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**The color of fresh pork: Consumers expectations, underlying farm-to-fork factors, myoglobin chemistry and contribution of proteomics to decipher the biochemical mechanisms**

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## Abstract

The color of fresh pork is a crucial quality attribute that significantly influences consumer perception and purchase decisions. This review first explores consumer expectations and discrimination regarding pork color, as well as an overview of the underlying factors that, from farm-to-fork, contribute to its variation. Understanding the husbandry factors, *peri*- and *post-mortem* factors and consumer preferences is essential for the pork industry to meet market demands effectively. This review then delves into current knowledge of pork myoglobin chemistry, its modifications and pork discoloration. Pork myoglobin, which has certain peculiarities compared to other meat species, plays a central role in determining pork color, and a thorough understanding of the biochemical changes it undergoes is crucial to preserving color stability. Furthermore, the growing role of proteomics as a high-throughput approach and its application as a powerful research tool in meat research, namely to decipher the biochemical mechanisms involved in pork color determination and identify protein biomarkers, are highlighted. Based on an integrative muscle biology approach, the available proteomics studies on pork color have enabled us to provide the first repertoire of pork color biomarkers, to shortlist and propose a list of proteins for evaluation, and to provide valuable insights into the interconnected biochemical processes implicated in pork color determination. By highlighting the contributions of proteomics in elucidating the biochemical mechanisms underlying pork color determination, the knowledge gained holds significant potential for the pork industry to effectively meet market demands, enhance product quality, and ensure consistent and appealing pork color.

**Keywords:** Pig meat quality; Visual appearance; Pig husbandry factors; Muscle biochemistry; Muscle proteomics; Biomarkers.

## 1. Introduction

The color of fresh pork is a paramount meat quality attribute that exerts a profound influence on consumer perception and purchase decisions (Mancini, 2009; Mancini & Hunt, 2005). The appealing visual appearance of pork is often associated with freshness, taste, and overall quality, while color defects in meat are often seen by consumers as indications of spoilage and unwholesomeness. Therefore, understanding the factors that contribute to the variation in pork color, from farm-to-fork, is of utmost importance for the pork industry to effectively meet market demands and ensure consumer satisfaction. Pork color can be objectively determined by instrumental evaluation using a chromameter or spectrophotometer and considering the  $L^*$ ,  $a^*$ ,  $b^*$  values in CIELAB color space (CIE, 1978). Therefore, the most important color traits of fresh meat at the time of sale are the lightness (paleness) measured by CIE- $L^*$ ; the redness measured by CIE- $a^*$ , and the yellowness measured by CIE- $b^*$  (King et al., 2023). Color can also be determined in the  $L^*C^*h^\circ$  color space (considering polar instead of Cartesian coordinates) in which  $L^*$  is lightness (as above),  $C^*$  is the chroma or saturation (calculated from square root of  $(a^{*2} + b^{*2})$ ) and  $h^\circ$  is the hue angle that corresponds to the angle formed between  $a^*$  and  $b^*$  axes (the lower the  $h^\circ$  value, the redder the meat) (Lebret, Prevolnik Povše, & Candek-Potokar, 2015). Lightness is related to myoglobin concentration as well as light scattering, while  $a^*$  and  $b^*$  coordinates are related to myoglobin concentration and its chemical state (Purslow, Gagaoua, & Warner, 2021; Suman & Joseph, 2013). Moisture on the meat surface and the presence of exudate also influence light scattering intensity and lightness measured with a chromameter or perceived by the human eye.

To broaden our knowledge on the complex and multifaceted aspects of pork color, this review aims to provide an overview of the consumer expectations and discrimination regarding pork color at purchase, the factors from farm-to-fork (from pig husbandry to *peri-* and *post-mortem* processes) that influence pork color, the biochemistry of pork color especially considering the chemistry and chemical modifications of myoglobin, and the complex interplay between all these aspects. Finally, this review emphasizes the growing role of proteomics in meat color research and showcases its valuable contributions in deciphering the interconnected biochemical processes implicated in pork color determination, beyond the traditional knowledge of myoglobin chemistry. Indeed, by gathering the available proteomics studies in the frame of an integromics meta-analysis, we provide the first repertoire of pork color biomarkers identified by proteomics and propose a list of candidate biomarkers for

further evaluation. The proteomics insights revealed in this review offer valuable guidance to the pork industry in meeting consumer demands and developing strategies to ensure consistent and visually appealing pork color. By integrating knowledge at different levels, this review endeavors to bridge the gap between the multiple factors at interplay in pork color development.

## 2. Consumers expectations and discrimination at purchase of pork color

Color of fresh red meat influences the consumers' purchase decisions at the point of sale (Mancini & Hunt, 2005). Using Grunert's Total Food Quality Model (Grunert, Larsen, Madsen, & Baadsgaard, 1995), the work by Bredahl, Grunert, and Fertin (1998) investigated the relationships between pork meat appearance and expected *versus* experienced eating qualities. Color had the strongest correlation to the expectation of good eating quality. Brewer and McKeith (1999) showed that a pale color of pork results in a lower intention to purchase by consumers than normal color. The rate and extent of pH decline during the muscle to meat conversion influence the appearance and quality of fresh pork and contribute to qualify defects such as dark, firm, dry (DFD), pale, soft, exudative (PSE), red, soft and exudative (RSE), and red, firm and non-exudative (RFN) conditions (Lopez-Pedrouso, Lorenzo, Gagaoua, & Franco, 2020; van Laack & Kaufman, 1999). The color of DFD pork was found to be equally preferred by consumers (Brewer & McKeith, 1999). Jeremiah (1994) also reported a preference for DFD pork chops and normal chops *versus* a discrimination against PSE chops, in terms of willingness to purchase, noting that there was some segmentation in preferences. This echoed an earlier study of actual purchases of PSE, normal color and DFD pork chops offered in self-selection market conditions (Wachholz, Kauffman, Henderson, & Lochner, 1978), where 57% of the 280 consumers chose normal-colored chops, while 48% purchased chops outside the normal color range (22% PSE, 26% DFD). The authors of this study correctly highlighted the possibility that aspects of the differences in appearance between PSE, normal and DFD pork other than color could contribute to discrimination between samples (Wachholz et al., 1978), a point that equally applies to other later studies (Brewer & McKeith, 1999; Jeremiah, 1994)

Using 16 photographs of pork chops showing a range of color, marbling and drip, researchers surveyed the preferences of 11,717 consumers from 22 countries (Ngapo, Martin, & Dransfield, 2007). They identified 4 distinct clusters of preference; 18% preferred light red pork, 29% preferred light red, lean pork without drip, 26% preferred lean meat, and 27%

preferred dark red pork. Thus, while other factors influenced consumer discrimination, pork color was a major discrimination factor. Preferences varied between country, socio-economic status and gender, but the overall conclusion was that consumers strongly depend on raw pork meat color to make purchase choices, but there is segmentation in preference, *i.e.*, some consumers prefer dark red pork, and other prefer a lighter shade. Other surveys using the same methodology in different countries: Greece and Cyprus (Fortomaris et al., 2006); South Korea (Cho et al., 2007); Taiwan (Chen, Guo, Tseng, Roan, & Ngapo, 2010); Canada (Ngapo, 2017) and Mexico (Ngapo, Rubio Lozano, & Braña Varela, 2018) reached similar conclusions on the importance of color in the acceptability of raw pork and the segmentation of preference, but with variations in segmentation depending on age, sex, socioeconomic status and geographic area. For example, gender generally had very little influence on the actual liking of pork (Aaslyng et al., 2007), but in terms of color female consumers preferred well-done meat with a light/grey color, whereas male consumers were not influenced by the appearance (Aaslyng et al., 2007). In fact, gender specific preferences and attitudes towards meat are very complex and need to be always contextualized depending on the type of product and origin of consumers (Kubberød, Ueland, Rødbotten, Westad, & Risvik, 2002).

Using the terminology of Grunert's Total Food Quality Model (Grunert et al., 1995), the cost cues, extrinsic quality cues and intrinsic quality cues (of which raw meat color is an important part) all influence willingness to purchase based on an expectation of quality, whereas a separate set of factors and cues combine to determine the experienced perception of quality on eating the cooked product (Brunsø, Grunert, & Fjord, 2002). The internal color of meat is taken by many consumers to be an indicator of whether it is safe to eat (King & Whyte, 2006). The color of cooked pork varies between muscles, with *post-mortem* storage and with end-point cooking temperature (Cauble et al., 2021; Honegger et al., 2022; Wilson et al., 2017). A retention of a pink color in cooked pork at a variety of end-point temperatures was found to be more prevalent in DFD *versus* PSE pork (Lien et al., 2002). Concerns about *Trichinella spiralis* have historically meant that recommendations for the cooking temperature of pork to be high (71°C), and retention of an internal pink color is deemed by many consumers to be an undesirable indicator of incomplete cooking (Lien et al., 2002). Authors examined the phenomenon whereby a pink color develops on brief storage of pork (return to red) in chops after cooking up to 76.6°C and noted that this redness is more pronounced at higher pH (Mancini, Kropf, Hunt, & Johnson, 2005). However, recent studies demonstrate a higher consumer acceptability of a pink color in pork cooked to the lower temperature of

63°C when confronted with improvements in tenderness, juiciness and flavor in sous-vide cooked pork (Honegger et al., 2022). Thus, traditional consumer preferences on the color of cooked pork may be overcome by other intrinsic eating qualities, if they have an extrinsic quality assurance that *Trichinella* risk is not a concern in a particular market.

### 3. Factors from farm-to-fork influencing pork color

The color of pork is determined by the concentration and redox state of pigments, and the muscle microstructure that influences the light-scattering properties of meat (Lindh, Lundström, & Tornberg, 2001; Ramanathan, Suman, & Faustman, 2020). The biomolecular interactions between myoglobin and muscle components (e.g., sarcoplasmic and/or myofibrillar proteins, mitochondria, and metabolites) in skeletal muscle matrix contribute to the redox state of the heme protein and appearance of fresh meat (Ramanathan, Hunt, et al., 2020; Suman, Wang, Gagaoua, Kiyimba, & Ramanathan, 2023). These biochemical and physical properties of meat result from complex interactions between husbandry (breed, muscle, diet, housing...), *peri-* and *post-mortem* factors (pig handling, transport, slaughtering, meat chilling and ageing, packaging and storage) within the whole value chain (Lebret & Čandek-Potokar, 2022; Mancini, 2009; Roløfsvold & Andersen, 2003; Sun & Berg, 2018; Tomasevic, Djekic, Font-i-Furnols, Terjung, & Lorenzo, 2021). In the following sections, these multifaceted factors (husbandry and *peri-* and *post-mortem* factors) that collectively contribute to the visually appealing color of pork on our plates are discussed. Due to the major effects of husbandry, pre-slaughter handling, slaughtering, meat storage and color measurement conditions (blooming, testing (replicates), equipment, procedure *i.e.* light source, illumination/viewing system, standard observer angle, diameter aperture...) on meat color coordinates (Gagaoua et al., 2020; Honikel, 1998; Lebret, Ecolan, Bonhomme, Méteau, & Prunier, 2015; Purslow et al., 2021; Tapp, Yancey, & Apple, 2011), only studies comparing factors with all other things and conditions equal were considered here. Furthermore, a summary of the factors considered in this section and how they impact fresh pork color is depicted in **Table 1**.

#### 3.1. Husbandry factors influencing pork color

##### a. Genotype, pig breed and sex

In pigs, the most important **genetic effect** on meat color is stress susceptibility and *post-mortem* muscle metabolism. Pork color especially from white muscles like *Longissimus* (LM)

is dramatically affected by the presence of “n” halothane allele (RYR1 locus) which induces an acceleration in *post-mortem* pH decline and leads to PSE meat defect (Barbut et al., 2008). PSE pork is mostly characterized as a result of a rapid pH decline *post-mortem* while carcass temperature remains high (Bowker, Grant, Forrest, & Gerrard, 2000). It has been described that a point mutation at the level of the 615 amino acid between Arginine (Arg) and cysteine (Cys), Arg615Cys, of the sarcoplasmic reticulum  $\text{Ca}^{2+}$  release channel is at the origin for the aberrant  $\text{Ca}^{2+}$  metabolism observed in *post-mortem* muscle (Monin, 2003). In the case of pigs with Arg615Cys mutation,  $\text{Ca}^{2+}$  is released at a rate that is around twice that of normal muscle (Mickelson & Louis, 1996). Furthermore, earlier studies evidenced that  $\text{Ca}^{2+}$  uptake is reduced in *post-mortem* muscle in pigs with this stress susceptibility (Küchenmeister, Kuhn, Wegner, Nürnberg, & Ender, 1999). The increase in the sarcoplasmic calcium activates muscle metabolism, hence accelerating the production of lactate and its accumulation in *post-mortem* muscle, therefore impacting meat color. Although the mutative “n” allele is recessive, LM from heterozygous pigs has intermediate  $L^*$  and  $b^*$  values between NN (normal) and nn genotypes (Salmi et al., 2010). Genotype at the *PRKAG3* gene (R200Q substitution in the *PRKAG3* gene), the other major gene affecting pork quality, also influences meat color with carriers of the RN<sup>+</sup> allele exhibiting higher muscle glycogen content and lower ultimate pH (acid meat), and lighter but also redder meat (higher  $a^*$  value) than the non-carriers (For review: (Ciobanu, Lonergan, & Huff-Lonergan, 2011)). Indeed, higher pigment content and  $a^*$  value have been found in the LM of Hampshire breed exhibiting a high frequency of the RN<sup>+</sup> allele compared to Swedish Landrace or Swedish Yorkshire breeds (Lindahl et al., 2001). In contrast, the favorable Ile199 allele in the *PRKAG3* gene, reported to be at high frequency in Berkshire breed, is associated with higher ultimate pH and better color scores, *i.e.* lower  $L^*$  and  $b^*$  values (Ciobanu et al., 2011).

Apart from these allelic effects, variations in color and pigment have been reported among **pig breeds**. For example, when comparing five pig breeding lines (n = 100 pigs per line considered as representative of each population) reared under the same environment and regimen in a breeding nucleus farm and slaughtered in the same abattoir, (Plastow et al., 2005) found the lowest and similar  $L^*$  average values in loin from Duroc, Large White and Piétrain (NN) lines whereas loin from Landrace had the highest  $L^*$  value, those from Meishan being intermediate. The lowest  $a^*$  average value of loin was found for Landrace and the highest for Piétrain and Meishan lines, the Large White and Duroc lines being intermediate. Regarding  $b^*$ , the lowest average values were found in the Large White and Duroc lines and



the highest in the Meishan and Landrace, the Piétrain being intermediate (Plastow et al., 2005). In another, smaller-scale study ( $n = 11$  to 18 pigs per line) therefore leading to less robust findings, chops from Duroc, Berkshire and Pietrain (nn) were found of less pink color than chops from Pietrain NN or Hampshire (either  $rn+$  or  $RN^-$ ) (Brewer et al., 2002). These authors reported that meat from Duroc, Pietrain NN and Hampshire ( $RN^-$ ) had the lowest  $a^*$  values, whereas  $L^*$  and  $b^*$  values did not differ according to genetic background, indicating a potential gap between visual appearance and physical color assessment. Additionally, pork from Hampshire animals demonstrated greater pigment content than the meat from Landrace (Lindahl et al., 2001) and Large White (Monin & Sellier, 1985) counterparts. In contrast with the above mentioned studies, a comparison of pork quality traits between pure Duroc and British Landrace (NN) pigs ( $n = 80$  pigs from 20 litters per breed allowing a good representativeness of these populations) showed lower  $L^*$  and higher  $a^*$  but similar  $b^*$  average values for chops from Duroc compared to Landrace pigs (Cameron, Warriss, Porter, & Enser, 1990). Studies comparing pork color from Duroc and Pietrain (NN) crossbred pigs generally report few if any color differences: similar  $L^*$ ,  $a^*$  and  $b^*$  values were reported in loin from Duroc and Pietrain NN crossbreeds by (Lowell et al., 2018) ( $n = 80$  pigs / genotype) and (Kowalski et al., 2020) ( $n = 40$  pigs / genotype). In a recent study we also found similar  $L^*$  but slightly lower  $a^*$  and  $b^*$  values in loin from Duroc vs Pietrain NN crossbred pigs, however these differences were not noticeable by human eyes as the score of red color intensity of raw meat assessed by a trained panel did not differ between genotypes (Lebret, Lhuisset, Labussiere, & Louveau, 2023). In contrast, greater differences in color coordinates especially  $L^*$  and  $a^*$  values are generally reported when comparing, within similar pig rearing, slaughtering and color measurement conditions, ‘local’ pig breeds that have not been selected for efficiency or lean growth rate, and ‘modern’ selected breeds (Lebret & Čandek-Potokar, 2022). Accordingly, a study on the LM of the French local Basque breed reported lower  $L^*$  and higher  $a^*$  values associated to higher red color intensity of raw meat compared with Large White breed (Lebret, Ecolan, et al., 2015). Based on another study conducted simultaneously on Danish Landrace pigs representing the years 1976 and 1995, it appears that the genetic selection for improved growth performance in this breed has resulted in lighter and less red meat and lower pigment content through correlated responses (Oksbjerg et al., 2000; Rosenvold & Andersen, 2003).

Influence of the **sex** of pigs on pork color is rather limited. In their meta-analysis, Trefan, Doeschl-Wilson, Rooke, Terlouw, and Bünger (2013), found no significant differences for  $L^*$ ,

$a^*$  and  $b^*$  values between non-castrated males, surgically castrated males and females but slightly higher  $L^*$  values for immune-castrated males. Such findings were in agreement with another earlier study (Pauly, Luginbühl, Ampuero, & Bee, 2012).

#### *b. Muscle type*

Irrespective of breed and age (discussed below), fresh pork color mainly depends on the **anatomical location and physiological function of muscles** (locomotive, support, *etc.*) that determines their fiber composition, and consequently their contractile and metabolic properties. In fact, muscle fibers are typically classified according to their contractile and metabolic properties into slow-twitch oxidative (SO), fast-twitch glycolytic (FG) and fast-twitch oxidative-glycolytic (FOG) fibers which differ, among others, in their pigment content (Lefaucheur, 2010; Listrat et al., 2016). The variation and distribution of oxidative (red) and glycolytic (white) fiber types is a key factor in *post-mortem* metabolism and pork quality, including for color (Lefaucheur, 2010). The myosin-1 (type IIX), myosin-4 (type IIB), related myosin light chains, troponin complex (fast), and metabolic enzymes are overexpressed in glycolytic fibers, while myosin-2 (type IIA), myosin-7 (type I), and myoglobin and mitochondrial oxidative metabolic enzymes were more importantly abundant in oxidative fibers (Kim, Yang, & Jeong, 2018; Picard & Gagaoua, 2020b). The high amount of oxidative fibers suggests, at first glance, higher pigment (*i.e.*, myoglobin) content and  $a^*$  value and lower  $L^*$ . This can be exemplified by findings that have been reported in *Biceps femoris* (BF) compared with LM, indicating the darker and redder meat of the BF (Heyer & Lebret, 2007; Lindahl et al., 2001). Loin and some hind leg muscles (*e.g.* *Semimembranosus*) have a more glycolytic metabolism, whereas foreleg, abdominal and neck muscles (*e.g.* *Semispinalis*) have more oxidative metabolism, inducing differences in meat color with in general lower  $L^*$  and higher  $a^*$  (mostly) and  $b^*$  values in the oxidative ones (Terlouw, Berne, & Astruc, 2009). Within-muscle variations in meat color, which can affect visual appearance and consumer acceptance of pork, can also occur. Variations in meat color ( $L^*$ ,  $a^*$ ) across zones of the *Semimembranosus* muscle have thus been reported and found to be associated to inherent differences in myoglobin abundance and metabolic properties (Kirkpatrick et al., 2022). On another hand, pork from extensively produced animals have been reported to be darker and redder due to greater myoglobin content (Gentry, McGlone, Miller, & Blanton, 2002). Variation on pork color can further be explained by *post-mortem* pH and its decline, mainly due to the variations induced in the rate of myoglobin oxidation. As previously mentioned, it is overall understood that low pH increases the rate of myoglobin denaturation and negatively

impacts the oxidation-reduction processes, destabilizing myoglobin and negatively impacting the final color of pork.

### *c. Feeding and rearing practices*

**Pig diet** can affect several muscle traits that influence pork color through their effects on glycogen stores, the rate and extent of *post-mortem* pH decline, the glycolytic potential or antioxidant status (Huff-Lonergan et al., 2002; Tikk, Lindahl, Karlsson, & Andersen, 2008). Finishing diets with a low digestible carbohydrate content reduced muscle glycolytic potential, thus decreasing meat lightness (Rosenvold et al., 2001). Dietary magnesium supplementation, from growing period onwards or only few days prior to slaughter, has been shown to increase meat darkness, as magnesium counteracts catecholamine effects in stress situations (Rosenvold & Andersen, 2003). Dietary manganese supplementation (80 ppm) also improved pork color, with reduced  $L^*$  value and increased subjective color scores, after 7 days of retail display of meat from supplemented pigs compared with controls (Apple et al., 2007). A preliminary study ( $n = 5$  pigs per group) suggested that feeding supranutritional concentrations of vitamin D<sub>3</sub> (80,000 IU/kg) to Duroc crossbred pigs would impact pork color with darker (lower  $L^*$  values) in the LM muscle of supplemented pigs compared with those fed with the control diet (Wilborn, Kerth, Gowsley, Jones, & Frobish, 2004). Earlier results further observed similar results when pigs are fed with moderate to high concentrations of vitamin D<sub>3</sub> for 7 or 10 days preceding slaughter by increasing the subjective color and firmness scores, while decreasing  $L^*$  values (Wiegand et al., 2002). Nevertheless, the improvement of pork color (less light meat) as a result of vitamin D<sub>3</sub> dietary supplementation remains to be confirmed on a larger scale, as another earlier study reported no effect (Swigert, McKeith, Carr, Brewer, & Culbertson, 2004). Besides, the underlying mechanisms on how feeding high concentrations of vitamin D<sub>3</sub> to pigs improves pork quality are not fully understood. We think that vitamin D<sub>3</sub> would impact the contractile and metabolic properties of the muscle with a shift to more oxidative muscle, hereby decreasing the rate and extent of pH decline, therefore improving the stability of color. Including extruded linseed in pig diet is a common practice to improve pork nutritional value (*i.e.* decreased n-6:n-3 polyunsaturated fatty acid ratio), but this increases the risk of lipid oxidation during meat storage which could in turn affect pork color. However, it has been shown that dietary supplementation of extruded linseed associated to antioxidants supplementation from either natural (polyphenol-rich grape skin extract) or synthetic origin ( $\alpha$ -tocopherol acetate) does not affect pork color parameters ( $L^*$ ,  $a^*$ ,  $b^*$ ) (Lebret et al., 2023; Minelli et al., 2020). Indeed, preventing effects of

various natural antioxidants (*e.g.* the dipeptide carnosine, naturally present in muscle tissue) on pork color changes during storage have been demonstrated (for review: (Jiang & Xiong, 2016)). Supplementing pig diet with antioxidants or their precursors can also influence the stability of meat color through their modulating effect on pigment oxidation (Phillips et al., 2001). For example, the supplementation with methionine (precursor of cysteine which forms the cellular antioxidant glutathione) two weeks prior to slaughter, limited meat discoloration and changes in lightness after 7 days storage (Lebret, Batonon-Alavo, Perruchot, Mercier, & Gondret, 2018). The effects of vitamin E supplementation on color stability of fresh pork are controversial, as both improvement and lack of effect have been reported in contrast with its general positive influence on color stability of beef (for review: (Rosenvold & Andersen, 2003)). These species-specific differences would rely on differences in the primary structure of myoglobin, making pork myoglobin less susceptible to oxidation (attack by reactive lipid oxidation products) than myoglobin from other animal species (Domínguez et al., 2019; Suman et al., 2023).

Increasing **slaughter age** of pigs can influence meat color, due to increased myoglobin content with animal age (Sun & Berg, 2016). This well described effect in cattle and lambs (della Malva et al., 2016; Gagaoua, Picard, & Monteils, 2018) can also occur in pigs when considering higher differences (*e.g.* 2 to 4 months) in slaughter age (Mayoral et al., 1999). A progressive increase in  $a^*$  value without changes in  $L^*$  and  $b^*$  values with increased slaughter age from 8 to 14 months was thus reported by (Ortiz et al., 2021). Increased intensity of visual red color associated to lower  $b^*$  and  $h^\circ$  values were found in the loin of Basque pigs slaughtered at 14 *versus* 10.5 months (Lebret, Ecolan, et al., 2015), even though in that case, age could be confounded with other factors such as differential pig physical activity in extensive *versus* 'conventional' farming system.

**Housing and rearing conditions** of animals can also influence meat color through their effects on muscle metabolic properties (myofiber typing, glycogen content and possibly pigment content) as a consequence of physical activity, ambient temperature and pig diet (Lebret & Čandek-Potokar, 2022; Millet, Moons, Van Oeckel, & Janssens, 2005). Indoor enrichment, *i.e.*, extra space and deep straw bedding *versus* slatted floor, has generally no effect on color parameters (for review: (Lebret, 2008)). Free-range rearing or access to outdoors has led to lower meat lightness and/or higher redness or yellowness (Gentry et al., 2002; Terlouw et al., 2009). However, these effects are not generic as reviewed in several earlier studies (Millet et al., 2005; Patton, Huff-Lonergan, Honeyman, Kerr, & Lonergan,

2008), because they depend on the overall influence of housing conditions on ante and *post-mortem* muscle metabolic traits. Moreover, effects of pig outdoor access on meat  $a^*$  and  $b^*$  can be greater in ham muscles than in loin (Lebret et al., 2011). Organic farming has generally weak effects on pork color – if any and these are largely dependent on the on-farm practices (including housing conditions) in interaction with pig genotype (Álvarez-Rodríguez et al., 2015; Prache et al., 2022; Tomažin, Batorek-Lukač, Škrlep, Prevolnik-Povše, & Čandek-Potokar, 2019). Besides, effects of rearing conditions on pork color appear to be muscle-dependent, with ham muscles generally being more affected than the loin (Lebret, 2008).

### 3.2. *Peri- and post-mortem factors influencing pork color*

**Pre-slaughter handling** of pigs can strongly affect the development of meat color through its influence on the rate and extent of *post-mortem* muscle pH decline. In pigs like in other animal species, an important stress during pre-slaughter handling or a high physical activity level during transport or due to fighting after mixing of animals for example, leads to low muscle glycogen content at slaughter and therefore high ultimate pH and risk of developing meat quality defects such as DFD meat (see above; (Faucitano & Nannoni, 2023; Rosenvold & Andersen, 2003; Terlouw et al., 2021)). By contrast, stress immediately before slaughter increases *peri-* and *post-mortem* muscle metabolism while muscle temperature is still high, leading to rapid pH decline and PSF meat exhibiting high paleness. This can occur in pigs as a consequence of inadequate immediate pre-slaughter conditions independently of their genotype at the RYR1 (halothane) locus. A high muscle temperature early *post-mortem*, induced by pre-slaughter stress, has major consequences on the development but also the stability of pork color (Lebret & Čandek-Potokar, 2022; Rosenvold & Andersen, 2003; Sun & Berg, 2018; Terlouw et al., 2021).

Slaughter, meat refrigeration and ageing conditions are also major factors influencing the stability and development of fresh pork color. The **stunning** method (carbon dioxide or electrical) and conditions (speed of the process) can affect the occurrence of PSE meat defect (Santé-Lhoutellier & Monin, 2014). A recent meta-analysis revealed that compared to carbon dioxide, electrical stunning is generally associated to higher meat lightness, especially in the case of head-to-back electrical stunning and conventional chilling of carcasses, whereas application of head-only electrical stunning and fast chilling of carcasses reduce the differences on pork lightness found between the stunning methods (Zybert, 2022). The **chilling rate of carcasses** is also a critical point, as fast chilling rate allows limiting the level

of muscle anaerobic activity and the occurrence of excessive meat paleness, especially in the glycolytic pig muscles (Savell, Mueller, & Baird, 2005). Thus, all other factors (*i.e.*, muscle metabolic properties, chilling conditions...) equally, carcass fatness, which depends on pigs genetics and management (especially feeding) strategies (Lebret & Čandek-Potokar, 2022) may influence chilling rate and therefore pork lightness. Overall, proper cold chain management and refrigerated temperatures during storage and display are key points to maximize shelf-life but also the color of pork (Santé-Lhoutellier & Monin, 2014). For pork, because of the impact of high muscle temperatures and low pH on the development of PSE meat, a more rapid chilling process is suitable to decrease PSE with the recommended internal muscle temperature of 10 °C at 12 h and 2–4 °C at 24 h (Savell et al., 2005).

**Freezing** is a common preservation method for pork, but requires control over temperature decline and water crystallization, frozen storage and thawing to preserve the original properties of meat as much as possible, especially color. Fast freezing, which leads to small ice crystals, combined with slow thawing are good conditions to minimize moisture loss and cell destruction, which favors oxidation and meat discoloration (Leygonie, Britz, & Hoffman, 2012). Supercooling storage, defined as lowering a product temperature below its freezing point without phase transition (*i.e.* water remains liquid) has potential value to extend shelf life of pork without structural damages and denaturation induced by ice crystal formation during the freezing process. However, supercooled products can be instable as nucleation can occur (SangYoon Lee et al., 2022). These authors developed and validated a stepwise method to reduce cooling rate and achieve storage temperature between -1°C and -3°C for supercooling of pork loin. After unfreezing following 16 d storage, supercooling increased meat color stability by reducing  $L^*$  changes and discoloration compared to conventional refrigeration (+3°C), with similar color values found for supercooled and conventionally frozen (-3°C or -18°C) pork. However, water holding capacity was higher for supercooled than frozen meat (Lee et al., 2022). Thus, supercooling appears as an effective method to maintain pork quality and color, provided that cooling procedures are controlled.

**Storage conditions** markedly influence the color of fresh pork, which has a major importance in consumer purchase decision; therefore, **meat packaging** and storage time are of utmost importance in the meat and pork value chain. Both packaging system and gas composition have been reported to influence fresh pork color quality (Gagaoua, Bhattacharya, et al., 2021; Mancini & Hunt, 2005). Traditional packaging (PVC overwrap) is very commonly used in retail display because of its low price; moreover, this packaging exposes



meat to the atmosphere and has a high oxygen permeability, allowing the development of the attractive bright-red oxymyoglobin on meat surface. However, oxidation of myoglobin would eventually occur, limiting retail shelf-life to around 5-7 days (Sun & Berg, 2018). Vacuum packaging allows extending color stability compared with PVC packaging and prevents oxidative rancidity. Moreover, the low myoglobin content and reduced formation of deoxymyoglobin in pork compared with beef (Suman et al., 2023), limits the risk for development of the unattractive purple color (reduced myoglobin) of vacuum-packed pork (Mancini, 2009; Sun & Berg, 2018). Modified atmosphere packaging (MAP) methods are also commonly used for pork. Overall, pork retailed and displayed in carbon monoxide MAP exhibited greater redness than their counterparts in high oxygen packaging (Krause, Sebranek, Rust, & Honeyman, 2003). In fact, MAP with high atmospheric oxygen (70-80%) promotes the formation of oxymyoglobin and its penetration into meat, thus delaying the migration of metmyoglobin to meat surface and leading to acceptable retail color for 10-14 days; however, the risk for lipid oxidation and altered flavor can be increased. In contrast, MAP containing 0.4% CO, 20-30% CO<sub>2</sub> and the rest as nitrogen, allows to stabilize meat color and to reduce lipid oxidation along the development of spoilage organisms and pathogenic bacteria (Sun & Berg, 2018). Thus, CO MAP allows shipment of fresh pork to distant markets without compromising color or shelf-life (Wilkinson, Janz, Morel, Purchas, & Hendriks, 2006). Further, *post-mortem* ageing can affect the color properties and stability of meat depending on its length and muscle type (Tikk et al., 2008). Thus, apart from packaging, edible coating of meat during storage is another way to preserve color and increase shelf life (Gagaoua, Bhattacharya, et al., 2021; Gagaoua, Pinto, et al., 2022). As example, coating of pork loin slices with chitosan/starch aldehyde-catechin conjugate, that were demonstrated to have antioxidant and antimicrobial actions, reduced the commonly observed increase in meat lightness during 14 d storage and led to higher  $a^*$  and lower  $b^*$  of pork from day 6, indicating this coating technique as effective to maintain pork color during storage compared with non-coated pork (Hu, Yong, Zong, Jin, & Liu, 2022). In general, pig genotype, muscle type and diet (potentially including antioxidants) are mutually interacting factors that need to be taken into consideration in the evolution of pork color, due to their effect on early *post-mortem* muscle temperature and metabolism, which determines color stability during retail display and ageing. Finally, **display lighting** (light source, direction of illumination) and background coloration of package tray, are other factors that influence consumer's perception of pork color (Barbut, 2001; Sun & Berg, 2018). Overall, pork color has been described to be more

desirable when presented under incandescent light, in comparison with either cool white or warm white fluorescent, but to be more desirable under deluxe cool white fluorescent.

#### 4. Myoglobin modifications and pork color stability

The most recent American Meat Science Association guidelines on measuring meat color clearly state that meat color revolves around the pigment myoglobin (King et al., 2023). Modifications of the primary structure influence the oxidative stability of myoglobin (Suman et al., 2023). Specifically, the binding of reactive lipid oxidation products to nucleophilic residues accelerates oxidation of myoglobin and compromises the heme protein's redox stability (Faustman, Sun, Mancini, & Suman, 2010; Ramanathan, Hunt, et al., 2020). Covalent modifications of histidine residues by reactive lipid oxidation products have been extensively documented in myoglobins from horse (Faustman, Liebler, McClure, & Sun, 1999), beef (Suman, Faustman, Stamer, & Liebler, 2006; Suman, Faustman, Stamer, & Liebler, 2007; Viana et al., 2020), pork (S. Lee, Phillips, Liebler, & Faustman, 2003; Suman et al., 2006; Suman et al., 2007), chicken (Navarro et al., 2010), ostrich (Nair, Suman, Li, Joseph, & Beach, 2014), and emu (Nair et al., 2014). Additionally, nucleophilic adduction of reactive aldehydes at the proximal (*i.e.*, position 93) and distal (*i.e.*, position 64) histidines, which are critical to myoglobin functionality, compromises redox stability of the heme protein and leads to the formation of brown metmyoglobin (Suman & Joseph, 2013). These covalent modifications in myoglobin are responsible for lipid oxidation-induced discoloration in fresh meats (Faustman et al., 2010; Ramanathan, Hunt, et al., 2020).

Dietary antioxidants, such as vitamin E, have been exploited as an effective pre-harvest strategy to prevent lipid oxidation and increase color stability in fresh red meats, as described above (Domínguez et al., 2019). Lipid-soluble vitamin E acts as a free radical scavenger and antioxidant in the cell membranes and inhibits oxidation of polyunsaturated fatty acids, subsequently minimizing oxidation of membrane lipids and myoglobin in *post-mortem* skeletal muscles (Domínguez et al., 2019; Faustman, Chan, Schaefer, & Havens, 1998; Sanders et al., 1997). While increased color stability and decreased lipid oxidation were observed in fresh beef from vitamin E-supplemented cattle muscles (Faustman et al., 1998; Sanders et al., 1997), a color stabilizing effect was not observed in pork from vitamin E-supplemented pigs, although lipid oxidation was minimized in pork (Cannon et al., 1996; Phillips et al., 2001). Pork lipids contain greater levels of unsaturated fatty acids than beef lipids. Therefore, logically, pork lipids would undergo lipid oxidation more readily and



generate reactive oxidation products rapidly than beef lipids. This increased oxidation of lipids could lead to a decline in color stability in fresh pork, which could be prevented by vitamin E supplementation in pigs. However, the independent reports that dietary vitamin E improved beef color stability (Faustman et al., 1998; Sanders et al., 1997), but exerted no influence on pork color (Cannon et al., 1996; Phillips et al., 2001) indicated that the susceptibility of beef and pork myoglobins to lipid oxidation products is different.

The molecular basis of species-specific effect of vitamin E on fresh beef and pork color has been examined in model systems using 4-hydroxy-2-nonenal (4-HNE, a reactive aldehyde product of lipid peroxidation) and myoglobin. Mass spectrometric investigations (Suman et al., 2006) observed that at meat storage conditions (pH 5.6 and 4°C), pork myoglobin is less susceptible to aldehyde adduction than beef myoglobin. Moreover, pork myoglobin formed only mono-adducts with 4-HNE after 3 days at meat storage conditions, whereas beef myoglobin formed both mono- and di-adducts with the aldehyde. While two histidines (at positions 24 and 36) were adducted in pork myoglobin by 4-HNE, four histidines (at positions 36, 81, 88, and 152) were adducted in beef myoglobin. These mass spectrometric data indicated that pork myoglobin is less susceptible to nucleophilic attack by reactive lipid peroxidation products than its beef counterpart and suggested that the primary structure of pork myoglobin decreased its susceptibility to reactive lipid oxidation products. Further studies employing quantitative proteomic techniques characterized the kinetics of 4-hydroxy-2-nonenal adduction in pork and beef myoglobins (Suman et al., 2007). While histidine 36 in pork myoglobin was the most reactive to the aldehyde, histidines 88 and 81 in beef myoglobin (in close vicinity to the heme pocket) were readily adducted by 4-HNE. Pork myoglobin has 9 histidines compared to 13 histidines in beef myoglobin; the lower number of histidines in pork myoglobin could be partially responsible for its susceptibility to lipid oxidation. The aforementioned investigations demonstrated that lipid oxidation is more critical to color stability in fresh beef than in pork and also explained why dietary vitamin E did not improve fresh pork color stability.

Interestingly, additional investigations (Yin et al., 2011) reported that the susceptibility of myoglobins to lipid oxidation-induced modification and subsequent oxidation is proportional to the number of histidines in the primary structure; myoglobins containing 9 histidines (pork, chicken, and turkey) were less susceptible to lipid oxidation-induced oxidation than their counterparts containing 11-13 histidines (beef, lamb, horse, and deer) explaining the biochemical basis for the species-specificity in lipid oxidation-induced meat discoloration. A

side-by-side comparison of 4-HNE adduction sites identified in myoglobins from various meat species (**Fig. 1**) indicated that at meat pH (pH 5.6) the susceptibility of pork myoglobin to lipid oxidation products is different from beef myoglobin but was similar to the myoglobins from non-ruminants and poultry (For review: (Suman et al., 2023)). These comparative data suggested that pork myoglobin is inherently less susceptible to modifications by lipid oxidation products than ruminant (beef, sheep, and deer) myoglobins and that myoglobin modifications may be minimally influential on pork color stability.

In agreement with the aforementioned and given this immense focus on myoglobin biochemistry in relation to meat color (including pork), it is noteworthy that the myoglobin gene (MB), although present in the analysis in **Table 2**, does not play a prominent role as revealed by the integrative proteomics analysis in the current review (see section 5.3 and onwards). Indeed, only one of the seven proteomics studies listed in **Table 2** (Kwasiborski et al., 2008) flags myoglobin as a significant contributor to fresh pork color. A possible inference from this is that the various factors affecting paleness of pork *via* the effect of *post-mortem* muscle metabolism and temperature, and the partial denaturation of sarcoplasmic proteins (mostly enzymes from the energy metabolism pathways) and myofibrillar proteins would play major roles in the determination of fresh pork color. Proteomics seemed as a useful tool to decipher such mechanisms and to broaden our knowledge on the underlying mechanisms. However, the large amount of data generated by meat research proteomics can be overwhelming. The integration of the proteomics findings and/or datasets through data-, text-mining and computational biology methods (bioinformatics) is a promising way to better understand the complex biological systems and refine the list of candidate biomarkers (Vaudel et al., 2016). In fact, data reuse is a cutting-edge, active, and evolving field (Griss, Perez-Riverol, Hermjakob, & Vizcaíno, 2015), recently successfully applied to meat research to rediscover and reshape the publicly available proteomics data to decipher the unknowns of meat quality determination (Gagaoua et al., 2020; Gagaoua, Terlouw, Mullen, et al., 2021). Therefore, the integration of fresh pork color proteomics studies and the related proteins in a unique database (comprehensive data repository) is more than needed to have a critical overview on the conducted studies. Furthermore, several attempts can be achieved through such integrative approach (**Fig. 2**), likely i) to provide, in the frame of knowledge discovery, a more holistic understanding of the biological processes and their interactions in underpinning pork color determination and variation; ii) to perform a cross-study analysis with the aim of identifying the consistent and commonly reported proteins related to pork color traits, and

accordingly develop an integrative meta-analysis approach to reveal new and robust features and/or generic molecular signatures of pork color beyond the scope of individual studies; iii) to perform a systems biology and network analysis with the aim of creating more comprehensive and accurate models of biological processes governing pork color determination; and (iv) to shortlist and propose, in the frame of biomarker discovery, potential candidate biomarkers related/correlated to pork color traits. Indeed, these approaches are a way to assess the consistency and reliability of findings across different proteomics experiments. In the following we aim to provide an overview of an integrative approach conducted on the available studies on pork color proteomics and a brief overview of how proteomics has been used and the main objectives.

## **5. Proteomics to better understand of pork color determination**

### ***5.1. Brief overview of proteomics as a powerful tool in meat research***

Proteomics, a high-throughput technology for the analysis of proteins, has emerged as an essential tool in functional genomics for effectively playing a key role in meat research, in addition of its usefulness in deciphering the molecular mechanisms underlying meat quality traits (Gagaoua & Picard, 2022). In the last two decades, proteomics has been a powerful approach used for the determination of meat quality evaluation, prediction and authenticity (Gagaoua & Picard, 2022; Gagaoua, Schilling, Zhang, & Suman, 2022). It was particularly important among other foodomics methods since it provides more information on the underlying biochemical mechanisms compared to traditional biochemistry methods (Purslow et al., 2021). Indeed, the global or partial study of the *post-mortem* proteome (myofibrillar, sarcoplasmic, mitochondrial protein extracts) makes it possible to propose explanatory mechanisms for the origin of variability in meat quality and/or to propose potential biomarkers. Therefore, the discovery of biomarkers enables, for example, the design of protein chips to predict meat quality (Gagaoua, Bonnet, De Koning, & Picard, 2018; Picard et al., 2018). Thus, one of the lofty goals is to explain and/or predict meat quality, by comparing its molecular signatures with those of reference groups gathered in databases and constituted on the basis of a targeted quality trait using specific thresholds. In pork, it was also successfully applied to study most of the farm-to-fork factors discussed above standing from productive (breed, diet, stress, ...) to technological (aging, cooking, ...) factors, including the assessment of multiple quality traits and quality defects of both fresh and processed pork products (for review: (Lopez-Pedrouso et al., 2020; Zhou, Pan, Cao, & Zhou, 2021)).

Referring to the early proteomics studies, they were mainly based on electrophoresis gels, where extracted muscle proteins are in general separated by one-dimensional electrophoresis according to the molecular mass of the proteins or by two-dimensional electrophoresis, in two successive steps. In the latter case, proteins are first separated in a first dimension according to their isoelectric point (electroisofocalization step, IEF) and then according to their molecular mass in the second dimension (Picard & Gagaoua, 2020a). In this way, the proteins separated constitute individual spots, with each spot most often corresponding to one major protein, but several proteoforms can be detected thanks to the new high accuracy liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) tools (della Malva et al., 2023) in comparison to earlier mass spectrometry methods such as matrix assisted laser desorption ionization-time of flight (MALDI-TOF) (**Table S1**). Image analysis is performed after staining to quantify the relative abundance of each protein. The comparison of spots (or protein bands) intensities using imaging and densitometry software is what allows determining whether certain proteins are different between the treatments. The spots (or protein bands) with differential abundances are cut from the gel and then treated with proteolytic enzymes, most often trypsin, to obtain protein-specific peptides after their digestion (For review about further details: (Gagaoua, Schilling, et al., 2022)). These peptides are then identified by mass spectrometry. In gel-free proteomics analysis, which is an emerging approach known also as label free or shotgun proteomics, the proteins present after extraction are digested by proteolytic enzymes, releasing peptide fragments. It is this set of peptides or fragments that is then analyzed by mass spectrometry, mainly by means of LC-MS/MS (Lamri, della Malva, Djenane, Albenzio, & Gagaoua, 2023; Lamri, della Malva, Djenane, López-Pedrouso, et al., 2023).

## ***5.2. Proteomics as an emerging tool in meat (pork) color research***

The application of proteomics in meat color research is very recent (Gagaoua et al., 2020; Joseph, Suman, Rentfrow, Li, & Beach, 2012; Suman et al., 2023) although the first studies on pork color that evaluated its potential to study color stability or determination were conducted few years before those on beef color (**Table S1**). Based on the seven studies available in the literature and gathered in **Table S1** (details are given later) that have used proteomics to correlate muscle proteome with pork color traits (D'Alessandro, Marrocco, Zolla, D'Andrea, & Zolla, 2011; Hwang, Park, Kim, Cho, & Lee, 2005; Kim, Jeong, Yang, & Hur, 2019; Kwasiborski, Rocha, & Terlouw, 2009; Kwasiborski et al., 2008; Żelechowska, Przybylski, Jaworska, & Santé-Lhoutellier, 2012; Zhang et al., 2014), we can distinguish four

major and complementary objectives of its applications. First, the main aim of using proteomics in pork color research is to better understand the biological mechanisms behind color variability, stability or development. Second, for the identification of color-related proteins that are associated with desirable or undesirable meat color traits through targeted comparisons of the meat samples proteomes with divergent color characteristics. Such knowledge can be used to deepen our understanding on the molecular mechanisms underlying color development and potentially develop strategies to manipulate or control the final pork color. Third, for the discovery of candidate biomarkers based for instance on correlations with specific pork color attributes, but mostly with  $L^*$ ,  $a^*$  and  $b^*$  (**Table 2**). This is one of the outcomes awaited by the pork industry in the context of using these biomarkers as indicators or predictors of pork color quality, to enable them to develop a rapid and objective assessment of pork color traits at an early *post-mortem* stage. The selected biomarkers can be further valuable in quality control and breeding programs aiming at improving pork color properties. Finally, the forth objective aims to decipher the *post-mortem* changes in the muscle/meat proteome and type of consequences on pork color development. In fact, the study of the proteome early *post-mortem* or during ageing/storage, allows the identification of proteins and pathways that undergo modifications (e.g., oxidation) and provide insights on their impact on pork color stability and appearance.

Compared to the proteomics research on beef color that mostly focused on identifying color-related biomarkers with the aim of better exploring the mechanisms underlying color stability difference in the lightness/darkness of meat (Gagaoua et al., 2020), in pork, the focus was mainly on the study of the differences in paleness/lightness of fresh meat (Joseph et al., 2012). The former study by Sayd et al. (2006) using 2DE-MS-based approach, compared the sarcoplasmic proteome of light *versus* dark pork. The authors identified 22 differentially abundant proteins from *Semimembranosus* muscle. The proteins belonging to response to stress (heat shock proteins) and oxidative metabolism (glucose-regulated protein 58 kDa, HSPB1 and CRYAB) were more abundant in darker meat, whereas those involved in oxidative stress (glutathione S-transferase) and glycolytic enzymes were overexpressed in lighter meat. The findings suggested that the predominant oxidative metabolism in dark meat delays *post-mortem* metabolism through the ATP depletion and lower pH decline rate, thereby leading to pork discoloration (Sayd et al., 2006). Similarly, PSE pork known to have weak water-holding capacity and very high lightness ( $L^* > 50$ ), was intensively investigated by proteomics to understand the proteome basis of PSE development (Lopez-Pedrouso et al.,

2020; Zequan et al., 2021). Overall, these studies targeted limited number of samples, but with divergent phenotypes to assess primarily the biochemical basis and underlying molecular pathways, and possibly propose candidate biomarkers. For example, a label-based proteomics (isotope labelling) conducted on 20 animals (10 PSE and 10 normal pork samples) taken from a batch of 150 pigs (Duroc x Landrace x Large White), reported candidate biomarkers of PSE pork in NN pigs at the RYR1 locus by the identification of proteins from glycolysis pathway to be more abundant compared to those from the Krebs cycle (TCA cycle) and oxidative phosphorylation (Zequan et al., 2021). Structural proteins, chaperones, and proteins involved in signal transduction were also identified to participate in PSE production. Other recent label-free proteomics studies provided more insights on this phenomenon by comparing the myofibrillar proteome of RFN and PSE pork (Liu et al., 2022; Liu et al., 2021). Several molecular signatures have been reported to be important pathways associated with the development of PSE pork, some of which are mostly involved in signaling pathways: calcium signaling, AMPK signaling, PI3K-Akt signaling and EIF-1 signaling, followed by others related to the regulation of actin cytoskeleton, glycolysis/gluconeogenesis and tight junction. However, further studies are needed to explore using high number of animals and under more complex experimental designs, to capture as much as possible the factors of variation (breed, sex, season, feeding and rearing practices, age at slaughter and carcass weight, ...) with the aim of validating the identified molecular signatures and the related protein candidates in pork research.

### **5.3. Overview of the available proteomics studies on pork color research**

Foreseeing the above and within the objective of gathering published proteomics studies on pork color research that aimed to identify biomarkers, an integrative muscle biology approach, in the frame of integromics studies, was applied as previously described (Gagaoua et al., 2020). Briefly, a computerized literature search (Scopus, WebOfScience and Google Scholar) was performed using the keywords “color or colour”, “proteom\*”, “protein”, “biomarker” in combination with “meat”, or “pork” or “pig” or “muscle”. Papers published up to June 2022 were identified and scrutinized. The inclusion/exclusion criteria were based on (i) proteomics on *Longissimus* muscle; (ii) only proteins that were significantly correlated at the level of 5% with color traits; and (iii) exclusion of studies on DFD or PSE pork quality defects. Papers that reported protein abundances only or comparisons between ageing times of pork and/or effect on pork color without correlation were not included, but considered in the discussion when relevant. This approach allowed retaining seven eligible studies



(D'Alessandro et al., 2011; Hwang et al., 2005; Kim et al., 2019; Kwasiborski et al., 2009; Kwasiborski et al., 2008; Żelechowska et al., 2012; Zhang et al., 2014) published between 2005 and 2019 across *Meat Science*, *Journal of Proteomics*, *Food chemistry* and *European Food Research Technology* and *Animal Genetics* journals. The studies used different breeds, genotypes and evaluated CIE  $L^*$ ,  $a^*$ ,  $b^*$  color coordinates and applied mostly for the separation of the muscle protein extracts two-dimensional electrophoresis gels for five studies, one-dimensional electrophoresis and label-free quantification before the identification of the proteins by means of MALDI-TOF and/or LC-MS/MS mass spectrometry (**Table S1**). Interestingly and in comparison with previous proteomics studies on beef color (For review: (Gagaoua et al., 2020)) or DFD beef (For review: (Gagaoua, Werner, et al., 2021)), the pork color proteomics studies used more animals/samples (average of 26 *versus* 15) for the proteomics analyses.

#### 5.4. Current list of biomarkers and molecular functions related to pork color determination

The data- and text-mining on the seven eligible studies permitted creating a database of 46 putative protein biomarkers of pork color (**Table 2**), which were identified by their gene names retrieved from UniProt (<https://www.uniprot.org/>). The database was subjected to bioinformatics analyses (Kiyimba, Gagaoua, Suman, Mafi, & Ramanathan, 2022) for protein-protein interactions using STRING database (<https://string-db.org/>), for Gene Ontology (GO) enrichment using the webservice tools Metascape® (<https://metascape.org/>) or ProteINSIDE (<http://www.proteinside.org/>) and for Voronoi treemap classification using Proteomaps (<https://bionic-vis.biologie.uni-goettingen.de/>). The proteins (given by their gene names and detailed in **Table 2**) had the highest number ( $n = 38$ ) of significantly correlated proteins with lightness ( $L^*$ ) (**Fig. 3**). This supports the knowledge evidence on the biological meaning and importance of lightness/paleness in pork color determination and the complexity of the biochemical processes at interplay, mainly in the frame of light scatter in meat cuts (Swatland, 2002a). In fact, the earlier Swatland's studies, postulated that pork paleness may be related to the refraction of light through myofibrils (Swatland, 2002a) and that the shape in muscle fibers and their type may further impact the bulk optical properties of pork color (Swatland, 2002b). For redness ( $a^*$ ) and yellowness ( $b^*$ ), a smaller number of putative biomarkers were identified counting 17 and 13 proteins, respectively (**Fig. 3**). The direction of the correlations (*i.e.*, positive, negative or in both directions) are given in **Fig. 4** together with the distribution and frequency of their identification among  $L^*$ ,  $a^*$  and  $b^*$  color traits and studies. The manual annotation and bioinformatics analyses allowed grouping the proteins into six distinct but

interconnected biochemical processes (**Fig. 5** and **Table 2**), these being (i) Catalytic, metabolism & ATP metabolic pathways (n = 17 proteins), (ii) Contractile & associated proteins (n = 12), (iii) oxidative stress & cell redox homeostasis (n = 5), (iv) chaperones & heat shock proteins (n = 4), (v) proteolysis (n = 1) and a final pathway grouping proteins involved in (vi) binding, cofactor & transport, signaling or apoptosis (n = 7). At first glance, these pathways are interestingly similar to those identified in earlier integromics studies from our group as being involved in determining beef color (Gagaoua et al., 2020) and tenderness (Gagaoua, Terlouw, Mullen, et al., 2021) but different from those involved in DFD beef defect (Gagaoua, Warner, et al., 2021). This suggests that the biochemical processes linked to variations in meat color are to some extent similar whatever the species, although differences exist in the contractile and metabolic properties among muscles and meat samples (Joseph et al., 2012; Picard et al., 2018; Tan et al., 2023).

### ***5.5. Lessons from the integromics database on the major proteins and molecular signatures involved in pork color determination***

From a pork color molecular signatures perspective and in line with the earlier integromics study on beef color (Gagaoua et al., 2020) the top two dominant biochemical pathways in pork color were catalytic and energy metabolism (half of which are glycolytic enzymes) and contractile and muscle structure, followed by protein folding and response to oxidative stress, binding and transport proteins, signaling, apoptosis and proteolysis (**Fig. 5**). Referring to the frequently identified putative protein biomarkers among the 7 studies irrespective of the color traits, 10 of them were identified in more than one study ( $\geq 2$  times): GAPDH, LDH, GPD1, CKM and PGM1 (energy metabolism), HSPB1 and HSPA2 (heat shock proteins), MLC1 and TNNT2 (muscle structure) and HBB (heme binding and oxygen transport). On another hand, four proteins: GPD1 (Glycerol-3-phosphate dehydrogenase [NAD(+)]), PKM (pyruvate kinase), TNNT2 (Troponin T) and MYH1 (Myosin-1) were commonly correlated with  $L^*$ ,  $a^*$ ,  $b^*$  color traits (**Fig. 3**).

TNNT2 and GPD1 are the only two biomarkers identified in more than one study among the other 10 proteins. These biomarkers are of particular interest (although the other 8 proteins could also be shortlisted) for further evaluation by initiating the first biomarker discovery pipeline for pork color, as has been done previously for beef color, for instance using targeted proteomics methods (Wu, Dai, & Bendixen, 2019). TNNT2 is a member of the Troponin T proteins encoding the cardiac isoform (myocardial subtype), which is described as a key



player in calcium regulation of actin thin filament function with essential roles in the contraction of striated muscles (Wei & Jin, 2016). TNNT2 seemed to be a negative biomarker for  $a^*$  and  $b^*$ , while it is in both directions for  $L^*$ . GPD1 seemed to be a negative biomarker of  $L^*$  and  $b^*$ , and in both directions for  $a^*$  (**Fig. 4**). The inversion in the direction of the correlations could occur when the biochemical mechanism underlying such correlation has a major contribution to the phenomenon under study (Terlouw et al., 2021), but other factors are also involved, such as animal age, breed, muscle type, husbandry factors, etc (For review: (Gagaoua et al., 2020)). The multifactorial and interdependent nature of the influence of *post-mortem* metabolism and oxidative properties observed in this database on meat color is well known (Gagaoua, Terlouw, & Picard, 2021; Suman et al., 2023). This may partially explain the inversion in the direction of the correlations observed in this pork color biomarkers database (**Table 2** and **Fig. 4**). For example, the *post-mortem* production of lactate under the anaerobic conditions of glycogen degradation, can influence the redox status of muscle, which may consequently have an impact on meat color (Suman & Joseph, 2013). ATP turnover results in muscle acidification, the rate and extent of which is dependent on muscle glycogen content at slaughter (Robergs, Ghiasvand, & Parker, 2004; Terlouw et al., 2021). Ultimately, the pH final and its final value would modulate the space between myofilaments and the diameter of myofibrils, hence affecting the lightness and paleness of pork meat through the achromatic processes of light diffusion (Purslow et al., 2021; Swatland, 2002a, 2002b).

Referring more to the two common proteins found to be correlated with the three color traits, GPD1 is involved in the catalysis of the oxidation and conversion of glycerol-3-phosphate (G3P) into dihydroxyacetone phosphate (DHAP) and utilized for gluconeogenesis (Sato, Yoshida, Morita, Mori, & Miura, 2016). More importantly and in link with the important role it may play in color determination, GPD1, the first isoform of GPD, is a  $\text{NAD}^+/\text{NADH}$  dependent enzyme located in the cytosol, while GPD2, not identified as a biomarker, is a  $\text{FAD}^+$  dependent mitochondrial enzyme (Mráček, Drahota, & Houštěk, 2013). It is worthy to note that both isoforms play pivotal roles as glycerol phosphate shuttles, transporting reducing equivalents to the mitochondria and improving the  $\text{NAD}^+/\text{NADH}$  ratio in the cytosol and the mitochondria with the known consequences on color stability and development (Ramanathan, Hunt, et al., 2020; Ramanathan, Suman, et al., 2020; Suman et al., 2023). In fact, *post-mortem* muscle is exposed to hypoxia, and the decrease in ATP levels causes the breakdown of muscle glycogen during anaerobic glycolysis to produce lactate, NADH, and ATP (Ouali et al., 2013). NADH accesses complex I and contributes to

movement of electron, thereby influencing meat color stability through increasing oxygen depletion or electron transference from NADH to oxidized myoglobin by metmyoglobin reductase (Ramanathan et al., 2011). Further, it has been evidenced that mitochondria are able to influence meat color by competition of oxygen with myoglobin or affecting metmyoglobin reduction (Mitacek et al., 2019; Ramanathan, Nair, Hunt, & Suman, 2019).

The GO analysis further evidenced “carbohydrate catabolic process” and “muscle system process” as the top enriched molecular signatures underlying pork color determination (**Fig. 6A,B**). The pivotal role of muscle structure and related proteins can be ascribed as mentioned above to the achromatic sources of variations of meat lightness, known also to be related to variations in the microstructure and amount of light scattering (Purslow et al., 2021; Purslow, Warner, Clarke, & Hughes, 2020). These results further demonstrated for the first time that various and interconnected GO terms (**Fig. 6C**) are affecting lightness of pork *via* the effect of *post-mortem* metabolism, *post-mortem* temperature and denaturation of muscle proteins (mostly enzymes from the energy metabolism and glycolysis), protein folding and cell death processes (apoptosis and autophagy) as major contributors to pork color variation. The higher number of proteins correlated with  $L^*$  than  $a^*$  and  $b^*$  (**Fig. 4**) supports such statement and asks for further investigations.

Overall, the proteomics strategies applied, in the case of pork color, compared extreme groups, with the aim of understanding the biochemical mechanisms and identify biomarkers. For the validation of the proteins and the molecular signatures we revealed in this paper, need to follow a specific workflow. In fact, the pipeline of biomarkers discovery requires a certain number of steps that need to be followed before the validation of the protein candidates and implementation at the industrial scale. Before that, two approaches are worthy to consider, these being integrative approaches to capture the most robust proteins (the purpose of this last section of the manuscript) or by running new experiments for validation of the former list of proteins. In the case of pork color, the current studies are all preliminary experiments and an extrapolation across pork is, at this stage, difficult and need further investigation and validation.

## 6. Conclusion and future prospects

The visual experience of fresh pork color is a complex and important quality trait for consumers that is influenced by a myriad of intrinsic and extrinsic factors standing from farm-to-fork (*i.e.* from pig genetics, housing system and management (feeding) strategies,

preslaughter handling, to slaughtering and meat storage conditions...) which can interact to determine the final color as perceived by consumers and/or assessed instrumentally mainly through CIE  $L^*$ ,  $a^*$  and  $b^*$  coordinates values. Factors such as sex and indoor space allowance have weak and no impact on  $L^*$ ,  $a^*$ ,  $b^*$  color parameters. However, factors such as muscle/meat cut and packaging and coating are key factors that strongly impact pork color parameters, which need to be well considered and/or managed. Moreover, the in-depth analysis of the myriad factors at interplay revealed that lightness is the major parameter that is impacted compared to redness and yellowness coordinates. This has been confirmed by the integromics meta-analysis, which identify that most of the candidate protein biomarkers (83%) are related to lightness compared redness (37%) and yellowness (28%). Pork color determination results from muscle biology and metabolism, muscle proteome profile, but to less extent myoglobin chemistry. The interactions of these multiple factors affect the structure and state of muscle proteomes, and involve sophisticated interconnected molecular signatures as underlying mechanisms in the development of fresh pork color. Thanks to the integrative proteomics approach we applied in this paper, we extended our knowledge on the interplay of multiple molecular and biochemical processes governing fresh pork color. First, it revealed a certain level of disparity among studies and suggest only 10 proteins candidates from the created repertoire for future evaluation following the pipeline of biomarkers discovery (Gagaoua, 2021), by using for instance targeted proteomics (Wu et al., 2019). Second, the multiple molecular signatures at interplay in pork color determination have been revealed, these being catalytic and energy metabolism, contractile and related muscle proteins, protein folding, oxidative stress, and signaling pathways, binding, transport and apoptosis. Further, the results confirmed that myoglobin is not a pivotal player in pork color stability. Integrating pork color proteomics profiling from the same samples with other omics methods such as transcriptomics and metabolomics, could offer much more insights and a promising alternative analytical platform to better understand the complex mechanisms underlying pork color variability produced within several farming systems and/or production conditions.

### **Declaration of Competing Interest**

No potential conflict of interest was reported by the authors.

### **CRedit authorship contribution statement**

**Mohammed Gagaoua:** Conceptualization, Methodology / Study design, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review and editing, Visualization, Project administration.

**Surendranath P. Suman:** Data curation, Visualization, Writing – review and editing.

**Peter P. Purslow:** Writing – review and editing.

**Bénédicte Lebret:** Data curation, Visualization, Writing – original draft, Writing – review and editing.

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**Table 1.** Summary of the factors from farm-to-fork influencing color traits (lightness ( $L^*$ ), redness ( $a^*$ ), and yellowness ( $b^*$ )) of fresh pork<sup>1</sup>

<b>Factors</b>	<b><math>L^*</math></b>	<b><math>a^*</math></b>	<b><math>b^*</math></b>
Breed/Genotype	+++	++	++
Sex	+	-	-
Muscle/meat cut	+++	+++	+++
Diet	+	+	+
Age/weight at slaughter	-	++	+
<b><i>Housing and rearing conditions</i></b>			
Indoor space allowance	-	-	-
Outdoor access or extensive system	++	++	++
Pre-slaughter handling	+++	+++	+++
Slaughter and carcass cooling conditions	+++	++	++
<b><i>Meat ageing/storage conditions and duration</i></b>			
Freezing	+++	++	++
Packaging/coating	+++	+++	+++
Display light	+	++	++
Storage duration (time)	+++	++	++

<sup>1</sup> Effect: none (-), weak (+), medium (++), strong (+++).

**Table 2.** List of the 46 putative protein biomarkers by biological family reported in seven proteomic-based studies to be significantly correlated with pork color traits from *Longissimus* muscle.<sup>1</sup>

Protein biomarkers names ( <i>genes</i> )	UniProt Accession	Meat color parameters	Direction <sup>2</sup>	References
<b>Chaperones &amp; heat shock proteins (n = 4; 9%)</b>				
Hsp27 ( <i>HSPB1</i> )	P04792	Lightness ( <i>L</i> *)	+	(Kim et al., 2019)
		Yellowness ( <i>b</i> *)	+	(Kim et al., 2019)
		Lightness ( <i>L</i> *)	-	(Hwang et al., 2005)
$\alpha$ B-crystallin ( <i>CRYAB</i> )	P02511	Lightness ( <i>L</i> *)	-	(Hwang et al., 2005)
Heat shock-related 70 kDa protein 2 Hsp72 ( <i>HSPA2</i> )	P54652	Lightness ( <i>L</i> *)	-	(Kwasiborski et al., 2008)
		Redness ( <i>a</i> *)	+	(Kwasiborski et al., 2009)
Heat shock protein HSP 90-alpha Hsp90 ( <i>HSP90AA1</i> )	P07900	Lightness ( <i>L</i> *) Yellowness ( <i>b</i> *)	-	(Zhang et al., 2014)
<b>Catalytic, metabolism &amp; ATP metabolic process (n = 17; 37%)</b>				
Enolase 1 ( <i>ENO1</i> )	P06733	Redness ( <i>a</i> *)	-	(Kwasiborski et al., 2008)
Enolase 3 ( <i>ENO3</i> )	P13929	Lightness ( <i>L</i> *)	-	(Kim et al., 2019)
Triosephosphate isomerase 1 ( <i>TP11</i> )	P60174	Lightness ( <i>L</i> *)	+	(Kim et al., 2019)
Glycogen phosphorylase ( <i>PYGM</i> )	P11217	Yellowness ( <i>b</i> *)	+	(Kim et al., 2019)
Glyceraldehyde-3-phosphate dehydrogenase ( <i>GAPDH</i> )	P04400	Yellowness ( <i>b</i> *)	+	(Żelechowska et al., 2012)
		Lightness ( <i>L</i> *)	-	(D'Alessandro et al., 2011)
Lactate dehydrogenase ( <i>LDH</i> )	P03338	Yellowness ( <i>b</i> *)	+	(Żelechowska et al., 2012)
		Lightness ( <i>L</i> *)	-	(Kim et al., 2019)
		Yellowness ( <i>b</i> *)	-	(Kim et al., 2019)
Glycerol-3-phosphate dehydrogenase [NAD(+)] ( <i>GPD1</i> )	P21695	Lightness ( <i>L</i> *)	-	(Kwasiborski et al., 2008)
		Redness ( <i>a</i> *)	+	(Kwasiborski et al., 2008)
		Redness ( <i>a</i> *)	-	(Kim et al., 2019)
		Yellowness ( <i>b</i> *)	-	(Kim et al., 2019)
Pyruvate kinase M 2 ( <i>PKM</i> )	P14618	Lightness ( <i>L</i> *)	-	(D'Alessandro et al., 2011)
		Redness ( <i>a</i> *)	-	(D'Alessandro et al., 2011)
		Yellowness ( <i>b</i> *)	-	(D'Alessandro et al., 2011)
Creatine kinase M type ( <i>CKM</i> )	P06732	Redness ( <i>a</i> *)	+	(Kwasiborski et al., 2008)
		Redness ( <i>a</i> *)	+	(D'Alessandro et al., 2011)
Phosphoglucosmutase 1 ( <i>PGMI</i> )	P36871	Redness ( <i>a</i> *)	-	(Kwasiborski et al., 2008)
		Lightness ( <i>L</i> *)	+	(Żelechowska et al., 2012)
		Lightness ( <i>L</i> *)	-	(Kim et al., 2019)
		Redness ( <i>a</i> *)	+	(Kim et al., 2019)
Retinal dehydrogenase 1 ( <i>ALDH1A1</i> )	P00352	Redness ( <i>a</i> *)	-	(Kwasiborski et al., 2008)

Fructose-bisphosphate aldolase A ( <i>ALDOA</i> )	P04075	Lightness ( $L^*$ )	-	(Kim et al., 2019)
Isocitrate dehydrogenase ( <i>IDH1</i> )	O75874	Lightness ( $L^*$ )	+	(Kwasiborski et al., 2008)
Cytosol aminopeptidase 3 ( <i>LAP3</i> )	P28838	Lightness ( $L^*$ ) Redness ( $a^*$ )	- -	(Kwasiborski et al., 2008) (Kwasiborski et al., 2008)
Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase ( <i>DHDH</i> )	Q9UQ10	Lightness ( $L^*$ )	-	(Kwasiborski et al., 2008)
Aminoacylase 1 ( <i>ACY1</i> )	Q03154	Lightness ( $L^*$ )	-	(Kwasiborski et al., 2008)
Pyridoxine-5'-phosphate oxidase ( <i>PNPO</i> )	Q9NVS9	Lightness ( $L^*$ )	+	(Kwasiborski et al., 2008)
<b>Oxidative stress &amp; cell redox homeostasis (n = 5; 11%)</b>				
DJ-1 ( <i>PARK7</i> )	Q99497	Lightness ( $L^*$ )	-	(Kwasiborski et al., 2008)
Phospholipid hydroperoxide glutathione peroxidase ( <i>GPX4</i> )	P36969	Lightness ( $L^*$ )	-	(Hwang et al., 2005)
Thioredoxin-dependent peroxide reductase ( <i>PRDX3</i> )	P30048	Lightness ( $L^*$ )	-	(Hwang et al., 2005)
Peroxiredoxin 6 ( <i>PRDX6</i> )	P30041	Redness ( $a^*$ )	-	(Kwasiborski et al., 2008)
Carbonic anhydrase ( <i>CA3</i> )	P07451	Lightness ( $L^*$ ) Redness ( $a^*$ )	+ -	(Kim et al., 2019) (Kim et al., 2019)
<b>Table 2. Continued.</b>				
<b>Contractile &amp; associated proteins (n = 12; 26%)</b>				
Myosin light chain 1 ( <i>MLC1</i> )	Q15049	Lightness ( $L^*$ ) Yellowness ( $b^*$ ) Lightness ( $L^*$ )	- - +	(Hwang et al., 2005) (Żelechowska et al., 2012) (Kim et al., 2019)
Myosin regulatory light chain 2, ( <i>MYL2</i> )	P10916	Lightness ( $L^*$ ) Redness ( $a^*$ )	+ -	(Kim et al., 2019) (Kim et al., 2019)
Myosin light chain 3 ( <i>MYL3</i> )	P08590	Yellowness ( $b^*$ )	-	(Kim et al., 2019)
Troponin C, slow skeletal and cardiac muscles ( <i>TNNC1</i> )	P63316	Lightness ( $L^*$ ) Yellowness ( $b^*$ )	- -	(Żelechowska et al., 2012)
Troponin T, cardiac muscle ( <i>TNNI2</i> )	P45379	Lightness ( $L^*$ ) Redness ( $a^*$ ) Lightness ( $L^*$ ) Yellowness ( $b^*$ )	- + + +	(Hwang et al., 2005) (Kwasiborski et al., 2008) (Żelechowska et al., 2012) (Żelechowska et al., 2012)
Troponin I, fast-twitch skeletal ( <i>TNNI2</i> )	P48788	Lightness ( $L^*$ )	-	(Kim et al., 2019)
Myosin-1 ( <i>MYH1</i> )	P12882	Lightness ( $L^*$ ) Redness ( $a^*$ ) Yellowness ( $b^*$ )	+ - +	(Kim et al., 2019) (Kim et al., 2019) (Kim et al., 2019)
F-actin-capping protein subunit beta ( <i>CAPZB</i> )	P47756	Lightness ( $L^*$ )	-	(Hwang et al., 2005)
Actin, alpha skeletal muscle ( <i>ACTA1</i> )	P68133	Lightness ( $L^*$ )	-	(Hwang et al., 2005)
Cofilin-2 ( <i>CFL2</i> )	Q9Y281	Lightness ( $L^*$ )	-	(Hwang et al., 2005)
Beta-tropomyosin ( <i>TPM2</i> )	P07951	Lightness ( $L^*$ )	+	(Kim et al., 2019)
Myozenin-1 ( <i>MYOZ1</i> )	Q9NP98	Yellowness ( $b^*$ )	+	(Kim et al., 2019)
<b>Proteolysis &amp; associated proteins (n = 1; 2%)</b>				
Calpain-1 catalytic subunit ( <i>CAPN1</i> )	P07384	Lightness ( $L^*$ )	+	(Żelechowska et al., 2012)



**Binding, cofactor & transport proteins, signaling or apoptosis (n = 7; 15%)**

Serotransferrin ( <i>TF</i> )	P02787	Lightness ( $L^*$ )	+	(Kwasiborski et al., 2008)
Hemoglobin subunit beta ( <i>HBB</i> )	P68871	Lightness ( $L^*$ ) Redness ( $a^*$ )	- +	(Hwang et al., 2005) (D'Alessandro et al., 2011)
Parvalbumin ( <i>PVALB</i> )	P20472	Lightness ( $L^*$ )	+	(Kwasiborski et al., 2008)
Calsequestrin ( <i>CASQ1</i> )	P31415	Lightness ( $L^*$ )	-	(Kim et al., 2019)
Myoglobin ( <i>MB</i> )	P02144	Lightness ( $L^*$ ) Redness ( $a^*$ )	+ +	(Kwasiborski et al., 2008) (Kwasiborski et al., 2008)
Phosphatidylethanolamine-binding protein 1 ( <i>PEBP1</i> )	P30086	Lightness ( $L^*$ ) Redness ( $a^*$ )	- -	(Kwasiborski et al., 2008) (Kwasiborski et al., 2008)
Methylthioribulose-1-phosphate dehydratase ( <i>APIP</i> )	Q96GX9	Redness ( $a^*$ )	-	(Kwasiborski et al., 2008)

<sup>1</sup> Papers that reported protein abundances only or comparisons between ageing times of pork and/or effect on pork color were not included in these relationships.

<sup>2</sup> (+) positively related; (-) negatively related;

## Figure captions

**Fig. 1.** Heat map on species-specificity of 4-hydroxy-2-nonenal (4-HNE) adduction at histidine residues in myoglobin at pH 5.6. The red color indicates histidines adducted by 4-HNE green color represents unadducted histidines, and the white color represent missing histidine residues due to species-specific differences in myoglobin primary structure. The heatmap was adapted with permission from Suman *et al.*, (2023).

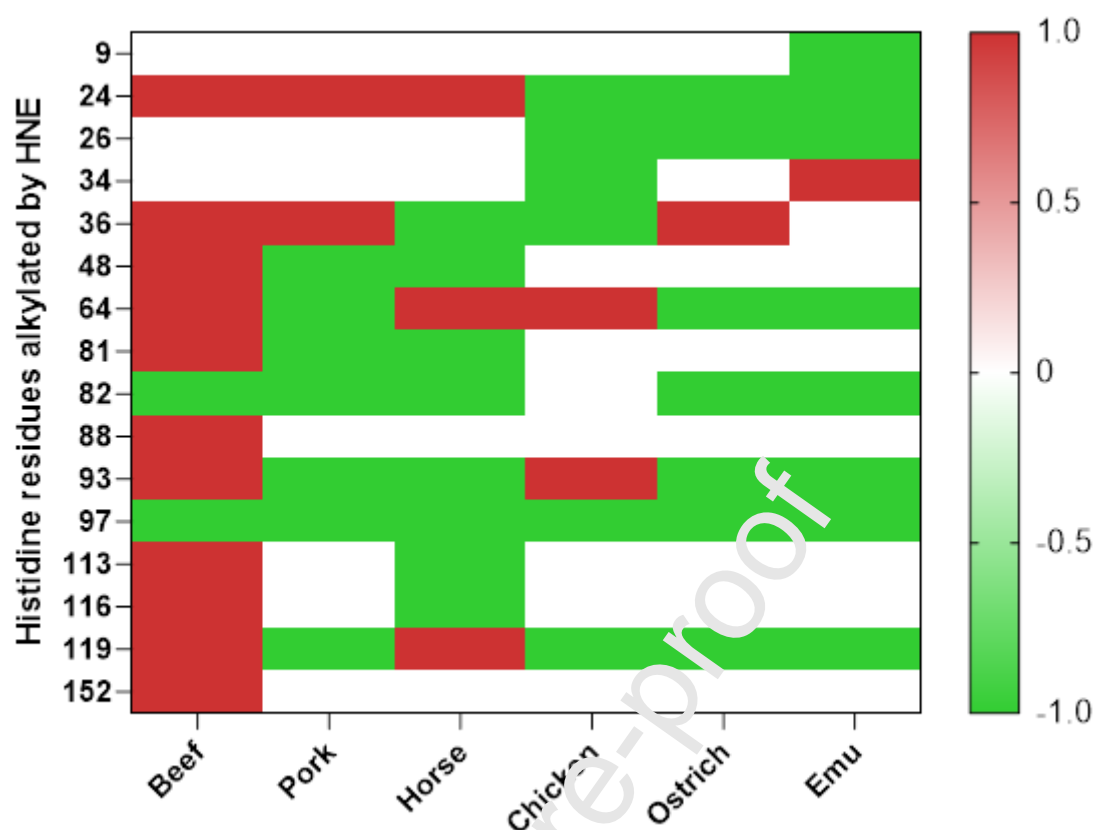
**Fig. 2.** Main objectives of integrating proteomics datasets through data mining and bioinformatics as a promising way to better understand complex biological systems and refine the list of pork meat quality (color) biomarkers.

**Fig. 3.** Venn diagram summarizing the distribution of the 46 putative proteomic markers and their correlations with pork  $L^*$  (lightness),  $a^*$  (redness) and  $b^*$  (yellowness) color coordinates. The total number of proteins for each color coordinate is given in white circles near each coordinate. The numbers of proteins specific to each color trait or common among the color traits are given in black bold type characters. The common proteins ( $n = 4$ ) to the  $L^*$ ,  $a^*$  and  $b^*$  color traits are given at the right of the Venn diagram. For the full protein names and Uniprot access IDs refer to **Table 2**.

**Fig. 4.** Summary of the direction of the correlations between the 46 putative biomarkers and pork *Longissimus thoracis* color traits identified from the seven proteomics studies. On the left panel, the negative correlations are highlighted in red, the positive in blue and those that are both negative and positive are highlighted in orange color (further details in **Table 2**). On the right panel, the black cells indicate presence of a particular protein and the white cells indicate the absence of the protein. The total number of identifications of a given protein (the same row) among the studies is shown at the last column and the total number of proteins per study in the bottom.

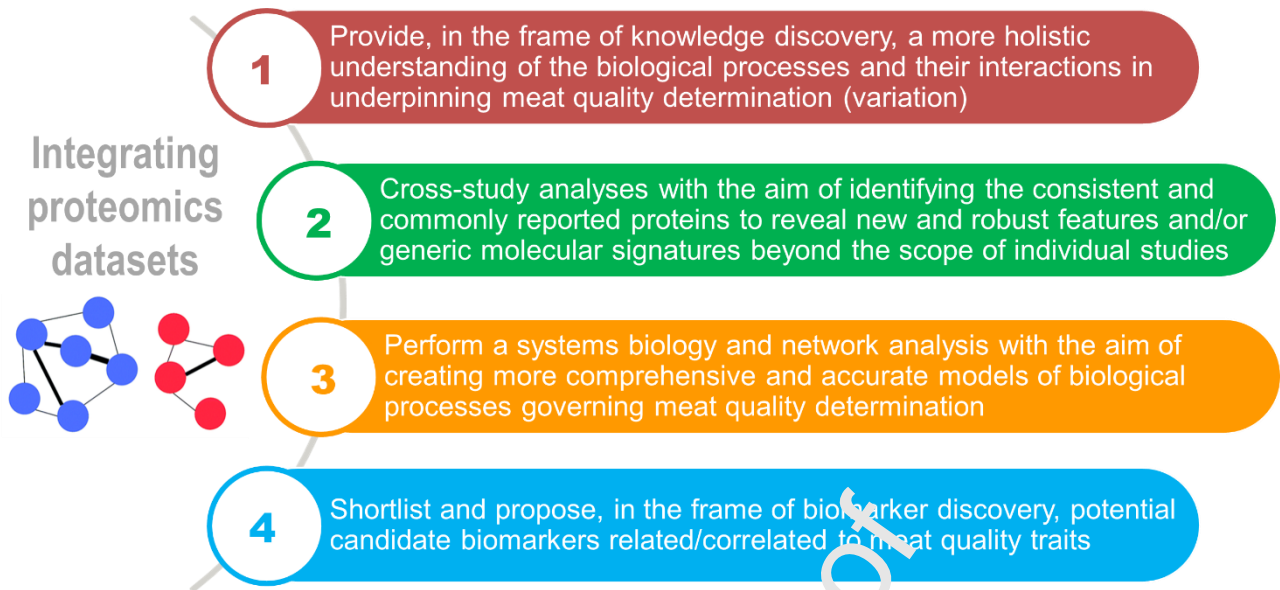
**Fig. 5.** A STRING functional interaction network linking the 46 proteins identified by proteomic studies to be related to porcine meat color traits (**Table 2**). The interaction map was generated from a web-based search of the STRING database (<http://string-db.org/>). Default settings of confidence of 0.5 and 4 criteria for linkage: Co-occurrence, experimental evidences, existing databases and text mining were used. Considering the limitation of the GO annotation of genes in porcine, we converted their Uniprot access IDs to orthologous human EntrezGene IDs using Biomart (<http://www.ensembl.org/biomart/>).

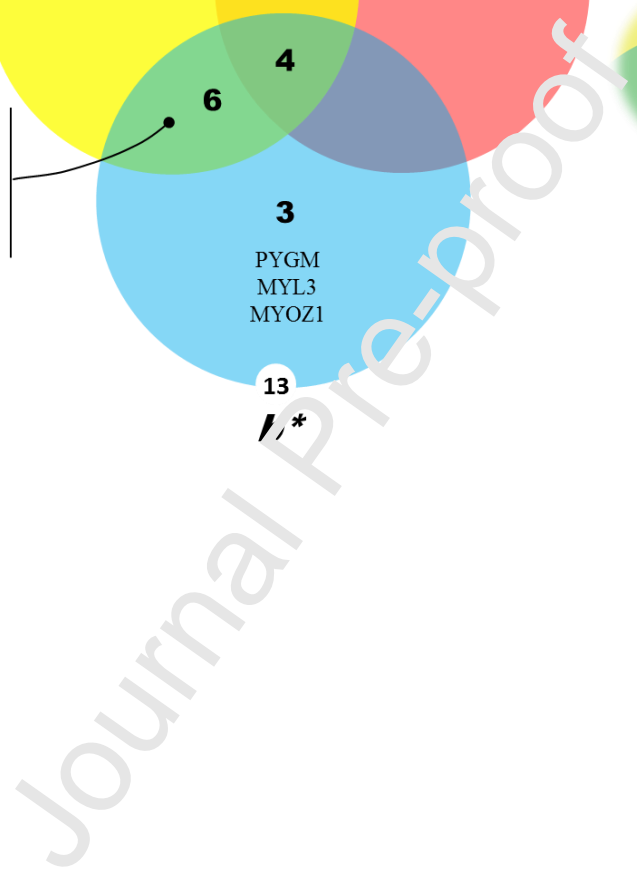
**Fig. 6.** Biological functions based on networks of pathways and process enrichment analyses on the 46 protein biomarkers of pork color. **A)** Voronoi treemap highlighting the biological functions and major proteins associated with pork meat color. The classification trees (graphs) were built in Proteomaps (<https://bionic-vis.biologie.uni-greifswald.de/>). Functional categories annotated based on KEGG orthology (left and middle panels) and related proteins (right panel) are shown by polygons. Areas of polygons illustrate protein abundance, weighted by protein size computed based on their number of identifications among studies. Functionally related function/proteins are arranged in common regions and coded using similar colors. **B)** Chart showing enriched pathways among pork meat color related 46 proteins. The depth of the color of the bar plot indicates the significance of  $p$ -value. Those enrichment terms including more nodes were more significant. **C)** Network of pathways and process enrichment cluster analysis. The network diagram is constructed with each enrichment GO term from the Top20 as a node and the similarity of the node as the edge. The nodes are colored by cluster ID, where nodes that share the same cluster ID are usually close



to each other. Both **B)** and **C)** graphs were generated with Metascape® (<https://metascape.org/>).

**Fig. 1**

**Fig. 2**



**Fig. 3**

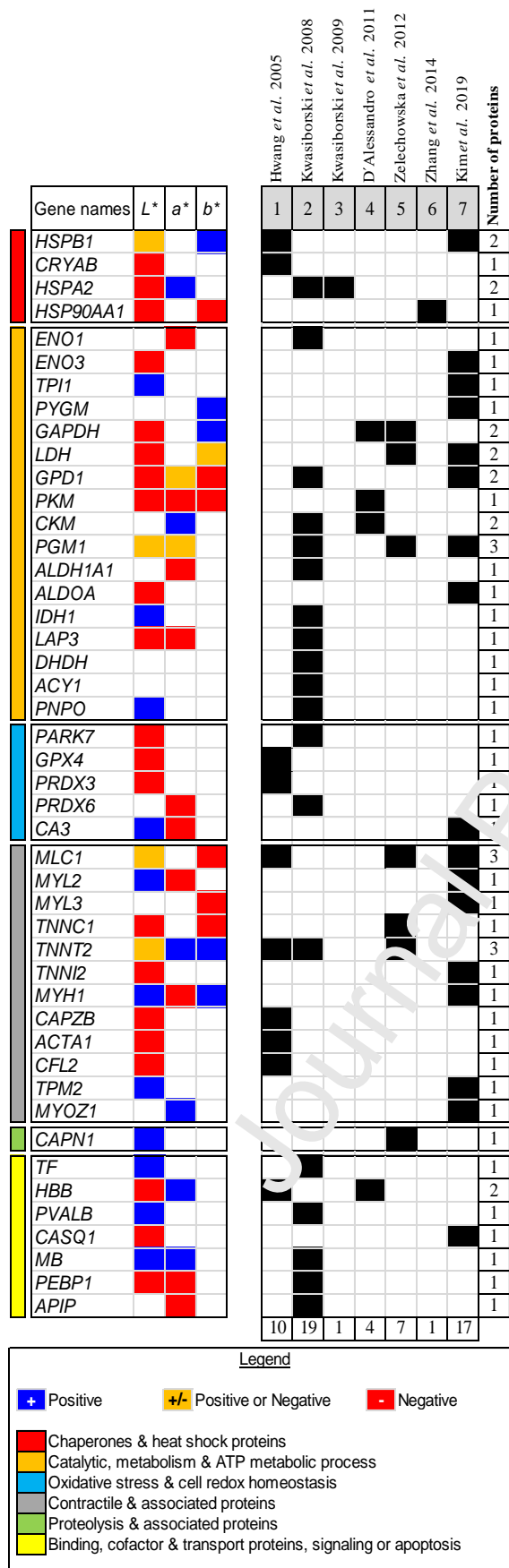


Fig. 4



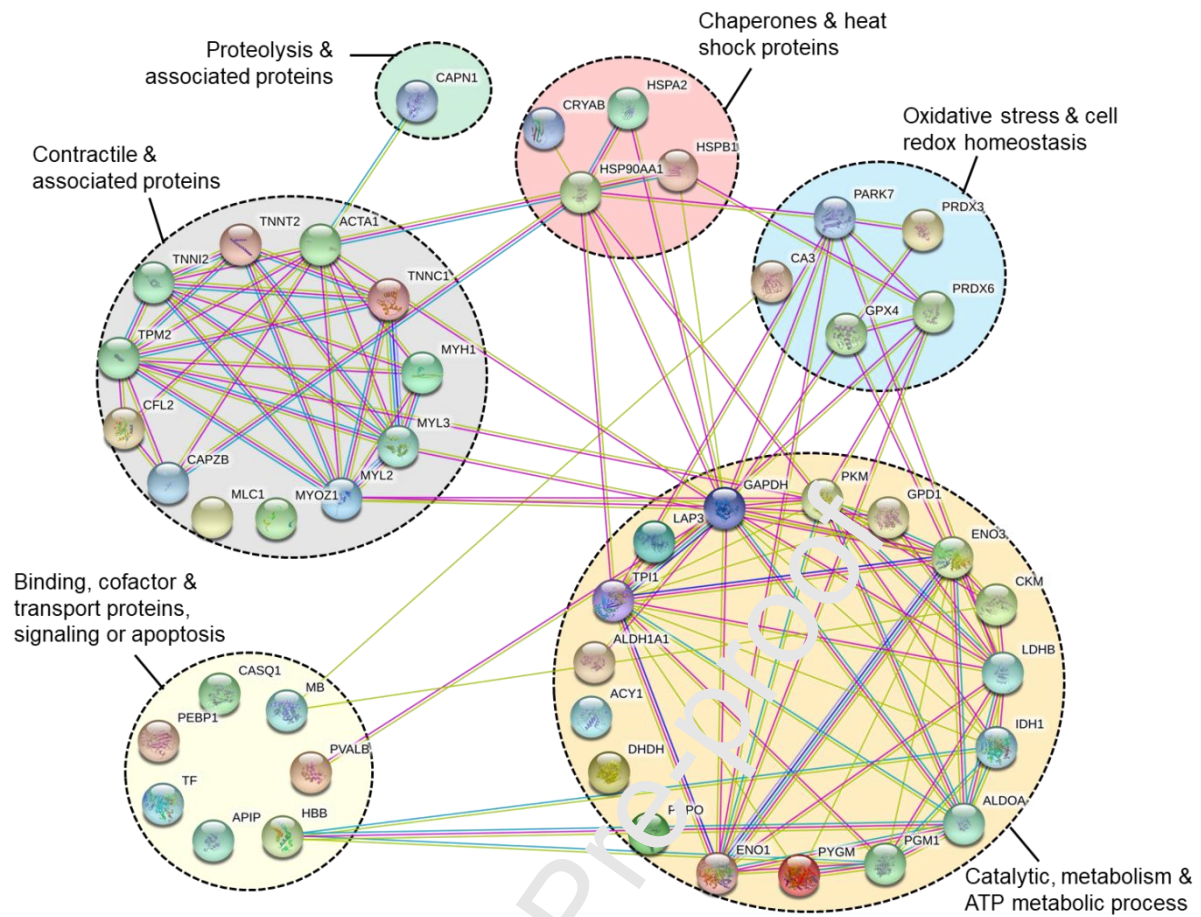
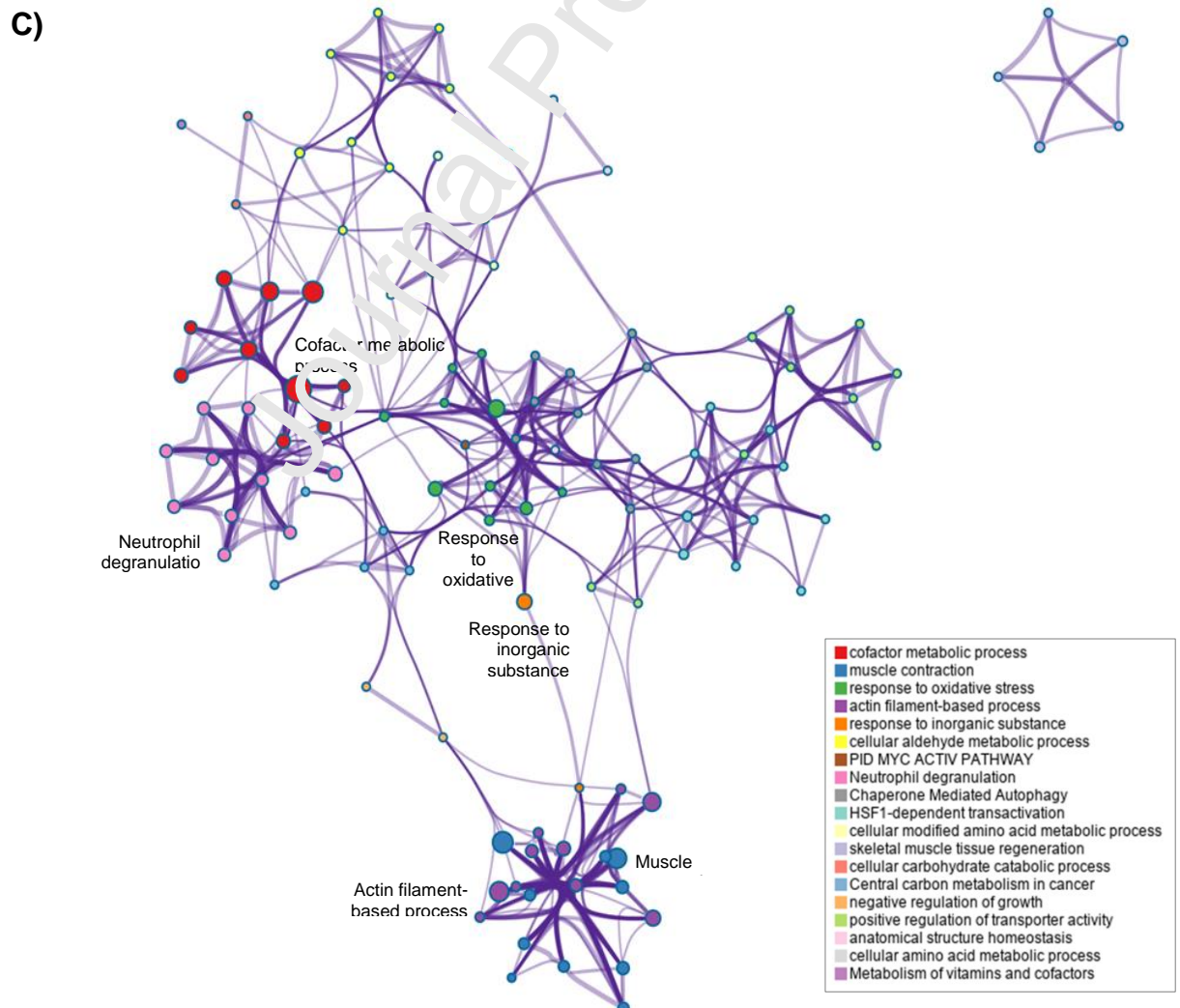
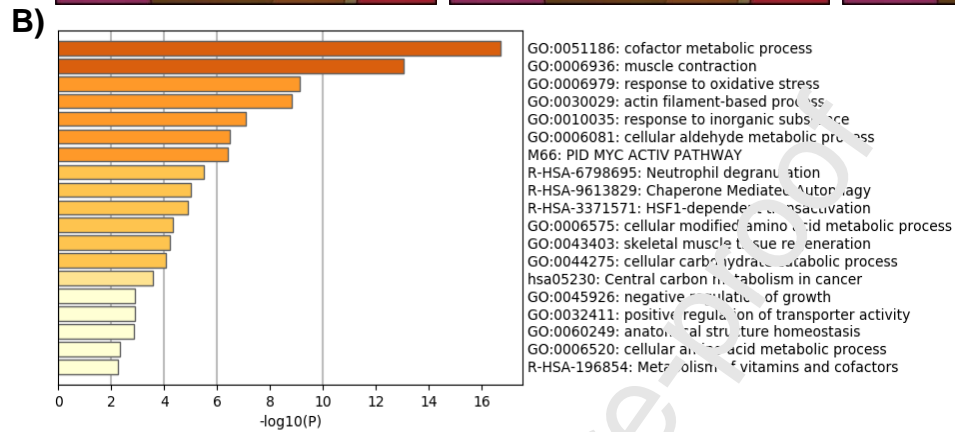
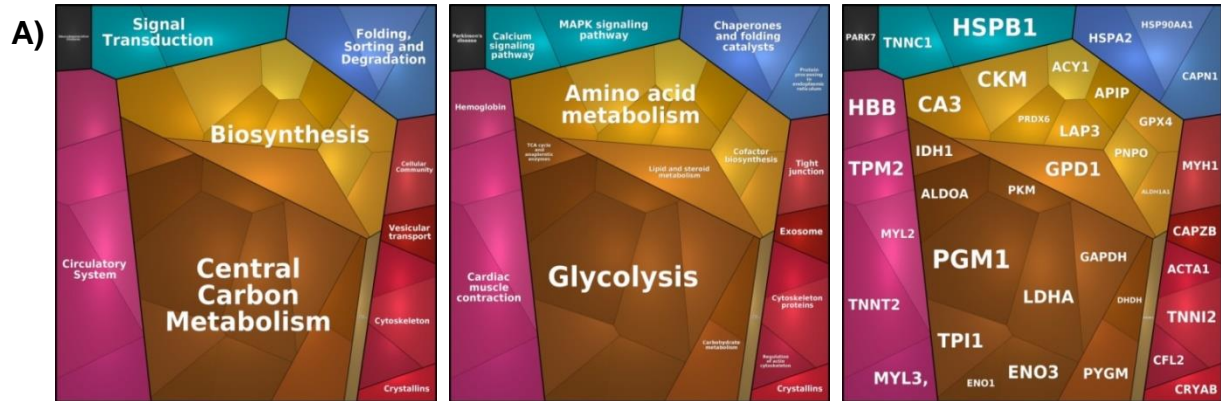


Fig. 5



**Fig. 6**

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**CRedit authorship contribution statement**

**Mohammed Gagaoua:** Conceptualization, Methodology / Study design, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review and editing, Visualization, Project administration, Supervision.

**Surendranath P. Suman:** Data curation, Visualization, Writing – review and editing.

**Peter P. Purslow:** Writing – review and editing.

**Bénédicte Lebre:** Data curation, Visualization, Writing – original draft, Writing – review and editing.

**Conflict of interest**

The authors declare no conflict of interest.

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