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

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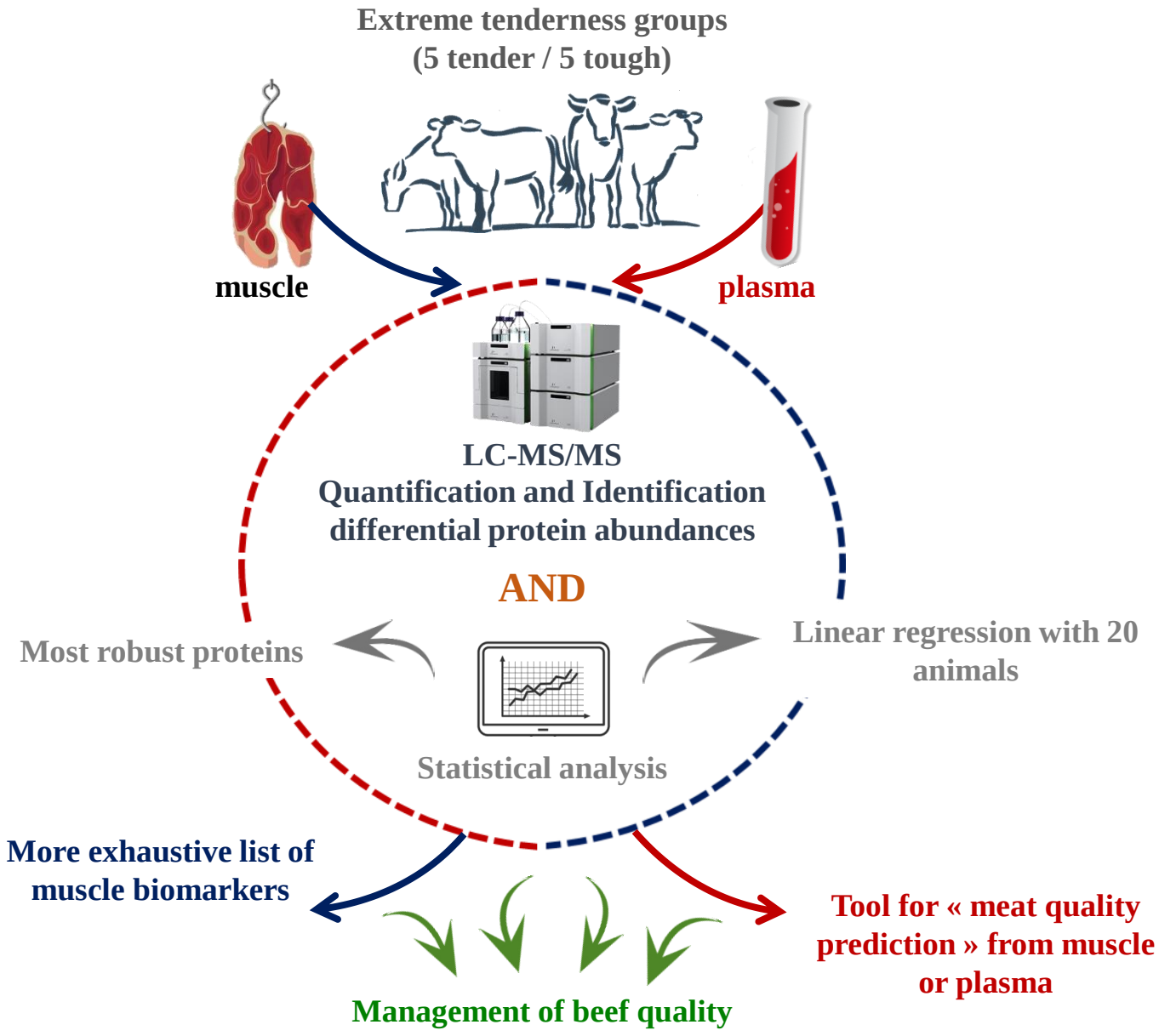
SIGNIFICANCE

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.

*Graphical Abstract



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

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ABSTRACT

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

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

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.

1. Introduction

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

Several omic approaches including proteomic technologies, developed over the last two decades have been conducted to identify biomarkers for meat tenderness and/or explore the tenderness process (see [6,7] for review). This approach allowed proposing a list of biomarkers and highlighted the interactions between them in the construction of tenderness [8]. Some muscle type or animal type specificities have been evidenced [9]. However, biomarker-based approach has some limitations since the analysis of muscle 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***

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

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

2.3. Tenderness evaluation and constitution of extreme groups

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

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

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

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

2.4. Proteins extraction

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

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

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

Plasma samples. In order to increase the chance of detecting proteins of interest, the ProteoMiner™ technology was used as protein enrichment approach (Large-Capacity Kit 163-3007, BioRad Inc., Hercule, CA, USA) [24] to concentrate mid- and low-abundance interest proteins such as albumins, immunoglobulins, fibrinogen or complement proteins [25]. To investigate the protein profile on SDS-PAGE before, plasma samples (1 mL) were loaded on a column with 100 µL ProteoMiner beads buffer (20% beads, 20% v/v aqueous EtOH) and incubated 2h at room temperature in order to allow peptides-ligands linkage. After two centrifugations (2x10000 rpm) and two washes (50 µL wash buffer (150 mM NaCl, 10 mM NaH_2PO4, pH 7.4) and 50 µL distillate water)) to eliminate highly abundant proteins, elution of the bound proteins was performed using elution reagent (5% acetic acid).

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

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

proteins (upper band) and low molecular weights (lower band). Excised bands were washed with 100 µL ammonium bicarbonate (50 mM-50% ethanol, 20 min at room temperature (RT)). After removal of the buffer, they were dehydrated with 100 µl of 100% ethanol (15 min). Reduction and alkylation reactions were performed during 30 min respectively with 10 mM DTT solution at 56°C and with 55 mM iodoacetamide solution protected from light. The bands were washed with 200 µL of 50 mM NH4HCO3 buffer for 15 min at room temperature and destained by 100 µL of 25 mM NH4HCO3 – 5% acetonitrile (v/v) for 15 min followed by three washing with 100 µL of 25 mM NH4HCO3 – 50% acetonitrile (v/v) for 30 min under agitation. Finally all bands were dehydrated with 100% acetonitrile (ACN). The samples were hydrolyzed during 5 hours at 37°C using 48 µl of a 25mM NH4HCO3 - 12.5 ng.µl-1 trypsin solution (V5111, Promega, Charbonnières-les-Bains, France) per band. Peptides were extracted from the gel bands in an ultrasonic field during 10 min with 38.4 µl of 99.9% acetonitrile /0.1% Formic Acid representing 80% of digestion volume. Supernatants were transferred in eppendorf vials and dried using Speed Vac for 60 min and adjusted to 50 µl with a solution (H2O/ ACN/TFA – 94.95/5/0.05). This solution was transferred into HPLC vials containing a 100µl glass insert before nano LC MS/MS analysis.

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

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

Data processing, protein identification and abundances. For raw data processing, MS/MS ion search was performed with Mascot v2.5 (http://www.matrixscience.com) against *bos taurus* database (i.e. ref_bos_taurus, 23970 sequences). The following parameters were considered for the search: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.5 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation (C), oxidation (M) and deamidation (NQ) set as variable modifications. Protein identification was validated when at least two peptides originating from one protein showed statistically significant identity above Mascot scores > 36 with a False Dicovery Rate of 1% (adjusted significance threshold $p < 0.0058$). Ions score is -log10 (P-value), where P is the probability that the observed match is a random event. Individual ions scores > 36 indicate identity or extensive

homology. All the proteins identified in this study correspond to one unique protein identifier as they were annotated by a minimum of two associated specific peptides.

For label-free protein quantification analysis, LC-Progenesis was used with the same identification parameters described above. All unique validated peptides of an identified protein were included and the total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. Statistical analysis was performed using the "between subject design" and p-values were calculated by a repeated measures analysis of variance using the normalized abundances across all runs.

2.6. Statistical analysis

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

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

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

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

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

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

In order to identify which proteins contributed the most to the models, the Variable Importance in the Projection (or VIP score) was calculated using the mixOmics package (vip function). The PLS_VIP values allow to define a hierarchy of the most explanatory proteins for each tenderness trait (WB, Tg, Idx). Only the 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

achieved in Human species. Human orthologs' annotations were privileged in order to get the most complete GO information, since bovine annotation remains limited. Muscle and plasma GO were considered independently for GO_BP analysis. Histogram representations of the GO_BP annotations were constructed using only specifications associated with P-value<0.001 with a minimum of annotated proteins≥2 (see supplementary data S1 and S2). The P-values were converted into –log10 (P-value) values, and classified in ascending order in an upward direction. Specifications were annotated as follows: GO annotation (Number of proteins included in the annotated in brackets) Proteins Gene Names tie to GO annotation separated by space. A table listing the major metabolic pathways was constructed using GO_BP TREE from the GO_BP annotations (P-value<0.001, minimum of annotated proteins≥2). In order to find specificity of metabolic pathways associated with the three tenderness quality traits, GO_BP analyses were performed respectively for each of the six datasets.

3. Results

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

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

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

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

3.2. Muscle candidate biomarkers

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

negatively with Tg and Idx on axis 1. On the the other hand, MYL2, TPM3, TNNC1, EEF1A2, ACTA1, CSRP3, and SMTNL1 were positively correlated with Tg and Idx and negatively with WB on axis 1. These results are in accordance with the values of the correlation coefficient illustrated in the Supplementary Data 3. The Heat Map representation of the 17 proteins shows a group of 10 proteins including ACTN3, ADSSL1, MYOZ3, GSTP1, MYOZ1, ALDOA, PDLIM7, GPI, PGAM2, and HPX as less abundant in the most tender LM and more abundant in the toughest. Conversely, 7 proteins including MYL2, TNNC1, TPM3, EEF1A2, ACTA1, SMTNL1, and CSPR3 were more abundant in the most tender LM and less abundant in the toughest LM. (Figure 3a)

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

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

Among the 71 differential proteins in LM muscle, 37 proteins were significantly differentially abundant between extreme groups of Idx tenderness (Table 2). The correlations between the abundance of these proteins and the three tenderness traits are illustrated in Supplementary Data 3. The PLS analysis revealed 15 proteins (VIP>1) (ACTN2, FABP3, FHL1, HSPB1, KLHL41, LMCD1, MYBPH, MYH1, OGN, OLA1, PARK7, PDLIM7, PGAM2, PGM1 and TNNT3) and 17 proteins (0.8<VIP<1) (ACTN3, AK1, ALDH1A1, ALDH2, ANKRD2, CFL2, CSRP3, EEF1G, ENO3, GLO1, GPI, HSP90AA1, LDHB, MGST3, MYL6B, MYOT and MYOZ2) which are considered as putative candidate proteins usable for meat tenderness prediction (Figure 2e). The PCA in Figure 2f illustrates that among the 32 proteins the most explicative of Idx tenderness (with VIP>0.8), PARK7, PGM1, TNNT3, PGAM2, PDLIM7, GLO1, ACTN3, AK1, MYBPH, ENO3, GPI, ALDH2, OGN, and MYH1 were positively correlated with WB and negatively with Tg and Idx on axis 1. On the other hand, ANKRD2, HSPB1, MYOZ2, CSRP3, MYL6B, LDHB, EEF1G, ACTN2, LMCD1, HSP90AA1, OLA1, MYOT, FABP3, KLHK41, CFL2, ALDH1A1, MGST3, and FHL1 were positively correlated with Tg and Idx and negatively with WB on axis 1. The Heat Map representation (Figure 3e) of the 32 muscle VIP's proteins illustrates, a group of 14 proteins including PGM1, TNNT3, PARK7, ALDH2, PGAM2, GLO1, GPI, AK1, ENO3, MYH1, MYBPH, PDLIM7, ACTN3, and OGN, less abundant in the most tender LM and more abundant in the toughest. Conversely, 18 proteins including ANKRD2, HSPB1, OLA1, LDHB, ACTN2, LMCD1, MYOZ2, MYL6B, EEF1G, CSRP3, KLKL41, HSP90AA1,

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

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

3.3. Plasma candidate biomarkers

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

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

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

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

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

4. Discussion

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

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

4.1. Candidate muscle biomarkers for tenderness

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

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

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

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

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

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

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

5. Conclusion

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

Author contributions

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

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

Conflict of interest

The authors declare no competing financial interest

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Tables and Figures

Figure captions

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

PGI (protected geographical indication)

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

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

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

Figure 3. Heat Map representation of the VIP's proteins identified in this study.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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**
Muscle contraction and structure					
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	
Metabolism, transport and cell signalling					
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	
Muscle energy metabolism					
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	
Regulation of cellular process (apoptosis, endocytosis, oxidative stress)					
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		
Autophagy					

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 though group minus the tender group.

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

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

Differential proteins			WB	Tg	Idx
Accession Number	**Gene Name**	**Full name**	**P-value**	**P-value**	**P-value**
Muscle contraction and structure					
F1MM07	MYH7	Myosin-7	4.91E-02		
P81948	TUBA4A	Tubulin alpha-4A chain	4.60E-02		
P60712	ACTB	Actin, cytoplasmic 1		3.47E-02	
Metabolism, transport and cell signalling					
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		
Muscle energy metabolism					
Q3ZC09	ENO3	Beta-enolase	5.74E-03		

P10096	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	4.47E-02	4.06E-03	
Immune system, cell defence and homeostasis (angiogenesis, fibrinolysis, blood coagulation, aging)					
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

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

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

Tissue	Tenderness	Protein biomarkers [a]	Model characteristics	
			R-squared	RMSE[b]
LM	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
	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
Plasma	WB	**MYH7 (-6.31) CFH (-23.59)** ENO3 (-2.84) PLA2G2D5 (-1.14)	0.52	24.05
	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

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 the 20 animals of the study for the three meat tenderness evaluations. WB stands for mechanical tenderness evaluation, Tg stands for sensory tenderness evaluation, Idx stands for synthetic tenderness index. RMSE: Root Mean Square Error. For each protein in prediction equation, the correlation coefficients are annotated in brackets.

Table

Click here to download Table: Boudon et al_ Review_Mascot protein identification.xlsx

Differential proteins			WB P-value
Accession Number	**Gene Name**	**Full name**	
Muscle contraction and structure			
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	
Metabolism, transport and cell signalling			
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	
Muscle energy metabolism			
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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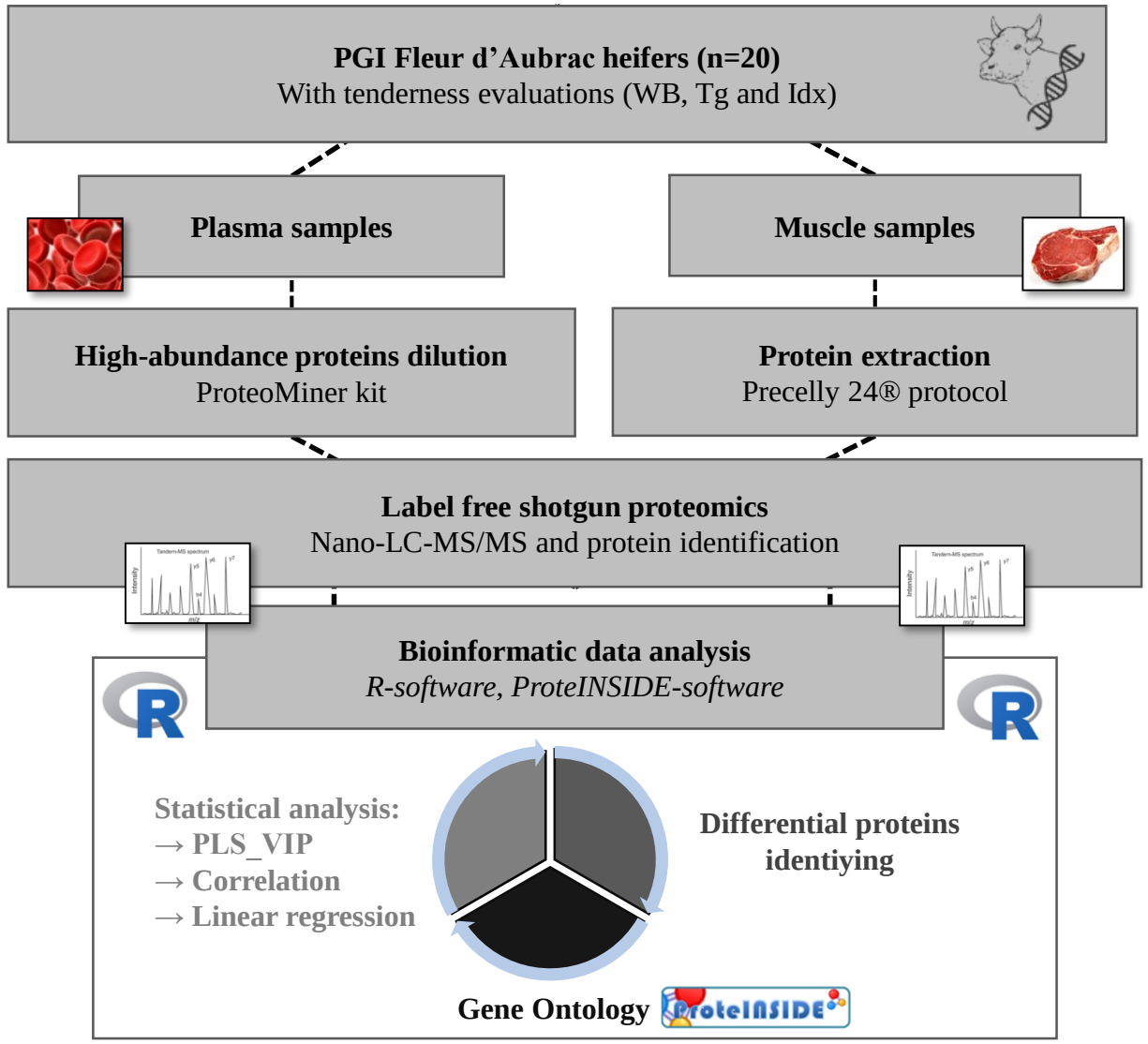

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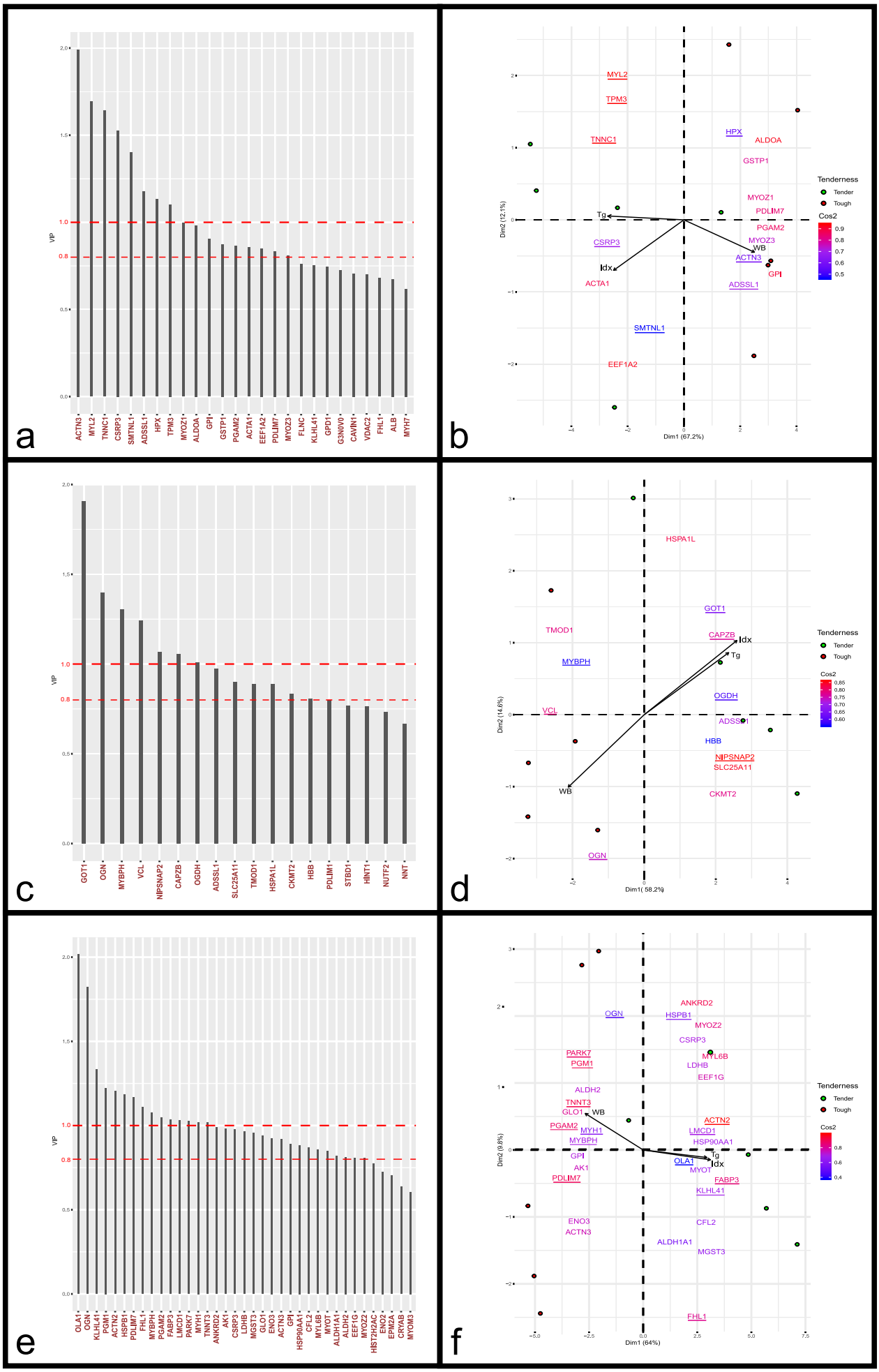

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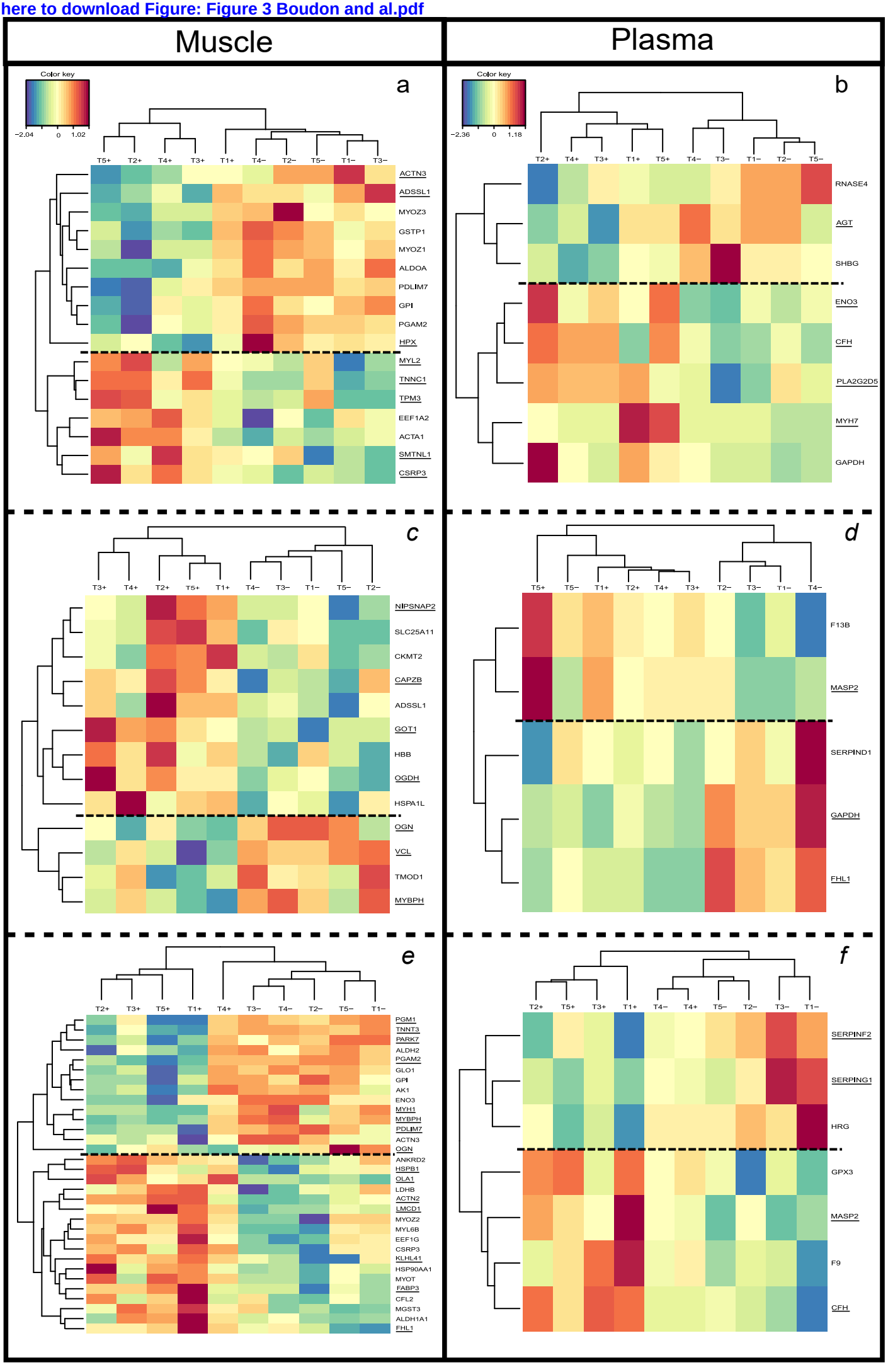

Figure

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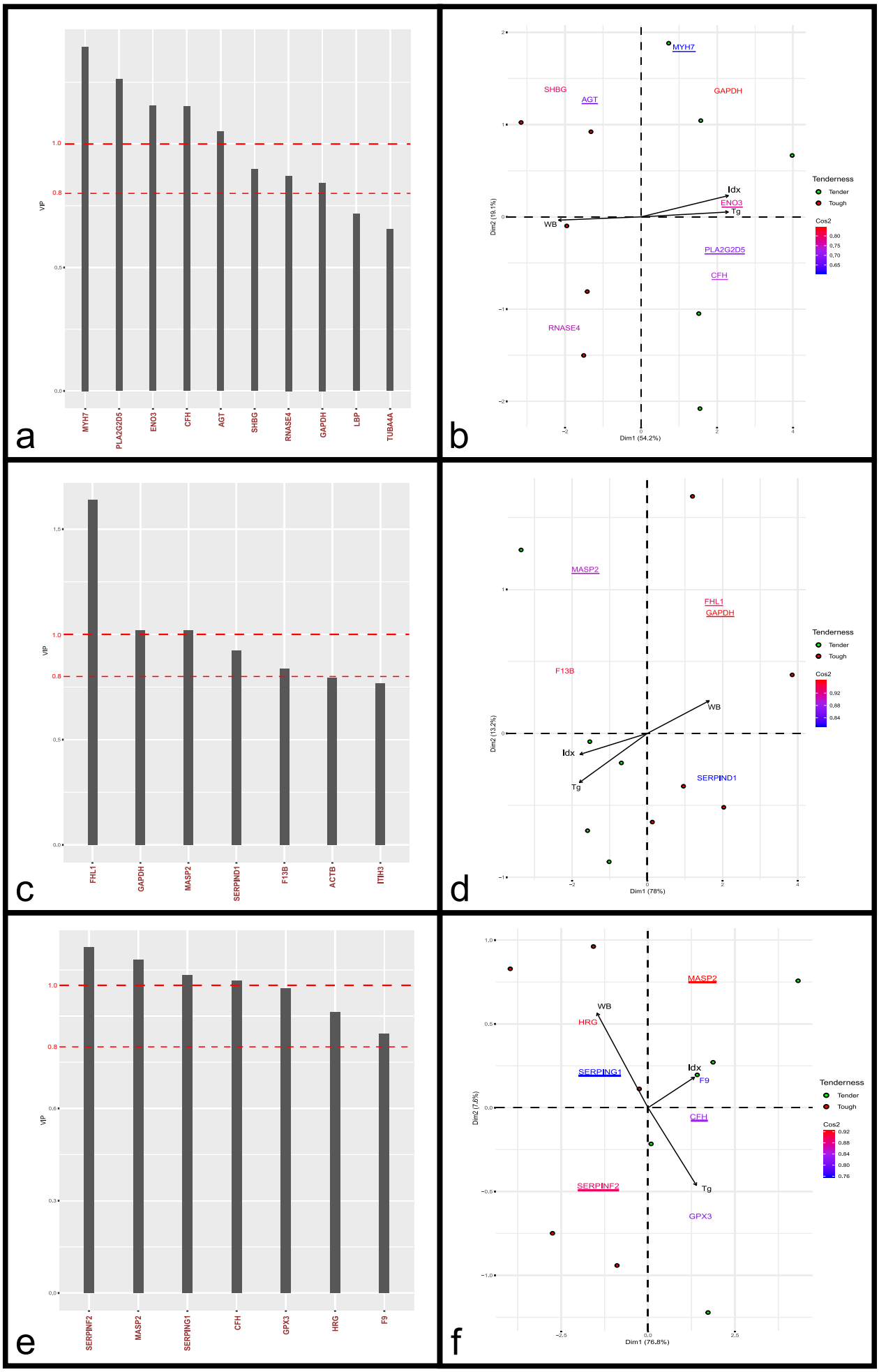

Figure

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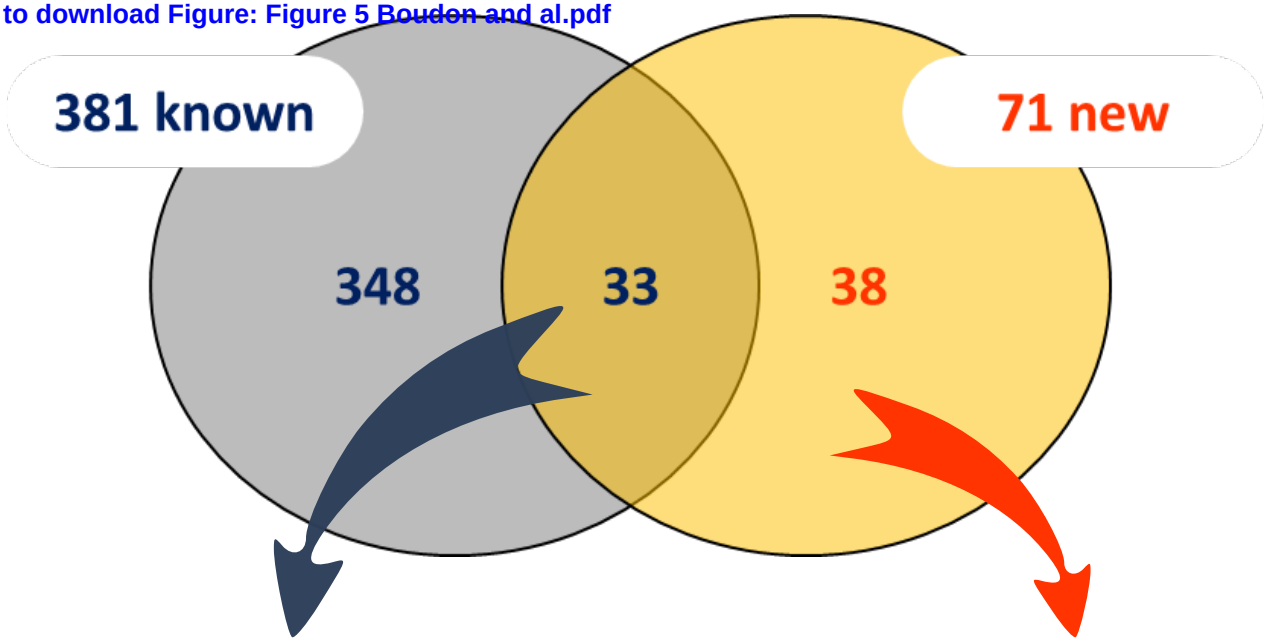

33 proteins reported in previous studies proteins	
Gene Name	QTL
Muscle contraction and structure	
ACTA1	
ACTN3	Tenderness score (Chr.29)
CAPZB	
CFL2	
CSRP3	Shear force (Chr.29)
KLHL41	
MYBPH	
MYH1	
MYH7	
MYL2	Shear force (Chr.17)
MYL6B	Shear force (Chr.5)
TNNT3	
TPM3	
Muscle energy metabolism	
AK1	
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)