

Quantification of biomarkers for beef meat qualities using a combination of Parallel Reaction Monitoringand antibody-based proteomics

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- **1** Quantification of biomarkers for beef meat qualities using a combination of parallel
- 2 reaction monitoring- and antibody-based proteomics
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26

27 ABSTRACT

We and others have identified biomarker candidates of tenderness or marbling, two major 28 attributes of bovine meat-eating qualities for consumers' satisfaction. In this study, reverse 29 phase protein arrays (RPPA) and targeted mass spectrometry assays using parallel reaction 30 monitoring (PRM) were developed to test whether 10 proteins pass the sequential 31 qualification and verification steps of the challenging biomarker discovery pipeline. At least 32 MYH1, TPI1, ALDH1A1 and CRYAB were qualified by RPPA or PRM as being 33 differentially abundant according to marbling values of longissimus thoracis and 34 semimembranosus muscles. Significant mathematical relationships between the individual 35 abundance of each of the four proteins and marbling values were verified by linear or logistic 36 37 regressions. Four proteins, TNNT1, MDH1, PRDX6 and ENO3 were qualified and verified for tenderness, and the abundance of MDH1 explained 49% of the tenderness variability. The 38 39 present PRM and RPPA results pave the way for development of useful meat industrial 40 multiplex-proteins assays.

Keywords: Proteins, Tenderness, Marbling, Reverse Phase Protein Array, Quantitative Mass
 Spectrometry, Targeted Proteomics

45

46 **1. Introduction**

The intramuscular adipose tissue, also called marbling, and tenderness are major 47 attributes that determine the meat-eating qualities of beef and contribute to the economic 48 value of carcasses and meat. For example, the grading of carcasses in North America is 49 50 influenced by the amount of marbling within certain limits, the more marbling the higher the grade. Tenderness is a top priority quality attribute that most eating quality systems focus on, 51 because it is a primary consideration in consumer satisfaction. The control of both tenderness 52 and marbling is thus a major concern for the beef sector, with the challenge of producing 53 tender and flavoursome beef. The gold standards for tenderness evaluation are the Warner 54 55 Bratzler Shear Force or the sensory panel evaluation, and marbling is assayed by the chemical analysis of intramuscular lipid content. Because these gold standards have significant 56 drawbacks, such as the time of analysis and the important amount of meat required for 57 analyses, investigations of higher throughput instrumental methods have been carried out to 58 manage and predict beef-eating quality. Several technologies such as tomography, ultrasound 59 and visible-near infrared spectroscopy have been developed to evaluate beef tenderness and 60 marbling, mainly on carcasses or muscle/meat as recently reviewed (Farmer & Farrell, 2018). 61 Few quantitative proteomics methods (Wu, Dai, & Bendixen, 2019) begins to exploit the 62 63 hundreds of markers of tenderness (Picard, Gagaoua, & Hollung, 2017) and marbling (Baik et al., 2017; Ceciliani, Lecchi, Bazile, & Bonnet, 2018) deciphered by high throughput 64 transcriptomic and proteomic assays in a perspective to develop methods usable on dead and 65 66 alive animals. Yet, molecular markers, as well as pipelines for biomarkers discovery and validation, were developed (Drabovich, Martinez-Morillo, & Diamandis, 2015; Rifai, Gillette, 67 & Carr, 2006) and used in Human medicine to improve diagnosis or guide molecularly 68

targeted therapies. We hypothesize that biomarkers of tenderness or marbling could be used to 69 70 manage and predict these beef-eating qualities. To date, proteomics assays comparing meat samples differing exclusively with respect to marbling or tenderness have revealed hundreds 71 72 of candidate protein biomarkers of tenderness (Picard, Gagaoua, & Hollung, 2017) and marbling (Baik et al., 2017; Ceciliani et al., 2018; Thornton, Chapalamadugu, Eldredge, & 73 Murdoch, 2017). However, they were not pursued to the next phases of "qualification" and 74 "verification" of the biomarker discovery pipeline. "Qualification" requires demonstration 75 that the differential abundance of a candidate biomarker observed in discovery is seen using 76 alternative, targeted methods. "Verification" is the extension to a larger number of samples 77 78 that closely represent the population in which a final test would be deployed (Rifai et al., 2006). A requisite to conduct these phases of "qualification" and "verification" is the 79 development of quantitative methods for candidate biomarkers of marbling and tenderness, 80 81 and the selection of biological materials/samples. Among the quantitative methods that are investigated here (Meyer & Schilling, 2017; Solier & Langen, 2014; Vidova & Spacil, 2017), 82 Reverse Phase Protein Array (RPPA) was presented by (Akbani et al., 2014) as a 83 "miniaturized antigen-down or dot-blot immunoassay suitable for quantifying the relative, 84 semi-quantitative or quantitative abundance of protein". Because of its multiplex capability, 85 RPPA allows the identification of tens of candidate biomarkers on a few hundreds of samples. 86 Another powerful antibody-free approach for the absolute quantification of proteins is 87 targeted proteomics coupled to isotope dilution. Among the panel of targeted proteomics 88 methods, parallel reaction monitoring (PRM) (Gallien et al., 2012; Peterson, Russell, Bailey, 89 Westphall, & Coon, 2012), performed on high-resolution/accurate-mass instruments, offers 90 high sensitivity and specificity, while covering a high dynamic range (Borràs & Sabidó, 2017; 91 Bourmaud, Gallien, & Domon, 2016; Shi et al., 2016). 92

The objective of the present study is thus to test the hypothesis that analytical
performances of PRM and RPPA would be suitable for "qualification" and "verification"
phases to prioritize candidate biomarkers. Therefore, we focused on ten muscular proteins
previously identified as candidate biomarkers of meat marbling or tenderness, and further
demonstrated the complementarity of PRM and RPPA assays.

98

99 2. Materials and Methods

All chemicals described in this section were purchased from Sigma-Aldrich (St QuentinFallavier, France) when the company is not specified.

102 2.1. Description of animals and muscles

103 In order to produce the biological material required for the "qualification" and 104 "evaluation" phases of the biomarker discovery pipeline, 43 muscles from 35 Rouge des Prés cows (n=30, mean slaughter age 63 months) and steers (n=5, mean slaughter age 33 105 106 months) were used as a subset of previously described samples (Picard, Gagaoua, Al Jammas, & Bonnet, 2019). Rouge des Prés breed was chosen as a meat and dairy breed, and cows are 107 108 reared for meat production and consumption (60% of consumed bovine meat) in France. All animal trials described herein were conducted according to relevant international guidelines 109 (European Union procedures on animal experimentation – Directive 2010/63/EU) for the use 110 111 of production animals in animal experimentation. The 43 samples consisted of 23 samples of semimembranosus (SM) and of 20 samples of longissimus thoracis (LT) muscles chosen for 112 their good representativeness of the biological variation of marbling and tenderness. Indeed, 113 114 the values of marbling, described by the IMF (Intra Muscular Fat) content, assayed according to the Soxhlet method described in (Couvreur, Le Bec, Micol, & Picard, 2019), ranged from 115 116 0.45 to 8.38% of fresh muscle with means of 4.04 and 4.39% for SM and LT muscles. The

117	value of Warner-Bratzler shear force assayed as described in (Couvreur, Le Bec, Micol, &
118	Picard, 2019) ranged from 23.63 to 76.91 N/cm ² with means of 47.73 and 31.40 N/cm ² for
119	SM and LT muscles (Supplementary Table 1).

120 2.2. Protein extraction

Proteins were extracted from frozen muscle samples by homogenizing the samples in the 121 "Precellys 24" tissue homogenizer (Bertin technologies, Saint Quentin-en-Yvelines, France). 122 Briefly, around 80 mg of frozen muscle for each animal was grinded using 1.4 mm ceramic 123 beads in a buffer containing 50 mM Tris (pH 6.8), 2% SDS, 5% glycerol, 2 mM DTT, 2.5 124 125 mM EDTA, 2.5 mM EGTA, 1x HALT Phosphatase inhibitor (Perbio Science, Villebon-sur-Yvette, France), Protease inhibitor cocktail complete MINI EDTA-free (Roche, Meylan 126 Cedex France, 1 tablet/10 mL), 2 mM Na3VO4 and 10 mM NaF. The extracts were then 127 boiled for 10 min at 100 °C, sonicated to reduce viscosity and centrifuged 10 min at 15000 128 rpm. The supernatants were collected and stored at -80 °C until further use. Protein 129 130 concentrations were determined with a commercial protein assay (Pierce BCA reducing agent 131 compatible kit, Thermo Scientific, Waltham, Massachusetts, United State) with BSA as standard. The same protein extract was used for PRM and RPPA assay. 132

133 2.3. Quantification of proteins using PRM mass spectrometry

134 2.3.1 Sample preparation

135 After protein concentration determination (RC-DCTM, Bio-Rad, Hercules, California,

136 United State), 30 µg of proteins were prepared in tube-gel, as described in Muller, Fornecker,

137 Van Dorsselaer, Cianférani, & Carapito (2016) with minor adaptations. Briefly, 42.25% H₂O,

138 25% acrylamide/bis-acrylamide, 0.25% TEMED were added to each sample for a final

volume of 100 μ L. Tubes were vortexed and centrifuged. Then 2.5% of 10% APS was added,

and tubes were rapidly vortexed and centrifuged before polymerization. Proteins were fixed in

50% ethanol (Carlo Erba Reagents, Val-de-Reuil, France) and 3% phosphoric acid, before
cutting in small pieces. Gel pieces were then washed three times in 75% ACN (LC-MS grade,
Fisher Chemical, Illkirch, France), 25% 25 mM NH₄HCO₃, and dehydrated twice in ACN,
before reduction and alkylation.

Eleven randomly chosen samples were mixed to be used as a representative matrix for
method development and external quality control during the analyses, and prepared as
described above.

A concentration-balanced mixture of 20 accurately quantified stable isotope-labelled
peptides (Spike Tides[™] TL, JPT Peptide Technologies, Berlin, Germany) corresponding to
proteotypic peptides of the 10 biomarker candidates was spiked into the samples before
overnight digestion at 37 °C using a solution of modified porcine trypsin (Promega, Madison,
Wiscosin, United State) at a 1:100 (w/w) enzyme-protein ratio.

Peptides were extracted twice for 1 h under agitation with 60% ACN, 0.1% formic acid
(FA) (Optima LC/MS grade, Fisher Chemical, Illkirch, France), followed by 100% ACN.
After vacuum drying, peptides were resolubilised in 2% ACN, 0.1% FA to obtain a final
concentration of 2 µg/µL.

157 2.3.2. NanoLC-PRM analyses

158 NanoLC-PRM analyses were performed on a NanoAcquity UPLC device (Waters,

159 Milford, Massachusetts, United State) coupled to a Q-Exactive Plus mass spectrometer

160 (Thermo Fisher Scientific, Bremen, Germany). Solvent system consisted of 0.1% FA in H₂O

- 161 (solvent A) and 0.1% FA in ACN (solvent B) (Optima LC/MS grade solvents, Fisher
- 162 Chemical, Illkirch, France). Peptides (500ng) were separated onto a Symmetry C18
- 163 precolumn ($20 \text{ mm} \times 180 \mu \text{m}$, $5 \mu \text{m}$ diameter particles; Waters, Milford, Massachusetts,
- 164 United State) over 3 min at 5 μ L/min with 1% solvent B. Peptides were eluted on a Acquity

165 UPLC BEH130 C18 column (250 mm × 75 μm, 1.70 μm particles; Waters, Milford,

Massachusetts, United State) at 0.45 µL/min with the following gradient of solvent B: from 166 1% to 3% in 0.5 min, linear from 3% to 26% in 54.5 min, linear from 26% to 35% in 5 min, 167 and up to 90% in 1 min. A scheduled PRM method consisting of one full MS1 scan and 16 168 targeted MS2 scans was developed. The full MS1 scan was collected from 300-1,800 m/z at a 169 resolution of 17,500 at 200 m/z (AGC target: 3e6, maximum IT: 50 ms). Targeted MS2 scans 170 171 were collected at a resolution of 35,000 at 200 m/z (AGC target: 1e6, maximum IT: 128 ms) and scheduled with 6-min time windows. Precursors were isolated within a 2-m/z window and 172 fragmented with a NCE of 27. 173

174 *2.4. Data treatment*

PRM data were processed with Skyline (version 3.7.1.11099) using appropriate
parameters (MacLean et al., 2010). Data were manually curated, and chromatographic peaks
manually integrated.

The limits of quantification of each targeted peptide, *i.e.* the lower limit of quantification 178 179 (LLOQ) and the upper limit of quantification (ULOQ), were determined using calibration curves. Eight different amounts of the concentration-balanced mixture of heavy-labelled 180 peptides were spiked into the representative matrix. The linear quantification range of each 181 peptide was determined by applying the following criteria: coefficient of variation (CV) \leq 182 20% between analytical triplicates, coefficient of determination (R^2) ≥ 0.99 between the 183 peptide signal and the injected quantity, $R^2 \ge 0.99$ between the back-calculated injected 184 quantity and the real injected quantity, and 80-120% accuracy by back-calculating the 185 expected injected quantity using the linear regression equation (Table 1). 186

187 Accurate quantification of the targeted peptides in the samples was performed by keeping188 at least three transitions per precursor. Peptide intensity was calculated by summing the

189 corresponding transition peak areas. After ensuring that peptide quantity is within its linear 190 range, the ratio between the endogenous and its heavy-labelled peptide counterpart was used 191 to determine the mol amounts of each endogenous peptide. The further calculated 192 concentration was expressed in fmol/µg of muscular protein (data not shown) and was also 193 reported as ng/µg of muscular protein (Supplementary table 1, for the 43 samples) when the 194 weight of the entire protein was considered. Results expressed as ng/µg of muscular protein 195 were used for statistical analyses.

196 2.5. Quantification of proteins using RPPA

All primary antibodies were validated for their selectivity/specificity by western blot. An
antibody was considered specific against the studied protein when only one band at the
expected molecular weight was detected by western blot in a selection of meat samples (data
not shown).

Briefly, for RPPA, the muscle extracts of all animals were printed onto nitrocellulose 201 covered slides (Supernova, Grace Biolabs, Bend, Oregon, United States) using a dedicated 202 Aushon 2470 arrayer (Quanterix Corp., Billerica, Massachusetts, United States). Four serial 203 dilutions, ranging from 2000 to 250 µg/ml, were printed for each sample, thus allowing to 204 cover the linear part of the antibody signal. In addition, two technical replicates per dilution 205 were printed to check for reproducibility. Arrays were labelled with 10 specific antibodies or 206 without primary antibody (negative control), using a DAKO Autostainer Plus (Agilent, Santa 207 208 Clara, United States). The slides were incubated with DAKO avidin, biotin and peroxidase blocking reagents (Agilent, Santa Clara, United States) before saturation with TBS containing 209 0.1% Tween-20 and 5% BSA (TBST-BSA). Slides were then probed overnight at 4 °C with 210 primary antibodies diluted in TBST-BSA. After washes with TBST, arrays were probed with 211 horseradish peroxidase-coupled secondary antibodies (Jackson Immuno Research 212 Laboratories, Newmarket, UK) diluted in TBST-BSA for 1 h at room temperature. To amplify 213

the signal, slides were incubated with Amplification Reagent (Bio-Rad, Hercules, California, 214 215 United States) for 15 min at room temperature. The arrays were washed with TBST, probed with Alexa647-Streptavidin (Invitrogen Molecular Probes, Thermo Fisher Scientific, 216 217 Waltham, Massachusetts, United States) diluted in TBST-BSA for 1 h and washed again in TBST. For staining of total protein, arrays were incubated 15 min in 7% acetic acid and 10% 218 219 methanol, rinsed twice in water, incubated 10 min in Sypro Ruby (Thermo Fisher Scientific, 220 Waltham, Massachusetts, United States) and rinsed again. The processed slides were dried by centrifugation and scanned using a GenePix 4000B microarray scanner (Molecular Devices, 221 San José, California, United States). Spot intensity was determined with MicroVigene 222 223 software (VigeneTech Inc, Carlisle, Massachusetts, Unites States).

Raw data quality control included semi-automated evaluation of the dilution curves for 224 each sample and each antibody to check for a correct sigmoid antibody response curve. 225 Technical replicates were checked for reproducibility. Samples with low overall intensity 226 227 were removed from further analysis because this can be indicative of protein degradation. 228 Antibodies were validated only if their median intensity over all samples was > 3 times higher 229 than the median intensity of the negative control slide. Lower limit of detection (LLOD) LLOQ and ULOQ were estimated for each antibody using a pool of all samples that was 230 231 printed in 10 serial dilutions ranging from 6700 to 13 ug/ml on each array (Table 2). The dynamic range, between the LLOQ and the ULOQ, covers the concentration range in which 232 the samples were printed (2000 to 250 µg/ml). Upper limit of detection (ULOD) could not be 233 assessed because of signal saturation. Indeed, scanner settings were chosen to attain saturation 234 235 for the first dilution of the pooled samples.

The relative abundances of proteins were determined according to the following
procedure. First, raw data were normalized using NormaCurve (Troncale et al., 2012), a
SuperCurve-based method that simultaneously quantifies and normalizes RPPA data for

fluorescent background per spot, a total protein stain and potential spatial bias on the slide.
Next, each RPPA slide was median centered and scaled (divided by median absolute
deviation). Remaining sample loading effects were corrected individually for each array by
correcting the dependency of the data for individual arrays on the median value of each
sample over all the arrays using a linear regression.

244 2.6. Descriptive and predictive statistical analysis

Statistical analyses were performed using R 3.5.1 software. Shapiro tests were performed 245 on IMF and shear force values as well as on protein abundances to check for adherence to 246 normality. For the qualification test, and because of the lack of normal distribution of some 247 values, a Wilcoxon test was done to identify differences between the high and low marbling 248 groups whatever the muscle (dataset 1), as well as between tender and tough LT (dataset 2) or 249 250 SM (dataset 3) muscles. Dataset 1 was composed of SM or LT muscles chosen to keep the 8 lowest and 8 highest values of IMF content. For the tenderness trait, a muscle effect on the 251 252 values was observed on shear force and of protein abundances (Supplementary Table 1). The 253 qualification test was therefore done separately for LT (dataset 2, 5 LT samples with the lowest and 5 with the highest values of shear force) and ST (dataset 3, 5 SM samples with the 254 lowest and 5 with the highest values of shear force) muscles. Differences were considered as 255 256 significant when p-value was < 0.05, and a tendency was recorded when p-value ranged from 0.05 to 0.15. 257

For the verification steps, the capacity to predict the IMF or shear force values using the abundance of one protein was tested. Data obtained for the 43 muscles without any classification were used to develop 54 different linear models to predict either IMF or shear force values. Each linear prediction model was composed of the abundance of one protein. For the shear force prediction, the muscle effect was added to the equation, in order to take into consideration the differences in shear force and protein abundances between muscles.

We also developed 54 binomial logistic regression with logit model to test the ability of 264 265 one protein to classify each muscle into lean, fat, tender or tough classes. Lean and fat classes consisted of muscles with IMF values lower and higher than 3.5%, respectively, which is the 266 267 threshold at which French meat consumers are able to distinguish lean from fat meat (Berthelot & Gruffat, 2018). Consequently, the lean (n = 21) and fat (n = 22) groups had 268 significant (P < 0.001) divergent values of IMF content (2.39 ± 0.94 and $6.08 \pm 1.02\%$, 269 270 respectively). The tender and tough groups consisted of muscles with shear force values lower 271 and higher than 42 N/cm², which corresponds to the threshold proposed by Destefanis, Brugiapaglia, Barge, & Dal Molin (2008). Consequently, the tender (n = 27) and though (n = 27)272 273 16) groups had significant (P < 0.001) divergent values of shear force $(31.81 \pm 4.07 \text{ and } 54.17)$ \pm 10.22 N/cm², respectively). Each logistic model predicted the probability of the muscle to 274 move from a reference cluster to a superior cluster of the predicted variable (*i.e.* tenderness or 275 276 adiposity). For all binomial logistic models predicting tenderness, the tough cluster was the 277 reference cluster, and for those predicting adiposity, the reference cluster was the lean cluster. 278 To predict the tenderness classes, the abundance of one protein and the muscle factor were included in each logistic model. To predict the adiposity classes, only the abundance of one 279 protein was included in each model. 280

The validation of each of the 54 linear and 54 logistic models was performed using a bootstrap procedure, because the number of muscle samples did not allow us to perform an external validation (Tan, Steinbach, & Kumar, 2006). The bootstrap procedure was repeated 500 times to generate 500 bootstrap samples, for each developed model. The number of times the protein coefficient in the model was significant was counted over the 500 repetitions. Only proteins for which \geq 60% of the bootstrap samples had a significant protein coefficient were selected for the next step, which is the quality evaluation of the models.

To evaluate the quality of the developed linear models, three criteria were considered: the 288 289 root mean square errors of prediction (Kobayashi & Salam, 2000), the mean prediction error (MPE) (Yan, Frost, Keady, Agnew, & Mayne, 2007) and R². The MPE and R² were used to 290 291 describe the prediction accuracy and the precision of the model, respectively. The prediction models were considered to have a high or good accuracy when MPE ranged from 0.10 to 0.30 292 and to have a high precision when the R² was the closest to 1. The quality of the developed 293 logistic models was assessed by four criteria: the success rate, the accuracy, the sensitivity 294 295 and the area under the curve (AUC). The AUC was measured using the "ROCR" package in R (Sing, Sander, Beerenwinkel, & Lengauer, 2005). The prediction models were considered 296 297 to have a good to high accuracy when the AUC was higher than 0.60, a threshold commonly used in biomarkers triage process (Capello et al., 2017; Yoneyama et al., 2016). Final models 298 were constructed with the mean of protein coefficients that were significant among the 500 299 300 repetitions (i.e. significant models over the 500 repetitions), and consequently metrics from these significant models were used to calculate mean criteria for linear and logistic models. 301

302

303 **3. Results**

304 *3.1.* Selection of biomarkers for testing in evaluation and verification steps

Potential muscular biomarkers for sequential validation studies were selected among
protein candidates that met at least two of the following criteria : 1) inclusion in the
compendium of potential biomarkers of marbling (Bazile, Picard, Chambon, Valais, &
Bonnet, 2019; Ceciliani et al., 2018; Thornton et al., 2017) or tenderness (Picard et al., 2017);
2) identified as differentially abundant by in-depth quantitative proteomic analyses of two
groups of Rouges des Prés bovine breed strongly divergent by marbling (Bazile et al., 2019)
or tenderness (Picard et al., 2019); 3) a dynamic muscular protein concentration range from

 10^2 and 10^5 or 0.1 to 100 ng/mg of protein assuming that the suitable minimal range of 312 313 detection proposed for plasma (Surinova et al., 2011) is accurate for muscular protein. As a result, a total of 10 potential biomarkers were selected for further qualification and 314 315 verification tests. The 10 proteins consisted of Malate dehydrogenase (MDH1), β-enolase 3 (ENO3), Retinal dehydrogenase 1 (ALDH1A1), Triosephosphate isomerase (TPI1), αB-316 crystallin (CRYAB), Heat shock protein beta-1 (HSPB1), Peroxiredoxin 6 (PRDX6), Myosin 317 318 1 (heavy chain-IIx, MYH1), Troponin T slow skeletal muscle (TNNT1), and Four and a half LIM domains 1 (FHL1). 319

320 *3.2. Quantitative PRM and semi-quantitative RPPA assay developments*

Two peptide analytes per protein (distinguished as P1 and P2 after the gene name of the 321 protein) were configured into a PRM-MS assay, and stable isotope-labelled peptides were 322 323 used as internal standards. The analytical performances of the PRM assays were determined by generating a response curve for each of the 20 targeted peptides. Performance 324 325 characteristics of the assays for the 10 candidate proteins are summarized in Table 1. The 326 medium analytical coefficient of variation at the lower limit of quantification (LLOQ) and across all peptides was 4.22%. The median lower limit of detection (LLOD) was 0.23 ng/µg 327 protein and the median LLOQ was $0.25 \text{ ng/}\mu\text{g}$ protein. When applied to the 43 muscular 328 329 samples, the absolute quantification failed for TNNT1_P2, and for ALDH1A1 with both peptides, because the quantitative values were below the LLOQ. The absolute quantification 330 failed for PRDX6 P1 due to the lack of a reliable calibration curve and consequently no 331 LLOQ has been calculated. Protein abundances were above the ULOQ for MYH1_P2 in half 332 of the 43 muscular samples (data not shown). 333

Performance characteristics of the RPPA assays for the 10 candidate proteins are
summarized in Table 2. The median analytical coefficient of variation at the LLOQ and across

all proteins was 13%. The median LLOD was 100 ng/µl of total protein and the median
LLOQ was 500 ng/µg of total protein (Table 2).

The quantitative values of the 9 proteins assayed by RPPA were correlated with the absolute PRM quantification for the 43 muscular samples. The values of the significant (P < 0.05) Pearson correlation coefficients were more than 0.60 for CRYAB, HSPB1 (both peptides) and MYH1_P1, and ranged between 0.34 and 0.60 for TNNT1, ENO3, TPI1 (both peptides) and MYH1_P2, indicating that the PRM and RPPA assays are in good and moderate agreement for 6 proteins. There was no agreement between the quantitative values assayed by PRM and RPPA for MDH1, FHL1 and PRDX6 (data not shown).

345 *3.3. Qualification of candidate biomarkers in extreme groups of marbling and tenderness.*

The candidate biomarkers were subjected to the qualification test using PRM, RPPA and sample sets specific for marbling or tenderness, in order to demonstrate that the differential candidate abundance observed in the discovery step is confirmed by at least an alternative targeted method, as requested within the biomarker pipeline (Rifai et al., 2006).

The marbling dataset 1 was composed of 16 muscles chosen for their high (7.06 ± 0.63) 350 mg/g of fresh muscle, n = 8) or low (1.26 ± 0.56 mg/g of fresh muscle, n = 8) values of IMF, 351 352 and as expected they were thus very divergent (P < 0.001) for marbling (Figure 1; Supplementary Table 2). When assayed by PRM, the abundances of MYH1 and TPI1 353 354 (especially when quantified using peptide 2) were significantly (P < 0.05) lower while the abundance of MDH1 was higher in the highly versus lightly marbled muscles. The 355 abundances of CRYAB and FHL1 quantified using peptides 1 tended (P < 0.13) to be the 356 357 highest in the highly marbled muscles. When assayed by RPPA, the abundances of ALDH1A1 and CRYAB were higher while the abundance of MYH1 was lower in highly 358 versus lowly marbled muscles. Moreover, the abundances of FHL1 and TNNT1 tended (P < 359

0.13) to be higher while the abundance of TPI1 tended to be lower in highly marbled muscles(Figure 1; Supplementary Table 2).

Two tenderness sample sets were composed of 10 LT (dataset 2) or 10 SM (dataset 3) 362 samples chosen for their high $(38.17 \pm 6.19 \text{ N/cm}^2 \text{ for LT}; \text{ or } 66.24 \pm 7.47 \text{ N/cm}^2 \text{ for SM}, \text{ n} =$ 363 5/per muscle, tough) or low (25.79 \pm 1.61 N/cm² for LT or 33.90 \pm 2.13 N/cm² for SM, n = 364 5/per muscle, tender) shear force values (P = 0.008, Figure 2; Supplementary Table 3), to take 365 366 into account a muscle significant effect on tenderness and on most of the protein abundances (Supplementary Table 1). As expected, the two groups of shear force were very divergent for 367 each muscle. For LT muscle, HSPB1, qualified both by PRM and RPPA assays, was the sole 368 369 protein differentially abundant between groups and as expected its abundance was the lowest in tender LT. A tendency (P = 0.12) for a higher abundance of TPI1 assayed by RPPA was 370 observed in tender LT. For SM muscle, MDH1 and TNNT1 were qualified when assayed by 371 PRM and their abundance was higher in the tender compared to tough SM. We recorded a 372 tendency (P < 0.15) for higher abundances of ENO3_P2, PRDX6_P2 assayed by PRM, and a 373 374 higher abundance of TNNT1 assayed by RPPA in the tender compared to tough SM (Figure 2; Supplementary Table 3). 375

376 *3.4. Verification studies for candidate biomarkers of marbling*

The PRM and RPPA assays were applied to the 43 muscular samples (whatever the muscle) to assess the performances of the biomarker candidates both for predicting the IMF values using linear regression, and to distinguish two classes (fat or lean class) of muscles according to their IMF content using univariate logistic regression.

381 Out of the protein abundances assayed, the abundance of MYH1_P1, MYH1_P2,

TPI1_P2 assayed by PRM as well as ALDH1A1, MYH1 or CRYAB assayed by RPPA

significantly predicted IMF values in the linear regression in at least 60% of the 500

repetitions done in the bootstrap procedure (see equations 6 to 11, Table 3). However, the precision of the predictions were low, since the R² ranged from 0.11 (for CRYAB) to 0.23 (for MYH1_P1), and the accuracies (MPE) were between 0.45 and 0.50. Nevertheless, these results highlighted that one protein abundance accounted for 11 to 23 % of the variability of IMF values in SM and LT muscles.

Logistic regression models based on the abundance of the 27 assayed proteins were developed to identify the proteins able to classify muscles to the lean or fat class. Only 3 abundances assayed by PRM yielded significant and good predictive models. The logistic regression model of TPI1_P2, MYH1_P1 and MYH1_P2 yielded an AUC of 0.75, 0.62 and 0.72, respectively (Table 4). The models yielded accuracies of 56 to 62% for fat class and sensitivities between 60 and 77%. For the lean class, accuracies of the models ranged from 64 to 69% and the sensitivities were between 49 and 67%.

396 *3.5. Verification studies for candidate biomarkers of tenderness*

The ability of one of the 27 protein abundances to predict shear force values thanks to a linear regression, or to distinguish two classes (tender or tough class) of muscles according to their shear force with univariate logistic regression was done for 43 samples of muscle but with the inclusion of a muscle factor.

401 Out of the 27 protein abundances assayed, the abundance of MDH1_P1, MDH1_P2, 402 ENO3_P2, PRDX6_P2 and TNNT1_P1 assayed by PRM were significant in the linear 403 regression to predict shear force values in at least 60% of the 500 repeats done in the bootstrap 404 procedure (see equations 1 to 5, Table 3). The precisions of the prediction (R²) ranged from 405 0.40 to 0.50 and the MPE values scored between 0.23 and 0.26. Thus, among these proteins 406 considered one by one, one protein abundance accounted for 40 to 50% of the variability of IMF values in SM and LT muscles. Any model by logistic regression for tenderness were
obtained probably due to the muscle effect that decreases the statistical power of the models.

409

410 **4. Discussion**

An initial set of 10 muscular biomarker candidates were selected, based on in-depth 411 proteomic analysis of muscles differing in marbling (Bazile et al., 2019) and tenderness 412 413 (Picard et al., 2019), and a review of literature (Ceciliani, Lecchi, Bazile, & Bonnet, 2018; Picard et al., 2017). The 10 candidate biomarkers were tested using a LC-MS/MS-based and 414 415 an antibody-based method for the qualification and verification steps of the biomarker discovery pipeline proposed by Rifai et al. (2006). Among these 10 muscular biomarker 416 candidates, 8 were qualified as biomarkers of marbling or tenderness, and the abundance of a 417 protein alone was shown to predict IMF content or shear force. Two biomarkers were shown 418 to distinguish classes of muscles with low and high levels of marbling. The high number of 419 biomarkers successfully confirmed by the present study may result from our choice to verify 420 few biomarker candidates, as well as from the relevant sample size (up to 10 samples per 421 group) and the stringent power calculation that we have applied during the discovery phases 422 (Bazile et al., 2019) in agreement with the recommendations from Drabovich et al. (2015). 423

424 *4.1. Biomarkers of marbling*

Considering the biomarker candidates of marbling, the present results qualified MYH1
and TPI1 both by PRM and RPPA methods, MDH1 by PRM, as well as ALDH1A1 and
CRYAB by RPPA. The differential abundance previously shown by proteomic studies in the
same breed but from divergent samples (Bazile et al., 2019) was thus confirmed using new
methods. Out of these 5 qualified biomarkers, MYH1, ALDH1A1, TPI1 or CRYAB were
verified as their abundance was able to explain the variability of IMF content values, and the

abundance of MYH1 or TPI1 was also able to distinguish lean or fat muscle groups. These 431 432 results are consistent with the muscular physiology and the molecular basis of lipid deposition in muscle. Indeed, muscles use both carbohydrates and fatty acids as energy sources for the 433 production of free energy and muscular contraction, according to a ratio that differs between 434 glycolytic and oxidative fibres. Oxidative muscles generally contain more lipids than 435 glycolytic muscles. Consistently, TPI1, a glycolytic enzyme, and MYH1, coding for the 436 437 myosin MyHC-2x isoform related to fast glycolytic fibre (Schiaffino & Reggiani, 2011), were less abundant in high marbled muscle, and were inversely related to IMF values in predictive 438 equations. The highest abundances of ALDH1A1 and CRYAB in highly marbled muscle is 439 440 also consistent with pathways related to lipid deposition. ALDH1A1 is a retinal dehydrogenase that irreversibly oxidizes retinaldehyde to retinoic acid (Takeda et al., 2016). 441 Retinoic acid was shown to have a pivotal regulatory role in the balance between lipid 442 443 synthesis (lipogenesis and fatty acid uptake) and degradation (lipolysis and fatty acid oxidation), at least in monogastric species. Indeed, as reviewed by Bazile et al. (2019), 444 445 retinoic acid was shown to stimulate lipid deposition in human adipose-derived stem cells, and lipid oxidation in mature adipocytes. Moreover, (Bazile et al., (2019) previously proposed 446 that an ALDH1A1-mediated increase in retinoic acid content mediates an increase in the 447 448 abundance of the small heat shock protein CRYAB, which is a retinoic acid-responsive gene (Takeda et al., 2016). Thus, the highest abundance of CRYAB in highly marbled Rouge des 449 Prés LT muscle first seen by Bazile et al. (2019), confirmed by the present results in LT and 450 451 SM muscles, may sustain the higher oxidative metabolism expected in highly marbled muscles. Lastly, the 4 biomarkers validated may be valuable biomarkers of marbling in other 452 breeds, since TPI1, MYH1, ALDH1A1 or CRYAB (for review, Bazile et al., 2019; Ceciliani 453 et al., 2018; Thornton et al., 2017) were reported as differentially abundant by proteomics 454 studies in up to four early-maturing bovine breeds known to deposit lipids. 455

Moreover, the present results suggest that TNNT1, FHL1 and MDH1 may be part of the 456 457 molecular signature of marbling, even if they were not retained as biomarkers of marbling because their abundance between highly and slightly marbled muscles tended to differ 458 459 (TNNT1, FHL1) or did not allow to predict IMF values (MDH1). In agreement with this hypothesis, among the 47 proteins that were differentially abundant across the adiposity 460 groups (Bazile et al., 2019), 4 were contractile or structural proteins classically used for the 461 462 muscular fibre classification. Of these, a higher abundance of slow-type proteins such as TNNT1 previously observed in Rouge des Prés cows (Bazile et al., 2019) as well as in others 463 breeds of cattle (raised in the Pacific Northwestern Unites States and Canada) divergent by 464 465 marbling (Thornton et al., 2017), tended to be confirmed in the present study. In bovine, we were the first to observe a tendency for a higher abundance of FHL1 assayed by shotgun 466 467 proteomics, in muscle of highly marbled Rouge des Prés bovines. An overexpression of the 468 FHL1 gene was also reported when obese-type pigs were compared to lean-type ones (Yang et al., 2017). A hypothetical functional link between FHL1 and marbling may be that FHL1 469 470 was shown to activate myostatin signalling, in skeletal muscle of mice (Lee, Lori, Wells, & Kemp, 2015). Moreover, in human, a deletion of the entire FHL1 gene was shown to induce a 471 muscle hypertrophy, a very mild fatty striation of the muscle shown by magnetic resonance 472 473 imaging scan and reduced subcutaneous fat (Willis et al., 2016). If we assume that similar FHL1 signalling occurs in bovine, a higher abundance of FHL1 may contribute to a higher 474 myostatin signalling and IMF deposition by promoting the commitment and differentiation of 475 476 multi-potent mesenchymal cell lines into the adipogenic lineage rather than the myogenic lineage (Bonnet, Cassar-Malek, Chilliard, & Picard, 2010). Lastly, the differential abundance 477 of MDH1 (cytoplasmic malate dehydrogenase) according to marbling was confirmed, 478 although its abundance alone was not able to predict IMF values or fatness classes. MDH1 is 479 involved in reduced nicotinamide adenine dinucleotide phosphate (NADPH) supply for de 480

novo fatty acid synthesis and is considered as a lipogenic enzyme. A significant correlation
between malic enzyme activity (MDH1) and IMF values in the *rectus abdominis* and *semitendinosus* muscles from Limousin, Angus and Japanese Black cross Angus steers was
previously reported (Bonnet et al., 2007). A higher MDH1 activity was also recorded in the
more oxidative and marbled *rectus abdominis* than in the *semitendinosus* (fast glycolytic)
muscle regardless of the breed (Bonnet et al., 2007).

487

4.2. Biomarkers of tenderness

Considering the biomarker candidates of tenderness, the present results qualified HSPB1 488 (in LT) and TNNT1 (in SM) assayed both by PRM and RPPA methods, and MDH1, PRDX6 489 and ENO3 assayed by PRM as biomarkers in SM muscle, since we have confirmed the 490 differential abundance previously shown by proteomic studies (Picard et al., 2017). Of these, 491 492 MDH1, PRDX6 TNNT1 and ENO3 were both differentially abundant between tender and tough SM and the abundance of one protein explained up to 50% of the variability of shear 493 494 force value. We validated PRDX6 as a biomarker of tenderness in the Rouge des Prés bovines, as in several other bovine breeds (Picard et al., 2017). It is a bifunctional protein with 495 two activities: glutathione peroxidase and phospholipase A2. Its implication in tenderness 496 could be through the *postmortem* detoxification of reactive oxygen species (ROS) in the cells. 497 498 Moreover, decrease of ROS has been described to enhance the function of µ-calpain involved in meat tenderization, which is in accordance with the correlation between PRDX6 and µ-499 calpain described by Picard et al., (2017). Our results also highlight a strong link between the 500 abundance of MDH1 and beef tenderness. Beside its lipogenic function, this protein was 501 described to play crucial roles in energy and cellular metabolic pathways, such as in the 502 503 malate-aspartate shuttle and in the citric acid cycle, to produce energy for many biological functions in aerobic tissues. To our knowledge, we are the first to propose MDH1 as a 504 positive biomarker and predictor of beef tenderness. A relationship between TNNT1 (or 505

TNNT3 fast isoforms) and tenderness has been described by several studies, and Troponin 506 507 was described as an indicator of tenderization since a fragment appears early *postmorten* (Picard et al., 2017). Glycolytic enzymes such as ENO3 (skeletal muscle isoform of enolase) 508 509 have also been associated with tenderness development in several breeds (Picard et al., 2017). In the present study, a higher abundance of ENO3 in the most tender SM was confirmed by 510 511 the PRM assay (with peptide 2) and this abundance was able to explain the variability of beef 512 tenderness. In line with these statistical relationships, glycolysis is the first biological pathway mobilized for *postmortem* production of ATP and its rate depends mainly on NAD⁺ 513 availability, involving enzymes such as MDH1 as described above. 514

515 Even if HSPB1 was confirmed as differentially abundant according to shear force values in LT, its abundance alone was not able to predict shear force values from the 2 studied 516 muscles. The HSPB1 protein appeared to be differentially abundant only in LT muscle, which 517 is consistent with a muscle type effect already described for small HSPs such as HSPB1 518 (Picard et al., 2017). This protein, involved in many molecular pathways such as response to 519 520 heat and stress, regulation and stabilization of myofibrillar proteins, protection of actin and 521 desmin, and negative regulation of apoptosis, has been proposed as a biomarker candidate of beef tenderness in several breeds as reviewed by Picard et al. (2017). However, the relation 522 523 between HSPB1 abundance and tenderness was different according to the contractile and metabolic properties of muscles. Most of the time, a positive relationship between HSPB1 and 524 525 tenderness was reported in the most glycolytic muscles, while a negative relationship was reported in the most oxidative ones (Picard et al., 2017). The lower abundance of HSPB1 in 526 527 the tender LT that we report in the present study is in line with this general rule, since Rouge 528 des Prés LT muscle was reported to have slow oxidative characteristics and to contain a low proportions of fast glycolytic fibres (Couvreur et al., 2019). A study in knock-out HSPB1 529 mice showed that calcium homeostasis, muscle structure and contraction and energy 530

metabolism were impacted by the absence of HSPB1, supporting a role of HSPB1 in
tenderization (Picard et al., 2016). In conclusion, the biomarkers of tenderness that we
validated in this study are coherent with the main biological functions involved in *postmortem*muscle modifications namely energetic metabolism, contraction, and cellular stress.

535 *4.3. Added-values of the combination of PRM and RPPA methods*

It is noteworthy that some biomarkers were qualified and verified either when assayed by 536 PRM, by RPPA or by both methods. Out of the 8 proteins that were shown to have statistical 537 relationships with marbling or tenderness, only MHY1 was selected in models for marbling 538 whatever the method of quantification. Despite the accuracies of the PRM and RPPA assays 539 reported here, methodological specificities have provided some differences in quantitative 540 values as exemplified by the lack of PRM results for ALDHI1A, or poor agreement for 541 542 MDH1 and PRDX6 candidates. Despite those discrepancies, we have chosen to combine PRM and RPPA results for two reasons : to take advantage of two orthogonal methods and 543 544 because both methods allow reaching sensitivities in the ng/mg to µg/mg range (Solier & 545 Langen, 2014), compatible with the expected abundances of the 10 selected candidate proteins. While PRM assays depend on the use of proteotypic standard peptides ensuring high 546 specificity and allowing an absolute quantification assuming an effective trypsin digestion, 547 548 RPPA assays rely on indirect fluorescent readout, require the use of a specific antibody against each protein and are highly dependent on the quality and the preservation of the 549 sample. Our choice was very efficient as out of the 6 proteins quantified with similar 550 abundance by PRM and RPPA, 5 proteins (MHY1, TPI1, CRYAB, HSPB1, TNNT1) were 551 qualified as biomarkers of tenderness or marbling by both methods. This result, together with 552 553 the 8 proteins that were verified for their statistical performance in the prediction of tenderness and marbling, demonstrate that the combination of both methods was very relevant 554 to screen for biomarker proteins in the present context. Strengths and weaknesses of antibody-555

versus MS-based validation methods have been largely reviewed and added-values and
discrepancies obtained when combining both methods have been previously illustrated in the
context of clinical studies (Drabovich et al., 2015; Solier & Langen, 2014; Surinova et al.,
2011; Yoneyama et al., 2016).

560 **5. Conclusion**

To conclude, we were able to quantify 9 and 10 candidate biomarkers by PRM and 561 RPPA, respectively. Of these, MDH1, TNNT1, ENO3, and PRDX6 were effective to predict 562 tenderness while marbling was predicted by TPI1, MYH1, and with lower efficacy by 563 ALDH1A1 and CRYAB, based on their confirmed differential abundances between divergent 564 groups and on their mathematical relationships with these meat-eating qualities. However, 565 these relationships are not precise enough to provide a specific test based on a single protein, 566 567 even if they showed that the abundance of one protein explains up to 50% of tenderness. Nevertheless, the PRM and RPPA assays developed in the present study constitute a very 568 569 promising starting point for a further large-scale sample screening for the final validation of 570 biomarkers of marbling and tenderness. First, because of the higher variability of tenderness 571 and protein abundances within SM muscle, SM should be more accurate than the LT muscle to screen meat-eating qualities. Second, the present PRM-related quantitative values provide 572 573 reference values to test simplest quantitative method, and RPPA assays are a first screening of specific antibodies that pave the way for next step of biomarker evaluation. Lastly, we will try 574 to combine up to 4 proteins to increase the power of the prediction, which implies using a 575 higher sample size than in the present study, the combination of several proteins being shown 576 to increase the predictive accuracy. Once a good level of prediction will be achieved by 577 578 considering the abundance of up to four proteins for marbling or tenderness, we will developed a multiplex protein quantification assay, in order to provide beef industry with an 579 original and low cost tool for meat grading. 580

581 Declaration of Competing Interest

582 The authors declared no conflicts of interest.

583

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591

592 Appendix A. Supplementary data

593 Were included after the tables

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Figure caption

Figure 1. Intramuscular fat content and protein abundances in longissimus thoracis (LT) or 725 *semimembranosus* (SM) muscles. The qualification step (1) was carried out by the 726 quantification of protein abundance according to two clusters divergent for marbling (sub 727 dataset 1). The verification step (2) was done by analysing the ability of one protein 728 abundance to predict marbling values (linear regression) or classes (logistic regression) on all 729 730 of the 43 samples. Protein abundances were evaluated by Parallel Reaction Monitoring (PRM) and Reverse Phase Protein Array (RPPA). *** P < 0.001, ** P < 0.01, * P < 0.05, 0.05 731 732 < *t* < 0.15.

733

Figure 2. Shear force and protein abundances in longissimus thoracis (LT) or 734 semimembranosus (SM) muscles. The qualification step (1) was carried out by the 735 quantification of protein abundance according to two clusters divergent for tenderness in LT 736 (sub dataset 2) and in SM (sub data 3). The verification step (2) was done by analysing the 737 ability of one protein abundance to predict tenderness values (linear regression) or classes 738 (logistic regression) on all of the 43 samples. Protein abundances were evaluated by Parallel 739 Reaction Monitoring (PRM) and Reverse Phase Protein Array (RPPA). *** P < 0.001, ** P < 740 0.01, * P < 0.05, 0.05 < t < 0.15. 741

742







Protein Name	Gene	Mass	Peptide Sequence	Peptide	LLOD (fmol)	LLOD (ng/µg)	LLOQ (fmol)	ULOQ	LLOQ	ULOQ	CV at
	Name	(kDa)		Name				(fmol)	(ng/µg)	(ng/µg)	LLOQ
											(%)
sp O77834 PRDX6_BOVIN	PRDX6	25.07	VIISLQLTAEK	PRDX6_P1	312.50	15.67	NQ	NQ	NQ	NQ	NQ
			LAPEFAK	PRDX6_P2	0.13	0.01	0.13	625	0.01	31.33	3.39
sp P02510 CRYAB_BOVIN	CRYAB	20.04	HFSPEELK	CRYAB_P1	0.63	0.03	6.25	3125	0.25	125.20	2.58
			FSVNLDVK	CRYAB_P2	312.50	12.52	625.0	3125	25.05	125.20	7.24
sp P48644 AL1A1_BOVIN	ALDH1A	54.81	QAFQIGSPWR	AL1A1_P1	3.13	0.34	3.13	312.5	0.34	34.25	1.71
			LECGGGPWGNK	AL1A1_P2	6.25	0.69	6.25	3125	0.69	342.54	2.05
sp Q3T145 MDHC_BOVIN	MDH1	36.44	VIVVGNPANTNCLTASK	MDHC_P1	0.31	0.02	0.31	1563	0.02	113.91	7.33
			LGVTSDDVK	MDHC_P2	3.13	0.23	3.13	1563	0.23	113.91	3.41
sp Q3T149 HSPB1_BOVIN	HSPB1	22.39	ALPAAAIEGPAYNR	HSPB1_P1	0.63	0.03	0.63	3125	0.03	139.96	0.42
			SATQSAEITIPVTFQAR	HSPB1_P2	31.25	1.40	31.25	3125	1.40	1396.96	8.07
sp Q3ZC09 ENOB_BOVIN	ENO3	47.10	TAIQAAGYPDK	ENOB_P1	6.25	0.59	6.25	3125	0.59	294.35	0.91
			VNQIGSVTESIQACK	ENOB_P2	0.63	0.06	0.63	3125	0.06	294.35	8.67
sp Q5E956 TPIS_BOVIN	TPI1	26.69	VVLAYEPVWAIGTGK	TPIS_P1	31.25	0.17	31.25	3125	0.17	16.68	5.23
			NNLGELINTLNAAK	TPIS_P2	6.25	0.03	62.50	3125	0.33	16.68	19.90
sp Q8MKH6 TNNT1_BOVIN	TNNT1	31.28	YEINVLYNR	TNNT1_P1	3.13	0.20	3.12	1563	0.20	97.79	2.78
			AQELSDWIHQLESEK	TNNT1_P2	62.50	3.91	62.50	625	3.91	39.10	5.80
sp Q9BE40 MYH1_BOVIN	MYH1	222.99	TLALLFSGPASGEAEGGPK	MYH1_P1	6.25	2.79	6.25	3125	2.79	1393.69	1.61
			GQTVEQVYNAVGALAK	MYH1_P2	312.50	139.37	625.0	3125	278.74	1393.69	4.22
tr F1MR86 F1MR86_BOVIN	FHL1	37.96	NPITGFGK	FHL1_P1	0.31	0.02	0.31	1563	0.02	118.67	8.09
			CLQPLASETFVAK	FHL1_P2	3.13	0.24	3.13	1563	0.24	118.67	10.00

Limits of quantification of the LC-PRM assay established with accurately quantified stable isotope-labelled peptides.

LLOD: lower limit of detection. LLOQ: Lower limit of quantification. ULOQ: Upper limit of quantification. NQ: Not quantifiable.

Limits of quantification of protein abundance measurements for each antibody by Reverse Phase Protein Array (RPPA), as determined on a pool of all samples that was printed in 10 serial dilutions ($6700 - 13 \mu g/ml$ of total protein).

name (gene) total protein) of total protein	of total protein)
ALDH 1A1 P48644 Po. anti-bovine Abcam ab23375 1/500 3000 500	100
CRYABP02511Mo. anti-bovine Assay Designs SPA-2221/10003000500	200
ENO3 P13929 Mo anti-human Abnova Eno3 (M01) clone 5D1 1/30 000 2500 400	100
	100
FHL1 Q3T173 Po. anti-human Sigma AV34378 1/5000 3500 400	50
HSPB1 P04792 Mo. anti-human Santa Cruz HSP27 (F-4):SC13132 1/3000 3000 400	100
MDH1 P40925 Mo. anti-pig Rockland 100-601-145 1/1000 3000 400	100
MYH1 P12882 Mo anti-boying Biocytex 8E4 1/500 3000 600	200
	200
PRDX6 P30041 Mo. anti-human Abnova PRDX6 (M01), clone 3A10-2A11 1/500 3000 600	100
TNNT1 Q8MKH6 Po. anti-human Sigma SAB2102501 1/4000 3000 600	200
TPI 1 Q5E956 Po. anti-human Novus NBP1-31470 1/50 000 2500 500	400

ULOQ: upper limit of quantification; LLOQ: lower limit of quantification; LLOD: lower limit of detection. ULOD is not indicated since it is defined by saturation of the signal.

Equation and performances for the linear predictive models of tenderness and marbling using the abundance of one protein, plus a muscle effect in the case of tenderness.

т.		Protein	N of significant models			R ²		MPE	
Item Protein used		abundance method	over 500 bootstraps	Model equat	ion	Mean	CI	Mean	CI
Shear	force								
(1)	MDH1_P1	PRM	445	LT SM	43.66 (± 3.44) - 47.13 (± 12.31) MDH1_P1 43.66 (± 3.44) - 47.13 (± 12.31) MDH1_P1 + 21.37 (± 3.10) SM	0.50	[0.24;0.65]	0.23	[0.18;0.30]
(2)	MDH1_P2	PRM	409	LT SM	49.44 (± 5.34) - 18.56 (± 5.24) MDH1_P2 49.44 (± 5.34) - 18.56 (± 5.24) MDH1_P2 + 21.87 (± 3.20) SM	0.48	[0.22;0.59]	0.24	[0.18;0.31]
(3)	TNNT1_P1	PRM	343	LT SM	42.69 (± 3.94) - 13.41 (± 4.46) TNNT1_P1 42.69 (± 3.94) - 13.41 (± 4.46) TNNT1_P1 + 16.61 (± 2.71) SM	0.40	[0.16;0.48]	0.26	[0.19;0.33]
(4)	ENO3_P2	PRM	335	LT SM	51.49 (± 6.19) - 1.57 (± 0.46) ENO3_P2 51.49 (± 6.19) - 1.57 (± 0.46) ENO3_P2 + 20.52 (± 2.78) SM	0.41	[0.09;0.49]	0.25	[0.19;0.33]
(5)	PRDX6_P2	PRM	305	LT SM	$\begin{array}{l} 48.05\ (\pm\ 3.90)\ \ 87.47\ (\pm\ 20.00)\ \mbox{PRDX6_P2} \\ 48.05\ (\pm\ 3.90)\ \ 87.47\ (\pm\ 20.00)\ \mbox{PRDX6_P2} + 19.78\ (\pm\ 2.99)\ \mbox{SM} \end{array}$	0.42	[0.23;0,46]	0.26	[0.19;0.33]
Intrar	nuscular fat								
(6)	MYH1_P1	PRM	454	LT and SM	6.35 (± 0.60) - 0.02 (± 0.004) MYH1_P1	0.23	[0.01;0.43]	0.45	[0.35;0.59]
(7)	ALDH1A	RPPA	427	LT and SM	4.90 (± 0.33) + 2.27 (± 0.49) ALDH1A	0.18	[0.02;0.32]	0.48	[0.37;0.62]
(8)	MYH1_P2	PRM	413	LT and SM	5.46 (± 0.42) - 0.001 (± 0.0003) MYH1_P2	0.19	[0.005;0.36]	0.49	[0.37;0.66]
(9)	TPI1_P2	PRM	434	LT and SM	5.44 (± 0.41) - 0.02 (± 0.004) TPI1_P2	0.18	[0.01;0.34]	0.48	[0.37;0.64]
(10)	MYH1	RPPA	351	LT and SM	4.15 (± 0.29) - 1.16 (± 0.28) MYH1	0.12	[0.005;0.22]	0.49	[0.39;0.62]
(11)	CRYAB	RPPA	334	LT and SM	$4.57 (\pm 0.30) + 1.60 (\pm 0.37) CRYAB$	0.11	[0.004;0.17]	0.50	[0.39;0.66]

The unit of protein abundances within the equation were ng/µg of muscular protein when assayed by Parallel Reaction Monitoring (PRM) and Arbitrary Unit

when assayed by Reverse Phase Protein Array (RPPA).

LT: longissimus thoracis muscle; SM: semimembranosus muscle; MPE: mean prediction error; CI: confidence interval.

Equations and performances for the logistic predictive models of marbling using the abundance of one protein assayed by PRM ($ng/\mu g$ of muscular protein)

Item	Protein used	Protein abundance	N of significant model over 500	ificant ver 500 Model equation after the bootstrap	Success rate (%)	AUC		Accuracy (%)		Sensitivity (%)	
		method	bootstraps			Mean	CI	Lean cluster	Fat cluster	Lean cluster	Fat cluster
Intran	nuscular fat										
(12)	TPI1_P2	PRM	322	- 1.79 (± 1.48) + 0.003 (± 0.003) TPI1_P2	61	0.75	[0.57;0.91]	69	58	49	77
(13)	MYH1_P1	PRM	304	- 2.77 (± 0.97) + 0.003 (± 0.009) MYH1_P1	63	0.62	[0.43;0.79]	65	56	58	65
(14)	MYH1_P2	PRM	313	- 1.58 (± 0.84) + 0.001 (± 0.001) MYH1_P2	61	0.72	[0.54;0.89]	64	62	67	60

AUC: area under the curve; CI: confidence interval.

Appendix A.

Supplemental Table 1

Trait descriptions and protein abundances assayed by Parallel Reaction Monitoring (PRM) and Reverse Phase Protein Array (RPPA) for the 43

samples composed of *longissimus thoracis* (LT) and *semimembranosus* (SM) muscles.

Item	Muscle										
	SM (n = 23)				LT (n = 20)			P		
	Mean	SD	Min	Max	Mean	SD	Min	Max			
Muscle traits											
Shear force (N/cm ²)	47.73	12.75	30.32	76.91	31.40	5.56	23.63	47.46	< 0.001		
Intramuscular fat (%)	4.02	2.19	0.98	8.39	4.39	2.02	0.45	7.48	0.44		
Protein abundances											
PRM method (ng/µg of p	proteins)										
CRYAB_P1	1.06	0.38	0.39	1.62	1.22	0.46	0.60	2.05	0.38		
CRYAB_P2	42.70	16.75	16.69	80.72	42.02	17.53	13.80	74.18	0.95		
ENO3_P1	36.34	7.31	23.24	55.34	33.86	7.31	16.08	50.64	0.30		
ENO3_P2	15.35	3.53	7.26	24.34	12.71	2.74	5.64	16.37	0.002		
FHL1_P1	1.56	0.65	0.59	3.05	2.20	0.81	0.67	3.88	0.01		
FHL1_P2	4.03	1.81	1.69	7.77	5.14	2.15	1.03	10.52	0.06		
HSPB1_P1	0.97	0.29	0.47	1.58	1.34	0.38	0.65	2.13	0.95		
HSPB1_P2	10.24	3.48	4.68	18.54	12.05	4.65	5.79	24.32	0.24		
MDH1_P1	0.36	0.14	0.12	0.55	0.26	0.08	0.09	0.43	0.01		
MDH1_P2	1.25	0.33	0.74	1.72	0.97	0.20	0.63	1.40	0.01		
MYH1_P1	113.27	43.97	60.25	252.54	90.76	52.51	10.35	261.51	0.03		
MYH1_P2	1516.60	1078.53	359.30	4302.60	895.80	719.84	128.60	2456.30	0.03		
PRDX6_P2	0.22	0.05	0.15	0.32	0.19	0.04	0.11	0.26	0.16		
TNNT1_P1	0.85	0.47	0.22	2.04	0.84	0.22	0.48	1.27	0.54		
TPI1_P1	7.79	3.49	1.67	18.29	5.93	2.69	1.41	13.63	0.03		
TPI1_P2	97.24	68.92	20.79	252.34	60.99	49.63	22.05	193.56	0.03		

RPPA method (AU)

ALDH1A	-0.38	0.37	-1.00	0.40	-0.22	0.48	-0.88	0.66	0.37
CRYAB	-0.35	0.53	-1.31	0.96	-0.15	0.56	-1.11	0.77	0.26
ENO3	0.48	0.58	-0.35	2.05	0.23	0.75	-1.65	1.20	0.48
FHL1	0.003	0.83	-2.05	1.63	0.15	0.69	-0.67	1.69	0.55
HSPB1	-0.41	0.48	-1.10	0.64	-0.18	0.57	-1.23	1.01	0.16
MDH1	-0.10	0.45	-1.05	0.64	0.10	0.35	-0.50	0.86	0.14
MYH1	0.15	0.61	-0.68	1.72	-0.20	0.92	-2.43	1.69	0.32
PRDX6	0.25	0.54	-1.40	1.02	-0.18	0.50	-1.34	0.48	0.003
TNNT1	-0.27	0.45	-1.42	0.59	-0.09	0.41	-0.82	0.83	0.32
TPI1	0.47	0.72	-0.72	1.90	0.15	0.81	-2.17	1.00	0.26

SD: Standard deviation. AU: Arbitrary Unit

The p-values were obtained from a X test.

Supplemental Table 2

Shear force, intramuscular fat content and protein abundances in *longissimus thoracis* (LT) or *semimembranosus* (SM) muscles according to two clusters divergent for marbling (dataset 1). Protein abundances were evaluated by Parallel Reaction Monitoring (PRM) and Reverse Phase Protein Array (RPPA).

Item	Clusters of intramuscular fat									
	Lean $(n = 8)$		Fat (n = 8)	Fat (n = 8)						
	Mean	SD	Min	Max	Mean	SD	Min	Max		
Muscle traits (LT and S	M)									
Shear force (N/cm ²)	50.71	18.87	28.74	76.91	37.17	8.85	25.36	54.45	0.19	
Intramuscular fat (%)	1.26	0.56	0.45	2.44	7.06	0.63	6.43	8.39	< 0.001	
Protein abundances										
PRM method (ng/ μ g of	proteins)									
CRYAB_P1	1.04	0.37	0.57	1.54	1.41	0.48	0.80	2.05	0.13	
CRYAB_P2	41.34	13.90	25.05	65.35	42.49	12.92	25.05	58.03	0.79	
ENO3_P1	34.71	9.26	23.24	50.12	33.55	6.79	20.99	43.03	0.96	
ENO3_P2	12.77	3.43	7.26	16.41	14.74	3.40	8.24	19.55	0.38	
FHL1_P1	1.39	0.47	0.76	2.120	2.33	1.23	0.59	3.88	0.10	
FHL1_P2	3.42	1.37	1.71	6.00	5.70	3.05	1.69	10.52	0.16	
HSPB1_P1	0.99	0.34	0.63	1.58	1.27	0.45	0.73	2.13	0.16	
HSPB1_P2	10.30	4.02	5.66	17.59	11.37	4.31	6.72	19.92	0.44	
MDH1_P1	0.23	0.09	0.12	0.41	0.31	0.07	0.20	0.43	0.04	
MDH1_P2	0.94	0.23	0.71	1.33	1.12	0.19	0.93	1.44	0.08	
MYH1_P1	150.34	77.21	61.50	261.51	76.34	24.99	27.69	105.44	0.04	
MYH1_P2	1818.20	1174.30	668.50	4302.60	396.90	87.72	278.70	516.20	< 0.001	
PRDX6_P2	0.17	0.03	0.14	0.22	0.20	0.04	0.15	0.26	0.38	
TNNT1_P1	0.57	0.23	0.22	0.92	0.77	0.27	0.40	1.24	0.23	
TPI1_P1	7.42	3.55	1.67	12.44	4.97	1.59	1.67	6.77	0.11	
TPI1_P2	113.71	72.95	29.66	245.06	30.62	9.77	20.79	51.19	0.003	
RPPA method (AU)										
ALDH1A	-0.63	0.30	-0.97	-0.11	0.005	0.46	-0.59	0.56	0.02	
CRYAB	-0.54	0.48	-1.16	0.13	0.14	0.51	-0.57	0.70	0.01	
ENO3	0.47	0.69	-0.09	2.05	0.16	0.88	-1.65	1.36	0.80	

FHL1	-0.19	0.79	-0.87	1.63	0.47	0.83	-0.67	1.42	0.13
HSPB1	-0.39	0.65	-1.23	0.64	-0.16	0.52	-1.05	0.75	0.50
MDH1	0.01	0.40	-0.51	0.44	-0.20	0.50	-1.05	0.59	0.38
MYH1	0.57	0.70	-0.53	1.72	-0.61	0.88	-2.43	0.21	0.01
PRDX6	-0.11	0.77	-1.22	0.91	-0.02	0.46	-0.86	0.69	0.96
TNNT1	-0.59	0.46	-1.42	-0.06	-0.20	0.41	-0.92	0.30	0.11
TPI1	0.56	0.75	-0.45	1.90	-0.23	0.89	-2.17	0.56	0.08

SD: Standard deviation.

The p-values were obtained from a Wilcoxon test.

Supplemental Table 3

Shear force, intramuscular fat content and protein abundances in longissimus thoracis muscle (LT) and *semimembranosus* muscle (SM) according to two clusters divergent for tenderness (dataset 2 for LT and dataset 3 for SM). Protein abundances were evaluated by Parallel Reaction Monitoring (PRM) and Reverse Phase Protein Array (RPPA).

	Tenderness clusters								
Item	Tender $(n = 5)$					Tough $(n = 5)$			
	Mean	SD	Min	Max	Mean	SD	Min	Max	
LT muscle traits									
Shear force (N/cm ²)	25.79	1.61	23.63	27.77	38.17	6.19	33.40	47.46	0.008
Intramuscular fat (%)	5.636	0.95	4.76	7.07	4.64	2.28	2.930	7.48	0.53
Protein abundances									
PRM method (ng/mg of proteins)									
CRYAB_P1	1.00	0.55	0.61	1.95	1.39	0.63	0.60	2.05	0.55
CRYAB_P2	32.30	15.45	18.52	58.03	41.87	23.30	13.80	74.18	0.55
ENO3_P1	33.67	2.33	32.25	37.80	36.47	11.13	20.99	50.64	0.69
ENO3_P2	12.89	2.01	11.52	16.37	11.98	3.36	8.24	16.31	1
FHL1_P1	1.89	0.50	1.22	2.60	2.31	1.27	0.67	3.88	0.69
FHL1_P2	4.51	1.55	3.04	7.15	5.03	2.96	1.03	8.80	0.84
HSPB1_P1	0.96	0.35	0.65	1.55	1.43	0.42	1.04	2.13	0.09
HSPB1_P2	8.89	2.66	6.67	13.11	13.57	4.44	8.47	19.92	0.05
MDH1_P1	0.26	0.05	0.21	0.34	0.20	0.07	0.09	0.26	0.22
MDH1_P2	0.97	0.12	0.82	1.11	0.87	0.14	0.65	1.01	0.55
MYH1_P1	78.24	13.30	69.35	101.68	90.85	44.57	60.01	169.46	1
MYH1_P2	583.00	114.57	485.90	732.30	694.90	575.72	220.30	1686.70	0.42
PRDX6_P1	1.56	0.43	1.15	2.22	1.83	0.76	0.94	2.85	0.55
PRDX6_P2	0.20	0.02	0.17	0.23	0.18	0.06	0.11	0.26	0.55
TNNT1_P1	0.82	0.07	0.71	0.89	0.78	0.30	0.48	1.27	0.55
TPI1_P1	5.32	0.15	5.19	5.55	4.73	2.00	1.67	7.22	0.42
TPI1_P2	38.26	5.51	31.23	43.96	42.87	19.29	22.05	61.79	0.69
RPPA method (AU)									
ALDH1A1	-0.30	0.43	-0.88	0.17	-0.10	0.57	-0.71	0.56	0.69
CRYAB	-0.51	0.37	-0.91	-0.12	0.17	0.73	-0.75	0.77	0.22
ENO3	0.68	0.42	0.20	1.10	-0.01	1.17	-1.65	1.20	0.55
FHL1	0.06	0.64	-0.67	0.88	0.51	0.94	-0.46	1.69	0.69
HSPB1	-0.54	0.34	-0.94	-0.11	0.17	0.42	-0.23	0.75	0.02
MDH1	-0.02	0.20	-0.29	0.23	0.004	0.48	-0.50	0.59	1
MyHC	-0.11	0.24	-0.39	0.14	-0.18	0.80	-1.12	0.64	0.69
PRDX6	0.00	0.27	-0.29	0.39	-0.32	0.77	-1.34	0.48	0.84

TNNT1	-0.07	0.28	-0.54	0.20	-0.11	0.48	-0.68	0.370	1
TPI1	0.68	0.31	0.28	1.00	-0.30	1.22	-2.17	0.93	0.12
SM mussle traits									
Shar force (N/am ²)	22.00	2.12	20.22	25 16	66.24	7 47	59 02	76.01	0.008
Intromuscular fat (0/)	2 17	2.13	1 29	5.40	2.08	2.09	J0.95 0.09	6.00	0.008
Drotain abundances	5.17	1.49	1.20	5.40	2.98	2.08	0.98	0.09	0.84
PDM method (ng/mg of proteins)									
CDVAD D1	1.20	0.42	0.65	1 61	0.015	0.54	0.20	154	0.21
CRIAD_FI CRVAD_D2	1.20	12.26	0.05	52.22	24.40	14 25	18.40	1.34	0.51
CNTAD_F2 ENO2_D1	40.42	15.20	21.04	32.33 27.96	21 60	14.55	18.40	32.31 42.77	0.09
ENO2_P1	34.72	4.03	20.72	57.80	51.00	1.44	23.24	42.77	0.42
ENU5_P2 EUL1_D1	15.08	2.30	11./5	17.72	11.72	4.20	7.20	10.41	0.15
FHLI_PI	1.70	0.52	1.15	2.31	1.23	0.37	0.84	1.05	0.22
FHLI_P2	4.57	1.50	2.93	6.04	2.93	1.07	1./1	4.30	0.22
HSPB1_P1	1.02	0.27	0.63	1.22	0.91	0.46	0.47	1.58	0.55
HSPB1_P2	9.44	2.35	6.26	11.69	8.51	3.98	4.68	14.62	0.42
MDHI_PI	0.43	0.14	0.19	0.55	0.21	0.07	0.12	0.28	0.05
MDH1_P2	1.42	0.36	0.78	1.64	0.87	0.12	0.74	1.07	0.09
MYHI_PI	115.75	38.87	80.27	172.48	124.96	76.86	61.50	252.54	0.84
MYH1_P2	1070.10	361.52	555.70	1517.20	1290.80	415.02	893.00	1794.40	0.69
PRDX6_P1	2.79	0.60	2.04	3.52	2.26	0.94	1.42	3.86	0.31
PRDX6_P2	0.26	0.07	0.16	0.32	0.18	0.02	0.15	0.20	0.09
TNNT1_P1	1.18	0.51	0.48	1.88	0.48	0.21	0.22	0.76	0.03
TPI1_P1	6.28	1.01	4.91	7.30	5.32	2.32	1.67	7.70	0.69
TPI1_P2	61.42	13.73	36.97	69.57	67.39	35.88	29.66	112.31	1
RPPA method (AU)									
ALDH1A1	-0.39	0.54	-0.99	0.40	-0.73	0.30	-1.00	-0.39	0.22
CRYAB	-0.24	0.73	-0.93	0.96	-0.59	0.57	-1.31	0.13	0.55
ENO3	0.156	0.41	-0.35	0.73	0.49	0.62	-0.09	1.19	0.60
FHL1	-0.27	1.18	-2.05	1.10	-0.55	0.34	-0.87	-0.03	0.55
HSPB1	-0.65	0.39	-1.10	-0.21	-0.30	0.60	-0.92	0.64	0.55
MDH1	-0.21	0.30	-0.48	0.29	0.14	0.35	-0.41	0.44	0.22
MyHC	0.16	0.92	-0.68	1.72	0.66	0.50	-0.06	1.14	0.21
PRDX6	0.26	0.39	-0.40	0.65	0.16	0.66	-0.79	1.02	0.84
TNNT1	-0.17	0.43	-0.92	0.10	-0.58	0.55	-1.42	-0.02	0.15
TPI1	0.23	0.62	-0.72	0.72	0.57	0.66	-0.45	1.27	0.31

SD: Standard deviation.

The p-values were obtained from a Wilcoxon test.

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