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The path from protein profiling to biomarkers: The potential of proteomics and data integration in beef quality research

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Abstract. This study aimed to provide an overview of the strategy of meat quality biomarkers identification from protein profiling to the establishment of putative protein biomarkers with a focus on beef tenderness and colour traits. Further, the current knowledge gained by data-integration, also known as integromics, of published meat proteomics studies during the last decade is briefly discussed in terms of the current list of protein candidate biomarkers revealed using different proteomics platforms and evaluated by proteomics-based approaches. The main biochemical pathways underlying the determination of tenderness and colour traits as important beef eating qualities revealed by bioinformatics analyses such as Gene Ontology annotations, pathway and process enrichments are further considered. This paper also addresses the potential of integromics and data-mining, in the era of big data and data analytics, to broaden our knowledge on the biochemical mechanisms underlying the conversion of muscle into meat and the consequences on beef sensory quality traits (tenderness and colour). Finally, the emerging interest of using such gathered and shortlisted protein biomarkers for first validation and then early *post-mortem* prediction of the potential quality of beef carcasses is highlighted.

1. Introduction

The production of meat, mainly beef, with consistent high quality is an ongoing challenge for farmers, meat industry stakeholders and meat research centres. In fact, the consistency of beef eating quality traits such as the appearance firstly judged at the point of sale by visual colour and degree of marbling and after cooking mainly evaluated by the three important beef palatability traits tenderness, juiciness and flavour intensity, directly influence the marketability of beef products and the re-purchase decisions by consumers [1-3]. However, several factors ranging from the farm-to-fork continuum [4, 5], including the interactions between the biological identities of the animal and the myriad biochemical muscle-to-meat conversion processes taking place after slaughter are known to affect the final beef sensory qualities [6, 7]. Many studies have revealed by means of conventional scientific methods some reasons of the variation in meat quality (for review: [7]) and certain of them proposed how to control during pre- or post-slaughter periods the biochemical processes occurring in the conversion of muscle to meat.

At the beginning of the 21st century, tremendous progress in high-throughput techniques (data-driven research), mainly OMICs known also as 'Foodomics' in the field of food sciences, for genes, proteins and metabolites analyses along with a considerable development of sophisticated statistical algorithms and bioinformatics tools allowed to explore meat quality and its development in ever greater detail and in a holistic manner [8, 9]. Therefore, OMICs techniques has the ability to generate large quantities of biological data about the genome, transcriptome, proteome, metabolome, etc. [10] from important

number of animals using tissue biopsies or fluids, to relate them with the important beef quality phenotypes including tenderness and colour to decipher the reasons of their quality variation. Among the OMICs approaches used in the field of meat science, proteomics seemed to be an effective quantitative analysis approach through the large-scale and systematic characterization of the global or partial analysis of the proteome (muscle tissue or fluids such as plasma) at given moment and environmental conditions to propose explanatory mechanisms at the origin of the variability of beef eating quality traits. Proteome analysis depends on five major steps; protein separation, identification, characterization, quantification and functional characterization, allowing the study of interactions between the proteins [11, 12]. The importance of proteomics in the field of meat science is illustrated by a large number of papers and reviews concerning its applications to characterize the cellular and molecular mechanisms behind most meat quality traits from all species, skeletal muscle in the context of livestock production, or biological traits and diseases in farm animals. Indeed, proteomics by means of gel-based and gel-free approaches coupled with mass spectrometry has emerged as a well-defined strategy in the field of meat science [13] which, through its in-depth characterization of the whole bovine muscle proteome (**Figure 1**), allowed to provide substantial data on the biochemical mechanisms underpinning important meat quality traits [8, 13, 14] including tenderness [12, 15-17], colour [18, 19], marbling [20], water-holding capacity [21, 22], and pH decline [23, 24], etc. In addition, proteomics was used to investigate the dynamic changes and modifications occurring in *post-mortem* muscle proteome [25, 26] and most importantly for the identification of protein biomarkers [6, 13].

In the following sections, the main steps of protein biomarkers discovery and current list of protein biomarkers and underlying pathways revealed by proteomics for the two major beef quality traits (tenderness and colour) are briefly summarised. Further, the potential of integromics in beef quality research to broaden our knowledge on the biochemical mechanisms behind the determination of beef tenderness and colour qualities is covered. The emerging interest in gathering published proteomics datasets on these two traits and the currently identified lists of putative protein biomarkers with the aim of creating unique repertoires is further discussed.

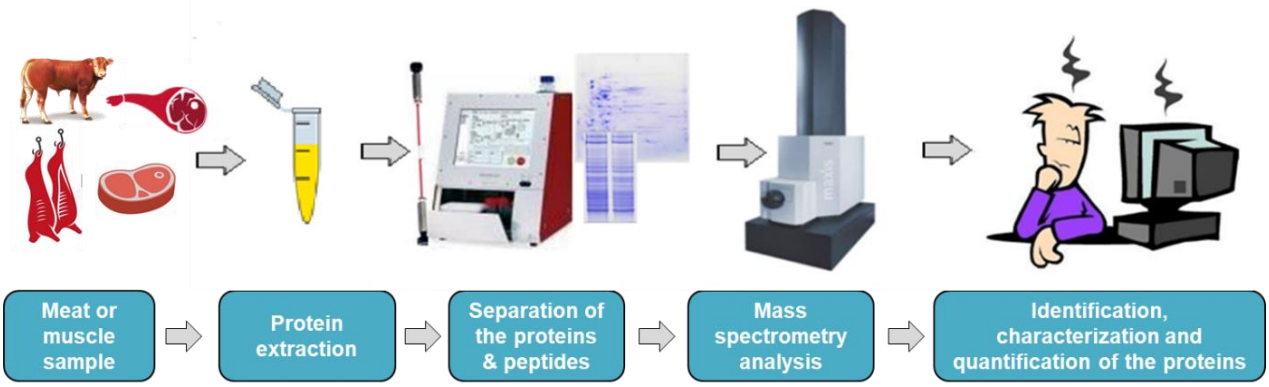

Figure 1. Main steps of proteomics analysis: from biological sample to identified proteins.
Schematically, proteomics analysis begins with the extraction and fractionation of proteins from a biological sample (biopsy of muscle taken early post-mortem or aged meat), followed by protein separation by electrophoresis using mono- or bi-dimensional gels and revelation (staining) of the separated proteins, then recovery of the bands or spots of interest and their identification by mass spectrometry after tryptic digestion. The peptides obtained are used for the identification, characterization and/or quantification of proteins by means of bioinformatics tools (in silico) as well as for statistical analyses to relate them with the phenotypes of interest.

2. The path from protein profiling (expressed proteins) to biomarkers: the input of proteomics

The main research area where beef research proteomics has been hugely applied is in the field of protein biomarkers discovery for which the pipeline and main phases are based on the strategy and path developed for biomarkers in medicine as described in several comprehensive reviews [27].

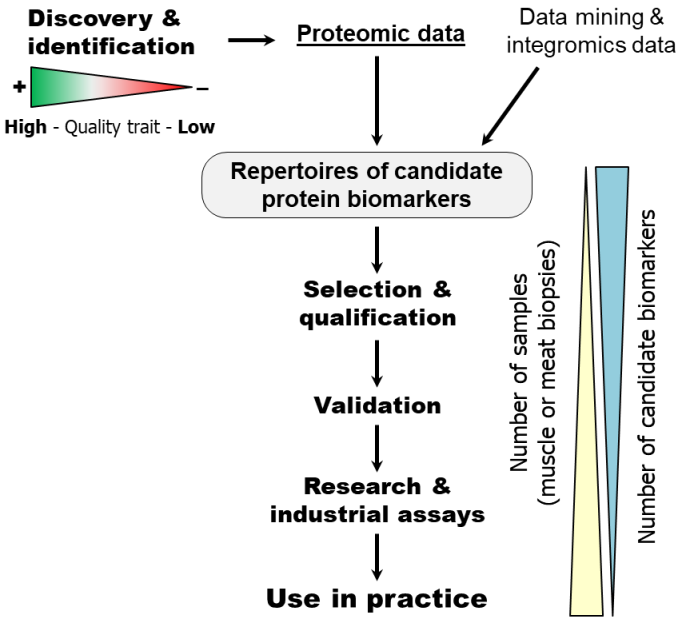

Figure 2. Brief description of the main phases of the meat quality protein biomarkers discovery path: from proteomic data generated on divergent quality traits (high quality *versus* low quality: tender *vs.* tough beef) to validation and development in practice for the carcasses and meat quality potential management/prediction.

During the discovery and identification phase, lists of 10 to 100 proteins with different abundances between two compared conditions (i.e., tender vs. tough) are identified before their qualification and validation to less than 10 biomarkers at the end of the path before practical use. Data mining of published proteomics play an important role in the preparation of the repertoires of candidate protein biomarkers to identify the robust ones (identified several times in independent meat proteomics experiments) that can be selected for qualification in the next steps.

The pipeline of biomarkers discovery was adapted to animal production and meat quality (**Figure 2**), more importantly in cattle for beef tenderness, and composed of six major phases including (i) discovery/identification, (ii) qualification, (iii) verification, (iv) research assay optimization, (v) industrial validation and (vi) commercialization [12, 15]. In all these phases, the quantification of the abundance of the proteins and their analyses using appropriate statistical algorithms along with bioinformatics tools is a pivotal step. Indeed, most research that is conducted on biological samples attempts to maximize the detection of differential proteins, although this is related to the sensitivity and precision of the instruments, while minimizing the number of samples required for analysis (**Figure 2**).

Therefore, the first phase consists in a comparison of groups of samples (mainly muscle biopsies and recently other biological sources such as plasma or meat exudates are tested) divergent for one phenotype, for example beef tenderness with high (tender) and low (tough) quality evaluated by sensory panellists or instrumental methods such as Warner-Bratzler shear force [12, 16]. The samples are mostly selected using specific thresholds that correspond to a consumer's acceptance or satisfaction with the quality of a meat product [28]. During the discovery or identification phase, the main objective remains an increase as much as possible of the number of candidates related to the variability of the beef trait of interest. Thus, the nature of the sample should be carefully selected to ensure an acceptable yield of candidate biomarkers on a maximum number of samples. The identification of new biomarkers thus consists in achieving a trade-off between the need to use a particular tissue or bio-fluid, and the need to identify the most exhaustive list of candidate protein biomarkers possible on a large number of samples. Accordingly, the samples are in general of the same conditions such as the same muscle (especially M. *longissimus thoracis*), same breed or gender, animals reared under the same production system, to analyse only the trait of interest. This allows reduce confused factors and bias, leading to accurate identification of proteins differentially abundant between the divergent meat quality groups [17].

In terms of methodology, the first discovery phase is composed of several steps from sampling and categorization of the samples based on the phenotype of interest at a specific threshold, protein extraction (using total protein extracts; sarcoplasmic proteins or myofibrillar proteins depending on the objective) till the identification of the differential proteins by mass spectrometry and their quantification. The current separation technologies are playing an important role in this respect by ensuring high reproducibility and sensitivity of the identification. In fact, the identification can be done either at the proteins or peptides levels after protein digestion of each sample mainly using trypsin, and is referred to as 'top-down' or 'bottom-up' approach, respectively. In the former approach, one- or two-dimensional electrophoresis coupled to mass spectrometry is the most common and successful method for analysis, separation and identification of the proteins. Although electrophoresis-based methods have been extensively used in the first years, the most suitable modern technology for protein biomarker discovery remains mass spectrometry in gel-free or label free manner [29]. Therefore, in the second approach and as an alternative to gel electrophoresis, the novel mass spectrometry platforms with very high sensitivity and resolution are used for the bottom-up approach allowing by means of shotgun proteomics better repeatability and complete analysis of the proteome or the targeted sub-proteomes (*e.g.* mitochondria).

When the candidate protein biomarkers have been identified and confirmed using several statistical and chemometrics analyses (*i.e.*, significant correlations with the phenotypes of interest; good splitters in decision trees; high importance in projection (VIP) scores in partial least squares; good separation of quality classes in clustering analyses…etc.), the next step is their evaluation (qualification) and validation in large scale. This phase requires high-throughput tools and if possible those that could be used in practice by the industry to manage meat quality of an animal or a carcass [12, 30]. The input of meat scientist will play another important role to shortlist a subset of biomarkers based on the biological knowledge of each protein, the ease of quantification and other technical aspects mainly tested in the qualification step. Overall, the qualification phase follows the same optimised steps of the discovery one, therefore ensuring that the differential expression of the protein is detectable by the assay that will be used for its evaluation. This consists for example of assaying fast techniques for the qualification of biomarkers in acceptable number of animals or carcasses, allowing the confirmation of the differential abundance of the protein using a method generally different from the one used for the identification of the candidate biomarker. In this context, the candidate protein biomarkers can be tested using immune-based techniques such as western blotting [31, 32], Dot-Blot [23, 33, 34] and Reverse Phase Protein Array [15, 35, 36]; or label-free gel mass spectrometry tools, namely Selected Reaction Monitoring (SRM), Sequential Window Acquisition of all Theoretical spectra (SWATH) [37] or Parallel Reaction Monitoring (PRM) [38]. These steps along with those of integromics based on previously published studies [12, 16, 18] will allow to shortlist a small number of highly qualified candidate biomarkers to move forward in the pipeline of biomarkers discovery through an external validation phase.

Finally and very briefly, during the validation and development phases, qualified and validated protein biomarkers are mainly measured on an important number of animals (thousands of samples) representative of the slaughtering routine in the context where the protein biomarkers should be validated. During the validation phase, a modification of the detection method can be necessary, mainly because mass spectrometry is not accepted as a validation assay method. At this level, the objective will consist of determining the reliability and robustness of the targeted biomarker, as well as of its internal validation on a larger number of animals of high heterogeneity and preferably under industrial conditions. For example, during this step one can perform an evaluation of the specificity and sensitivity of the biomarker by means of appropriate methods using receiver operating characteristic curves among others [39]. To conclude, the advances on analytical methods and bioinformatics tools will enable in the future further access to detailed information on enzymatic events, physiological responses or metabolic status that would allow a better optimisation of the robustness and accuracy of the biomarkers.

3. Proteomics and beef tenderness biomarkers

Tenderness is considered as one of the most important palatability traits of cooked beef and a known driving factor that affects its acceptability along with the buying decisions by consumers who are willing

to pay more for tender cuts [40, 41]. However, variability in beef tenderness still occur and is related to several factors ranging from farm-to-fork [5, 9, 28], also described to be a result of complex interconnected molecular pathways [16]. In fact, the inherent variability of tenderness among beef cuts is mostly understood by consumers who associate it for example to the cooking methods or to the price of the meat cuts. However, the variability from the same meat cut, especially the valuable pieces such as ribeye, are a serious concern for both consumers and meat industry. Moreover, there is currently no consistent conclusion about the mechanisms of beef tenderization, but several studies have recognized that the meat tenderizing process involves myriad pathways such as the degradation of structural proteins, energy metabolism pathways, response to stress and oxidative stress, apoptosis and signalling pathways [6, 14] as confirmed recently by the integromics study of Gagaoua and co-workers [16].

In our quest for beef tenderness biomarkers, more than 100 proteomics studies were conducted in the last 20 years for several objectives. It is beyond the scope of this short paper to review the entire range of proteomics studies on beef quality and the reviews that were conducted. However, it is important to mention the recent ground-breaking integromics meta-analysis performed by our group on 28 eligible beef tenderness proteomics experiments (22 papers) from the literature. This analysis allowed to propose the first comprehensive list of 124 biomarkers (**Figure 3**) from which 64 were found in a minimum of two studies, allowing then to shortlist for future validation a robust panel of 33 biomarkers (bold and italics gene names in **Figure 3**) that were identified in at least four independent experiments [16].

ACTA1	**TNNT1**	MYL6B	DES	*VCL*	**CKM**	**TPI1**	IDH1	*OGDH*	**PARK7**
CAPZB	**TNNC1**	*ACTB*	*WDR1*	*COL1A1*	**ENO1**	LDHA	PYGM	*SLC25A11*	**SOD1**
FHL1	**TNNI2**	*CAPZA3*	MYOZ1	*COL1A2*	**ENO3**	*GPI*	UQCRC1	NDUFS1	**PRDX6**
MYH7	**MYLPF**	TPM3	*MYOZ3*		**GAPDH**	PGAM2	GPD1	ALDH1A1	*SOD2*
MYH1	*FLNC*	ACTN3	*PDLIM7*		**ALDOA**	*SDHA*	*AKR1B1*	*GOT1*	*PRDX1*
MYBPH	KLHL41	CSRP3	*PDLIM1*		**MDH1**	*PDHB*	*DLST*		*PRDX2*
MYL1	MYL3	*ACTN2*	*SMTNL1*		**PKM**	AK1	ATP5B		GSTP1
TNNT3	MYL2	MYH2	*TMOD1*	**A**	**PGM1**	*ACBD6*	*NNT*	**B**	MSRA
CA3	*RABGGTA*	EEF1A2	*VDAC1*	*HPX*	*STBD1*	*CALM2*	**HSPB1**	**HSPA9**	*PDIA3*
GDI2	HINT1	HBB	VDAC2	*CAVIN1*	*ANKRD2*	*FABP3*	**HSPB6**	**YWHAE**	*SH3BGRL3*
ALB	*AAMDC*	*NUTF2*	*TF*	*ADSS1*	*KCNJ15*	*SHC4*	**CRYAB**	*HSPA2*	*CSN2*
RAB21	*ZNF197*	*HIP1R*	*POR*	HIST2H2AA3	*ANXA6*	TP53	**HSPA1A**	*CCT8*	
PARP1	*ZFHX4*	*MPHOSPH9*	*MB*	LGALS1	TRIM72	*AHCY*	**HSPA8**	*STIP1*	
ITPR1	**E**			*PSMB2*	*PSMC2*	**F**	**HSPA1B**	**C**	**D**

A. n = 35	Muscle contraction, structure and associated proteins	**D.** n = 11	Oxidative stress proteins
B. n = 29	Energy metabolism	**E.** n = 36	Regulation of cellular processes, binding and transport
C. n = 11	Heat shock proteins	**F.** n = 2	Proteolysis

Figure 3. List of the 124 putative protein biomarkers of beef tenderness identified in the integromics meta-analysis of Gagaoua *et al.* [16] based on 28 proteomics-based studies performed on M. *Longissimus thoracis*.

The proteins are organised and highlighted by different colours according to their functional pathways; blue = muscle contraction, structure and associated proteins; orange = energy metabolism proteins; red = heat shock proteins; green = oxidative stress proteins; yellow = proteolysis; and black/grey = regulation of cellular processes, binding, apoptosis and transport proteins. The full protein names are given in detail in [16]. The proteins (gene names) in bold character are robust candidate protein biomarkers identified in a minimum of 4 independent proteomics studies. Those in italic were identified in one study only. The others with normal characters were identified 2 or 3 times.

Briefly, this meta-analysis allowed to identify in a robust manner the importance of the changing integrity of muscle contractile and structure proteins, energy metabolism enzymes, response to stress and oxidative stress proteins in the determination of beef tenderness, in that order of importance. Moreover, protein networks analysis delivered a functional annotation of the 124 proteins from M. *longissimus thoracis* and provided key insights into the interconnectedness among various pathways and processes in the muscle which are pivotal in producing high quality beef. Therefore, six interconnected pathways were identified to play a pivotal role in the determination of beef tenderness these being: (i)

Muscle contraction and structure development; (ii) Energy metabolism; (iii) Cellular responses to stress; (iv) Response to oxidative stress; (v) Proteolysis and (vi) Regulation of cellular processes, binding, apoptosis and transport. In addition, it seemed from the protein-protein interactomics that these six pathways and most of the proteins directly or indirectly impinge on apoptosis onset in *post-mortem* muscle confirming the importance of this early *post-mortem* phenomenon [6] in muscle to meat conversion and consequences on beef palatability traits, likely tenderness. The meta-analysis study further revealed the importance of mitochondria, thus suggesting to conduct more studies in the future to understand the specific role of mitochondrial metabolism *post-mortem* on tenderness development, and how this is related to differences between muscles with different proportions of fibre types [42], where mitochondrial metabolism differs.

On another hand, similar integromics meta-analysis of published proteomics studies were recently conducted by our group to create new biomarkers repertoires for beef colour [18] and the quality defect of dark, firm, and dry (DFD) beef, otherwise termed dark-cutting beef [43]. From our data integration experience, it seemed that meta-analysis and data-mining of published proteomics studies are very useful tools to gather and compare meat eating quality OMICs studies conducted under various platforms [16, 18, 44] or even from a single laboratory using the same proteomic platform [12] in the frame of integrated animal systems biology to draw robust conclusions. These analyses allowed also to shortlist protein biomarkers for validation along with broadening our knowledge on the underlying mechanisms compared to conventional (traditional) studies.

Further, in the era of big data and data analytics, and in a time when OMICs data is easier than ever to collect, an important limitation on fully elucidating molecular signatures of relevance relates to the 'shape' of data that is commonly generated. Within proteomics datasets, and OMICs data in general, the number of data recorded for each sample being vastly greater than the number of samples / phenotypes in a traditional (conventional) study and this challenges statistical inference. For the future, multi-OMICs approaches and sophisticated statistical and bioinformatics would allow further development in this topic to allow further progress in the pipeline of biomarkers discovery as well that of a better understanding of the mechanisms behind beef tenderization.

4. Proteomics and beef colour biomarkers

Lean beef colour is critical to fresh meat marketability as it often influences consumer purchasing decisions (indicator of freshness and wholesomeness) and attractiveness at the point of sale. Historically, the role of muscle proteins in meat colour have been identified including the major role of muscle fibre type [42], glycolysis and sarcoplasmic proteins [19], oxidation and myofibrillar structure [7, 45]. Proteomics has only recently been used to study the muscle proteome associated with beef colour stability and/or variation [46], allowing new insights about the underlying mechanisms. Indeed, proteomics was mainly used to investigate the biochemical basis of pre- and post-harvest aspects affecting colour and to identify predictive candidate protein biomarkers for colour stability [3]. Currently, there exist around 13 proteomics studies that aimed to study beef colour with the goal of identifying biomarkers that are correlated with one of the colour parameters [18]. Using an integromics data-mining approach, these studies were gathered by Gagaoua and co-workers in a unique repertoire of 79 protein biomarkers for 5 different muscles (**Figure 4**) with a major number of them (n = 59) from M. *longissimus thoracis* [18]. This protein list was also subjected to bioinformatics and pathway process enrichments, therefore allowing to confirm several biological pathways previously known to be involved in meat colour development, including energy metabolism (mainly glycolysis), contractile and associated proteins, proteolysis, chaperones and heat shock proteins, oxidative stress and cell redox homeostasis, and binding, cofactor and transport proteins, including signalling or apoptosis (**Figure 4**).

The analysis of the pathways governing colour stability showed that similar mechanisms are shared with tenderness, however glycolysis and other associated energy metabolism functional pathways are predominant for colour while muscle structure and contractile proteins were for beef tenderness determination [16, 18]. For beef colour proteomics and with a cut-off level ≥3 studies that identified a given protein, 27 potential biomarkers were shortlisted as robust from which β-enolase (ENO3),

peroxiredoxin 6 (PRDX6), HSP27 (HSPB1), phosphoglucomutase 1 (PGM1), superoxide dismutase (SOD1) and μ-calpain (CAPN1) were consistently reported (cut-off level ≥5) by multiple studies as being differentially expressed and having a pivotal role in beef colour.

The two integromics allowed to confirm the central role of energy metabolism in determining beef colour and tenderness [16, 18], as well as its role in determining *post-mortem* pH [43]. Apart from the role of energy metabolism in determining meat colour, the study highlighted the potential of integromics to reveal the importance of oxidative stress, cell redox and contraction pathways, and particularly their interactions, in beef colour. In fact, the changes in muscle structure and contractile proteins were proposed to have their role through light scattering from structural elements and the paleness of the meat surface. The oxidative/redox proteins were proposed to have a role in the onset of oxidation *post-mortem*, hence impacting beef colour, and importantly, colour stability during storage and retail display.

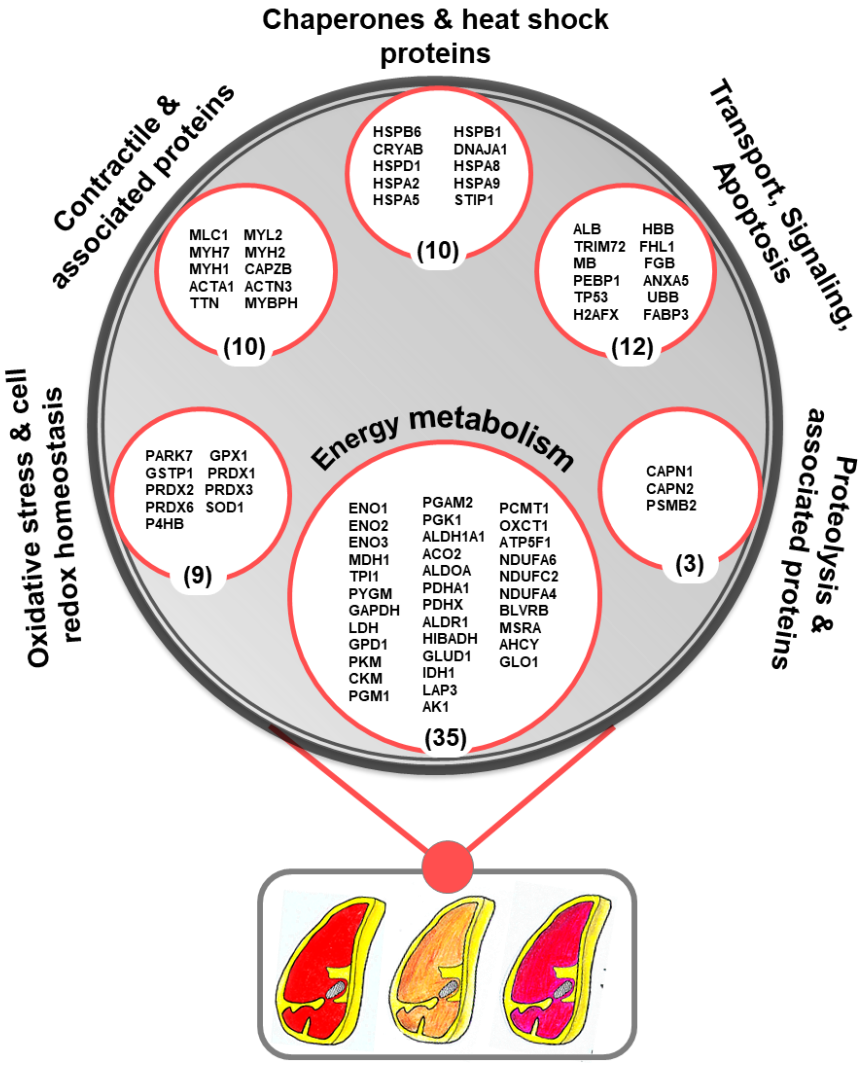

Figure 4. List of the protein biomarkers candidates (n = 79) of beef colour across five bovine muscles, but mainly *Longissimus thoracis* (n = 59), gathered in the integromics meta-analysis of Gagaoua *et al.* [18] to be correlated with colour parameters (*L**, *a**, *b**, *C**, *h**, MRA, OCR, RCR and R630/580). *Proteins were detected by gel-based and/or gel-free approaches coupled to mass spectrometry and organised by biological pathways and molecular functions to which they belong. The number of protein biomarkers for each biological pathway is shown in brackets. The full names of each protein (gene name) can be found in the original paper [18]. The graph is reproduced with permission from [18].*

5. Conclusion

To summarise, proteomics has provided during the last decade substantial data in terms of protein biomarkers related to or explaining the development of beef tenderness and colour stability. The recent integromics meta-analyses were valuable sources that synthesized the current knowledge and existing studies and beef proteomics datasets, hence allowing the building of reference databases (repertoires) of putative protein biomarkers of beef tenderness and colour traits. These repertoires allowed an in-depth description of the main biological pathways and mechanisms involved in the determination of these major sensory beef quality traits. In fact, such protein biomarkers repertoires may be enriched with newly identified proteins in future proteomics work, to allow further insights into the biological pathways involved in each meat quality trait and also to shortlist robust and generic biomarkers for validation and use for routine. For the future research, multi-OMICs will further increase our knowledge about these two quality traits by producing robust datasets to perform new types of analyses such as OMICs multi-layered networks. Also, more applications for data integration and analysis in the field of beef research would be demonstrated.

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