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Prevalence of H63D, S65C, and C282Y hereditary hemochromatosis gene variants in Madeira Island (Portugal)

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Title: Prevalence of H63D, S65C and C282Y hereditary hemochromatosis gene variants in Madeira Island (Portugal).

Article Type: Original Article

Keywords: HFE; H63D; C282Y; S65C; Madeira Island; Hereditary Hemochromatosis.

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Order of Authors: Carla Spínola; António Brehm; Helder Spinola

Abstract: Hereditary HFE Hemochromatosis is an inherited disorder of iron metabolism that result from mutations in the HFE gene. Almost all patients with Hereditary Hemochromatosis show a C282Y mutation in homozygosity or in compound heterozygosity with H63D. Also, the mutation S65C has been shown to be associated to a milder iron overload. Since allele and genotype frequencies of these three variants of the HFE gene vary between populations, the determination of their prevalence in Madeira Island will clarify the population susceptibility to Hereditary Hemochromatosis. One hundred and fifty four samples from Madeira Island were genotyped for the three most common HFE gene mutations, H63D, C282Y and S65C, by Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphism analysis. Results have shown a prevalence of 20.5%, 0.33% and 1% for H63D, C282Y and S65C, respectively. Accordingly to our estimates, both genotypes associated to Hereditary Hemochromatosis, C282Y homozygotes and C282/H63D compound heterozygotes, could be present in Madeira Island population in 1,648 individuals, which represents 0.65% of the total population.

Response to Reviewers: . In this revised version we included the four references recommended by reviewers:

AS REFERENCE 3

Aranda N, Viteri FE, Montserrat C, et al. Effects of C282Y, H63D, and S65C HFE gene mutations, diet, and life-style factors on iron status in a general Mediterranean population from Tarragona, Spain. *Ann Hematol (Germany)*, Aug 2010, 89(8) p767-73

AS REFERENCE 5

Pedersen P, Milman N. Genetic screening for HFE hemochromatosis in 6,020 Danish men: penetrance of C282Y, H63D, and S65C variants. *Ann Hematol (Germany)*, Aug 2009, 88(8) p775-84

AS REFERENCE 12

Mendes AI, Ferro A, Martins R, et al. Non-classical hereditary hemochromatosis in Portugal: novel mutations identified in iron metabolism-related genes. *Ann Hematol (Germany)*, Mar 2009, 88(3) p229-34

AS REFERENCE 15

Pedersen P, Melsen GV, Milman N. Frequencies of the haemochromatosis gene (HFE) variants C282Y, H63D and S65C in 6,020 ethnic Danish men. *Ann Hematol (Germany)*, Sep 2008, 87(9) p735-40

***Conflict of interest**

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Arnold Ganser, M.D.
Editor-in-Chief
Annals of Hematology

We would like to re-submit the revised article entitled "Prevalence of H63D, S65C and C282Y hereditary hemochromatosis gene variants in Madeira Island (Portugal)" (Ref.: Ms. No. AOHE-D-10-00266), to be considered for publication in Annals of Hematology. In this revised version we considered the remarks pointed by reviewer #1:

Reviewer #1: In the title I should prefer the word "variants" instead of "mutations".

Authors: Done.

Reviewer #1: In the abstract it should say "Hereditary HFE haemochromatosis...." as there now exists many genetic forms of haemochromatosis.

Authors: Done.

Reviewer #1: The introduction to a large extent repeats well known facts about HFE hemochromatosis and should be shortened at least by 3/4 page.

Authors: we shortened the introduction.

Reviewer #1: There are several spelling errors.

Authors: We made a spelling revision.

Reviewer #1: In recent years, Annals of Hematology has published many papers on HFE haemochromatosis, among others by the authors Remacha AF, Pedersen P, Milman N, ect. I strongly suggest the authors make a screening on PubMed to identify these AOH papers and include them in the reference list.

Authors: We added a paper from Remacha published in Annals of Hematology.

Best regards,

Hélder Spínola

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4 Short Communication

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7 **Prevalence of H63D, S65C and C282Y hereditary hemochromatosis gene**
8 **mutations-variants in Madeira Island (Portugal)**
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15 Carla Spínola¹, António Brehm¹, Hélder Spínola^{1*}
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40 **Short title:** HFE mutations in Madeira Island.
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4 **Abstract**

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6 Hereditary HFE Hemochromatosis is an inherited disorder of iron metabolism that
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8 results from mutations in the HFE gene. Almost all patients with Hereditary
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10 Hemochromatosis show a C282Y mutation in homozygosity or in compound
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12 heterozygosity with H63D. Also, the mutation S65C has been shown to be associated to
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14 a milder iron overload. Since allele and genotype frequencies of these three variants of
15
16 the HFE gene vary between ~~different~~ populations, the determination of their prevalence
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18 in Madeira Island will clarify the population susceptibility to Hereditary
19
20 Hemochromatosis.

21
22 One hundred and fifty four samples from Madeira Island were genotyped for the three
23
24 most common HFE gene mutations, H63D, C282Y and S65C, by Polymerase Chain
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26 Reaction followed by Restriction Fragment Length Polymorphism analysis. Results
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28 have shown a prevalence of 20.5%, 0.33% and 1% for H63D, C282Y and S65C,
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30 respectively. Accordingly to our estimates, both genotypes associated to Hereditary
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32 Hemochromatosis, C282Y homozygotes and C282/H63D compound heterozygotes,
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34 could be present in Madeira Island population in 1,648 individuals, which represents
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36 0.65% of the total population.

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43 **Keywords:** HFE; H63D; C282Y; S65C; Madeira Island; Hereditary Hemochromatosis.
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Introduction

Genetic mutations found in [the](#) HFE locus, located in the short arm of chromosome 6 in the Human Leukocyte Antigen (HLA) complex, are associated with Hereditary Hemochromatosis [1, 2, 3]. Hereditary Hemochromatosis results from an excessive iron accumulation leading to injuries and organ failures that varies in clinical severity ~~from fatigue, hiperpigmentation and cardiac arrhythmias to arthritis, heart failure, diabetes mellitus and hepatic cirrhosis, among others~~ [4, 52]. This inherited disorder was first identified in 1976 [and found](#) to be in linkage with some HLA alleles [6]. ~~and, t~~ twenty years later, two mutations on HFE locus were confirmed to be linked to the majority of the disease cases [1, 43, 74].

The mutation most associated to Hereditary Hemochromatosis is a G-to-A transition at nucleotide 845 (G845A) in the exon 4 of [the](#) HFE locus, which substitutes a tyrosine for a cysteine at amino acid position 282 and is designated as C282Y. The other mutation associated to this iron metabolic disorder is a C-to-G at the nucleotide position 187 (C187G) in the exon 2 of [the](#) HFE locus, ~~that it~~ substitutes ~~an~~ aspartic acid ~~for to~~ histidine at amino acid position 63 and is designated as H63D [85]. Eighty to 90% of patients with Hereditary Hemochromatosis show the C282Y ~~mutation~~variant, especially in homozygosity, and 3 to 5% are compound heterozygotes between C282Y and H63D [96]. Another mutation on the exon 2 of the HFE locus, the S65C, a A-to-T transition at the position 193 that leads to a serine-to-cysteine substitution, have been shown to be associated with milder iron overload [107, 118]. ~~Other mutations, namely in other genes, have shown a modest contribution on the Hereditary Hemochromatosis development [12].~~ ~~revealing their modest contribution for the development of this phenotype~~

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4 The product of the HFE locus is a transmembrane glycoprotein that is thought to
5 regulate iron uptake through a mechanism that involves its binding to the transferrin
6 receptor in the cell membrane [9]. The mutation C282Y described above, when
7 inherited in homozygosity or in compound heterozygosity with other mutations, could
8 increase iron uptake leading to the development of Hereditary Hemochromatosis. Since
9 the identification of this association, Hereditary Hemochromatosis have been found to
10 be one of the most common genetic disorders in northern European descends [10].
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19 The prevalence of the HFE mutations varies ~~accordingly to the~~among populations.
20 Mutation C282Y shows a higher prevalence in Ireland (14%), United Kingdom (8.1%),
21 Faroe Islands (8%), France (7.7%) and Norway (6.6%) but for H63D ~~the higher-highest~~
22 prevalence's ~~have been found go to in~~ Spain Basque (30.4%), Northern Spain (20%),
23 Portugal (23%) and Bulgaria (23%). ~~The mutation~~S65C has~~ve~~ been found only at low
24 frequencies, reaching the highest values in the Saami people from Sweden (3%) ~~and but~~
25 also in France (1.95), ~~and~~ Croacia (1.8%) ~~and Denmark (1.8%)~~ [134, 14, 152]. A
26 population from the North of Portugal ~~had show edn~~ a 23% high prevalence of H63D
27 ~~mutation (23%)~~ but a much lower value for C282Y (2.8%) [163].
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39 The Madeira Island (Portugal), located in the North Atlantic Ocean, has never been
40 included in any characterization study for HFE mutations. This Island, uninhabited until
41 the 15th century, was settled mainly by Portuguese but also by sub-Saharan, brought as
42 slaves, and also by different Europeans (Spaniards, Italians, French, and English) [174,
43 185]. Some previous studies on Y chromosome, mitochondrial DNA and HLA had
44 shown that the Madeiran-Madeira population genetic profile, besides the European
45 influence, is also characterized by a valuable sub-Saharan imprint [196-224].
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4 The aim of the present study is to characterize, for the first time, the prevalence of
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6 C282Y, H63D and S65C HFE [mutations-variants](#) and their genotype combinations in
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8 the Madeira Island [population](#) in order to discuss about population susceptibility for
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10 Hereditary Hemochromatosis. We [intend](#) also [intend](#) to search for any differences
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12 between the Madeira population and other Portuguese regions since a previous study
13
14 had shown a clear north-south gradient on Portugal mainland for the C282Y [mutation](#)
15
16 [variant](#) [2320].
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19 **Materials and Methods**

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21 The samples, collected after informed consent, consisted of 154 unrelated males, with
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23 age of 18 to 23 years old, whose parents and grandfathers were born and living in
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25 Madeira Island, where the population has approximately 253,000 inhabitants. Genomic
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27 DNA was isolated from whole blood containing EDTA using a salting-out procedure
28
29 accordingly to Miller and colleagues, with some modifications [24]. C282Y, H63D
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31 and S65C [mutations-variants](#) were typed by PCR followed by restriction fragment
32
33 length polymorphism (RFLP) analysis [118, 252]. The region containing the S65C and
34
35 H63D mutations, located in the exon 2, was amplified with primers 5'-
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37 ACATGGTTAAGGCCTGTTGC-3' (forward) and 5'-
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39 CTTGCTGTGGTTGTGATTTCC-3' (reverse) giving a PCR product of 294 base pairs
40
41 (bp). Those amplification products containing [the](#) H63D mutation, when digested with
42
43 *MboI*, gave restriction fragments of 237 and 57 bp, while wild-type will result in 3
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45 fragments: 138, 99 and 57 bp. To detect the S65C mutation, the same PCR products
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47 were digested with *HinfI*, resulting in two fragments (147 and 61 bp) for the wild-type
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49 or in one (208 bp) for the mutation. For C282Y typing, exon 4 was amplified using
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51 primers 5'-CAAGTGCCTCCTTTGGTGAAGGTGACACAT-3' (forward) and 5'-
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4 CTCAGGCACTCCTCTCAACC-3' (reverse), which gives a PCR product of 343 bp
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6 that result~~ing~~ in 203, 111, and 29 bp fragments for the mutation and 203 and 140 bp
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8 fragments for the wild-type, when digested with *RsaI*.
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10 11 Analysis

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13 Allele and genotype frequencies were calculated by gene counting and respective
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15 confidence intervals through Exploratory Software for Confidence Intervals (ESCI
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17 JSMS, La Trobe University, Melbourne, Australia) available online
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19 (<http://www.latrobe.edu.au/psy/esci/>) [~~263~~]. The expected numbers of HFE genotypes
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21 in Madeira Island population were extrapolated through observed genotype frequencies
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23 on the samples studied, except for C282Y and S65C homozygotes that were estimated
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25 from the Hardy–Weinberg equilibrium statistical formula ($p^2+2pq+q^2=1$). Comparisons
26
27 with other populations were performed using Fisher Exact Test as defined by Agresti
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29 [~~274~~]. Hardy-Weinberg equilibrium was evaluated through Arlequin software [~~285~~].
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Field Code Changed

32 33 Results and Discussion

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36 Tables 1 and 2 show allele and genotype frequencies in [the](#) Madeira Island population.
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38 H63D, with 20.5%, was the mutation most prevalent, followed by S65C (1%) and
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40 C282Y (0.33%), all showing to be in Hardy-Weinberg equilibrium. Except for Algarve,
41
42 in the extreme south of the mainland Portuguese territory, with 0.9% ($p=0.4$), C282Y
43
44 prevalence in Madeira was significantly lower than in all regions of Portugal (2-6%,
45
46 $p<0.04$), including Terceira Island in the Azores archipelago. However, the H63D and
47
48 the S65C allele frequency in Madeira Island did not show significant differences with
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50 other Portuguese populations ($p>0.1$) [~~2320, 296, 28~~].
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4 | The low C282Y allele frequency found in Madeira Island is consistent with the north-
5 | south decreasing cline that has been found in European countries and even in mainland
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7 | Portugal [134, 2320]. This result could be also related to the genetic background that is
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9 | in the basis of the origin of the Madeira population, which, besides Portugal, includes an
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11 | important influence from sub-Saharan populations [2047]. The high H63D allele
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13 | prevalence in Madeira is also consistent with the frequencies found in the South of
14 |
15 | Europe and especially in the Iberia Peninsula. The S65C ~~mutation-variant~~ in Madeira
16 |
17 | has a similar frequency than in several other countries in Europe [134].
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20 | H63D heterozygotes were found in Madeira Island population at 32%, the same
21 |
22 | prevalence as in Terceira Island in the Archipelago of Azores and similar to the
23 |
24 | different regions in mainland Portugal ($p>0.1$). Its compound heterozygote with C282Y
25 |
26 | was 0.65%, similar to the different regions of Portugal (0.71%-3.8%, $p>0.07$). H63D
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28 | homozygotes reach 3.9%, which is also similar to other Portuguese regions (2.3%-6.2%,
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30 | $p>0.27$). No samples were identified as homozygous for C282Y or S65C ~~mutations~~
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32 | ~~variants~~ and neither as compound heterozygote between both of them, a genotype that
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34 | had been also associated to a mild form of iron overload [118].
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37 | Accordingly to our estimates, both genotypes associated to Hereditary
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39 | Hemochromatosis, C282Y homozygotes and C282/H63D compound heterozygotes,
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41 | could be present in the Madeira Island in 1,648 individuals, representing 0.65% of the
42 |
43 | total population (Table 2). The prevalence of these genotypes is similar to the one found
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45 | in other different regions of Portugal (0.75%-3.1%, $p>0.1$), except for the Centre where
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47 | they have a statistical significantly higher frequency (4.6%, $p=0.04$).
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4 | In conclusion, the prevalence of H63D, C282Y and S65C ~~mutations-variants~~ in Madeira
5 | Island is, in general, similar to other Portuguese regions, especially the Algarve in the
6 | extreme south of the mainland territory, since the other regions show a statistically
7 | significant higher prevalence on the C282Y ~~mutation~~. The data presented here is a
8 | contribution to better understand HFE mutation distribution in the world and, especially,
9 | in Portugal. It gives also a perception about the number of individuals from Madeira
10 | Island that could be most susceptible to developed Hereditary Hemochromatosis. Future
11 | studies to clarify the penetrance of the se three HFE ~~gene mutations-variants~~ in
12 | Hereditary Hemochromatosis patients from Madeira Island will be important to better
13 | understand the clinical implications of the present data, namely their consequences on
14 | iron overload.

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19 | **Acknowledgments:** This research was supported by Intervir+ program from Madeira
20 | Autonomous Region (Portugal) and European Community, as well as BIOFORMA and
21 | SANTOQUEIJO.

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Table 1. Allele frequencies of H63D, S65C and C282Y mutation in the *HFE* gene in Madeira Island population.

| HFE alleles | Frequency | 95% CI |
|-------------|-----------|--------|
| H63D | 20.5% | 16-25 |
| S65C | 1% | 0-2.1 |
| C282Y | 0.33% | 0-1 |

Table 2. Genotype frequencies of H63D, S65C and C282Y mutations-variants in *HFE* gene in Madeira Island population. The presence or absence of the indicated mutation variant is shown by (+) or (-), respectively. CI- Confidence Interval. *1 Value calculated from Hardy-Weinberg Equilibrium formula ($p^2+2pq+q^2=1$). *2We assume zero since lower limit of CI 95% was a negative value.

| Genotypes | | | Observed (n=154) | Expected (n=253,000) | |
|-----------|------|-------|------------------|----------------------|---------------------------|
| H63D | S65C | C282Y | n | n | % (95% CI) |
| -/- | -/- | -/- | 95 | 156,101 | 61.7 (54-69.4) |
| +/- | -/- | -/- | 49 | 80,454 | 31.8 (24.4-39.2) |
| +/+ | -/- | -/- | 6 | 9,867 | 3.9 (0.84-7) |
| -/- | +/- | -/- | 2 | 3,289 | 1.3 (0 ² -3.1) |

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|--------------|-----|-----|-----|-------------------|--------------------------------|
| +/- | +/- | -/- | 1 | 1,645 | 0.65 (0 ² -1.9) |
| +/- | -/- | +/- | 1 | 1,645 | 0.65 (0 ² -1.9) |
| -/- | -/- | +/- | 0 | 653 ^{*1} | 0.26 (0 ² -1.1) |
| -/- | +/+ | -/- | 0 | 25 ^{*1} | 0.01 (0 ² -0.17) |
| -/- | -/- | +/+ | 0 | 3 ^{*1} | 0.0011 (0 ² -0.053) |
| Total | | | 154 | | |