# MC4R gene polymorphism associated with hematology parameters of Awassi and Arabi Iraqi sheep

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## ABSTRACT

Blood parameters are among the basic growth traits that need to be studied because they are useful for assessing the physiological condition of breeds that are different according to the breed, sex, physiological status, and genotype of the animal. Therefore, this study aims to investigate the possibility of an association between the polymorphism of the *MC4R gene* (exon 1) with hematology parameters in Iraqi Awassi and Arabi sheep. A total of 150 sexually mature and healthy sheep aged between 2 to 3 years were included in this study. DNA samples were extracted from each blood sample sheep and the genetic analysis were included PCR-SSCP and direct sequencing. Result of the present study identified three genotypes for the *MC4R* gene (exon 1) (AA, AC, and GC). Two missense SNPs g.59349260G>C and g.59349291A>C were identified in exon1 *MC4R* gene that was responsible for the observed heterogeneity in both breeds. The amount of RBC, Hb, and PCV%, WBC, lymphocyte, granulocyte, lymphocyte%, and granulocyte % were significantly higher ($p<0.05$) in awassi than Arabi breed and sex male than female. The genotype AC showed higher accounts of RBC, Hb, PCV%, MCHC, accounts of WBC, lymphocyte, lymphocyte% and granulocyte % relative to other genotypes. In summary, Awassi breed and the sex factor are effecting on blood parameters. The two novel SNP (107G/C and 138 A/C) were highly associated with hematology parameters. The AC genotype was preponderant genotype affecting the sheep growth traits and recommended to be selected and fixed in sheep production.

**Keywords:** *MC4R* gene, polymorphism, blood parameters, sheep

# تعدد الاشكال لجين الميلانوكورتين وارتباطها بمعايير الدم في الاغنام العواسي والعرابي العراقية

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*الملخص*

تعد معاييرالدم من بين صفات النمو الاساسية التي بجب دراستها لانها مفيدة لتقييم الحالة الفسيولوجية للسلالات والتي تختلف باختلاف السلالة والجنس والحالة الفسيولوجية والنمط الوراثي للحيوان. لذالك تهدف هذه الدراسة الى التحري عن احتمالية وجود علاقة بين تعدد الاشكال لجين الميلانوكورتين *MC4R* (اكسون 1) مع معايير الدم في الاغنام العواسي

والعرابي العراقية . في هذه الدراسة تم تضمين 150 من الاغنام الناضجة جنسياً وصحياً وبعمر تراوح بين (3-2) سنوات . تم استخراج الحامض النووي من كل عينة دم للاغنام والتحليل الجيني تضمن تعدد الاشكال للشريط المفرد المتشكل PCR-SSCP والتسلسل المباشر. بينت نتائج هذه الدراسة ثلاثة انماط جينية لجين *MC4R* (اكسون 1) (AC, AA و GC).تم تحديد اثنين من الطفرات المغلوطة في جين *MC4R* (اكسون 1) g.59349260G>C و g.59349291A>C والتي كانت مسؤولة عن التغايير الملحوظ في كلا السلالتين . كمية كريات الدم الحمراء , تركيز الهيموغلوبين, وحجم الكريات المضغوط , قياس تركيز الهيموكلوبين، كريات الدم البيضاء, الخلايا اللمفية, النسبة المئوية للخلايا اللمفية والنسبة المئوية للخلايا المحببة بشكل كبير($p<0.05$) في الاغنام العواسي من العرابي وفي الذكور من الاناث. اظهر النمط الجيني AC كميات من كريات الدم الحمراء , تركيز الهيموغلوبين, وحجم الكريات المضغوط, كريات الدم البيضاء, الخلايا اللمفية, الخلايا الحبيبة ,نسبة الخلايا اللمفية و النسبة المئوية للخلايا الحبيبة اكبر مقارنة مع الانماط الوراثية الاخرى. وباختصار، فان سلالة العواسي وعامل الجنس يؤثران على معايير الدم . الطفرتين (107G/C و 138 A/C) ارتبطت ارتباطاً كبيراً بمعايير الدم . النمط الوراثي AC كان النمط الوراثي المتفوق الذي يؤثر على خصائص نمو الاغنام والذي يوصي باختياره وثباته في انتاج الاغنام.

الكلمات المفتاحية: جين الميلانوكورتين *MC4R*, تعدد الاشكال , معايير الدم , الاغنام

**البحث مستل من رسالة ماجستير للباحث الثالث**

## 1. Introduction

The hypothalamic–melanocortin system plays an important role in the regulation of energy balance, reproduction and growth traits via the melanocortin receptors (1). Five melanocortin receptors (MCRs) mediate the actions of the melanocortin system. They are classified MC1R to MC5R according to their sequence (2). Of the five-melanocortin receptors, melanocortin-4 receptor (MC4R) is a G-protein-coupled receptor with seven transmembrane domains that is received more attention (3). MC4R was detected in the pituitary and ovaries (4), and is expressed in the appetite-regulating areas of the brain that involved in food intake and can be a mediator between appetite and reproduction (5). Blood parameters are among the basic growth traits that need to be studied because they are useful for assessing the physiological condition and knowing the animal's health status and are related to the suitability of breeds under certain environmental conditions (6,7**)**. Blood parameters of animals are different due to different factors including breed, age, sex, physiological status, and genotype of the animal (8). Few reports studied the association of breed, sex and gene polymorphism with hematology parameters. Egbe-Nwiyi(9) studied the effect of sex on the hematological values of goats and sheep in Nigeria. Badawi and AL-Hadithy (10) revealed a significant difference ($P<0.05$) in some hematological values of Awassi sheep in Iraq. Regarding the genotype effect, many variants were identified in the *MC4R* gene, which is associated with growth traits in Langshan chickens (11), in pigs of Russia (12) and sheep (3). Only one study revealed that the animals with MN *MspI* CAST genotype had significantly ($p<0.05$) higher neutrophil percentage and neutrophil to lymphocyte ratio than NN *MspI* CAST genotype in Awassi sheep, indicate that *CAST* gene heterozygous individuals are healthier than the homozygous individual**.** Based on the above consideration, no research yet on the association of the *MC4R* gene with the hematology parameters have been reported in Awassi and Arabi sheep. Thus, the current study aimed to evaluate the association of single nucleotide polymorphism (SNPs) in the *MC4R* gene on hematology parameters in Awassi and Arabi sheep.

## 2. Materials and Methods

Animals**,** blood collection and hematological examination

This study was conducted according to regulations of the international recommendations for the care and use of animals under Al-Qasim Green University approval (Agri, No. 015,3,12), at the College of Agriculture /department of Animal Resources for the period from January 2018 to August 2018 on Awassi and Arabi sheep. A total of 150 sexually mature and healthy sheep aged between 2 to 3 years including Awassi (n = 75) (22 male: 53 female) and Arabi (n = 75) (15 male: 60 female), were included in this study. Animals were collected randomly from three Station for raising sheep (Babylon, Karbala, Kufa). Animals were kept on natural pasture during summer, while in winter; animals were kept indoors and fed about 2.5% of their live body weight daily, comprising a mixture of barely (59%), bran (40%), and salt (1%) concentrates. Blood samples were collected from the sheep, using vacutainer tubes with EDTA. Hematology analyzer (vet.18, mythic company) measured hematological parameters. These parameters included erythrocyte constituents, total platelet count, and white blood cell constituents.

***Genomic DNA extraction, Primers design, and PCR***

Genomic DNA from blood was isolated by the high salt method of (Al-Shuhaib, 2017).

**Table(1) The sequence of the primer used in this study as follows:**

| Set | Primer code | Primer sequence (5′ → 3′) | Product size | Annealing temp. |
|---|---|---|---|---|
| 1 | MC4R,exo1-F | GTCACAAACACCTCGGGAGA | 181 bp | 57.8°C |
| | MC4R,exo1-R | TCCAGAGGGGACCTGAATCC | | |

**Table (2)** Recommended thermal cycling conditions for PCR amplification for the MC4R gene. These guidelines were tested for Eppendorf thermal cycler :

| **Ste Step Step p** | **Tm(°C)** | **Time** | **No.of cycle** |
|---|---|---|---|
| Initial denaturation | 95 | 4 minutes | ×1 cycle |
| Denaturation | 94 | 30 sec. | ×30 cycles |
| Gradient annealing | | | |
| Ovine *MC4R*(exon1)<br>*MC4R* (exon 2.1)<br>*MC4R* (exon 2.2)<br>*MC4R*(exon 2.3) | 57.8<br>57.8<br>60.4<br>59.1 | 30 sec. | |
| Extension | 72°C | 30 sec. | |
| Final extension | 72°C | 5 minutes | ×1 cycle |
| Holding | 4°C | Indefinite | ×1 cycle |


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***Single-strand conformation polymorphism (SSCP) and Sequencing analysis***

The initial denaturation of the PCR amplicons, as well as SSCP protocol, were performed according to Al-Shuhaib et al. (2018) protocol. For single-strand conformation polymorphism (SSCP) analysis, 10 µL of each amplification product was mixed with 10 µL of denaturing buffer (98 % formamide, 0.025 % bromophenol blue, 0.025 % xylene cyanol FF, 10 mmol=L Ethylenediaminetetraacetic acid (EDTA) (at pH 8.0) and 2 % glycerol), heated for 7 min at 95 ◦C and then cooled on ice for 7 min. Denatured PCR products were as follows:

| Set | Amplicons | Gel Concentration | Running temperature | Running time | Running voltage | Running amperage |
|---|---|---|---|---|---|---|
| 1 | *MC4R*,exo1 | 10% | 10°C | 5.0 hr | 200V | 105 mA |

SSCP patterns on the gels were visualized by silver staining according to the protocol of Byun (13). For each genotype, the PCR products were sent for purification and sequencing of multiple sequence alignment program, according to DNA Star, EditSeq. / ClustalW, with the sequences published in the GenBank database taken as a reference to identify the polymorphisms. The observed mutations were visualized and annotated by SnapGene Viewer, ver. 4.0.4. (GSL. Biotech. LLC). The novelty of the observed variants was checked by the deposited variants of the ovine *MC4R* gene database in the Ensemble genome browser 96 (https://asia.ensembl.org/index.html).

***Statistical analyses***

The allele and genotype frequencies observed heterozygosity (*Ho*), and expected heterozygosity (*He*), were analyzed using PopGen32 software, v. 1.31 (14). The significant effect of breed, sex, and on the various parameters studied were analyzed by Statistical Package for the Social Sciences (SPSS) software version 23.0., with the general linear model:

$$Y_{ijkl} = \mu + B_i + S_j + G_k + e_{ijkl}$$

where $Y_{ijkl}$ = phenotypic traits, µ = overall mean, $B_i$ = fixed effect of $i^{th}$ breed (*i* = Awassi, Arabi), $S_j$ = fixed effect of $j^{th}$ sex (*j* = male, female), $G_k$ = fixed effect of $k^{th}$ genotype, and $e_{ijkl}$ = random error associated with $Y_{ijkl}$ observation and assumed to be NID (0, $\sigma^2 e$). Means were compared using Tukey-Krammer test with a significance level of ($P<0.05$). Preliminary statistical analysis indicated the effect of factor interaction, age, season and station did not have a significant effect on phenotypic traits, so are not matched in the general linear model.

***3. Results and Discussion***

***The genetic polymorphism***

In the present study, Single-strand conformation polymorphism ( SSCP) analysis reveals three genotypes (AA, AC , and GC) in all scanned *MC4R* (exon 1) of sheep (Figure 1). The overall ratio of the genotypes AC was the highest (56 and 60 %) in Arabi and Awassi sheep respectively (Table3). Two missense SNPs g.59349260G>C and g.59349291A>C were identified in exon1 *MC4R* gene that was responsible on the observed heterogeneity in both breeds and the genotype nominated according to this substitution. According to the value of Chi-square, the population under study was not in Hardy–Weinberg equilibrium (HWE), which was statistically significant at ($P < 0.05$).

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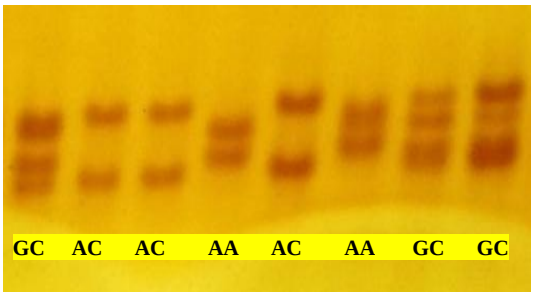


**Figure (1):** SSCP non-denaturing polyacrylamide gel electrophoresis of the *MC4R gene* (exon 1) PCR fragments showed three genotypes (AA, AC, and GC). Electrophoresis conditions: Polyacrylamide gel concentration 10%, power applied: 200V (7.5V/cm) – 100mA, time to run: 4 hr. Staining method; Silver nitrate.

**Table(3).** Genotype, allele frequencies and genetic diversity parameters in the *MC4R* gene for both Awassi and Arabi breeds.

| | Genotypes (N) | Genotype frequencies | Allele | Allele frequencies | *χ2* | *Ho* | *He* |
|---|---|---|---|---|---|---|---|
| **A) Arabi** | AA(7) | 0.09 | A | 0.37 | 55. | 0.906 | 0.629 |
| | AC(42) | 0.56 | G | 0.17 | 733 | | |
| | GC(26) | 0.35 | C | 0.45 | | | |
| **B) Awassi** | AA(12) | 0.16 | A | 0.46 | 46.127 | 0.840 | 0.601 |
| | AC(45) | 0.60 | G | 0.12 | | | |
| | GC(18) | 0.24 | C | 0.42 | | | |

**Abbreviations: χ2 – chi-square, Ho – observed heterozygosity, He – Expected heterozygosity.* All Chi-square tests have two degrees of freedom and within the significance level P<0.05.**

***Assessment of MC4R polymorphism and association analysis***

Association analysis of *MC4R* polymorphism refer to numerous physiological changes occurs in this study. Table 4 shows the least-square means of erythrocyte constituents and platelets as affected by breed and sex. The amount of RBC, Hb, and PCV% were significantly higher ($p<0.05$) in awassi than Arabi breed and sex male than female while there was no significant difference ($p>0.05$) for other parameters. The numbers of erythrocytes, Hb and PCV% were higher in awassi (9.461 ×106/µl), (9.552) and (31.317 %) respectively than Arabi. The same pattern was seen in male than female for the same parameters. Similarity, according to the leukocyte constituents, the Awassi breed and sex male showed higher WBC, lymphocyte, granulocyte, lymphocyte% and granulocyte % than Arabi breed and the sex female, while no statistically significant difference was observed for the other leukocyte profile ($P>0.05$) (Table6). The genotype AC showed higher accounts of RBC, Hb, PCV% and MCHC relative to other genotypes (Table4). Table 7 shows the effect of SNP on leukocyte constituents. The sheep with AC genotype showed higher accounts of WBC, lymphocyte, lymphocyte% and granulocyte % than AA and GC genotypes.

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**Table (4): Least square Mean ± SE of erythrocyte constituents and platelets for the breed and sex effects.**

| Indices | | RBC ($\times10^6$/µl) | Hb (g/dl) | PCV (%) | MCV (fl) | MCH (pg ) | MCHC (g/dl) | PLT ($\times10^3$/µl) |
|---|---|---|---|---|---|---|---|---|
| Breed | Arabi | 8.167 ± 0.593 [b] | 8.061 ± 0.752 [b] | 28.833 ± 1.786[b] | 36.139 ± 1.198 [a] | 10.072 ± 0.731 [a] | 27.956 ± 2.315 [a] | 575.278 ± 10.148 [a] |
| | Awassi | 9.461 ± 0.557 [a] | 9.552 ± 0.706 [a] | 31.317 ± 1.676 [a] | 34.049 ± 1.125 [a] | 10.029 ± 0.686 [a] | 29.550 ± 2.173 [a] | 526.097 ± 14.953 [a] |
| Sex | Male | 9.228 ± 1.027[a] | 8.733 ± 1.303[a] | 32.117 ± 3.093 [a] | 36.217 ± 2.076 [a] | 10.183 ± 1.266 [a] | 28.550 ± 4.009 [a] | 507.167 ± 25.194 [a] |
| | Female | 8.377 ± 0.395[b] | 7.585 ± 0.501[b] | 27.107 ± 1.190 [b] | 33.036 ± 0.799 [a] | 9.993 ± 0.487 [a] | 28.993 ± 1.542 [a] | 493.177 ± 27.398 [a] |

**RBC, red blood cell; Hb, the concentration of hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets. Different superscript in the same column within each classification indicate significant differences (P<0.05).**

**Table (5): Least square Mean ± SE of erythrocyte constituents and platelets for the *MC4R* polymorphism effects in Awassi and Arabi breeds.**

| Genotype | RBC ($\times10^6$/µl) | Hb (g/dl) | PCV (%) | MCV (fl) | MCH (pg ) | MCHC (g/dl) | PLT ($\times10^3$/µl) |
|---|---|---|---|---|---|---|---|
| AA | 7.861 ± 0.726 [b] | 7.967 ± 0.921 [b] | 27.208 ± 2.187 [b] | 34.983 ± 1.468 [a] | 10.500 ± 0.489 [a] | 28.350 ± 1.735 [b] | 576.000 ± 12.881 [a] |
| AC | 8.531 ± 0.602 [a] | 8.778 ± 0.762 [a] | 31.958 ± 1.851 [a] | 33.600 ± 1.266 [a] | 9.994 ± 0.400 [a] | 33.989 ± 1.417 [a] | 616.667 ± 8.997 [a] |
| GC | 7.203 ± 0.557 [b] | 7.240 ± 0.706 [b] | 28.430 ± 1.676 [a] | 34.045 ± 1.125 [a] | 10.625 ± 0.686 [a] | 29.292 ± 2.173 [b] | 604.972 ± 14.953 [a] |

**RBC, red blood cell; Hb, the concentration of hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets. Different superscript in the same column within each classification indicate significant differences (P<0.05).**

**Table (6): Least square Mean ± SE of the constituents of white blood cell count for the breed and sex effects.**

| Indices | | WBCs ($\times10^3$/µl) | Lymphocytes ($\times10^3$/µl) | Monocytes ($\times10^3$/µl) | Granulocytes ($\times10^3$/µl) | Lymphocytes (%) | Monocytes (%) | Granulocytes (%) |
|---|---|---|---|---|---|---|---|---|
| Breed | Arabi | 9.656 ±1.594[b] | 5.589 ± 1.168[b] | 0.833 ± 0.017[a] | 1.928 ± 0.063[b] | 55.761 ± 5.517[b] | 11.167 ± 0.997[a] | 27.028 ± 3.959[b] |
| | Awassi | 15.883 ±1.373[a] | 10.563 ± 2.974[a] | 1.307 ± 0.016 [a] | 3.999 ± 0.059[a] | 62.301 ± 5.179[a] | 10.608 ± 0.936 [a] | 32.424 ± 3.716[a] |
| Sex | Male | 13.717 ± 0.224 [a] | 7.400 ± 0.488 [a] | 1.033 ± 0.030 [a] | 3.283 ± 0.105 [a] | 66.167 ± 9.555 [a] | 10.750 ± 1.727 [a] | 33.017 ± 6.856 [a] |
| | Female | 10.813 ± 1.395[b] | 5.443 ± 0.111 [b] | 1.133 ± 0.011 [a] | 2.103 ± 0.425 [b] | 58.831 ± 3.676 [b] | 10.887 ± 0.664 [a] | 28.949 ± 2.638 [b] |

**WBC, white blood cell; Different superscript in the same column within each classification indicate significant differences (P<0.05).**

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**Table (7): Least square Mean ± SE of the constituents of white blood cell count for the *MC4R* polymorphism effects in Awassi and Arabi breeds.**

| Genotype | WBCs ($\times10^3$/µl) | Lymphocytes ($\times10^3$/µl) | Monocytes ($\times10^3$/µl) | Granulocytes ($\times10^3$/µl) | Lymphocytes (%) | Monocytes (%) | Granulocytes (%) |
|---|---|---|---|---|---|---|---|
| **AA** | 10.875 ± 1.238[b] | 8.867 ± 0.880[b] | 1.017 ± 0.021[a] | 3.008 ± 0.078[a] | 54.108 ± 6.757[b] | 12.417 ± 1.221[a] | 30.475 ± 4.848[b] |
| **AC** | 13.344 ± 1.460[a] | 9.088 ± 0.108[a] | 1.094 ± 0.157[a] | 3.161 ± 0.059[a] | 65.472 ± 4.277[a] | 11.844 ± 0.856[a] | 34.639 ± 3.659[a] |
| **GC** | 13.078 ± 0.386[b] | 8.746 ± 0.974[b] | 1.300 ± 0.016[a] | 3.400 ± 0.059[a] | 58.667 ± 5.179[b] | 10.167± 0.936[a] | 28.123 ± 3.716[b] |

**WBC, white blood cell; Different superscript in the same column within each classification indicate significant differences (P<0.05).**

The result refers to the presence of significant differences (P< 0.05) in hematology parameters between Awassi and Arabi breed. Erythrocyte and leucocyte cell distribution is affected by breed (15). There was a significant influence of age, sex, and breed on lymphocyte count (16). This study consistent with the study of Oramari (8) that showed that the Karadi sheep had higher value of HB (9.36 g/dl) and PCV (28.73%) than Awassi and Naimy sheep. Concerning the effect of gender, results in table 4 and 6 indicated that sex male showed higher blood cell count than the sex female. The differences in hematological values between males and females may be due to the negative influence of estrogen on erythropoiesis of females in comparison with the positive influence of androgen in males (10). These results were consistent with the findings obtained by Badawi and AL-Hadithy, ( 10) and Al- Samarai and Al-Jbory (16) who reported that males have significantly (P<0.05) higher mean values of PCV, Hb, and RBC compared with females values. For the MC4R polymorphism, the result of the present study showed that the genotype AC had higher accounts of RBC, Hb, PCV%, MCHC, accounts of WBC, lymphocyte, lymphocyte% and granulocyte % than AA and GC genotypes. Melanocortins play critical roles in inducing erythroid differentiation including enucleation through the MCRs that are differentially expressed in human erythroblasts depending on the differentiation stages (18). In adults, MCRs have been reported to be expressed in lymphocytes, macrophages, and neutrophils (18, 19), to reduce inflammatory cytokine production and leukocyte trafficking (20).

**4. Conclusion**: Awassi breed and the sex factor are effecting on blood parameters. The two novel single nucleotide polymorphism (SNP) (107G/C and 138 A/C) were highly associated with hematology parameters. The AC genotype was preponderant genotype affecting the sheep growth traits and recommended to be selected and fixed in sheep production.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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