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Reply to HEP 16-0784.R1 and HEP 16-0898:

1) *GNPAT* polymorphism rs11558492 is not associated with increased severity in a large cohort of *HFE* p.Cys282Tyr homozygous patients; and 2) *GNPAT* p.D519G variant and iron metabolism during oral iron tolerance test

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To the Editor

Tchernitchko and colleagues evaluated the relationship of *GNPAT* p.D519G (rs11558492) with iron phenotypes in *HFE* p.C282Y homozygotes identified in a phlebotomy program of the French Blood Agency. They described the prevalence of p.D519G positivity among p.C282Y homozygotes with intermediate degrees of iron overload, a subgroup that was not represented in our report.[1] Failure to find an effect could be the result of low power, suggested by large confidence intervals (their Table 2, erratum in press). Their confidence intervals include biologically and clinically relevant effect sizes. The upper 95% confidence limit of 1.73 indicates a 73% greater amount of iron removed [AIR] among p.D519G homozygous males compared to p.D519G wt/wt males. If the values of e^B in Table 2 of 1.11 and 1.28 represent a biological association for men (e.g., 11% and 28% greater AIR for p.D519G heterozygotes and homozygotes, respectively), a simulation study, using the authors' distributional information and methods, shows only 21% and 39% power to detect these effects at the 0.05 significance level. Control for alcohol use was not described for the association analyses. Table 2 shows statistically and clinically significant associations between higher alcohol consumption and higher serum ferritin (SF) and AIR. Failure to control for alcohol consumption can bias the genetic effect size estimates downward and decrease power to detect any genetic association.

Using an extreme phenotypes sample designed to have a large effect size if a true association exists, we examined p.C282Y homozygous men who consumed little or no alcohol. The low expressers had mean SF of 302 μg/L; 31% did not require phlebotomy. The mean iron removed to achieve depletion in the remainder was 1.5 g.[1] High expressers had mean SF of 2,391 μg/L and a mean iron removed of 15 g.[1] Tchernitchko et al. identified 32 men and 5 women with >10 g mobilized body iron but did not report the proportion with p.D519G positivity. Thus, a comparison between their subgroup and ours is not possible.

Tchernitchko et al. also describe a subgroup of male p.C282Y homozygotes with alcohol consumption <20 g/d. The p.D519G allele frequencies in both 37 "high" (>5 g iron removed) and 38 "low" expressers (<3 g iron removed) were similar to those reported in the Exome Aggregation Consortium. Their threshold of mobilizable iron indicates that their "high" expresser subgroup included patients whose iron phenotypes would have been classified between our extreme phenotypes. The authors did not state the duration of phlebotomy therapy in their "high" expressers. Besson-Fournier et al. defined high expressers as patients with >5 g iron removed within the first year of phlebotomy treatment, demonstrating that among these high expressers the proportion with p.D519G positivity was significantly higher than that among 4300 European Americans (Exome Variant Server).[2]

The background genomic structure can lead to different results in different populations. Given the uncertain differences in iron measurements that might be associated with *GNPAT* in the overall p.C282Y homozygous population, along with the complexities of differing population backgrounds, functional studies are more promising and can show cause and effect. To this end, Tchernitchko et al. tested for differences in serum hepcidin levels between p.D519G-positive and -negative p.C282Y homozygotes (~equal numbers of men and women), excluding patients with alcohol consumption ≥20 g/d. Mean hepcidin levels

did not differ significantly between patients with at least one p.D519G allele and those homozygous for the wild-type *GNPAT* allele, but the authors provided no power estimates, used a low-powered, non-parametric test, and did not provide confidence intervals to facilitate interpretation. The data available to Tchernitchko et al. provide a basis for power calculations over a range of effect sizes in the general population of p.C282Y homozygotes that could be used in designing future studies. Based on the evidence presented, their results are not incompatible with ours.

Rametta et al. examined p.D519G positivity in European subjects without hemochromatosis-associated *HFE* genotypes. Serum iron levels and transferrin saturation (TS) after an oral iron challenge were significantly higher in p.D519G-positive than in p.D519G-negative subjects. The rise in serum hepcidin levels in proportion to TS was significantly smaller in subjects with p.D519G. Rametta et al. concluded that p.D519G augments iron absorption by reducing hepcidin release. These results agree with those of Hsiao et al.[3] who observed that p.D519G positivity in healthy Taiwanese women (in whom *HFE* p.C282Y is not found) was associated with higher serum iron levels and TS after an oral iron challenge. Consistent with the results of Rametta et al., we observed that siRNA *GNPAT* knockdown decreased hepcidin mRNA expression in HepG2/C3A cells.[1] Taken together, the results of Rametta et al., Hsaio et al.,[3] and our *in vitro* experiments[1] provide substantial evidence that p.D519G reduces hepcidin release and increases iron absorption.

The studies of Tchernitchko et al. and Rametta et al. contribute to our understanding of the role of p.D519G in modulating iron phenotypes in p.C282Y homozygotes and controlling intestinal iron absorption. We previously suggested that p.D519G might identify p.C282Y homozygotes at increased risk of developing severe iron overload.[1] Tchernitchko et al. have observed that many p.C282Y homozygotes with intermediate degrees of iron overload also have p.D519G. On the other hand, some high expressers we studied did not have p.D519G,[1] suggesting they may have other modifiers. Multiple genetic modifiers and environmental triggers likely contribute to expression of *HFE* hemochromatosis. When other alleles that modify iron phenotypes of p.C282Y homozygotes are identified, assessing the risk of severe iron overload may require considering polymorphisms in multiple genes.

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Abbreviations

SF	serum ferritin
TS	transferrin saturation

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