Assessment of haptoglobin alleles in autism spectrum disorders

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Gene-environment interactions, by means of abnormal macromolecular intestinal adsorption, is one of the possible causes of autism spectrum disorders (ASD) predominantly in patients with gastrointestinal disorders. Pre-haptoglobin-2 (zonulin), encoded by the Haptoglobin (HP) allele-2 gene, enhances the intestinal permeability by modulation of intercellular tight junctions. The two alleles of HP, HP1 and HP2, differ for 2 extra exons in HP2 that result in exon duplication undetectable by classic genome-wide association studies. To evaluate the role of HP2 in ASD pathogenesis and to set up a method to discriminate HP alleles, Italian subjects with ASD (n = 398) and healthy controls (n = 379) were genotyped by PCR analysis; subsequently, the PCR results were integrated with microarray genotypes (Illumina Human Omni 1S-8), obtained using a subset from the same subjects, and then we developed a computational method to predict HP alleles. On the contrary to our expectations, there was no association between HP2 and ASD (p > 0.05), and there was no significant allele association in subjects with ASD with or without gastrointestinal disorders (p > 0.05). With the aid of bioinformatics analysis, from a window frame of ~2 Mb containing 314 SNPs, we obtain imputation accuracy (r2) between 0.4 and 0.9 (median 0.7) and correct predictions were between 70% and 100% (median 90%). The conclusions endorse that enhanced intestinal permeability in subjects with ASD should not be imputed to HP2 but to other members of the zonulin family and/or to environmental factors.

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders with a complex and heterogeneous etiology that appears within the 3rd year of subjects and affects mainly males (ratio at 8 years of age male:female 4.5:1). Subjects with ASD are characterized by impaired interpersonal relationship, verbal and non-verbal communication difficulty, restricted and repetitive behavior.

ASD are often associated with comorbidities such as mental retardation, epilepsy, metabolic impairment, autoimmune and gastrointestinal disorders (GIDs) with microbial dysbiosis and enhanced intestinal permeability. Over the last decades, a dramatic increase of the disorder has occurred. This is justifiable to a limited extent, due to changes in diagnostic criteria in the Diagnostic and Statistical Manual of Mental Disorders (DSM) over the years. Genetics play a key role in autism pathogenesis and hundreds of genes have been associated to ASD, only a mere 20–25% of autistic subjects carry a causative genetic variant. A gene-environment interaction has been proposed for the remaining cases, especially in cases with GIDs that correlate with the severity of ASD symptoms. GIDs cases are often associated to “leaky gut” and the alteration in normal commensal gut microbiota (dysbiosis) leading to an increase in inflammation, abnormal macromolecule trafficking, potential translocation of intestinal microorganisms to lamina propria and blood. Furthermore, dysbiosis is coupled with the production of toxins and an adsorption dysfunction. Many environmental factors including intestinal microorganisms (i.e. Candida

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*albicans*), bacterial toxins such as zonula occludens toxin (Zot) secreted by the pathogen *Vibrio cholerae* as well as food mycotoxins can alter the intestinal permeability. Zot is a well-known toxin that induces severe intestinal permeability, that leads to the reversible opening of intestinal intercellular tight junctions via dissociation of Zonula Occludens-1 from its transmembrane binding partners occludin and claudin-1. A human protein called zonulin has been identified and resembles similarities with Zot. The human zonulin, that corresponds to pre-haptoglobin 2 (pre-HP2), shares a common motif with the active fragment of Zot that is critical for intestinal receptor binding. Pre-HP2 increases intestinal permeability in *ex vivo* and *in vivo* murine intestine in a dose-dependent manner, but not mature HP12. For these reasons, the role of human pre-HP2 in modulation of intestinal permeability is suggested due to its similarity with Zot in subjects with HP2 allele.

Mature HP is an acute phase protein with a haemoglobin-binding function. Its presence is abundant in plasma where it acts as a free haemoglobin scavenger preventing oxidative damage between heme iron and protein or lipids. It also has an important role as an angiogenic, anti-inflammatory and immune modulator factor. HP is mainly expressed in the liver and its synthesis is induced by cytokines, especially interleukin (IL) -6, IL-1 and tumour necrosis factor (TNF)13.

In humans, HP (UniProt accession number P00738; GI: 386783) is encoded by 2 alleles, *HP1* and *HP2* (Gene ID: 3240) yielding three different genotypes: *HP1*-1, *HP1*-2 and *HP2*-2. *HP1* and *HP2* alleles differ for the duplication of exons 3 and 4, resulting in exons 5 and 6 in *HP2* (Fig. 1). The precursor protein (pre-HP) includes signal peptide (amino-acids position: 1–18) and mature protein sequences, containing both alpha and beta chains. The proteolitically cleaved beta chain is common in HP from *HP1*-1 and *HP2*-2 alleles, while alpha chain differs from *HP1* to *HP2*: the alpha chain from *HP2* (alpha-2 chain) includes amino-acids at position 19–160, while alpha chain from *HP1* (alpha-1 chain), lacks of residues 29–87 in comparison to alpha-2 chain, and includes residues from 19 to 101 (Fig. 1). The exon 3 contains the protein multimerization domain, allowing quaternary structure formation. Alpha and beta chains combine as tetramer to obtain HP. As a consequence of HP allelic variant, the structure of HP in *HP1*-1 phenotype is (alpha-1-beta)2, that of *HP1*-2 are (alpha-1-beta)2 and (alpha-2-beta)n, and that of *HP2*-2 is (alpha-2-beta)n (Fig. 1). HP1, on the other hand, displays fast (HP1F) and slow (HP1S) isoforms of the alpha chain, depend on their fast (F) or slow (S) electrophoretic motilities. These two forms differ for the presence of an amino-acid substitution (Lys54 of HP1S is replaced by Glu in the HP1F) and originate from different allelic subtypes: *HP1*F, *HP1*S, *HP2*F, *HP2*S and *HP2*S (very rare). Because of the structure of

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**Figure 1.** *HP* alleles and protein structures. (a) *HP1* and *HP2* allele structure: *HP1* allele contains 5 exons, while *HP2* is made up of 7 exons: exons 1 to 4 correspond to *HP1*, exons 5 and 6 is the duplication of exon 3 and 4, and exon 7 corresponds to exon 5 in *HP1*. (b) *HP* isoform and protein structure. *HP1* allele encode for alpha-1 and beta chain (in blue and orange, respectively), while *HP2* allele for alpha-2 and beta chain (green and orange respectively). The quaternary structures of *HP* genotypes are also illustrated (modified from17). (c) *HP* gene encode for signal peptide, from amino-acid residues 1–18, and mature protein sequence from 19 to 347 or 406, depending on the allele of origin: *HP1* allele encoding for alpha-1 chain (residues 19–101) and beta chain (d), *HP2* allele for alpha-2 chain (residues 19–160) and beta chain (e). Alpha-1 chain lacks of residues 29–87 of alpha-2 chain, and beta chain is common to *HP1* and *HP2* alleles.
the two alleles, it is very difficult to recognize them by means of classic genome-wide association studies (GWAS), so most of the genotyping studies are limited to small size populations analyzed by PCR or quantitative RT-PCR.

HP1 and HP2 proteins are functionally different. HP-1 protein has a superior antioxidant capacity than HP-2 and induces a more rapid free hemoglobin degradation20-21, HP1 carriers also have a higher HP concentration than HP2, with HP1-1 > HP1-2 > HP2-221.

Due to the protective role of HP1, HP1-1 genotype has a significant association to longevity22, on the other hand, HP2-2 has a higher susceptibility to immune-mediated diseases, such as celiac disease, Rheumatoid Arthritis, Type 1 diabetes, Systemic Lupus Erythematosus23-26 and cancer.

In 2000, Wang and collaborators reported that an 8 amino-acid sequence in N-terminal of human pre-HP2 (residues 8–15: G G V L V Q P G) shared a common motif with the active fragment of Zot (residues 291-298: G R L C V Q D G) from which originated the alternative name “zonulin”. The common amino-acid motif is: non-polar (G), variable, non-polar, variable, non-polar (V), polar (Q), variable, non-polar (G)13. In 2018, Scheffler and collaborators identified another member of the zonulin family, properdin, a mannose-associated serine protease27.

Zonulin induces the disassembling of intercellular tight junctions in many epithelial and endothelial barriers, including the “leaky gut” and enhancing the intestinal trafficking of microorganisms and macromolecules that, in sequence, challenge the immune response involving different tissues and organs in genetically susceptible subjects22,26,28. Of interest, a significant high level of zonulin (pre-HP2) in a small cohort of subjects with ASD vs healthy controls was found29, as well as an association between HP2 allele and autism with GID comorbidity30.

In this study, we genotyped a larger cohort of patients and controls to test the hypothesis that HP2 allele is associated with ASD development at least with GID in autism. We assessed the HP alleles in a batch of Italian subjects with ASD (n = 398) and healthy controls (n = 380) by PCR analysis. Because of the internal exon duplication of HP, it is very difficult to distinguish the two different alleles by means of standard bioinformatics analysis, and PCR techniques limit the size of tested subjects. We integrated PCR and microarray genotyping data, previously obtained on a subgroup of patients and controls, to impute and discriminate HP alleles by GWAS. Thus, we provide a bioinformatic reference useful for further HP prediction on publicly available GWAS data.

Materials and Methods

Ethics committees.

- Ethics committee IRCCS Eugenio Medea – Scientific section of the association ”La Nostra Famiglia” 2010, IRCCS Eugenio Medea – La Nostra Famiglia, Bosio Parini (LC), Italy;
- Ethics committee of the hospital institutions (CEIOC) IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli 50/2008, IRCCS Istituto Centro S. Giovanni di Dio Fatebenefratelli, Brescia, Italy;
- Local ethics committee, Prot. 567/06 dated 28-09-2006 and document integration dated 09-10-2006, Azienda Ospedaliera Universitaria Senese, Siena, Italy;

All Ethics Committees of involved Institutions approved the study and enrolled subjects that signed informed consents. Informed signed consents were collected from parents and/or legal guardians for subjects under the age of 18 years. All methods were performed in accordance with relevant guidelines and regulations regarding observational studies.

Subjects. A total sample-set consisting of 398 Italian subjects with ASD [338 males (85%) and 60 females (15%)], and 379 unrelated Italian controls [170 males (45%) and 209 females (55%)] were recruited and peripheral blood samples collected.

Patients. IRCCS Eugenio Medea – La Nostra Famiglia recruited 206 subjects with ASD [171 males (83%) and 35 females (14%)] ranging between 2 and 12 years old. The diagnosis for autism (70.3%), Asperger’s syndrome (3.5%) or pervasive developmental disorder not otherwise specified (26.2%) were performed according to DSM, fourth edition, text revision (DSM-IV TR) (American Psychiatric Association [APA], 2000) and by the Autism Diagnostic Observation Scale 2 (ADOS-2)31 and Autism Diagnostic Interview - Revised (ADI-R)32. Subjects with genetic syndromes, epilepsy and neuroradiological confirmed disorders were excluded. Clinical data about the presence/absence of GIDs were evaluated in a sub-group of 157 subjects with ASD [135 males (86%) and 22 females (14%)].

The University of Siena recruited 192 subjects with ASD [167 males (87%) and 25 females (13%)] ranging from 1 to 3 years old. The diagnosis for autism (82%), Asperger’s syndrome (0,5%) and pervasive developmental disorders not otherwise specified (17%) were performed according to the DSM-IV TR (APA, 2000), and by the ADOS-2 and ADI-R. Within these patients and clinical data about the presence/absence of GIDs were evaluated in a sub-group of 8 male subjects.

Controls. IRCCS Eugenio Medea – La Nostra Famiglia recruited 25 unrelated children (12 males and 13 females) evaluated by Child Behavior Checklist as neurotypical controls; the University of Brescia and IRCCS Istituto Centro S. Giovanni di Dio, Fatebenefratelli of Brescia enrolled 259 healthy subjects [109 males (42%) and 150 females (58%); mean age 51.6 ± 15.5]; 191 (73.7%) were unrelated non-affected volunteers who were screened for DSM-IV Axis I disorders by expert psychologists using the Mini-International Neuropsychiatric Interview33. Only subjects without a history of drug or alcohol abuse/dependence and without a personal or first-degree family history of psychiatric disorders were enrolled in this study. Furthermore, subjects who obtained a score lower than 27/30 at the Mini Mental State Examination34 were excluded. The University of Siena recruited 95 healthy...
controls [45 females (47%) and 50 males (53%), aged between 20 years and 61 years, mean age of 41 years]. The subjects were healthy adults who were not evaluated for neuropsychiatric conditions.

**DNA isolation.** Genomic DNA was extracted from peripheral blood leukocytes, using commercial kits and relative protocols. Quality (280/260 ratio) and quantity of DNA was checked by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE USA).

**Haptoglobin genotyping.** PCR - HP genotypes were determined by PCR amplification devised by Koch and collaborators with minor modifications. Briefly, new PCR primers (Table 1) were designed on HP1 and HP2 specific sequences M69197.1 (EMBL-GeneBank Data Libraries) and 30 ng of genomic DNA was amplified in 10 µl of reaction mixture (Platinum™ SuperFi™ PCR Master Mix, for primers NewA and NewB, and Platinum® PCR SuperMix, High Fidelity, for primers NewC and NewD- Invitrogen™, Thermo Fisher Scientific, Wilmington, DE USA) as suggested by the supplier. Two couples of primers, NewA with NewB and NewC with NewD were used separately, to analyze every sample and each analysis was duplicated. Primers NewA (5′-GGGTTTCCCTGGCAGAAATGA-3′) and NewB (5′-CCCTGCGCTGGTGAACCTGTATT-3′) can produce, contextually, a 1775 bp and/or a 3487 bp specific product for HP1 and HP2 alleles, respectively. Primers NewC (5′-ATGCCAACCTGCTGTATT-3′) and NewD (5′-CGAACCGAGTGCTCCACATA-3′) amplify a 360 bp HP2 allele specific fragment.

After denaturation for 30 seconds at 98 °C, thermo-cycling profile consisted of denaturation for 12 seconds at 98 °C, annealing for 10 seconds at 65.4 °C and extension for 1 minute and 40 seconds at 72 °C repeated for 35 cycles when using primers NewA and NewB, while denaturation for 2 minutes at 94 °C, thermo-cycling profile for 1 minutes at 94 °C, 40 seconds at 68 °C and 50 seconds at 72 °C repeated for 32 cycles, and extension for 2 minutes at 72 °C for primers NewC and NewD. PCR products obtained by NewA-B and NewC-D couples of primers were run on 1% and 2% agarose gel (E-Gel agarose gels, Invitrogen™, Thermo Fisher Scientific) respectively. In HP1-1 we detected the NewA-B PCR product of 1775 bp length, but not NewC-D PCR product; in HP2-2 we found the NewA-B PCR product of 3487 bp and NewC-D 360 bp PCR product; in HP1-2 subjects we identified both 3487 bp and 1775 bp amplicons, and the 360 bp PCR product (Fig. 2).

**Sanger sequencing.** To check the PCR products, two PCR products for each type of HP genotype obtained by NewA and –B amplification (HP1-1, HP1-2, HP2-2) as well as those obtained by NewC and -D, were purified (CleanSweep PCR Purification Reagent, Applied Biosystems, Thermo Fisher Scientific, Wilmington, DE USA) and sequenced by Sanger method using NewC and NewD respectively for forward and reverse sequencing. Results were compared to HP1 and HP2 sequences (NCBI Reference Sequence: NG_012651.1).

**Data analysis.** The association between HP alleles and ASD was calculated considering subjects with ASD vs all the analyzed controls and subjects with ASD without GID vs subjects with ASD with GID. Chi-square with Yates correction test was applied to evaluate statistical significance. We sub-classified the controls in two groups referring to the enrollment screening test: ASDs negative controls (n = 188), referred as non-ASD (NASD) controls, and those negative for ASD and DSM-IV Axis I disorders (n = 191), referred as super-controls. The HP allelic distribution was evaluated in all subjects with ASD and controls and in all sub-groups. Possible effect of sex on HP allele distribution was investigated in patients with ASD and in healthy subjects, considering the sub-classification of controls.

Due to the small size of controls enrolled in this study we implemented our data by a meta-analysis including HP genotyping of healthy Italians, healthy Caucasians (Table 2).

**Microarray.** Microarray analysis was performed on a subset of samples (n = 318) collected by IRCCS Eugenio Medea – La Nostra Famiglia using Human Omni 1S-8 v 1.0 Illumina chip and iScan Illumina (Illumina, San Diego, CA, USA) according to the manufacturer’s protocols, and a selection of 230 subjects were genotyped by microarray and PCR.

Among controls collected by the University of Brescia, 177 subjects (87 females and 90 males) were genotyped by Affymetrix Human Mapping GeneChip 6.0 array (Affymetrix, Thermo Fisher Scientific, Wilmington, DE USA) according to the manufacturer’s protocols and applying quality control procedures described by Sacchetti and colleagues, and 103 of them were genotyped for HP gene by PCR.

**Bioinformatics.** Genetic data for 1,185,076 SNPs in 230 subjects were collected from “Top” alleles of the Illumina’s Genome Studio Final Report, generated using HumanOmni1S platform and GRCh37.p13 genome assembly. Standard quality control procedures were used to exclude individuals with discordant sex and call rates

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**Table 1.** PCR primers sequence and amplicon length.

| Primer | Primer sequence | Amplicon length |
|--------|-----------------|-----------------|
| NewA   | 5′-GGGTTTCCCTGGCAGAAATGA-3′ | 1775 bp 3487 bp |
| NewB   | 5′-CCCTGCGCTGGTGAACCTGTATT-3′ | — 360 bp |
| NewC   | 5′-ATGCCAACCTGCTGTATT-3′ | — |
| NewD   | 5′-CGAACCGAGTGCTCCACATA-3′ | — |

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**Table 1.** PCR primers sequence and amplicon length.
below 98% and filter SNPs with MAF < 1%, Hardy Weinberg p < 1 × 10⁻⁴ and a call rate < 99%. A total of 696,849 markers and 293 subjects fulfill quality control requirements. The analysis of genetic similarity among individuals revealed the presence of several outliers. Only individuals belonging to the most populated cluster were considered for further analyses. The quality control procedures repeated on this subgroup determined the exclusion of some additional markers. A total of 690'215 markers and 282 individuals were retained.

Genotypes detected by microarray and HP genotypes detected by PCR were phased using Beagle38 against the haplotypes provided by the 1000 Genomes project (Phase 3). HP alleles were imputed using Beagle v4.139. A total of 100 cross-validation trials were run, randomly assigning each time 90% of the subjects to the “reference” group and the remaining 10% to the “test” group.

**Results**

**HP allele distribution in patients and controls.** As for PCR HP genotyping the length of NewA-B PCR products reflected the expected lengths (HP1 = 1775 bp; HP2 = 3487 bp) and included the duplication breakpoint sequence only in NewC-D products (360 bp) and in NewA-B products corresponding to HP2 allele (Fig. 2).

**Figure 2.** HP alleles structure and PCR primers. HP2 allele contains a duplication of 1700 bp, corresponding to extra exons 3 and 4. Due to this duplication, PCR amplification with NewA and NewB primers (narrowly indicate 5’ > 3’ orientation) amplification gives 1775 bp length amplicons in HP1 allele (a), and 3487 bp length amplicons in HP2 allele (b). PCR products obtained with primers pairs NewC/NewD are 360 bp long in HP2 allele (b), while in HP1 no amplicon is given (c). Agarose gels, 1% (d) and 2% (e), are used to detect the three different genotype for both primer pairs. In the case of NewA and NewB in HP1-1 subjects only 1775 bp amplicon is present, in HP1-2 both amplicons are present and in HP2-2 only 3487 bp amplicon is detected (d). For NewC/NewD primer pairs 360 bp amplicons is detected only in HP1-2 and HP2-2 genotypes (e). Gels images (d,e) are cropped from images of full-length gels (Supplementary Fig. S1).
Table 2. HP allele distribution in subjects with ASD and controls. Data on neurotypical Italian population were from the total of controls enrolled by Bottini and collaborators and Napolioni and co-worker. Genotyping data on Caucasian healthy subjects were from Koch and collaborators, and HP allele frequency of individuals sampled by the 1000 Genomes Project was calculated in the study of Boettiger and co-worker. HP2 allele is highly represented in subjects with ASD and controls. HP1 allele increases significantly in patients when compared to total and non-ASDs controls enrolled in this study and to controls from 1000 Genomes projects. In contrast, HP1 allele did not increase significantly when compared to the other controls. P value was calculated with Chi-square with Yates correction test (*P < 0.05; **P < 0.005).

| Allele frequency | Subjects with ASD | Controls from literature | P value |
|------------------|-------------------|--------------------------|---------|
|                  | HP1-1              | Total Super controls NASD control |         |
|                  | n=398             | n=379 n=191 n=188 | n=2130 n=2059 n=2318 n=249 n=76 n=89 |
| HP1-1            | 2318 (69.3%)      | 233 (30.7%) 129 (34.3%) | 104 (27.2%) 151 (35.2%) 233 (30.7%) 104 (27.2%) 129 (34.3%) |
| HP1-2            | 293 (36.8%)       | 233 (30.7%) 129 (34.3%) | 104 (27.2%) 151 (35.2%) 233 (30.7%) 104 (27.2%) 129 (34.3%) |
| HP2              | 2509 (64.8%)      | 2318 (69.3%) 129 (34.3%) | 104 (27.2%) 151 (35.2%) 233 (30.7%) 104 (27.2%) 129 (34.3%) |
| P value          | 0.0134**          | 0.0014** 0.4429 | 0.4169 0.2480 0.3904 0.5673 0.8542 |

HP genotypes of the 398 subjects with ASD were HP1-1 = 59 (14.8%), HP1-2 = 175 (44.0%) and HP2-2 = 164 (41.2%), and the frequencies of HP1 and HP2 were 293 (36.8%) and 525 (63.2%), respectively. The distribution of genotype frequencies is in Hardy Weinberg equilibrium (p ≈ 0.3; χ² test).

The genotypes of all the controls (n = 379) were: HP1-1 = 35 (9.2%), HP1-2 = 163 (43.0%) and HP2-2 = 181 (47.8%); HP1 frequency = 233 (30.7%) and HP2 = 525 (69.3%); and are in Hardy Weinberg equilibrium (p ≈ 0.9; χ² test). Considering the two control groups separately, the allelic distribution of HP1 was 34.3% in NASD controls and decreased to 27.2% in super-controls, while HP2 was 65.7% and 72.3%, respectively (Table 2).

The HP1 increased in subjects with ASD when compared to the total number of controls: Chi-square with Yates correction test was calculated and a significant increase of HP1 allele frequency was found in subjects with ASD compared to total controls (NASD controls + super controls) (P < 0.05) and super-controls (P < 0.005). Consequently, the association between HP1 and ASD disappeared (P > 0.05) excluding super-controls and adding to NASD controls, Italian and Caucasian healthy subjects (Table 2). Considering only subjects with ASD with clinical GID data (n = 157), the allelic distribution in subjects with ASD with GID (n = 89; HP1 37.1% and HP2 62.9%) and without GID (n = 76; HP1 35.4% and HP2 64.6%) did not differ (P > 0.05) (Table 2).

The sex ratio differs from the subjects with ASD and controls, and this could influence the HP allele distribution. For this reason, we also investigated HP genotype related to sex. HP allele distribution did not show statistically significant imbalanced sex ratio in patients with ASD (P > 0.1), in the total number of controls (P > 0.5), and in the sub-groups NASD controls and super controls (P > 0.1) (Supplementary Table S1). To know more about the consequence of the sex imbalance within controls and subjects with ASD we also re-analyzed data from the study of Brackenridge that found a significant sex imbalance in HP1-2 distribution within Australian population. HP allele distribution was calculated for healthy Australian population (Supplementary Table S1) and we did not find significant difference in HP distribution between males and females by Fisher test (P > 0.5). HP alleles imputation from SNP haplotypes. To assess whether HP alleles can be predicted by the haplotype of surrounding SNPs we performed a series of cross-validation trials, in which we split the autist population genotyped with the Illumina array into two groups (“reference” and “test”) panels, and imputed (Beagle v4.1) the HP alleles of test subjects, using the multi-SNP haplotypes surrounding HP and HP alleles detected by PCR in subjects assigned to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group. We considered the SNPs occurring up to 2 Mb around HP1 and HP2 detected by PCR and added to the reference group.

The same approach was applied to healthy subjects, genotyped by the Affymetrix platform (Table 4). We observed a slightly lower accuracy (e.g. 0.89 using 76 SNPs; 0.5 Mb window), but its trends relative to the number of surrounding SNPs considered and to HP genotypes were similar to those observed in autistic subjects.

Discussion
Wang and colleagues first found, in human intestinal tissue, a protein that they called zonulin, sharing a common amino-acid motif with Zot, the Vibrio cholerae toxin acting on the intestinal wall disassembles tight junctions with consequent intestinal permeability. A following study identified human zonulin, as pre-HP2. Since an involvement of zonulin has already been demonstrated in many immune mediated diseases with GID, and many subjects with ASD suffer from GID and “leaky gut”, a possible role of zonulin in ASD pathogenesis was assumed. Nevertheless, because of its structure containing a duplication of 2 exons, HP gene is difficult to genotype by classic genome-wide studies and HP available data are limited to a very small amount of cases.

To understand the role of zonulin in ASD etiology and to provide a bioinformatics method to genotype HP using or re-using newly generated or archival data, respectively, we i) analyzed, by PCR, the HP allele distribution

Table 2. HP allele distribution in subjects with ASD and controls. Data on neurotypical Italian population were from the total of controls enrolled by Bottini and collaborators and Napolioni and co-worker. Genotyping data on Caucasian healthy subjects were from Koch and collaborators, and HP allele frequency of individuals sampled by the 1000 Genomes Project was calculated in the study of Boettiger and co-worker. HP2 allele is highly represented in subjects with ASD and controls. HP1 allele increases significantly in patients when compared to total and non-ASDs controls enrolled in this study and to controls from 1000 Genomes projects. In contrast, HP1 allele did not increase significantly when compared to the other controls. P value was calculated with Chi-square with Yates correction test (*P < 0.05; **P < 0.005).
pared to those of previous study of Bottini and collaborators and Napolioni and co-worker. Consequently, the allelic distribution by Fisher test between Australian males and females, revealed no significant differences in then again, the re-analysis of the data reported by Brackenridge showing that HP1-2 has different distribution the study of Zhao and colleagues declared no significant sex-based difference in the allelic distribution of HP. The present study showed no significant association between age and genotype in healthy subjects. Taking together these findings, we conclude that allele distribution is not related to sex imbalance between controls and patients with ASD.

Moreover, no allelic association was found between subjects with ASD and patients suffering from GID. This indicates that the HP genotype does not represent a risk factor for ASDs pathogenesis, and that HP does not

### Table 3. Bioinformatics imputation analysis, Illumina platform. Results of HP alleles imputation from SNP haplotypes genotyped using an Illumina array. Best prediction accuracy results are achieved in increments of 0.5 windows (in bold).

| Window (Mb) | Number of markers | Accuracy of prediction |
|-------------|-------------------|------------------------|
|             |                   | HP1-1  | HP1-2  | HP2-2  | Mean   |
| 0.1         | 16                | 0.34   | 0.63   | 0.96   | 0.73   |
| 0.25        | 51                | 0.70   | 0.93   | 0.96   | 0.91   |
| 0.5         | 79                | 0.80   | 0.92   | 0.97   | 0.92   |
| 1           | 124               | 0.73   | 0.94   | 0.96   | 0.91   |
| 1.5         | 203               | 0.76   | 0.93   | 0.98   | 0.92   |
| 2           | 313               | 0.79   | 0.92   | 0.97   | 0.92   |

### Table 4. Bioinformatics imputation analysis, Affymetrix platform. Results of HP alleles imputation from SNP haplotypes genotyped using an Affymetrix platform. Best prediction accuracy results are achieved in increments of 0.5 windows (in bold).

| Window (Mb) | Number of markers | Accuracy of prediction |
|-------------|-------------------|------------------------|
|             |                   | HP1-1  | HP1-2  | HP2-2  | Mean   |
| 0.1         | 11                | 0.26   | 0.38   | 0.94   | 0.67   |
| 0.25        | 40                | 0.64   | 0.87   | 0.97   | 0.89   |
| 0.5         | 76                | 0.59   | 0.89   | 0.97   | 0.89   |
| 1           | 126               | 0.68   | 0.90   | 0.95   | 0.89   |
| 1.5         | 185               | 0.61   | 0.85   | 0.95   | 0.87   |
| 2           | 256               | 0.67   | 0.89   | 0.96   | 0.90   |
phytase 1, regulatory (inhibitor) subunit 3 (PPP1R3F) from which rare mutations have been found in autism.\(^4\)

Datasheets were corrected reporting zonulin or HP as target protein. (Immundiagnostik AG, Germany), and only after the publication of the study of Scheffler and colleagues, the Esnafoglu and colleagues used the Human Zonulin ELISA Kit, Elabscience\(^2\) to quantify zonulin in the serum of literature. Indeed, zonulin quantification using commercial Human Zonulin ELISA kits was widely reported in literature. Gelolin \(\text{[i.e. Human Zonulin ELISA Kit, Elabscience, (Wuhan, Hubei Province, China) and IDK® Zonulin ELISA}}\) and NCBI (Gene ID 3234), report both HP and zonulin among "names", while only Nextprot (NX_P00738) and NCBI (Gene ID 3234), report both HP and zonulin among "names", while only pre-HP2 belongs to the zonulin family. This has induced some authors to consider zonulin and HP as the same protein. Furthermore, the zonulin ELISA commercial kits and antibodies also reported indifferently HP or zonulin [i.e. Human Zonulin ELISA Kit, Elabscience, (Wuhan, Hubei Province, China) and IDK® Zonulin ELISA (Immundiagnostik AG, Germany)], and only after the publication of the study of Scheffler and colleagues, the datasheets were corrected reporting zonulin or HP as target protein.

Considering zonulin a family of proteins instead of a single one\(^2\) modifies the interpretation of some previous literature. Indeed, zonulin quantification using commercial Human Zonulin ELISA kits was widely reported in different pathological conditions and the conclusions drawn on the basis of HP-zonulin identity. For instance, Esnafoglu and colleagues used the Human Zonulin ELISA Kit, Elabscience\(^9\) to quantify zonulin in the serum of subjects with ASD. The capture antibody of this kit recognizes a sequence within residues 118–281 of pre-HP2 (Fig. 3). Then again, the capture antibody of the IDK® Zonulin ELISA, recognizes a portion of zonulin previously reported by Wang and colleagues\(^13\), (datasheet http://www.immundiagnostik.com/fileadmin/pdf/Zonulin_K5601.pdf). Since this portion is not included in HP sequence, the antibody may recognize another zonulin family member, as suggested by Scheffler and colleagues\(^9\). Indeed, these authors, which integrated antibody capture experiments, mass spectrometry, and Western blot analysis, demonstrated that the IDK® Zonulin ELISA (based on a polyclonal antibody anti-zonulin) mainly recognize properdin, a potential second member of the zonulin family\(^2\). Interestingly, properdin (P27918 [https://www.uniprot.org/uniprot/P27918]) maps on Xp11.23 making male subjects more susceptible to its allelic or variant effect. Furthermore, this chromosome region includes three other genes related to autism: the calcium channel, voltage-dependent, alpha 1 F (CACNA1F) associated with syndromic autism and schizophrenia\(^4\), the phosphatase 1, regulatory (inhibitor) subunit 3 F (PPP1R3F) from which rare mutations have been found in autism\(^5\) and histone deacetylase 6 (HDAC6) from which a partial skipping of exon 3 was found in a subject with ASD\(^6\).

Then again, gut dysbiosis has been described in subjects with ASD\(^7\)–\(^10\) and gut microbial imbalance, that increases inflammation and microbial toxic metabolites production, is the strongest stimulus for the activation of the zonulin pathway with consequent intestinal permeability and trafficking of macrophages through intestinal wall\(^11\). Moreover, gut microbes strongly participate in "microbiota-gut-brain-axis" (the microbiota-CNS cross-talk)\(^12\) modulating inflammatory cytokines, neurotransmitters production and epigenetic factors such as RNA interference, DNA methylation and histone modification. So, microbiota alteration can produce negative effects on brain function via "gut-brain axis" dysregulation and, indeed, dysbiosis has been reported in many psychiatric conditions\(^13\) including ASD\(^7\)–\(^10\). Recently, the role of mycotoxins has also been proposed in ASD pathogenesis\(^14\). Mycotoxins, worldwide contaminants of food with toxicological effects, induce intestinal permeability and inflammation and interact with gut microbiota resulting in impairment of gut health\(^15\).
It is well known that the HP CNV is not in strong linkage disequilibrium with any individual SNP and therefore it is not successfully genotyped by SNP–array technologies. This is likely due to the fact that HP1 allele arose from recurrent deletions in HP2 allele. In addition, HP1F and the left copy of HP2FS share a 300 bp sequence identical to a segment of Haptoglobin-Related Protein (HPR) gene. This HPR sequence that contains many derived variants was probably transferred into HP gene through a paralogous conversion event making HP genotyping unpredictable by classic GWAS. Thus, PCR or Real Time-PCR is generally performed for HP genotyping on very small cohorts.

Although HP1 allele arose many times, it has been shown that it is possible to impute HP alleles from SNP haplotypes with a high level of accuracy. Indeed, alleles that are old and common today segregate on characteristic SNP haplotypes. In this paper, we assessed the performance of HP alleles imputation from SNP haplotypes in our cohort. Overall, we observed a high accuracy in the prediction of HP genotypes in both autistic and healthy subjects screened by Illumina and Affymetrix platforms. In both groups, the prediction of HP2-2 genotype is more successful (accuracy is 0.97 in both platforms), on the other hand HP1-1 genotype is lower (0.80 and 0.59, respectively).

Beagle prediction based on the Illumina microarray platform gives acceptable results and should be useful to genotype HP for a large scale of subjects. Due to the lower accuracy of HP1-1 prediction, even if it represents a good accomplishment, a PCR genotyping to confirm HP1-1 prediction should be useful.

It is important to highlight that the intention of our study was to verify the distribution of HP2 allele in subjects with ASD and to relate this with GID comorbidity, also driven by the consideration of the increased levels of pre-HP2/zonulin in subject with ASD, measured by ELISA. The unexpected results addressed the question on the possible dissimilarity between zonulin and HP2. During this investigation Scheffler and colleagues and Ajamian and colleagues preceded thus we confirm their statements.

Conclusion
Zonulin is a family of structurally and functionally associated proteins including pre-HP2 and properdin. On the contrary to our expectations, no correlation between HP alleles and Italian ASD patients or between subjects with ASD and those patients suffering from GID was found. These results further support a recent study of Scheffler and colleagues. They demonstrated that, within zonulin family members, properdin protein, rather than haptoglobin, is similar to Zot and possibly is involved in intestinal permeability. Interestingly, properdin maps on Xp11.23 show that male subjects are more prone to its effects. Further efforts should be dedicated to genotype and/or to sequencing this gene in subjects with ASD.

However, additional investigations with a wider number of cases and controls are necessarily required to confirm these results. For this purpose, the study proposes a bioinformatics method to predict HP allele distribution starting from GWAS data.

Moreover, many issues concerning HP and zonulin definition and detection must still be thoroughly studied. In conjunction with new genetic and/or environmental and predisposing factors which may lead to or provoke leaky gut in autistic subjects.

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References
1. Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Investigators; Centers for Disease Control and Prevention. Prevalence of autism spectrum disorders—Autism and Developmental Disabilities Monitoring Network, 14 sites, United States, 2008. MMWR Surveill Summ. 61, 1–19 (2012).
2. Smaga, J. L. et al. Oxidative stress as an etiological factor and a potential treatment target of psychiatric disorders. Part 2. Depression, anxiety, schizophrenia and autism. Pharmacol. Rep. 67, 569–80 (2015).
3. Rosenfeld, C. S. Microbiome Disturbances and Autism Spectrum Disorders. Drug Metab. Dispos. 43, 1557–71 (2015).
4. de Magistris, L. et al. Alterations of the intestinal barrier in patients with autism spectrum disorders and in their first-degree relatives. J. Pediatr. Gastroenterol. Nutr. 51, 418–24 (2010).
5. de Magistris, L. et al. Antibodies against food antigens in patients with autistic spectrum disorders. Biomed. Res. Int. 729349 (2013).
6. Berg, J. M. & Geschwind, D. H. Autism genetics: searching for specificity and convergence. Genome Biol. 13, 247 (2012).
7. Adams, J. B., Johansen, L. J., Powell, L. D., Quig, D. & Rubin, R. A. Gastrointestinal flora and gastrointestinal status in children with autism—comparisons to typical children and correlation with autism severity. BMC Gastroenterol. 16, 11–22 (2011).
8. Buie, T. et al. Recommendations for evaluation and treatment of common gastrointestinal problems in children with ASDs. Pediatrics. 125, S19–29 (2010).
9. Hisao, E. Y. Gastrointestinal issues in autism spectrum disorder. Harv. Rev. Psychiatry. 22, 104–11 (2014).
10. Akbari, R. et al. The intestinal barrier as an emerging target in the toxicological assessment of mycotoxins. Arch. Toxicol. 91, 1007–29 (2017).
11. Goldblum, S. E. et al. The active Zot domain (aa 288–293) increases ZO-1 and myosin 1C serine/threonine phosphorylation, alters interaction between ZO-1 and its binding partners, and induces tight junction disassembly through proteinase activated receptor 2 activation. FASEB J. 25, 144–58 (2011).
12. Tripathi, A. et al. Identification of human zonulin, a physiological modulator of tight junctions, as prehaptoglobin-2. Proc. Natl. Acad. Sci. USA 106, 16799–804 (2009).
13. Wang, W., Uzzau, S., Goldblum, S. E. & Fasano, A. Human zonulin, a potential modulator of intestinal tight junctions. J. Cell Sci. 113, 4435–40 (2000).
14. Quaye, J. K. Haptoglobin, inflammation and disease. Trans. R. Soc. Trop. Med. Hyg. 102, 735–42 (2008).
15. Raynes, J. G., Eagling, S. & McAdam, K. F. Acute-phase protein synthesis in human hepatoma cells: differential regulation of serum amyloid A (SAA) and haptoglobin by interleukin-1 and interleukin-6. Clin. Exp. Immunol. 83, 488–91 (1991).
16. Yang, F., Brune, J. L., Baldwin, W. D., Barnett, D. R. & Bowman, B. H. Identification and characterization of human haptoglobin cDNA. Proc. Natl. Acad. Sci. USA 80, 5875–9 (1983).
17. Melamed-Frank, M. et al. Structure-function analysis of the antioxidant properties of haptoglobin. Blood. 98, 3693–8 (2001).
18. Van Vlierberghe, H., Langlois, M. & Delanghe, J. Haptoglobin polymorphisms and iron homeostasis in health and in disease. Clin. Chim. Acta. 345, 35–42 (2004).
19. Black, J. A. & Dixon, G. H. Amino-acid sequence of alpha chains of human haptoglobins. Nature. 218, 736–41 (1968).
20. Buehler, P. W. et al. Haptoglobin preserves the CD163 hemoglobin scavenger pathway by shielding hemoglobin from peroxidative modification. Blood. 113, 2578–86 (2009).
21. Langlois, M. R. & Delanghe, J. R. Biological and clinical significance of haptoglobin polymorphs in humans. Clin. Chem. 42, 1589–600 (1996).
22. Napolioni, V., Gianni, P., Carpi, F. M., Concetti, F. & Lucarini, N. Haptoglobin (HP) polymorphisms and human longevity: a cross-sectional association study in a Central Italy population. Clin. Chim. Acta. 412, 574–7 (2011).
23. Levy, A. P. et al. Haptoglobin: basic and clinical aspects. Antioxid. Redox Signal. 12, 293–304 (2010).
24. Pavin, E. J. et al. Proteomic analysis of plasma from patients with systemic lupus erythematosus: increased presence of haptoglobin alpha2 polypeptide chains over the alpha1 isoforms. Proteomics. 6, S282–92 (2006).
25. Dayan, L. et al. Haptoglobin genotype and endothelial function in diabetes mellitus: a pilot study. Eur. J. Appl. Physiol. 106, 639–44 (2009).
26. Fasano, A. Zonulin, regulation of tight junctions, and autoimmunity diseases. Ann. N. Y. Acad. Sci. 1258, 25–33 (2012).
27. Scheffler, L. et al. Widely used commercial ELISA does not detect precursor of Haptoglobin2, but recognizes Properdin as a potential second member of the Zonulin family. Front. Endocrinol. (Lausanne). 9, 42 (2018).
28. Fasano, A. Intestinal permeability and its regulation by zonulin: diagnostic and therapeutic implications. Clin Gastroenterol Hepatol. 10, 1096–1100 (2012).
29. Esnafort, E. et al. Increased serum Zonulin levels as an intestinal permeability marker in autistic subjects. J. Pediatr. 188, 240–44 (2017).
30. Rose, D. R. et al. Differential immune responses and microbiota profiles in children with autism spectrum disorders and co-morbid gastrointestinal symptoms. Brain Behav. Immun. 70, 354–68 (2018).
31. Lord, C. et al. ADOS-2—Autism Diagnostic Observation Schedule, 2nd ed.; Lord, C., & Rutter, M., Eds.; Western Psychological Services: Torrance, CA, USA (2012).
32. Lord, C., Rutter, M. & Le Couteur, A. Autism Diagnostic Interview-Revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. J. Autism Dev. Disord. 1994 24, 659–85 (1994).
33. Sheehan, D. V. et al. The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. J. Clin. Psychiatry. 59 Suppl 20, 22–33. quiz 34–57 (1998).
34. Folsite, M. F., Folsite, S. E. & McHugh, P. R. Mini-mental state: A practical method for grading the cognitive state of patients for the clinician. J. Psychiatr. Res. 12, 189–98 (1975).
35. Koch, W., H. et al. Genotyping of the common haptoglobin Hp1/2 polymorphism based on PCR. Clin. Chem. 48, 1377–82 (2002).
36. Bottini, N. et al. Haptoglobin genotype and natural fertility in humans. Fertil. Steril. 72, 293–6 (1999).
37. Sacchetti, E. et al. The GRM7 gene, early response to risperidone, and schizophrenia: a genome-wide association study and a confirmatory pharmacogenetic analysis. Pharmacogenomics J. 17, 146–54 (2017).
38. Browning, S. R. & Browning, B. L. Rapid and accurate haplotype phasing and missing-data inference for whole-genome association studies by use of localized haplotype clustering. Am. J. Hum. Genet. 81, 1084–97 (2007).
39. Browning, B. L. & Browning, S. R. Genotype Imputation with Millions of Reference Samples. Am. J. Hum. Genet. 98, 116–26 (2016).
40. Brackenridge, C. J. A sex-dependent difference in the haptoglobin distributions of some Australian urban population samples, with a note on the method of Haldane for estimating the significance of the logarithm of a ratio of frequencies. J. Exp. Biol. Med. Sci. 49, 525–9 (1971).
41. Vanuytsel, T., Vermeire, S. & Ceynken, I. The role of Haptoglobin and its related protein, Zonulin, in inflammatory bowel disease. Tissue Barriers. 1, e27321 (2013).
42. Carter, K. & Worwood, M. Haptoglobin: a review of the major allele frequencies worldwide and their association with diseases. Int. J. Lab. Hematol. 29, 92–110 (2007).
43. Zhao, H., Zhang, G., Duan, Y. & Yu, S. Haptoglobin types in Chinese ethnic groups. Hum. Hered. 43, 131–3 (1993).
44. Wei, J. & Hemmings, G. P. A further study of a possible locus for schizophrenia on the X chromosome. Biochem. Biophys. Res. Commun. 344, 1241–5 (2006).
45. Piton, A. et al. Systematic resequencing of X-chromosome synaptic genes in autism spectrum disorder and schizophrenia. Mol. Psychiatry. 16, 867–80 (2011).
46. Piton, A. et al. Analysis of the effects of rare variants on splicing identifies alterations in GABAA receptor genes in autism spectrum disorder individuals. Eur. J. Hum. Genet. 21, 749–56 (2013).
47. Williams, B. L. et al. Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances. Plos One. 6, e24585 (2011).
48. Strati, F. et al. New evidences on the altered gut microbiota in autism spectrum disorders. Microbiome. 5, 24 (2017).
49. Campon, D., Fonzo, P., Alessandria, C., Saracco, G. M. & Balzola, F. The role of microbiota in autism spectrum disorders. Minerva Gastroenterol. Dietol. 64, 333–50 (2018).
50. Pulikkann, I. et al. Gut microbial dysbiosis in Indian children with autism spectrum disorders. Microb. Ecol. 76, 1102–18 (2014).
51. Valitutti, F. & Fasano, A. Breaking down barriers: how understanding celiac disease pathogenesis informed the development of novel treatments. Dig. Dis. Sci. 64, 1748–58 (2019).
52. Roman, P., Rueda-Ruzafa, L., Cardona, D. & Cortes-Rodriguez, A. Gut-brain axis in the executive function of autism spectrum disorder individuals. Behav. Pharmacol. 29, 654–63 (2018).
53. Alam, R., Abdulmaleky, H. M. & Zhou, J. R. Microbiome, inflammation, epigenetic alterations, and mental diseases. Am. J. Med. Genet. B. Neuropsychiatr. Genet. 174, 651–60 (2017).
54. De Santis, B. et al. Study on the association among mycotoxins and other variables in children with autism. Toxins (Basel). 9, E203 (2017).
55. De Santis, B. et al. Role of mycotoxins in the pathobiology of autism: A first evidence. Nutr. Neurosci. 10, 1–13 (2017).
56. Liew, W. P. & Mohd-Redzwan, S. Mycotoxin: its impact on gut health and microbiota. Front Cell Infect Microbiol. 8, 60 (2018).
57. Cahill, E. L. et al. Currently available versions of genome-wide association studies cannot be used to query the common haptoglobin copy number variant. J. Am. Coll. Cardiol. 62, 860–1 (2013).
58. Boetger, L. M. et al. Recurring exon deletions in the HP (haptoglobin) gene contribute to lower blood cholesterol levels. Nat. Genet. 48, 359–66 (2016).
59. Ahamian, M., Steer, D., Rosella, G. & Gibson, P. R. Serum zonulin as a marker of intestinal mucosal barrier function: may not be what it seems. Plos One. 14, e0210728 (2019).

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**Author contributions**
F.A.C. participated in wet and statistical analysis, wrote the paper; E.M. bioinformatics analyses; M.G., C.M. generated SNP-array genotypes of the Brescia cohort; M.M. bioinformatics infrastructure development; M.L., N.G. wet analyses; A.B. patients recruitment; L.V. and M.M. ASD diagnosis; M.E.R. recruitment coordination; A.R. and F.C. provided DNA and diagnosis of Telethon patients; A. Mi. evaluation and recruitment of “super controls”; A.Ma. P.I. of the project funded by Italian Ministry of Health; L.M. responsible of the Italian Flagship-project InterOmics (PB05), A.Me. conceived and designed the study, participated in wet and statistical analysis, wrote the paper. F.A. data interpretation and final revision. All the authors critically revised the manuscript.

**Competing interests**
The authors declare no competing interests.

**Additional information**
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