Inverse relationships between biomarkers and beef tenderness according to contractile and metabolic properties of the muscle

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Abstract

In previous proteomic analyses, we established a list of proteins biomarkers of beef tenderness. In the present study we quantified the relative abundance of 21 of these proteins by Dot-blot technique in the *Longissimus thoracis* and *Semitendinosus* muscles of 71 young bulls from three breeds: Aberdeen Angus (AA), Limousin (LI) and Blond d'Aquitaine (BA). For both muscles overall tenderness was estimated by sensory analysis, shear force was measured with a Warner-Bratzler instrument, and an index combining sensory and mechanical measurements was calculated. Multiple regressions based on relative abundances of these proteins were used to propose equations of prediction of the three evaluations of tenderness. Hsp70-1B appeared a good biomarker of low tenderness in the three breeds and in the two muscles. Proteins such as Lactate dehydrogenase-B, Myosin heavy chain IIx, small Heat Shock Proteins (Hsp27, Hsp20 and αB-crystallin) were related to tenderness but inversely according to the muscle and breed. The results demonstrate that prediction of tenderness must take into account muscle characteristics and animal type.

Key words

Biomarkers, beef tenderness, skeletal muscle, heat shock proteins, prediction, Dot-blot

INTRODUCTION

Beef quality includes sensory quality traits (tenderness, flavour, juiciness, colour, etc), nutritional value, healthiness and technological quality (which all refer to intrinsic quality traits) as well as issues like animal welfare, environmental concerns, traceability, etc (which refer to extrinsic quality traits). A major cause of consumer dissatisfaction is the high and uncontrolled variability in sensory beef quality, especially tenderness [1]. Muscle characteristics (fiber type, collagen, intramuscular lipids) can only explain up to 30% of the variability in tenderness [2-4]. Another problem is that meat intrinsic quality can only be determined at the time of eating, i.e. after slaughter and cooking, which hampers the production of beef of consistent good quality. In order to better control sensory quality, it is necessary to have tools to predict eating quality, especially tenderness, in live animals. Therefore, the beef sector is looking for biological or molecular indicators to identify live animals with desired quality attributes, to help beef producers to choose the most appropriate production system, animal types and markets. To meet this objective, several genomics programs combining genomics, transcriptomics, proteomics, computational biology and biochemistry have been carried out over the past years (for review: [5-9]). Using 2-D electrophoresis techniques, comparisons of two groups of high *versus* low tenderness allowed the identification of proteins of which abundance was associated with tenderness [6, 8, 10, 11]. These proteins are representative of several biological functions: muscle structure, contraction, energetic metabolism, cellular stress and proteolysis [7, 12]. The objective of the present study was to test the predictive power of the 21 proteins most strongly associated with tenderness in another group of experimental young bulls. To do so, we analysed the relationships between protein abundances and tenderness in young bulls from three beef breeds differing in their precocity and physiological characteristics. The French Blond d'Aquitaine breed is highly muscled with low intramuscular fat content [13], the Angus breed is known to be fat, producing marbled meat, while Limousin French breed has intermediate properties [14]. Two muscles with differences in metabolic characteristics and tenderness: *Longisssimus thoracis* (LT) and *Semitendinosus* (ST) were studied. The abundances of the 21 proteins were quantified by the immunological technique Dot-blot developed by Guillemin *et al.* [15] allowing the simultaneous analysis of large number of samples for one protein. Relationships between tenderness traits and protein relative abundance were evaluated using multiple regression analyses.

MATERIAL AND METHODS

The study was carried out in compliance to the current French welfare recommendations for the use of experimental animals.

Animals and samples

The study was conducted using 71 young entire males of three pure breeds: Aberdeen Angus (AA) (n =21), Limousin (LI) (n =25) and Blond d'Aquitaine (BA) (n =25). Animals (12 month-old at start) were assigned to a 100 day finishing period before slaughter. They were housed in groups of 4 animals of the same breed in 6 x 6 m pens with straw bedding, individually fed and weighed every 2 weeks. Diets consisted of concentrate (75 %) and straw (25 %). Animals were slaughtered at the same age (around 17 months) and final live weight (around 665 kg) in order to avoid weight and age effects on muscle characteristics and beef meat quality.

All bulls were directly transported in a lorry (3 x 2 m) from the experimental farm to the experimental abattoir situated at 1 km from the rearing building, with 2 bulls of the same home pen per transport to avoid social isolation stress. After unloading, they were slaughtered within 3 min in the slaughterhouse of INRA institute (Saint-Genès-Champanelle, France) in compliance with the current ethical guidelines for animal welfare. Bulls were stunned by captive bolt prior to exsanguination.

Muscle samples from *Longissimus thoracis* (LT, mixed fast-oxido-glycolytic muscle 23% of type I fibers, 36 % IIA and 39 % of IIX) and *Semitendinosus* (ST, mixed fast glycolytic 8 % of type I fibers, 24 % of IIA and 64 % of IIX), were excised from the carcass of each animal within 15 minutes after slaughter. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until protein extraction for protein markers quantification. Samples of the two muscles for sensory evaluation and mechanical measurement were cut into steaks 24 hours after slaughter and placed in sealed plastic bags under vacuum and kept between 2–4°C for 14 days for ageing, then frozen and stored at –20°C until analysis.

Meat quality evaluation

LT and ST samples were grilled on a preheated grill at 310°C, resulting in an internal cooked temperature of 55°C. For sensory analysis, a trained sensory panel (12 experienced panellists) evaluated the steak samples of the same muscle. The panel evaluated overall tenderness attribute on a continuous and unstructured scale with scores from 0 to 10 (0 = hard – 10 = tender) [16, 17].

Toughness of cooked meat was further evaluated instrumentally by Warner-Bratzler shear force (WBSF) using INSTRON 5944 as described by Lepetit and Culioli [18]. Force at rupture during shear compression testing was expressed in N/cm².

Sensory and mechanical values of tenderness were used to compute a synthetic tenderness index by combining standardized normal sensory and mechanical tenderness/toughness values[19]. This was calculated for each muscle as the difference within each breed between the standardized values of tenderness score minus the standardized value of the WBSF measure. This index was suggested to take into account the very close genetic correlation underlying the moderate phenotypic correlation observed between both traits [20], suggesting both traits are under the control of common genes.

Dot-Blot analysis

The 21 proteins analysed are described in **Table 1**. The conditions for use and specificity of primary antibodies against these 21 proteins in bovine muscle have been determined previously by Guillemin *et al.* [21] using western blot techniques (**Table 2**).

Total protein extractions were performed according to Bouley *et al.* [22] in a denaturation extraction buffer (8.3M urea, 2M thiourea, 1% DTT, 2% CHAPS). The protein concentration was determined using the Bradford protein assay [23]. Protein extractions were stored at –20°C.

Relative abundances of proteins were evaluated following the Dot-blot technique as described by Guillemin *et al.* [21]. Briefly, protein samples were spotted in quadruplicate on a nitrocellulose membrane with the Minifold I Dot blot from Schleicher & Schuell Biosciences (Germany) and hybridised with the specific antibody of each protein, with conditions described in **Table 2**. Secondary fluorescent-conjugated IRDye 800CW antibodies (anti-mouse, anti-sheep and anti-rabbit) were supplied by LI-COR Biosciences (Lincoln, Nebraska, USA) and used at 1/20000. Subsequently, membranes were scanned by an Odyssey (LI-COR Biosciences, Lincoln, NA, USA) scanner at 800 nm. Protein relative abundance for each sample, given in arbitrary units, was normalised by comparison to a reference sample constituted by mixing all samples from young bulls from this experiment in equal proportions. The technical coefficient of variation of this technique is in average of 9% [21].

Statistical analysis

Analysis of variance was performed using the GLM procedure of SAS for repeated measured (Version 9.1, 2002; SAS Institute Inc.). The effects of breed, muscle-type (LT *vs*

ST) and breed x muscle-type interaction are reported. When significant effects were detected, differences were evaluated by the PDIFF option of SAS.

Principal component analyses were performed using the factor procedure of SAS to study the overall relationships between the studied variables (tenderness traits and protein relative abundances).

Multiple regression analyses were carried out using XLStat 2009 software to explain overall tenderness, WBSF and tenderness index traits for the two muscles separately, presenting the 21 protein biomarkers as potential explanatory variables, as well as breed. The 'optimal model' explaining maximal variability option was used with 'maximal 4 variables'. The percentage of variability in meat quality parameter explained by proteins is based on the adjusted r^2 value of the regression analysis x 100%.

Absence of colinearity was verified for each model. Condition indices and variance proportions were produced using the COLLIN option of SAS, with components identified as collinear if they possessed both a high condition index greater than 10 and a proportion of variation greater than 0.5 for two or more variables.

RESULTS

Tenderness traits

The values of tenderness evaluated by three ways are presented in **Table 3**. For the LT muscle, significant breed effects were found for overall tenderness only, AA bulls being more tender than BA and LI bulls (**Table 3**). In the ST muscle, significant differences between breeds were observed for the WBSF and the tenderness index. A significant muscle x breed interaction was observed for WBSF and tenderness index. Limousin ST muscle being tougher than BA, while AA had intermediate values (**Table 3**). Overall tenderness (**Table 3**) was much higher in the LT. For WBSF, this was only a tendency.

Protein relative abundances

Significant breed effects were found for at least one of the two muscles for most of the proteins except for Hsp70-1B, MyBP-H, and SOD1 (**Table 4**). The two muscles of AA bulls presented higher levels of αB-crystallin, Hsp27, Eno3 and PRDX6 and lower levels of MyHC-IIx. Most proteins showed an effect of muscle or a muscle x breed interaction, apart from MyLC-1F, MyBP-H, SOD1 and µ-calpaïn (**Table 4**).

Relationships between biomarkers and tenderness traits

Descriptive analysis

The principal component analysis (PCA) illustrating the relationships between relative abundances of protein biomarkers and tenderness traits evaluated by i) sensory analysis (overall tenderness), ii) Warner-Bratzler shear force (WBSF) and iii) tenderness index are presented in Figure 1 (a-d). In the two muscles, overall tenderness and tenderness index were positively correlated, and both were negatively correlated with the WBSF

- ***LT muscle***

As illustrated in **Figure. 1a,** high overall tenderness scores were positively associated with small Hsp proteins (Hsp27, Hsp20, αB-crystallin), Eno3, structural protein CapZ-β and antioxidants PRDX6 and DJ-1 when considered across breeds. Low tenderness scores were associated with proteins of the Hsp70 family (Hsp70-8, Hsp70-1A/B and Hsp70/Grp75), or related to fast glycolytic muscle fibres (e.g. LDH-B, MyHC-IIx). The average loadings of the different breeds **(Figure. 1b)** differed on the first axis **(**BA<LI<AA; p=0.0001) and on the second axis, the AA bulls differed from the other breeds (AA>LI, BA; p=0.001). Compared to LI and BA, LT muscles of AA bulls were characterised by higher abundances of small Hsp (Hsp27, Hsp20, αB-crystallin), Hsp40, Eno3, CapZ-β, PRDX6, DJ-1 and lower abundances of LDH-B and MyHC-IIx (cf **Table 4**).

- ***ST muscle***

In the ST muscles, overall tenderness scores were positively associated with LDH-B and MyHC-IIx **(Figure. 1c)** and negatively with small Hsp's (Hsp27, Hsp20, αB-crystallin), Hsp40, Eno3, MyHC-I, CapZ-β, PRDX6, DJ-1. On the first axis **(Figure. 1d)**, AA differed significantly ($p < 0.0001$) from the other breeds (LI, BA < AA) and on the second axis all breeds differed significantly (AA < LI < BA; $p < 0.01$). As in the LT AA were characterised by higher abundances of small Hsp, Hsp40, Eno3, MyHC-I, CapZ-β, PRDX6, DJ-1 and lower abundances of LDH-B and MyHC-IIx.

- ***Comparison of the PCA's of the LT and ST muscles***

The PCA demonstrates inverse relationships between some proteins and tenderness in the two muscles. MyHC-IIx and LDH-B were positively associated with tenderness in ST muscle and negatively in LT. In contrast, proteins from the small Hsp family, Eno3, Hsp40, CapZ-β, PRDX6 and DJ-1 were negatively correlated with tenderness in ST and positively in

LT muscle. In addition, the graphs show that the Angus breed was associated with high tenderness values in LT muscle and with low tenderness in the ST muscle.

Regression analyses

Multiple regression analyses carried out on the pooled data of the three breeds showed that, for the LT and ST muscles, the proteins explained only 10 - 17% of the variability of the three tenderness measurements (**Tables 5-7**). The models for overall tenderness retained (**Table 5**) LDH-B, PRDX6 and Hsp20, Hsp70-1A/B for the ST muscle; Hsp20 and MyHC-IIx, Hsp70-1B for LT muscle. The models for WBSF retained Hsp70-1B for the two muscles and MyHC-IIx for the ST muscle (**Table 6**). The models of the tenderness index retained MyHC-IIx, for the ST and for the LT muscle. The LT tenderness index was further correlated with α-actin and with Hsp70-1A/B (**Table 7**).

When breeds were considered separately, the prediction power (adjusted r^2) improved, although it varied according to breed and tenderness measurement (**Tables 5 to 7**). The models for LI bulls had most predictive power. The model of overall tenderness of the ST muscle of LI bulls retained (**Table 5**) fast MyHC, Hsp70/Grp75 and PRDX6 explaining 53% of the variability between animals. MyLC-1F and Hsp70-1A/B explained 35% of variability in overall tenderness of the LT muscle between LI bulls. The model for WSBF explained even 60% of the variability of the LI breed in both muscles (**Table 6**). Proteins retained in the models were MyHC-IIx and -II for the ST muscle and LDH-B, Hsp70-1A/B and MDH1 for the LT muscle (**Table 6**). The model for the tenderness index for both muscles had high predictive power, explaining 73 and 66% of the variability for the ST and LT, respectively (**Table 7**).

Correlations between predicted and measured values for each tenderness trait among the two studied muscles are shown (**Figure 2, a-f)**.

For the AA bulls, none of the 21 quantified protein biomarkers could predict WBSF of LT muscle (**Table 6**). In the ST muscle, WBSF was predicted by Eno3 and Hsp70-1A/B (positively) and by αB-crystallin (negatively). Overall tenderness and tenderness index were better predicted in the ST than in the LT muscle.

For the BA bulls, prediction powers were similar in the two muscles with better prediction for overall tenderness by Hsp70-1A/B (negatively) in ST and by DJ-1 (positively) and MyBP-H (negatively) in LT muscle (**Table 5**). The predictions of WBSF and tenderness index were lower than in the two other breeds (**Table 6 and 7**).

Overall, results demonstrate that among the 21 quantified proteins, Hsp70-1A/B was often retained in the prediction models of the different breeds, and negatively correlated with the different tenderness measurements in both muscles. Proteins representing fast glycolytic fibre types such as MyHC-IIx or LDH-B were correlated with tenderness in the two muscles for the three tenderness traits but the direction of the correlation depended on the muscle, as also illustrated in the PCA (**Figure 1**): they were positively correlated with tenderness measurements in the ST and negatively in the LT muscle. The glycolytic Eno3 enzyme was correlated with the three tenderness indicators in the ST muscle of the AA bulls. PRDX6 was correlated with ST overall tenderness and tenderness index but never with WBSF.

DISCUSSION

The first objective of this study was to determine in two different muscles of young Aberdeen Angus, Limousin and Blond d'Aquitaine bulls the relationships between tenderness indicators and several protein biomarkers previously identified as good predictors of meat tenderness [6]. The second objective was to propose prediction equations of tenderness based on the abundances of the biomarkers. Among the quantified proteins, Hsp70-1A/B was the only protein associated with the different tenderness indicators in the two muscles and the three breeds. Other proteins including LDH-B, MyHC-IIx, and various small Hsp's were associated with tenderness, but oppositely in the ST compared to the LT muscle. These data suggest that different mechanisms underlie tenderness some which are common between both muscles and while others differ. The underlying mechanisms appear to be related to the contractile and metabolic properties of the muscle and they differ in the sense that the direction of the correlation with tenderness depends on the muscle.

Muscle and breed specific biomarkers of tenderness

Our findings show that MyHC-IIx and LDH-B are positively and negatively correlated with tenderness of the ST muscle (fast glycolytic) and LT muscle (fast oxido-glycolytic, respectively. These opposite correlations are in agreement with earlier studies. For example, Chaze *et al.* [19] showed in young bulls from three main French beef breeds that in the LT muscle several proteins representing fast glycolytic properties were negatively correlated with tenderness and several proteins corresponding to slow oxidative properties were positively correlated with tenderness. Studying the same muscle in young Blond d'Aquitaine, Morzel *et al.* [24] found Succinate dehydrogenase, an oxidative enzyme, to be a good marker of tenderness. In Charolais young bulls, fast proteins such as Troponin T fast isoforms,

phosphoglucomutase, fast MyHC, glycogen phosphorylase were found to be potential biomarkers of toughness and slow MyHC, ATP synthase β, ApoBEC were associated with increased tenderness [12]. Other studies based on fibre types and enzyme activities showed positive relationships between slow oxidative fibre types and tenderness in the LT and a negative relationships in the ST muscle [2]. This was further confirmed in a meta-analysis combining more than 332,000 data on fibre types and enzyme activities of these two muscles in several types of cattle [4]. The present results indicate also that in the case of a fast glycolytic muscle such as ST, muscles containing a greater proportion of fast glycolytic fibres will produce more tender meat and in the case of a more oxidative muscle such as LT, muscles containing a greater proportion of slow oxidative fibres will produce more tender meat. In the present study, LT meat of AA bulls was more tender, and ST meat was less tender compared to the LI and BA breeds. This is coherent with the opposite correlations described above. Irrespectively of breed, for the LT muscle, the more glycolytic it is, the less tender it is, and for the ST muscle, the more glycolytic it is, the more it is tender. The LT of AA was less glycolytic compared to the other breeds, and consequently more tender. The ST of AA was less glycolytic compared to the other breeds, and consequently, less tender. Other factors, such as lipid content, may of course also play a significant role.

The positive correlation between MyHC-IIx and tenderness observed in the LT muscle of the AA bulls may seem to contrast with the above idea. Other authors found similarly, positive relationships between fast glycolytic type and tenderness in LT muscle from animals with muscles with oxidative characteristics. For example, D'Allessandro *et al.* [25] in *Longissimus dorsi* from Chianina beef cattle observed that the tender meat group on the basis of WBSF was characterized by higher levels of glycolytic enzymes.

Overall, existing results demonstrate that the contractile and metabolic properties of muscle play a major role in the elaboration of tenderness.

The present study found other opposite associations between proteins and tenderness according to the muscle or breed. Thus, proteins from the small Hsp family (Hsp20, 27 and αB-crystallin) were inversely associated with tenderness depending on two muscles. These proteins were negatively associated with tenderness in the ST muscle and positively in the LT muscle. These data are in agreement to the results of Guillemin *et al.* [26] which demonstrated that in ST muscle, Hsp from both Hsp70 family and small sHsp family were inversely correlated with tenderness as observed in the present study. Nevertheless, these Hsp's were not correlated with tenderness of the LT muscle. In a previous experiment with Blond

d'Aquitaine young bulls we found inverse relationships between sHsp abundances and tenderness in ST and LT muscles [10]. This could demonstrate that the functions of sHsp depend on the contractile and metabolic properties of the muscle. Guillemin *et al.* [27] showed that slow oxidative fibres have the highest abundance of αB-crystallin. This is in accordance with our data showing higher abundances in LT than in ST muscle and earlier data reported by Guillemin *et al.* [26] showing higher abundances in Charolais young bulls and AA than in LI and BA.

Overall, these results allow understanding why the correlations between one biomarker and tenderness could be inversed as described in the literature. Our results give explanations as described in **Figure 3**. These relationships according to muscle and breed types need to be confirmed in other muscles and animal types.

In the present study, PRDX6, an antioxidant enzyme was associated with tenderness mainly of the ST muscle, but in opposite directions, depending on the breed. This is in accordance with earlier results. Guillemin *et al.* [27] found that PRDX6 is a biomarker of low tenderness of the ST muscle in young Charolais bulls while Jia *et al.* [28] reported an over expression of PRDX6 in tender meat in young Norwegian Red bulls. The antioxidant action of PRDX6 is based on the hydrolysis of hydrogen peroxides and by facilitating repair of damaged cell membranes *via* reduction of peroxidised phospholipids [7,29]. Antioxidant enzymes such as PRDX6 and also SOD1 are involved in the protection of the cell against oxidative stress which is causal of free radicals of oxygen, resulting in formation of protein aggregates [30]. These aggregates may hamper the tenderization process of the meat. In agreement with this finding, D'Alessandro *et al.* [25] proposed that oxidative stress promotes meat tenderness and elicits heat shock protein responses. Ouali and co-workers [7, 31] proposed that the first step of the conversion of muscle into meat is the onset of apoptosis. This death process is energy dependent and involve a large number of proteins included Hsp families. D'Alessandro *et al.* [25] discusses the possible causes of opposite relationships between abundances of oxidative enzymes and tenderness. The authors speculate that *postmortem* metabolism in tender and tough meat is subtly modulated *via* higher levels of specific enzymes and amino acidic residue phosphorylation in a breed-specific fashion. Results of the present study showing that PRDX6 is positively associated with overall tenderness in AA and negatively in LI young bulls are in accordance with this suggestion. Guillemin *et al.* [27] showed that the relationship between antioxidant enzymes such as PRDX6 and also SOD1 was more significant in Charolais steers than in young bulls, suggesting that gender may also

play a role. They showed that SOD1 was correlated with tenderness only in steers, which may explain the absence of an association between tenderness and SOD1 in the present study.

Overall, these data demonstrate that the contractile and metabolic properties of muscle play a major role in the elaboration of tenderness. It is likely that mechanisms underlying the determinism of tenderness involve several biological processes such as apoptosis, oxidative stress, and proteolysis which depend probably on these muscle properties.

Hsp70-1A/B a good candidate biomarker of meat tenderness in the three breeds

Proteins from the Hsp70 family (Hsp70-1A/B, Hsp70-8, GRP 75) were recently found to be related to tenderness [8]. In the present study, among Hsp70's, only Hsp70-1A/B (gene HSPA1B) also called Hsp70-2 depending on species, was negatively associated to overall tenderness and tenderness index and positively with WBSF in the two muscles across the three breeds.

The members of the Hsp70 family serve a variety of roles: i) they act as molecular chaperones facilitating the assembly of multi-protein complexes, ii) they participate in the translocation of polypeptides across cell membranes and to the nucleus, and iii) they help in the proper folding of nascent polypeptide chains [32]. In the *Diaphragma* muscle from Holstein-Friesian cattle, Sugimoto *et al.* [33] showed that misfolding of energy-related proteins due to Hsp70 deficiency might lead to protein aggregation and muscle fiber degeneration. This is in coherence with the negative association with tenderness observed in the present study. Moreover, the study of Crawford and Horowits [34] showed that in particular scaffolding proteins and chaperone proteins such as Hsp90 and 70 are required for individual steps in the assembly of myofibril. The study of Miyabara *et al.* [35] showed that Hsp70 improves structural and functional recovery of skeletal muscle after disuse atrophy. This is in agreement with several studies showing that Hsp70 is one of the most important heat shock protein for maintenance of cell integrity during normal cellular growth as well as under pathophysiological conditions [36,37]. These data suggest that Hsp70 is important for maintaining structural, ultrastuctural and functional properties of skeletal muscle. Possibly, Hsp70-1A/B plays also an important role in structural modifications during *post-mortem* ageing. Hsp70-1A/B has further an anti-apoptotic role in skeletal muscle. Gao *et al.* [36] reported a function of Hsp70-1A/B in regulating TNF-α-induced cell apoptosis. By forming a complex Hsp70/CHIP/ASK1, Hsp70 promotes ASK1 proteasomal degradation and prevents TNF-α-induced cell apoptosis. Hsp70 proteins are also known to sequester pro-apoptotic

353 factors such as BCL-2 [38]. These data are in agreement with the important role of apoptosis in
354 meat ageing [7, 39].

355 The present study found that in contrast to Hsp70-1A/B, the association between the
356 relative abundance of the other Hsp70's and tenderness traits differed according to breed and
357 muscle. This may be explained by different regulation of the expression of these Hsp70's in
358 the muscle. In the present study, Hsp70/Grp75 and Hsp70-8 were less abundant in AA than in
359 BA, particularly in the LT muscle. However, Hsp70-1A/B abundance did not differ between
360 breeds, in the two muscles. These data are in agreement with the results of Guillemin *et al.* [27]
361 who showed no effect of gender on Hsp70-1A/B abundance between steers and young bulls.
362 This would explain the association between Hsp70-1A/B and tenderness across muscles and
363 breeds observed in the present study. The lack of consistent associations between tenderness
364 and Hsp70/Grp75, may be explained by the fact that it is exclusively expressed in the
365 mitochondrial matrix, and that it is involved in the translocation and folding of nascent
366 polypeptide chains of both nuclear and mitochondrial origin [40].

367 These findings suggest that Hsp70-1A/B could be a relatively general biomarker of
368 tenderness in different muscles and breeds compared to proteins discussed earlier which
369 appear to be quite strongly muscle or breed specific.

370 In conclusion, this study shows that some biomarkers of tenderness such as MyHC IIx,
371 LDH-B and small Hsp are dependent of the contractile and metabolic properties of the muscle
372 (cf **Figure 3**), explaining their opposite relationships with tenderness in the ST compared to
373 the LT muscle and in AA compared to LI and BA bulls. It further shows that Hsp70-1A/B is a
374 biomarker of low beef tenderness across the breeds and muscles studied. All these biomarkers
375 need further testing in various muscles from cattle of different genders and breeds before they
376 can be used as biomarkers in routine immunological testing.

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Figure captions

Figure 1. Principal component analysis. **a)** and **c)** Distribution of protein abundance and meat tenderness traits (overall tenderness, WBSF and tenderness index) for the LT and ST muscles respectively; **b)** and **d)** Distribution of the three breeds on the first two principal axes (mean ± standard errors) for LT and ST muscles respectively

Figure 2. Correlations between predicted and measured beef tenderness traits (for tenderness, WBSF and tenderness index) using best models for the three breeds among LT (a, b and c) and ST muscles (d, e, f). Adjusted R-squares corresponding to the prediction equations shown in Tables (5 – 7) for each breed are given. Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin.

Figure 3. Schematic illustration of the relationships between muscle metabolic and contractile properties and tenderness depending on the muscle.
Left: For the *Longissimus thoracis* (fast oxido-glycolytic muscle), higher degrees of fast glycolytic properties are associated with lower tenderness. Right: For the *Semitendinous* (fast glycolytic muscle) higher degrees of fast glycolytic properties are associated with higher tenderness.
Consequently biomarkers associated with contractile and metabolic properties of the muscles such as MyHC IIx and LDH-B, are positively or negatively associated with tenderness of the ST and LT muscles, respectively. The opposite is found for other biomarkers such as small Hsp's (Hsp20, Hsp27, αB-crystallin).
ST and LT muscles from Angus breed (AA) have lower levels of fast glycolytic properties than those of Limousin (LI) and Blond d'Aquitaine (BA) breeds. Therefore, their LT and ST muscles are more and less tender, respectively, than those of the LI and BA breeds.

Table 1. List of the 21 protein biomarkers of beef tenderness investigated in this study[1].

Protein name	Gene	UniProt ID
Heat Shock Proteins		
αB-Crystallin	CRYAB	**P02511**
Hsp20	HSPB6	**O14558**
Hsp27	HSPB1	**P04792**
Hsp40	DNAJA1	**P31689**
Hsp70-1A/B	HSPA1B	**P08107**
Hsp70-8	HSPA8	**P11142**
Hsp70-Grp75	HSPA9	**P38646**
Metabolism		
Eno3 (Enolase 3)	ENO3	**P13929**
LDH-B (Lactate Dehydrogenase Chain B)	LDHB	**P07195**
MDH1 (Malate Dehydrogenase 1)	MDH1	**P40925**
Structure		
CapZ-β (F-actin-capping protein subunit β)	CAPZB	**P47756**
α-actin	ACTA1	**P68133**
MyLC-1F (Myosin Light Chain 1F)	MYL1	**P05976**
MyBP-H (Myosin Binding Protein H)	MYBPH	**Q13203**
MyHC-I (Myosin Heavy Chain-I)	MYH7	**P12883**
MyHC-II (MyHC IIa+IIx+IIb))	MYH2	**Q9UKX2**
MyHC-IIx (Myosin Heavy Chain-IIx)	MYH1	**P12882**
Oxidative resistance		
DJ-1 (Parkinson disease protein 7)	PARK7	**Q99497**
PRDX6 (Cis-Peroxiredoxin)	PRDX6	**P30041**
SOD1 (Superoxide Dismutase Cu/Zn)	SOD1	**P00441**
Proteolysis		
μ-calpain	CAPN1	**P07384**

[1] List of protein biomarkers of beef tenderness established by our group in previous proteomic studies (For review: Guillemin *et al.*, 2011; Picard *et al*,. 2010; Picard *et al*,. 2012a,b; 2013)

Table 2. Suppliers and conditions for each primary antibody used in this study.

Target protein	Antibody references	Dilution[1]
Heat Shock Proteins		
αB-crystallin	Monoclonal anti-bovine Assay Designs SPA-222	1/500
Hsp20	Monoclonal anti-human Santa Cruz HSP20-11:SC51955	1/200
Hsp27	Monoclonal anti-human Santa Cruz HSP27 (F-4):SC13132	1/3000
Hsp40	Monoclonal anti-human Santa Cruz HSP40-4 (SPM251):SC-56400	1/250
Hsp70-1A/B	Monoclonal anti-human Abnova HSPA1B (M02), clone 3B7	1/2000
Hsp70-8	Monoclonal anti-bovine Santa Cruz HSC70 (BRM22):SC-59572	1/250

Hsp70-Grp75	Monoclonal anti-human RD Systems Clone 419612	1/250
Metabolism		
Eno3	Monoclonal anti-human Abnova Eno3 (M01), clone 5D1	1/45000
LDH-B	Monoclonal anti-human Novus LDHB NB110-57160	1/50000
MDH1	Monoclonal anti-pig Rockland 100-601-145	1/1000
Structure		
CapZ-β	Monoclonal anti-human Abnova CAPZB (M03), clone 4H8	1/250
α-actin	Monoclonal anti-Rabbit Santa Cruz α-actin (5C5):SC-58670	1/1000
MyLC-1F	Polyclonal anti-human Abnova MYL1 (A01)	1/1000
MyBP-H	Monoclonal anti-human Abnova MYBPH (M01), clone 1F11	1/4000
MyHC-I	Monoclonal anti-bovine Biocytex 5B9	1/2000
MyHC-II[2]	Monoclonal anti-bovine Biocytex 15F4	1/4000
MyHC-IIx	Monoclonal anti-bovineBiocytex 8F4	1/500
Oxidative resistance		
DJ-1	Polyclonal anti-human Santa Cruz DJ-1 (FL-189):SC-32874	1/250
PRDX6	Monoclonal anti-human Abnova PRDX6 (M01), clone 3A10-2A11	1/500
SOD1	Polyclonal anti-rat Acris SOD1 APO3021PU-N	1/1000
Proteolysis		
μ- calpain	Monoclonal anti-bovine Alexis μ-calpain 9A4H8D3	1/1000

[1] Dilution of each antibody was defined according to Guillemin *et al.* (2011).

[2] MyHC II corresponds to fast MyHC: IIa+IIx+IIb isoforms

Table 3. Effect of breed and muscle on beef tenderness trait evaluated using three analyses methods.

		Breed (B) [1]				Muscle (M) [3]		Significance [4]		
Variables	Muscle	AA *21*	BA *25*	LI *25*	SEM[2]	LT 70	ST 68	**B**	**M**	**B x M**
Overall tenderness	LT[5]	5.27[a]	4.85[b]	4.75[b]	0.08	4.94[a]	4.58[b]	*	**	ns
	ST	4.58	4.66	4.49	0.05			ns		
WBSF[6]	LT	40.62	44.24	41.69	1.27	42.27	44.90	ns	t	*
	ST	45.91[a,b]	41.35[b]	47.80[a]	1.01			*		
Tenderness index	LT	0.67	-0.14	-0.26	0.20	0.04	-0.03	ns	ns	*
	ST	-0.14[a,b]	0.58[a]	-0.56[b]	0.18			*		

[1] Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin.
[2] standard error of mean
[3] Muscle designation: LT: *Longissimus thoracis,* ST: *Semitendinosus*
[4] t<0.1, * <0.05, ** < 0.001
[5] Least square means in the same row for breed and muscle effects not followed by a common letter (a-c) differ significantly: $P < 0.05$.
[6] Warner-Bratzler Shear Force test.

Table 4. Breed, muscle and breed x muscle interaction effects on the 21 protein biomarkers of beef tenderness.

		Breed (B)[1]				Muscle (M)[3]		Significance[4]		
Variables	Muscle	AA 21	BA 25	LI 25	SEM[2]	LT 71	ST 69	**B**	**M**	**B x M**
αB-crystallin[5]	LT	26.5 a	16.1 b	18.4 b	0.84	20.0 a	8.9 b	***	***	*
	ST	12.5a	7.0b	7.9b	0.45			***		
Hsp20	LT	20.2	17.5	17.9	0.51	18.5 a	13.2 b	t	***	ns
	ST	13.6	12.9	13.3	0.35			ns		
Hsp27	LT	28.7 a	19.0 b	21.4 b	1.08	22.7 a	16.0 b	**	***	ns
	ST	21.0a	13.7b	14.3b	0.60			***		
Hsp40	LT	18.1 a	17.1[a,b]	16.1 b	0.26	17.0 a	13.0 b	**	***	ns
	ST	13.8	12.8	12.6	0.22			*		
Hsp70-1A/B	LT	17.6	17.8	19.3	0.46	18.3 a	12.5 b	ns	***	ns
	ST	12.0	12.6	12.8	0.29			ns		
Hsp70-8	LT	15.8 b	17.5 a	16.6[a,b]	0.22	16.7 a	15.6 b	*	**	ns
	ST	15.0	16.0	15.8	0.19			t		
Hsp70/Grp75	LT	9.1 c	16.6 a	12.6 b	0.44	13.0	12.6	***	ns	***
	ST	13.3	12.6	12.0	0.24			ns		
Eno3	LT	17.5 a	14.9[a,b]	13.4 b	0.56	15.1 a	13.4 b	*	*	ns
	ST	15.5a	13.3b	12.9b	0.35			**		
LDH-B	LT	10.2 c	19.1 a	14.2 b	0.59	14.7	13.4	***	t	**
	ST	11.2c	15.0a	13.5b	0.33			***		
MDH1	LT	12.3 b	15.4 a	14.1[a,b]	0.45	14.1	14.9	*	ns	*
	ST	15.4	15.1	14.2	0.37			ns		
CapZ-β	LT	19.3 a	15.9 b	15.4 b	0.42	16.7 a	14.0 b	**	***	*
	ST	14.9	13.4	13.8	0.28			ns		
α-actin	LT	16.9 b	19.5 a	16.3 b	0.40	17.6	17.6	**	ns	**
	ST	17.5	17.4	17.9	0.31			ns		
MyLC-1F	LT	14.9[a,b]	15.5[a]	14.3[b]	0.20	14.9	15.2	*	ns	ns
	ST	15.0	15.7	15.0	0.18			ns		
MyBP-H	LT	14.9	13.3	13.7	0.84	14.0	12.7	ns	ns	ns
	ST	13.8	12.4	12.0	0.36			ns		
MyHC-I	LT	17.7	16.9	18.4	0.33	17.7 a	12.4 b	ns	***	**
	ST	14.3[a]	10.2[c]	12.4[b]	0.35			***		
MyHC-II	LT	13.7[c]	16.5[a]	15.0[b]	0.30	15.1 b	16.8 a	**	***	*
	ST	16.3	16.9	17.2	0.22			ns		
MyHC-IIx	LT	4.2[c]	23.3[a]	14.3[b]	1.08	14.5[b]	24.34[a]	***	***	**
	ST	18.4[c]	28.6[a]	24.8[b]	0.74			***		
DJ-1	LT	17.1	16.0	16.6	0.33	16.5 a	13.5 b	ns	***	ns
	ST	14.6a	13.1b	13.0b	0.26			*		
PRDX6	LT	15.5 a	13.4 b	13.3 b	0.22	14.0 b	16.1 a	***	***	ns
	ST	17.7a	15.6b	15.2b	0.23			***		
SOD1	LT	17.6	15.8	15.6	0.42	16.3	16.7	ns	ns	ns
	ST	16.1	10.2	12.4	1.22			ns		
μ-calpaïn	LT	14.2 b	15.9 a	14.0 b	0.28	14.7	14.4	**	ns	ns
	ST	13.6b	15.2a	14.1a,b	0.26			*		

[1] Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin.
[2] standard error of mean
[3] Muscle designation: LT: *Longissimus thoracis*, ST: *Semitendinosus*
[4] t<0.1, * <0.05, ** < 0.001, ***<0.0001
[5] Least square means in the same row for breed and muscle effects not followed by a common letter (a-c) differ significantly: $P < 0.05$.

Table 5. Equations of best models (parameters including the direction and level of significance) to predict overall tenderness across breeds and for each breed

Breeds[1]	Parameter 1	*P*- value[2]	Parameter 2	*P*- value	Parameter 3	*P*- value	Parameter 4	*P*- value	Predictive power[3]	*P*- value model
					Semitendinosus muscle					
AA	+ PRDX6	**	– Eno3	*	– α-actin	≠	-	-	43	*
BA	– Hsp70-1A/B	*	+ Replicate	*	-	-	-	-	39	*
LI	+MyHC-II	***	– PRDX6	**	+ Hsp70/Grp75	‡	-	-	53	**
All breeds	– Hsp20	*	+ LDH-B	≠	+ PRDX6	≠	– Hsp70-1A/B	*	14	*
					Longissimus thoracis muscle					
AA	– MyLC-1F	**	+ MyHC-IIx	≠	+ Replicate	‡	-	-	35	≠
BA	+ DJ-1	***	– MyBP-H	≠	+ Replicate	*	-	-	40	*
LI	– Hsp70-1A/B	*	+ MyLC-F1	*	-	-	-	-	35	*
All breeds	– MyHC-IIx	≠	+ Hsp20	≠	– Hsp70-1A/B	≠	-	-	17	*

[1] Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin
[2] Significance of differences: ‡: P=0.06; ≠: P<0.05; *: P<0.01; **: P<0.001; ***: P<0.0001
[3] (%) of variability between animals explained by the model.

Table 6. Equations of best models (parameters including the direction and level of significance) to predict WBSF across breeds and for each breed

Breeds[1]	Parameter 1	*P*- value[2]	Parameter 2	*P*- value	Parameter 3	*P*- value	Predictive power[3]	*P*- value model
			Semitendinosus muscle					
AA	– αB-Crystallin	≠	+ Eno3	*	+ Hsp70-1A/B	≠	36	≠
BA	– MyHC-IIx	±	+ Eno3	‡	– α-actin	≠	22	≠
LI	– MyHC-IIx	*	– MyHC-II	≠	– Replicate	***	60	***
All breeds	– MyHC-IIx	*	+ Hsp70-1A/B	≠	-	-	15	*
			Longissimus thoracis muscle					
AA	-	-	-	-	-	-	-	-
BA	– α-actin	≠	-	-	-	-	21	≠
LI	+ LDH-B	**	– MDH1	**	+ Hsp70-1A/B	**	60	***
All breeds	+ Hsp70-1A/B	*	+ Replicate	≠	-	-	10	*

[1] Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin
[2] Significance of differences: ‡: P=0.1; ≠: P<0.05; *: P<0.01; **: P<0.001; ***: P<0.0001
[3] (%) of variability between animals explained by the model.

Table 7. Equations of best models (parameters including the direction and level of significance) to predict tenderness index across breeds and for each breed

Breeds[1]	Parameter 1	*P*- value[2]	Parameter 2	*P*- value	Parameter 3	*P*- value	Parameter 4	*P*- value	Predictive power[3]	*P*- value model
					Semitendinosus muscle					
AA	+ αB-Crystallin	≠	+ PRDX6	≠	– Eno3	*	-	-	47	*
BA	– Hsp70-1A/B	≠	+ Replicate	≠	-	-	-	-	24	*
LI	– αB-Crystallin	*	+ Hsp40	*	+ MyHC-II	**	– PRDX6	**	73	***
All breeds	+ MyHC-IIx	≠	+ Replicate	*	-	-	-	-	12	*
					Longissimus thoracis muscle					
AA	– Eno3	≠	+ Hsp27	*	– CapZ-β	+	-	-	29	*
BA	– CapZ-β	≠	+ Hsp40	‡	+ α-actin	*	– Hsp70-8	*	28	≠
LI	– LDH-B	***	+ MDH1	**	+ CapZ-β	*	– Hsp70-1A/B	***	66	***
All breeds	+ α-actin	≠	– Hsp70-1A/B	*	– MyHC-IIx	*	-	-	17	**

[1] Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin
[2] Significance of differences: ‡: P=0.1; ≠: P<0.05; *: P<0.01; **: P<0.001; ***: P<0.0001
[3] (%) of variability between animals explained by the model.

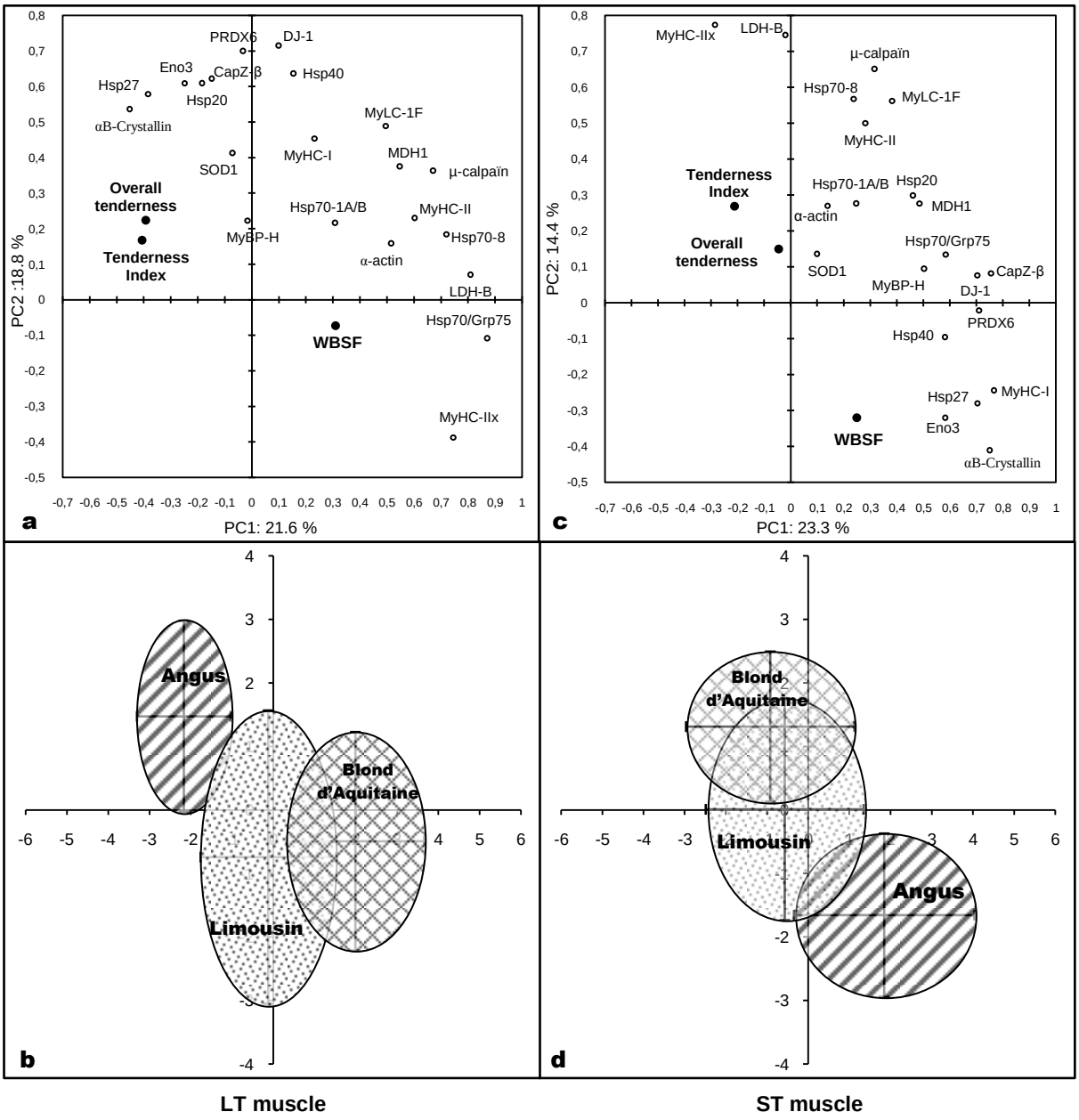

a
PC2 :18.8 %
PC1: 21.6 %
PRDX6
DJ-1
Eno3
CapZ-β
Hsp40
Hsp27
Hsp20
αB-Crystallin
MyLC-1F
MyHC-I
MDH1
SOD1
μ-calpaïn
Overall tenderness
Hsp70-1A/B
MyHC-II
MyBP-H
Hsp70-8
Tenderness Index
α-actin
LDH-B
Hsp70/Grp75
WBSF
MyHC-IIx
c
PC2: 14.4 %
PC1: 23.3 %
MyHC-IIx
LDH-B
μ-calpaïn
Hsp70-8
MyLC-1F
MyHC-II
Tenderness Index
Hsp70-1A/B
Hsp20
MDH1
α-actin
Overall tenderness
Hsp70/Grp75
SOD1
CapZ-β
MyBP-H
DJ-1
PRDX6
Hsp40
Hsp27
MyHC-I
Eno3
WBSF
αB-Crystallin
b
Angus
Blond d'Aquitaine
Limousin
d
Blond d'Aquitaine
Limousin
Angus
LT muscle
ST muscle

Figure 1.

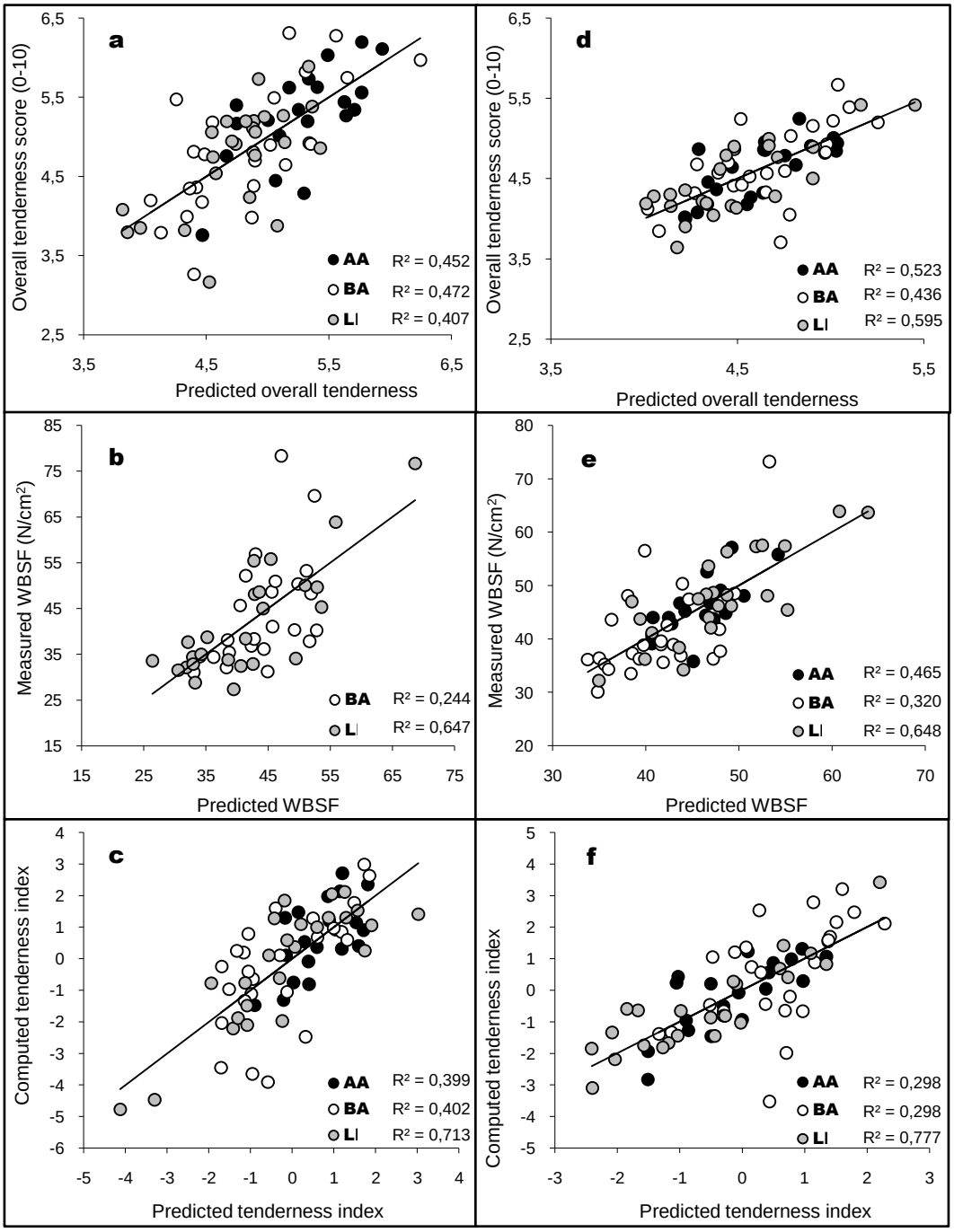

a
Overall tenderness score (0-10)
Predicted overall tenderness
AA R² = 0,452
BA R² = 0,472
LI R² = 0,407
d
Overall tenderness score (0-10)
Predicted overall tenderness
AA R² = 0,523
BA R² = 0,436
LI R² = 0,595
b
Measured WBSF (N/cm²)
Predicted WBSF
BA R² = 0,244
LI R² = 0,647
e
Measured WBSF (N/cm²)
Predicted WBSF
AA R² = 0,465
BA R² = 0,320
LI R² = 0,648
c
Computed tenderness index
Predicted tenderness index
AA R² = 0,399
BA R² = 0,402
LI R² = 0,713
f
Computed tenderness index
Predicted tenderness index
AA R² = 0,298
BA R² = 0,298
LI R² = 0,777
LT muscle
ST muscle

Figure 2.

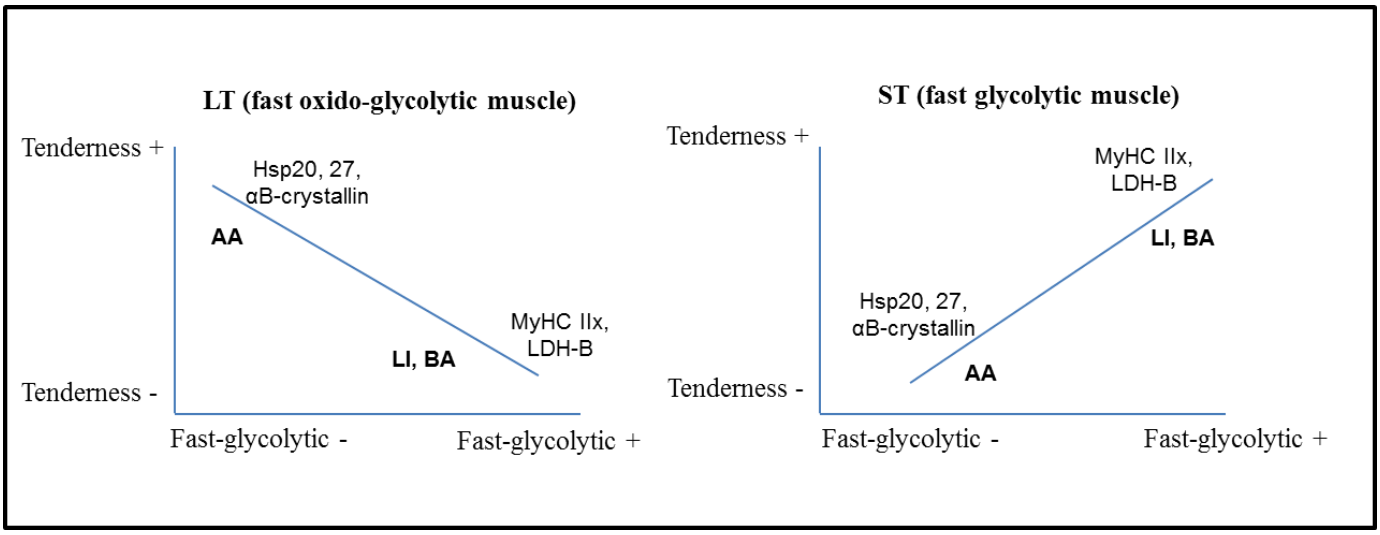
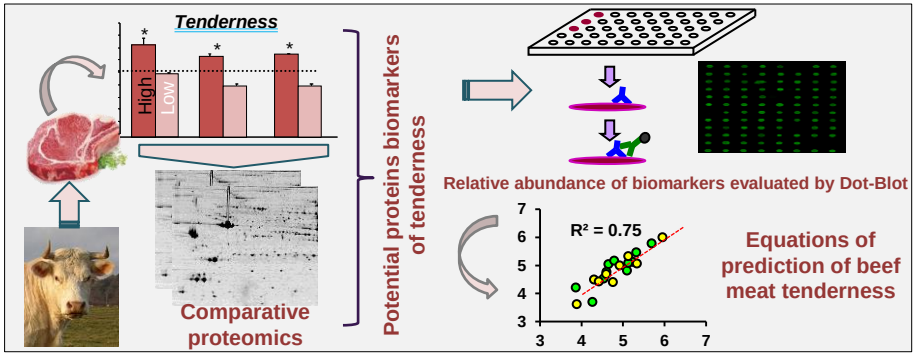

LT (fast oxido-glycolytic muscle)
Tenderness +
Hsp20, 27,
αB-crystallin
AA
MyHC IIx,
LDH-B
LI, BA
Tenderness -
Fast-glycolytic -
Fast-glycolytic +
ST (fast glycolytic muscle)
Tenderness +
MyHC IIx,
LDH-B
LI, BA
Hsp20, 27,
αB-crystallin
AA
Tenderness -
Fast-glycolytic -
Fast-glycolytic +

Figure 3.


Tenderness
High
Low
Comparative proteomics
Potential proteins biomarkers of tenderness
Relative abundance of biomarkers evaluated by Dot-Blot
R² = 0.75
Equations of prediction of beef meat tenderness