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Title:
HFE p.C282Y homozygosity predisposes to rapid serum ferritin rise after menopause: A genotype-stratified cohort study of hemochromatosis in Australian women

Date:
2017-04-01

Citation:
Warne, C. D., Zaloumis, S. G., Bertalli, N. A., Delatycki, M. B., Nicoll, A. J., McLaren, C. E., Hopper, J. L., Giles, G. G., Anderson, G. J., Olynyk, J. K., Powell, L. W., Allen, K. J., Gurrin, L. C., Bahlo, M., Fletcher, A. R., Forrest, S. M., Osborne, N. J., Vulpe, C. D. & Turkovic, L. (2017). HFE p.C282Y homozygosity predisposes to rapid serum ferritin rise after menopause: A genotype-stratified cohort study of hemochromatosis in Australian women. Journal of Gastroenterology and Hepatology Australia, 32 (4), pp.797-802. https://doi.org/10.1111/jgh.13621.

Abstract

Background and Aim

Women who are homozygous for the p.C282Y mutation in the *HFE* gene are at much lower risk of iron overload-related disease than p.C282Y homozygous men, presumably due to the iron-depleting effects of menstruation and pregnancy. We used data from a population cohort study to model the impact of menstruation cessation at menopause on serum ferritin (SF) levels in female p.C282Y homozygotes, with p.C282Y/p.H63D simple or compound heterozygotes and those with neither p.C282Y nor p.H63D mutations (*HFE* wild-types) as comparison groups.

Methods

A sample of the Melbourne Collaborative Cohort Study (MCCS) was selected for the "HealthIron" study (n=1,438) including all *HFE* p.C282Y homozygotes plus a random sample stratified by *HFE*-genotype (p.C282Y and p.H63D). The relationship between the natural logarithm of SF and time since menopause was examined using linear mixed models incorporating spline smoothing.

Results

For p.C282Y homozygotes, SF increased by a factor of 3.6 (95% CI (1.8, 7.0), $p < 0.001$) during the first ten years post menopause, after which SF continued to increase but at less than half the previous rate. In contrast, SF profiles for other *HFE* genotype groups increase more gradually and did not show a distinction between pre- and post-

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jgh.13621

menopausal SF levels. Only p.C282Y homozygotes had predicted SF exceeding 200 µg/L post-menopause, but the projected SF did not increase the risk of iron-overload related disease.

Conclusions

These data provide the first documented evidence that physiological blood loss is a major factor in determining the marked gender difference in expression of p.C282Y homozygosity.

Key Words

hereditary hemochromatosis, *HFE* p.C282Y homozygosity, iron accumulation, menopause, iron overload-related disease, women's health.

Introduction

Hereditary hemochromatosis is an iron overload disorder with clinical complications resulting from the deposit of excess iron throughout the body. The health consequences can be serious, with untreated cases at risk of hepatic cirrhosis and hepatocellular carcinoma (1-4). The discovery of the *HFE* gene in 1996 identified homozygosity for the p.C282Y *HFE* mutation as the major risk factor for hemochromatosis, which accounts for over 90% of clinical cases (5). Genetic susceptibility is most common among people of Northern European descent, with at least 1 in 200 homozygous for the p.C282Y mutation. The phenotypic expression or *penetrance* of p.C282Y homozygosity for both elevated serum iron indices and disease manifestations is incomplete. That is, many p.C282Y homozygotes do not develop iron overload-related disease (1).

Epidemiological studies have consistently shown a relationship between p.C282Y homozygosity and elevated body iron stores, with elevated serum ferritin (SF) concentration (a sensitive indicator of body iron stores (3, 4)) found in 40-60% of female homozygotes and 75-100% of male homozygotes (1, 6). The lower incidence of elevated iron in women compared to men has been widely attributed to the iron-depleting effects of menstrual blood loss and pregnancy (5, 7), as suggested by a number of studies showing that pre-menopausal women have lower SF levels than post-menopausal women and men. (7-12). Some studies have reported increases in SF levels after menopause followed by a plateau at 6-10 years (9-11), but these studies did not stratify by *HFE* genotype. Moreover, they provided only descriptive results by plotting

SF levels against categorical time intervals since menopause, or used regression models that did not go beyond testing for linear trends. No study has previously compared pre- and post-menopausal iron loading stratified by *HFE* genotype including a comparison group representing the general population. Elucidating this relationship may suggest reasons why p.C282Y homozygous women are at low risk of iron overload-related disease.

We investigated the relationship between time since menopause and SF concentration and whether this varies between HFE genotypes: p.C282Y homozygotes, p.C282Y/H63D compound heterozygotes (at intermediate risk of iron overload (13)), and the remaining three HFE genotype groups (p.C282Y and p.H63D heterozygotes and p.H63D homozygotes). The latter three groups confer no increased risk of iron overload compared with those with no HFE mutations (2, 6, 14, 15). This was achieved by using data from female participants in the "HealthIron" study.

Methods

The Melbourne Collaborative Cohort Study (MCCS)

Between 1990 and 1994, 41,514 people (24,469 females) aged between 27 and 75 years (99% aged 40 to 69 years) were recruited to the Melbourne Collaborative Cohort Study (MCCS) (16). Participants were recruited through the Australian Electoral Roll (voting is compulsory in Australia) and as part of the study completed a questionnaire about

diet, lifestyle factors, medical history and reproductive history (for women), had a physical examination and provided a fasting blood sample.

The HealthIron study

Beginning in 2004, blood samples from 31,192 MCCS participants (17,951 female) of Northern European descent were genotyped for the p.C282Y mutation. Those with one copy of the p.C282Y mutation were then genotyped for the p.H63D mutation and classified as simple (one copy of the p.C282Y mutation) or compound (one copy each of the p.C282Y and p.H63D mutations) heterozygotes. All 203 participants homozygous for the p.C282Y mutation (108 female) were invited to attend HealthIron follow-up clinics, together with a random sample stratified by *HFE*-genotype, including 242 compound heterozygotes (125 female) and 361 participants (197 female) wild-type for both mutations. Of the 1,438 subjects invited, 269 were lost to follow-up and 113 were deceased, leaving 1,056 in the study and an overall participation rate of 73.2% or 79.4% excluding those already deceased.

HealthIron follow-up clinics were held between 2004 and 2006. Participants completed a questionnaire on medical history (including diagnosis and treatment of hemochromatosis), blood donation, and reproductive history for women. Participants also underwent a physical examination, provided a blood sample and provided a checkbrush swab to confirm baseline *HFE* genotype. Blood samples were paired for analysis with stored baseline samples collected between 1990 and 1994 for each

participant and analysed for SF, transferrin saturation and a selection of liver enzymes (1).

All participants gave written informed consent to participate in both the MCCS and the HealthIron sub-study. Both protocols were approved by the Cancer Council Victoria's Human Research Ethics Committee.

Definitions and exclusion criteria

Menopausal status and age at menopause were assessed at baseline and follow-up by asking women whether they had had a menstrual period in the last 12 months, and if not, how old they were at their last menstrual period and the reason their menstrual periods ceased (naturally, hysterectomy, other). If women were post-menopausal or had had a hysterectomy before baseline they were not asked again at follow-up.

Time since menopause was calculated as the number of years between a women's age at baseline (or follow-up) and her reported age at natural menopause or hysterectomy. In subsequent analyses we considered time since natural menopause and time since hysterectomy equivalent in that both result in the cessation of regular menstrual blood loss and were therefore physiologically equivalent.

All analyses were stratified by four groups of *HFE* genotype: p.C282Y homozygote, p.C282Y/H63D compound heterozygote, Other *HFE* genotype (includes p.C282Y heterozygotes, p.H63D heterozygotes and p.H63D homozygotes) and HFE wild-type (neither p.C282Y nor p.H63D mutation). For all analyses, we used *HFE* wild-types as the control group due to their low risk of iron overload. Any SF measurements from participants made after therapeutic venesection were excluded from analyses.

Statistical methods

The relationship between the natural logarithm of SF (log(SF)) and time since menopause was examined using linear mixed models (17). This incorporated both spline smoothing (18) and correction of parameter estimates, and their standard errors, for repeated measures at baseline and follow-up separately for each *HFE* genotype group, excluding measurements post-venesection. These models were used to generate curves for the mean predicted SF levels (with 95% confidence intervals) from 10 years pre-menopause to 30 years post-menopause for each *HFE* genotype group. To quantify the change in SF levels post-menopause, the ratio of mean predicted SF level post-menopause to mean predicted SF level at menopause (the SF ratio) was calculated by exponentiating the difference in the corresponding post-menopause and menopause log(SF) values at intervals post-menopause of 5, 10, 15 and 20 years. All analyses were performed in Stata version 13.1 (19).

Results

Across all *HFE* genotype groups, 692 women participated in the HealthIron study. Nineteen p.C282Y homozygotes and one p.C282Y/H63D compound heterozygote were excluded due to being therapeutically venesected during the study period, leaving 89 p.C282Y homozygotes, 124 compound heterozygotes, 197 *HFE* wild-types and 262 other *HFE* genotypes available for analysis.

Table 1 shows the characteristics of selected variables across *HFE* genotype groups for the 672 women included in the analytic sample, of which 539 were post-menopausal at follow-up. The mean age of women at baseline, menarche and age of menopause were similar for the four *HFE* genotype groups. Similarly, there was no statistically significant difference in the number of post-menopausal women at follow-up between the genotype groups.

Table 2 shows estimated SF ratios at five-year intervals post menopause for each of the four *HFE* genotype groups. For p.C282Y homozygotes, SF rose by a factor of 3.6 (95% CI (1.8, 7.0), $p < 0.001$) after the first ten years post menopause, after which SF continued to increase but at less than half the previous rate (Table 2 and Figure 1). In contrast, SF profiles for other *HFE* genotype groups (Table 2 and Figure 1) increased only gradually and did not show a distinction between pre- and post-menopausal SF levels. Only p.C282Y homozygotes had predicted SF exceeding 200 μg/L post-

menopause, but, based on previously reported analyses from the same cohort, the projected SF did not increase the risk of iron-overload related disease (20).

This analysis could not examine SF changes in detail during the 5-year window after menopause because the mean age of post-menopausal women at baseline was 61 years (SD=5), and consequently, baseline SF measurements were taken on average 11 years after menopause (SD=6).

Discussion

This is the first longitudinal study to investigate the role of menstruation cessation in iron accumulation, as measured by SF, in women with a genetic pre-disposition to iron overload-related disease. This was achieved by analysing longitudinal data from female participants in the HealthIron study to examine differences in the trajectory of pre- and post-menopausal SF levels between *HFE* groups. The results of spline smoothing of the logarithm of SF concentration against time since menopause suggested that there was no evidence of SF increasing substantially beyond 5-10 years after menopause or hysterectomy in each *HFE* group and that the majority of post-menopausal iron-loading occurred near the time of menopause.

The observation that women accumulate iron once their regular menstrual cycles finish is well documented in population studies from Australia, the USA, France, the UK and

Denmark (7, 8, 10-12). A small number of studies have described the association between SF and time since menopause or hysterectomy, yet none have explored this relationship between different *HFE* genotypes. An analysis by Liu, et al. (21) of single SF measurements from 620 post-menopausal women in the Nurse's Health Study found time since menopause was linearly associated with higher SF concentrations when controlling for age, BMI, physical activity, dietary factors and excluding women who had used post-menopausal hormones (PMH). Whitfield, et al. (11) analysed single blood samples from 2,039 women in the Australian Twin Registry reporting that SF increases above pre-menopausal levels in the first five years after menopause, before reaching a plateau at approximately 6-10 years. In a study of single measurements of iron indices from a random sample of 1,359 Danish women, (10) a similar trend of rapid SF increase in the first five years after menopause before a levelling out at 6-10 years was reported. A significant correlation (r=0.18) between SF and the number of post-menopausal years in non-blood donors was found. The authors noted similar SF levels were seen when studying healthy 85-year-old women in Copenhagen County (22), and suggested that after 7-10 years of post-menopausal iron accumulation, SF levels stabilise to their new steady-state level and remain fairly constant. This idea has also been put forward by Garry, et al. (9) that an individual's "theoretical setpoint of iron stores" occurs around the age of 40 years for men and after menopause for women. They discuss this in light of their finding that age was associated with SF in a cross-sectional analysis of 276 post-menopausal women when adjusting for BMI and dietary factors, arguing that not

enough time had elapsed for post-menopausal women to reach their steady-state iron level.

Previous studies have reported that age, alcohol intake, body mass index (BMI) and energy intake are positively correlated with SF levels in post-menopausal women, whereas blood donation / therapeutic venesection and hormone replacement therapy (HRT) are negatively correlated with SF levels (8, 9, 11, 21, 23-25). The effect of dietary heme-iron intake on SF remains controversial; the UK Women's Cohort Study (8), Nurses' Health Study (21) and a Danish cohort study (24) reported positive correlations, yet longitudinal and cross-sectional analyses of participants in the New Mexico Aging Process Study (9) reported no relationship. Extending the statistical methods used here to accommodate covariates when generating SF profiles will be the subject of future research.

One potential limitation of this study was the possibility that individuals with iron overload-related disease were less likely to participate in follow-up. However, there is good evidence that this was not the case, given that participation rates were comparable between p.C282Y homozygotes (72%) and the overall cohort (73%), and the similarity of baseline iron indices between participants (male and female) lost to follow-up (data not shown). The use of self-reported age at menopause was considered appropriate given that menopause was well defined in the questionnaire and is a significant life event the timing of which most women would be expected to remember.

In conclusion, our demonstration that SF approaches steady-state levels 5-10 years after menopause in all *HFE* groups offers an explanation as to why female p.C282Y homozygotes remain at low risk of iron overload-related disease. It is acknowledged that the iron depleting effects of menstruation and pregnancy result in female homozygotes not being exposed to elevated SF levels until much later in life (7). Our study extends this finding by showing that while steady-state SF levels after menopause were higher in p.C282Y homozygotes than in other *HFE* genotypes, there was no evidence that SF levels increased beyond this point. This pattern of post-menopausal iron accumulation is similar to that reported for non-genotyped study populations (10, 11), and is consistent with the notion that iron accumulation is physiologically self-limiting and that women reach their steady-state iron levels after menopause (3, 9, 26) even if age rather than the interval since menopause is used as the time scale (27). The observation that SF levels plateau at steady-state levels in male and female p.C282Y homozygotes (28, 29), combined with growing evidence that female homozygotes don't reach this point until about 10 years after menopause, or at roughly 60 years of age, suggests that female homozygotes have approximately 20 years less exposure to elevated iron stores than male homozygotes. It is, therefore, plausible that female homozygotes are not exposed to elevated SF levels for long enough to ever put them at increased risk of iron overload-related disease.

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Table 1: Sample characteristics

	n	Age at baseline Mean (SD)	Age at menarche Mean (SD)	Post-menopausal at follow-up n (%)	Age at menopause Mean (SD)[&ℓ]	At least one SF measure n	Two SF measures n

p.C282Y homozygotes[†]	89	56.1 (8.6)	13.2 (1.5)	77 (87%)	49.0 (5.6)	66	49
Compound heterozygotes[‡]	124	55.1 (9.3)	13.2 (1.5)	103 (83%)	47.7 (6.6)	97	67
HFE wild-type	197	53.8 (9.2)	12.9 (1.6)	156 (79%)	46.8 (6.5)	181	148
Other *HFE*	262	54.0 (9.3)	13.1 (1.5)	203 (78%)	48.6 (5.3)	228	166

[†] Excludes 19 participants therapeutically venesected

[‡] Excludes 1 participant therapeutically venesected

[&] For women post-menopausal at follow-up

[ℓ] Comparison mean age at menopause *HFE* wild-type and Other *HFE* p= 0.009, although times to menopause were not statistically significantly different using the log-rank test to compare either p.C282Y/H63D compound heterozygotes (p = 0.48) or p.C282Y homozygotes (p = 0.85) to Other *HFE* genotypes (p.C282Y heterozygotes, p.H63D heterozygotes and p.H63D homozygotes).

Table 2: Estimated SF ratios (95% CI) for 5, 10, 15 and 20 post-menopause

	Time since menopause (years)

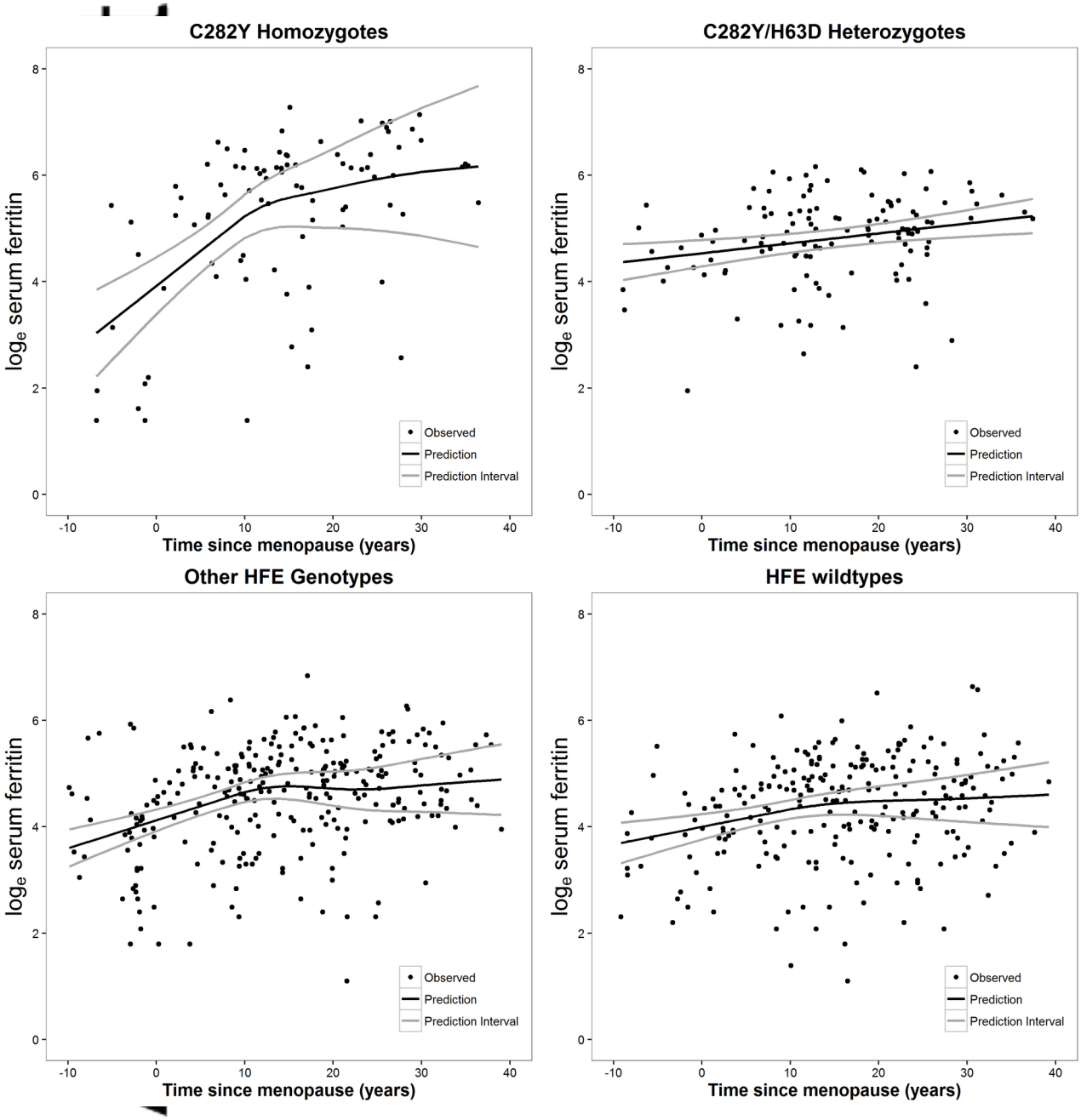
	n	5	10	15	20
p.C282Y homozygotes[†]	89	1.93 (1.5, 2.47)	3.71 (2.26, 6.09)	5.23 (2.49, 11.02)	6.25 (2.32, 16.86)
Compound heterozygotes[‡]	124	1.1 (1.03, 1.17)	1.21 (1.07, 1.36)	1.32 (1.1, 1.59)	1.45 (1.14, 1.85)
HFE wild-type	197	1.18 (1.07, 1.3)	1.39 (1.15, 1.68)	1.55 (1.16, 2.06)	1.62 (1.11, 2.37)
Other *HFE*	262	1.3 (1.18, 1.43)	1.7 (1.4, 2.06)	1.89 (1.41, 2.52)	1.81 (1.23, 2.66)

[†] Excludes 19 participants therapeutically venesected

[‡] Excludes 1 participant therapeutically venesected

Figure Legend

Figure 1: Log serum ferritin (SF) against time since menopause for *HFE* p.C282Y homozygotes, *HFE* p.C282Y/H63D compound heterozygotes, Other *HFE* genotypes (p.C282Y heterozygotes, p.H63D heterozygotes and p.H63D homozygotes) and *HFE* wildtypes (no p.C282Y or p.H63D).



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Title

HFE p.C282Y homozygosity predisposes to rapid serum ferritin rise after menopause: a genotype-stratified cohort study of hemochromatosis in Australian women

Running title

HFE p.C282Y homozygosity, iron accumulation and menopause

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Disclosure Statement

$ The contribution of Charles D. Warne to this work was completed while he was studying for a Master of Epidemiology at the University of Melbourne and pre-dates his employment with Roche Products Ltd. The views and opinions expressed in this paper are those of the authors alone, and do not necessarily reflect the views, policies or any position of Roche Products Ltd. The remaining authors have no conflict of interest to declare.

Acknowledgements

This study was funded by National Institute of Diabetes, Digestive and Kidney Diseases (US) (1 RO1 DK061885-01 A2), National Health and Medical Research Council

(Australia) (Grants No. 251668, 209057). Cohort recruitment was funded by VicHealth and The Cancer Council Victoria. The NHMRC provided funding support for the following authors: LCG, KJA, MBD, JKO, GJA, JLH. Dr Sue Forrest from the Australian Genome Research Facility, Melbourne, supervised *HFE* genotyping of the cohort samples performed at the Australian Genome Research Facility. Andrea A Tesoriero with M.C.S. supervised DNA extraction. Ashley Fletcher provided assistance with study co-ordination and sample retrieval. This study was made possible by the contribution of many people, including the original investigators and the diligent team who recruited the participants and who continue working on follow-up. Finally, we would like to express our gratitude to the many thousands of Melbourne residents who continue to participate in the study.