menopause of these women were also included in the analyses because, although they were potentially less informative, they did contribute phenotypic information. Ten women did not resume menstruation after discontinuation of OCs before or at age 45 years and were assigned a menopausal age of 1 year earlier.

Table 3 shows the distribution of the phenotypic values of both the total study population and of the group containing only the concordant early and discordant sib-pairs. The median age at menopause in the concordant early and discordant pairs was 44 years.

**TABLE 3**

Distribution of phenotypes in families.

| No. of phenotypic values per family | All families | | Early/discordant families | |
|---|---|---|---|---|
| | No. of families | No. of phenotypic values | No. of families | No. of phenotypic values |
| 2 | 72 | 144 | 39 | 78 |
| 3 | 37 | 111 | 14 | 42 |
| 4 | 14 | 56 | 5 | 20 |
| 5 | 2 | 10 | 2 | 10 |
| 6 | 1 | 6 | 1 | 6 |
| Total | 126 | 327 | 61 | 156 |

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A quantitative trait locus approach is the most sensible choice for investigating linkage of age at menopause as this phenotype is measured on a continuous scale. Artificially dichotomizing a continuous phenotype would require determination of a cutoff point for normal and abnormal age at menopause. In addition, in this nonparametric approach unknown parameters like mode of inheritance and penetrance can be ignored.

The mean information content (single-point) of the markers was 0.59. The mean information content (multipoint) over the six markers was 0.82. Multipoint analysis including all possible sib-pairs yielded a maximum LOD score of 0.08. The second analysis, including only the early concordant and discordant sib-pairs under the hypothesis that the FSH-receptor gene would determine predominantly early age at menopause, resulted in a maximum LOD score of 0.03. This means that in both analyses, none of the markers showed evidence in favor of linkage with overall age at menopause or early age at menopause (Table 4).

# DISCUSSION

We investigated the FSH-receptor region as a candidate gene in age at natural menopause using a sib-pair-based linkage approach. None of the markers in the candidate region showed statistical significance for linkage in this

**TABLE 4**

Maximum LOD scores by Haseman-Elston regression.

| | Single point | Location in cM (marker) | Multipoint | Location in cM |
|---|---|---|---|---|
| Early/discordant pairs | 0.009 | 73.1 (D2S1352) | 0.03 | 75 |
| All pairs | 0.22 | 73.1 (D2S1352) | 0.08 | 74 |

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sample. The phenotypes previously associated with mutations in the FSH-receptor gene are quite severe, ranging from ovarian dysgenesis with primary amenorrhea to having normally sized ovaries and menopause at an extremely young age. Although different locations of mutations in the gene seem responsible for the range of severity of phenotypes, age at natural menopause in the more or less normal range might not be part of this spectrum of phenotypes.

A limitation in this study concerns the precision of the phenotyping. The phenotype, age at menopause, was self-reported through a questionnaire and may be affected by recall bias (34). Incorrect phenotypic values can be problematic if discrepancies exist in the magnitude and direction of error between the sisters. This would influence the squared difference in menopausal age of a sib-pair, which is the dependent variable in Haseman-Elston regression. However, it is unlikely that these errors are nonrandom.

The distribution of menopausal age deviates from normality by a significant skewness to the left side. Previous studies tested for heterogeneity in large population-based samples and found that the observed distribution is better explained by two or more overlapping distributions (35,36) (unpublished data). This could imply that women classified with idiopathic POF based on the definition that they reached menopause before age of 40 years, actually belong to the left end of the distribution of normal menopausal age, suggesting that early menopause is probably genetically more heterogeneous than late menopause. Our null findings might be explained by a wide spread genetic heterogeneity in the left tail of the distribution of menopausal age. This has possibly led to loss of power, especially in the analysis of early age at menopause.

Haseman-Elston regression uses the phenotypic sib-pair difference, which is less powerful than using the true values like in a maximum likelihood quantitative trait locus variance components approach (37). However, where Haseman-Elston is robust, variance components models rely heavily on normality of the phenotype, which is by definition absent when using extreme sampling. Applying a variance components model would, in this case, lead to unreliable results due to incorrect estimates of the mean and variance of the phenotype.

In conclusion, although we found no evidence for linkage of (early) age at menopause with markers in the FSH-receptor region, we cannot completely rule out involvement of the FSH-receptor gene in (early) age at menopause because we consider genetic heterogeneity in the early part of the distribution to be a major complicating factor.

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