# PROTEOMIC ANALYSIS OF EFFECT OF POLYMOPHISMS IN GLUCO- AND MINERALOCORTICOID RECEPTORS GENES IN BOVINE MEAT

Mirele D. Poleti[1*], Cristina T. Moncau[1], Fábio M. Moraes[1], Alessandra F. Rosa[1], Saulo L. Silva[2] and Júlio C. C. Balieiro[1]

[1] Department of Basic Sciences, College of Animal Science and Food Engineering, University of São Paulo, Pirassununga,São Paulo, Brazil;

[2] Department of Animal Science, College of Animal Science and Food Engineering, University of São Paulo, Pirassununga,São Paulo, Brazil

**Abstract – The glucocorticoids receptors (GR) and mineralocorticoids receptors (MR) are essential in the coordination of responses to stress of the hypothalamic-pituitary-adrenal axis, due to its intrinsic relations of the cortisol responses to restore homeostasis. In this study, the functional proteomic analysis was used as a tool to promote more comprehension of the effect of two single nucleotide polymorphisms (SNP), the NR3C2_1 in MR and NR3C1_1 in GR, on the protein profile of bovine meat. *Longissimus dorsi* muscle was analyzed from homozygotes and heterozygotes Nellore cattle for SNPs collected 24 hours *post-mortem*. Proteins were separated by two-dimensional electrophoresis technique and identified by MALDI TOF/TOF MS/MS. Ten proteins were significantly altered by polymorphisms in 24 hours samples. Four proteins were changed by the interaction between genotype combination of MR and GR. The most proteins were altered by SNP in MR. Among the identifications are proteins involved in muscle contraction, metabolism, cellular defense and others. The results suggest that the GR and MR are involved in various functions, which may be targets for studies aimed at understanding the complexity of the conversion of muscle in meat.**

**Key Words – Single nucleotide polymorphisms, Hypothalamic-pituitary-adrenal axis, Two-dimensional electrophoresis**

## I. INTRODUCTION

The effect of pre-slaughter stress on muscle pH *post-mortem* and meat quality is well reported in the literature [1]. Stress promotes decrease of glycogen in muscle and, consequently, leads to changes in the meat, as the condition DFD (dark, firm and dry) [2,3,4].

The main regulatory stress axis is Hypothalamic-Pituitary-Adrenal (HPA), which begins and ends the reactions in order to restore homeostasis. Cortisol is the main hormone this axis and influence on muscle glycogenolysis [ 1,5,6].

The action of this hormone is regulated by two receptors, the mineralocorticoid (MR) and glucocorticoid (GR) receptors, which are located in the cell cytoplasm and act on the transcription of some genes [7]. Single nucleotide polymorphisms (SNPs) present in these receptors have shown to affect theirs functionality and influence metabolic pathways [8].

In this context, the objective of this study was to evaluate the translation effects of polymorphisms related to stress axis. More specifically, evaluate the possible implications of polymorphisms, NR3C1_1 G.3293 A>G in the GR and NR3C2_1 g.115 T>C in the MR, in protein profile of bovine meat.

## II. MATERIALS AND METHODS

### *Muscle samples*

*Longissimus dorsi* muscle was sampled 24 hours after slaughter from Nellore cattle homozygote and heretozygote for SNPs in the GR (NR3C1_1 g.3293A>G; ss528103353) and MR (NR3C2_1 g.115 T>C; ss528103346). Both SNPs result in non-synonymous mutations and are associated with changes in hormone levels of cortisol and meat quality characteristics, respectively (date not published). A total of 27 animals were available, *i.e.* five animals per genotype combination, except for one genotype combination, which was found only two animals (Table 1).

The samples collected were immediately frozen in liquid nitrogen and kept at -80ºC until subsequent analysis.

Table 1 Number of individuals (N) distributed in different combinations of genotypes between single nucleotide polymorphisms (SNP) in the genes of the glucocorticoid receptor (GR) and mineralocorticoid (MR)

| SNP | | GR NR3C1_1 g.3293A>G | |
|---|---|---|---|
| | Genotype | AA (N) | GA (N) |
| MR NR3C2_1 g.115 T>C | CC | Pool 1 (5) | Pool 4 (5) |
| | TC | Pool 2 (5) | Pool 5 (5) |
| | TT | Pool 3 (5) | Pool 6 (2) |

*Extraction of muscle proteins*

Sample preparation was performed according Bouley *et al.* [9]. Briefly, frozen muscle tissue from five animals was pooled to obtain one sample per genetic type. The samples was homogenized in a lysis buffer containing 8 M Urea, 2 M Thiourea, 1% DTT, 2% CHAPS and 2% IPG buffer pH 4-7 and centrifuged at 10 000 x *g* for 30 min. at 4ºC. The supernatant was harvested and protein concentration determined (PlusOne 2-D Quant Kit; GE Healthcare).

*2-DE and data analysis*

IPG strips (pH 4-7, 13 cm) containing 500 µg of protein were subjected to IEF (19,603Vh) in a Ettan IPGphor 3 (GE Healthcare) gel apparatus at a temperature of 20ºC. Proteins were separated in the second dimension on SDS-PAGE 12,5% using a SE 600 Ruby system (GE Healthcare). 2-D gels were stained using a colloidal Coomassie Blue G-250 procedure. Triplicate gels from each protein extract were analysed. Spot detection and quantification were performed with ImageMaster 2-D Platinum software. 2-D gel data were normalized, by dividing each spot volume by the total volume of all the matched spots in the 2-D gel image, to obtain a normalized spot volume value. The gels were realized in triplicate and a protein spot was shown with the mean spot volume value from triplicate 2-D gels. The resulting value was submitted to ANOVA using PROC MIXED procedure of Statistical Analysis System (SAS), version 9.1.3, to test the effects of genotype combinations. Due to the high number of spots to be evaluated, it was applied Bonferroni correction to control the level of significance set at 5%.

*Protein isolation and identification by MS*

The spots identified as differentially expressed in statistical analyzes were excised by hand from stained 2-D gels, and peptides were extracted by according to the methodology described by Shevchenko [10].
The peptide solution was combined with an equal volume of a-cyano-4, 4-hydroxycinnamic acid and spotted onto a MALDI sample plate. Peptide masses were determined in the positive-ion reflector mode in a Matrix-Assisted Laser Desorption/Ionization - Time Off Flight Mass Spectrometer (AB Sciex-5800, Framingham, MA, USA). Peptide mass fingerprints were compared to *Bos taurus* databases (SWISS-PROT)

## III. RESULTS AND DISCUSSION

The 2-D gels produced high resolution of muscle proteins and allow us detect in mean 548 spots (Figure 1). After a comparative study (match) between genotypes, 278 spots were detected as common protein spots between replicate gels of each sample. Bouley *et al.* [9] detected in 2-D gels from the same muscle tissue approximately 500±50 *spots*.

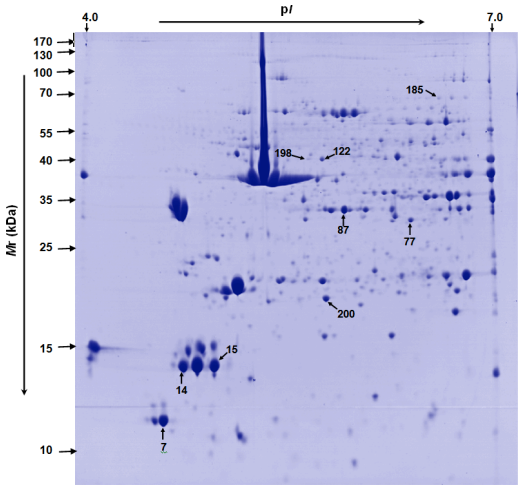


Figure 1. Representative 2-D gel image of *Longissimus dorsi* muscle. Arrow show 9 spots that are significantly changed in abundance by polymorphisms in MR and GR gene.

The statistical analysis (ANOVA, $P \leq 0.0002$) to determine the significance of changes in protein expression between genetic types revealed 9 spots significantly altered: (i) five spots were altered between the different genotypes for marker in MR gene, (ii) one spot that had abundance altered in MR also had its abundance altered between genotypes for the marker in GR gene, and (iii) four spots altered its expression by the effect of interaction of genotype MRxGR.

These observed results are extremely relevant since it shows the influence of genotype on the MR and GR in expression of certain proteins, and that some proteins are expressed independently for each marker or by their interaction .

For subsequent MALDI-TOF/TOF MS/MS all spots were successfully identified.

The changes in protein expression recurrent of the effect of SNP present in GR gene were observed in only one protein. The Troponin T slow skeletal muscle fibers (sTnT, spot 77) expression was down-regulated for homozygote genotype (Figure 2).

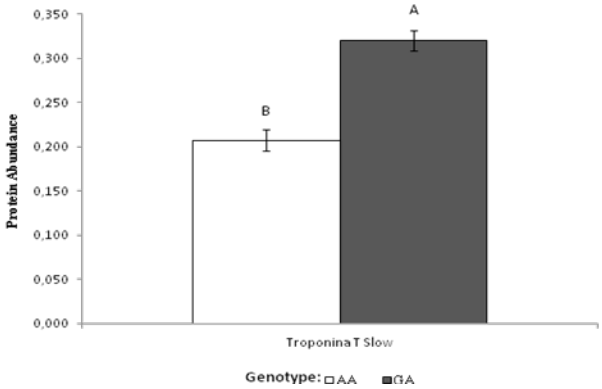


Figure 2. Estimates of the protein abundance significant in the analysis of variance between different genotypes for the polymorphism in glucocorticoid receptor gene.

For the SNP in the MR gene it was observed that two spots identified as Myosin light chain 2 - MLC2 – (spots 14 and 15) presented different profiles of expression in relation to genotype, as well as for sTnT (spots 77 and 87), suggesting the presence of isoforms with differences in molecular weight and isoelectric point. The presence of isoforms can be attributed the occurrence of co- or post-translation modifications, for example phosphorylation, glycosylation and proteolytic cleavage. The protein Cytocrome b-c1 complex subunit 1 was more expressed in the TT genotype (Figure 3).

For MLC2 the phosphorylation is a important regulatory mechanism, which could increase the calcium sensitivity and to determine the variation in strength of muscle fibers [11]. According to Bouley *et al.* [9] the isoform pattern of TnT is complex and a large number of isoforms are generated by alternative splicing of three isogenes characteristics of cardiac, slow and fast skeletal muscle fibers.

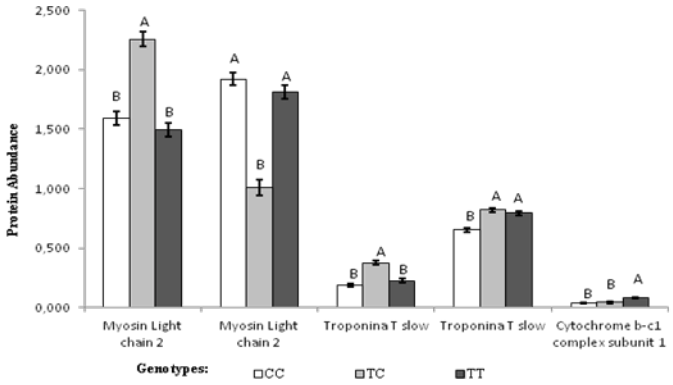


Figure 3. Estimates of the protein abundance significant in the analysis of variance between different genotypes for the polymorphism in mineralocorticoid receptor gene.

Regarding interaction effect between markers was observed that four proteins altered expression due to the genotype combination MRxGR (Table 2).

In the present study it was possible observed that a part of the proteins identified belong to the group of contractile apparatus such myosin and troponin and another with oxidative phosphorylation metabolism and transport of iron ion, demonstrating the effect of these receptors about important proteins in muscle and highly associated in several studies with tenderness.

Consequently allow us to infer the importance of genetic variability in these genes with meat quality traits.

Table 2 Estimates of means and its standard error (SE) of the intensities of the spots significant for main effect of interaction between markers NR3C1_1 g.3293A>G (ss528103353) in GR (glucocorticoid receptor) x NR3C2_1 g.115 T>C (ss528103346) in MR (mineralocorticoid receptor) for beef samples

| *Number of Spot / Protein* | *MR NR3C2_1 g115 T>C* | **GR NR3C1_1 g.3.293A>G** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **AA** | | | **GA** | | |
| | | **Estimate** | **SD** | | **Estimate** | **SD** | |
| **7** - Myosin Light chain 1 | CC | 1,318 | 0,035 | B | 1,532 | 0,035 | A |
| | TC | 1,380 | 0,043 | A | 1,119 | 0,035 | B |
| | TT | 1,189 | 0,035 | B | 1,677 | 0,035 | A |
| **122** - Cytochrome b-c1 complex subunit 1 | CC | 0,144 | 0,006 | B | 0,186 | 0,001 | A |
| | TC | 0,169 | 0,008 | A | 0,177 | 0,006 | A |
| | TT | 0,169 | 0,006 | A | 0,077 | 0,006 | B |
| **185** - Serotransferrin | CC | 0,043 | 0,003 | A | 0,035 | 0,003 | A |
| | TC | 0,044 | 0,003 | A | 0,052 | 0,003 | A |
| | TT | 0,071 | 0,003 | A | 0,030 | 0,003 | B |
| **200** - Myosin light chain 6B | CC | 0,172 | 0,022 | B | 0,411 | 0,022 | A |
| | TC | 0,429 | 0,027 | B | 0,629 | 0,022 | A |
| | TT | 0,372 | 0,022 | A | 0,336 | 0,022 | A |

## IV. CONCLUSION

The SNPs in MR and GR gene altered the expression of some proteins. The most proteins are altered by the SNP present in the MR gene relative to the SNP present in GR gene.

The proteins altered by these markers were found to be involved in the process of muscle contraction and energy metabolism, indicating that these genes are potential candidates for studies of beef quality.

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