Interaction between the melanocortin 1 receptor (MC1R) and agouti signalling protein genes (ASIP), and their association with black and brown coat colour phenotypes in peruvian alpaca

Stefano Pallotti**, Bathrachalam Chandramohan**, Dario Pediconi*, Cristina Nocelli*, Antonietta La Terza* and Carlo Renieri*

*Scuola di Scienze del Farmaco e dei Prodotti della salute, University of Camerino, Camerino, Italy; **National Institute of Science Education and Research (NISER) Bhubaneswar P.O. Jatni, Khurda, India; *Scuola di Bioscienze e Medicina Veterinaria, University of Camerino, Camerino, Italy

ABSTRACT
The aim of this work was to assess the interaction between MC1R and ASIP genes in determining coat colour variation in black and brown Peruvian alpacas. Skin biopsies were collected from 25 black alpacas and 15 brown alpacas. Genomic DNA was isolated, amplified and sequenced. Black alpacas were dominant homozygous (E/E) or heterozygous (E/e) at the MC1R locus while brown alpacas were heterozygous at the locus. All the black animals were heterozygous for the non-functional alleles at ASIP locus (aH–aD57–aht). On the contrary, brown alpacas were dominant homozygous (A/A) or heterozygous (A/aht) at this locus. When the combined genotypes at MC1R and ASIP were considered together, alpacas with black coats had four possible genotype combinations namely aH/aD57–E/E, aH/aht–E/E, aH/aD57–E/e and aH/aht–E/e. The brown alpacas were found to have the A/aht–E/e and the A/A–E/e genotype. Our results show that the two genes interact to synergically modulate the amount of melanin synthesised in alpaca fibre. In black coat alpacas in fact, the expression of non-functional ASIP results in the failure of pheomelanin production while the functional MC1R allows the synthesis almost exclusively of eumelanin. On the other hand, the brown coat phenotype is produced when the animal possesses at least one functional copy of the gene at both ASIP and MC1R loci. In fact, MC1R binds both ASIP and α-MSH allowing a mixed melanogenesis characterised by the synthesis of eumelanin and pheomelanin granules in a similar ratio.

HIGHLIGHTS
- The income of thousands of Peruvian alpaca farmers depends mainly on the sale of fibre in high demand on the global textile market.
- A wide variety of colours exists, therefore this fibre is preferred by the ecologically sustainable industry for organic textile products.
- Our results provide information for selection breeding program of coloured alpaca helpful to improve the condition of welfare of Andean populations.

Introduction
Coat colour and pattern in mammals is a complex trait controlled by over 300 genetic loci at over 150 genes (Rochus et al. 2019). The relative amounts of melanin pigments in mammals are mainly controlled by the melanocortin-1-receptor (MC1R), encoded by the Extension (E) locus, and its peptide antagonist agouti-signalling-protein (ASIP), encoded by the Agouti (A) locus (Lu et al. 1994). In the hair-bulb melanocytes, the MC1R is mutually bound by ASIP and the α-melanocyte stimulating hormone (α-MSH) which lead to the synthesis of pheomelanin (yellow–red pigments) and eumelanin (dark pigments), respectively. In other words, ASIP acts as an antagonist of MC1R by nullifying the action of α-MSH, therefore, loss-of-function of MC1R results in pheomelanin whereas loss-of-function
of ASIP leads to the production of the black eumelanin (Barsh 1996). The effect of two genes upon hair colour and skin pigmentation has been extensively drawn from scientific literature dealing with genetic of coat colour. Actually, several ASIP and MC1R mutations responsible for coat colour variation have been identified in wild mammals (Liu et al. 2016; Reissmann et al. 2020) as well as in domestic animals including equids (Rieder et al. 2001; Shang et al. 2019), ruminants (Reni et al. 2008; Henkel et al. 2019; Matsumoto et al. 2020), companion animals (Dreger et al. 2013), rabbits and rodents (Lai et al. 2019; Letko et al. 2020). To date, the two genes were addressed separately although there is a low number of paper focussed on the interaction between MC1R and ASIP in modulating the hair pigmentation. So far, this interaction has been explored in camel (Alshanbari et al. 2019), dromedary (Almathen et al. 2018), llama (Daverio et al. 2016), sheep (Fontanesi et al. 2011; Rochus et al. 2019), pig (Shi et al. 2006), horse (Rieder et al. 2001; Shang et al. 2019) and dog (Oguro-Okano et al. 2011). The alpaca is a South American camelid which produce a soft and lustrous fibre in high demand on the global textile market (McGregor et al. 2012). A wide variety of colours exists, therefore, this fibre is preferred by the ecologically sustainable industry for organic textile products (Chandramohan et al. 2013). It is known that the differences between black and brown hair shaft colour are due to both melanogenesis intensity variation and the presence of distinctive melanosomes displaying specific morphological features (Renieri et al. 1991; Cecchi et al. 2004, 2011). Candidate polymorphisms at MC1R and ASIP genes which may impair the structure and functionality of both proteins have been already shown to affect coat colour in this species (Powell et al. 2008; Feeley and Munyard 2009; Bathrachalam et al. 2011; Feeley et al. 2011; Guridi et al. 2011; Chandramohan et al. 2013, 2015). In fact, the Agouti locus in white and coloured (black and brown) alpacas has been found to be affected by three different mutations consisting in two independent SNPs and one deletion of 57 bp (Feeley et al. 2011; Chandramohan et al. 2013). On the other hand, a total of eight mutations were genotyped at MC1R locus in white and coloured alpacas, of which seven were independent SNPs and one was a 4 bp deletion (Powell et al. 2008; Feeley and Munyard 2009; Chandramohan et al. 2015). Moreover, other tree mutations (SNPs) have been describe in this locus which are associated with fibre lightness although without any correlation with colour (Guridi et al. 2011). Recently both genes have been mapped in alpaca. Through the use of FISH-mapping in fact, MC1R was assigned to chr21 and ASIP to chr19 (Alshanbari et al. 2019). Although evidences suggest an interaction between the two genes, none of these paper has shed light on this. Here, we assessed the interaction between MC1R and ASIP genes in determining coat colour variation in black and brown Peruvian alpacas.

### Materials and methods

All the extended protocols and methodology were described in detail in Chandramohan et al. (2013, 2015). Briefly, skin biopsies from 25 black alpacas and 15 uniform brown alpacas (without any particular coat colour patterns such as black and tan or black face and extremities) were collected by disposable biopsy punch (8 mm diameter) and stored in RNAlater (SIGMA, Neustadt, Germany). Genomic DNA was isolated using a DNAeasy tissue kit (Qiagen S.A., Courtaboeuf, France). The extended protocols used for the amplification of genomic DNA, sequencing and sequence analysis are fully described in Chandramohan et al. (2013, 2015).

### Results

Four different alleles were found at ASIP locus while only two alleles were characterised at MC1R locus

| Table 1. ASIP and MC1R alleles in Peruvian alpacas. |
|-------------|-------------|-------------|-------------|-------------|
| Allele | ASIP | MC1R |
| g3836C > T pr98C | g3866-3923del57 C109_R127del | g3896G > A pR118H |
| g82A > G pT28A | g259A > G pV87M | g376A > G pG126S | g901C > T pR301C |
| A | g3836C | g82A | A |
| aH | C | A |
| aHe | T | G |
| a57 | C | Yes |
| ASIP: agouti signalling protein; MC1R: melanocortin 1 receptor. |
resulting in the brown hair phenotype (Klungland et al. 1995).

In our study, all the animals genotyped from the ten black families were heterozygous for the mutated ASIP allele (Table 3), suggesting that these mutations lead to a complete loss-of-function of the translated protein, and possessed at least one functional copy of MC1R (E/E or E/e genotype). In black coat alpacas, the non-functional ASIP results in the failure of the protein to properly bind to the MC1R receptor preventing the initiation of the pheomelanin synthesis in the melanocytes. At the same time, the functional MC1R binds the α-MSH allowing the synthesis of the round and oval-shaped mature eumelanosomes (black pigment granules) which display transverse striations that confer the black colour to the hair shaft (Renieri et al. 1991). It is known that the eumelanosomes production in alpaca increases as the colour deepens from white to black. This implies that black fleece is characterised by the almost exclusive presence of eumelanin pigment while a very small amount of pheomelanin is produced in the black phenotype (Renieri et al. 1991; Shang et al. 2019). Similarly, the black coat colour in the Massese sheep breed are determined by non-functional ASIP alleles (Rieder et al. 2001; Shang et al. 2019). 

The interaction between the two loci we describe here has been already observed to lead to the black phenotype in others species. Studies conducted on several equine breeds found that all the black animals were homozygous for the mutated ASIP alleles and had at least one functional MC1R alleles. Black coat horses, in fact, possess the a/a genotype at the ASIP locus and E/E or E/e genotype at the MC1R locus (Rieder et al. 2001; Shang et al. 2019). Similarly, the black coat colour in the Massese sheep breed are mainly derived by combining ASIP and MC1R mutations being determined by non-functional ASIP alleles (recessive) and by the dominant MC1R allele (Fontanesi et al. 2011). Recently, the interaction

**Table 2. Alpaca ASIP-MC1R genotype combinations in the two coat colours.**

| Colour   | ASIP   | MC1R |
|----------|--------|------|
| Black    | \(d^I/d^{AS} \) | E/E  |
| Black    | \(d^I/d^H \) | E/e  |
| Brown    | A/d^H  | E/e  |
| A/A      |        |      |

*ASIP: agouti signalling protein; MC1R: melanocortin 1 receptor.*

(Table 1). These alleles have been already described by Chandramohan et al. (2015, 2013).

As shown in Table 2, both black and brown alpacas were heterozygous at MC1R locus. On the contrary, the dominant homozygous genotype was only in black animals while none of the brown alpacas were homozygote for the dominant or recessive alleles. All the black animals were heterozygous for non-functional alleles at ASIP locus. On the other hand, the dominant wild allele was observed only in brown alpacas for which the genotypes found were A/A and A/d^H. It should be noted that black genotypes are characterised by a high recessive allele heterogeneity (\(d^I, d^{AS}, d^H\)). Conversely, a single recessive allele was found to be responsible for the brown phenotype (\(a^{HT}\)). Therefore, we proceed the analysis in order to understand how the Asip alleles segregate in a full black population (Table 3).

When the combined genotypes at MC1R and ASIP were considered together, alpacas with black coat had four possible genotype combinations viz. \(d^I/d^{AS} - E/E\), \(d^I/a^{HT} - E/E\), \(d^I/a^{AS} - E/e\) and \(d^I/a^{HT} - E/e\) (Tables 2 and 3) For brown alpacas, the animals were found to have the A/a^{HT} - E/e and A/A - E/e genotype.

**Table 3. Crosses involving 5 full black sires and 10 dams.**

|             | Black ram genotype | Black dam genotype | Black offspring genotype |
|-------------|--------------------|--------------------|-------------------------|
| d^I/d^{HT} - EE | d^I/d^{HT} - E/e | d^I/d^{HT} - E/E | d^I/d^{HT} - E/E |
| d^I/d^{ST} - EE | d^I/d^{ST} - E/e | d^I/d^{ST} - E/E | d^I/d^{ST} - E/E |
| d^I/d^{ST} - EE | d^I/d^{ST} - E/e | d^I/d^{ST} - E/E | d^I/d^{ST} - E/E |
| d^I/a^{ST} - EE | d^I/a^{ST} - E/e | d^I/a^{ST} - E/E | d^I/a^{ST} - E/E |
| d^I/a^{ST} - EE | d^I/a^{ST} - E/e | d^I/a^{ST} - E/E | d^I/a^{ST} - E/E |

**Discussion**

The aim of this paper was to investigate the interaction between ASIP and MC1R in determining coat colour in Peruvian alpacas. It is clear that in mammal the black and brown coat pigmentation is the result of the interaction between these two loci. Eumelanin and pheomelanin synthesis, is determined by the MC1R which acts as a molecular switch in melanin production. If this receptor is bounded by α-MSH, eumelanin synthesis is triggered resulting in black hair. On the other hand, pigment production switch to pheomelanine if the receptor is bounded by ASIP
between the two loci were also studied in some camelid species. In dromedaries a deletion and a SNP in ASIP have been found to be associated with the black/dark brown colour (Almathen et al. 2018; Alshanbari et al. 2019). Polymorphisms in ASIP and MC1R were identified also in llama, however none of which were completely associated to a particular colour phenotype (Daverio et al. 2016).

From our study, brown coat alpacas were found heterozygous or homozygous for the dominant ASIP alleles and heterozygous at MC1R locus (Table 2). This result clearly shows that the brown coat phenotype is produced when the animal possesses at least one functional copy of the gene at both ASIP and MC1R loci. In brown coat alpaca melanocytes in fact, MC1R binds both ASIP and α-MSH allowing a mixed melanogenesis characterised by the synthesis of eumelanin and pheomelanin granules in a similar ratio. The brown hair shaft, in fact, is characterised by the presence of a mixed melanosome population at different maturation stages leading to the expression of the brown phenotype (Renieri et al. 1991; Cecchi et al. 2011). Similarly, the E/E – A/a genotype has been found to be the advantage genotype for brown coat colour in horse (Shang et al. 2019). In the same species, the dark bay coat colour, classified as sub-phenotype of brown coat colour, has been found to be expressed by the E/E – A/A, E/e – A/a or E/e – A/A genotypes (Rieder et al. 2001; Shang et al. 2019).

**Conclusions**

In this study, we investigated the interaction between the MC1R and ASIP, and their association with black and brown coat colour phenotypes in Peruvian alpaca. Taken together, MC1R and ASIP genotypes show that the two genes interact to sinergically modulate the amount of melanin synthesised in alpaca fibre. The black coat colour is the result of the synthesis almost exclusively of eumelanin due to lack of ASIP functional allele. On the other hand, the brown coat phenotype is due to the expression of both genes which leads to the synthesis of the same proportion of eumelanin and pheomelanin. In light of this, both genes have to be considered in the development of marker assisted breeding program for coat colours in alpaca.

**Ethical approval**

All experiments were approved and performed in 2008 according to the guidelines of the Animal Ethics Committee of the University of Camerino.

**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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**ORCID**

Stefano Pallotti http://orcid.org/0000-0003-3196-151X
Bathrachalam Chandramohan http://orcid.org/0000-0002-3464-7553
Dario Pediconi http://orcid.org/0000-0001-8734-3070
Cristina Nocelli http://orcid.org/0000-0003-1376-0538
Antonietta La Terza http://orcid.org/0000-0002-1244-1503
Carlo Renieri http://orcid.org/0000-0003-4816-228X

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