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Label free shotgun proteomics for the identification of protein biomarkers for beef tenderness in muscle and plasma of heifers

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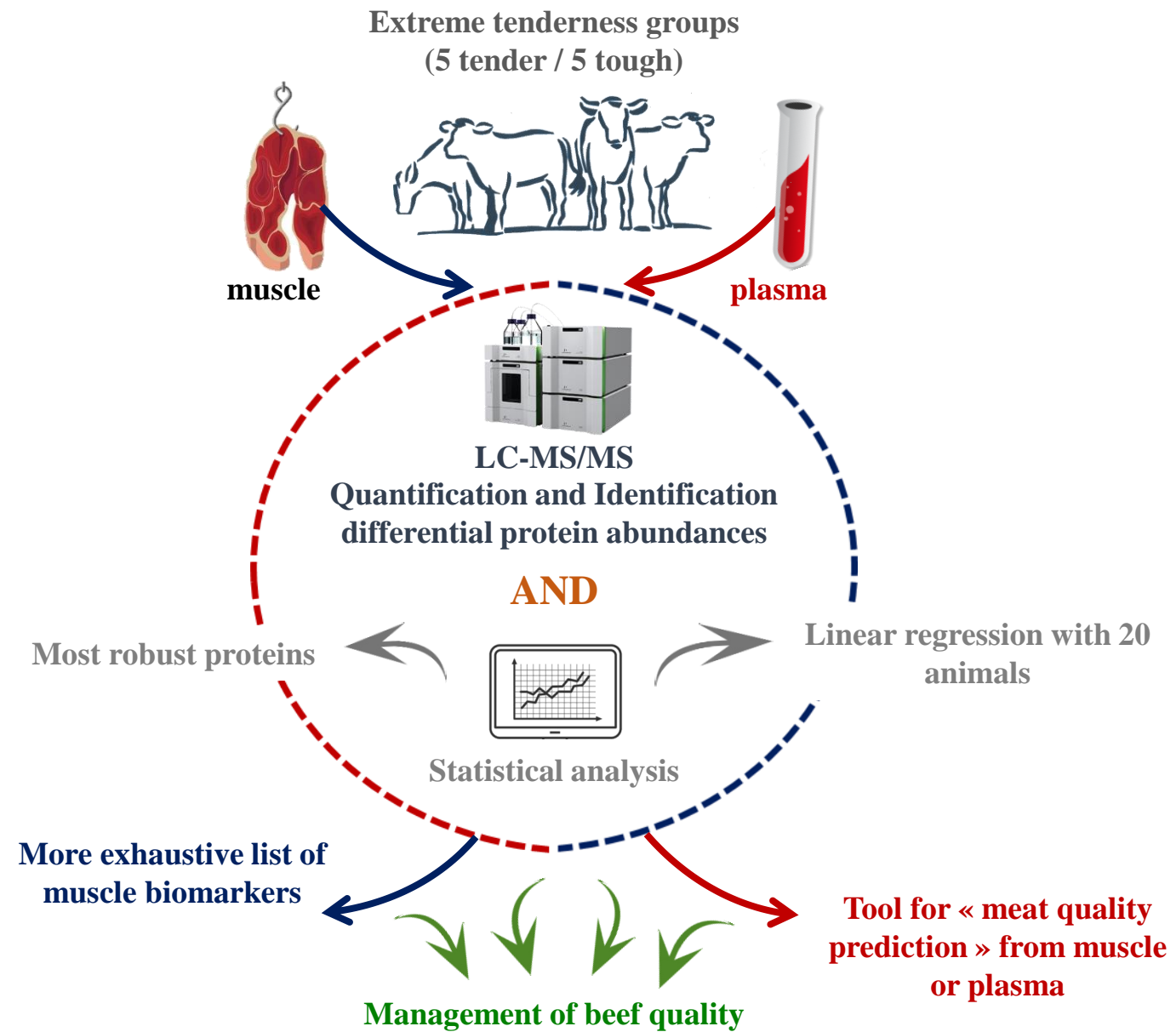
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SIGNIFICANCE

The label free proteomic approach used in this study allowed to complete the atlas of biomarkers of tenderness of the *Longissimus* muscle. This innovative proteomic approach applied on plasma samples allowed to identify circulating candidate biomarkers for beef tenderness. This low-invasive approach constitutes an interesting alternative to evaluate early the “beef meat potential” of living animals in farm or of the carcass in slaughterhouses.



HIGHLIGHTS

- Shotgun allowed to complete the list of tenderness biomarkers in *Longissimus* muscle
- Biomarkers for *Longissimus* tenderness were identified for the first time in heifers
- For the first time biomarkers for tenderness identified in plasma
- Biological pathways involved in tenderness similar in heifer and young bulls
- FHL1, ENO3, MYH7: tenderness biomarkers measurable in plasma and *Longissimus* muscle

1 **Label free shotgun proteomics for the identification of protein biomarkers for**
2 **beef tenderness in muscle and plasma of heifers**

3

4 Sabrina Boudon¹ (ORCID 0000-0003-0854-3432), Daoud Ounaissi¹ (ORCID 0000-
5 0002-0576-5059), Didier Viala^{2,3}, Valérie Monteils¹ (ORCID 0000-0002-9234-3451),
6 Brigitte Picard¹ (ORCID 0000-0002-8075-6718) Isabelle Cassar-Malek¹ (ORCID
7 0000-0001-7407-1408)

8 ¹Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122
9 Saint-Genès-Champanelle, France

10 ² UR370, Qualité des Produits Animaux, INRAE, F-63122 Saint-Genès-Champanelle,
11 France

12 ³ Metabolomic and Proteomic Exploration Facility (PFEM), INRAE, F-63122 Saint-
13 Genès-Champanelle, France

14 *Corresponding author: isabelle.cassar-malek@inrae.fr

15 Phone number: +33 4 73 62 46 57

16

17 **ABSTRACT**

18 Meat quality prediction is a priority for the beef industry. Label free shotgun
19 proteomics was performed on *Longissimus* muscle and plasma from 20 crossbred
20 Charolais x Aubrac beef heifers, classified as subgroups of 5 extreme tender and 5
21 extreme tough meat according to sensory evaluation, Warner Bratzler shear force, and
22 a synthetic tenderness index. This technique identified 268 proteins in muscle and 136
23 in plasma. Among them, 71 muscle proteins and 21 plasma proteins discriminated
24 tender and tough groups. These proteins were analyzed to select the most correlated
25 and explicative ones which were used in a linear regression on the 20 heifers. The
26 results validated in heifers 33 muscle proteins previously identified as related with
27 tenderness, and revealed 38 new candidates. Twelve are localized in shear force or
28 tenderness score QTL. Among them ACTN2, ADSSL1, GOT1, HPX, OGDH, OGN,
29 TNNC1 and VCL are proposed as robust candidates with 3 other proteins known to be
30 related with tenderness (MYBPH, CAPZB, MYH1). Examination of the plasma
31 proteome showed 8 putative biomarkers (MYH7, CFH, ENO3, PLA2G2D5, FHL1,
32 GAPDH, MASP2 and SERPINF2). Three of them (MYH7, ENO3 and FHL1) were
33 identified as discriminative of tenderness both in *Longissimus* muscle and in plasma.

34

35 **Keywords:** LC-MS/MS mass spectrometry, proteome analysis, beef tenderness,
36 biomarkers, muscle, plasma.

37

38 **SIGNIFICANCE**

39 The label free proteomic approach used in this study allowed to complete the atlas of
40 biomarkers of tenderness of the *Longissimus* muscle. This innovative proteomic
41 approach applied on plasma samples allowed to identify circulating candidate
42 biomarkers for beef tenderness. This low-invasive approach constitutes an interesting
43 alternative to evaluate early the “beef meat potential” of living animals in farm or of
44 the carcass in slaughterhouses.

45

46 **1. Introduction**

47 The beef meat consumers place a high expectation on the tenderness and solving
48 inconsistent tenderness is a top-priority for the meat sector. For more than 30 years
49 meat scientists have worked on the biomarkers identification for meat tenderness to be
50 integrated into phenotyping tools. Meat tenderness has a multifactorial origin depending
51 on muscle characteristics (fiber, sarcomere, collagen) [1,2], on the animal (age, gender,
52 genetic type) and its rearing management [3,4], but also dependent on the post-mortem
53 proteolysis and key proteins associated [5]. Thus, a combination of biomarkers is
54 necessary to predict this quality trait. Up to now, the search for biomarkers for
55 tenderness has been carried out on muscle samples only.

56 Several omic approaches including proteomic technologies, developed over the last
57 two decades have been conducted to identify biomarkers for meat tenderness and/or
58 explore the tenderness process (see [6,7] for review). This approach allowed proposing
59 a list of biomarkers and highlighted the interactions between them in the construction of
60 tenderness [8]. Some muscle type or animal type specificities have been evidenced [9].
61 However, biomarker-based approach has some limitations since the analysis of muscle
62 is done after slaughter or on muscle obtained by biopsy from live animal, which is

63 invasive. In this way, blood-based biomarkers offer an alternative low-invasive strategy
64 to predict the meat quality potential on living animals.

65 Circulating blood, notably through the plasma fraction, is a useful source of
66 biomarkers and can be collected easily through minimal-invasive procedure [10]. In
67 medical research, many studies have investigated blood-based proteomic biomarkers to
68 distinguish healthy and diseased or damaged tissues [11–13]. Nowadays, plasma
69 biomarker researches are tending to spread in a wide variety of domains. Indeed, in the
70 livestock sector, biomarkers were identified for stress in the serum of pigs housed at
71 different stocking rate [14], or Copper deficiency in cattle [15,16]. The aim of the
72 present study was to identify putative plasma biomarkers for meat tenderness and to
73 enrich the list of muscle tenderness biomarkers using the label free shotgun technique
74 on heifers.

75

76 **2. Materials and Methods**

77 Figure 1 presents the workflow used in this study.

78 **2.1. Animals**

79 Twenty crossbreed Charolais x Aubrac heifers of the French protected
80 geographical indication (PGI) Fleur d'Aubrac issued from the protocol described by
81 Soulat et al. [17] were used. The heifers were born between December 2012 and May
82 2013; they were managed according to the specifications of the PGI and slaughtered
83 between February 2015 and June 2016 in a single industrial slaughterhouse (Abattoir
84 du Gévaudan, Antrenas, France) as described in [17].

85

86 **2.2. Sample collections**

87 **Muscle sampling.** *Longissimus* muscles (LM) were removed from the 5th and 4th ribs,
88 24 h *post-mortem* for this study. Samples for proteomics were frozen in liquid
89 nitrogen and stored at -80°C until analysis. Samples for tenderness evaluation were
90 vacuum packaged and aged for 14 days at 4°C, then frozen at -20°C until tenderness
91 evaluation as described in [18]. The choice of LM is justified by the fact that this
92 muscle, considered as an international reference for meat science, has been analysed
93 in previous studies investigating tenderness biomarkers in cattle mainly in young bulls
94 and using two-dimensional electrophoresis.

95 **Plasma sampling.** Blood samples were collected from the tail vein (EDTA tubes) in
96 farm before transport to the slaughterhouse and stored at 4°C until processing. After
97 sample centrifugation at 4500 rpm for 20 min at RT, plasma fraction was transferred
98 to another tube with 10 µL PMSF (10mg/mL) and kept at -80°C in aliquot fraction
99 for further analysis.

100

101 **2.3. Tenderness evaluation and constitution of extreme groups**

102 Tenderness evaluation was performed with two methods as described in Soulat et
103 al. [18]. Sensory evaluation (global tenderness, Tg) was performed thanks to trained
104 panellists according to [19]. LM samples were grilled in a double-face grill at 300°C
105 during 1 min 45 sec resulting an internal cooked temperature of 55°C. The
106 mechanical tenderness evaluation was further instrumentally evaluated through
107 Warner-Bratzler shear force measurement (WB, expressed in N/cm²) using MTS
108 Synergie 200 equipment [20].

109 Also, as suggested by [21] for considering the genetic/phenotypic close
110 correlation observed between the sensory and mechanical tenderness, a synthetic
111 tenderness index (called Idx) was calculated by combining the difference between the

112 standardized sensory and the instrumental values: $((Tg \text{ score} - \text{means } Tg) / \text{standard}$
113 $\text{error } Tg) - (\text{WB measurement} - \text{means WB}) / \text{standard error WB}$) as described in [22].
114 The added value of Idx is to combine both mechanical and sensory tenderness to
115 create a more accurate evaluation of the tenderness phenotype. This Idx was already
116 used in the literature [9,22].

117 Among the 20 heifers, extreme groups were defined for muscle and plasma
118 samples according to the WB, Tg, and Idx tenderness quality traits respectively.
119 These groups contained the samples with the 5 highest tenderness values or the 5
120 lowest tenderness values for each trait. One sample was shared between the extreme
121 groups for WB, Tg and Idx; 3 were shared between groups for WB and Idx, 6
122 between groups for Tg and Idx, and 2 between groups for WB and Tg. All further
123 analyses performed were achieved for each of the six groups. The mean values of
124 tenderness scores for WB, Tg and Idx and their significant differences are presented
125 in Table 1.

126

127 **2.4. Proteins extraction**

128 In order to investigate the protein profile of muscle and plasma samples by LC-
129 MS/MS, appropriated extractions were performed depending on the studied tissue.

130 **Muscle samples.** Muscle proteins were extracted using Precelly 24® tissue
131 homogenizer protocol (Berton technologies, Saint Quanetin-en-Yvelines, France).
132 Frozen muscle tissue (80 mg) stored at -80°C was mixed in a buffer containing 50
133 mM Tris (pH 6.8), 2% SDS, 5% glycerol, 2 mM DTT, 2.5 mM EDTA, 2.5 mM
134 EGTA, 2x HALT phosphatase inhibitor (Perbio 78420), Protease inhibitor cocktail
135 complete MINI EDTA-free (Roche 1836170, 1 tablet/10 mL), 4mM Orthovanadate de
136 sodium (NA₃VO₄) and 10 mM Fluorure de sodium (NaF). The mixtures were then

137 boiled for 3x15 sec / 30 sec break, incubated 10 min at 100°C and centrifuged 15 min
138 at 13000 rpm (15-20°C). The supernatants containing protein lysate were collected
139 and stored at -20°C until further use. Determination of protein concentration was
140 performed with the Pierce commercial assay (Pierce BCA reducing agent compatible
141 kit) with BSA as standard [23].

142 **Plasma samples.** In order to increase the chance of detecting proteins of interest, the
143 ProteoMiner™ technology was used as protein enrichment approach (Large-Capacity
144 Kit 163-3007, BioRad Inc., Hercules, CA, USA) [24] to concentrate mid- and low-
145 abundance interest proteins such as albumins, immunoglobulins, fibrinogen or
146 complement proteins [25]. To investigate the protein profile on SDS-PAGE before,
147 plasma samples (1 mL) were loaded on a column with 100 µL ProteoMiner beads
148 buffer (20% beads, 20% v/v aqueous EtOH) and incubated 2h at room temperature in
149 order to allow peptides-ligands linkage. After two centrifugations (2x10000 rpm) and
150 two washes (50 µL wash buffer (150 mM NaCl, 10 mM NaH₂PO₄, pH 7.4) and 50
151 µL distillate water)) to eliminate highly abundant proteins, elution of the bound
152 proteins was performed using elution reagent (5% acetic acid).

153

154 **2.5. Nano-LC-MS/MS and protein identification**

155 **Sample preparation.** In order to achieve LC-MS/MS analysis, 100 µg of muscle or
156 plasma protein extracts were concentrated at the interface between 12 % resolving/4%
157 stacking acrylamide gels of 1D/SDS-PAGE. The migration of proteins was performed
158 during 15 min at 80V on Mini-Protean II electrophoresis unit (BioRad, Marnes-La-
159 Coquette, France). Then, gels were stained using colloidal Coomassie blue staining R-
160 250 and two bands at this interface between stacking and resolving gels were excised.
161 These bands included two protein fractions: high molecular weight myofibrillar

162 proteins (upper band) and low molecular weights (lower band). Excised bands were
163 washed with 100 μ L ammonium bicarbonate (50 mM-50% ethanol, 20 min at room
164 temperature (RT)). After removal of the buffer, they were dehydrated with 100 μ L of
165 100% ethanol (15 min). Reduction and alkylation reactions were performed during 30
166 min respectively with 10 mM DTT solution at 56°C and with 55 mM iodoacetamide
167 solution protected from light. The bands were washed with 200 μ L of 50 mM
168 NH_4HCO_3 buffer for 15 min at room temperature and destained by 100 μ L of 25 mM
169 NH_4HCO_3 – 5% acetonitrile (v/v) for 15 min followed by three washing with 100 μ L
170 of 25 mM NH_4HCO_3 – 50% acetonitrile (v/v) for 30 min under agitation. Finally all
171 bands were dehydrated with 100% acetonitrile (ACN). The samples were hydrolyzed
172 during 5 hours at 37°C using 48 μ L of a 25mM NH_4HCO_3 - 12.5 ng. μ L⁻¹ trypsin
173 solution (V5111, Promega, Charbonnières-les-Bains, France) per band. Peptides were
174 extracted from the gel bands in an ultrasonic field during 10 min with 38.4 μ L of
175 99.9% acetonitrile /0.1% Formic Acid representing 80% of digestion volume.
176 Supernatants were transferred in eppendorf vials and dried using Speed Vac for 60
177 min and adjusted to 50 μ L with a solution ($\text{H}_2\text{O}/\text{ACN}/\text{TFA}$ – 94.95/5/0.05). This
178 solution was transferred into HPLC vials containing a 100 μ L glass insert before nano
179 LC MS/MS analysis.

180 ***Nano-LC-MS/MS analysis.*** Peptides mixtures were analysed by nano-LC-MS/MS
181 (Thermo Fisher Scientific) using an Ultimate 3000 system coupled to a LTQ Orbitrap
182 Velos mass spectrometer (MS) with a nanoelectrospray ion source. For each sample,
183 two microliters of peptide mixture were first preconcentrated and desalted at a flow
184 rate of 30 μ L/min on a C18 pre-column 5 cm length X 100 μ m (Acclaim PepMap 100
185 C18, 5 μ m, 100A nanoViper) equilibrated with Trifluoroacetic Acid (TFA) 0.05% in
186 water. After 6 min, the pre-column was switched online with the analytical C18

187 column (Acclaim PepMap 100 - 75 μm inner diameter \times 25 cm length; C18 - 3 μm -
188 100 \AA) equilibrated with 96 % solvent A (99.9 % H₂O, 0.1 % formic acid) and 4 %
189 solvent B (99.9 % ACN, 0.1 % formic acid). Peptides were eluted at a 300 nL/min
190 flow rate according to their hydrophobicity using a 6 to 24% gradient of solvent B for
191 114 min. Eluates were electro-sprayed in positive-ion mode at 1.6 kV through a
192 nanoelectrospray ion source heated to 250°C. The LTQ Orbitrap Velos MS was used
193 in CID top 15 mode (i.e. 1 full scan MS and the 15 major peaks in the full scan were
194 selected for MS/MS). The parameters of mass spectrometry analysis were as follow:
195 Full-enhanced-scan MS spectra realized in the FTMS ion trap at a resolution of 60000
196 (tolerance 10 ppm) acquired with 1 microscan (m/z 300 – 1400), dynamic exclusion
197 used with 2 repeat counts, 20 sec repeat duration and 60 s exclusion duration. For
198 MS/MS, isolation width for ion precursor was fixed at 2 m/z, single charged species
199 were rejected; fragmentation used 37% normalized collision energy as the default
200 activation of 0.25 and 10 ms activation time.

201 ***Data processing, protein identification and abundances.*** For raw data processing,
202 MS/MS ion search was performed with Mascot v2.5 (<http://www.matrixscience.com>)
203 against *bos taurus* database (i.e. ref_bos_taurus, 23970 sequences). The following
204 parameters were considered for the search: precursor mass tolerance of 10 ppm and
205 fragment mass tolerance of 0.5 Da, a maximum of two missed cleavage sites of
206 trypsin, carbamidomethylation (C), oxidation (M) and deamidation (NQ) set as
207 variable modifications. Protein identification was validated when at least two peptides
208 originating from one protein showed statistically significant identity above Mascot
209 scores > 36 with a False Discovery Rate of 1% (adjusted significance threshold $p <$
210 0.0058). Ions score is $-\log_{10}$ (P-value), where P is the probability that the observed
211 match is a random event. Individual ions scores > 36 indicate identity or extensive

212 homology. All the proteins identified in this study correspond to one unique protein
213 identifier as they were annotated by a minimum of two associated specific peptides.
214 For label-free protein quantification analysis, LC-Progenesis was used with the same
215 identification parameters described above. All unique validated peptides of an
216 identified protein were included and the total cumulative abundance was calculated by
217 summing the abundances of all peptides allocated to the respective protein. Statistical
218 analysis was performed using the “between subject design” and p-values were
219 calculated by a repeated measures analysis of variance using the normalized
220 abundances across all runs.

221

222 ***2.6. Statistical analysis***

223 Shotgun analyses were annotated "M_LM" for LM muscle and "P_LM" for the
224 plasma samples on the same heifers. For further statistical analysis, M_LM samples
225 and P_LM were tested independently based on the three extreme groups defined by
226 WB, Tg and Idx.

227 ***Dataset normalization.*** In order to minimize intra-group variance, the most
228 appropriate normalized method of each of the six datasets was determined using R
229 software (version 3.5.1; 2018-07-02) and NormalyzerDE. The efficiency of the 8
230 normalization methods tested (Median intensity, Mean, Quantile, Variance
231 Stabilizing Normalization (VSN), log2, Robust Linear Regression (RLR), Global
232 Intensity (GI) and CycLoess,) was tested (NormalyserDE package, Normalyzer
233 function, version 1.1.16). For each dataset, the most appropriate normalization
234 method was selected according to several quantitative and qualitative statistical
235 data described in [26]. The optimal normalization was the Quantile method for LM
236 Tg and Idx and the CycLoess method for LM WB. For the plasma, the VSN method

237 was retained for Tg and Idx and the Quantile for WB. Raw proteins abundances of
238 each of the six datasets were normalized using the selected method.

239 ***Differential abundance proteins analysis.*** Identification of the differential protein
240 abundances from each of the six datasets was performed using the Normalyzer tool
241 Differential Expression (DE). Only the proteins with differential abundance (P-
242 value \leq 0.05) were considered for further analysis.

243 ***Correlation analysis between differential proteins and tenderness evaluations.***
244 Relationships between tenderness traits, - WB, Tg and Idx respectively- and the
245 differential proteins were determined by means of regression and Spearman
246 correlation analyses using the Hmisc package. Correlation analysis was performed
247 from the extreme groups to find the most correlated proteins with tenderness (P-
248 value \leq 0.05).

249 ***Identification of the most explanatory proteins.*** A Partial Least Squares (PLS)
250 statistical analysis (R-software, mixOmics package, pls function, version 6.6.2) was
251 used to identify how the set of differential proteins, which constitutes the variables,
252 was associated to the tenderness quality traits (WB, Tg, and Idx respectively). Filter
253 method associated with PLS analysis was applied to identify the variable importance
254 in the projection (VIP) in order to select the most important variables (or most
255 explanatory proteins) in the model [27,28].

256 In order to identify which proteins contributed the most to the models, the
257 Variable Importance in the Projection (or VIP score) was calculated using the
258 mixOmics package (vip function). The PLS_VIP values allow to define a hierarchy of
259 the most explanatory proteins for each tenderness trait (WB, Tg, Idx). Only the
260 variables with VIP $>$ 0.8, and more interestingly variables with VIP $>$ 1 (underlined in

261 the illustrations) were considered to identify putative candidate proteins usable for
262 meat tenderness prediction as previously applied in [29].

263 The selection of the proteins with the $VIP > 0.8$ related to each of the 6 datasets
264 was performed using the VIP Barplot representation. To illustrate the most
265 explanatory proteins correlated with the three tenderness quality traits (WB, Tg, and
266 Idx), a Principal Component Analysis (PCA) of the VIP's proteins selected was
267 performed using the mixOmics package (pca function, $VIP > 1$ underlined). In order to
268 visualize the group of proteins positively or negatively correlated with WB, Tg and
269 Idx, a Heat map representation of the VIP's protein abundance was performed using
270 the mixOmics package (cim function, $VIP > 1$ underlined).

271 ***Linear multiple regression models and validation of proteins in 20 heifers.*** Multiple
272 regression analyses were performed using the modvarsel package (choicemod
273 function, version 0.0.2) [30] to find the most robust proteins correlated with
274 tenderness in the whole dataset of 20 heifers. Muscle, plasma and the three tenderness
275 evaluation traits (WB, Tg, Idx) were considered as dependent variables, the VIP's
276 proteins ($VIP > 0.8$) were considered as independent variables. The regression
277 coefficients were calculated and the highest coefficients were marked in bold. The
278 regression analyses conducted with WB, Tg and Idx were performed to reveal the
279 most robust candidate proteins related to meat tenderness quality traits.

280

281 ***2.7. Gene ontology analysis***

282 In order to understand the biological functions in which differential proteins are
283 involved, Gene Ontology (GO) analysis was performed with the ProteINSIDE
284 webservice (<http://www.proteinside.org>; Database is 1.2.11 / last update 16-May-
285 2019) [31]. GO annotation enrichment analysis (Biological Process (BP)) was

286 achieved in Human species. Human orthologs' annotations were privileged in order to
287 get the most complete GO information, since bovine annotation remains limited.
288 Muscle and plasma GO were considered independently for GO_BP analysis.
289 Histogram representations of the GO_BP annotations were constructed using only
290 specifications associated with P-value<0.001 with a minimum of annotated proteins \geq 2
291 (see supplementary data S1 and S2). The P-values were converted into $-\log_{10}$ (P-
292 value) values, and classified in ascending order in an upward direction. Specifications
293 were annotated as follows: GO annotation (Number of proteins included in the
294 annotated in brackets) Proteins Gene Names tie to GO annotation separated by space.
295 A table listing the major metabolic pathways was constructed using GO_BP TREE
296 from the GO_BP annotations (P-value<0.001, minimum of annotated proteins \geq 2). In
297 order to find specificity of metabolic pathways associated with the three tenderness
298 quality traits, GO_BP analyses were performed respectively for each of the six
299 datasets.

300

301 **3. Results**

302 *3.1. Differential proteins between extreme groups of tenderness and their GO* 303 *annotations*

304 Shotgun analysis allowed to quantify 268 muscle proteins with a unique ID and
305 identified with at least 2 peptides in the LM and 136 proteins in the plasma of the
306 same heifers.

307 Among these proteins, 71 proteins in LM had significant differences (P<0.05) in
308 abundance between extreme groups of tenderness according to WB, Tg, and Idx
309 respectively (Table 2). In the plasma, 21 proteins were differential between the same
310 extreme groups of tenderness (Table 3).

311 Further investigation of pathways related to the proteins differentially abundant
312 was carried on by retrieving their Gene ontology (GO) annotation. GO analysis
313 revealed the biological pathways of these differential proteins in LM and in plasma as
314 illustrated in the Supplementary Data 1 and 2 respectively. The major biological
315 pathways are showed in Table 2 and Table 3 respectively. The five major metabolic
316 pathways associated with the 71 differential muscle proteins were "muscle contraction
317 and structure", "metabolism, transport and cell signalling", "muscle energy
318 metabolism", "regulation of cellular process (apoptosis, endocytosis, oxidative
319 stress)", and "autophagy". The four major metabolic pathways associated with the 21
320 differential plasma proteins were "muscle contraction and structure", "metabolism,
321 transport and signalling", "muscle energetic metabolism", and "immune system, cell
322 defence and homeostasis (angiogenesis, fibrinolysis, blood coagulation, aging)".

323

324 ***3.2. Muscle candidate biomarkers***

325 Among the 71 differential proteins in LM muscle, 26 proteins were significantly
326 differentially abundant between extreme groups of WB (P-value<0.5) (Table 2).
327 Supplementary Data 3 illustrates the values of the correlations (P-value<0.5) between
328 these proteins and the three tenderness traits. Among the 26 proteins the PLS analysis
329 revealed 8 proteins (VIP>1) (ACTN3, ADSSL1, CSRP3, HPX, MYL2, SMTNL1,
330 TNNC1 and TPM3) and 9 proteins (0.8<VIP<1) (ACTA1, ALDOA, EEF1A2, GPI,
331 GSTP1, MYOZ1, MYOZ3, PDLIM7 and PGAM2) which are considered as putative
332 candidate proteins usable for meat tenderness prediction (Figure 2a). The PCA in
333 Figure 2b illustrates that among the 17 proteins the most explicative of WB
334 tenderness (with VIP>0.8), ALDOA, PGAM2, GPI, GSPT1, MYOZ1, PDLIM7,
335 MYOZ3, ADSSL1, HPX, and ACTN3 were positively correlated with WB and

336 negatively with Tg and Idx on axis 1. On the the other hand, MYL2, TPM3, TNNC1,
337 EEF1A2, ACTA1, CSRP3, and SMTNL1 were positively correlated with Tg and Idx
338 and negatively with WB on axis 1. These results are in accordance with the values of
339 the correlation coefficient illustrated in the Supplementary Data 3. The Heat Map
340 representation of the 17 proteins shows a group of 10 proteins including ACTN3,
341 ADSSL1, MYOZ3, GSTP1, MYOZ1, ALDOA, PDLIM7, GPI, PGAM2, and HPX as
342 less abundant in the most tender LM and more abundant in the toughest. Conversely,
343 7 proteins including MYL2, TNNC1, TPM3, EEF1A2, ACTA1, SMTNL1, and
344 CSRP3 were more abundant in the most tender LM and less abundant in the toughest
345 LM. (Figure 3a)

346 Among the 71 differential proteins in LM muscle, 18 proteins were significantly
347 differentially abundant between extreme groups of Tg tenderness (Table 2). The
348 correlations between these proteins and the three tenderness traits are illustrated in
349 Supplementary Data 3. The PLS analysis revealed proteins ($VIP > 1$) (CAPZB GOT1,
350 MYBPH, NIPSNAP2, OGDH, OGN and VCL) and 6 proteins ($0.8 < VIP < 1$)
351 (ADSSL1, CKMT2, HBB, HSPA1L, SLC24A11 and TMOD1) which are considered
352 as putative candidate proteins usable for meat tenderness prediction (Figure 2c). The
353 PCA in Figure 2d illustrates that among these 13 proteins the most explicative of Tg
354 tenderness (with $VIP > 0.8$), VCL, TMOD1, OGN, and MYBPH were positively
355 correlated with WB and negatively with Tg and Idx on axis 1. Conversely,
356 NIPSNAP2, SLC24A11, HSPA1L, CAPZB, CKMT2, ADSSL1, GOT1, OGDH and
357 HBB were positively correlated with Tg and Idx and negatively with WB on axis 1.
358 The Heat Map representation (Figure 3c) of the 13 muscle VIP's proteins correlated
359 with Tg, a group of 4 proteins including OGN, VCL, TMOD1, and MYBPH as less
360 abundant in the most tender LM and more abundant in the toughest. On the contrary,

361 9 proteins including NIPSNAP2, SLC25A11, CKMT2, CAPZB, ADSSL1, GOT1,
362 HBB, OGDH, and HSPA1L were more abundant in the most tender LM and less
363 abundant in the toughest.

364 Among the 71 differential proteins in LM muscle, 37 proteins were significantly
365 differentially abundant between extreme groups of Idx tenderness (Table 2). The
366 correlations between the abundance of these proteins and the three tenderness traits
367 are illustrated in Supplementary Data 3. The PLS analysis revealed 15 proteins
368 ($VIP > 1$) (ACTN2, FABP3, FHL1, HSPB1, KLHL41, LMCD1, MYBPH, MYH1,
369 OGN, OLA1, PARK7, PDLIM7, PGAM2, PGM1 and TNNT3) and 17 proteins
370 ($0.8 < VIP < 1$) (ACTN3, AK1, ALDH1A1, ALDH2, ANKRD2, CFL2, CSRP3,
371 EEF1G, ENO3, GLO1, GPI, HSP90AA1, LDHB, MGST3, MYL6B, MYOT and
372 MYOZ2) which are considered as putative candidate proteins usable for meat
373 tenderness prediction (Figure 2e). The PCA in Figure 2f illustrates that among the 32
374 proteins the most explicative of Idx tenderness (with $VIP > 0.8$), PARK7, PGM1,
375 TNNT3, PGAM2, PDLIM7, GLO1, ACTN3, AK1, MYBPH, ENO3, GPI, ALDH2,
376 OGN, and MYH1 were positively correlated with WB and negatively with Tg and Idx
377 on axis 1. On the other hand, ANKRD2, HSPB1, MYOZ2, CSRP3, MYL6B, LDHB,
378 EEF1G, ACTN2, LMCD1, HSP90AA1, OLA1, MYOT, FABP3, KLHL41, CFL2,
379 ALDH1A1, MGST3, and FHL1 were positively correlated with Tg and Idx and
380 negatively with WB on axis 1. The Heat Map representation (Figure 3e) of the 32
381 muscle VIP's proteins illustrates, a group of 14 proteins including PGM1, TNNT3,
382 PARK7, ALDH2, PGAM2, GLO1, GPI, AK1, ENO3, MYH1, MYBPH, PDLIM7,
383 ACTN3, and OGN, less abundant in the most tender LM and more abundant in the
384 toughest. Conversely, 18 proteins including ANKRD2, HSPB1, OLA1, LDHB,
385 ACTN2, LMCD1, MYOZ2, MYL6B, EEF1G, CSRP3, KLHL41, HSP90AA1,

386 MYOT, FABP3, CFL2, MGST3, ALDH1A1, and FHL1 were more abundant in the
387 most tender LM and less abundant in the toughest LM.

388 Seven of these proteins (FHL1, CSRP3, ACTN3, PDLIM7, PGAM2, KLHL41,
389 GPI) were common to the WB and Idx, one protein (ADSSL1) was common to the
390 WB and Tg, and two proteins (MYBPH, OGN) were common to the Tg and Idx. As
391 expected, the correlation tables (Supplementary Data 3) and PCAs (Figures 2) showed
392 an opposition between WB and Tg and Idx tenderness. They also showed a positive
393 correlation between Idx and Tg and a negative correlation between Idx and WB.
394 Interestingly, the proteins identified as differential for a tenderness trait are correlated
395 (inversely) with the three tenderness traits (Supplementary Data 3) which confirms
396 their involvement in tenderness whatever the method of evaluation.

397

398 **3.3. Plasma candidate biomarkers**

399 Among the 21 differential proteins in the plasma, 10 were significantly (P-
400 value<0.5) differentially abundant between extreme groups of WB tenderness (Table
401 3). The coefficients of correlation (P-value<0.5) between the abundances of these
402 proteins and the three tenderness traits are illustrated in Supplementary Data 4. The
403 PLS analysis revealed 5 proteins (VIP>1) (MYH7, PLA2G2D5, ENO3, CFH and
404 AGT) and 3 proteins (0.8<VIP<1) (SHBG, RNASE4 and GAPDH) which are
405 identified as putative candidate protein usable for meat tenderness prediction (Figure
406 4a). The PCA in Figure 4b illustrated that among the 8 proteins the most explicative
407 of WB tenderness (with VIP>0.8), SDHB, RNASE4, and AGT were positively
408 correlated with WB and negatively with Tg and Idx on axis 1. On the other hand,
409 GAPDH, ENO3, CFH, PLA2G2D5, and MYH7 were positively correlated with Tg
410 and Idx and negatively with WB on axis 1. The Heat Map representation (Figure 3b)

411 of the 8 plasma VIP's proteins correlated with WB shows a group of 3 proteins
412 including RNASE4, AGT and SHBG less abundant in the most tender LM and more
413 abundant in the toughest. Conversely, 5 proteins including ENO3, CFH, PLA2G5D5,
414 MYH7 and GAPDH were more abundant in the most tender LM and less abundant in
415 the toughest LM.

416 Among the 21 differential proteins in the plasma, 7 proteins were significantly
417 (P-value<0.5) differentially abundant between extreme groups of Tg tenderness
418 (Table 3). The coefficients of correlation (P-value<0.5) between the abundances of
419 these proteins and the three tenderness traits are illustrated in Supplementary Data 4.
420 The PLS analysis revealed 3 proteins (VIP>1) (FHL1, GAPDH and MASP2) and 2
421 proteins (0.8<VIP<1) (SERPIND1 and F13B) which are identified as putative
422 candidate proteins usable for meat tenderness prediction (Figure 4c). The PCA in
423 Figure 4d illustrated that among the 5 proteins the most explicative of Tg tenderness
424 (with VIP>0.8), FHL1, GAPDH, and SERPIND1 were positively correlated with WB
425 and negatively with Tg and Idx on axis 1. On the contrary, F13B and MASP2 were
426 positively correlated with Tg and Idx and negatively with WB on axis 1. The Heat
427 Map representation (Figure 3d) of the 5 plasma VIP's proteins correlated with Tg
428 shows a group of 3 proteins including SERPIND1, GAPDH, and FHL1 were less
429 abundant in the most tender LM and more abundant in the toughest. Conversely,
430 F13B and MASP2 were more abundant in the most tender LM and less abundant in
431 the toughest LM.

432 Among the 21 differential proteins in the plasma, 7 proteins were significantly
433 (P-value<0.5) differentially abundant between extreme groups of Idx tenderness
434 (Table 3). The coefficients of correlation (P-value<0.5) between the abundances of
435 these proteins and the three tenderness traits are illustrated in Supplementary Data 4.

436 The PLS analysis revealed 4 proteins (VIP>1) (SERPINF2, MASP2, SERPING1 and
437 CFH) and 3 proteins (0.8<VIP<1) (GPX3, HRG and F9) which are identified as
438 putative candidate proteins usable for meat tenderness prediction (Figure 4e). The
439 PCA in Figure 4f illustrated that among the 7 proteins the most explicative of Idx
440 tenderness (with VIP>0.8), SERPINF2, HRG, and SERPING1 were positively
441 correlated with WB and negatively with Tg and Idx on axis 1. On the contrary,
442 MASP2, CFH, GPX3 and F9 were positively correlated with Tg and Idx and
443 negatively with WB on axis 1. The Heat Map representation (Figure 3f) of the 7
444 plasma VIP's proteins correlated with Idx shows a group of 3 proteins including
445 SERPINF2, SERPING1, and HRG were less abundant in the more tender LM and
446 more abundant in the toughest. Conversely, 4 proteins including GPX3, MASP2, F9,
447 and CFH were more abundant in the most tender LM and less abundant in the
448 toughest LM.

449 One of these proteins CFH was differentially abundant between extreme groups
450 of WB and Idx, one protein GAPDH was differentially abundant between extreme
451 groups of WB and Tg, and one protein MASP2 differentially abundant between
452 extreme groups to Tg and Idx.

453

454 ***3.4. Linear multiple regression models***

455 The most explanatory proteins of tenderness with VIP >0.8 have been used in a
456 linear multiple regression to predict each tenderness trait in the whole dataset from 20
457 heifers. The Table 4 illustrates the most accurate equations and the proteins retained
458 in these equations.

459 ***Muscle most robust tenderness proteins.*** For WB, a total of 7 proteins are involved in
460 the linear multiple regression models (SMTNL1, ADSSL1, HPX, ACTN3, TPM3,

461 CSRP3, and TNNC1) of which 5 proteins were identified with a high weight of
462 tenderness explanation, putative candidate biomarkers as they explained alone a large
463 part of WB tenderness variability (Table 4). For Tg, a total of 6 proteins are involved
464 in the linear multiple regression models (OGN, MYBPH, GOT1, VCL, OGDH, and
465 CAPZB), of which 4 proteins were identified with a high weight for tenderness
466 explanation, putative candidate biomarkers explaining alone a large part of Tg
467 tenderness variability (Table 4). For Idx, a total of 5 proteins are involved in the linear
468 multiple regression models (MYH1, ACTN2, MYBPH, OGN and PGAM2), of which
469 one protein (PGAM2) was identified with a high weight for tenderness explanation,
470 putative candidate biomarker as they explained alone a large part of Idx tenderness
471 variability (Table 4).

472 ***Plasma most robust tenderness proteins.*** For WB, a total of 4 proteins are involved
473 in the linear multiple regression models (ENO3, PLA2G2D5, CFH and MYH7), on
474 which 2 proteins (CFH and MYH7) were identified with a high weight of tenderness
475 explanation, putative candidate biomarkers explaining alone a large part of WB
476 tenderness variability (Table 4). For Tg, a total of 3 proteins are involved in the linear
477 multiple regression models (FHL1, GAPDH and MASP2), of which MASP2 protein
478 was identified with a high weight of tenderness explanation, putative candidate
479 biomarker explaining alone a large part of Tg tenderness variability (Table 4). For
480 Idx, a total of 3 proteins are involved in the linear multiple regression models
481 (MASP2, CFH and SERPINF2), of which the 2 proteins (CFH and SERPINF2) were
482 identified with a high weight for tenderness explanation, putative candidate
483 biomarkers explaining alone a large part of Idx tenderness variability (Table 4).

484 These results obtained on 20 samples validated the results obtained with the
485 comparison of the 5-5 extreme groups. They allowed identifying among the candidate

486 biomarkers the most robust ones and therefore the most promising ones for the three
487 tenderness traits.

488

489 **4. Discussion**

490 The prediction and management of the phenotypic traits related to meat production
491 and quality, especially meat tenderness, are a top priority for the beef meat industry.
492 Previous studies have investigated the potential of muscle-derived protein biomarkers
493 for meat quality prediction. However, the muscle biomarkers may not be exhaustive
494 nor generic for predicting the tenderness [32,33].

495 The search for meat tenderness biomarkers carried out in the last two decades was
496 based mainly on 2-DE- proteomics of muscle samples (see [34] for review). However,
497 although the 2-DE is a very resolute method and allows to reveal post-translational
498 modifications, it has some limitations [35]. It allows the separation of complex
499 mixtures of proteins according to their isoelectric point, molecular mass, solubility,
500 and relative abundance. Thus, it enables detecting exclusively acidic or basic proteins.
501 Very hydrophobic proteins, and in particular membrane proteins are under-
502 represented in 2-DE, as well as the extremely acid or basic proteins (with extreme
503 isoelectric points) [36]. The shotgun method helps overcome the limitations described
504 above, by enabling the detection of a greater range of acidic, basic and hydrophobic
505 proteins simultaneously [37,38]. Therefore, we assumed that the shotgun method
506 would allow to identify a more exhaustive list of muscle biomarkers and also be more
507 suitable for the identification of tenderness biomarkers from plasma, which would
508 allow us to overcome the muscle type effect reported for several biomarkers.

509

510 **4.1. Candidate muscle biomarkers for tenderness**

511 As illustrated in Figure 5, the analysis of LM proteins by shotgun proteomics in
512 the present study allowed to confirm some biomarkers (n=33) and to identify proteins
513 (n=38) which were not reported previously in the literature as related with tenderness.
514 The Venn diagram (Figure 5) illustrates the comparison of the list of putative
515 candidate biomarkers of tenderness published in the literature, identified by proteomic
516 or transcriptomic analysis (Boudon, personal communication), with the list of the 71
517 candidate biomarkers of tenderness arising from of the present study.

518 The new candidate biomarkers are mainly involved in muscle contraction and
519 structure but also in muscle energy metabolism, *post-mortem* muscle proteolysis
520 (apoptosis, autophagy) and oxidative stress processes (Figure 5). These results are in
521 accordance with the knowledge gained from previous studies [7,33,39]. The new
522 proteins correspond mainly to isoforms of cytoskeletal proteins, new proteins
523 involved in the biological pathways cited previously but also several new proteins
524 involved in metabolism, transport and cell signalling, pathways more generally. These
525 proteins enriched existing knowledge for a better understanding of the mechanisms
526 involved in beef tenderness determinism.

527 The differences between the results of the present study and the data of the
528 literature could be explained by the proteomic technique used but also by the type of
529 animal studied. The innovative shotgun approach used here for quality traits
530 investigation seems to be a good method to obtain a more exhaustive list of muscle
531 putative tenderness biomarkers. Moreover, the present study was carried on heifers,
532 while the majority of published studies related to meat tenderness were carried on
533 young male and few in cows or steers [40]. The present study shows that the
534 candidate biomarkers of tenderness quantified in the *Longissimus* muscle are in
535 common between heifers and other bovine types. These results validate the list of 33

536 previously identified proteins as good potential biomarkers in the *Longissimus* muscle
537 of heifers. In addition, for 11 of these candidate biomarkers were detected as located
538 in a bovine QTL for shear force or tenderness score (results of ProteINSIDE analysis
539 with the private module proteoQTL, <http://www.proteinside.org/>) (Figure 5), which
540 reinforces the relevance of our results.

541 Among the 33 proteins previously reported as tenderness biomarkers (Figure 5), 7
542 proteins (CAPZB, MYBPH, MYH1, ACTN3, CSRP3, PGAM2 and TPM3) could be
543 proposed as robust candidates because they were both identified with a differential
544 abundance between extreme groups of tenderness, significantly correlated with
545 tenderness, identified as VIP's proteins and found in the linear regression performed
546 on the 20 animals of the study. More specifically, the F-actin-capping protein subunit
547 β (CAPZB) identified as positively related with Tg is a capping protein of the thin
548 actin filament which plays a role in thin filament organisation [41,42]. The CAPZB
549 protein was previously described as positively correlated with LM tenderness in
550 bovine and porcine species [9,43,44] in accordance with our study. The Myosin
551 Binding Protein H (MYBPH) identified as negatively related with Tg and Idx was
552 previously described to be negatively correlated with LM tenderness [9,45,46] as
553 observed in the present study. This sarcomere protein known to interact with the thick
554 myosin filament is higher expressed in fast glycolytic fibres. So the negative relation
555 with tenderness found here is consistent with the negative relation observed for
556 Myosin heavy chain-IIx (MYH1), expressed in fast glycolytic fibres as MYBPH [47]
557 This contractile protein, fragmented and released during aging and tenderization, was
558 previously described as a putative proteolysis indicator [48]. As found in the present
559 study, MyHC IIx was described to be negatively correlated with LM tenderness in
560 Charolais and Maine-Anjou cows breeds [40]. However, in the *Semitendinosus* (ST)

561 muscle from French beef breeds it was described to be positively correlated with
562 tenderness. This inverse relationships between some contractile proteins and
563 tenderness have been validated by several experiments [9,40]. The α -actinin 3
564 (ACTN3) identified as positively related with WB and negatively with Idx is an actin-
565 binding protein specifically expressed in fast skeletal muscle fibres. In young bulls,
566 the direction of the correlation depends on muscle type: positively correlated in the
567 fast glycolytic muscle (ST) and negatively correlated in the fast oxido-glycolytic
568 muscle (LT) [9,22] as mentioned previously for the two other fast glycolytic proteins.
569 The Cysteine and glycine-rich protein 3 (CSRP3) identified as negatively related with
570 WB and positively with Idx in the present study was previously described as
571 unfavourable for beef quality including tenderness, juiciness and flavour by a
572 transcriptomic approach in LM from Charolais young bulls [49]. However, this
573 protein has never been found in two-dimensional electrophoresis (2-DE). It is the first
574 time that we described a relation between its abundance and the tenderness. This
575 protein regulates the control of muscle structure, development and cellular
576 differentiation processes. Interestingly, by combination with data about published
577 tenderness QTL (ProteoQTL module of ProteINSIDE), ACTN3 is annotated as
578 included in a tenderness score QTL (Chromosome 29) and CSRP3 in a Shear force
579 QTL (Chromosome 29) (Figure 5) supporting that these two proteins would be a good
580 predictor for beef tenderness. Finally, the Tropomyosin α -3 chain (TPM3) identified
581 as negatively correlated with WB (positively with tenderness) is one of the
582 myofibrillar proteins expressed in slow skeletal muscle [60]. This protein composing
583 a dimer with the TPM2 isoform was described in [58] as favourable with high sensory
584 tenderness quality trait, in accordance with our study. The phosphoglycerate mutase 2
585 (PGAM2) identified as positively related with WB and negatively with Idx was also

586 found to be related with tenderness of *Longissimus* from steers Aberdeen Angus cattle
587 [50]. This protein plays an important role in coordinating energy production with
588 generation of reducing power and the biosynthesis of nucleotide precursors and amino
589 acids. A link of PGAM2 with carcass traits, and especially *post-mortem* maturation
590 processes, was described in an association study performed in 15 breeds of cattle
591 using 389 SNP belonging to 206 candidate genes known to be involved in muscle
592 development, metabolism and structure [51].

593 These data confirm a positive relationship between tenderness and some
594 contractile proteins of the slow oxidative type and a negative one with some
595 contractile proteins of the fast glycolytic type in the *Longissimus* muscle. They
596 validate also the high implication of contractile proteins in the tenderness of LM
597 comparatively to ST as described in [40]. Furthermore, the results showed that these
598 relationships are the same in heifers and in other animal types.

599 Among the 38 muscle proteins newly identified in this study thanks to shotgun
600 proteomics (Figure 5), 8 proteins (ACTN2, ADSSL1, GOT1, HPX, OGDH, OGN,
601 TNNC1 and VCL) are proposed as robust candidates because they are identified as a
602 differentially abundant between extreme groups of tenderness, but also significantly
603 correlated with tenderness, identified as VIP's proteins and found in the linear
604 regression performed on all 20 animals (Supplementary Data 4). More specifically,
605 the α -actinin 2 (ACTN2), identified as positively correlated with Idx, is an actin-
606 binding protein as ACTN3 but expressed in both slow and fast skeletal muscle fibres.
607 This protein was described as negatively related with intramuscular fat content [52].
608 The Adenylosuccinate synthetase isozyme 1 (ADSSL1), identified as positively
609 related with WB, is a protein linked to glycolytic energy metabolism and is described
610 as overabundant in *Longissimus lumborum* muscle of Chinese cattle during *post-*

611 *mortem* periods [53]. The Glutamic-oxaloacetic transaminase 1 (GOT1), identified as
612 positively correlated with Tg, plays a role in amino acid metabolism, glutamate
613 synthesis and the urea and tricarboxylic acid cycles. [54]. Also associated with lipid
614 metabolism and deposit [55], GOT1 would be a good biomarker for meat tenderness
615 (positive correlation) since intramuscular lipid depots are generally positively
616 correlated with meat tenderness [1,56]. The Hemopexin protein (HPX), identified as
617 positively related with WB (negatively with tenderness), is the main vehicle for the
618 heme transport in the plasma which allows to prevent the heme-mediation oxidative
619 stress [57]. Also associated with iron metabolism, this protein was proposed as a
620 biomarker for water-holding capacity in pork meat [58]. Lastly, the Mimecan protein
621 (OGN), identified as negatively related with Tg and Idx, is associated with regulation
622 of the type I collagen fibrillogenesis and its modulation [59]. The protein is produced
623 by muscle tissues and is putatively a crucial humoral bone anabolic factor [60]. OGN
624 is located in a region which corresponds to the QTL interval for several carcass traits
625 significant in pork [61,62]. Interestingly, OGN is also included in a Shear force QTL
626 in cattle as HPX and TNNC1 (Figure 5) supporting that these proteins would be good
627 candidate biomarkers of *Longissimus* meat tenderness.

628 **4.2. Putative plasma biomarkers of tenderness**

629 In the present study we report for the first time 21 proteins with differential
630 abundance in the plasma of heifers differing by the tenderness of their *Longissimus*
631 muscle.

632 Overall view of the major biological pathways related to these plasma proteins
633 shows the several pathways identified in previous research performed from muscle
634 samples and described above [5,6,44,63–69] but also the signalling pathways of the
635 complement system and coagulation which has been expected considering plasma

636 fluid. However, we hypothesized that the cytoskeletal proteins such as Myosin heavy
637 chain-I (MYH7), Tubulin alpha-4A chain (TUBA4A), or β -actin (ACTB) may be
638 proteolytic fragments of proteins excreted by muscle cells. These proteins are not
639 soluble proteins and by consequent are likely not secreted through conventional
640 secretory pathways. However it cannot be excluded that they were secreted through a
641 new secretory pathway. According to [70], muscle cells would be able to secrete
642 proteins through a newly described secretory membrane-derived vesicles shedding
643 and addressed to the plasma membrane [70–72]. During the last decade, the
644 extracellular vesicles (EVs) whether as micro-particles (MPs; 150–300 nm size) or
645 exosomes, 50–100 nm size) have emerged as an important mechanism involved in
646 inter-cellular communication in normal physiologic condition (e.g. heart and muscle
647 development, angiogenesis, and vesicle formation during reticulocyte maturation [73–
648 76]) but also in pathophysiological conditions [77–79]. Recent studies have shown
649 that skeletal muscle is also able to release EVs into the extracellular space [70,80].
650 According to the authors, muscles crosstalk with tissues and organs through this
651 mechanism and participate to maintain muscle physiology and whole-body
652 homeostasis [81–83].

653 Among the 21 plasma proteins identified as discriminant between two groups of
654 tenderness, 8 appeared to be robust proteins (CFH, ENO3, FHL1, GAPDH, MASP2,
655 MYH7, PLA2G2D5 and SERPINF2) because identified as differential abundant
656 proteins, but also significantly correlated with tenderness, identified as VIP's proteins
657 and found in the linear regression performed on all 20 animals. More specifically, the
658 complement factor H (CFH), identified as negatively related with WB and positively
659 correlated with Idx, was linked to the signalling pathways of the complement system
660 and coagulation. The β -enolase protein (ENO3) identified as negatively correlated

661 with WB (positively with tenderness) and the Four and a half LIM domains 1 protein
662 (FHL1), identified as negatively correlated with Tg, showed an inverse relationship
663 compared to the muscle sample. The β -enolase protein (ENO3), and the
664 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), identified as negatively
665 related with Tg, are glycolytic enzymes described in muscle as energy metabolism
666 *post-mortem* indicator in the same direction [84,85]. Interestingly, a physiological
667 target of serpin C1-inhibitor, the Mannan-binding lectin serine protease 2 (MASP2),
668 identified as positively correlated with Tg an Idx, is involved in the activation of the
669 complement cascade via the Mannan-binding Lectin (MBL) pathway, a part of the
670 innate immune defence [86]. No previous study has reported any association of
671 MASP2 with muscle phenotype nor for meat tenderness in livestock. The Calcium-
672 dependent phospholipase A2 (PLA2G2D5), identified as negatively correlated with
673 WB (positively with tenderness), is a myotoxic protein previously identified in snake
674 venom and able to affect the sarcoplasmic reticulum *in vivo*. This protein binds to
675 several receptors in muscle plasma membrane and disrupts them but also induces
676 myofibrillar alterations [87]. With regards to tenderness evaluation, this protein, also
677 called PLA2s, seems to be a good putative biomarker for meat tenderness prediction.
678 Finally, among the number of differential Serine Protease Inhibitors (SERPINs)
679 isoforms found in this analysis, the most robust Alpha-2-antiplasmin protein
680 (SERPINF2) is identified as negatively related with Idx. The SERPINF2, a paralog of
681 the SERPING1, is involved in regulation of proteolysis in response to heat stress. The
682 SERPINs act as inhibitors of their target proteases by a specific mechanism and some
683 isoforms (SERPINA3 notably) were described as related to meat tenderness in
684 previous studies [16,88–90].

685 Interestingly, the three proteins ENO3, FHL1 and MYH7 are found in muscle
686 but also in plasma. As described previously these proteins are identified as tenderness
687 biomarkers. Although we observed an inverse correlation with tenderness between
688 muscle and plasma abundances, we can hypothesize that these observations are
689 probably due to putative complex regulation in organism, but could be reflect of the
690 status of tenderness biomarker in muscle. The perspectives of this study are to test the
691 relationship between abundances of these proteins and tenderness ranking on a larger
692 panel of individuals.

693

694 **5. Conclusion**

695 This study is one of the first to use shotgun proteomic approach for the identification
696 of muscle biomarkers related to meat tenderness and the first to research candidate
697 tenderness biomarkers in the plasma. It is also to our knowledge, the first proteomic
698 study in heifers. The main results allowed to validate and complete the list of putative
699 biomarkers of tenderness in LM. Among the 38 new candidates, 8 are proposed as
700 robust candidates for further analysis. The most original result of this study is the
701 detection of 21 proteins of which the abundance in the plasma is related with LM
702 tenderness. Among them 9 proteins are considered as robust candidates. Further
703 analyses are needed to evaluate on a large scale the relationship between their
704 abundance in the plasma and the tenderness of LM. Finally the validated biomarkers of
705 tenderness could be used in diagnostic tool to evaluate or predict the potential of
706 tenderness on living cattle.

707 **Author contributions**

708 SB, ICM and BP defined the experimental design, managed the experiment, co-wrote
709 the paper, and approved the final draft of the manuscript. SB managed and analyzed the

710 data, prepared figures and/or tables. DO and SB performed the statistical analysis. VM
711 designed and co-managed the experiments. DV coordinated shotgun analysis. All
712 authors collaborated to interpretation and discussion of the results. All authors have
713 given approval to the final version of the manuscript.

714 **Conflict of interest**

715 The authors declare no competing financial interest

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1058

1059

Tables and Figures

1060 **Figure captions**

1061 **Figure 1.** Flowchart of the workflow applied for the identification of candidate
1062 biomarkers for beef tenderness in the muscle and plasma samples of heifers using a
1063 Label free shotgun proteomics.

1064 PGI (protected geographical indication)

1065

1066 **Figure 2.** PLS_VIP analysis and principal component analysis of the muscle VIP's
1067 proteins identified in LM between extreme groups of tenderness.

1068 PLS_VIP analyses are shown in a, c, and e. Principal Component Analyses (PCA) are
1069 shown in b, d, and f. Only the proteins with VIP >0.8 are illustrated in PCA, the VIP's
1070 proteins >1 are underlined. Distribution of the VIP's proteins was performed for the
1071 three meat tenderness evaluations. WB: a, b ; Tg: c, d ; Idx: e, f. WB stands for

1072 mechanical tenderness evaluation, Tg stands for sensory tenderness evaluation, Idx
1073 stands for synthetic tenderness index.

1074

1075 **Figure 3.** Heat Map representation of the VIP's proteins identified in this study.
1076 VIP's proteins from muscle are shown in a, c and e. VIP's proteins from plasma are
1077 shown in b, d and f. Only the proteins with VIP >0.8 are illustrated in Heat Map, the
1078 VIP's proteins >1 are underlined. Heat Map representation was performed for the three
1079 meat tenderness evaluations. WB: a, b; Tg: c, d; Idx: e, f. WB stands for mechanical
1080 tenderness evaluation, Tg stands for sensory tenderness evaluation, Idx stands for
1081 synthetic tenderness index. Hierarchical clustering of standardized proteomic data
1082 enables to visually group of proteins showing an increased abundance in the high
1083 tenderness group, or a decreased abundance in the low tenderness group. The color
1084 code allowed to visualize highly-abundance proteins in red and low-abundance
1085 proteins in blue.

1086

1087 **Figure 4:** PLS_VIP analysis and principal component analysis of the plasma VIP's
1088 proteins between extreme groups of tenderness.

1089 PLS_VIP analysis are shown in a, c and e. Principal Component Analysis (PCA) are
1090 shown in b, d and f. Only the proteins with VIP >0.8 are illustrated in PCA, the VIP
1091 proteins >1 are underlined. Distribution of the VIP's proteins was performed for the
1092 three meat tenderness evaluations. WB: a, b; Tg: c, d; Idx: e, f. WB stands for
1093 mechanical tenderness evaluation, Tg stands for sensory tenderness evaluation, Idx
1094 stands for synthetic tenderness index.

1095

1096 **Figure 5.** Comparison of the list of proteins identified in this study with the list of
1097 candidate biomarkers reported in previous studies.

1098 The Venn diagram shows the intersect of an atlas of proteins related to tenderness in
1099 the literature with the list of differential proteins reported in Table 2. The proteins in
1100 common to both datasets are validated as good candidate biomarkers in heifers. The
1101 proteins specific to the present study are new promising candidate biomarkers.

1102 Query of genetic information was performed with the ProteQTL module included in
1103 ProteINSIDE in order to retrieve information on the location of the genes encoding
1104 proteins of interest within published Quantitative trait *loci* (QTL) for tenderness. This
1105 module interrogates a publicly available QTL library in Animal QTL database [91]
1106 that contains cattle QTL and the published data associated.

1107 **Supplementary data 1.** Gene Ontology enrichment analysis of the 71 differential
1108 muscle proteins.

1109 **Supplementary data 2.** Gene Ontology enrichment analysis of the 21 differential
1110 plasma proteins.

1111 **Supplementary data 3.** Table of Spearman correlations between the differential
1112 abundance of the muscle proteins identified in this study.

1113 Correlation analysis was performed with the differential proteins detected in the LM.
1114 The correlation was performed on the groups of extreme (5 tender /5 tough) for the
1115 three meat tenderness evaluations (WB, Tg and Idx). WB stands for mechanical
1116 tenderness evaluation, Tg stands for sensory tenderness evaluation, Idx stands for
1117 synthetic tenderness index.

1118 **Supplementary data 4.** Table of Spearman correlations between the differential
1119 abundance of the plasma proteins identified in this study.

1120 Correlation analysis was performed with the differential proteins detected in the
1121 plasma. The correlation was performed on the groups of extreme (5 tender /5 tough)
1122 for the three meat tenderness evaluations (WB, Tg and Idx). WB stands for
1123 mechanical tenderness evaluation, Tg stands for sensory tenderness evaluation, Idx
1124 stands for synthetic tenderness index.

1125 **Table 1.** Description of extreme groups of tenderness for the three considered traits: Warner-Bratzler shear-force (WB), score of global
 1126 tenderness evaluated by sensory analysis with trained panellists (Tg), and a synthetic index combining the both (Idx).

Tenderness evaluation	T+ group (n=5) (Mean \pm σ)	T- group (n=5) (Mean \pm σ)	P-values
WB	33.74 \pm 3.47	69.73 \pm 11.94	4.30E-04
Tg	7.72 \pm 0.16	6.31 \pm 0.25	3,05E-06
Idx	1.37 \pm 0.15	-1.58 \pm 0.44	1,78E-05

1127

1128 Values are expressed as the means \pm standard deviation (σ). P-values were calculated by Student's t-test between the tough groups vs. the tender
 1129 groups. T+/ T- stands for the tender and tough groups respectively, n : number of heifers.

1130

1131 **Table 2.** List of the 71 differential proteins detected in the *Longissimus* muscle using the shotgun technique. The major Gene Ontology
 1132 annotation terms were retrieved using ProteINSIDE.
 1133

Differential proteins			WB	Tg	Idx
Accession Number	Gene Name	Full name	P-value	P-value	P-value
<i>Muscle contraction and structure</i>					
P68138	ACTA1	Actin, alpha skeletal muscle	1.35E-02		
Q3ZC55	ACTN2	Alpha-actinin-2			2.53E-03
Q0III9	ACTN3	Alpha-actinin-3	4.71E-02		4.22E-02
P79136	CAPZB	F-actin-capping protein subunit beta		5.69E-03	
Q148F1	CFL2	Cofilin-2			4.62E-02
Q4U0T9	CSRP3	Cysteine and glycine-rich protein	2.90E-03		5.70E-03
E1BE25	FLNC	Filamin-C	3.49E-02		
A4FV78	KLHL41	Kelch-like protein 41	7.75E-03		9.19E-03
G3X6W9	MYBPH	Myosin-binding protein H		7.54E-03	1.91E-03

Q9BE40	MYH1	Myosin-1			5.68E-03
Q9BE39	MYH7	Myosin-7	3.22E-02		
F1ME15	MYL2	Myosin regulatory light chain 2	3.93E-02		
Q148H2	MYL6B	Myosin, light chain 6B			6.58E-03
E1BCU2	MYOM3	Myomesin-3			4.31E-02
F1MPU4	MYOT	Myotilin			4.28E-02
Q8SQ24	MYOZ1	Myozenin-1	2.37E-02		
Q5E9V3	MYOZ2	Myozenin-2			1.56E-02
F1N0W6	MYOZ3	Myozenin-3	3.14E-02		
A6H7E3	PDLIM1	PDZ and LIM domain 1		1.05E-02	
Q3SX40	PDLIM7	PDZ and LIM domain protein 7	2.53E-02		6.88E-03
E1BPV6	SMTNL1	Smoothelin-like protein 1	4.21E-02		
A0JNC0	TMOD1	Tropomodulin-1		4.24E-02	
P63315	TNNC1	Troponin C, slow skeletal and cardiac muscles	5.79E-03		
Q8MKH7	TNNT3	Troponin T, fast skeletal muscle			1.44E-03
Q5KR47	TPM3	Tropomyosin alpha-3 chain	4.59E-02		

F1N789	VCL	Vinculin		3.86E-02	
<i>Metabolism, transport and cell signalling</i>					
A1L578	CAVIN1	Caveolae-associated protein 1	3.40E-02		
Q32PH8	EEF1A2	Elongation factor 1-alpha 2	2.44E-02		
F1MG05	EEF1G	Elongation factor 1-gamma			3.10E-02
F1MR86	FHL1	Four and a half LIM domains protein 1	2.66E-02		9.95E-03
A4FUZ1	GLO1	Lactoylglutathione lyase			2.26E-02
A1A4R1	HIST2H2AC	Histone H2A type 2			4.65E-02
Q17QE2	LMCD1	LIM and cysteine-rich domains protein 1			6.40E-03
Q32KP9	NUTF2	Nuclear transport factor 2		4.06E-02	
<i>Muscle energy metabolism</i>					
P00570	AK1	Adenylate kinase isoenzyme 1			4.88E-02
P48644	ALDH1A1	Retinal dehydrogenase 1			1.90E-02
P20000	ALDH2	Aldehyde dehydrogenase, mitochondrial			3.89E-02
A6QLL8	ALDOA	Fructose-bisphosphate aldolase	4.80E-02		
F1MJT6	CKMT2	Creatine kinase S-type, mitochondrial		4.78E-02	

A6QR19	ENO2	Gamma-enolase			1.95E-02
Q3ZC09	ENO3	Beta-enolase			2.02E-02
P10790	FABP3	Fatty acid-binding protein, heart			4.25E-03
Q5EA88	GPD1	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	2.62E-02		
Q3ZBD7	GPI	Glucose-6-phosphate isomerase	2.35E-02		2.17E-02
Q5E9B1	LDHB	L-lactate dehydrogenase B chain			2.83E-02
Q08DP0	PGM1	Phosphoglucomutase-1			2.02E-02
Q3SWX4	NIPSNAP2	Protein NipSnap homolog 2		1.01E-02	
P11024	NNT	NAD(P) transhydrogenase, mitochondrial		4.90E-02	
Q148N0	OGDH	2-oxoglutarate dehydrogenase, mitochondrial		1.19E-02	
Q2HJ33	OLA1	Obg-like ATPase 1			5.32E-03
F1N2F2	PGAM2	Phosphoglycerate mutase	2.02E-02		1.00E-02
P22292	SLC25A11	Mitochondrial 2-oxoglutarate/malate carrier protein		1.17E-02	
<i>Regulation of cellular process (apoptosis, endocytosis, oxidative stress)</i>					
A5PJR4	ADSSL1	Adenylosuccinate synthetase isozyme 1	2.72E-02	3.94E-02	
A0A140T897	ALB	Serum albumin	4.21E-02		

F1MX12	ANKRD2	Ankyrin repeat domain-containing protein 2			4.08E-02
P02510	CRYAB	Alpha-crystallin B chain			4.28E-02
G3N0V0	G3N0V0	Uncharacterized protein	1.34E-02		
P33097	GOT1	Aspartate aminotransferase, cytoplasmic		5.51E-03	
P28801	GSTP1	Glutathione S-transferase P	3.91E-02		
P02070	HBB	Hemoglobin subunit beta		1.30E-02	
P62958	HINT1	Histidine triad nucleotide-binding protein 1		3.89E-02	
Q3SZV7	HPX	Hemopexin	9.51E-03		
Q76LV2	HSP90AA1	Heat shock protein HSP 90-alpha			4.50E-02
P0CB32	HSPA1L	Heat shock 70 kDa protein 1-like		4.05E-02	
Q3T149	HSPB1	Heat shock protein beta-1			2.76E-02
Q3T100	MGST3	Microsomal glutathione S-transferase 3			9.03E-03
G3N088	OGN	Mimecan		6.42E-03	3.15E-02
Q5E946	PARK7	Protein DJ-1			1.22E-02
P68002	VDAC2	Voltage-dependent anion-selective channel protein 2	3.59E-02		
<i>Autophagy</i>					

A5PK37	EPM2A	Laforin			3.84E-02
E1BAJ4	STBD1	Starch-binding domain-containing protein 1		3.23E-02	

1134

1135 We report muscle proteins identified with a significant differential abundance (P-value < 0.05) between the tough group minus the tender group.

1136 WB stands for mechanical tenderness evaluation, Tg stands for sensory tenderness evaluation, Idx stands for synthetic tenderness index.

1137 **Table 3.** List of the 21 differential proteins detected in the plasma using the shotgun technique. The major Gene Ontology annotation terms were
 1138 retrieved using ProteINSIDE.
 1139

Differential proteins			WB	Tg	Idx
Accession Number	Gene Name	Full name	P-value	P-value	P-value
<i>Muscle contraction and structure</i>					
F1MM07	MYH7	Myosin-7	4.91E-02		
P81948	TUBA4A	Tubulin alpha-4A chain	4.60E-02		
P60712	ACTB	Actin, cytoplasmic 1		3.47E-02	
<i>Metabolism, transport and cell signalling</i>					
F1MR86	FHL1	Four and a half LIM domains 1		6.31E-04	
F1MQ77	PLA2G2D5	Phospholipase A(2	1.57E-02		
Q58DP6	RNASE4	Ribonuclease 4	4.07E-02		
<i>Muscle energy metabolism</i>					
Q3ZC09	ENO3	Beta-enolase	5.74E-03		

P10096	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	4.47E-02	4.06E-03	
<i>Immune system, cell defence and homeostasis (angiogenesis, fibrinolysis, blood coagulation, aging)</i>					
Q3SZH5	AGT	Angiotensinogen	4.04E-02		
Q28085	CFH	Complement factor H	4.88E-02		7.98E-03
Q2TBQ1	F13B	Coagulation factor XIII		2.51E-02	
F1MBC5	F9	Coagulation factor IX			4.09E-02
F1MKS5	HRG	Histidine-rich glycoprotein			4.95E-02
F1MNN7	LBP	Lipopolysaccharide-binding protein	3.20E-02		
E1BJ49	MASP2	Mannan-binding lectin serine protease 2		1.66E-02	3.48E-02
A6QPP2	SERPIND1	Heparin cofactor 2		3.79E-02	
P28800	SERPINF2	Alpha-2-antiplasmin			2.30E-02
E1BMJ0	SERPING1	Plasma protease C1 inhibitor			3.57E-02
A5PKC2	SHBG	Sex hormone-binding globulin	3.72E-02		
G3X8D7	GPX3	Glutathione peroxidase			9.41E-03
P56652	ITIH3	Inter-alpha-trypsin inhibitor heavy chain		4.56E-02	

1140

1141 We report plasma proteins identified with significant differential abundance (P-value < 0.05) between the tough group minus the tender group
1142 defined on LM. WB stands for mechanical tenderness evaluation, Tg stands for sensory tenderness evaluation, Idx stands for synthetic
1143 tenderness index.

1144

1145 **Table 4.** Linear multiple regression models for the three evaluations of beef tenderness.

Tissue	Tenderness	Protein biomarkers ^a	Model characteristics	
			R-squared	RMSE ^b
	WB	HPX (6.14) ACTN3 (14.49) TPM3 (8.12) SMTNL1 (-2.33) CSRP3 (-9.05) ADSSL1 (2.02) TNNC1 (-16.41)	0.70	86.00
LM	Tg	GOT1 (0.59) VCL (-0.55) OGN (-0.037) OGDH (0.13) MYBPH (-0.076) CAPZB (0.16)	0.58	0.022
	Idx	MYH1 (-0.71) ACTN2 (-0.54) MYBPH (0.041) OGN (-0.61) PGAM2 (-4.40)	0.56	0.33
	WB	MYH7 (-6.31) CFH (-23.59) ENO3 (-2.84) PLA2G2D5 (-1.14)	0.52	24.05
Plasma	Tg	FHL1 (-0,14) APDH (0,11) MASP2(0,39)	0.38	0.023
	Idx	CFH (-1.55) SERPINF2 (-0.72) MASP2 (0.35)	0.42	0.085

1146

1147 The linear regression analysis of the plasma VIP's proteins (VIP>0.8) identified in LM (at the top) and plasma (on the bottom) was performed on
1148 the 20 animals of the study for the three meat tenderness evaluations. WB stands for mechanical tenderness evaluation, Tg stands for sensory
1149 tenderness evaluation, Idx stands for synthetic tenderness index. RMSE: Root Mean Square Error. For each protein in prediction equation, the
1150 correlation coefficients are annotated in brackets.

1151
1152

Table
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Differential proteins			WB
Accession Number	Gene Name	Full name	P-value
<i>Muscle contraction and structure</i>			
P68138	ACTA1	Actin, alpha skeletal muscle	1.35E-02
Q3ZC55	ACTN2	Alpha-actinin-2	
Q0III9	ACTN3	Alpha-actinin-3	4.71E-02
P79136	CAPZB	F-actin-capping protein subunit beta	
Q148F1	CFL2	Cofilin-2	
Q4U0T9	CSRP3	Cysteine and glycine-rich protein	2.90E-03
E1BE25	FLNC	Filamin-C	3.49E-02
A4FV78	KLHL41	Kelch-like protein 41	7.75E-03
G3X6W9	MYBPH	Myosin-binding protein H	
Q9BE40	MYH1	Myosin-1	
Q9BE39	MYH7	Myosin-7	3.22E-02
F1ME15	MYL2	Myosin regulatory light chain 2	3.93E-02
Q148H2	MYL6B	Myosin, light chain 6B	
E1BCU2	MYOM3	Myomesin-3	
F1MPU4	MYOT	Myotilin	
Q8SQ24	MYOZ1	Myozenin-1	2.37E-02
Q5E9V3	MYOZ2	Myozenin-2	
F1N0W6	MYOZ3	Myozenin-3	3.14E-02
A6H7E3	PDLIM1	PDZ and LIM domain 1	
Q3SX40	PDLIM7	PDZ and LIM domain protein 7	2.53E-02
E1BPV6	SMTNL1	Smoothelin-like protein 1	4.21E-02
A0JNC0	TMOD1	Tropomodulin-1	
P63315	TNNC1	Troponin C, slow skeletal and cardiac muscles	5.79E-03
Q8MKH7	TNNT3	Troponin T, fast skeletal muscle	
Q5KR47	TPM3	Tropomyosin alpha-3 chain	4.59E-02
F1N789	VCL	Vinculin	
<i>Metabolism, transport and cell signalling</i>			
A1L578	CAVIN1	Caveolae-associated protein 1	3.40E-02
Q32PH8	EEF1A2	Elongation factor 1-alpha 2	2.44E-02
F1MG05	EEF1G	Elongation factor 1-gamma	
F1MR86	FHL1	Four and a half LIM domains protein 1	2.66E-02
A4FUZ1	GLO1	Lactoylglutathione lyase	
A1A4R1	HIST2H2AC	Histone H2A type 2	
Q17QE2	LMCD1	LIM and cysteine-rich domains protein 1	
Q32KP9	NUTF2	Nuclear transport factor 2	
<i>Muscle energy metabolism</i>			
P00570	AK1	Adenylate kinase isoenzyme 1	
P48644	ALDH1A1	Retinal dehydrogenase 1	
P20000	ALDH2	Aldehyde dehydrogenase, mitochondrial	
A6QLL8	ALDOA	Fructose-bisphosphate aldolase	4.80E-02
F1MJT6	CKMT2	Creatine kinase S-type, mitochondrial	
A6QR19	ENO2	Gamma-enolase	

Figure

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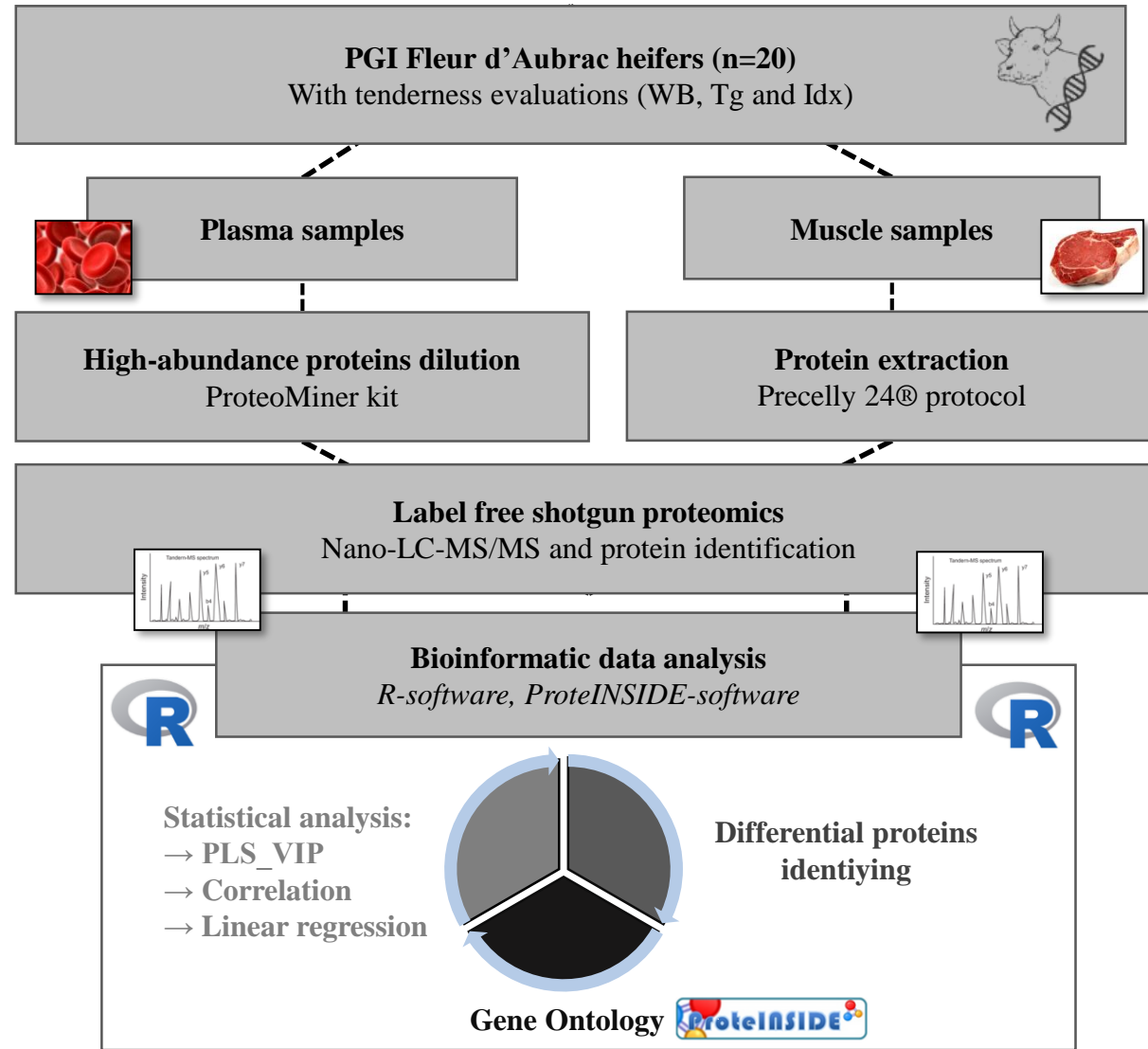
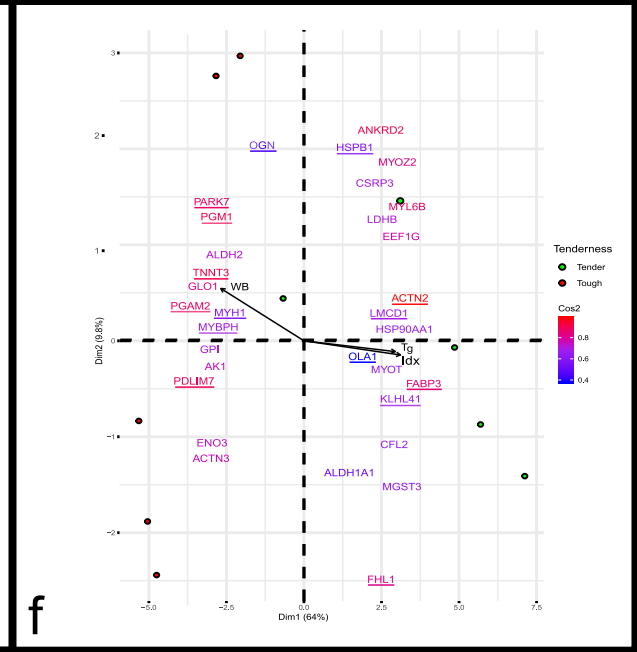
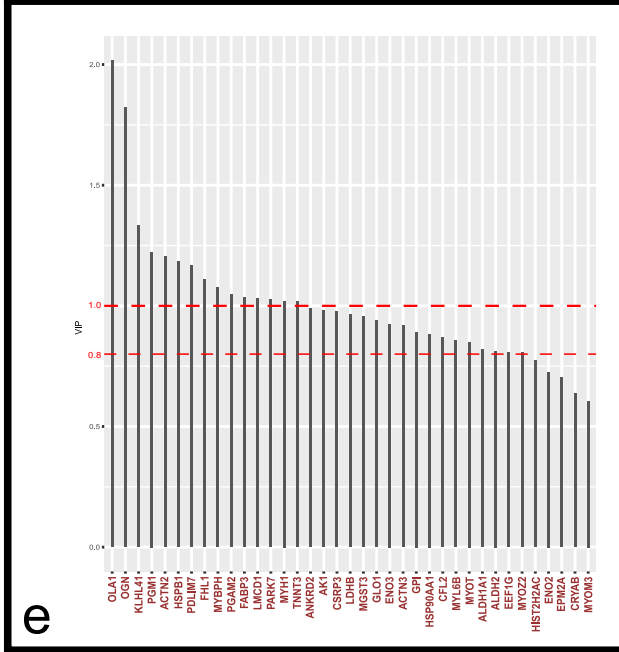
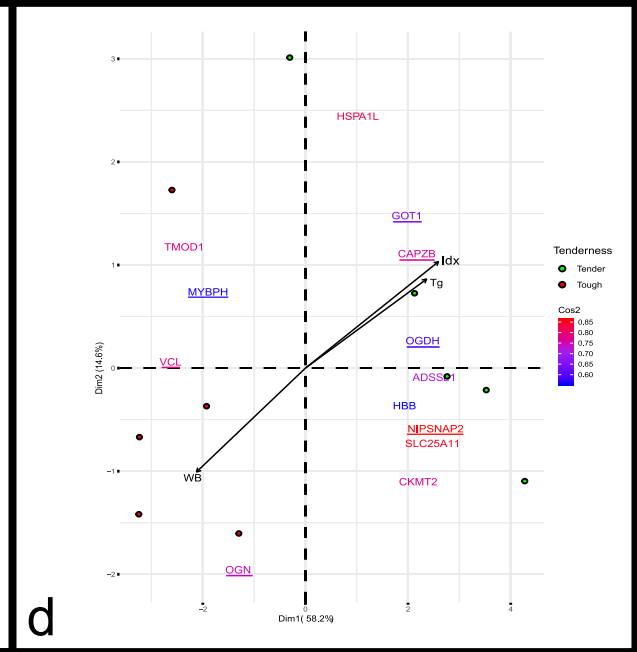
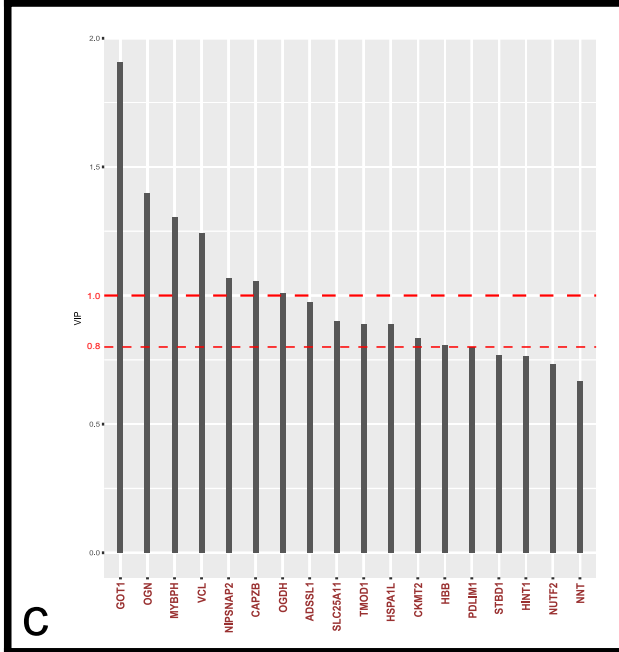
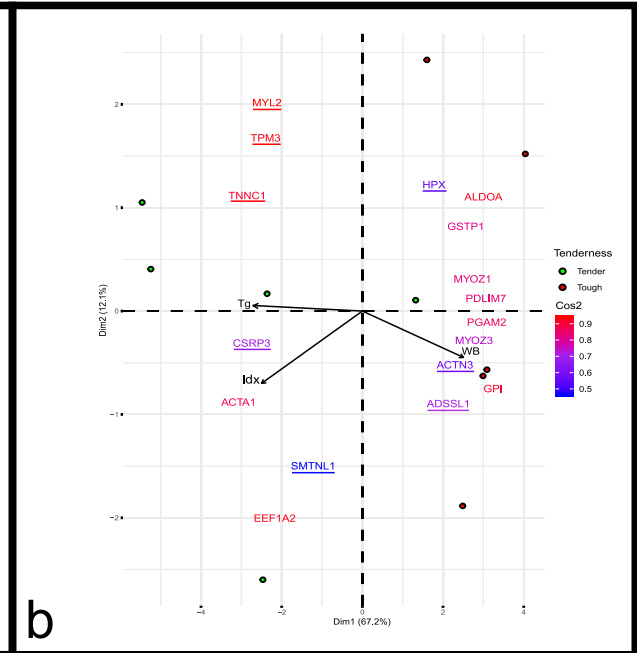
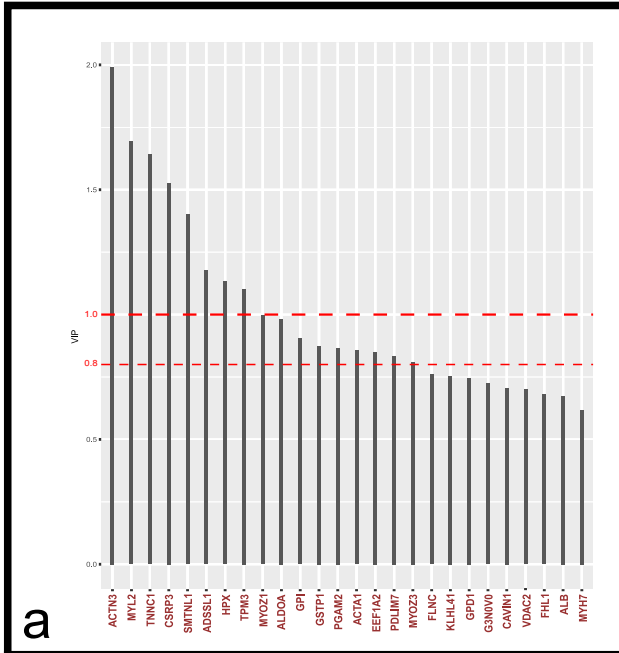


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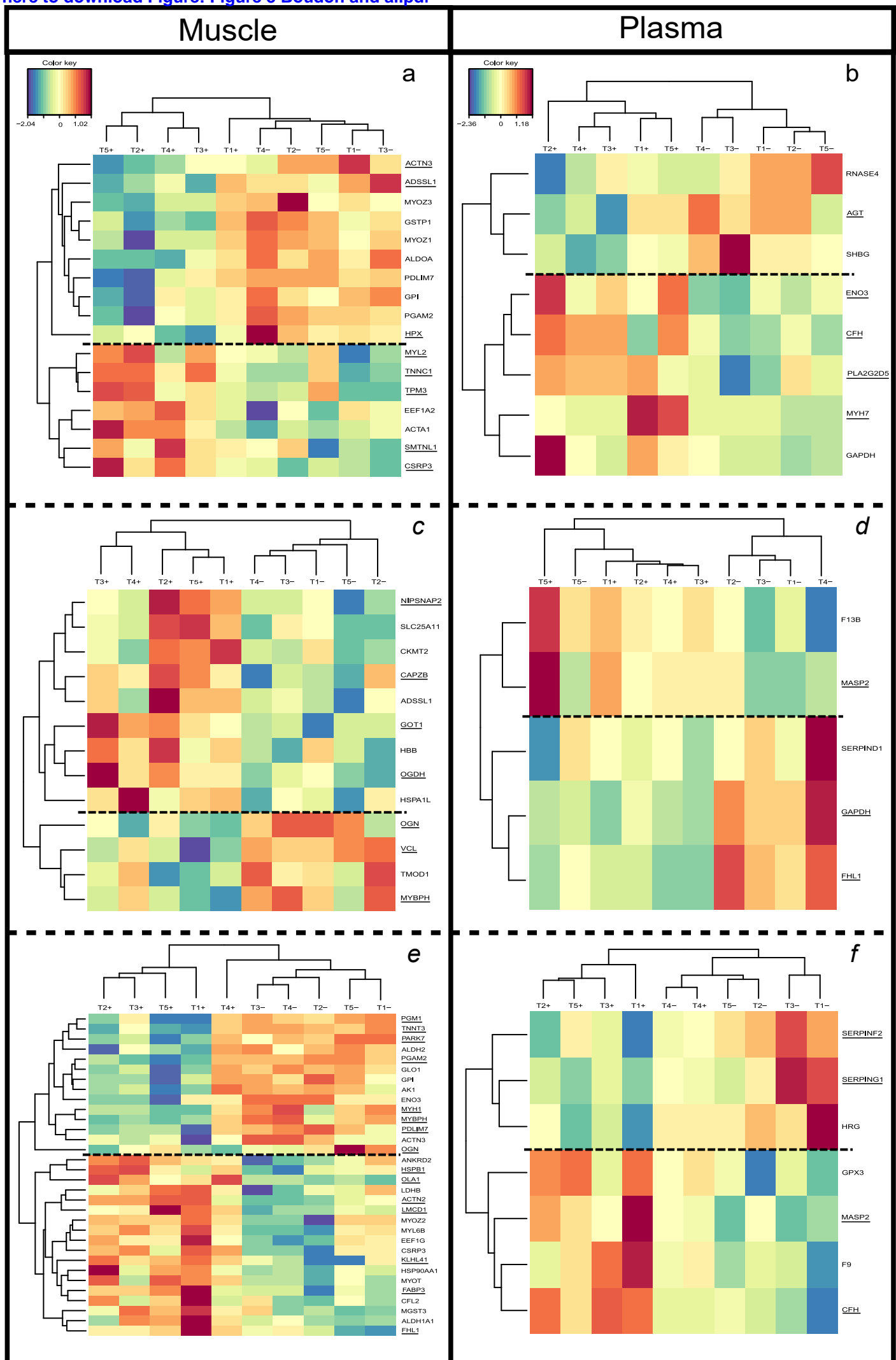
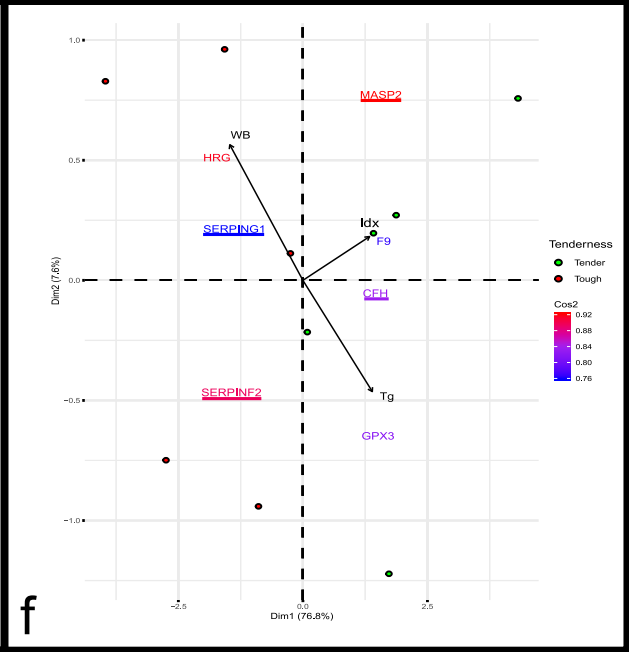
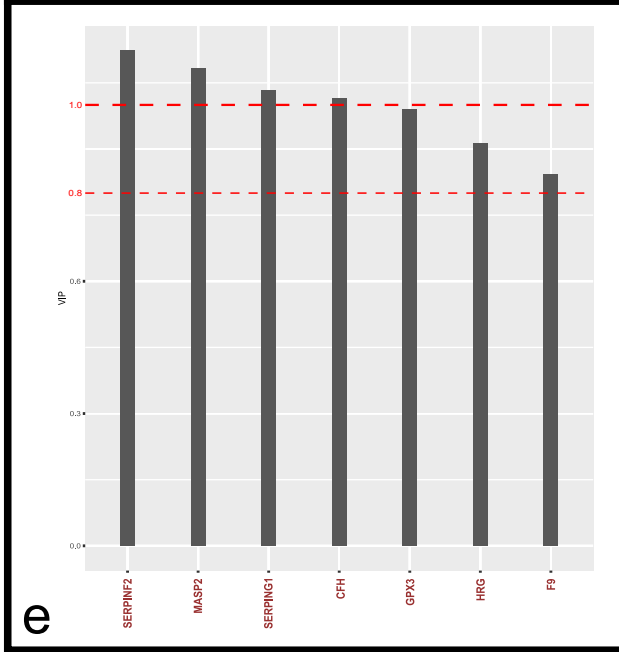
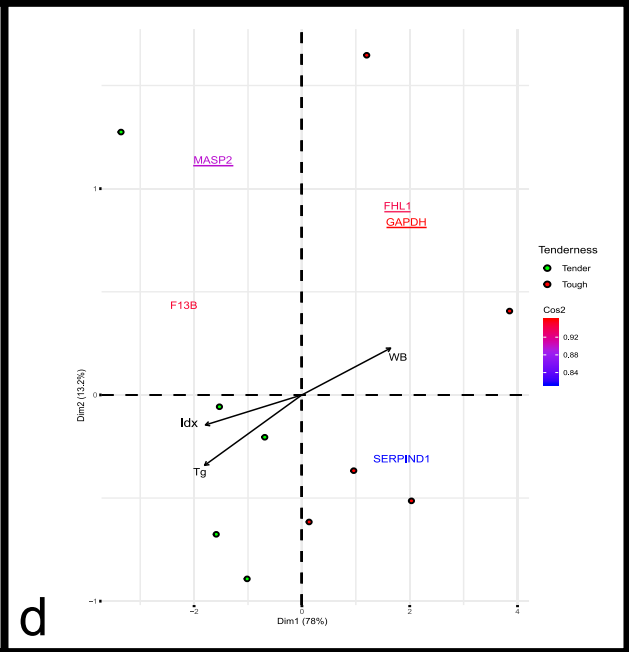
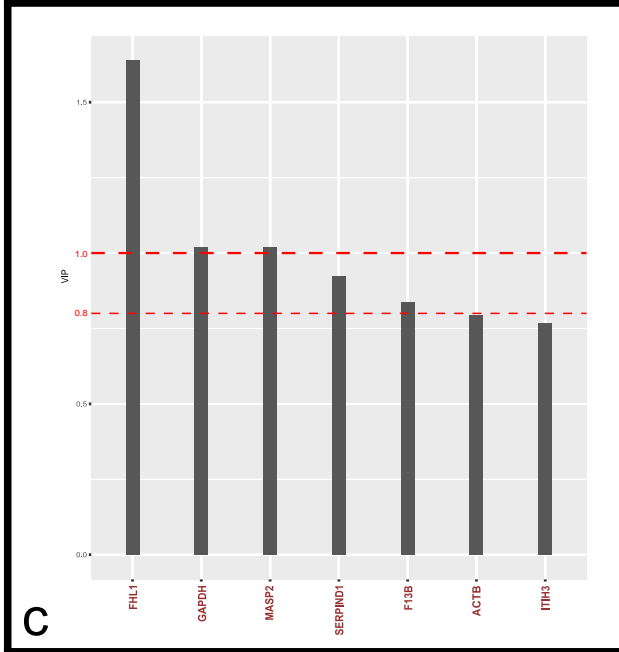
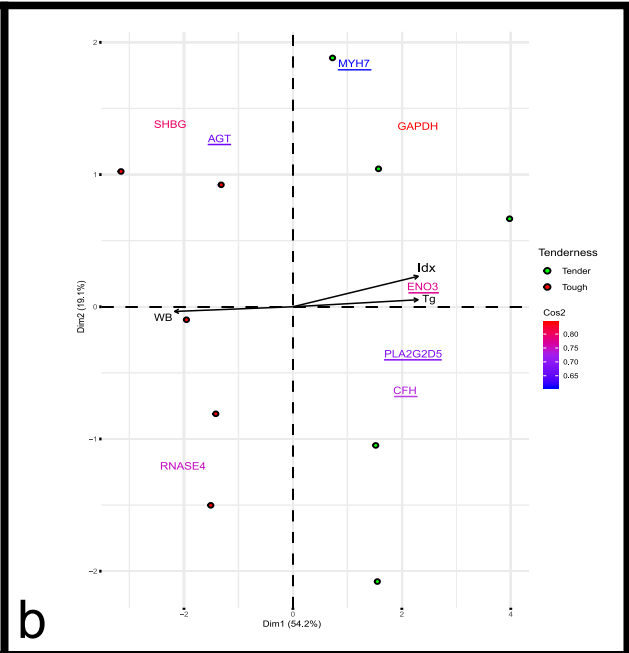
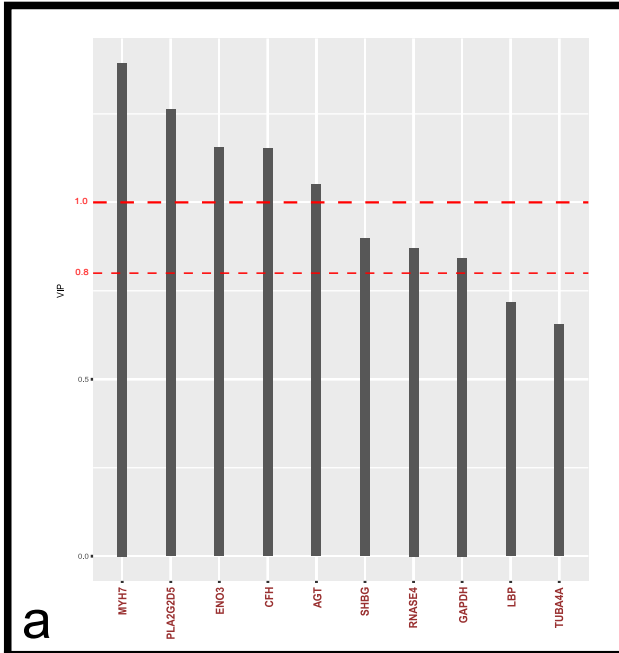
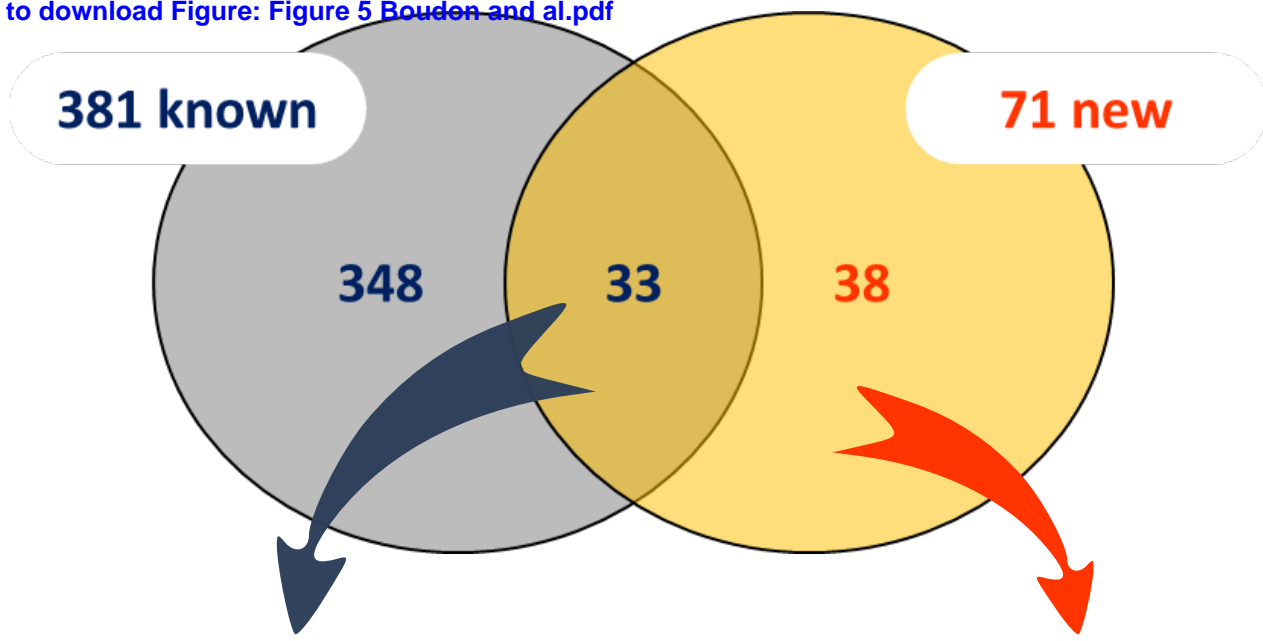


Figure
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Figure

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33 proteins reported in previous studies proteins	
Gene Name	QTL
Muscle contraction and structure	
ACTA1	
ACTN3	Tenderness score (Chr.29)
CAPZB	
CFL2	
CSRP3	Shear force (Chr.29)
KLHL41	
MYBPH	
MYH1	
MYH7	
MYL2	Shear force (Chr.17)
MYL6B	Shear force (Chr.5)
TNNT3	
TPM3	
Muscle energy metabolism	
AK1	
ALDH2	Shear force (Chr.17)
ALDOA	
CKMT2	Shear force (Chr.7)
ENO3	
FABP3	
GPD1	
LDHB	Shear force (Chr.5)
PGM1	Shear force (Chr.3)
PGAM2	
Metabolism, transport and cell signaling	
no protein	
Regulation of cellular process (apoptosis, oxidative stress)	
ALB	
ANKRD2	
CRYAB	
GSTP1	Tenderness score (Chr.29)
HBB	Shear force (Chr.15)
HINT1	
HSP90AA1	Shear force (Chr.21)
HSPB1	
PARK7	
VDAC2	Tenderness score (Chr.28)

38 new proteins	
Gene Name	QTL
Muscle contraction and structure	
ACTN2	
FLNC	
MYOM3	
MYOT	Shear force (Chr.7)
MYOZ1	
MYOZ2	
MYOZ3	Shear force (Chr.7)
PDLIM1	
PDLIM7	Shear force (Chr.7)
SMTNL1	
TMOD1	Shear force (Chr.8)
TNNC1	Shear force (Chr.22)
VCL	
Muscle energy metabolism	
ALDH1A1	Shear force (Chr.8)
ENO2	
GPI	
NIPSNAP2	
NNT	
OGDH	
OLA1	
SLC25A11	
Metabolism, transport and cell signaling	
CAVIN1	
EEF1A2	Shear force (Chr.13)
EEF1G	Tenderness score and Shear force (Chr.29)
FHL1	
GLO1	
HIST2H2AC	
LMCD1	
NUTF2	
Regulation of cellular process (apoptosis, oxidative stress)	
ADSSL1	
G3N0V0	
GOT1	
HPX	Shear force (Chr.15)
HSPA1L	
MGST3	
OGN	Shear force (Chr.8)
Autophagy	
EPM2A	Tenderness score (Chr.9)