Genetic markers associated with resistance to infectious diseases have no effects on production traits and haematological parameters in Italian Large White pigs

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Highlights:

- Four markers associated with disease resistance were analyzed in several pig populations
- Two *GBP1* SNPs were not in complete linkage disequilibrium in most pig breeds
- *MUC4*, *FUT1* and *GBP1* SNPs did not affect any production traits in Italian Large White pigs
- SNP allele frequencies did not substantially change over 20 years of selection in Italian Large White boars
- Marker-assisted selection based on these SNPs should not affect genetic gain for production traits

Abstract

Infectious diseases have large economic impacts on the pig breeding industry worldwide. A few genetic markers associated to disease resistance have been recently identified and used in marker-assisted selection (MAS) in a few pig populations, as part of disease control programs. Neonatal Diarrhea (ND), Post-weaning Diarrhea (PWD), caused by enterotoxigenic *E. coli*, and Porcine Reproductive and Respiratory Syndrome, caused by PRRS virus (PRRSV), are diseases with high priority for the pig industry. Since interactions or antagonism between growth, innate immunity and disease resistance traits could exist, this work investigated if four disease resistance gene markers [mucin 4 (*MUC4*), rs338992994 associated with resistance to ND; fucosyltransferase 1 (*FUT1*), rs335979375 associated with resistance to PWD; and guanylate binding protein 1 (*GBP1*), rs340943904 and rs80800372 (also known as WUR10000125) associated with resistance to PRRSV] could affect seven production traits (average daily gain, back fat thickness, lean meat cuts, feed gain ratio, ham weight, visible intermuscular fat and ham weight loss at first salting) and 15 haematological parameters in about 550 performance tested Italian Large White pigs. We also monitored allele frequencies of the same markers on Italian Large White boars sampled under the national selection breeding program over a 20 years-period. Moreover, we evaluated allele frequencies of these polymorphisms in four Italian local pig breeds (Apulo-Calabrese, Casertana, Cinta Senese and Nero Siciliano). The frequency of the resistance-associated alleles for the four polymorphisms was usually higher in all local pig breeds, indirectly supporting a higher rusticity of autochthonous breeds, compared to commercial populations. The two *GBP1* polymorphisms were not in complete linkage disequilibrium in all breeds, except in Apulo-Calabrese. No significant allele frequency change for the investigated markers occurred over 20 years in the Italian Large White boar population in three of these markers. Only *FUT1* showed a modest but significant change of allele frequencies over this period. Association analyses carried out in Italian Large White pigs for production traits, meat quality or haematological parameters under investigation showed no significant effect of any genotyped polymorphisms. Our results indicate that implementing MAS programs in Italian Large White pigs

with polymorphisms associated with disease resistance have no direct effects on production traits. The selection program running for this heavy pig breed might not negatively impact disease resistance derived by the investigated major genes.

Keywords: association study; *FUT1*; *GBP1*; *MUC4*; pig breeding; WUR10000125.

1. Introduction

Infectious diseases are the most important causes of economic losses for the pork industry worldwide. Selection and breeding for disease resistance is considered one possible approach to mitigate this problem (Mellencamp et al., 2008). Based on their relevance and potential responsiveness to genetic selection, diarrhea caused by *Escherichia coli* and Porcine Reproductive and Respiratory Syndrome (PRRS) are classified among the infectious diseases with the highest priorities in pig breeding strategies (Davies et al., 2009).

Different Enterotoxigenic *E. coli* (ETEC) strains are among the prevalent etiological agents responsible for the incidence of diarrhea in piglets during the neonatal or post-weaning periods (Chan et al., 2013). Genetic resistance to diarrhea caused by ETEC is determined by the presence or the absence of host intestinal cell surface receptors for bacterial fimbriae which are the most important factors in conferring virulence to *E. coli* strains (Schroyen et al., 2012). Using invasive *in vitro* adhesion assays, resistant pigs can be identified by a much lower number of bacteria attached to the intestinal cell surface compared to susceptible ones (Sellwood et al., 1975). ETEC strains presenting F4 fimbriae with different antigenic variant combinations have been shown to be the most prevalent strains causing Neonatal Diarrhea (ND) in pigs (Moon et al., 1999). A single nucleotide polymorphism (SNP) in intron 7 (rs338992994, NC_010455.5:g.134226654C>G) of the mucin 4 (*MUC4*) gene (which encodes a large membrane-bound mucin) is in linkage disequilibrium with the receptor for ETEC strain exhibiting F4 fimbriae with both antigenic variants *ab* or *ac*. Allele G of this marker is associated with the presence of the receptor that confers susceptibility to ND and is presumed to be dominant over the resistance allele C, associated with the absence of the receptor (Jorgensen et al., 2004). This marker, not completely concordant with the adhesion assay results (Rasschaert et al., 2007), still remains the most reliable polymorphism used in marker-assisted selection (MAS) programs to reduce ND incidence in piglets (Goetstouwers et al., 2014).

Post-Weaning Diarrhea (PWD) is determined by *E. coli* strains exposing F18 fimbriae, that seem more prevalent than ETEC F4 (Moon et al., 1999). A nucleotide substitution in the alpha (1,2)-

fucosyltransferase 1 (*FUT1*) gene (rs335979375, NC_010448.4:g.54079560A>G) has been found to be in high linkage disequilibrium with the gene encoding for the receptor for ETEC strain exhibiting F18 fimbriae with *ab* antigenic variant (ETEC F18ab). The recessive A allele is associated with the absence of ETEC F18ab receptor, that in turn, determines resistance to PWD (Meijerink et al., 1997). *FUT1* gene encodes an enzyme that catalyzes an intermediary step in the formation of antigens pertaining to the porcine AO blood group system. The indicated polymorphism in this gene is considered thus the most reliable discovered marker for the identification of PWD resistant pigs and for this reason has been included in MAS programs (Meijerink et al., 1997; Coddens et al., 2007, 2008).

PRRS is responsible for gross production losses due to by late-term reproductive failure in sows and respiratory illness in growing pigs. The etiological agent is an Arteriviridae positive-sense RNA virus with two main different strains showing only about 60% of sequence identity (Kappes and Faaberg, 2015; Neumann et al., 2005). Genetic variation in the host response to this disease was first observed in the late ‘90s (Halbur et al., 1998) and was subsequently investigated by a series of challenging studies conducted by the PRRSV Host Genetic Consortium (PHGC) (Lunney et al., 2011) leading to the discovery of an important QTL on porcine chromosome (SSC) 4 responsible for 15% of the genetic variation of the host’s immune response to this infection (Boddicker et al., 2012). This QTL region includes five genes encoding guanylate binding protein (GBP) family members, that are mediators of the proinflammatory immune response post viral or bacterial infections (Koltes et al., 2015, Pilla et al., 2014). Two SNPs have been found to be in linkage disequilibrium with this SSC4 QTL. One SNP (rs340943904, NC_010446.5:g.127301202G>T), located in intron 9-10 of the *GBP1* gene (encoding a negative regulator of T-cell responses), has been identified as the putative causative mutation responsible of this QTL (Koltes et. al, 2015). The presence at this locus of nucleotide G introduces a splice acceptor site that leads to the addition of 5 nucleotides into intron 9-10. This causes, in turn, the shift of the reading frame and the formation of a premature stop codon in *GBP1* sequence with the transcription of non-functional products. However, only pigs with GG genotype

produce entirely non-functional transcripts and are regarded as susceptible, suggesting a dominant effect of the resistance allele T (Koltes et al., 2015). The second SNP, WUR10000125 (rs80800372, NC_010446.5:g.127441677A>G), is a polymorphism located next to a putative polyadenylation site in the 3'-untranslated region of the *GBP1* gene which can potentially affect transcript stability, with consequences on protein synthesis and expression (Gol et al., 2015). A few studies have been carried out in different pig populations to investigate the role of WUR10000125 in PRRSV resistance and its possible impact on performance traits (Boddicker et al., 2014; Niu et al., 2015, 2016; Abella et al., 2016; Dulkenberger et al., 2017a; Waide et al., 2017). This polymorphism is currently included in commercial porcine SNP genotyping chips. Pigs with unfavorable A allele at this marker showed higher viremia and lower weight gain following infection whereas pigs carrying the G variant, which might be the dominant allele, reported a lower viremia and showed higher growth rate under PRRSV infection (Boddicker et al., 2012). The same allele has been also associated with lower porcine circovirus type 2b (PCV2b) virus load and might be useful to improve host response on coinfection of PRRSV with PCV2b (Dunkelberger et al., 2017b).

Since previous studies have suggested a negative correlation between growth traits and disease resistance and a few of these markers have been suggested to have antagonistic pleiotropic effects on these economically relevant aspects (Doeschl-Wilson et al., 2009; Fontanesi et al., 2012; Huang et al., 2008), investigating the effects of these markers on growth, carcass and meat quality and production traits is essential for their proper use in MAS programs.

In this study, we genotyped *MUC4*, *FUT1* and *GBP1* gene markers i) to compare allele frequencies distribution in Italian local pig breeds and Large White pigs, ii) to retrospectively investigate allele frequency changes of these markers over two decades of selection among Italian Large White boars and iii) to evaluate the effect of these polymorphisms on performance, carcass and meat quality traits and haematological parameters (some of which related to the immune response) in Italian Large White pigs.

2. Materials and methods

2.1. Animals and traits

All animals used in this study were not raised for any experimental purposes. They were kept according to Italian and European legislation for pig production and all procedures described were in compliance with national and European Union regulations for animal care and slaughtering.

Three groups of pigs were included in this study. Group 1 was made by Italian local pig breeds. Groups 2 and 3 were made by Italian Large White pigs. Group 1 included a total of 117 unrelated animals of four Italian local pig breeds (Apulo-Calabrese, Casertana, Cinta Senese and Nero Siciliano; 15-30 pigs per breed) that were used for allele frequency analysis and comparison across breeds.

Group 2 consisted of 189 Italian Large White boars born and raised from 1992 to 2012 under the national heavy pig selection scheme of this breed (Fontanesi et al., 2015; Schiavo et al., 2016). Heavy pigs are slaughtered at about 160 kg live weight at nine months of age. These boars were used for the evaluation of allele frequency changes over years. The animals were selected among all Italian Large White boars born from 1992 to 2012 (n = 5983) and approved for reproduction based on their genetic merit after evaluation by the National Pig Breeders Association (ANAS). The boars were ranked according to the reliability of their estimated breeding values (EBVs; calculated in 2012) and those with the highest reliability (n = 189) were selected and used to constitute eight groups (n = 19-28 boars per group) based on the years in which they were born (groups were constituted using 2-4 years windows; 2011-2012 did not have boars with high EBV reliability, thus this window was not included in the two-decades representation). More information about these boars are reported in Fontanesi et al. (2015).

Group 3 consisted of 557 performance tested Italian Large White pigs (382 gilts and 175 castrated males). These animals were used in the association studies between gene markers and phenotypic traits (see below). These pigs were part of the sib-testing program of the Italian Large White population for which triplets of pigs from the same litter (2 females and 1 castrated male) were

individually performance tested at the ANAS Central Test Station for the genetic evaluation of a boar from the same litter (sib-testing). The test period started when piglets were 30-45 days old and ended when they reached 155 ± 5 kg live weight at approximately nine months of age. The nutritive level was *quasi ad libitum*, meaning that about 60% of the pigs were able to ingest the entire supplied ratio (Fontanesi et al., 2008, 2012). During the test period feed intake was recorded daily, body weight was measured bimonthly, and then average daily gain (ADG, in g) and feed:gain ratio (FGR) were calculated. At the end of the testing period, animals were slaughtered in a commercial abattoir after electrical stunning and following standard procedures. These pigs were slaughtered in 18 different days in the years 2012-2013. After slaughtering, weight of lean cuts (LC, the weight of neck and loin in kg), weight of the hams (HW, in kg) and backfat thickness (BFT, determined at the level of *Musculus gluteus medius* and expressed in mm) were measured on the carcasses. Visible intermuscular fat (VIF) was scored on the exposed muscles of the legs as previously described (Fontanesi et al. 2017b; Bertolini et al., 2018). Ham weight loss at first salting (HWLFS, in g) was calculated during the first week of seasoning (Fontanesi et al., 2017a; Bertolini et al., 2018). Haematological parameters were determined on blood samples, collected just after jugulation and exsanguination, in EDTA added tubes (Vacutest Kima s.r.l.). A total of 15 haematological parameters (erythrocyte traits: red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and red cell distribution width; leukocyte traits: white blood cell count, lymphocyte count, neutrophil count, eosinophil count, basophil count and monocyte count; platelet traits: platelet count and mean platelet volume) were measured on an Olympus AU 400 (Beckman Coulter) automated analyzer at the Veterinary Haematological Laboratory of the University of Bologna under standard procedures.

2.2. SNP genotyping

Genomic DNA was extracted from blood using the NucleoSpin®Tissue commercial kit (Macherey-Nagel, Düren, Germany). Genotyping was obtained for four SNPs in three genes already

188 reported to be associated with resistance to porcine bacterial or viral determined diseases:
189 rs338992994 (g.134226654C>G) in the *MUC4* gene; rs335979375 (g.54079560A>G) in the *FUT1*
190 gene; rs340943904 (g.127301202G>T) and rs80800372 (WUR10000125 or g.127441677A>G) in
191 the *GBP1* gene. PCR primers and genotyping protocols are reported in Supplementary Table 1. PCR-
192 RFLP was used for the genotyping of three SNPs (rs338992994, rs335979375 and rs340943904)
193 whereas results for the WUR10000125 marker were obtained from the genotyping of the
194 PorcineSNP60 BeadChip (Illumina Co., St. Diego, CA, USA) SNP chip, version 1 (as reported by
195 Fontanesi et al. 2017a,b). PCR was carried on a 2720 Thermal Cycler (Applied Biosystems) in a total
196 volume of 20 μL that included 10 pmol of each primer, 2.5 mM each dNTP, 2 μL of KAPA Taq
197 Buffer 10x (containing 1.5 mM of $MgCl_2$ at 1X), 0.5 U of KAPA Taq DNA Polymerase (KAPA
198 Biosystems, Boston, MA, USA). Digested PCR products were visualized by electrophoresis on 2.5%-
199 3.0% agarose gels in TBE (Tris-Borate-EDTA) 1X with the addition of GelRed Nucleic Acid Gel
200 Stain (Biotium Inc., Hayward, CA, USA).
201
202 **2.3. Statistical analyses**
203 Allele and genotype frequencies were obtained by counting. Hardy-Weinberg equilibrium
204 (HWE) was evaluated using the HWE software program (Linkage Utility Programs, Rockefeller
205 University, New York, NY). For each breed, PLINK software v1.07 (Purcell et al., 2007) was used
206 to calculate the linkage disequilibrium (LD, here reported by using the r^2 measure) and PHASE
207 software v2.1.1 (Stephens et al., 2001) was used to identify haplotypes between the two SNPs
208 (rs340943904 and WUR10000125) genotyped in the *GBP1* gene (1000 iterations were set as
209 parameter for computing haplotypes).
210 Allele frequency changes over years of the genotyped SNPs in the first group of Italian Large
211 White pigs (group 2) was modeled using a logistic regression of the number of copies of the minor
212 allele on time.

213 The second group of Italian Large White performance tested pigs (group 3) was used in the
214 association analysis between the recorded phenotypic traits and the genotyped SNPs. Association
215 with production traits was obtained using Random Residuals (RRs) as defined by Fontanesi et al.
216 (2010) on traits (ADG, BFT, LC, FGR, HW, VIF and HWLFS) measured by ANAS, as described
217 above. RRs were calculated including in the model sex, batch on trial, inbreeding coefficient of the
218 animal, interaction of sex × age at slaughtering, date of slaughtering as fixed effect, and litter and
219 animal as random effect (Fontanesi et al., 2010). As previously reported, the use of RRs reduces the
220 type I error and the overestimation of the effects that would be obtained by using EBVs (Fontanesi et
221 al., 2013). Haematological parameters were normalized in R (packages "MASS" and "CAR") by
222 applying the Box–Cox transformation as previously described by Bovo et al. (2016).
223 Association analysis was carried out in GEMMA (Zhou and Stephens, 2012) by fitting linear
224 mixed models, using GEMMA option -lmm and including a centered genomic matrix K (computed
225 with GEMMA software using 45952 SNPs). The assessment of the association between each SNP
226 and trait was obtained by testing the null hypothesis H_0:$\beta = 0$, where β is the effect size of the marker.
227 Additive genetic model, assuming a trend per number of copies of the minor allele, was used to
228 specify the dependency of trait on genotype categories. The model for the (normalized)
229 haematological traits included as covariates sex (coded as dummy variable), date of slaughtering
230 (coded with a set of N-1 dummy variables; N=18) and carcass weight (continuous variables). Because
231 of the estimation of RR for each trait already corrects for possible variables, these covariates were
232 not added while fitting the linear model. *GBP1* haplotypes were not used in the association analysis
233 as recombinant haplotypes were carried by few animals, preventing a correct estimation of their
234 effect. Significance was tested by using the Wald test. SNPs were defined to be significantly
235 associated if their P nominal values were below 5.68×10^{-04} (Bonferroni corrected $P < 0.05$). This
236 threshold was determined by multiplying the four SNPs by the 22 traits under investigation (seven
237 production traits and 15 haematological traits). Suggestively significant results were considered if *P*
238 nominal values were below 0.05.

3. Results

Allele and genotype frequencies for the investigated SNPs are reported in Table 1. Only *FUT1* polymorphism in the Italian Large White population (group 3 of pigs) significantly deviated ($P < 0.05$) from HWE (Supplementary Table 2). All other investigated polymorphisms in the analyzed breeds were in HWE (Supplementary Table 2).

Frequency of the *MUC4* C allele (conferring resistance to ND) was 0.57 in the Italian Large White population, the lowest frequency in the analyzed breeds. This allele was always the major allele in all local breeds in which its frequency ranged from 0.72 (Nero Siciliano) to 1.00 (Cinta Senese).

Allele A of the *FUT1* marker (the allele associated with resistance to PWD) was the minor allele in all breeds. Its frequency in Italian Large White pigs was similar to that of most local breeds apart Casertana which showed the highest frequency of this allele among all investigated breeds (0.554).

The frequency of allele T of the *GBP1* rs340943904 marker (the dominant allele associated with resistance to PRRS) was low (0.087) in the Italian Large White population. Its frequency was higher in the Italian local breeds, except in Apulo-Calabrese where it was 0.125. Cinta Senese was again the breed that showed the highest frequency of the resistance allele (0.437).

Allele G of WUR10000125 (*GBP1* rs80800372) SNP (associated with resistance to PRRS) was low in all breeds and ranged from 0.1 (Casertana) to 0.168 (Cinta Senese). It was 0.07 in the commercial Italian Large White breed.

The two analyzed *GBP1* SNPs were in complete LD only in Apulo-Calabrese pigs ($r^2 = 1.00$). The value of r^2 was 0.133 in Cinta Senese, 0.698 in Nero Siciliano and 0.722 in Casertana. In the Italian Large White breed r^2 was 0.696. Table 2 reports the *GBP1* haplotype frequencies observed in the analyzed breeds. The haplotype derived by the combination of the two susceptibility alleles (i.e. GA, respectively for rs340943904 and WUR10000125) was, on average, the most frequent overall

investigated pigs (0.80). The haplotype derived by the two resistance associate alleles (i.e. TG at the same positions) showed the lowest frequency in the Italian Large White population.

Figure 1 shows the frequencies of the resistance associated alleles for the four SNPs under investigation in this study in the Italian Large White boars over two decades. The plot describes a retrospective analysis of frequency changes in this timeframe in the whole Italian Large White population (Fontanesi et al., 2015). This overview might give a general impression of slow reduction of the frequency of the resistance alleles for all analysed markers (Fig. 1). However, the logistic regression model indicated that only for *FUT1* SNP there was a significant change of allele frequencies over the investigated 20 years (P = 0.027) with a gradual reduction of the resistance associated allele (0.25 in 1996-1997 and 0.06 in 2008-2010). *MUC4* and *GBP1* rs80800372 and rs340943904 allele frequency distribution did not significantly change in the considered timeframe (Table 3). For these investigated markers, there was a peak for the resistance alleles in the 1996-1997 years that was not subsequently confirmed by the trends of the following years (Fig. 1).

Overall allele frequencies of all four investigated SNPs in the 2008-2010 window was a little bit lower than the frequency observed in the second group of Italian Large White pigs used in the association studies, which were born in the years 2011-2012 (as they were slaughtered in 2012-2013; Supplementary Table 3). This result may indicate, indirectly, that allele frequencies in the sib-tested population tend to fluctuate, a few years later, around values already observed in the boar population in the considered timeframe.

Results of association analysis between the analyzed SNPs and ADG, BFT, FGR, LC, HW, VIF and HWLFS are reported in Table 4. None of the four SNPs was associated with any production traits both considering a Bonferroni corrected threshold (significant association) and a P nominal value of 0.05 (suggestive association).

Association study for the haematological traits showed only four suggestive associations (P nominal value < 0.05; Supplementary Table 4). Table S5 reports β estimates and Stantard Errors (SE) for each association. *MUC4* was suggestively associated with mean platelet volume and lymphocyte

count, *FUT1* was suggestively associated with red blood cell count and *GBP1* rs80800372 was suggestively associated with monocyte count.

4. Discussion

A few gene markers have been already shown to be associated with resistance to some of the most important diseases that have a negative economic impact on the pig production chain worldwide (Vögeli et al., 1997; Huang et al., 2008; Boddicker et al., 2012, Schroyen et al., 2016). Based on these works that prompted the use of polymorphisms in the *MUC4*, *FUT1* and *GBP1* genes in MAS to increase disease resistance in several pig populations, it is important to first evaluate allele frequency distribution in other populations that were not part of these investigations and to verify if these markers are associated with other production traits. This is essential to evaluate if antagonist effects of these genes might reduce the genetic progress on economic traits that are currently used in the breeding program of commercial populations.

This study investigated the allele frequency distribution of *MUC4*, *FUT1* and *GBP1* polymorphisms in an important heavy pig breed (Italian Large White) and in several Italian local breeds (Apulo-Calabrese, Casertana, Cinta Senese and Nero Siciliano) which complemented the results on the commercial breed. The limited number of animals analysed in the local breeds might provide a first allele frequency distribution that should be further investigated in larger samples. The Italian Large White breed was also investigated to evaluate if allele frequencies of the analyzed markers changed over a period of 20 years of selection for animals with carcass and meat quality traits more adapted to the dry-cured ham production chain (Bosi and Russo, 2004). None of the four investigated SNPs were associated with any traits included in the national selection program of the Italian Large White breed. In addition, none of the haematological traits (some of which might be important to define indirectly the potential immune response of the animals) was significantly associated with the same markers, suggesting that other biological mechanisms might be involved in explaining the reported effect on disease resistance.

316 The results of the association studies with production traits are in line with the general stable
317 allele frequencies of these markers over two decades of selection in the Italian Large White breed that
318 significantly increased average daily gain and improved all meat and carcass traits needed for the
319 production of dry-cured hams (Fontanesi et al., 2015). Had the analyzed gene markers strongly
320 affected the traits already used in the selection programs of this breed, a strong allele frequency
321 modification over time should have been expected. This was already found for other gene markers
322 already shown to be associated with important production traits (i.e. *IGF2*, *FTO*, *MC4R* and *VRTN*;
323 Fontanesi et al. 2015). Only the *FUT1* showed a significant allele frequency change in the investigated
324 timeframe, probably due to genetic drift or a small effect (not detected in the association analysis) on
325 traits under direct or indirect selection in this population. In this context, despite the results were not
326 statistically significant, it is worth to note that there was a general, even small, decrease, of allele
327 frequency of the resistance associate alleles at all loci. Considering that there would be no detrimental
328 effect on any production traits (according to the results obtained in the association study we carried
329 out), it should be important to re-consider the selection program of this breed to reverse this trend by
330 adopting a targeted MAS program that aims to increase the frequency of the favorable alleles
331 (associated to resistance to ND, PWD and PRRS) at these markers and, indirectly, increase disease
332 resistance in this population. This could be important considering that the favorable allele at these
333 markers was in almost all cases the minor allele in the Italian Large White population (except for the
334 *MUC4* SNP). In contrast, in most local breeds, the favorable allele usually showed a higher frequency
335 than in the Italian Large White population. This aspect might confirm the higher rusticity (partly
336 derived by a lower susceptibility to infection diseases) that is usually attributed to local pig breeds
337 compared to commercial populations. In these autochthonous breeds, the effect of natural selection,
338 and thus adaptation to harsh environmental conditions, could have increased the frequency of disease
339 resistance alleles.
340 In a previous study that was based on extremely divergent Italian Large White pigs for
341 estimated breeding values for a few production traits, the same *MUC*4 SNP showed a significant

342 association between ADG and the resistance allele that was more frequent in slow-growing pigs
343 (Fontanesi et al., 2012). The design of that experiment was probably more powerful and was able to
344 capture more subtle effect on growth traits that were not evident in the current study which was based
345 on animals coming from a more homogeneous sib-tested population (born in the years 2011-2012).
346 The general direction of the effect seems however, even not significant, the same as that previously
347 observed (Fontanesi et al., 2012), i.e. genotype CC grew slower than the other genotypes (data not
348 shown). In another study, Yan et al. (2009) reported faster growth rates during the fattening period
349 for animals presenting ETEC F4ab and F4ac receptors. Yan et al. (2009) also indicated that
350 susceptible pigs with F4abR demonstrated higher values of ADG during the suckling period. These
351 results might confirm, to some extent, a small or population dependent effect of this marker on
352 production traits.

353 The *FUT1* marker that, in this study, did not affect any traits in the Italian Large White
354 population was, instead, associated with growth rate (with a positive effect of allele A) in a Swiss pig
355 population (Kadarmideen, 2008). Similar results were also obtained by Bao et al. (2012) in a study
356 on Sutai pigs (hybrids of Duroc and Meishan pigs) where animals with the AA genotype had the
357 highest ADG value before weaning.

358 Allele frequencies of the WUR10000125 (*GBP1* rs80800372) polymorphism obtained in this
359 study were similar to those reported by other works which showed that the unfavorable A allele is the
360 most frequent one in commercial lines selected for increased growth rate (ranging from 0.7 to 0.9;
361 Boddicker et al., 2012; Abella et al., 2016). In a recent study on nonchallenged animals, Dunkelberger
362 et al. (2017a) estimated the effect of WUR10000125 on traits under selection in four different
363 purebred pig lines. Even if the effect of this marker acted differently among commercial lines,
364 nonsignificant effect was reported for most of the traits and for overall selection index value,
365 suggesting that selecting for the resistance allele should result in pig resistant to PRRS without
366 consequences on productive traits in nonchallenging conditions.

The linkage between the two *GBP1* markers associated to PRRS resistance (i.e. rs340943904 and rs80800372) was previously evaluated only by Koltes et al. (2015) in a commercial crossbred population who reported that the two markers were in perfect LD. Our results contrast with what was reported by Koltes et al. (2015). LD varied among breeds with extreme values in local pig populations (r^2 = 1.00 in Apulo-Calabrese and r^2 = 0.133 in Cinta Senese). Therefore, more than two haplotypes were identified in Italian Large White, Casertana, Cinta Senese and Nero Siciliano breeds suggesting that previous association studies based on WUR10000125 should be revised including the genotyping of the other associated *GBP1* SNP in populations in which these two markers are not in complete LD.

5. Conclusions

This study showed that implementing a MAS program in the Italian Large White pig breed, aiming to indirectly increase disease resistance, might not have any relevant adverse effect on all other performance, carcass and meat quality traits included in the genetic merit index used in the Italian national selection scheme. The alleles in the *MUC4*, *FUT1* and *GBP1*, that have been shown by other studies to be associated to disease resistance, did not have any negative effects on the investigated traits. In our case, before implementing MAS based on these genes, further studies should be carried out to investigate their effect on reproduction traits in this heavy pig breeds that is usually used as maternal line in the heavy pig breeding programs. Other studies are also needed to evaluate the level of LD between *GBP1* SNPs and the effect of their haplotypes on production traits for the subsequent use of these markers in MAS to reduce susceptibility to PRRS.

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this work.

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Table 1. Allele and genotype frequencies of the analyzed polymorphisms in the Italian Large White population and in the autochthonous pig breeds.

	***MUC4*[1] rs338992994**						***FUT1* rs335979375**						***GBP1* rs340943904**						***GBP1 rs80800372***					
Breeds	No. of Pigs	Allele Frequency		Genotype Frequency			No. of pigs	Allele Frequency		Genotype Frequency			No. of pigs	Allele Frequency		Genotype Frequency			No. of pigs	Allele Frequency		Genotype Frequency		
		C	G	CC	CG	GG		A	G	AA	AG	GG		T	G	TT	TG	GG		G	A	GG	AG	AA
Italian Large White[2]	500	0.575	0.425	0.328	0.494	0.178	510	0.115	0.885	0.026	0.178	0.796	557	0.087	0.913	0.013	0.149	0.838	557	0.070	0.930	0.009	0.120	0.871
Apulo Calabrese[3]	15	0.830	0.170	0.000	0.333	0.667	25	0.060	0.940	0.000	0.120	0.880	28	0.125	0.875	0.000	0.250	0.750	28	0.125	0.875	0.000	0.250	0.750
Casertana[3]	27	0.910	0.090	0.000	0.185	0.815	28	0.554	0.446	0.393	0.321	0.286	30	0.134	0.866	0.000	0.267	0.733	30	0.100	0.900	0.000	0.200	0.800
Cinta Senese[3]	22	1.000	0.000	1.000	0.000	0.000	29	0.069	0.931	0.000	0.862	0.138	16	0.437	0.563	0.188	0.500	0.312	16	0.168	0.832	0.000	0.188	0.812
Nero Siciliano[3]	30	0.720	0.280	0.100	0.366	0.534	23	0.108	0.892	0.000	0.217	0.783	26	0.230	0.770	0.077	0.308	0.615	26	0.160	0.840	0.077	0.192	0.731

[1]Frequencies reported from Fontanesi et al. (2012) except for Italian Large White pigs, which are from this study.

[2]Total individuals were 557 but for *MUC4* and *FUT1* genotyping was missing for some individuals. Allele and genotype frequencies were reported here only for the second group of Italian Large White pigs, which included performance tested pigs born in the years 2011-2012.

[3]Genotyping results in the local breeds were obtained for 15-30 pigs each.

576 **Table 2.** Haplotype frequencies in the Italian Large White and Italian local pig breeds for the two
577 markers in the *GBP1* gene.

Breed[1]	***GBP1* haplotypes**[2]	**Haplotype frequency**
Italian Large White (557)	TG	0.066
	TA	0.024
	GG	0.005
	GA	0.905
Apulo Calabrese (28)	TG	0.125
	GA	0.875
Casertana (30)	TG	0.100
	TA	0.033
	GA	0.867
Cinta Senese (16)	TG	0.094
	TA	0.343
	GA	0.563
Nero Siciliano (26)	TG	0.173
	TA	0.058
	GA	0.769

578 [1] The number of pigs is included in parenthesis.
579 [2] The first and the second nucleotides are for the rs340943904 and rs80800372 SNPs, respectively.

582 **Table 3.** Effect of time on resistance allele frequency changes in candidate boars over 20 years.

Gene/SNPs	**Allele**	**Chi square value**	**P value**
MUC4/ rs338992994	C	0.600	0.423
FUT1/ rs335979375	A	5.000	0.027
GBP1/ rs340943904	T	1.260	0.263
*GBP1/*rs80800372	G	0.650	0.422

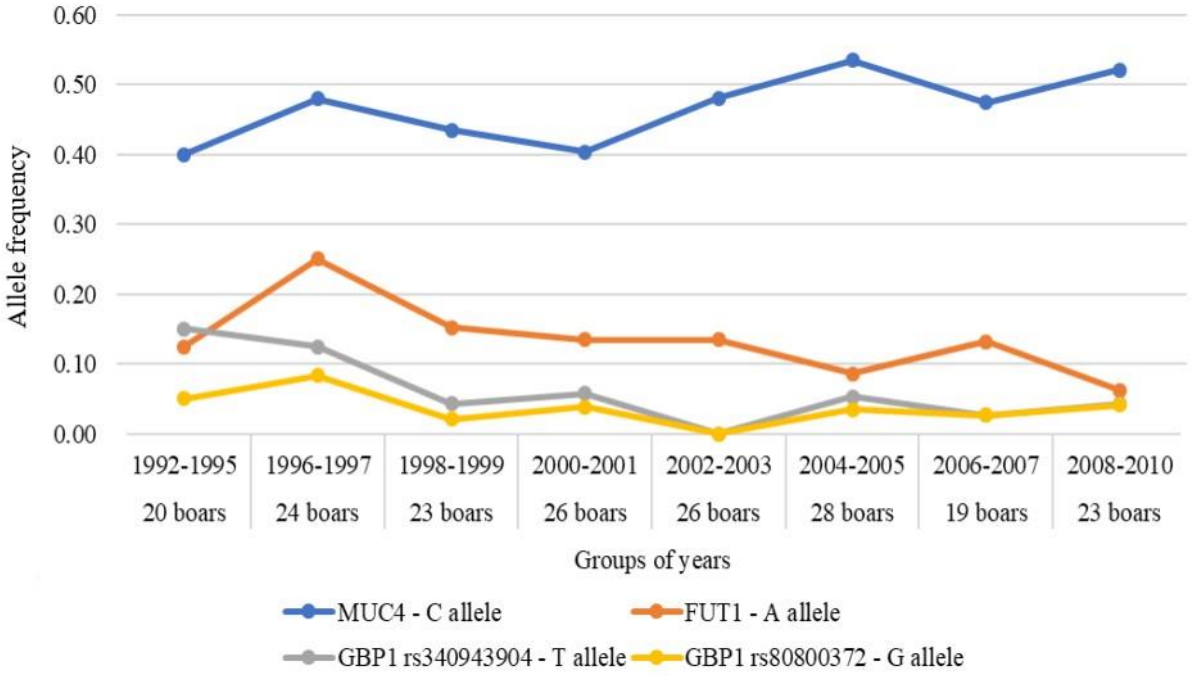
583

584 **Table 4**. Results of the association study between the analyzed markers and production, carcass and
585 meat quality traits in the second group of Italian Large White pigs. The table reports the calculated P
586 nominal value.

Traits[1]	***MUC4* rs338992994**	***FUT1* rs335979375**	***GBP1* rs340943904**	***GBP1* rs80800372**
ADG	0.420	0.337	0.291	0.101
BFT	0.068	0.730	0.118	0.236
LC	0.133	0.534	0.768	0.633
FGR	0.577	0.308	0.738	0.605
HW	0.910	0.878	0.639	0.976
VIF	0.678	0.143	0.731	0.953
HWLFS	0.388	0.491	0.160	0.261

587 [1]ADG: average daily gain, BFT: back fat thickness; LC: lean meat cuts; FGR: feed gain ratio; HW:
588 ham weight; VIF: visible intermuscular fat; HWLFS: ham weight loss at first salting.

Figure 1. Allele frequency trends of four single nucleotide polymorphisms associated to disease resistance in Italian Large White boars under the national selection program over about two decades. *GBP1* haplotype frequency changes were not evaluated due to the low frequencies of three haplotypes out of four reported in this population (see Table 2 for the haplotype frequencies in this breed).



Supplementary Table 1. PCR conditions and genotyping protocols.

Genes/markers	**PCR primers**	**Temp.**[1]	**Product size**[2]	**RFLP** [3]
FUT1	F:5'-CTTCCTGAACGTCTATCAAGACC-3' R:5'-CTTCAGCCAGGGCTCCTTTAAG-3'	62.5	421	*Hha*I; Alelle A: 328+93; Allele G: 241+93+87
MUC4	F:5'-GTGCCTTGGGTGAGAGGTTA-3' R:5'-CACTCTGCCGTTCTCTTTCC-3'	57.0	367	*Xba*I; Allele C: 367; Allele G: 216+151
GBP1 rs340943904[4]	F:5'-TCCTGATAGAATCTTTGCCGC-3' R:5'-GGAGGGAGAAGGATGGGTAC-3'	60.0	218	*Fsp*BI; Allele T: 113+29+23; Allele G: 136+29

[1] Annealing temperature in °C.

[2] Amplified fragment size (in bp).

[3] Restriction enzymes used in the RFLP methods are indicated together with the resulted fragments of the two alleles obtained after enzyme digestion (in bp).

[4] The *GBP1* rs80800372 marker was analyzed using the Illumina PorcineSNP60 BeadChip.

610 **Supplementary Table 2.** Hardy-Weinberg Equilibrium (HWE) test for all gene markers genotyped in five pig breeds. Numbers of animals are reported
611 in the correspondence of the SNP genotypes.

Gene marker	Genotypes	Italian Large White	Apulo-Calabrese	Casertana	Cinta Senese	Nero Siciliano
MUC4	GG	89	10	22	0	16
	CG	247	5	5	0	11
	CC	164	0	0	22	3
	P value	0.810	0.315	0.474	-	0.599
FUT1	GG	406	22	8	25	18
	AG	91	3	9	4	5
	AA	13	0	11	0	0
	P value	0.012	0.661	0.062	0.586	0.434
GBP1 rs340943904	GG	467	21	22	5	16
	TG	83	7	8	8	8
	TT	7	0	0	3	2
	P value	0.168	0.316	0.266	0.949	0.508
GBP1 rs80800372	AA	485	21	24	13	19
	AG	67	7	6	3	5
	GG	5	0	0	0	2
	P value	0.162	0.316	0.413	0.577	0.127

612

Supplementary Table 3. Resistance allele frequency values of four gene markers in Italian Large White boars under the national selection program over about two decades.

Groups of years	***MUC4* – C rs338992994**	***FUT1* – A rs335979375**	***GBP1* - T rs340943904**	***GBP1* – G rs80800372**
1992-1995	0.40	0.13	0.15	0.05
1996-1997	0.48	0.25	0.13	0.08
1998-1999	0.43	0.15	0.04	0.02
2000-2001	0.40	0.13	0.06	0.04
2002-2003	0.48	0.13	0.00	0.00
2004-2005	0.53	0.09	0.05	0.03
2006-2007	0.47	0.13	0.03	0.03
2008-2010	0.52	0.06	0.04	0.04

Supplementary Table 4. Results of the association study between the analyzed gene markers and haematological traits in the second group of Italian Large White pigs. The table reports the calculated P nominal value.

Haematological traits[1]	***MUC4* rs338992994**	***FUT1* rs335979375**	***GBP1* rs340943904**	***GBP1* rs80800372**
HGB	0.927	0.205	0.876	0.408
HCT	0.942	0.119	0.869	0.498
RBC	0.823	0.030	0.881	0.414
MCV	0.917	0.662	0.764	0.821
MCHC	0.971	0.485	0.939	0.660
MCH	0.898	0.647	0.701	0.912
RDW	0.322	0.416	0.108	0.537
MPV	0.012	0.703	0.584	0.463
PLT	0.222	0.977	0.343	0.454
WBC	0.577	0.803	0.992	0.154
LINFO	0.012	0.821	0.142	0.216
EOSINO	0.069	0.677	0.803	0.447
NEUTRO	0.750	0.931	0.466	0.558
BASO	0.436	0.697	0.329	0.112
MONO	0.622	0.252	0.309	0.017

[1] HGB: Hemoglobin; HCT: Hematocrit; RBC: Red blood cell count; MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; MCH: Mean corpuscular hemoglobin; RDW: Red cell distribution width; MPV: Mean platelet volume; PLT: Platelet count;WBC: White blood cell count; LINFO: Lymphocyte count; EOSINO: Eosinophil count; NEUTRO: Neutrophil count; BASO: Basophil count; MONO: Monocyte count.

Supplementary Table 5. Statistic parameters obtained in the association analyses: β estimates and Standard Errors (SE) for each analyzed trait.

Traits[1]	***MUC4* rs338992994**		**FUT1 rs335979375**		**GBP1 rs340943904**		**GBP1 rs80800372**	
	β estimate	**SE**	**β estimate**	**SE**	**β estimate**	**SE**	**β estimate**	**SE**
ADG	3.78E+00	4.69E+00	-6.36E+00	6.62E+00	-7.67E+00	7.25E+00	-1.38E+01	8.41E+00
BFT	-6.17E-01	3.38E-01	1.63E-01	4.71E-01	-8.07E-01	5.15E-01	-7.31E-01	6.16E-01
LC	2.46E-01	1.63E-01	-1.37E-01	2.21E-01	-7.16E-02	2.43E-01	-1.44E-01	3.01E-01
FGR	-9.15E-03	1.64E-02	2.36E-02	2.31E-02	-8.49E-03	2.53E-02	1.54E-02	2.97E-02
HW	9.02E-03	7.93E-02	-1.67E-02	1.08E-01	5.58E-02	1.19E-01	4.45E-03	1.45E-01
VIF	-9.94E-03	2.39E-02	-4.92E-02	3.36E-02	1.27E-02	3.68E-02	2.56E-03	4.33E-02
HWLFS	1.85E+00	2.14E+00	-2.06E+00	2.98E+00	4.60E+00	3.27E+00	4.36E+00	3.88E+00
HGB	9.12E-02	9.93E-01	1.81E+00	1.42E+00	-2.38E-01	1.53E+00	1.45E+00	1.75E+00
HCT	-4.79E-01	6.62E+00	1.49E+01	9.58E+00	-1.69E+00	1.03E+01	7.92E+00	1.17E+01
RBC	-5.81E+07	2.59E+08	7.98E+08	3.67E+08	5.93E+07	3.96E+08	3.72E+08	4.55E+08
MCV	-9.67E+01	9.23E+02	-5.66E+02	1.29E+03	-4.18E+02	1.39E+03	-3.66E+02	1.62E+03
MCHC	-2.01E+00	5.60E+01	-5.68E+01	8.13E+01	-6.70E+00	8.70E+01	4.35E+01	9.88E+01
MCH	-3.37E+00	2.63E+01	-1.69E+01	3.70E+01	-1.53E+01	3.97E+01	-5.11E+00	4.61E+01
RDW	-1.33E-06	1.35E-06	1.55E-06	1.90E-06	3.29E-06	2.04E-06	1.46E-06	2.37E-06
MPV	3.05E-04	1.22E-04	6.68E-05	1.75E-04	1.03E-04	1.88E-04	1.60E-04	2.17E-04
PLT	-3.73E+03	3.05E+03	-1.29E+02	4.42E+03	4.49E+03	4.73E+03	4.04E+03	5.39E+03
WBC	1.15E-01	2.06E-01	7.29E-02	2.92E-01	-3.12E-03	3.14E-01	-5.18E-01	3.62E-01
LINFO	3.41E-01	1.35E-01	4.43E-02	1.96E-01	-3.09E-01	2.10E-01	-2.99E-01	2.41E-01
EOSINO	4.09E-02	2.25E-02	-1.41E-02	3.37E-02	-9.00E-03	3.60E-02	-3.06E-02	4.02E-02
NEUTRO	-2.68E-01	8.42E-01	1.04E-01	1.21E+00	9.43E-01	1.29E+00	-8.72E-01	1.49E+00
BASO	1.06E-02	1.36E-02	-7.73E-03	1.98E-02	-2.07E-02	2.12E-02	-3.83E-02	2.41E-02
MONO	4.99E-03	1.01E-02	-1.69E-02	1.47E-02	-1.61E-02	1.58E-02	-4.25E-02	1.79E-02

[1] ADG: average daily gain, BFT: back fat thickness; LC: lean meat cuts; FGR: feed gain ratio; HW: ham weight; VIF: visible intermuscular fat; HWLFS: ham weight loss at first salting; HGB: Hemoglobin; HCT: Hematocrit; RBC: Red blood cell count; MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; MCH: Mean corpuscular hemoglobin; RDW: Red cell distribution width; MPV: Mean

platelet volume; PLT: Platelet count;WBC: White blood cell count; LINFO: Lymphocyte count; EOSINO: Eosinophil count; NEUTRO: Neutrophil count; BASO: Basophil count; MONO: Monocyte count.