SKIN LESION VITALITY ASSESSMENT FOR FORENSIC SCIENCE: CURRENT RESEARCH AND NEW PERSPECTIVES

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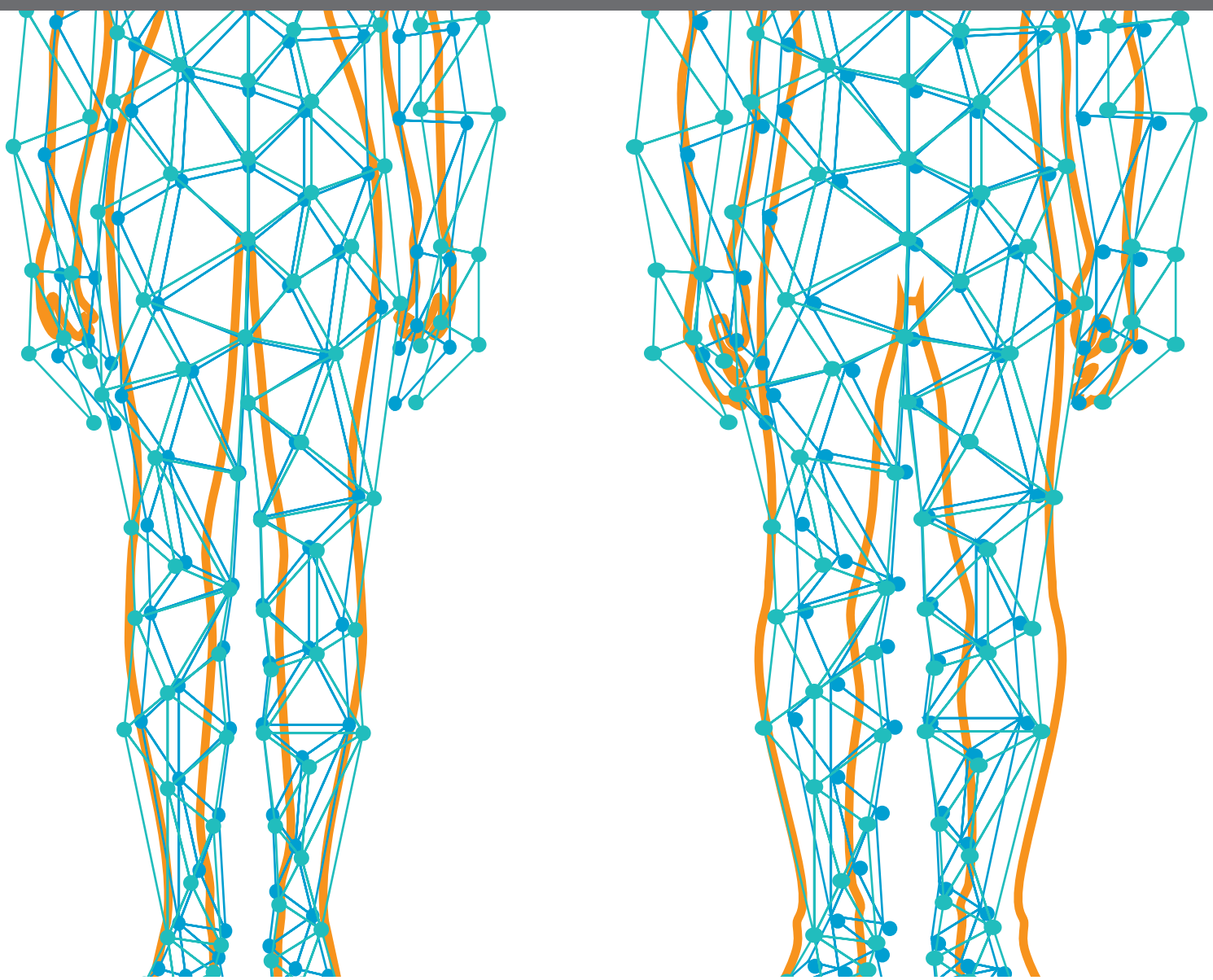

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SKIN LESION VITALITY ASSESSMENT FOR FORENSIC SCIENCE: CURRENT RESEARCH AND NEW PERSPECTIVES

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Editorial: Skin lesion vitality assessment for forensic science: Current research and new perspectives

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Editorial on the Research Topic
Skin lesion vitality assessment for forensic science: Current research and new perspectives

Lesion vitality demonstration is one of the most challenging topics in forensic pathology (1–5). The demonstration of viability refers to determining whether an injury was caused ante- or post-mortem, so that it can be asserted with a high degree of probability that injuries objectified on a cadaver may be the basis of the cause of death (6–10). This specific field of research has been much studied over the years, and today the literature consists of a large body of in-depth studies that span three major strands. Quantitative analysis in biological fluids and tissues of various markers, immunohistochemistry, and ribonucleic acids studies particularly on epigenetics.

This editorial summarizes the contributions to the Frontiers Research Topic "*Skin Lesion Vitality Assessment for Forensic Science: Current Research and New Perspectives*" appearing in the Frontiers in Medicine section Translational Medicine. In particular, this Research Topic collects various contributions highlighting new types of analysis and methods to deal more efficiently with issues relating to the vitality of the lesion, a very important topic of forensic analysis (11–13). To better address and clarify the issues relating to this research field, the topic consists of a part dedicated to reviews on general or specific arguments in another containing research articles on specific topics.

Review of general arguments

The first article in this collection by Collados Ros et al. reviews the forensic impact of the omics sciences involved in wounds to clarify different aspects of the diagnosis of vital injuries, time of appearance, age estimation, and the wound's vitality. Light is shed on the role of omics research during wounding, identifying different cytokines and other inflammatory mediators and cells involved in the specific stage of the wound healing process, showing great utility in estimating the age of a wound.

If this review addresses general issues relating to the vitality of the lesion, Maiese et al. review current knowledge about the vitality of ligature marks since this argument is a challenge for the forensic pathologist. The authors conclude that to ensure high reliability in court cases, forensic investigation in hanging should rely on modern and proven markers. Furthermore, given the difficulties of detecting vitality in a hanging groove, the authors recommend the use of various techniques using different markers and comparing the results obtained.

Review of specific arguments

Concerning cellular tools in wound vitality, Ishida et al. review the role of bone marrow-derived cells as promising markers of potential usefulness in forensic applications. However, suggest the authors, since the purpose of wound age estimation is to present objective evidence in court when only a single marker is investigated, contradictory results are often obtained, eventually confusing the interpretation of data. Consequently, various populations of bone marrow-derived cells should be investigated during forensic analysis.

Examining the use of molecular tools in wound vitality, Prangenberg, Doberentz, Madea et al. reviewed the forensic value of aquaporins, their range of applications, current limits, and future implementations. The authors offer an overview of fields of application and applicable aquaporins as well as supplementary biomarkers. Thus, aquaporins are studied not only for skin injury, age of wound, and viability, but also for what emerges in literature on this subject concerning drowning, burns, trauma, suffocation/occlusion, intoxication, sudden cardiac death, and SIDS. A combination of immunohistochemistry and gene expression analysis seems useful to increase statistical significance in each case.

The same authors Prangenberg, Doberentz, Mawick, Madea et al. review the use of heat shock proteins in the practice of forensic analysis. These proteins act as molecular chaperones with cytoprotective functions that support cell survival under lethal conditions. Besides, they are expressed in specific cellular compartments and have many functions. In forensic analysis, results have indicated that Hsp70 is a helpful marker to estimate survival time. Therefore, this area of research appears to be potentially crucial in studies relating to lesion vitality.

De Simone et al. review the role of miRNAs as novel molecular biomarkers for dating the age of wound production. Some studies highlight whether the animal died during the day or at night, considering the modification of other miRNAs. miR-200c is a critical determinant that inhibits cell migration during skin repair after injury and may contribute to age-associated alterations in wound repair. miRNAs play an essential role in moderating cellular adaptations in drug abuse and addiction. For this reason, an exciting field of application of miRNAs is as an anti-doping marker.

Research articles on specific topics

Bertozzi et al. investigated the possibility of using immunohistochemistry in the putrefied body. The results showed that most of the tested markers were highly expressed in putrefied skin for up to 15 days from death, leading to the conclusion that the use of various cellular markers allows only a qualitative evaluation of their expression, and consequently, other studies are necessary to deepen the issues considered.

Zhang et al., investigated the immunohistochemical expression of ubiquitin, which is involved in heat shock response and could be regarded as a valuable marker for diagnosing traces of antemortem compression in hanging furrows. The authors demonstrate that the depletion of ubiquitin expression revealed in neck compression may be caused by the impaired conversion of conjugated to free ubiquitin and failure of *de novo* ubiquitin synthesis. Therefore, the ubiquitin expression in the ligature mark can be considered a valuable marker for diagnosing traces of antemortem compression.

Gauchotte et al. evaluated the sensitivity and specificity of CD15 and myeloperoxidase (MPO), co-stained with glycophorin C, compared to standard histology, in a series of fresh medico-legal wounds and post-mortem controls in human experimental surgical model perspective. The detection of an inflammatory infiltrate based on histology is the gold standard, being highly specific but showing very low sensitivity in fresh wounds. The double staining for MPO or CD15/glycophorin C is a novel and interesting technique for detecting early vital reactions.

Conclusions

The compilation of this Research Topic provides an occasion for the meeting of expert scientists, from different schools in the world, whose research is aimed toward lesion vitality. The published paper reviews are timely in clarity and exposition and the research articles propose new methods and fields of research that will certainly be deepened in the near future. In conclusion, from this collection emerges the idea that the issue of the

vitality of the lesion is extremely significant, creating occasions to propose new molecular techniques to understand the role of the genome, proteome, and metabolome, adding to consolidated methods and opening new horizons to clarify different aspects of the diagnosis of vital injuries, time of appearance, age estimation, and the wound's vitality (14).

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Mini Review: Forensic Value of Aquaporines

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Forensic pathologists are routinely confronted with unclear causes of death or findings. In some scenarios, it can be difficult to answer the specific questions posed by criminal investigators or prosecutors. Such scenarios may include questions about wound vitality or causes of death when typical or landmark findings are difficult to find. In addition to the usual subsequent examinations to clarify unclear causes of death or special questions, immunohistochemical analysis has become increasingly important since its establishment in the early 40s of the 20th century. Since then, numerous studies have been conducted to determine the usefulness and significance of immunohistochemical investigations on various structures and proteins. These proteins include, for example, aquaporins, which belong to the family of water channels. They enable the transport of water and of small molecules, such as glycerol, through biological channels and so far, 13 classes of aquaporins could have been identified in vertebrates. The classic aquaporin channels 1, 2, 4 and 5 are only permeable to water. The aquaporin channels 3, 7, 9, and 10 are also called aquaglycerolporins since they can also transport glycerol. This mini review discusses the immunohistochemical research on aquaporins, their range of applications, and respective forensic importance, their current limitations, and possible further implementations in the future.

Keywords: aquaporins (AQPs), immunohistochemistry, wound vitality, drowning, SIDS

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INTRODUCTION

Aquaporins (AQPs) belong to the family of water channels and enable the transport of water and small molecules, such as glycerol, through biological channels in many epithelial and endothelial cells (1–4). So far, 13 classes of AQPs have been identified in vertebrates. The classic AQPs (AQP1, 2, 4 and 5) are only permeable to water. The AQP channels 3, 7, 9, and 10 are also called aquaglycerolporins since they can also transport glycerol (5). AQP1 is located around the dermal capillaries (6). AQP3 is expressed in epidermal keratinocytes (7); the stratum corneum of the epidermis does not contain keratinocytes and AQP3-channels (8). AQP1, AQP4, and AQP9 are the best described AQPs in the brain (9), with AQP4 being the main water channel. They have a significant role in water homeostasis and neural signal transduction in the brain (10, 11), and their expression is rapidly induced by several stimuli, such as osmolarity (12, 13), mechanical, or chemical stress (14–16). In lung tissue, AQP5 represents the major water channels (17, 18). Although AQP5 expression appears to be induced by hypertonic stress (19) and suppressed by freshwater drowning (20) in murine lungs, its immunohistochemical expression patterns remain inconclusive in human lungs (20). In terms of forensic significance, AQP1 and AQP3 have been the most intensively researched AQPs in human skin to date. AQP1 is localized in fibroblasts, capillaries, and Langerhans and dendritic cells (21, 22). AQP3 is found in hypodermal adipocytes,

dermal fibroblasts, epidermal keratinocytes, melanocytes, and dendritic and Langerhans cells and capillaries (21, 23–26). In particular, AQP3 has often been a central focus of forensic research in the past, as it appears to have an overriding function in skin hydration, epidermal barrier repair, and wound healing (26–31). AQP5, 7, 9, and 10 are also found in the skin and perform important functions but have not been systematically investigated forensically. In this mini review, we discuss the immunohistochemical research on aquaporins, their range of applications and respective forensic importance, their current limitations, and possible further implementations in the future.

METHODS

We reviewed the Medline dataset for studies published between 2009 and September 15, 2021 for AQPs in forensic context based on the updated 2020 PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) (32) for methodology and reporting. The words "aquaporin" and "forensic" were used to identify studies examining AQPs in forensic context. The following combination of Medical Subject Heading terms and Boolean operators was applied in our Medline search: "aquaporin" AND "forensic." The reference lists of the included articles were manually searched for further studies. Two authors (J.P. and B.M.) conducted eligibility assessment and data extraction, assessment, and management independently. Only original research articles on human specimens published in English or German were considered for review. Eligibility of the article was determined by screening of titles and abstracts.

RESULTS

The initial search identified 49 studies, and after screening of the title, 23 studies remained for further inspection. After reviewing the abstracts, five studies were excluded because four were studies on animals and one did not present sufficient data for further analysis. Through manual searching of the reference lists, no further articles that matched the criteria could have been detected. The search eventually identified a total of 18 eligible studies between 2009 and 2021. Considering the study period, an average of 1.4 studies on AQPs in a forensic context have been published per year. Most studies were published in 2014, 2018, and 2021 ($n = 3$ each); moreover, almost half (44%) of the total identified studies were published between 2018 and 2021. The majority of studies addressed skin injuries and drowning ($n = 5$ each), followed by sudden infant death syndrome (SIDS) ($n = 4$). The remaining four studies covered different topics (pulmonary injury, traumatic brain injury, intoxication, discriminating between smothering and choking from sudden cardiac death). AQP4 was the most frequent subject of the studies ($n = 6$), followed by AQP1 ($n = 5$), AQP3, and AQP5 ($n = 4$ each). It was also striking that more than half of the studies ($n = 11$) on AQP were published in the I*nternational Journal of Legal Medicine*. Fourteen of the included studies were two-group designs, and five were multigroup designs. All studies combined had a total of 1,119 study specimens and 1,093 control specimens.

TABLE 1 | Overview of aquaporins and their respective potential applications.

Aquaporin	Potential field of application
1	Pulmonary injuries, skin injuries, wound age, and vitality
2	Drowning
3	Burn injuries, skin injuries, wound age, and vitality
4	SIDS, intoxication, trauma, drowning
5	Drowning, smothering, chocking, sudden cardiac death
9	SIDS

An overview of the identified aquaporins and their respective potential applications is shown in above **Table 1**.

DISCUSSION

SIDS

The four studies that focused on SIDS investigated gene variations mainly by genetic analysis. AQP4 was predominantly studied. A decrease in AQP4 expression was observed in infants >12 weeks old. AQP4 expression was lower in infants and children with the rs2075575 CT/TT genotype than in those with the CC genotype (33). No differences in allele frequencies of the three AQP4 single-nucleotide polymorphisms (SNPs) previously shown to be associated with SIDS in Norwegian infants (rs2075575), severe brain edema (rs9951307), and increased brain water permeability (rs3906956) have been found between SIDS children and adult controls (34). Regarding the AQP1 gene, a significant association was found between the rs17159702 CC/CT and SIDS genotypes ($p = 0.02$). In the AQP9 gene, the combination of a TT genotype of rs8042354, rs2292711, and rs13329178 was more common in SIDS cases than in controls ($p = 0.03$). In the SIDS group, an association was found between genetic variations in the AQP1 gene and maternal smoking and between the 3×TT combination in the AQP9 gene and the finding of lifeless infants in the prone position (35). For AQP4, one study found an association between the T allele and CT/TT genotypes of rs2075575 and SIDS (C vs. T, $p = 0.01$; CC vs. CT/TT, $p = 0.03$), but none for the other three SNPs. For the SNP = rs2075575, an association between the brain/body-weight ratio and genotype was also found in SIDS patients at 0.3–12 weeks of age ($p = 0.014$, median ratio of CC = 10.6, CT/TT = 12.1) (36).

We concluded that specific variations in the genes of AQP1, 4, and 9, along with external risk factors and probably other genetic factors, represent a genetic predisposition that make an infant vulnerable to sudden death. Furthermore, the AQP4 CT/TT genotypes appear to be associated with an increased brain-to-body-weight ratio in infants (35, 36). Additionally, AQP4 expression in infants may be influenced by both age and genotype, but the role of AQP4 in the pathogenesis of SIDS remains to be elucidated (33). Yet another study concluded that variations in the AQP4 gene were of limited importance as predisposing factors in Caucasian SIDS children (34).

Drowning

Five studies have examined AQP expression in drowning by immunohistochemistry and gene expression analysis.

In addition, studies focused on the distinction between freshwater drowning (FWD) and saltwater drowning (SWD). Intrapulmonary gene expression of AQP5 was significantly decreased in FWD relative to that in SWD and other cases, which may be due to suppressed AQP5 expression in type I alveolar epithelial cells by hypotonic water to prevent hemodilution from a physiological perspective (20). Another study found that there was no statistically significant difference in lung-tissue AQP5 between SWD, FWD, and controls (37). In the kidneys, there were no significant differences in the expression of AQP1 and AQP4 between the FWD, SWD, and control groups. Immunohistochemically, AQP2 was predominantly expressed in the apical plasma membrane of collecting duct principal cells in all kidney samples from FWD and SWD. Morphometrically, there was significantly increased AQP2 expression in the apical plasma membrane of collecting ducts in the SWD group compared with the FWD and control groups (38). Brain samples showed that the mean value of AQP4-positive astrocytes was significantly higher in the FWD group than in the SWD and control groups. In addition, AQP4 expression was significantly lower in the SWD group than in the control group ($p < 0.05$) (39). For AQP2 (as well as arginine vasopressin), there was stronger statistically significant expression in renal tissue in the SWD group ($p < 0.05$) than in the FWD and control groups (40). The authors of the respective studies concluded that immunohistochemical detection of AQP2 in the kidney and AQP4 detection in the brain could be valuable markers for differentiating between FWD and SWD. Two studies of AQP5 expression in lung tissue yielded conflicting results.

Skin Injuries

Five studies investigated AQP expression in different types of skin lesions, mainly by immunohistochemistry. In the central portions of burn wounds where the epidermis and dermis are destroyed, no AQP3 was found in one study, but strong AQP3 staining was detected along the edge of the burn wound. Western blot analysis also showed stronger staining along the burn wound than in unburned control skin. Quantification showed significantly more AQP3 along the burn wound than in unburned skin and no AQP3 expression in the center of the burn wound (41). Examination of the expression of AQP1 and AQP3 in skin samples of the neck in cases of neck compression showed no significant difference in the AQP1 expressions in dermal capillaries between the study and control groups. In contrast, weak positive signals for AQP3 were detected in uninjured skin samples, and the positive signals again appeared more intense in keratinocytes in the compression regions. Morphometric analysis revealed that the proportion of AQP3-expressing keratinocytes was significantly increased in the neck compression regions relative to that in the control groups (42). The same authors studied the expression of AQP1 and AQP3 in human skin wounds that were classified into different groups according to their post-infliction interval. In uninjured skin samples, AQP1 and AQP3 were detected in dermal vessels and keratinocytes, respectively, at low levels, and the percentage of AQP1-positive vessels and number of AQP3-positive keratinocytes appeared to increase with wound age (43).

Another study combined gene analysis and immunohistochemistry to assess the expression of AQP1 and AQP3 in the skin of forensic autopsy cases and its value in the differential diagnosis of antemortem and post-mortem burns. AQP3 gene expression was significantly higher in the skin of antemortem burn cases than in post-mortem burn cases, mechanical wounds, and control cases. In contrast, immunohistochemical evaluation showed no differences in AQP3 expression patterns between control, antemortem, and post-mortem burn skin. This finding was attributed to a probable increase in dermal AQP3 gene expression to maintain water homeostasis in response to dehydration caused by the burn (44). In another study, the expression of AQP1 and AQP3 was investigated in various skin injuries caused by blunt, sharp, and thermal force trauma; strangulation marks; gunshot wounds; and frostbite. In another study, the expression of AQP1 and AQP3 was investigated in various skin injuries caused by blunt, sharp, and thermal force trauma; strangulation marks; gunshot wounds; and frostbite. There was no correlation between AQP3 expression and age, sex, body mass index, duration of agony, and post-mortem interval. For AQP1, there were no differences between injured and uninjured skin (45).

To summarize the conclusions of these five studies, immunohistochemical detection of AQP3 in neck skin could be valuable as a forensic marker for the diagnosis of antemortem compression or as a vital signs marker. Furthermore, immunohistochemical analyses of AQP1 and AQP3 in human skin wounds seem to be capable of supporting the objective accuracy of wound-age determination and determination of AQP3 gene expression seems to be useful for the forensic molecular diagnosis of antemortem burn wounds.

Other Research Areas

Four other studies investigated different aspects of AQP expression. One study compared intrapulmonary expressions of AQP1 and AQP5 via mRNA quantification as markers of water homeostasis between cases with smothering and choking or strangulation and with sudden cardiac death and acute brain injury. AQP5, but not AQP1, showed suppressed expression in smothering compared with expression in strangulation and sudden cardiac death and death from acute brain injury (46). Furthermore, molecular pathological analysis of post-traumatic alveolar injury and systemic responses affecting pulmonary edema, including AQP1 and APQ5 mRNA, expression of AQP1 in lung tissue was significantly higher in subacute sharp force injury than in the other groups. Regarding AQP5 mRNA expression, there were no differences among all groups. On immunohistochemical examination, AQP1 was clearly detectable in all vascular endothelial cells but showed no differences in distribution and intensity. AQP5 was weakly detectable in a linear pattern in type-1 alveolar epithelial cells and sporadically in interstitial macrophages, as shown in the other study by this group (47). A study on post-mortem brain mRNA and immunohistochemical expressions, including AQP4, in

TABLE 2 | Overview of fields of application and the respective applicable aquaporins as well as supplementary biomarkers.

Potential field of application	Aquaporin	Supplementary biomarkers
Skin injuries, wound age, and vitality	1, 3	MMP1, MMP9 (50), EPC (51), Tryptase, IL-15, CD15 (52), VEGF (53), Ubiquitin (54), IL-1α (55), IL-8, MCP-1, MCP-1α (56)
Drowning	1, 2, 4, 5	SP-A (37), AVP (40)
Burn injuries	3,	HIF (57)
Trauma	1, 4, 5	MMP2, MMP9, ICAM-1, Claudin-5 (47), GFAP, HIF-1α, IBA-1, CD68 (49), VEGF (16)
Smothering/chocking	5	Claudin-5 (46)
Intoxication	4	MMP2, MMP9, Claudin-5 (48)
Sudden cardiac death	5	
SIDS	1, 4, 9	

MMP, metalloproteinase; VEGF, vascular endothelial growth factors; EPC, endothelial progenitor cells; IL-15, Interleukin-15; SP-A, Surfactant protein A; AVP, Hormone Arginine Vasopressin; HIF, Hypoxia-inducible Factors; GFAP, Glial fibrillary acidic protein; IBA, Ionized calcium-binding adaptor molecule; ICAM, Intercellular adhesion molecule.

forensic autopsy cases of carbon monoxide methamphetamine and phenobarbital intoxications compared with different cases of traumatic injury showed higher expression of AQP4 in methamphetamine intoxications. Immunostaining results showed substantial interindividual differences between groups, with no apparent differences in distribution or intensity between all causes of death (48). Another study examined expression of AQP4 and correlation with hypoxia and neuroinflammation in human traumatic brain injury. AQP4 showed a significant and progressive increase between the control group and groups 2 (one-day survival) and 3 (3-day survival) from the acute stages of traumatic insult. In addition, there was an increase in AQP4 immunopositivity in groups 4 (7-day survival), 5 (14-day survival), and 6 (30-day survival), which may indicate upregulation of AQP4 at 7–30 days relative to that on day 1 (49).

In summary, mRNA quantification of AQP5 could distinguish smothering from choking and sudden cardiac death. Systematic analysis of gene expression, including of AQP4, via real-time polymerase chain reaction could be a useful procedure in forensic death investigations of methamphetamine intoxications since AQP4 might be upregulated in the brain during this kind of intoxication. Furthermore, AQP4 might be useful for estimating the time of survival in traumatic brain injuries.

CONCLUSIONS

In the 13-year period studied, relatively few studies were published that addressed the forensic significance of AQPs. The main focus of these studies was on SIDS, skin injuries, and drowning, in particular, on the distinction between FWD and SWD. The studies on SIDS mainly involved gene analysis, whereas the other two main topics involved either immunohistochemistry or a combination of the two. Specific gene variations of AQP1, 4, and 9, in combination with other influencing factors, might make infants more susceptible to onset of SIDS, although the importance of AQPs, especially AQP4, remains largely unclear. In drowning, AQP2 in the kidney and APQ4 in the brain appear to be useful with respect to distinguishing FWD from SWD, although such a distinction will probably only have relevant applications at a few forensic institutions with appropriate geographic settings. For skin injuries, AQP3 in particular seems to be a possible complementary test; e.g., to detect the vitality of (burn) wounds or antemortal skin compressions and to narrow the wound age. In addition, AQP5 could be used to distinguish smothering from sudden cardiac death, and AQP4 could be used to temporally delineate survived traumatic brain injury. Interesting research approaches could be found in the respective studies indicating that investigation of AQPs potentially can provide considerable added value for answering some questions. A combination of immunohistochemistry and gene expression analysis appears to be useful in each case to increase statistical significance. An overview of the complementary biomarkers is shown in **Table 2**.

This mini-review was limited by the fact that only studies on human material were included. Experimental studies or animal studies were deliberately omitted. Emphasis was placed on studies where immediate practical application is possible and where the results may, at best, add value to criminal investigations or court proceedings.

However, it is striking that there are only a few, if any, follow-up studies to the respective studies and that these were often conducted by the same research group. It must be noted, therefore, that the authors' frequent claims that further research is needed to evaluate the value and applicability of AQPs in the forensic context have gone largely unheeded by the scientific community.

Nevertheless, this mini review certainly shows the potential of AQPs in forensics and, despite the relatively few studies that have been conducted on human specimen to date, that there are very interesting and potentially relevant research approaches worth pursuing.

AUTHOR CONTRIBUTIONS

JP: conception and design of study and drafting the manuscript. JP and BM: acquisition of data. JP, BM, and ED: analysis and/or interpretation of data, revising the manuscript critically for important intellectual content, and approval of the version of the manuscript to be published. All authors contributed to the article and approved the submitted version.

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Wound Vitality in Decomposed Bodies: New Frontiers Through Immunohistochemistry

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Background: The question about wound vitality and the estimation of wound age of production are two of the classic investigation fields of forensic sciences. To answer this, the techniques most frequently used in research studies are immunohistochemistry (IHC), molecular biology, and biochemistry. Despite the great data on the literature about the usefulness of IHC in forensic pathology, there is always a request for further studies, especially on tissues altered by putrefactive phenomena. In fact, the degradation of the tissues is intended as the main limiting factor to the use of this technique.

Scope: The aim of this pilot study was to evaluate the immunohistochemical behavior of samples collected from decomposed bodies (in different putrefaction phases) and to relate these findings to wound vitality and postmortem interval.

Materials and Methods: Samples of skin and soft tissues were collected during autopsies, which were executed on decomposed bodies, whose cause of death was concluded to be traumatic. An immunohistochemical study was performed using antibodies against CD15, CD45, IL-15, tryptase, and glycophorin-A MMPs (endopeptidases involved in degrading extracellular matrix proteins: MMP-9 and MMP-2). An immunohistochemistry (IHC) reaction was evaluated according to a qualitative method as the following legend: (0): not expressed, (+): isolated and disseminated expression, (++): expression in groups or widespread foci, and (+ + +): widespread expression.

Results: Most of the tested markers (tryptase, glycophorin, IL15, CD 15, CD 45, and MMP9) showed to be highly expressed in the tissue of putrefied skin for 15 days.

Discussion and Conclusion: Although certainly inconclusive, this experimental application demonstrated that a nonexclusive but combined use of multiple antibodies is appropriate to verify wound vitality in decomposed bodies. Among them, GPA exhibited major reliability.

Keywords: wound vitality, decomposed body, IHC, GPA, MMP-9

INTRODUCTION

Assessment of the wound vitality is a long-standing question for forensic investigations to ascertain violent modality or supposed ones (e.g., in case of corpses found in open spaces, hypothetically wounded by local fauna after death) (1). To answer this, the most frequently used techniques in research studies are biochemistry molecular biology and immunohistochemistry (IHC) (2). Biochemical methods take advantage of the chemical and physics techniques. In particular, microspectrophotometry, microfluorimetry, and spectrophotometry have been used to assess concentration levels of vasoactive amines, although contradictory results emerged; also, atomic absorption spectrometry has been used to evaluate the diagnostic value of standalone ions and the ions ratio in skin wounds (3, 4).

Some authors found increased Fe concentrations in antemortem wounded skin and muscle, but no difference in Zn and Mg ions. Moreover, the K/Na ratio was found to be reduced in antemortem muscle samples, but not in the skin samples (5, 6).

On the other hand, molecular biology techniques have been applied rather onto the wound age estimation than on vitality (7–9). However, evaluation of mRNA levels of cytokines and enzymes throughout PCR technique has its rationale in the occurring changes of mRNA levels, after wounding, sooner than protein levels and histomorphology alterations (1, 10, 11). Nevertheless, the degradation of RNA caused by post-mortem effects is the most probable occurrence in some days. Hence, by measuring specific mRNA levels into the known decay time, it is possible to estimate the wound age estimation.

However, among all the techniques, IHC provides a great deal of evidence in the literature, demonstrating to be a valuable choice in determining, with a wide variety of markers (tissue molecules, cytokines, and growth factors), if a lesion is vital or not (2, 12). Furthermore, the IHC, if compared to other techniques, has proved to be more useful not only for its ease of application and its high reliability but, above all, for the possibility to analyze the localization of the molecules of interest (13). In this context, even if some markers are promising, prior to their application in daily routine, their use needs to be confirmed with other studies.

However, although these techniques are continuously studied in the forensic field of vitality on samples collected from fresh cadavers, there are not many applications on the decomposed bodies due to degeneration of microstructures investigated through routinely accessible methods. In particular, the skin samples are harder to be studied because of the ease of putrefaction in comparison to the muscle tissues located in the interior of the body (14). Indeed, to our knowledge, no biochemical method-based study has been performed extensively on putrefied specimens, as well as there are no molecular biology universal applications. Even with the IHC efficacy, wound vitality evaluation in putrefied corpses varies among authors in literature, mainly depending on each marker sensibility and specificity; moreover, the leakage of searched antigens normally contained into known structures produces a lack of specificity (15).

In this context, the aim of this pilot study was to evaluate the immunohistochemical behavior of samples collected from the decomposed bodies (in different putrefaction phases) and to relate these findings to wound vitality and post-mortem interval (PMI).

MATERIALS AND METHODS

Case Selection

The cases for the present study were selected as follows: (i) cadavers with residual skin; (ii) cadavers not subjected to special transformative processes (such as saponification and/or corification); and (iii) cadavers whose evidence gathered from the circumstantial data, crime scene investigation, external inspection until the mascroscopic and microscopic autoptic findings, oriented toward a traumatic death. Therefore, a total of 7 case studies, with different stages of decomposition between a few hours and 15 days after death, were elected. A negative control (NC) case (a decomposed body with no-traumatic injuries but with skin losses thorough feeding activity of the local macro- and microfauna) was also included. The result of the selection is summarized in **Table 1**.

IHC

All the samples of tissue have been fixed in formalin 10% for 48 h, then processed and included in paraffin. For each sample, 4 micron thick sections have been carried out; one section has been stained with Haematoxylin and Eosin (H&E). On the other sections, an immunohistochemical study has been performed using a panel of antibodies; details as summarized in the following **Table 2**.

The sections in paraffin have been rehydrated and incubated for 20 min in methanol, containing 10% of H_2O_2 to block endogenous peroxidases. The sections have been pre-treated to facilitate antigen retrieval and to increase membrane permeability to antibodies, then incubated with the primary antibody (**Table 1**). The utilized detection system was a refined avidin–biotin system in which a biotinylated secondary antibody reacts with several peroxidise-conjugated streptavidin molecules. The positive reaction was visualized by 3,3′-diaminobenzidine (DAB) peroxidation, according to standard methods. The sections were counter-stained with Mayer's hematoxylin, dehydrated, cover-slipped, and observed with an optical microscope.

Histologic examination was based on a semiquantitative screening, of which we report below the gradation of positive reaction:

- [0]: not expressed,
- (+): isolated and disseminated expression,
- (++): expression in groups or widespread foci,
- (+ + +): widespread expression.

TABLE 1 | Cases and control characteristics.

Case	Historical data	Intrinsic features	Environmental condition	PMI	Cause of death attributed	Sample
CASE 1	Suicide	Nomal adipose panniculus; dressed	Housing; door and windows closed	24–36 h from death	Gunshot	Lacerated wound of the frontal region
CASE 2	Fight among partners	Normal adipose panniculus; dressed	Housing; door and windows closed	3–6 days from death	Strangulation	Anterior region of the neck
CASE 3	suicide	Nomal adipose panniculus; dressed	Countryside, outdoors; dry and ventilated climate	3–6 days from death	Hanging	Anterior region of the neck
CASE 4	Family mourning	Aboundant adipose panniculus; dressed	Countryside, outdoors; humid and unventilated climate	7–10 days from death	Slaughtering	Incised wound of the neck
CASE 5	Agricultural worker	Normal adipose panniculus; dressed	Countryside, outdoors; dry and ventilated climate	7–10 days from death	Massive fracture head injury	Lacerated wound of the scalp
CASE 6	Frequent allegations of assault	Normal adipose panniculus; dressed	Countryside, outdoors; dry and unventilated climate	10–15 days from death	Burst head injury	Lacerated wound of the scalp
CASE 7	Disappearance by family members reported	Aboundant adipose panniculus; dressed	Mountain forest, outdoors; dry and ventilated climate	10–15 days from death	Slaughtering	Incised wound of the neck
CASE 8 (NC)	Disappearance by family members reported	Aboundant adipose panniculus; dressed	Countryside, outdoors; dry and ventilated climate	3–6 days from death	Sudden cardiac death	Skin losses by macro- and micro-fauna from the arms

TABLE 2 | A panel of antibodies in study.

Tryptase			
(mouse monoclonal antibody, sc-59587, Santa Cruz, CA, USA)	Proteinase K (T: 20 °C for 15 min)	120 min, 20°C	1:1000
IL-15 (mouse monoclonal antibody, sc-8437, Santa Cruz, CA, USA)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:50
CD 15 (mouse monoclonal antibody, sc-53290, Santa Cruz, CA, USA)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:50
MMP 2 (mouse monoclonal antibody, sc-53630, Santa Cruz, CA, USA)	boiling in 0.1 M Citric Acid buffer.	120 min, 20°C	1:100
MMP 9 (mouse monoclonal antibody, sc-21733,Santa Cruz, CA, USA)	boiling in 0.1 M Citric Acid buffer.	120 min, 20°C	1:100
Glycophorin A (GPA - mouse monoclonal antibody, sc-59181, Santa Cruz, CA, USA)	boiling in 0.25 mM EDTA buffer	120 min, 20°C	1:500
CD 45 (mouse monoclonal antibody, sc-19664, Santa Cruz, CA, USA)	boiling in 0.25 mM EDTA buffer.	120 min, 20°C	1:600

RESULTS

The microscopic analysis of the skins' preparations showed a different reactivity in various stages of putrefaction. Among the different tested antibodies, only some showed a remarkable reactivity on the very particular putrefied tissue of skin (**Table 2**). Based on this first observation, we selected some markers, which were expressed in the putrefied tissues. Most of the tested markers (tryptase, GPA, IL15, CD 15, CD 45, and MMP9) were shown to be highly expressed in the tissue of putrefied skin for up to 15 days. The reaction against antibody anti-CD 15, CD45, and GPA is localized on the cellular membrane; tryptase, IL15 MMP2, and MMP9 antibodies showed cytoplasmic staining. All measurements were done on the same magnification of image (40 x) and by three different examiners (**Table 3**). A correlation between the IHC pattern and the PMI is also provided in **Figure 1**.

DISCUSSION

Diagnosis of wound vitality and wound-age estimation represent crucial, and, still open, questions for forensic pathologists. Vitality refers to a set of physiological processes, such as erythrocyte extravasation or inflammation, which prove the injury was inflicted when the individual was still alive. Typically, the vitality of a lesion was assessed with the standard hematoxylin-eosin stain to detect erythrocyte extravasation. However, this evidence alone does not represent a reliable sign of vitality, as some studies have suggested that red blood cells extravasations can occur even after death (16, 17).

Wound-age estimation remains an unsolved problem that is limited by the non-specificity and low reproducibility of biomarkers, as well as the ethical limitations related to the impossibility of excising wound samples in living subjects (1).

TABLE 3 | IHC reaction evaluation according to the qualitative method selected by three different examiners.

Antibody	CASE 1	CASE 2	CASE 3	CASE 4	CASE 5	CASE 6	CASE 7	CASE 8
CD 15	+++	+++	+++	++	++	++	++	-
IL-15	+++	+++	+++	++	++	++	++	-
Tryptase	+++	+++	+++	++	++	++	++	-
CD 45	+++	+++	+++	++	++	+	+	-
MMP 2	++	+	+	+	+	+	+	-
MMP 9	+++	+++	+++	+++	+++	+++	+++	+
GPA	+++	+++	+++	+++	+++	+++	+++	-

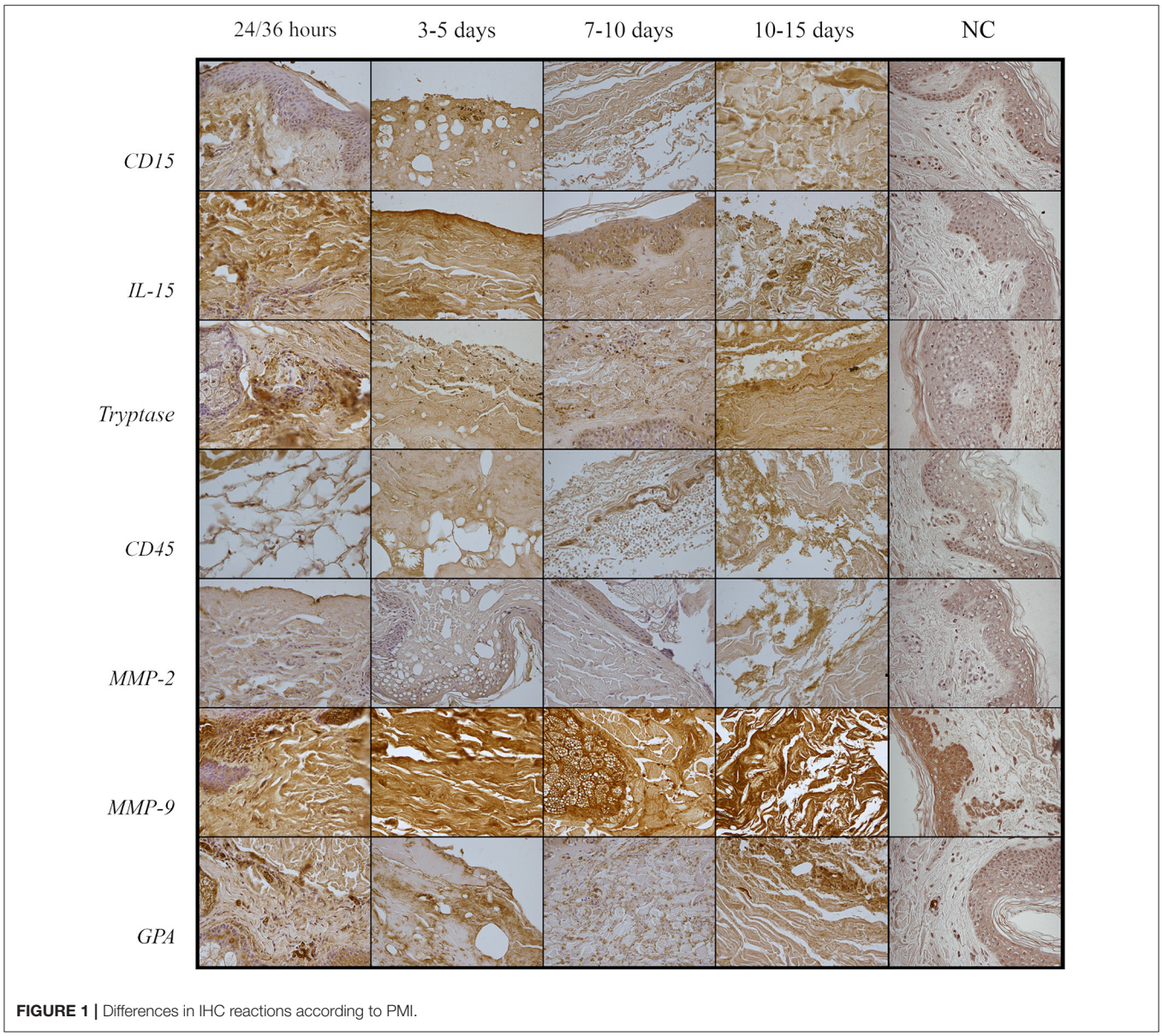

FIGURE 1 | Differences in IHC reactions according to PMI.

The numerous studies published in the literature have identified immunohistochemistry, molecular biology, and biochemical tests as the tools to solve these questions. Immunohistochemistry is the most widely used method due to its ability to detect the location of antigens, ease application on formalin-fixed paraffin-embedded tissue, and reproducibility (1).

The forensic science community has focused its attention on various immunohistochemical markers to differentiate pre- and

post-mortem injuries and to estimate the time interval between the infliction of the wound and the death. Among the investigated molecules are inflammatory cytokines, coagulation factors, metal ions, structural proteins of erythrocytes, and proteolytic enzymes that were involved in the physiological response of a living tissue to external stimuli (6, 18–20). Immunohistochemical methods gave satisfying results in recently deceased bodies; however, their use is challenging in putrefied corpses, as the alteration of the tissues can compromise the interpretation of the results. The main limitation linked to putrefaction is the degradation of the protein molecules, which alters the antigens and determines their migration to a different site than the original one. In addition, a false positivity may arise in putrefied tissues due to the increased binding of antibodies to the altered epitopes. Nevertheless, several studies have tested the applicability of immunohistochemistry on decomposed corpses in different tissues and have found that some antigens can be identified despite the tissue alteration. The most significant applicability of immunohistochemistry was observed within a post-mortem interval of 3 days (21).

Glycophorin A (GPA), an integral membrane protein of erythrocytes, is a widely used bleeding marker. In putrefied subjects, it can highlight bleeding in the skin, bone, and muscle tissue. According to Tabata and Morita, immunohistochemical detection of GPA in the putrefied corpse allows discrimination between hemoglobin diffusion and bleeding (1, 22). The present study has tested the possibility of applying immunohistochemistry on skin samples taken from traumatic lesions of corpses in different stages of putrefaction, using a panel of markers involved in the mechanisms of inflammation and wound repair: metalloprotease 2 and 9 (MMP-2 and MMP-9), interleukin 15 (IL-15), tryptase, and leukocyte differentiation clusters 15 and 45 (CD15 and CD45); in addition, GPA was used to identify bleedings.

MMPs are proteolytic enzymes expressed proactively in tissues, which are converted into active form after injury. They take part in a variety of processes, including remodeling of extracellular matrix and cell repair. The MMP-2 and MMP-9 are implicated in collagen, elastin, fibronectin, and laminin degradation, and have pro- and anti-inflammatory roles in numerous tissues (23). The MMPs are proteolytic enzymes proactively expressed in tissues, which are converted into active form after injury. In particular, MMP-2 and MMP-9, respectively known as gelatinases A and B, take part in a variety of processes, including: (i) remodeling of extracellular matrix and cells repair; (ii) degradation of collagen, elastin, fibronectin, and laminin; (iii) digesting various inflammation-involved molecules, such as pro-TNF, TGF-β, pro-IL-1β, and pro-IL-8; and (iv) processing of various pro- and antiangiogenic factors during wound healing (24, 25). Although they are not reliable in the diagnosis of viability as they do not differ significantly in pre- and post-mortem lesions, several studies emphasize their usefulness in determining the wound age (26). Indeed, increased expression of MMP-2 can be detected in the connective tissue at the edge of acute wounds during all stages of the healing process and remained fairly stable until the phase of re-epithelialization. The MMP-9 contributes to healing wound by the initiation of keratinocytes migration and mobilization of endothelial progenitor cells from the bone marrow (27, 28).

The present study findings show that MMP-2 is widely expressed in the recently deceased corpse, while it decreases between 3 and 6 days, respectively, after death, up to be weak/absent in the 10–15 days post-mortem interval. Conversely, MMP-9 expression is unvaried in the different putrefaction phases.

The IL-15 is a cytokine with a wide range of functions. It is involved in the recruitment of mononuclear cells, deposition of fibrous tissue, angiogenesis, and regulation of the phenotype of lymphocytes and monocytes. Together with CD-15 (an antigen expressed by leukocytes, involved in cell adhesion), IL-15 appears to be a reliable indicator for assessing the viability of lesions (29), although there are no studies on its applicability in putrefied bodies (30).

Gauchotte et al. indicated that CD-15 positive cells can be passively released from the vessels in putrefied samples, thus giving false positives (15). This research results highlight a widespread expression of IL-15 and CD15 of up to 6 days after death, which decreases with the advancement of putrefactive phenomena.

The CD45 is a leukocyte antigen used to assess the viability of the lesion, as its immunohistochemical detection localizes migrated white blood cells to the site of inflammation. Furthermore, it is a reliable marker for estimating the time of the injury (31). The present results demonstrate strong CD45 expression within a 6-day post-mortem interval, which progressively decreases over time.

Tryptase is a protease contained in mast cell granules, involved in the inflammatory and anaphylactic response. Its immunohistochemical identification suggests the activation and consequent degranulation of mast cells, and, therefore, is an indicator of the vitality of the lesion (32). According to this study, tryptase is strongly expressed within 6 days of death and weakens as the post-mortem interval increases. Furthermore, the expression of GPA remains constant despite the time progresses.

However, this study does not lack limitations, represented, above all, by the small number of samples included and by the non-uniformity of the environmental conditions at the time of the discovery of the corpses. Moreover, the present work has focused its attention on a post-mortem interval of 15 days; thus, the results do not allow for considerations beyond this time.

CONCLUSION

The present study demonstrates the possibility of investigating wound vitality through the IHC method, despite the limitations inherent to the technique itself and relative to its application in samples from decomposed bodies. Indeed, the positivity of the selected antibodies has been noticed for up to 15 days from death, except for MMP-2, which was definitely positive in the only corpse with the most recent PMI. Furthermore, the positivity obtained turns out to be inversely proportional to the PMI. Therefore, to obtain a diagnosis of vitality as accurate and reliable as possible, a non-exclusive but combined use of these markers is

recommended in decomposed bodies. On the other hand, when selecting the other antibodies to be used, it is necessary to keep in mind the non-unique interpretation of the data provided by the literature review, particularly regarding the use of MMP-9. Besides, the CD15-positive cells could passively disseminate from the vessels altered by the decomposition process. However, in this integration of evidence, the antibody that was proven to be more reliable, and should be routinely used is GPA. Further studies are, therefore, needed to standardize the antibody pattern to be used.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LC and GB: conceptualization and supervision. GB and MN: methodology. MF and RLR: validation. Autoptic cases from SDS, LC, MN. MF, GG, and GP: literature review. PF, LA, and MN: IHC investigation. GB, MF, GG, and GP: writing of original draft preparation. RLR, PF, LC, and MN: writing, reviewing, and editing. All authors have read and agreed to the published version of the manuscript.

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frontiers in Medicine

SYSTEMATIC REVIEW
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Forensic Impact of the Omics Science Involved in the Wound: A Systematic Review

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Background: In forensic autopsies, examining the wounds is one of the most critical aspects to clarify the causal relationship between the cause of death and the wounds observed on the corpse. However, on many occasions, it is difficult to differentiate antemortem injuries from post-mortem injuries, mainly when they occur very close to the moment of death. At present, various studies try to find biomarkers and clarify the molecular mechanisms involved in a wound due to the high variability of conditions in which they occur, thus being one of the most challenging problems in forensic pathology. This review aimed to study the omics data to determine the main lines of investigation emerging in the diagnosis of vital injuries, time of appearance, estimation of the age and vitality of the wound, and its possible contributions to the forensic field.

Methods: A systematic review of the human wound concerning forensic science was carried out by following PRISMA guidelines.

Results: This study sheds light on the role of omics research during the process of wounding, identifying different cytokines and other inflammatory mediators, as well as cells involved in the specific stage of the wound healing process, show great use in estimating the age of a wound. On the other hand, the expression levels of skin enzymes, proteins, metal ions, and other biomarkers play an essential role in differentiating vital and post-mortem wounds. More recent studies have begun to analyze and quantify mRNA from different genes that encode proteins that participate in the inflammation phase of a wound and miRNAs related to various cellular processes.

Conclusions: This study sheds light on the role of research in the molecular characterization of vital wounds, heralding a promising future for molecular characterization of wounds in the field of forensic pathology, opening up an important new area of research.

Systematic Review Registration: URL: https://www.crd.york.ac.uk/prospero/#myprospero, Identifier: CRD42021286623.

Keywords: human skin wounds, omics sciences, vital wounds, forensic sciences, age wound

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INTRODUCTION

Estimating the age and vitality of human skin wounds in the living and dead is essential in forensic practice (1, 2). Due to supravital reactions and minor morphological changes evident during this time, immunohistochemical parameters for age assessment and vitality for human skin wounds remain challenging (3–6).

Antemortem wounds elicit vital reactions that do not occur in post-mortem wounds, so the demonstration of a vital injury is sufficient to affirm that the injury occurred before death. On the other hand, vital reactions follow regular and time-dependent courses, which allows a reliable temporal classification of wound healing (7). Extravasations of red blood cells and hemoglobin to the wound were once considered a vital reaction indication, but considerable research have contradicted these findings, indicating that it cannot be utilized as a good marker in wound vitality diagnosis (8).

In this context, forensic molecular pathology includes applying omics sciences to investigate the genetic basis and the cause of death at the molecular biological level. Today many genomic investigations carry out an analysis of the genetic background (9–11), study the dynamics of gene expression (transcriptomics) is also playing an important role (12–14), as well as vital phenomena involving activated biological mediators and their degenerative products (proteomics) (15), and finally, the analysis of the different metabolites involved (metabolomics) (16–18).

Post-mortem biochemistry and experimental research propose the use of molecular biology techniques in the context of forensic pathology to detect functional changes involved in the death process, which cannot be detected morphologically (1, 19). In this context, different studies have shown that many cytokines, growth factors, and proteases are involved in the healing process of a wound, their study being practical to determine the vitality of the wound or its age in forensic medicine (20).

Besides, there are different approaches to assess the vitality of a lesion, from macromorphology to the level of mRNA through histology and protein. However, in the last 30 years, immunohistochemical techniques have been the method of choice to study wound vitality and age (21). However, elucidating the link between wounds and mortality causes utilizing specific vitality indicators with an adequate and consistent strategy remains a subject of controversy (6, 22).

The main objective of this systematic review was to compile the main lines of research on age and differentiation of vital and post-mortem human wounds that have emerged in recent years and their relationship with omics sciences, as well as the possible contributions or limitations in the field of forensic sciences.

SYSTEMATIC REVIEW

The methods used for this systematic review (covering 1992 to July 2021) were developed by reference to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (23) for studies published in accordance with the methods detailed in the Cochrane Handbook for Systematic Reviews of Interventions (24). Before commencement, the protocol for this systematic review was registered with the International Prospective Register of Systematic Reviews (PROSPERO, CRD42021286623).

Inclusion Criteria

All studies exploring the vitality and the age of wounds in human forensic science in subjects aged 0–97 years old were included. The articles were chosen according to three main inclusion criteria: (i) studies of the age of vitality wounds, (ii) differentiation of vital and post-mortem wounds, and (iii) wounds of human origin.

Search Strategy

Literature search strategies were developed in collaboration with a health sciences librarian using two scientific electronic databases (PubMed and Scopus) and keywords.

For the articles included in the review, the key characteristics of the studies were identified: topic discussed, first author, and year. The following keywords and subject heading terms were used: [[(wounds) OR (injuries)] AND (skin)] AND (forensic). The search in the two scientific electronic databases (PubMed and Scopus) was limited to articles published in English and studies conducted in humans. Two independent reviewers revised titles and abstracts and then full-text publications concerning the inclusion criteria. Study selection interrater agreement between the two reviewers was calculated as the proportion of favorable agreement (25).

Data Extraction

Two independent testers retrieved duplicate data using Microsoft Excel. We checked and compared multiple reports from the same study and extracted them where specific data existed. For all studies that met the inclusion criteria, the following data were extracted: authors, year of publication, geographic location, study population, study design, sample size, age range, post-mortem interval, gender, type of wound, biomarker detected, and technique used.

Risk of Bias Assessment

The risk of bias was assessed for each sample by comparison with the Cohort Research Checklist of the Critical Assessment Skills Program (CASP) (26). The following confounding variables within the CASP checklist were evaluated: sample size, age, post-mortem interval, gender, and analyses technique. Based on the CASP checklist, study output was graded as "bad," "fair," or "good." The overall quality of the proof was rated as high, moderate, weak, or extremely low (27).

Descriptive Studies

A total of 3.265 studies were identified in the two scientific electronic databases, PubMed (1.634) and Scopus (1.631) (**Figure 1**). A total of 3.059 duplicates and non-relevant studies were eliminated, and 206 studies were reviewed to assess their relevance. A total of 140 studies were excluded by these criteria:

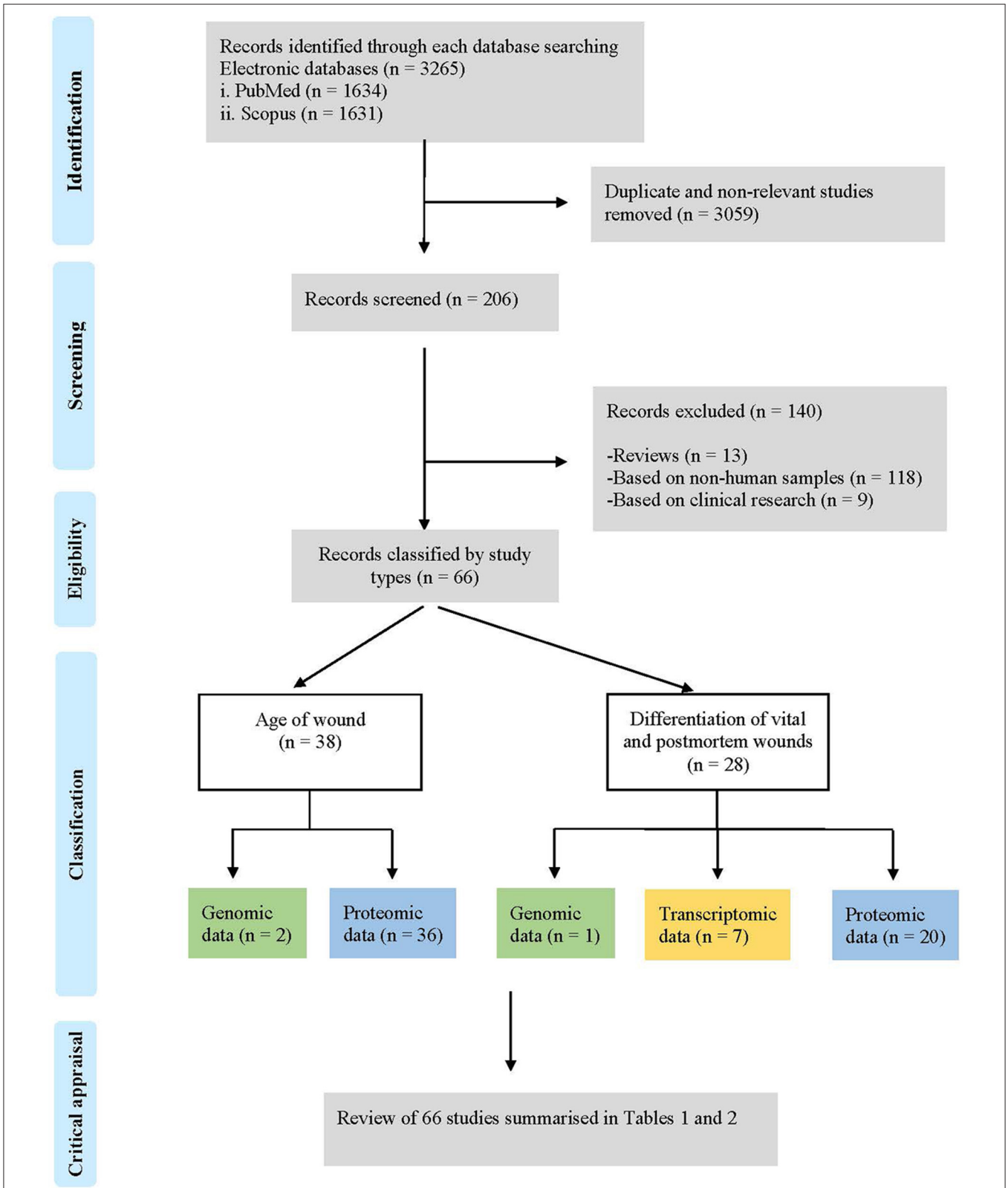

FIGURE 1 | Flow chart of the literature search process and study selection according to PRISMA (Preferred reporting items for systematic reviews and meta-analysis) guidelines.

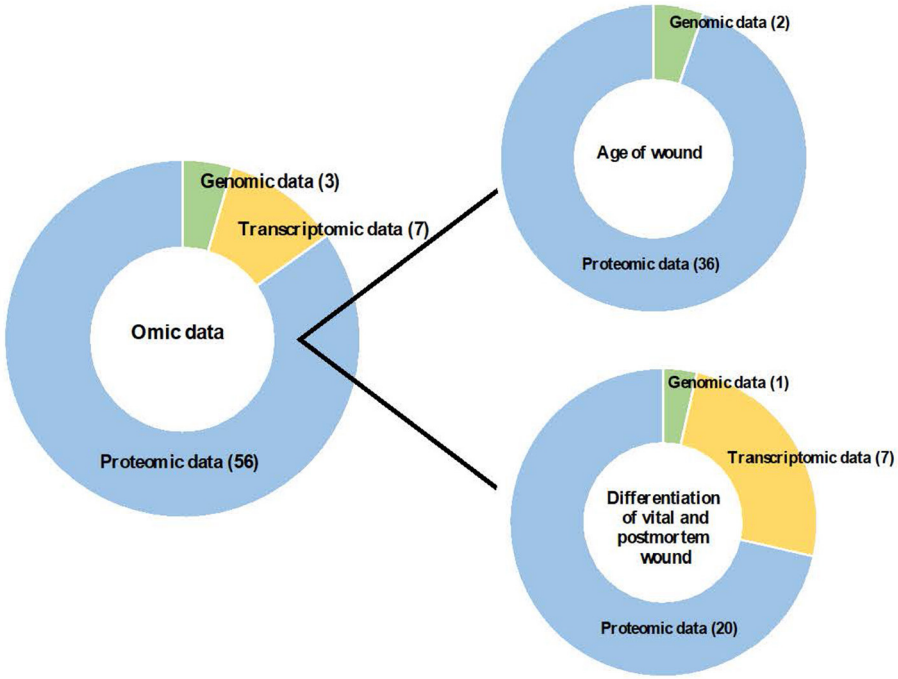

FIGURE 2 | Contribution of omic sciences to the study of age of wound and differentiation of vital and post-mortem wound.

(i) reviews ($n = 13$); (ii) based on non-human samples ($n = 118$) and (iii) based on clinical research ($n = 9$).

Finally, this search strategy identified 66 descriptive studies according to the following classification: (i) age of wounds ($n = 38$): genomic data ($n = 2$) and proteomic data ($n = 36$); and (ii) differentiation of vital and post-mortem wounds ($n = 28$): genomic data ($n = 1$), transcriptomic data ($n = 7$) and proteomic data ($n = 20$); that were included in this systematic review (**Figure 2**).

Risk of Bias Assessment

According to the CASP risk of bias assessment, most studies (57.58%) were judged as "good" due to the considered variables, while 42.42% were judged as "poor" or "moderate," primarily due to confounding variables not being considered (**Tables 1**, **2**). Participants were recruited from few geographic regions, making it difficult to generalize beyond these regions. Overall, the quality of the literature was good.

Laboratory Methods

The methods used varied between studies (**Tables 3**, **4**; **Figure 3**). To determine the age of the wound, most studies (30/38) used inmunohistochemical analysis to detect different biomarkers involved in the healing wound. Two studies (28, 29) used *In Situ* Labeling of DNA fragments (ISEL) to detect DNA fragments. The other two studies used ELISA (45, 47), and the other two used immunofluorescence analysis (51, 52). Finally, a study (54) used Cytochemistry analysis, and another study (63) used enzyme histochemical analysis (**Table 3**). On the other hand, most studies (14/28) used inmunohistochemical analysis to differentiate between vital and post-mortem wounds. Five studies (65, 66, 69–71) used RT-qPCR and two studies (67, 68) used RT-qPCR and western blot to detect transcriptomic data. Other seven studies used different techniques: q-DNA analysis (64), enzyme histochemical analysis (79), histological analysis (85), atomic absorption spectrometry (84), liquid chromatography (86), multiplex sandwich immunoassay (88) and western blot (89) (**Table 4**).

WOUND CHARACTERIZATION IN POST-MORTEM FORENSIC STUDIES

Age of Wounds

When faced with a wound, one of the main tasks of the forensic pathologist is to determine how long the victim survived after the wound was inflicted (92). Healing of a skin wound begins immediately after injury and consists of three phases: inflammation, proliferation, and maturation, which involve interactions between various types of cells and soluble factors (20, 93–96).

A total of 38 descriptive studies on the determination of the age of vital wounds have been reviewed (**Table 3**). The studies that place the appearance and/or quantification of specific markers on a timeline referring to the age of the wound in comparison with the group used as control have been collected in **Figure 4**.

Two studies analyze genomic data using *in situ*-end labeling (ISEL). They detect and quantify nuclear DNA fragments that appear as a consequence of direct cell injury or during

TABLE 1 | Risk of bias assessment: age of wounds[a].

Omics studies	Address a clearly focused issue	Acceptable cohort recruitment	Exposure accurately measured	Outcome accurately measured	Important confounding factors identified	Important confounding factors accounted for	Precise results	Believable results	Results fit with other available data	Overall quality score
Genomic data										
Suárez-Peñaranda et al. (28)	●	●	●	●	●	●	●	●	●	●
Betz et al. (29)	●	●	●	●	●	●	●	●	●	●
Proteomic data										
Hausmann et al. (30)	●	●	●	●	●	●	●	●		●
Tarran et al. (31)	●	●	●	●	●	●	●	●	●	●
Betz et al. (32)	●	●	–	–	–	●	●	●	●	●
Betz et al. (33)	●	●	●	●	●	●	●	●	●	●
Balazîc et al. (34)	●	●	●	●	●	●	●	●	●	●
Van de Goot et al. (35)	●	●	●		●	●	●	●	●	●
Guler et al. (36)	●	●	●	●	●	●	●	●	●	●
Kondo et al. (37)	●	●	●	●	●	●	●	●	●	●
Fieghut et al. (38)	●	●	●	●	●	●	●	●	●	●
Dreßler et al. (39)	●	●	●	●	●	●	●	●	●	●
Dreßler et al. (40)	●	●	●	●	●	●	●	●	●	●
Dreßler et al. (41)	●	●	●	●	●	●	●	●	●	●
Dreßler et al. (3)	●	●	●	●	●	●	●	●	●	●
Dreßler et al. (42)	●	●	●	●	●	●	●	●	●	●
Betz et al. (43)	●	●	●	●	●	●	●	●	●	●
Fronczek et al. (44)	●	●	●	●	●	●	●	●	●	●
Grellner et al. (45)	●	●	●	●	●	●	●	●	●	●
Grellner et al. (46)	●	●	●	●	●	●	●	●	●	●
Birincioglu et al. (47)	●	●	●	●	●	●	●	●	●	●
Takamiya et al. (48)	●	●	●	●	●	●	●	●	●	●
Kondo et al. (49)	●	●	●	●	●	●	●	●	●	●
Ishida et al. (50)	●	●	●	●	●	●	●	●	●	●
Ishida et al. (51)	●	●	●	●	●	●	●	●	●	●
Kuninaka et al. (52)	●	●	●	●	●	●	●	●	●	●
Jebur et al. (53)	●	●	●	●	●	●	●	●	●	●
Bonelli et al. (54)	●	●	●	●	●	●	●	●	●	●
Kondo et al. (55)	●	●	●	●	●	●	●	●	●	●
Grellner et al. (4)	●	●	●	●	●	●	●	●	●	●
Yagi et al. (56)	●	●	●	●	●	●	●	●	●	●
Hayashi et al. (57)	●	●	●	●	●	●	●	●	●	●

(Continued)

TABLE 1 | Continued

Omics studies	Address a clearly focused issue	Acceptable cohort recruitment	Exposure accurately measured	Outcome accurately measured	Important confounding factors identified	Important confounding factors accounted for	Precise results	Believable results	Results fit with other available data	Overall quality score
Ishida et al. (58)	●	●	●	●	●	●	●	●	●	●
Ishida et al. (59)	●	●	●	●	●	●	●	●	●	●
Ishida et al. (60)	●	●	●	●	●	●	●	●	●	●
Ishida et al. (61)	●	●	●	●	●	●	●	●	●	●
Dachun and Jiazhen (62)	●	●	●	●	●	●	●	●	●	●
Betz (63)	●	●	●	●	●	●	●	●	●	●

[a]*Data based on CASP-based risk of bias assessment.*

Green, good risk of bias; Orange, moderate risk of bias; Red, low risk of bias.

programmed cell death (apoptosis), thus providing information on the wound healing process and, as a consequence, on its age. Suárez-Peñaranda et al. (28) limited their research to incisional lesions, showing that the finding of apoptosis in keratinocytes of the dermis can be an early vitality marker. However, they did not find variations in the level of positive cells when the age of the wound increases. Therefore, the validity of this marker to determine the age of the wound is doubtful. On the contrary, another study (29) analyzed lacerations, stab wounds, and surgical wounds, concluding that a rapid increase in the number of fibroblasts occurs during wound healing after ∼3 weeks.

Thirty-six studies associate wound age estimation with the detection and quantification of different proteins (proteomics data) mainly involved in the inflammation phase of the wound healing process. Two studies analyzed the expression of the protein p53, a protein responsible for stopping the cell cycle when it detects DNA damage, thus allowing the repair of damaged DNA or inducing apoptosis.

Hausmann et al. (30) analyzed lacerations, stab wounds, and surgical wounds and stated that it could be expected to find a significant number of fibroblasts positive to p53 ($r \geq 0.2$) in wounds of at least 3 days of age, while when the values of are >0.5, the post-infliction interval is at least 8 days. On the other hand, Tarran et al. (31) explored the expression of p53 in antemortem and post-mortem material, concluding that more studies are necessary to determine the minimum survival period necessary for a burn wound to express the protein mentioned above and to determine if its expression can occur in uninjured skin such as a result of agonizing stress.

Two studies of Betz et al. (32, 33) analyze two glycoproteins of the extracellular matrix, fibronectin, and tenascin, respectively, in surgical wounds, stab wounds, lacerations, bruises, and abrasions. These glycoproteins support the adhesion of different cells such as fibroblasts and endothelial cells, participating in the early stages of the wound healing process. The immunohistochemical investigations of these authors demonstrated that fibronectin allows differentiation between wounds of less than two and more than 3 weeks, while tenascin appears for the first time 2 days after the injury, around fibroblastic cells, observing a decrease in the intensity of tenascin staining with increasing age, tenascin still being present in wounds up to 1.5 months. Similarly, Balažic et al. (34) also studied fibronectin's expression, but unlike the previous ones, they analyzed gunshot wounds. Their results indicate that fibronectin is a reliable marker of the vitality and age of wounds with a short survival time (few minutes).

van de Goot et al. (35) analyzed the fibronectin glycoprotein together with CD62p and coagulation factor VIII. Fibronectin is responsible for forming a clot at the site of the injury, promoting the spread of platelets, as well as the migration of neutrophils, monocytes, fibroblasts, and cells endothelial cells, CD62p and factor VIII being present on the surface of the latter a few minutes after a wound occurs. They observed a significant increase of the three markers in wounds of 15–30 min compared to the uninjured control samples. Another study (36) studies the expression of tenascin together with that of ubiquitin, a cytokine-like protein with anti-inflammatory properties expressed in neutrophils, leukocytes, macrophages, and fibroblasts in the

TABLE 2 | Risk of bias assessment: differentiation of vital and post-mortem wounds[a].

Study	Address a clearly focused issue	Aceptable cohort recruitment	Exposure accurately measured	Outcome accurately measured	Important confounding factors identified	Important confounding factors accounted for	Precise results	Believable results	Results fit with other available data	Overal quality score
Genomic data										
Grellner and Benecke (64)	🟢	🔴	🔴	🔴	🔴	🔴	🟢	🟢	🟢	🟠
Transcriptomic data										
Xu et al. (65)	🟢	🔴	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟠
Ye et al. (66)	🟢	🔴	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟠
Qu et al. (67)	🟢	🔴	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟠
He et al. (68)	🟢	🔴	🔴	🔴	🔴	🔴	🟢	🟢	🟢	🟠
Liapi et al. (69)	🟢	🔴	🔴	🔴	🔴	🔴	🟢	🟢	🟢	🟠
Neri et al. (70)	🟢	🟢	🔴	🔴	🔴	🔴	🟢	🟢	🟢	🟠
Kubo et al. (71)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Proteomic data										
Ishida et al. (72)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Prangenberg et al. (73)	🟢	🔴	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Bonelli et al. (74)	🟢	🔴	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Oehmichen et al. (75)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Turillazzi et al. (76)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Gauchotte et al. (77)	🟢	🟢	🔴	🔴	🔴	🔴	🟢	🟢	🟢	🟠
Legaz et al. (78)		🔴	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Hernández-Cueto et al. (79)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Montisci et al. (80)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🔴	🔴
Ortiz-Rey et al. (81)	🟢	🔴	🟢	🟢	🟢	🟢	🟢	🟢	🔴	🔴
Ortiz-Rey et al. (82)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Legaz Pérez et al. (83)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Yu-Chuan et al. (84)	🟢	🔴	🔴	🔴	🔴	🔴	🟢	🟢	🟢	🟠
Ali (85)	🟢	🔴	🔴	🔴	🔴	🔴	🟢	🟢	🟢	🟠
He and Zhu (86)	🟢	🔴	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Bacci et al. (87)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Peyron et al. (88)	🟢	🔴	🔴	🔴	🔴	🔴	🟢	🟢	🟢	🟠
Kimura et al. (89)	🟢	🔴	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟠
Bacci et al. (90)	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢
Focardi et al. (91)	🟢	🔴	🟢	🟢	🟢	🟢	🟢	🟢	🟢	🟢

[a] *Data based on CASP-based risk of bias assessment.*
Green, good risk of bias; Orange, moderate risk of bias; Red, low risk of bias.

TABLE 3 | Age of wound in post-mortem studies[a].

Study	*N*	Age (years)		PI (hours)		Male/Female	Population analyzed	Type of wound	Biomarker analyzed	Analyses technique
		Range	Mean	Range	Mean					
Genomic data										
Suárez-Peñaranda et al. (28)	30	28–76	46.7	n.i.	n.i.	18/12	Spain	Wounds inflicted with a scalpe.	Apoptotic keratinocytes	ISEL
Betz et al. (29)	56	17–75	52	<72	n.i.	n.i.	Germany	Lacerations, stab wounds and surgical wounds.	Apoptotic fibroblastic cell	ISEL
Proteomic data										
Hausmann et al. (30)	82	17–75	52	24–96	n.i.	n.i.	Germany	Lacerations, stab wounds and surgical wounds.	p53	IC
Tarran et al. (31)	13	2–70	38	n.i.	n.i.	6/5	Australia	Thermal burns	p53	IC
Betz et al. (32)	53	15–92	54	<72	n.i.	n.i.	Germany	Surgical wounds, lacerations, stab wounds, heamtomas and abrasions.	Fibronectin	IC
Betz et al. (33)	56	15–92	54	<72	n.i.	n.i.	Germany	Surgical wounds, stab wounds and lacerations.	Tenascin	IC
Balazîc et al. (34)	48	n.i.	n.i.	n.i.	n.i.	n.i.	Slovenia	Gunshot wounds	Fibronectin	IC
Van de Goot et al. (35)	322	0–95	n.i.	0–48	n.i.	n.i.	Netherlands	Only skin wound samples that are the result of "blunt force trauma".	Fibronectin, CD62p and Factor VIII	IC
Guler et al. (36)	170	15–85	39.44	4–24	18	74/15	Turkey	Gunshot wounds, blunt injuries, Sharp weapon injuries, and surgical excisions.	Tenascin and ubiquitin	IC
Kondo et al. (37)	55	8–75	40.6	<72	n.i.	n.i.	Germany	Stab wounds, incised wounds, surgical wounds and lacerations.	Ubiquitin	IC
Fieguth et al. (38)	38	12–89	44	24–48	n.i.	21/17	Germany	Neck soft tissue	Myoglobin, Fibronectin, C5b-9, MRP14	IC
Dreßler et al. (39)	132	n.i.	n.i.	n.i.	n.i.	n.i.	Germany	Injured skin.	ICAM-1 (CD54)	IC
Dreßler et al. (40)	65	n.i.	n.i.	n.i.	n.i.	42/23	Germany	Lacerated wounds, incised wounds and excoriations.	ICAM-1 (CD54)	IC
Dreßler et al. (41)	97	n.i.	49.5	n.i.	n.i.	n.i.	Germany	Lacerated/contused wounds, incised wounds, and excoriations.	Selectins	IC

(Continued)

TABLE 3 | Continued

Study	*N*	Age (years)		PI (hours)		Male/Female	Population analyzed	Type of wound	Biomarker analyzed	Analyses technique
		Range	Mean	Range	Mean					
Dreßler et al. (3)	97	n.i.	n.i.	n.i.	n.i.	n.i.	Germany	Injured skin	VCAM-I	IC
Dreßler et al. (42)	194	n.i.	n.i.	n.i.	n.i.	n.i.	Germany	Injured skin	VCAM-I, ICAM-1, P-selectin, E-selectin and L-selectin	IC
Betz et al. (43)	74	n.i.	n.i.	n.i.	n.i.	n.i.	Germany	Surgical wounds, lacerations, and stab wounds after surgical treatment.	Collagen types I and VI	IC
Fronczek et al. (44)	101	17–80	37	n.i.	n.i.	52/49	Netherlands	Bruises, abrasions, bites, stabs, scratches, and firework.	Collagen III, collagen IV and α-smooth muscle actin.	IC
Grellner et al. (45)	48	n.i.	45.8	n.i.	n.i.	33/15	Germany	Stab and incised wounds.	IL-1β, IL-6, TNF- α	ELISA
Grellner et al. (46)	105	3–93	51.1	4–192	56.8	n.i.	Germany	Skin wounds are caused by sharp force.	IL-1β, IL-6, TNF- α	IC
Birincioglu et al. (47)	50	10–80	41.12	n.i.	n.i.	44/6	Turkey	Firearms, penetrating trauma by sharp objects and blunt trauma.	IL-1β, IL-6, TNF-α and EGF.	ELISA
Takamiya et al. (48)	121	n.i.	n.i.	n.i.	n.i.	58/63	Japan	Incised wounds, stab wounds, laceration and contusions.	IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ and TNF- α	IC
Kondo et al. (49)	50	7–77	48.8	<72	n.i.	n.i.	Germany	Skin wounds.	IL-8, MCP-1, and MIP-1α	IC
Ishida et al. (50)	53	8–75	40.6	<72	n.i.	n.i.	Germany	Stab wounds, incised wounds, surgical wounds and lacerations.	CD45 and collagen type 1	IC
Ishida et al. (51)	52	8–75	40.6	<72	n.i.	n.i.	Germany	Stab wounds, incised wounds, surgical wounds and lacerations.	CD34/Flk-1	IF
Kuninaka et al. (52)	53	8–75	40.6	0–72	n.i.	n.i.	Germany	Stab wounds, incised wounds, surgical wounds or lacerations.	CD11c and HLA-DRα	IF
Jebur et al. (53)	88	n.i.	n.i.	n.i.	n.i.	n.i.	Irak	Lacerated skin wound.	Tryptase, IL-1 and IL-6	IC
Bonelli et al. (54)	75	10–97	n.i.	n.i.	n.i.	55/20	Italy	Surgical wounds, lacerations and abrasions.	Tryptase and cymase	C

(Continued)

TABLE 3 | Continued

Study	*N*	Age (years)		PI (hours)		Male/Female	Population analyzed	Type of wound	Biomarker analyzed	Analyses technique
		Range	Mean	Range	Mean					
Kondo et al. (55)	40	8–75	40.6	<72	n.i.	28/12	Germany	Stab wounds, incised wounds, surgical wounds and lacerations.	IL-1α	IC
Grellner et al. (4)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	Germany	Skin wounds.	TGF-α, and TGF-β	IC
Yagi et al. (56)	44	0–86	56.2	<72	n.i.	n.i.	Japan	n.i.	CD14	IC
Hayashi et al. (57)	53	8–75	40.6	<72	n.i.	n.i.	Germany	Stab wounds, incised wounds, surgical wounds and lacerations.	VEGF	IC
Ishida et al. (58)	58	8–75	48.6	<72	n.i.	n.i.	Germany	Stab wounds, incised wounds, surgical wounds and lacerations.	ORP150	IC
Ishida et al. (59)	55	7–83	45.8	<72	n.i.	n.i.	Germany	Stab, incised, surgical or laceration wounds	MMP-2 and MMP-9	IC
Ishida et al. (60)	60	7–83	46.5	<72	n.i.	n.i.	Germany	Stab wounds, incised wounds, surgical wounds and lacerations.	Cyclooxygenase-2	IC
Ishida et al. (61)	55	7–83	45.8	<72	n.i.	n.i.	Germany	Stab wounds, incised wounds, surgical wounds or lacerations.	Aquaporin-1 and aquaporin-3	IC
Dachun and Jiazhen (62)	8	n.i.	n.i.	n.i.	n.i.	n.i.	China	Gunshot wounds, lacerations and incisions.	Non-specific esterase	IC
Betz (63)	221	15–94	50	<96	n.i.	148/73	Germany	Lacerations, surgical or stab/cut wounds.	Nonspecific esterases, Acid phosphatase, ATPase, Aminopeptidase and Alkaline phosphatase.	EH

[a]*n, number of individuals or samples.*

PI, post-mortem interval; n.i., not indicated; ISEL, In situ end-labeling of DNA fragments; IC, Immunohistochemical Analysis; IF analysis, Immunofluorescence analysis; C, Cytochemistry analysis; EH, Enzyme histochemical analysis; ELISA, Enzyme-Linked Immunosorbent Assay.

TABLE 4 | Differentiation of vital and post-mortem wound in post-mortem studies[a].

Omic studies	*N*	Age (years)		PI (hours)		Male/Female	Population analyzed	Type of wound	Biomarker analyzed	Analyses technique
		Range	Mean	Range	Mean					
Genomic data										
Grellner and Benecke (64)	24	n.i.	47.8	n.i.	n.i.	20/4	Germany	Strangulation marks	DNA	q-DNA
Transcriptomic data										
Xu et al. (65)	6	26–52	37.7	28–46	35.5	3/3	China	Traumatic injuries of traffic accident	Cxcl1, Jun, Fos, IL-6, and Sfrp2.	RT-qPCR
Ye et al. (66)	21	18–56	31.2	31–72	52.4	11/10	China	Traumatic injuries of traffic accident.	IL-6 and IL-20	RT-qPCR
Qu et al. (67)	6	27–60	41.5	39–96	64.2	3/3	China	Traumatic injuries	ATF3 and BTG2	RT-qPCR and WB.
He et al. (68)	5	n.i.	n.i.	n.i.	n.i.	n.i.	China	Traumatic injuries of traffic accident	CXCL1 and CXCR2	RT-qPCR and WB.
Liapi et al. (69)	19	n.i.	n.i.	n.i.	n.i.	n.i.	Germany	Stab wounds.	GAPDH, PGK1, YWHAZ and PPIA.	RT-qPCR
Neri et al. (70)	64	n.i.	n.i.	12–24	n.i.	n.i.	Italy	Ligature marks of suicidal hanging.	miRNA 92a-3p, 125a-5p, 214-3p, 125b-5p, 103a-3p	RT-qPCR
Kubo et al. (71)	48	20–88	63.1	<72	n.i.	26/22	Japan	Burned skins, abrasion and bruise skins.	Aquaporin-3	RT-qPCR
Proteomic data										
Ishida et al. (72)	56	0–89	56.1	8–72	30.5	33/23	Japan	Ligature marks of suicidal hanging and strangulation.	Aquaporin-1 and aquaporin-3	IC
Prangenberg et al. (73)	30	19–95	54.6	n.i.	n.i.	19/11	Germany	Dried skin abrasions, frost erythema, laceration, stab wound, gunshot wound, and strangulation mark.	Aquaporin-1 and aquaporin-3	IC
Bonelli et al. (74)	20	22–79	48.3	n.i.	n.i.	19/1	Italy	Surgical wounds, lacerations and abrasions.	Tryptase and cymase	IC
Oehmichen et al. (75)	64	6–71	36.75	<48	n.i.	35/29	Germany	n.i.	Tryptase and esterase NAS-DCIAE	IC

(Continued)

TABLE 4 | Continued

Omic studies	*N*	Age (years)		PI (hours)		Male/Female	Population analyzed	Type of wound	Biomarker analyzed	Analyses technique
		Range	Mean	Range	Mean					
Turillazzi et al. (76)	70	20–50	29.16	n.i.	n.i.	40/30	Italy and Spain	Ligature marks of suicidal hanging.	Tryptase, CD15 and IL-15.	IC
Gauchotte et al. (77)	92	n.i.	n.i.	n.i.	n.i.	n.i.	France	Stab.	FVIII, CD15 and tryptase	IC
Legaz et al. (78)	15	21–47	33.6	19–36	n.i.	12/3	Spain	Ligature mark of suicidal hanging.	Fibronectin, cathepsin-D, and P-selectine.	IC
Hernández-Cueto et al. (79)	53	14–82	36.7	n.i.	n.i.	46/7	Germany	Incision wounds.	Cathepsin D	EH
Montisci et al. (80)	40	36–80	58.48	n.i.	n.i.	25/15	Italy	Skin fragmens.	Cathepsin-D	IC
Ortiz-Rey et al. (81)	24	35–76	57.1	n.i.	n.i.	13/11	Spain	Surgical incisions.	P-selectin	IC
Ortiz-Rey et al. (82)	48	33–76	59.2	2–16	7.7	27/21	Spain	Incised wounds	Fibronectin and tenascin	IC
Legaz Pérez et al. (83)	71	12–82	35.7	19–36	n.i.	61/10	Spain	Ligature mark of suicidal hanging	Fe, Zn, Mg, Ca, P-selectin, and cathepsin D.	IC
Yu-Chuan et al. (84)	14	20–45	n.i.	24–72	n.i.	12/2	China	Lacerations, contusions and stab wounds.	Fe, Zn, Mg, Cu, K, and Na	AAS
Ali (85)	19	n.i.	n.i.	n.i.	n.i.	n.i.	UK	Ligature marks, electric marks, edges of stab wounds, burns, and bruises.	Collagen	H
He and Zhu (86)	7	10–65	29	4–24	13,29	5/2	China	Lacerations and skin incisions.	LTB_4	HPLC
Bacci et al. (87)	40	19–79	n.i.	n.i.	n.i.	30/10	Italy	Skin lesions	TNF-α	IC
Peyron et al. (88)	24	n.i.	n.i.	n.i.	n.i.	n.i.	France	Lacerations, stab wounds and gunshot wounds.	IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13,TNF-α.	MSI
Kimura et al. (89)	4	16–61	39.5	≤24	24	3/1	Japan	Compression mark, stab wound, and ligature mark.	LC3-II and p62	WB
Bacci et al. (90)	80	3–86	50	24–48	n.i.	56/24	Italy	Car accident, fall, homicide, or hanging	MHC-II and CD1a	IC
Focardi et al. (91)	20	3–89	n.i.	24–48	41	14/6	Italy	Hanging mark wounds.	MHC-II and CD1a	IC

[a] *n, number of individuals or samples.*

PI, post-mortem interval; n.i., not indicated; q-DNA analysis, quantitative DNA analysis; IC analysis, Immunohistochemical Analysis; EH analysis, Enzyme histochemical analysis; WB, western blot; AAS, Atomic absorption spectrometry; H analysis, Histological analysis; HPLC, liquid chromatography; MSI, Multiplex sandwich immunoassay.

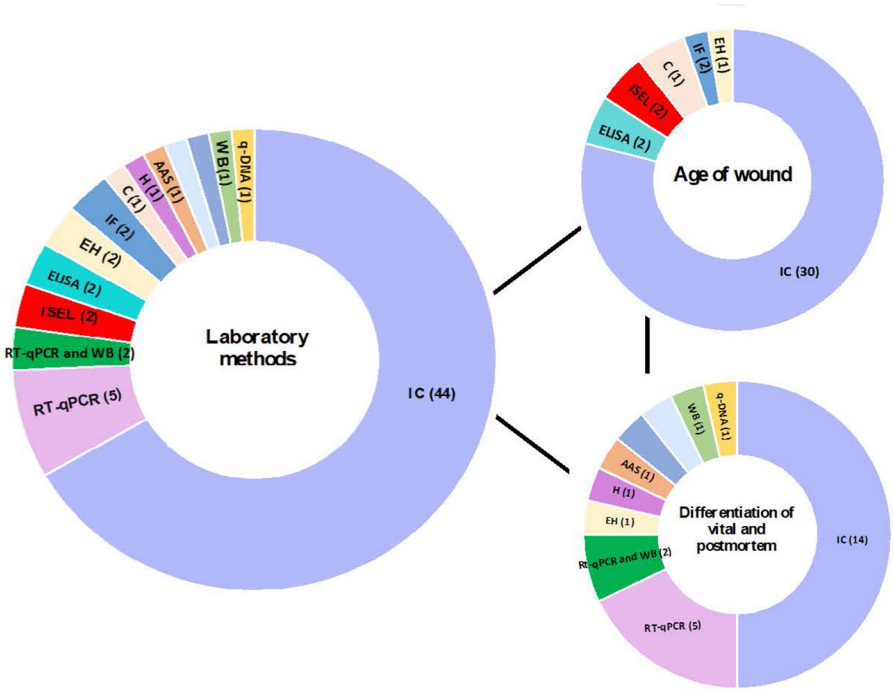

FIGURE 3 | Contribution of different laboratory techniques to the analysis of age of wound and differentiation of vital and post-mortem wound. q-DNA analysis, quantitative DNA analysis; IC analysis, Immunohistochemical Analysis; EH analysis, Enzyme histochemical analysis; WB, western blot; AAS, Atomic absorption spectrometry; H analysis, Histological analysis; HPLC, liquid chromatography; MSI, Multiplex sandwich immunoassay.

wound area. They analyzed gunshot wounds, blunt injury, and sharp injury, showing no relationship between the type of wound and the determination of age by tenascin and ubiquitin. They found a positive correlation between the number of positive cells for both markers and the age of the wound. Tenascin was positive in 91.8% of the cases with a wound age $>$ 24 h and negative in 98.3% of the cases with a wound age $<$ 24 h. In contrast, ubiquitin was positive in 4.25% of the cases with a wound age $<$ 24 h and in 26.14% of the cases with a wound age $>$ 24 h. When the wound age was $>$ 40 days, the fibroblasts still expressed ubiquitin, but not tenascin.

Kondo et al. (37) found significant differences in the expression of ubiquitin between different age groups of the wound, the wounds between 7 and 14 days old showed the highest expression of ubiquitin, decreasing this from day 17. Other authors (38) examined lesions in the neck, indicating that an accumulation of myoglobin indicates a lesion with a survival time of a few minutes. In contrast, if fibronectin is also detected in the same lesion, the post-infliction interval is several minutes. Positive C5b-9 reactions indicate that death did not occur during strangulation but occurred afterward.

Different investigations of Dreßler et al. (3, 39–42) analyzed the expression of different adhesion molecules: ICAM-1, VCAM-1 and selectins. VCAM-1, ICAM-1, P-selectin, and E-selectin are endothelial adhesion molecules whose expression requires activation by lipopolysaccharides and cytokines, especially IL-1β and TNF-α, which are released in the wound healing process. Therefore, these molecules are essential in the inflammation phase of the wound. Immunohistochemical investigation did not reveal strong expression of ICAM-1 by endothelial cells and keratinocytes until approximately a minimum of 1.5 h after injury and up to a maximum of 3.5 days, the intensity of VCAM-1 increased with the increasing number of blood vessels, observing a strong intensity 3 h after the infliction of the wound and up to 3.5 days later. On the other hand, they also determined that L-selectin is not valid for estimating age, while P-selectin was found in an interval between 3 min and 7 h after injury, and E-selectin was between 1 h and 17 days.

Two more studies look at collagen to estimate the age of the wound. On the one hand, Betz et al. (43) state that network structures that react positively for type I or VI collagen indicate a wound age of at least 5–6 days and 3 days, respectively. On the contrary, Fronczek et al. (44) studied the presence of type I collagen in blood vessels for wound estimation, instead of network structures, in addition to the presence of type III, IV, and Alpha-smooth muscle actin collagen.

Two studies (45, 46) analyzed IL-1β, IL-6, and TNF-α by ELISA and immunohistochemical techniques, respectively, obtaining more precise results with immunohistochemical techniques. The three proinflammatory cytokines proved to be useful markers for determining a lesion onset interval of up to a few hours. The first increase in reactivity could be noted for IL-1β, IL-6, and TNF-α, almost simultaneously after 15 $\pm$ 20 min, their expression changing after $\sim$1 $\pm$ 1.5 h.

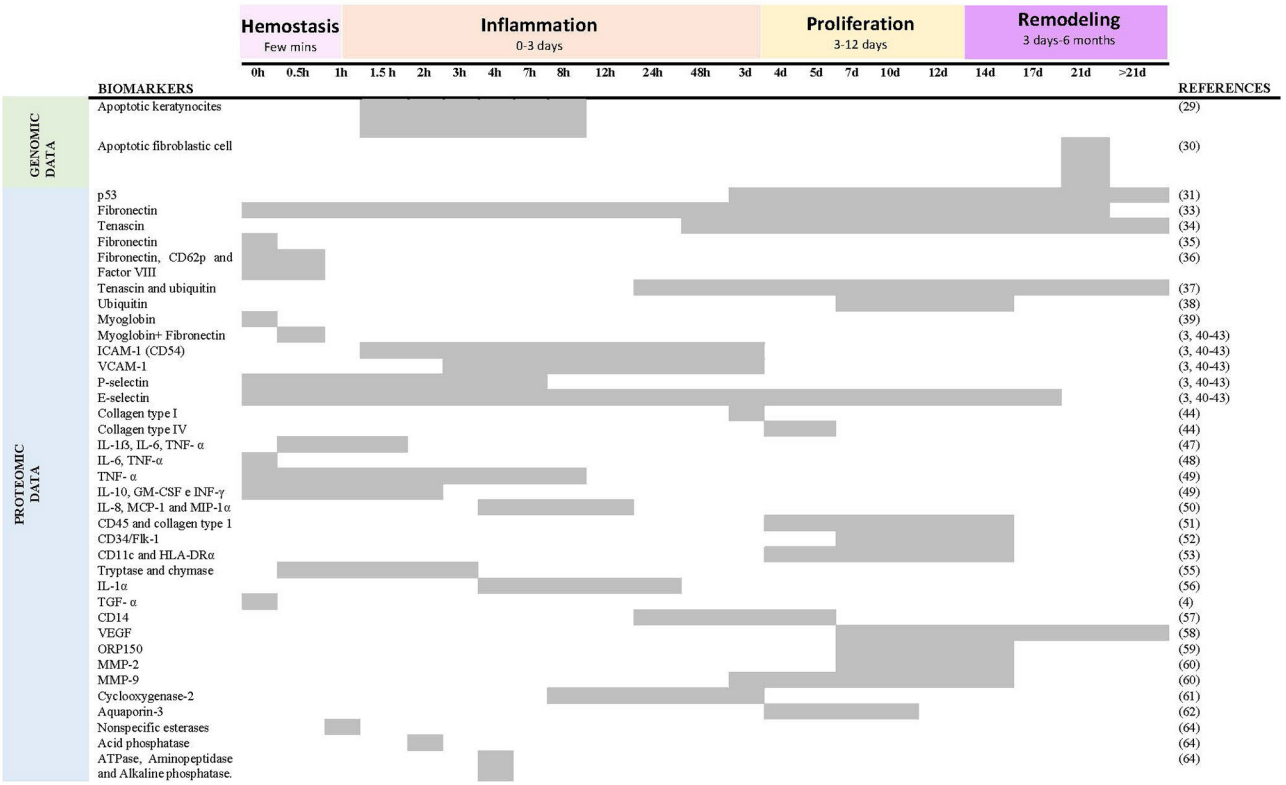

FIGURE 4 | Summary of reactivity of age biomarkers respect to time after skin injury. Five studies (31, 44, 45, 53, 62) were excluded because they do not place the appearance and/or quantification of a marker on a timeline about the age of the wound.

A more recent study (47) indicates IL-6 and TNF-α as early phase markers indicating a wound age of <30 min. However, they consider that the usefulness of IL-1β and EGF should be reevaluated.

In correlation with the previous studies, another study (48) showed a significant increase in TNF-α in survival times of <30 min. IL-8, the most abundant cytokine in this study, has been shown to originate from keratinocytes, fibroblasts, endothelial cells, and neutrophils. In addition, IL 8 proliferates keratinocytes and acts as a potent chemokine for neutrophils and lymphocytes. IL-2 promotes T lymphocyte proliferation and interacts with IFN-γ in the production of IL-8 mRNA in keratinocytes. On the other hand, IL-4 is believed to proliferate fibroblasts. Significant expressions of IL-6, IL-8, IFN-γ, and TNF-α have significant effects on dermal wound healing.

A sample of 50 wounds of different ages from autopsies observed polymorphonuclear cells with positive reactions to IL-8 and the inflammatory proteins MCP-1 and MIP-1alpha in wounds of 4–12 h (49).

Fibrocytes are mesenchymal progenitors that co-express cell antigens and fibroblast products such as CD45 and type I collagen and are involved in tissue repair after injury. Ishida et al. (50) consider that the determination of fibrocytes from the CD45 and type I collagen markers have great precision and objectivity when estimating the age of a wound. They demonstrated the appearance of fibrocytes in human skin wounds with a wound age of at least 4 days, suggesting a number of fibrocytes > 15 and a wound age of 9–14 days. After demonstrating the usefulness of fibrocytes in determining wound age, a study by the same authors (51) investigated the utility of endothelial precursor cells (EPC) that contribute to vasculogenesis, a process essential for the survival of growing, injured and ischemic tissue. Thus, after analyzing 52 skin wounds from autopsies using immunofluorescence analysis, they determined that the number of CPE was significantly high in wounds between 7 and 14 days with more than 20 CPE in most cases, while it decreased below 15 CPE in wounds of more than 14 days. Third, these researchers (52) hypothesized that dendritic cells (DCs) were closely related to the onset of the immune response after injury, and again by immunofluorescence, they determined that a count > 50 DC in a wound sample would indicate an age of same between 4 and 14 days.

A more recent study (53) studied tryptase as a marker of mast cell activation as a factor of wound vitality and two proinflammatory cytokines (IL-1 and IL-6 to determine the age of the wound. Using immunohistochemical techniques, they found a positive correlation between infiltration with mast cells (mast cells tryptase) and the passage of time.

A total of 75 vital skin lesions were examined by Bonelli et al. (54). Their investigations demonstrated a progressive and

significant increase in the number of neutrophils with the time elapsed between injury and death. Mast cell density progressively increased to a maximum in vital injuries between 1 and 3 h before death, decreasing after that.

On the other hand, a group of authors (55) stated that in six out of ten wounds aged between 4 h and 1 day, the proportions of infiltrating cells positive for IL-1α were higher than 30%, being lower than the said percentage in wounds between 1.5 and 21 days.

The stimulation and regulation of angiogenesis are among the most important effects of TGF-α in the wound healing process. At the same time, TGF-β1 has chemotactic activity and promotes extracellular matrix synthesis (collagen, fibronectin, tenascin), present in the three phases of healing. Grellner et al. (4) observed an increase in TGF-α reactivity from a wound age of 10–20 min, especially in the middle epidermal areas of the spinous layer. Otherwise, TGF-β1 was detected in all phases of wound repair, except for the lesions with the shortest survival time.

Another study (56) indicated that CD14 might be a helpful marker for estimating wound age between 1 and 5 days in forensic practice.

In cutaneous wound healing, the vascular endothelial growth factor (VEGF) plays a vital role as it is a crucial angiogenic factor for forming new granulation tissue in the proliferative phase. Some authors (57) suggest that a VEGF positive ratio $>$ 50% indicates a wound age $>$ 7 days.

Overexpression of the ORP150 gene by adenovirus vectors has accelerated wound healing by modulating VEGF (14). Ishida et al. (58) suggested that a positive ORP150 ratio $>$ 50% indicates a wound age of 7–14 days.

In addition to growth factors, cytokines, and adhesion molecules, wound healing processes also involve matrix metalloproteases (MMPs). MMP-2 and MMP-9 bind gelatin, collagens, and laminin, with MMP-9 participating in the epithelialization process and early repair events, while MMP-2 plays a key role in the prolonged remodeling stage. A study (59) has shown that probably several MMP-2 positive macrophages $>$ 20 indicate a wound age of between 7 and 12 days, while many MMP9- $+$ cells $>$ 30 would indicate a wound age between 3 and 14 days. Therefore, MMP-2 and MMP-9 would be useful markers of the proliferative phase of skin wound healing. On the other hand, IL-1 could positively regulate the gene expression of these two metalloproteases. One more study by Ishida et al. (60) suggested that a positive COX-2 ratio $>$ 40% indicates a wound age of 8 h−3 days. The same authors (61) immunohistochemically examined the expression of AQP-1 and AQP-3 in human skin wounds by showing their participation in the migration of keratinocytes and endothelial cells, among others. Thus, they determined that a number of AQP-3 $+$ cells $>$ 300 possibly indicates a wound age of between 5 and 10 days.

Two studies analyze non-specific esterases for determining the age of the wound. On the one hand, some authors (62) quantified non-specific esterase (NSE) in injured skin using a microspectrophotometric scanning technique, indicating that it applied to medico-legal practice for determining the age of the wound. On the other hand, another study (63) showed that NSE activity increased ∼1 h after injury, followed by changes in acid phosphatase at ∼2 h, and changes in aminopeptidase, ATPase, and alkaline phosphatase activity at ∼4 h, changes that did not they were evidenced in post-mortem wounds.

Differentiation of Vital and Post-mortem Wounds

To assess the survival time from wound age estimation, the forensic pathologist must differentiate antemortem from post-mortem wounds. In this field, scientists investigate relevant markers of vital origin (22). Therefore, 28 articles have been selected that analyze different vitality markers to differentiate between injuries before and after death (**Table 4**).

Grellner and Benecke (64) used a genomic technique such as DNA quantification to analyze strangulation marks, concluding that quantitative changes in the DNA content of the grooves are not significant as a sign of vitality in strangulation.

On the other hand, seven studies analyzed transcriptomic data using the RT-qPCR technique. A study (65) discussed the possibility of using this technique to reveal differentially expressed genes (DEGs) as possible markers of vital reactions. They evaluated the results by studying five DEGs in wounds from human autopsies, observing how RNA expression levels of Cxcl1, Jun, Fos, and IL-6 increased in post-mortem human skin wounds compared to intact skin the Sfrp2 expression.

Another study (66) investigated IL-6 and IL-20 mRNA expression in mouse and human skin wounds. In animals, they found that the expression of IL-6 and IL-20 was more regulated in the contused area of the skin than in intact skin and post-mortem bruised skin. These results were validated by examining post-mortem human skin tissues, in which they were level. IL-6 and IL-20 mRNA were significantly higher in injured regions compared to intact ones.

Qu et al. (67) also analyzed the expression levels of ATF3 and BTG2 in human and mouse skin wounds. The protein levels examined by western blot showed no changes in the expression levels of both proteins between wounded and intact skins. However, the mRNA levels demonstrated a higher ATF3 and BTG2 in mouse skins with an antemortem contusion than intact skin and with post-mortem contusion. In human skin samples from forensic autopsies, increased levels of ATF3 mRNA were detected up to 48 h after the autopsy, but no differences were found between injured and intact skin for BTG2. ATF3 can be considered a potential marker for a vital skin contusion reaction, but BTG2 cannot.

Another study (68) analyzed mRNA levels in skin wounds in mice and humans, in this case, CXCL1 and CXCR2 proteins. As in the previous study, the western blot analysis of protein levels did not show differences between wounded and intact skin. The mRNA levels demonstrated higher CXCL1 and CXCR2 in bruised mouse and human skin compared to intact skin.

Liapi et al. (69) examined the effect of RNA integrity on reference gene expression stability for future normalization of relative qPCR data from intact skin and post-mortem wounds. Thus, GAPDH and PGK1 were classified as two reference genes stably expressed in post-mortem skin tissues, while YWHAZ and

PPIA increased the variation in gene expression, so they should be excluded as reference genes.

Other authors (70) demonstrated an increase in the expression of different miRNAs recognized as regulators of the inflammatory response in skin lesions in wounded skin from people who died by hanging compared to healthy skin. Their data confirm that miRNA expression in traumatic skin wounds is related to an act of regulation of the inflammatory phase aimed at inhibiting intracellular signals activated by the production of inflammatory cytokines, even in cases of lesions that develop in a short time.

Another study (71) observed that, both in animal models and in cases of human autopsies, there was a significant difference in the expression of the AQP3 gene between pre and post-mortem burned skin. They, therefore, suggested that the expression of the dermal gene AQP3 was increased to maintain water homeostasis in response to dehydration from burns.

Twenty studies analyzed proteomic data to study vital markers in the differentiation of vital and post-mortem wounds. Ishida et al. (72) studied the expression of aquaporins AQP1 and AQP3 in suicide hanging and strangulation ligation marks using immunohistochemical techniques. The authors found no difference in AQP1 expression between compressed neck skin and uninjured skin. However, they observed that AQP3 was expressed in antemortem ligation mark keratinocytes obtained from forensic cases' autopsies compared to intact skin. Other authors (73) also analyzed AQP1 and AQP3 in strangulation marks and examined thermal injuries, gunshot wounds, and frostbite erythema. Like the previous ones, they did not find significant differences between injured and non-injured skin about the expression of AQP1. However, they did find a higher expression of AQP3 in epidermal keratinocytes in all types of lesions.

Bonelli et al. (74) determined that the mast cell density (positive tryptase and cymase) in vital lesions, observing that it was significantly higher in healthy controls and in post-mortem lesions.

Another study (75) analyzed skin wounds from 64 human cadavers to determine whether skin mast cells are activated during the very early phase of human wound healing. He compared the number of tryptase-reactive mast cells, which do not lose all their enzymatic activity during the degranulation process, with the number of naphthol AS-D chloroacetate esterase (NAS-DCIAE) positive mast cells, which lose their total enzymatic activity. In victims who survived the injury for $<$60 min, the average number of NAS-DCIAE-reactive mast cells along the wound margin was significantly lower than the number of tryptase-reactive mast cells. The findings of this study show that mast cells experience a very early loss of NAS-DCIAE activity at wound margins; thus, it appears to be an early cellular marker of wound survival.

On the other hand, Turillazzi et al. (76) investigated the immunohistochemical expression of a panel of cytokines and inflammatory cells in skin samples from an autopsy of hanging cases to evaluate whether the mark and signs of hanging occurred before or after the death of the victim. They conclude that tryptase and IL-15 can complement the determination of the vitality of CD15-based ligation marks with the precision necessary for forensic purposes. Another study (77) affirmed the usefulness of CD15 and tryptase as markers to differentiate recent antemortem wounds from post-mortem ones; however, they denied the usefulness of FVIIIra as a vitality marker.

Legaz et al. (78) show an increase in fibronectin and cathepsin-D immunoreactivity and a decrease in P-Selectin in skin wounds from marks from suicide hangings with a post-mortem interval of 19–36 h. However, they state that a limitation of their study could be that the samples were not collected at the time of death, which could influence the immunoreactivity of the proteins studied. Another study (79) demonstrated the usefulness of cathepsin-D as a wound vitality marker. However, Montisci et al. (80) found high levels of cathepsin-D in post-mortem lesions compared to vital wounds collected from living subjects, thus ruling out any usefulness of histochemical quantification of this enzyme for the differentiation between vital and post-mortem lesions. Regarding Selectin-P, some authors (81) found no significant differences in the P-selectin immunoreactivity analysis between vital and post-mortem skin wounds, for which they state that P-selectin is not a specific marker of vital lesions. In a previous study (82), these same authors studied the expression of fibronectin and tenascin in 48 vital wounds and ten post-mortem wounds. They observed a lattice staining for fibronectin at the wound edge and in the dermis of 50% of vital samples, compared to 0% of post-mortem samples, while tenascin was negative in all samples. In contrast, the vital bleeding and post-mortem areas showed positivity for fibronectin and tenascin, so they cannot be considered helpful vitality markers.

Legaz Pérez et al. (83) determined that Fe and Zn concentrations were significantly higher in injured skin from suicide-hanging ligation marks than healthy skin. Furthermore, Ca and Zn decreased, while Fe increased with the severity of the neck injury. On the other hand, they observed a high percentage of negative and moderate expression of cathepsin D and selectin P in damaged skin, correlated with a low iron concentration. Based on these data, the study of proteins and metal ions can be helpful in the characterization and differentiation of injured and uninjured skin. Previously, other authors (84) studied the diagnostic value of ions to differentiate antemortem and post-mortem wounds using atomic absorption spectrophotometry. They found higher Fe concentrations in the skin and muscle of antemortem wounds compared to the control, while the K/Na ratio concentrations were significantly reduced in the antemortem wounds compared to the controls. For this reason, the Fe concentration and the K/Na ratio can be helpful for the differentiation of wounds produced before and after death.

Ali (85) found that altered collagen can also be formed after death, when post-mortem injuries occur, probably due to the physical-chemical changes that collagen fibers undergo, so the presence of altered collagen is not necessarily a sign of vitality.

Other authors (86) detected by HPLC an inflammation mediator, LTB_4, in an antemortem wound but did not detect it in post-mortem samples. Therefore, their results suggest the detection of LTB_4 as a useful method to distinguish between antemortem and post-mortem wounds.

Bacci et al. (87) found that the number of mast cells stained for TNF-α increased progressively and significantly over time

and became significantly different from controls when the time elapsed after injury was more than 15 min. Furthermore, the post-mortem samples had significantly fewer mast cells and fewer TNF-α positive cells than the antemortem and control sample groups.

Another more recent study (88) used multiplex sandwich immunoassay to analyze different cytokines to discriminate between vital and post-mortem wounds. Cytokine levels (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α) were significantly higher in vital wounds than post-mortem, except for IFN - γ and IL-2. IL-8 was the cytokine that showed the best results for wound differentiation.

Kimura et al. (89) investigated autophagy in human and mouse skin wounds using western blotting. They found a marked reduction in LC3-II and an increase in p62 in antemortem wounds, both human and mouse, with a post-infection interval greater than or equal to half an hour, compared to non-injured skin. However, there were no notable changes in LC3-II and p62 levels in post-mortem wounds.

Finally, two studies (90, 91) suggest the usefulness of the markers CD1a and MHC-II (dendritic cells and Langerhans cells) distinguish between vital and post-mortem injuries, as well as to estimate the interval between injury and death.

FUTURE CHALLENGES IN THE CHARACTERIZATION OF HUMAN WOUNDS IN FORENSIC SCIENCES

In this systematic review, the main results were obtained from studies that attempt to relate different biomarkers with the characterization of wounds, both for estimating the age and for the differentiation between vital and post-mortem wounds. Together, these studies evaluate the potential and limitations of the different biomarkers analyzed for their future use as a forensic tool.

In most of the studies analyzed, immunohistochemistry is the primary method of choice, the basis of this method being the immunological reaction between an antigen present in the study tissue and an applied antibody (6). This method serves to detect vitality and wound markers at the protein level. However, in the review, we have also seen more recent studies that analyze the earliest stage of a reaction at the mRNA level. These are morphological methods such as *in situ* hybridization and molecular biology techniques such as RT-qPCR.

MicroRNAs (miRNAs) are promising biomarkers in forensic sciences because of their small size and their value for degraded or complex samples, which are very common in this field (12). In the articles reviewed in this systematic review, only one analyzed miRNAs in forensic samples, which suggests that the analysis of miRNAs in human samples for the study and characterization of vital wounds is novel and requires more research.

Approximately 30 years have passed since the publication of the first research analyzed in this review, but the molecular characterization of vital wounds remains a topical issue. Estimating the age of vital wounds and the differentiation of vital and post-mortem wounds require further investigation of both biomarkers to analyze and new molecular biology techniques that allow the detection of earlier stages of reactions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

ACR and IL participated in designing the review supervising the data generation, analyzing the data, and writing the manuscript. AL, IL, and SB participated in data generation, organization, writing, and manuscript discussion. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Mini Review: The Forensic Value of Heat Shock Proteins

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Forensic pathologists are routinely confronted with unclear causes of death or related findings. In some instances, difficulties arise in relation to questions posed by criminal investigators or prosecutors. Such scenarios may include questions about wound vitality or cause of death where typical or landmark findings are difficult to ascertain. In addition to the usual examinations required to clarify unclear causes of death or address specific questions, immunohistochemistry and genetic analyses have become increasingly important techniques in this area since their establishment last century. Since then, many studies have determined the usefulness and significance of immunohistochemical and genetic investigations on cellular structures and proteins. For example, these proteins include heat shock proteins (Hsp), which were first described in 1962 and are so called based on their molecular weight. They predominantly act as molecular chaperones with cytoprotective functions that support cell survival under (sub) lethal conditions. They are expressed in specific cellular compartments and have many divergent functions. Central family members include, Hsp 27, 60, and 70. This mini review investigates recent research on the Hsp family, their application range, respective forensic importance, and current limitations and provides an outlook on possible applications within forensic science.

Keywords: heat shock proteins, immunohistochemistry, gene expression, forensics, legal medicine

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INTRODUCTION

Molecular chaperones are present in all cells and compartments of the body and contribute to protein biosynthesis (1–3), newly synthesized protein folding, and transport to sites of action (2, 4). Heat shock proteins (Hsps) are one such family of chaperones; they are highly conserved, and their expression is increased after heat exposure (5). Increased protein expression is also linked to responses to various stimuli, including hypo- or hyperthermal conditions, oxidative stress, energy deficiency, or ischemia (6–9). Hsp nomenclature refers to the respective molecular weight; therefore, Hsp70 weighs 70 kDa. Well-known family members include, Hsp27, Hsp60, and Hsp70, which are expressed in different cellular compartments with different functions. In forensic science, Hsp70 has been used as a marker of cellular stress upon heat exposure of burn victims and in tissues of the upper respiratory tract, lungs, and kidneys (10, 11). However, detailed studies on Hsp expression in other forensically relevant areas are scarce. Therefore, this mini review addresses current immunohistochemical research on Hsps, their scope, respective forensic relevance, and current limitations. Finally, the review provides an outlook on potential future applications.

MATERIALS AND METHODS

We reviewed Medline (https://pubmed.ncbi.nlm.nih.gov) for studies published between January 1, 2000 and the September 30, 2021, for Hsp research in a forensic context. For methodology and reporting, we used the updated 2020 Preferred Reporting Items for Systematic Reviews and Meta-Analysis guidelines (12). The words "Heat Shock Proteins" and "Forensic" were used to identify studies examining Hsp research in a forensic context. The following Medical Subject Heading combination terms and Boolean operators were applied during our search: "Heat Shock Proteins" AND "Forensic." Bibliographies of selected articles were manually reviewed for further studies. Two authors (J.P. and A.M.) independently conducted eligibility assessments and managed data extraction. Only original research articles on human specimens published in English or German were considered for review. Article eligibility was determined based on the screening of titles and abstracts.

RESULTS

The initial search identified 126 studies. After screening titles, 44 studies remained for further review. After reviewing abstracts and checking for availability in English or German, 27 studies were further excluded. After manually searching bibliographies, three additional articles that matched the criteria were captured. The search eventually identified 20 eligible studies conducted between 2000 and 2021. Thus, over this period, an average of 1.11 Hsp forensic studies were published per year. Most studies were published in the years, 2006, 2012–2014, and 2017 (10%, $n = 2$ each). A small majority of studies addressed fire, hypothermia, and sudden infant death syndrome (SIDS)/peripartum deaths (15%, $n = 3$ each), followed by cardiac deaths, traumatic injuries, excited delirium (ED), and drowning (10%, $n = 2$ each). Three other studies examined acute lung injury, Hsp detection in formalin-fixed human brain tissue, and methamphetamine intoxication. One study addressed traumatic injury, asphyxia, and sudden cardiac death.

Hsp70 was the most frequent study subject (95%, $n = 19$), followed by Hsp27 (25%, $n = 5$). Hsp60 and Hsp90 were investigated in two studies each. In addition, single studies dealt with Hsp72 and Hsp110. Approximately one third (30%, $n = 6$) of the selected studies were published in the International Journal of Legal Medicine, and one quarter (25%, $n = 5$) were published in Forensic Science International. Fourteen studies used a two-group design, five used multiple-group designs, and one used a one-group design. All studies had a combined total of 1,223 study specimens and 1,178 control specimens. An overview of the identified heat shock proteins, their coding genes and their respective potential applications is shown in **Table 1**.

DISCUSSION

Cardiac Death

A polymorphic study on the HSPA1B gene (rs3036297), encoding Hsp70 (member 1B), in the Chinese population reported that individuals with an insertion allele had a comparatively lower risk of sudden cardiac death compared with individuals with a deletion allele. Thus, it was hypothesized that the rs3036297 variant regulated HSPA1B expression *via* microRNA binding and HLA-DRB5 expression *via* long-range promoter interactions, thereby contributing to a susceptibility to sudden cardiac death. Therefore, rs3036297 is a potential marker for the molecular diagnostics and genetic counseling of sudden cardiac death (13).

In blood samples collected up to the first day after an acute myocardial infarction and analyzed by enzyme-linked immunosorbent assay, Hsp70 levels were reportedly twice as high as in control patients with angina. Moreover, peak Hsp70 levels 6 h after infarction correlated significantly with creatine kinase and cardiac troponin T levels, as well as with interleukin-6 (IL-6) and IL-8. Thus, circulating Hsp70 could be a suitable marker of myocardial damage and may play a role in inflammatory responses after acute myocardial infarction (14).

Fire

Hsp70 was identified as a reliable marker for the antemortem impact of fire or hot vapor inhalation. Protein expression was significantly increased in the epiglottis, trachea, main bronchi, and peripheral bronchi of burn victims compared with control cases. In this regard, Hsp70 expression was particularly evident not only in blood vessels but also in seromucosal secretory cells, ciliated epithelial cells, smooth muscle cells, and alveolar cells, suggesting a (supra-) vital response to hot vapor inhalation (10).

Another study confirmed a general tendency for high Hsp27 and Hsp70 expression levels in lung tissue, particularly in central airways, renal vasculature, and renal tubule cells, in fire fatalities. However, no differences in Hsp expression were observed depending on the burn degree. Therefore, Hsp27 and Hsp70 expression, particularly in lung and kidney tissues, may be used to determine vitality in fire or heat death. In particular, absent or low-level expression may have indicated the deceased was likely subjected to heat after death (11).

Furthermore, different Hsps may be used to estimate survival time since Hsp27 is expressed within seconds or minutes of a stressful exposure and in large amounts to protect cells, whereas Hsp70 takes up to 1 h to reach optimal expression levels (15).

Hypothermia

In a study by Preuss et al. in 2008, approximately one fifth of fatal cases of hypothermia showed varying levels of Hsp70 expression in tubular epithelial cells and glomeruli in renal tissue, whereas the majority of control cases showed no or low Hsp70 expression. However, Hsp70 expression did not show a strong correlation with Wischnewski's spots (16).

A subsequent 2013 study did not confirm the findings that expression of Hsp70 is absent in control groups. Moreover, Hsp70 in glomerular podocytes indicated that expression was predominantly in the nucleolus, which appeared to be characteristic of hypothermia deaths. Therefore, analysis of Hsp70 expression patterns in glomeruli is potentially useful in forensic diagnostics to determine whether the ambient temperature was antemortem-low. A combination of immunohistochemical and real-time polymerase chain reaction (RT-PCR) studies also showed that Hsp70 was rapidly

TABLE 1 | Overview of mentioned heat shock proteins and their respective potential applications.

Heat shock protein	Coding gene	Potential field of application
27	HSPB	Perinatal hypoxia/ischemia, fire deaths, hypothermia, SIDS
60	HSPD1	Traumatic injury, mechanical asphyxiation, sudden cardiac death
70	HSPA1A, A1B, A2, A7, A8, A12B and A13 (Hsp70 member 1A, 1B, 2, 7, 12B, and 13, respectively)	Drowning, drug abuse, myocardial ischemia and sudden cardiac death, fire deaths, hypothermia, excited delirium, traumatic injury, acute lung injury, mechanical asphyxiation, SIDS, formalin fixation
90	HSP90A and HSP90B	Perinatal hypoxia/ischemia, traumatic injury, asphyxiation, sudden cardiac death
110	HSPH1	Traumatic injury, asphyxiation, sudden cardiac death

translocated to podocyte nuclei after cold exposure without new protein biosynthesis (17).

Besides renal tissue, pituitary gland showed slightly increased Hsp27 expression levels in hypothermia death cases compared with control cases, whereas Hsp70 showed no expression in either group. However, to identify hypothermia deaths, it is more appropriate to assess fatty degeneration by Sudan staining, which is used to assess hypothermia in almost half of the cases (18).

Trauma

During traumatic injury to the frontal cortex, HSPA12B, the gene encoding HSP family A (Hsp70, member 12B), appears to be downregulated. Moreover, the combination of HSPA12B and FOSB gene expression diagnostically distinguished traumatic brain injury from control cases (mainly fatal cardiac events) (19). Additionally, HSPA7 and A13 gene transcripts appeared to be much higher in cases of traumatic injury than cases of mechanical asphyxia and sudden cardiac death (20).

SIDS/Peripartum Deaths

Based on the hypothesis that SIDS may be associated with a decreased ability to respond to external stressors, a PCR analysis of Achilles tendon samples from SIDS cases indicated that HSPA1B (Hsp70) and HSPD1 (Hsp60) expression was increased in response to thermal stress. Furthermore, in SIDS cases where the infant was found in a prone position, lower HSPA1B expression was detected compared with cases where the infant was found on the side or the back (21). In contrast, an immunohistochemical study investigating the role of hyperthermia as a pathogenic factor for SIDS and Hsp27 expression analysis in the kidney, heart, and lung tissue revealed no meaningful differences between SIDS and control cases. Hsp70 was consistently negative in both groups and examined tissues (22).

An immunohistochemical study of brain and brainstem sections from 47 peripartum deaths showed increased Hsp70 and Hsp90 responses in the cytoplasm of neurons in non-acute cases of hypoxic-ischemic insults, whereas only mild responses were observed in isolated fields in acute cases. These observations could indicate that Hsp70 and Hsp90 are strongly expressed as later reactions in neurons (23).

Excited Delirium

ED is one of several terms describing a syndrome characterized by delirium, agitation, and combativeness. HSPA1B transcript (Hsp70) expression was increased 1.8- to 4-fold in post-mortem brain tissue samples of ED cases. The mean core body temperature of cases, when recorded, was 40.7°C. Elevated Hsp70 levels in autopsy brain specimens may be considered to confirm that hyperthermia was an associated symptom, and often a forerunner, of death in these cases. Thus, a two-protein biomarker signature (in this study, HSPA1B and dopamine transporter levels) may serve as reliable forensic tools to identify ED at autopsy (24).

A later study addressing Mash et al. (24), quantified HSPA1A and HSPA1B gene transcript (encoding Hsp70) abundance in midbrain samples from a series of cocaine-related deaths and corresponding drug-free controls. Hsp70 expression was significantly increased in the cocaine-dependent group compared with controls, whether or not an ED was present. Elevated Hsp70 expression levels were predictive of a documented survival period between cocaine-use and death that included medical and/or police intervention, regardless of the presence of the ED syndrome. This study suggested that elevated Hsp70 expression was more likely to be related to survival times after drug use and/or medical and police intervention than to the presence or absence of ED *per se* (25).

Drowning

Hsp70 expression in the neurons of the brain stem hypoglossal nucleus appeared to be significantly higher in drowning cases than other causes of asphyxia (hanging, strangulation, suffocation, asphyxia, and respiratory failure), suggesting that drowning caused more severe damage to the neurons of the hypoglossal nucleus. Furthermore, no correlations were identified between the rate of immunoreactivity and the post-mortem interval or survival in these cases. Thus, immunohistochemical examination of the hypoglossal nucleus could provide useful information to determine the cause of asphyxia (26).

In the lung tissue of freshwater and saltwater drowning and control cases, no statistically significant differences in Hsp70 expression levels were detected between respective groups. Only

one case out of 10 cases each for freshwater and saltwater drowning displayed strong Hsp70 expression (27).

Other Research Areas

Immunohistochemical examination of kidneys from forensic autopsy cases where methamphetamine was detected showed positive staining for Hsp70 in approximately one fifth of the cases, with antemortal hyperthermia confirmed in two cases. This suggested that heat stress may have led to Hsp70 expression. Interestingly, in Hsp-negative cases, methamphetamine levels in the blood were on average twice as high as Hsp-positive cases and almost 13 times as high in urine (28).

Individuals who died from mechanical asphyxia had higher mRNA levels of HSPA2, encoding Hsp70 (member 2), in the occipital region of the cerebral cortex compared with individuals who died from a traumatic injury. Hence, Hsp mRNA levels, as potential forensic biological markers in the occipital lobe, may provide clues to the cause and progression of death (20).

In cases of acute lung injury, extracellular Hsp72, encoded by the HSPA1A gene, was present in plasma and pulmonary edema fluid. Further, extracellular Hsp72 levels were highest in pulmonary edema fluid of patients with acute lung injury and preserved alveolar fluid clearance (29).

The findings of Preusse-Prange et al. (30) may provide some perspective to the aforementioned studies. These authors investigated the effects of formalin treatment using various protein detection methods on some members of the Hsp70 superfamily (HSPA1A and HSPA8). Their western blot analyses of formalin-fixed tissues failed to reliably detect proteins in cerebral and cerebellar tissue samples. In contrast, reproducible detection by immunohistochemistry was possible even after 1 month of incubation. However, protein detectability decreased proportionally to the fixation time. Therefore, only samples with a known fixation time should be used, and a fixation time longer than 1 month may lead to false-negative results.

Sample preparation and fixation times may explain why some studies conclude contrary results, and why in some studies, no Hsp expression is detected. Especially with regard to study comparability, a uniform approach may be useful. While reviewing the studies, we observed that methodologies, if specified, differed considerably. In gene analysis studies, the methodology primarily stated that samples were frozen at −70°C (20, 29) or −80°C (13, 14, 19, 21) immediately after collection. In two studies, the storage temperature was not specified (24, 25), and in one study, samples were submerged in RNA stabilization solution (17).

In reviewing Preusse-Prange et al., differences were apparent in immunohistochemical studies, which may have significantly limited comparability in some cases. With regard to fixation times, they were only stated in three studies. Fixation times were 24 h (27), 48 h (23), and between a few days and several years (16). The remaining studies did not report a fixation duration for their samples.

We also observed differences with respect to formalin concentrations. Results from Preusse-Prange et al. were based on a 10% fixation concentration. This was used in only two studies (17, 23). One study used 4% formalin (27) and four studies used 8–10% formalin (11, 15, 16, 18). Four studies did not report the formalin concentration (10, 22, 26, 28). Even though formalin fixation times appeared to have a significant influence on Hsp stainability, especially in the case of a prolonged fixation, it remains unclear whether these statements can also be applied to other, especially lower, formalin concentrations. This would require further investigation. However, this factor should be accounted for and considered as a possible source of negative results.

CONCLUSIONS

In this 21-year range of selected studies, few provided absolute numbers; however, they dealt with different topics exploring the forensic significance of Hsp. There was no single focus; approximately equal attention was paid to fire deaths, hypothermia, SIDS/peripartum deaths, drowning, ED, and cardiac deaths. The ratio of immunohistochemical and gene analysis studies was approximately equal. Hsp70 was by far the most studied protein, followed by Hsp27.

These studies yielded several interesting findings with potentially relevant implications for forensic science. In fire deaths, consistent results indicated that Hsp27 and Hsp70 were useful markers for detecting (supra-) vitality; moreover, these markers could be used to estimate survival time. Hsp70 determination in blood may be a suitable marker for acute myocardial infarction and may be an exciting forensic approach for unclear deaths with suspected myocardial infarction or coronary insufficiency. For hypothermic deaths, Hsp70 displayed characteristic expression patterns in the kidney, which may be used diagnostically. In addition, the combined assessment of HSPA12B and FOSB gene expression may distinguish traumatic brain injury from control cases. HSPA1B and HSPD1 showed increased expression in response to thermal stress in SIDS cases. In ED, increased Hsp70 expression was demonstrated in the brain although the underlying pathophysiology remained unclear. Yet, in methamphetamine-related deaths, antemortal heat stress may have led to increased Hsp70 expression in the kidneys. Furthermore, drowning cases showed significantly higher Hsp70 expression levels in the neurons of the nucleus hypoglossus of the brainstem compared with other fatal asphyxia events. In addition, increased HSPA2 mRNA levels were observed in the cerebral cortex during mechanical asphyxia compared with traumatic injury.

However, it was striking that there were few, if any, follow-up studies on the respective research areas. Furthermore, a uniform approach to future studies should be considered. This factor could relate in particular to formalin concentrations and fixation times. This approach would not only avoid potentially false-negative results but also significantly increase comparability between respective studies.

This mini-review was limited by the fact that only studies on human material were included. Emphasis was placed on studies where immediate practical application is possible and where the

results may, at best, add value to criminal investigations or court proceedings. Therefore, animal or experimental studies were deliberately excluded.

Nevertheless, our mini review highlighted the significant potential of Hsps and the established Hsp investigations in the forensic field. Our review indicated that despite the few studies available, those that were selected were very interesting in their own right, and the identified research approaches/areas require further study. Furthermore, it should be noted that, according to the defined criteria, no studies could be found that dealt with Hsp expression in the skin from a forensic point of view. Thus, it represents a vastly unexplored and potentially promising area of research.

AUTHOR CONTRIBUTIONS

JP: conception and study design and drafting the manuscript. JP and AM: acquisition of data. JP, BM, AM, and ED: analysis and/or interpretation of data, revising the manuscript critically for important intellectual content, and approval of the version of the manuscript to be published. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Role of miRNAs as New Molecular Biomarkers for Dating the Age of Wound Production: A Systematic Review

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Background: The timing of wounds production is a significant issue in forensic pathology. Although various methods have been evaluated, obtaining an accurate dating of lesions is still a challenge. The pathologist uses many parameters to value wound age, such as histological and immunohistochemical. In recent years, there have been many studies regarding the use of miRNAs in wound-age estimation; indeed, miRNAs have multiple potential uses in forensic pathology.

Scope: This review aims to verify the efficacy and feasibility of miRNAs as a tool for determining the timing of lesions.

Materials and Methods: The authors conducted the systematic review according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines. PubMed was used as a search engine to find articles published between January, 1st 2016 and October, 1st 2021, to evaluate the current state of the art regarding wound-age estimation.

Results: A total of 256 articles were collected; after screening according to PRISMA guidelines, the systematic review included 8 articles. The studies included in this review were all Original articles evaluating the use of biomarkers for wound-age determination.

Discussion and Conclusion: The literature review showed that analysis of miRNA is an innovative field of study with significant potentiality in forensic pathology. There are few studies, and almost all of them are at an early stage. The challenge is to understand how to standardize the samples' selection to obtain reliable experimental data. This observation represents a necessary prerequisite to planning further clinical trials.

Keywords: wound-age estimation, wound-age determination, postmortem miRNA, miRNA, post-mortem investigation

INTRODUCTION

The timing of wounds production is a significant issue in forensic pathology. Although various methods have been evaluated, obtaining an accurate dating of lesions is still a challenge (1).

After an injury, the pathologists must consider many vital processes (e.g., hemorrhage, inflammatory cells migration, infiltration, development of granulation tissue) for an accurate wound-age estimation (2).

Morphological and macroscopic analysis was one of the first methods used (3). But it is not of practical use because it cannot be standardized.

Histological evaluation (with Haematoxylin-Eosin staining) can detect the presence of inflammatory cells or substances secreted during the inflammatory process (4). Different types of inflammatory cells reach a peak with exact timing, allowing age estimation of the lesion [neutrophilic granulocytes reach the maximum levels after 24 h (4, 5); macrophages after 2-3 days (4); lymphocytes at least 20 h; fibroblastic cells after 6 days or more (4)].

Immunohistochemical methods can also detect different substances secreted during the inflammatory process (6) [esterase and adenosine triphosphatase increase 1 h after injury; aminopeptidase reaches a peak at about 2 h; acid phosphatase at 4 h; alkaline phosphatase at 8 h (7)] or analyze the expression of Fibronectin, CD62p, and Factor VIII, which are markers involved in coagulation and inflammation processes. These, significantly increase in wounds produced close to death (15–30 min old) (8). Also, the number of matrix metalloproteinase-2 (+) macrophages increases in accordance with wound ages (9).

Histamine is a mast cell degranulation product, used as a marker in forensics (e.g., for asphyxiation) (10). Histamine levels in the skin vary appreciably after wounding; these levels increase significantly between 5 min and 3 h after trauma and decrease until 24 h. The transient infiltration of mast cells occurs in the dermis for 3 h upon vital lesions and is followed by a protracted decrease until 24 h (11, 12).

The real-time polymerase chain reaction (PCR) measures mRNA levels (13). PCR can detect the mRNA levels of inflammatory cytokines and wound-healing factors [e.g., caspase level 3, 8, and 9 expressions in tissues (14); VEGF and TGFb1 mRNA have high grades from the early stages, reaching the peak at day 7 (5)]. As mRNA is not very stable due to the action of ribonucleases (15), it is not very useful for forensic purposes. The stability, moreover, presents significant differences depending on the tissue analyzed: skeletal muscle, heart, and brain are more stable than the pancreas (13).

According to the studies on this topic a single parameter is not sufficient for estimating wound-age; indeed, combining parameters can reduce error.

Therefore, in recent years, many studies have been performed regarding the use of miRNAs in wound-age estimation. Indeed, the researchers found microRNAs (miRNAs) expression at 0, 24, and 48 h after death. Especially high levels of miRNA-205 and miRNA-21 24 h after death were observed (16).

MiRNAs were first described in 1993 by Lee et al. (17), and the term microRNA was born in 2001 (18).

MiRNAs are small non-coding RNA molecules containing about 21–25 nucleotides (19) that interact with the 3′ untranslated region (3′ UTR) of target mRNAs to induce their degradation. They can also interact with the 5′ UTR region, coding sequence, gene promoters and activate translation or regulate transcription (20).

The interaction of miRNAs with their target genes is dynamic. It depends on many factors, such as the subcellular location of miRNAs, and both the abundance of miRNAs and target mRNAs. miRNAs can also be secreted into extracellular fluids and operate as chemical messengers to mediate intercellular communication (20).

About 1-3% of the genome encodes miRNAs, which regulate more than 30% of protein-coding genes (17). Many studies have demonstrated that miRNAs play a significant role in biological processes, including cell proliferation, differentiation, apoptosis, organ development, pathogenesis, metabolic control, and antiviral defense (21).

However, the cellular localization of miRNAs is still widely unappreciated (22).

For this reason, many studies have been carried out in recent years to identify some tissue-specific miRNAs (21, 22). MiRbase, the bioinformatic database of miRNA sequences, reports the 1917 miRNAs discovered and encoded by the human genome [www.mirbase.org].

Microarray or Next Generation Sequencing (NGS) techniques can achieve the analysis of miRNAs. After their identification, the researcher can perform the assay by Real-Time quantitative PCR (qRT-PCR) (23).

Modification in the normal miRNA expression pathway can affect normal cellular physiology and lead to different pathologies.

Chronic lymphocytic leukemia was the first human disease known to be related to miRNA deregulation. Human cancer can be associated with changes in miRNA expression by deregulation of oncogenes and oncosuppressor genes (24–26).

MiRNAs' dysregulation is also involved in other diseases like cardiac hypertrophy and heart failure (27–30), chronic kidney disease (31), obesity (32), and various neurological and neuropsychiatric disorders (33).

The aim of this review is to verify the usefulness of miRNAs as a tool for determining the age of lesions. In our opinion it is necessary to analyze the current state of the art on wound-age estimation before to plan additional experimental studies on these topics.

MiRNAs have multiple potential uses in forensic pathology. In fact, unlike mRNA, they are less sensitive to degradation (due to environmental factors, such as UV light or heat, and the action of cellular ribonucleases) (34) and more stable than mRNA. They can also be extracted together with the DNA profile, representing a double test, useful in case of shortage of biological material (35).

MiRNAs are helpful for the recognition of biological fluids found at a crime scene. Nowadays, only a few miRNAs are recognized as specific for a single biological fluid (especially for blood and seminal fluid, while miRNAs specific for venous and menstrual blood are yet to be determined) (36).

Many authors focalized their research on the study of wound vitality (37). Some authors have focused on differentiating ante- and post-mortem lesions through miRNAs (38), but studies are still few and at an early stage.

Determining the age of production of the wounds is a relevant topic to forensic purposes (39). Over the years, many authors studied how putrefaction affects the concentration of proteins, DNA and RNA (40–43). Maiese et al. (44) analyzed the progress made in recent years, showing that some miRNAs are stable and reasonably reliable as markers. Wang et al. (45) demonstrated that three miRNAs (miR-122, miR-150, miR-195) are stable in the first 24 h of PMI (Post-Mortem Interval), declining after that time. Some studies remarked whether the animal died during the day or at night, considering the modification of other miRNAs (miR-541 and miR-142-p) (46).

MiRNAs have an essential role in moderating cellular adaptations occurring in case of drug abuse and addiction (47). The alteration of regular miRNAs expression occurs even in response to substances' abuse, such as nicotine, cocaine, morphine, and alcohol (48–50).

A further field of application for miRNAs is as anti-doping marker (51, 52).

An innovative field of application for miRNAs is the estimation of wound-age, as miRNAs are variously involved in wound healing physiology and physiopathology from injury to re-epithelialization (53, 54).

Aunin et al. (55) investigated the expression of miR-200 in skin wounds of subjects of different ages, marking its role in wound repair. The study identified miR-200c as a critical determinant that inhibits cell migration during skin repair after injury and may contribute to age-associated alterations in wound repair.

Ibrahim et al. (16) studied the post-mortem expression of miR-21 and miR-205 on incisional wounds on a cohort of 18 female albino rats. The animals were injured and then sacrificed, and divided into three groups. In the first group, the samples were taken immediately after death, in the second group after 24 h, and in the third one after 48 h. The researchers analyzed the expression of miRNAs in each sample, observing higher expression in the second group than in the other two. The examined miRNAs were still expressed 48 h after death, although at a lower level. Despite these exciting results, the population studied is too restricted to be considered of meaningful impact and further studies are necessary.

MATERIALS AND METHODS

The authors conducted the systematic review according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guideline (56). The quality assessment of this study was evaluated using the Checklist for Systematic Reviews and Research Syntheses recommended by the Joanna Briggs Institute (JBI).

PubMed and Scopus were used as a search engines to find articles published between 1 January 2016 and 1 October 2021, to evaluate the current state of the art regarding wound-age estimation. The Medical Subject Heading (MeSH) thesaurus was used for the following word: "(wound-age estimation) and (wound healing)"; "(wound-age estimation) and (post-mortem)"; "(wound-age estimation) and (miRNA)."

Inclusion and Exclusion Criteria

The inclusion criteria were: Article in English, Case report; Case series; Original Article; *in vivo* studies (both animals and human); Studies with searching for specific biomarkers.

The exclusion criteria were: Article not in English; Abstract; Poster; Proceedings; Review; Meta-Analysis. The researchers decided to exclude reviews and meta-analysis to give an experimental attitude to the article, analyzing only experimental research.

The methodology of the search strategy is presented in **Figure 1**.

Methodological Evaluation

Methodological evaluation of each study was conducted according to the PRISMA standards, including assessment of bias. Data collection involved study selection and data extraction. Three researchers (S.D.S., M.A.B., E.G.) independently reviewed those documents whose title or abstract appeared to be relevant and selected those who analyzed the wound-age estimation.

Risk of Bias

This systematic review focuses on articles published in the last 5 years, doing specific research using a few keywords. This topic represents a restricted research field, in which few experiments have been carried out. The limited number of articles available can be a source of error.

RESULTS

A total of 285 articles were collected, removing 47 duplicates. 238 papers were screened, and 222 did not meet the inclusion criteria; 15 pertinent articles were in Chinese. In conclusion, the systematic review included 16 articles. The studies included in this review were all Original articles ($n = 16$) considering biomarkers for wound-age determination (**Table 1**).

DISCUSSION

The wound-age estimation is still a challenge for forensic pathologists worldwide. They often attend crime scenes, in which bodies show many injuries, whose production time is only conceivable (73). The relevance of this argument encourages more studies on this topic.

In one of the latest reviews in the literature, Li et al. (2) examined the publications made in the years 2010-2016. They stated that there are no standardized and reliable biomarkers to estimate wound-age, despite the study of many molecules. The use of multiple markers could make dating more reliable.

From 2016, as emerged from our literature analysis, only a few studies have been published. All these papers specify a need for further researches.

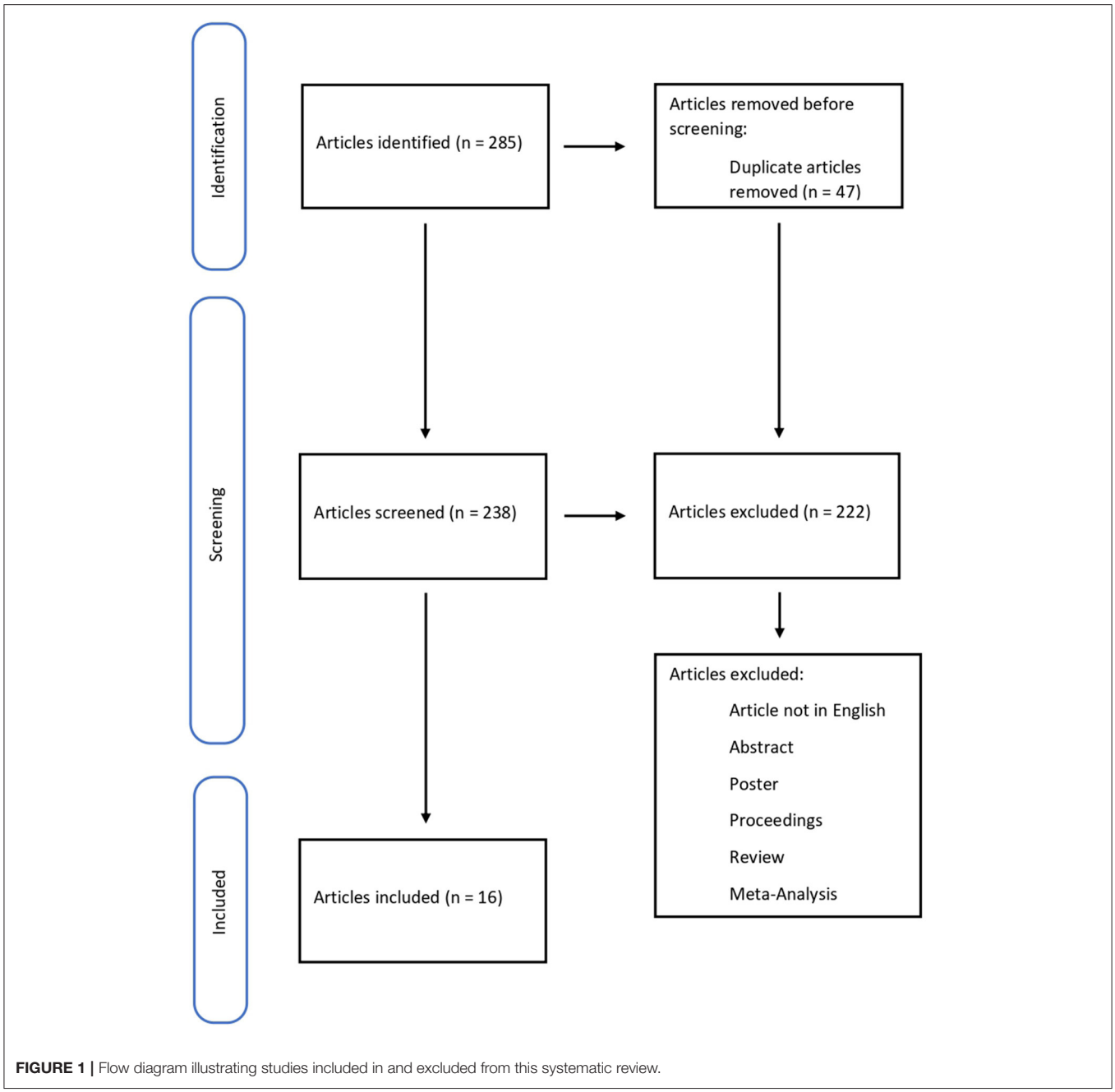

FIGURE 1 | Flow diagram illustrating studies included in and excluded from this systematic review.

Yuan et al. (57) investigated the role of miR-203 and IL-8 in the healing process of diabetic foot ulcers in rats. MiR-203 is involved in wound repair and promotes apoptosis, while IL-8 promotes cell proliferation and survival. MiR-203 directly downregulates mRNA expression for IL-18, inhibiting the wound healing process. However, the authors do not evaluate miR-203 or IL-18 expression timing, highlighting only greater miRNA expression in diabetic tissues.

Niedecker et al. (58) studied markers such as matrix metalloproteinases (MMP) 2 and 9 and tissue inhibitors of matrix metalloproteinases 1 (TIMP-1) in human and animal tissues. The authors collected samples of human injured skeletal muscle, injured human myocardium, rat myocardium both with vital and post-mortem injuries. Immunohistochemical investigations showed that these markers did not help wound-age estimation, as the samples did not show positivity (or weak positivity in older wounds).

The research of Kuninaka et al. (59) examined dendritic cells. Dendritic cells (DCs) are cells involved in the immune response and healing the burned skin. The authors collected 53 samples of injured human skin whose production time was known (from a few hours to 21 days). The CD11c and HLA-DRα markers

TABLE 1 | Summary of the reviewed literature.

Biomarker	Lesions	Sample source	Cohort number	References
miR-203, IL-18	Foot ulcers	Male Sprague-Dawley diabetic rats	12	Yuan et al. (57)
MMP2, MMP9, TIMP-1	Human injured skeletal muscle, injured human myocardium, rat heart with vital injuries, and rat heart with injuries inflicted after death	Humans and rats	141 autopsies. Not specified rats number.	Niedecker et al. (58)
Dendritic cells	Stab wounds, incised wounds, surgical wounds, lacerations	Humans	53	Kuninaka et al. (59)
Receptor for advanced glycation end products (RAGE)	Incisional skin wounds	Diabetic rats	Not specified	Ji et al. (60)
iNOS, IL-6	Burned skins	Male albin rats	50	El Noor et al. (61)
mRNA encoding SFRP5, FZD4, Fosl1	Contused muscle	Male Sprague-Dawley rats	78	Zhu et al. (62)
miR-21	Excisional skin wounds	Mice	Not specified	Long et al. (63)
Abhd2, Rael, Asb5, Slfn3, Samd4b, Prr5, Arid5a, Ier3, Cdc40, CD68, Tbx18, Ipo4, Fam210a, Tmem100, Sc65, Legend, Mad212, Rcc1l, Anxa11, Hst6st1, Leprot, Trit1, Polpid3, Lin37, Prrx2, Fbxw4, Prr3, Dclre1b, Impact, Dennd5a, Teme45b, Myg1, Lrrc4l, Rabepk, Rhbdd3	Contused muscle\Rats	Male Sprague-Dawley rats	108	Du et al. (64)
CCL4, CXCL5, IL-1β, IL-6, IL-7	Incisional skin wounds	Male BALB/c mice	72	Gaballah et al. (65)
mRNA encoding TnI, tPA	Contused muscle	Female albino rats	25	Ibrahim et al. (66)
mRNA encoding PUM2, TAB2, GJC1, and CHRNA1	Contused muscle	Male Sprague-Dawley rats	78	Sun et al. (67)
Fosl1	Contused muscle	Male Sprague-Dawley rats	126	Sun et al. (68)
IL-1b, IL-6, TNF-a, IFN-g, MCP-1, CXCL12, VEGF-A, EGF, KGF, pro-col Ia2 and pro-col IIIa1	Excisional skin wounds	Male BALB/c mice	60	Wang et al. (69)
CD14	Skin wounds Postmortem wounds (not specifid type of injury)	Male BALB/c mice Human skin wound	34 97	Yagi et al. (70)
Pax7, MyoD	Contused muscle	Male Sprague-awley rats	40	Tian et al. (71)
3,522 genes	Contused muscle	Male Sprague-Dawley rats	33	Li et al. (72)

detect the presence of DCs. Wounds aged 4 to 7 days showed low DCs, similar to 9 to 14 days. The samples aged 4-14 days showed the presence of more than 50 DCs per observation field. In the samples aged 17-21 days, this number gradually decreased.

This study indicates that DCs could be a good marker for wound-age estimation, even if the time interval of expression is too broad. Also, Bacci et al. (74) performed a study on DCs cells, measuring their concentration within hours of injury (a short range of time).

Ji et al. (60) made incisional injuries on diabetic mice that they killed after a defined time (6 h to 14 days), finally sampling the lesions. Subsequently, they looked for the expression of the receptor for advanced glycation end products (RAGE) expressed mainly by polymorphonuclear cells. There was an upregulation of RAGEs by 60% between 7 and 10 days after the wounds. However, the authors suggest cautiously interpreting these results, as the diabetic mice used for the study had persistent hyperglycemia from 4 weeks of age and never received treatment.

El-Noor et al. (61) studied the expression of inducible nitric oxide synthase (iNOS) and IL-6 in burned rat skin samples. The authors caused burns on the skin of the mice, sampling them after a defined time (1, 3, 5, 7, 9, 11, 13, 15, and 21 days after the burn). Six hours after the death of the rats, they inflicted another burn, also sampled. The study of markers in ante-mortem wounds revealed that iNOS becomes positive between 3 and 5 days, peaking on day 7, after which it begins to decline. The expression of IL-6 is also time-dependent, starting on day one and peaking on day 3, remaining high until day 5. The positivity begins to decrease from day 7 until it disappears by day 21. In the post-mortem wounds, both markers were weakly positive.

Zhu et al. (62) studied the expression of mRNAs encoding frizzled-related protein 5 (SFRP5), frizzled class receptor 4 (FZD4), and Fos-link antigen 1 (Fosl1) in bruised muscles of rats.

These proteins are involved in the healing of injured muscle. The authors monitored their expression for up to 48 h; all mRNAs showed changes, although very different. These trends need further studies to understand if the coding proteins could be considered as useful markers.

Research by Long et al. (63) focused on studying the expression of mir-21 on the skin of female rats with excisional wounds. The authors demonstrated that miR-21 expression improved cutaneous wound repair. However, the purpose of this paper was not to use miR-21 to date the injuries production, so it cannot have forensic application.

Du et al. (64) studied the expression of 35 wound healing-related genes on contused muscle tissue samples from rats through PCR. The authors divided the samples into three groups time-based. Of the 35 genes, 14 showed particular utility in discriminating between the three age groups based on their expression time.

Gaballah et al. (65) studied the expression of five cytokines from the measurement of their RNA through qRT-PCR: chemokine ligand 4 (CCL4), chemokine ligand 5 (CXCL5), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-7 (IL-7). The authors performed incisional wounds on the hamstring muscle of 72 mice, divided into five groups based on the time of wound collection (6, 12, 24, 36, and 48 h post-injury). CXCL5 was the most upregulated molecule with higher expression 6-36 h after injury, such as CCL4 and IL-1β. IL-6, on the other hand, peaked at 6 h post-injury, to significantly decrease at 12 h. IL-7 showed a slow and steady increase over time up to 48 h after injury. Immunohistochemical staining of the post-injury samples showed a gradual increase in intensity around the wound edges. In light of these promising results, this study points out that since post mortem RNA degrades quickly, the application of protein markers is more reliable.

Ibrahim et al. (66) analyzed skeletal troponin I mRNA (TnI) and tissue plasminogen activator (tPA) mRNA expression after inflicting a bruised wound on 25 rats. The researchers divided the rats into five groups based on the injury time. tPA expression levels significantly decreased at 1, 6, and 30 h after contusion. In contrast, TnI expression levels increased at 1 and 6 h post-traumatic, then gradually reduced to normal levels at 24 h, and they assumed significantly lower levels at 30 h after the contusion.

Sun et al. (67) developed an "up, no change or down" system to study the time-dependent expression of PUM2, TAB2, GJC1, and CHRNA1 mRNAs in bruised skeletal muscle of rats. The rats were divided into 12 groups based on the time of wound production. Subsequently, the authors combined the levels of mRNAs. The mRNA levels of PUM2, TAB2, and GJC1 decreased, while CHRNA1 mRNA levels increased. The proposed system can detect the injury time for short periods.

In another research article, Sun et al. (68) such as Zhu et al., studied Fosl1 mRNA and protein in contused skeletal muscle. The researchers divided the rats into different groups (control, contused, and postmortem). They then examined the expression of Fosl1 mRNA and the coding protein in bruised muscle, uninjured contralateral, and postmortem femoral muscle samples. Both mRNA and protein levels of Fosl1 exhibited time-dependent expression during contused skeletal muscle healing. There was no significant difference in Fosl1 protein levels among the postmortem muscle and control specimens, which means that Fosl1 protein levels remained stable in the postmortem samples within 24 h. The study suggests that the expression of Fosl1 mRNA was susceptible to the degree of injury. Fosl1 mRNA could be a promising marker to determine the force that caused the wound but is not stable enough to estimate the duration of the wound.

Wang et al. (69) studied several molecules, including IL-1b, IL-6, TNF-a, IFN-g, MCP-1, CXCL12, VEGF-A, EGF, KGF, pro-col Ia2, and pro-col IIIa1. The authors inflicted skin wounds and subsequently removed them after standardized times. The expression of each molecule was increased over a different time range, depending on their role in wound repair.

Yagi et al. (70) looked for CD14 expression in mouse wounds and postmortem humans. In particular, in humans, they evaluated CD14 in combination with other proteins, such as CD32B and CD68. CD14 appears to be a valuable marker of wound age, 1–5 days postinfliction. However, the combination of several markers increases the sensitivity of the method.

Tian et al. (71) investigated the time-dependent expression of transcription factor 7 (Pax7) and myoblast-determining protein (MyoD) during skeletal muscle wound healing. The experiment consisted of injuring the right limb of rats. Pax7 protein peaked 5 days after injury and subsequently declined, while MyoD mRNA expression peaked 3 days after injury. In conclusion, Pax7 and MyoD expression are time-dependent upregulated during skeletal muscle wound healing, suggesting that they may become potential markers for estimating wound age in skeletal muscle.

Li et al. (72) collected samples of animal injured skeletal muscle taken from the right posterior limb and investigated differentially expressed genes (DEGs) using microarray analyses. A total of 2,844 and 2,298 DEGs involved in muscle repair were identified in the mild and severe contusions, respectively. These DEGs overlap during the early stages after injury (within 48 h). During later stages (at 168 h) there is an expression of only 29 genes. The genes showed time-dependent patterns of expression, which provide a basis for further studies of wound age estimation.

There are not many recent studies on wound-age estimation, probably because of sampling difficulties. Most studies regarded animals but in a low number cohort, summarized in **Table 1**. Studies on human tissues are often problematic to apply because of ethical requirements (75). Most of the studies also concern protein markers and their mRNA, analyzed by immunohistochemistry, Western-Bloth, and PCR.

From a forensic point of view, it is challenging to select a homogeneous sample of patients due to the difficulty of knowing the lesion's production time. It is essential to know the timing of lesions to experimentally study the expression trend of a potential marker.

Only Kuninaka et al. (59) and Yagi et al. (70) performed studies sampling human wounds with known production time. However, in the studies on rats, the production time was established by the researchers.

CONCLUSION

The wound-age estimation is an essential issue in forensic pathology. Pathologists need sensitive and specific markers to conduct an objective assessment and to obtain scientifically and validated evidences. The literature review showed that analysis of miRNA is an innovative field of study with significant potentiality in forensic pathology. The review on the wound-age estimation revealed that research has not progressed in the past 5 years. There are few studies, and almost all of them are at an early stage. On the other hand, the field of wounds' dating in forensic pathology is essential. The main problem in obtaining sufficient data for validation is the difficulty of collecting adequate and homogeneous samples. Several studies recommend the use of multiple methods to get reliable results. In our opinion, the challenge is to understand how to standardize the samples' selection to obtain reliable experimental data. This observation represents a necessary prerequisite to planning further clinical trials.

AUTHOR CONTRIBUTIONS

SD, MF, and EG: analyzed the literature. SD and MB: writing. LC and RL: review and editing. GB and SV: language supervision. All authors have read and agreed to the published version of the manuscript.

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The reviewer AM declared a shared affiliation, with no collaboration, with one of the authors SV to the handling editor at the time of the review.

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An Overview on Actual Knowledge About Immunohistochemical and Molecular Features of Vitality, Focusing on the Growing Evidence and Analysis to Distinguish Between Suicidal and Simulated Hanging

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In forensic practice, the pathologist is often asked to determine whether a hanging was committed as suicide or as a simulated hanging (when a dead body is suspended after death). When exterior evidence of violence is absent and the crime scene investigation fails to identify useful proof, it is nearly impossible to tell whether the dead body was suspended or not. As a result, determining whether the ligature mark was created during life or not should rely on the research and demonstration of vital reactions on the ligature mark. The main purpose of this review article is to provide a summary of current knowledge about the histological and immunohistochemical characteristics of vitality in hanging. The authors also aim to identify the most significant vitality markers on ligature marks for further scientific validation and to propose a standardized diagnostic protocol for hanging. The study was conducted according to the Preferred Reporting Items for Systematic Review (PRISMA) Protocol. Relevant scientific papers were found from PubMed up to April 2021, using the following keywords: hanging AND skin AND vitality. Three main points were studied: ligature mark dehydration, immunological response to mechanical injury, and apoptosis induction as a result of the previous points. An increase in apoptosis is evident in the ligature mark (due to physical and chemical processes involved), as demonstrated by FLICE-inhibitory protein (FLIP) depletion. Immunohistochemical detection of Aquaporin 3 (AQP3) and increase in the concentration of different electrolytes rely solely on ligature mark dehydration. Also, microRNAs (MiRNAs) could become reliable forensic biomarkers for ligature mark vitality diagnosis in the near future. To ensure high reliability in court cases, forensic investigation in hanging should rely on modern and proven markers, even a mix of several markers.

Keywords: hanging, autopsy, vitality, immunohistochemistry, ligature mark, skin

INTRODUCTION

One of the most challenging issues in forensic medicine is to answer the question of whether a hanging was perpetrated by suicide or is a simulated hanging (when a dead body is suspended after death).

Although most hangings are suicidal, homicidal hanging can rarely occur. Typically, when a victim is conscious, a forensic pathologist will inevitably find signs of resistance on the dead body, such as bruises or signs of binding the arms, wrists, or legs (1, 2).

When such external signs are absent, and crime scene investigation is unsuccessful (e.g., no signs of suicidal ideation), it is almost impossible to distinguish whether the dead body was suspended or not. Therefore, it is pivotal to determine whether the ligature mark was produced during life or not, thus analyzing vital reactions. The term "vital reaction" refers to effects in, at, or by the body following trauma and allows the assumption that the trauma occurred during life (3).

It is crucial to remember that in hanging, the mere presence of erythrocytes extravasation in neck tissues must not be considered as a vital sign (4).

In hanging, classic signs of asphyxia (hypostasis in legs and hands, congestion) are often absent.

The classic Simon sign, hemorrhage of lumbar spine anterior ligament due to violent hyperextension, is identified in only 34% of hanging (5). Although petechial hemorrhages can be frequently found on the skin, mucosal surfaces (6), and subconjunctiva (7), they are not significant as typically associated to any asphyxia death. Acute pulmonary emphysema can be present, but not exclusively (e.g., drowning) (8).

When analyzing neck tissues in hanging, there may be surprisingly little to find. Hence, the inspection should focus on ligature mark features, especially on histologic ones (9).

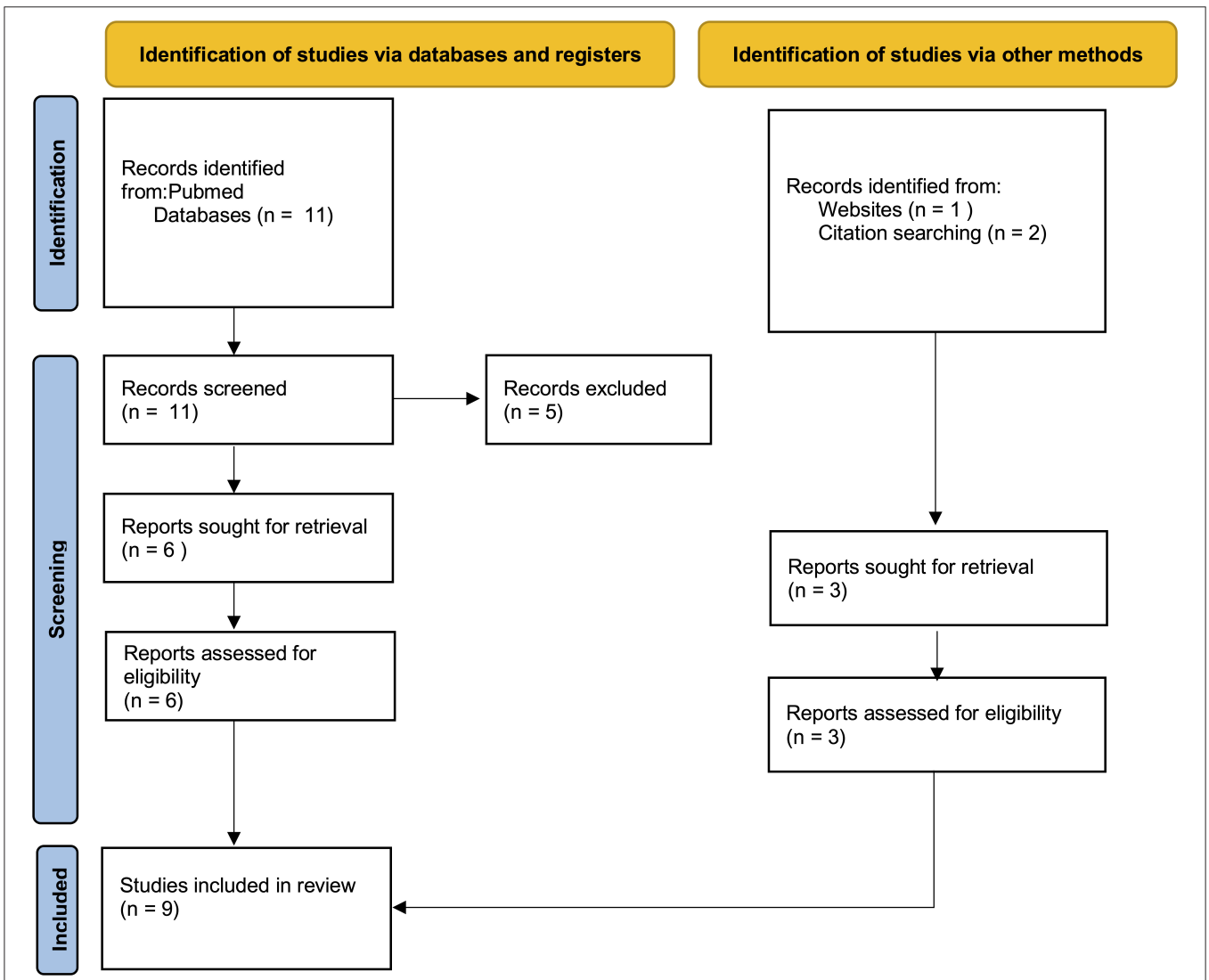

FIGURE 1 | The selection of appropriate scientific papers was performed; 9 articles met the inclusion criteria and were included.

On the basis of ligature mark macroscopic characteristics, it is substantially impossible to distinguish between a vital or non-vital one (10). Histologic features typically linked to vital reactions in ligature marks are vesicles, detachment of the epidermis outermost layer (stratum corneum), and nuclei thinning. On inner layers, a hemorrhage of clavicle insertion of sternocleidomastoid muscle can be found. Such sign is widely discussed, though: the scientific community does not agree with its capability to act as a vitality indicator due to the infrequency of extensive neck stretching (11–13).

The non-specificity of external and internal signs in hanging imposes the need for more reliable data, especially when dealing with suicidal or simulated hanging (14).

Differential diagnosis between such events should be built on the research and demonstration of vital reactions on ligature marks, analyzing both cutaneous and inner neck structures.

The aim of this literature review is to provide an update on relevant vital signs of ligature marks, focusing on immunohistochemically and molecular pathology detectable vital reactions. The present review article also intends to provide a differentiation between ligature mark features and surrounding non-injured skin.

MATERIALS AND METHODS

The primary endpoints of the present study are to provide an overview of actual knowledge about histological and immunohistochemical features of vitality, focusing on forensically relevant data able to distinguish between suicidal and simulated hanging. Also, to propose the most significant vitality markers on ligature marks for further scientific validation. Finally, to propose a standardized diagnostic protocol for hanging (crime scene inspection—circumstantial data analysis—post-mortem imaging—macroscopic external and internal signs—histology, immunohistochemistry, and laboratory tests). The study was conducted according to the Preferred Reporting Items for Systematic Review (PRISMA) Protocol. Relevant scientific articles were identified from PubMed up to April 2021, using the following keywords: hanging AND skin AND vitality (1, 15).

Papers presenting the following features were included:

1. Cohort or retrospective studies on biomolecular and immunohistochemistry analysis of ligature mark.
2. Suicide by hanging as the manner of death.
3. Comparative studies on suicidal and simulated hanging, non-injured skin in deceased by hanging, the skin of subject's dead by other causes.
4. Submitted and already published articles, excluding non-published ones.

Meta-analysis, reviews, systematic reviews, case reports were excluded to avoid repetitions and data duplication. All data were extracted from suitable articles. Papers from the authors' personal archive and those extracted from articles' references were also added. The selection of papers using PubMed provided a total of 11 articles, 5 of which were excluded since not suitable for the purpose of this review (**Figure 1**). Three other papers were included, for a total of 9 suitable articles (**Table 1**).

RESULTS

A description of tested molecules is provided, focusing on useful immunohistochemical markers for ligature mark vitality (**Table 2**). Data on molecular expression, comparison of markers expression with non-injured surrounding tissues, comparison with deceased by other causes, comparison with cases of simulated hanging are discussed. Not all listed cases present a comparison with post-mortem suspended cadavers.

Ishida et al. (18) tested 35 cases of hanging and 21 cases of strangulation, immunostaining skin samples with Aquaporin 1 (AQP1) (dermal capillaries) and Aquaporin 3 (AQP3) (epidermidis). Samples were collected within 72 h from death. In each case (where the forensic autopsy was performed), the cause of death was diagnosed on macroscopic and microscopic findings along with toxicological data. Authors incubated collected sections with anti-AQP1 or anti-AQP3 antibodies and after, they incubated biotinylated secondary antibodies.

Sections were later incubated with rabbit serum, and no positive signal could be detected, indicating the specificity of the antibodies. AQP1 was expressed in dermal capillaries of both injured and uninjured skin samples. AQP3 was greatly expressed on ligature marks skin. Since epidermal cells of ligature marks are significantly dehydrated, it is possible to suppose that AQP3 expression was significantly enhanced by neck compression (24). In one case, AQP3 immunostaining revealed the vitality of different skin wounds produced before suicidal hanging. AQP3 tested positive only in vital wounds (25).

TABLE 1 | The selection of appropriate scientific papers was performed; nine articles met the inclusion criteria and were included.

Selected articles			
References	**Molecule**	**Technique**	**Sample**
De Matteis et al. (16)	TnI	IHC	Human muscle
Maiese et al. (17)	FLIP	IHC	Human skin
Ishida et al. (18)	AQP3/AQP1	IHC	Human skin
Balandiz et al. (19)	IL-1β	IHC	Wistar albino Rat's skin
Legaz Pérez et al. (20)	Ca, Mg, Fe, Zn P-Selectin, Cathepsin D	ICP-AES; IHC	Human skin
Turillazzi et al. (21)	Tryptase, CD15, IL-15	IHC	Human skin
Focardi et al. (22)	AVIDIN CD1A MHC Class II	Cryosection – Immunofluorescence	Human skin
Neri et al. (23)	miR-146a-5p, miR125a-5p, miR125b-5p, miR103a-3p, mR92a-3p, miR21	miRNA PCR	Human skin

TABLE 2 | Selected articles.

				Selected markers				
Molecule	**N° hanged**	**N° cadaver suspension**	**N° control group**	**Expression in full of the loop***	**Hanged vs. cadaver suspension**	**Ligature mark vitality vs. non-injured skin**	**Hanged vs. Control**	**Ligature mark vs. pm wounds (including cadaver suspension)**
TnI	21	N/A	10	Cervical muscles	N/A	N/A	N/A	N/A
FLIP	21	3	13 (overdose $n = 2$, car accident n = 3, SCD $n = 5$, post-mortem suspension $n = 3$)	Negative epidermidis (intracytoplasmic depletion of FLIP)	0/21 vs. 3/3	***P* < 0.05**	***P* < 0.01**	***P* < 0.01**
AQP3	56 mechanical asphyxia (35 hanging - 21 strangulation)	N/A	56 (non-injured skin)	Positive epidermidis	N/A	***P* < 0.01**	***P* < 0.01**	N/A
AQP1	//	N/A	//	Dermal capillaries	N/A	–	–	N/A
IL-1β	10	10	N/A	Epidermal, annexal and subepidermal cells (100% PMI = 2 h)	***P* < 0.005**	N/A	N/A	N/A
[Fe]; [Zn]; Fe/Ca; Fe/Mg;	71	N/A	71	N/A	N/A	***P* <0 .05,** low concentration in ligature mark	N/A	N/A
Cathepsin D	71	N/A	71	Epidermidis	N/A	***P* < 0.05** (if compared with low [Fe])	N/A	N/A
P-Selectin	71	N/A	71	Epidermidis	N/A	**//**	N/A	N/A
Tryptase	49	7	21	Derma	N/A	N/A	***P* <0 .001**	N/A
CD15	49	7	21	Derma	N/A	N/A	***P* < 0.001**	N/A
IL-15	49	7	21	Dermal capillaries and sub-dermal capillaries	N/A	N/A	***P* < 0.001**	N/A
AVIDIN CD1A MHC Class II	10	N/A	10	Skin Derma Derma	N/A	***P* < 0.01** Positive ligature mark Negative Skin	Both positive	N/A
miR-146a-5p, miR125a-5p, miR125b-5p, miR103a-3p, mR92a-3p, miR21	36	N/A	28	Skin	N/A	***P* < 0.05**	N/A	N/A

*Including human and non-human samples and different testing techniques. ICP-AES, inductively coupled plasma-atomic emission spectrometry; IHC, immunochemistry. Selected molecules are singularly described and data are illustrated according to relevance. SCD, sudden cardiac death; ABI, Acute brain injuries; "–" , non-significant or P > 0.05; *where non-specified, consider positive; PMI, post-mortem interval; "//", same as precedent; "N/A", not applicable. Bold values are those statistically significant.*

De Matteis et al. (16) evaluated the use of Troponin I—fast skeletal muscle (TNNI2) to perform differential diagnoses about vitality in suicide by hanging and simulated hanging. TNNI2 is a 21.3 kDa protein able to rapidly bind ATP, enabling a rapid oxygen muscular exchange. The authors assumed that ligature neck compression produces muscular tissue ischemia. The study was carried out on sternocleidomastoid and infrahyoid muscles: sampling from deceased by hanging (21 subjects) was carried out at the level of the "full of the loop." Ten cases of rapid death were chosen as the control group. An evident intracytoplasmic depletion of Troponin I was observed in most hanging cases. A quantitative score (ranging from −3 to +3) was used to evaluate the staining intensity. Two cases out of 21 tested negative (score 0): both were women who used a soft ligature. Although interesting, the results did not show a statistical significance. TNNI2 did not present variability according to hanging methods or neck muscles depth. It is therefore evident the need to enlarge this case study, to obtain more significant and reliable results on Troponin I.

Pérez et al. (20) studied the concentration of iron (Fe), zinc (Zn), magnesium (Mg), and calcium (Ca) and the expression of P-selectin (present in thrombocyte α-granules and Wiebel-Palade bodies) and cathepsin D (a lysosomal enzyme activated at the site of wounding in the low pH environment induced by hypoxia and necrosis) in the ligature marks in a cohort of 71 suicidal hangings. Inductively coupled plasma-atomic emission spectrometry (ICP-AES) revealed high Ca and Mg concentrations. However, higher Fe and Zn concentrations were statistically significant in injured skin samples. Immunohistochemical analysis revealed positivity for cathepsin D in 51.3% of tested specimens, and for P-selectin in 47.2%. A higher frequency of cells positive to cathepsin D and P-selectin was found in subcutaneous injured skin. In injured skin, a higher concentration of Fe correlated to high cathepsin D- and P-selectin expression.

A further study (19) was conducted on a total of 20 Wistar albino rats, divided into 2 groups randomly: group A, antemortem hanging group ($n = 10$), in which rats were anesthetized, and group B, post-mortem hanging group ($n = 10$), in which rats were killed by giving them an overdose of anesthesia. Skin samples were taken as follows: in group A, abdominal skin samples were taken as control before hanging; ligature mark skin samples were taken 2, 24, and 72 h after hanging. In group B, abdominal skin samples were taken as control before hanging, and hanging mark skin samples were taken 2 h after the hanging process. Contrary to Chandrakanth et al. (26) and Samanta and Nayak (27) results, histological tests showed no morphological or histological difference between group A and group B. Both groups presented typical hanging features: neck skin thinning, epithelium cells were elongated and hyperchromatic, thinning and flattening of cutaneous adnexal cells, vascular dilatation, neutrophils, erythrocytes, and hemosiderin-laden macrophages. Authors immunostained interleukin-1β, based on data related to sharp force injuries vitality (28). Surprisingly, Interleukin 1β (IL-1β) showed positive immunostaining in the second-hour antemortem hanging group; in the post-mortem control group and second-hour hanging mark samples, there were almost no immunostaining. The comparison of the antemortem control group and antemortem second hour hanging mark group was statistically significant ($P = 0.002$). The authors concluded by highlighting the promising role of Interleukin-1β immunostaining of epidermal cells as a tool to discriminate antemortem and post-mortem hanging.

Turillazzi et al. (21) studied the immunohistochemical expression of a panel of cytokines and inflammatory cells in skin samples in autopsy cases of death due to hanging: the authors selected 21 cases in which a soft ligature was used, 28 cases in which the chosen material was hard, and 21 cases for the control group (4 cases of sudden cardiac death and 7 cases of post-mortem hanging). The immunohistochemical investigation was performed using antibodies anti-tryptase, fibronectin, Tumor Necrosis Factor α (TNFα), IL-6, IL-8, IL-10, MCP-1, IL-15, IL-1ß, CD45, CD4, CD3, CD8, CD68, CD20, and CD15. Statistical analysis showed a great positivity for Tryptase, CD15, and IL15 in ligature mark cells (stain score + + +: immunopositivity in up to half of the cells (50%) and + + ++: strong immunopositivity in the majority or 100% of cells). Immunostaining resulted in negative in the control group and in cases of post-mortem hanging. The authors highlighted the reliability of tryptase, IL-15, and CD15 in the determination of ligature marks' vitality, especially when dealing with soft marks.

Maiese et al. (17) focused on the FLICE-inhibitory protein (c-FLIPL) expression in 21 cases of death from suicidal hanging (11 of which used a soft ligature, 10 a hard one). The control group consisted of 2 cases that died from opioid overdose, 3 cases of traumatic death (car accident), 5 cases of sudden cardiac death, and 3 cases of post-mortem suspension of bodies. All deaths were characterized by their rapidity. The immunohistochemical analysis was performed based on the model of previously published studies (16, 21). Preliminarily, the authors confirmed the positivity of the vital ligature marks with already established methods, such as the study of tryptase, CD15, and Troponin I fast skeletal muscle. In all cases of subjects who died by hanging, a clear and evident intracytoplasmic depletion of FLIP was observed in the epidermal layers of ligature mark (average value of intensity −2.71, statistically significant ($P < 0.05$). In post-mortem injuries and in uninjured skin specimens of the control skin, the authors revealed a lack of depletion of the anti-FLIP antibody and preservation of epidermal layers morphology.

Focardi et al. (22) conducted a pilot study on the immunohistochemical expression of CD1a + Langerhans cells, Avidin/Tryptase + mast cells, and Major Histocompatibility Complex (MHC II) dendritic cells, performed on the deceased by hanging fatalities. Skin samples were taken 20 cm below the ligature marks of the hanging fatality cases (group 1); the second group consisted of skin removed from the neck where the hanging mark was the deepest. Group 3 consisted of samples of vital skin lesions (surgical wounds, abrasions, and lacerations of knee or ankles), and group 4 of samples of post-mortem wounds. Microscopic analysis revealed the reliability of selected markers on vital injuries. The expression of dendritic MHC-II class cells and CD1A+ cells-Langerhans cells was significantly higher in ligature marks and vital lesions. The degranulation of mast cells also showed higher values in ligature marks and vital lesions when compared with other groups.

MicroRNAs (MiRNAs) have been studied as hallmarks and biomarkers in inflammation and wound healing processes (29–31). MiRNAs' role is to regulate the expression of many genes in several biological processes during the post-transcriptional phase. Since mRNA has scarce stability, it could act as a potentially good marker for wound age evaluation in forensic pathology (32, 33).

Neri et al. (23) assessed the application of miRNA expression in forensic in cases of hanging, applying the method on skin samples. Authors studied microRNAs expressed in frozen samples of skin from the hanging ligature marks and compared results to control group skin samples: 36 skin samples from ligature marks and 28 samples from non-injured skin of subjects who had died by suicidal hanging were tested. Results showed an increase in the expression of miRNAs known as regulators of the inflammatory response

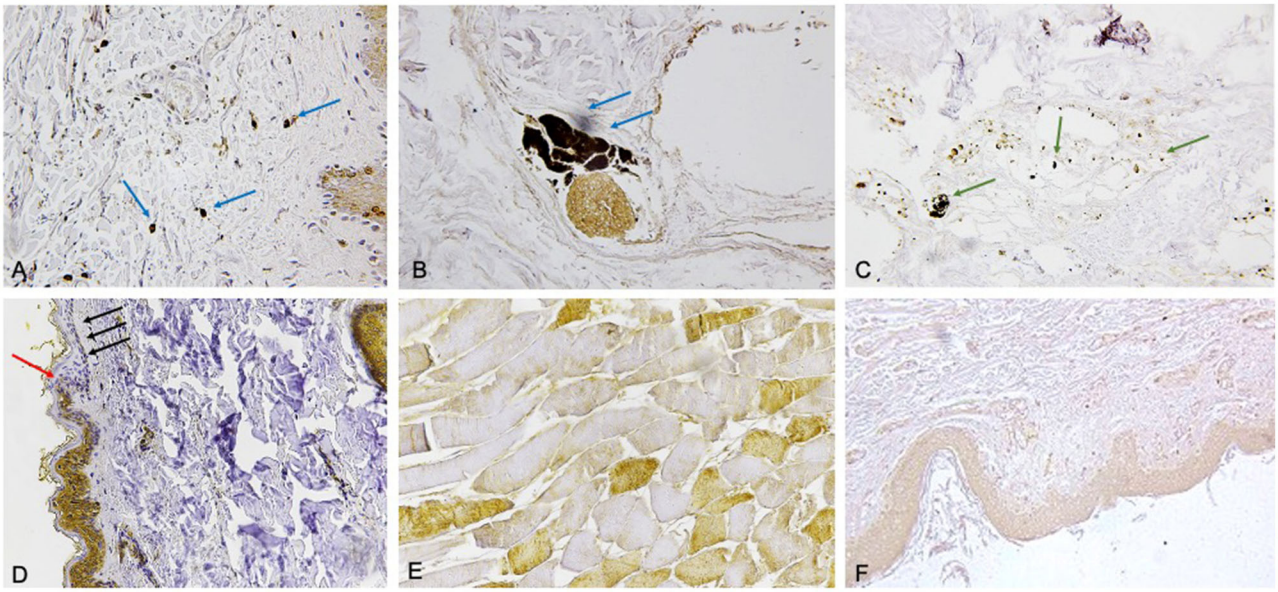

FIGURE 2 | (A) Mast cells tagged by tryptase reaction (blue arrows) (×60). **(B)** The immunodetection of interleukin (IL)-15 (blu arrows) is typical of most perivasal spaces (×60). **(C)** CD15 reaction (green arrows) to demonstrate a small number of neutrophils near the vessels (x60). **(D)** Evident intracytoplasmic depletion of FLIP was appreciated in the epidermal layers with the coexistence of epidermal flattening especially marked in the basal and spinous strati (×60). The passage from the uninjured skin to the compression zone (ischemia) is clearly distinguishable (red arrow indicates the passage of the clear "ax blow" hypo-expression). The coexistence of epidermal flattening is especially marked in the basal and spinous strati (black arrows). **(E)** Scarce positive TnI fast (brown) intracytoplasmic staining (×100). **(F)** The expression of aquaporin 3 (AQP3) in the keratinocytes in the skin ligature marks (×60).

in skin lesions (34) such as miR125a-5p and miR125b-5p. Overexpression of other miRNAs—miR214a-3p, miR128-3p, miR130a-3p, and miR92a-3p—with anti-inflammatory activity was highlighted. miR103a-3p ($P < 0.05$), miR214-3p, and miR92a-3p ($P < 0.01$) showed statistical significance to the control skin samples. Such interesting results suggest different miRNA expression profiles in skin samples with hanging ligature marks, highlighting the expression of miRNAs related to inflammation processes. Further investigation on this subject is needed to validate the reliability of such data.

DISCUSSION

Medico-legal diagnosis of death is pivotal in forensic pathology and great challenges emerge when dealing with differential diagnosis of injury inflicted ante- or post-mortem. Numerous authors focused their scientific work on the determination of the vitality of an injury. It is obvious that copious questions arise when a forensic pathology is asked to distinguish whether a hanging ligature mark was produced ante-mortem or pre-mortem.

Recently, research into the various biological molecules involved in the process of injuries determination in hanging has been carried out, aiming to identify reliable markers able to distinguish whether they are due to hanging or post-mortem suspension of the body. Scientific research in forensic medicine should identify proper markers—especially histological and immunohistochemical—characterized by a high probative value for their useful application in judicial trials (3, 35).

Estimating the vitality of lesions typically associated with death by hanging is nowadays based on the study of circumstantial data, crime scene investigation, the forensic study of the deceased (1) along with the histopathological analysis of ligature marks (3).

From the available literature data emerged two interesting cohort studies (17, 19) comparing vitality markers on injured skin, thus allowing further validating studies. The first evaluates IL-1β expression in ante-mortem and post-mortem hanged Wistar albino rats; the second observed a clear and evident intracytoplasmic depletion of FLIP in the epidermal layers of ligature mark of subjects who died by hanging. Evidence of strong reliability of tested markers clearly emerges from these studies, and it is therefore desirable that further and larger standardized studies with human samples be conducted.

Six papers examined and confronted non-injured and injured skin from the same suspended dead bodies (simulated hanging). The expression of FLIP, AQP3, [Fe], [Zn], Fe/Ca ratio; Fe/Mg ratio, Cathepsin D, P-Selectin, Avidin, CD1A MHCII, miR-146a-5p, miR125a-5p, miR125b-5p, miR103a-3p, mR92a-3p, and miR21 resulted statistically significant (18, 20, 22–25).

In particular, the FLICE-inhibitory protein (c-FLIPL) expression, relates to negativity in ligature marks (17).

Selected markers were compared to skin samples obtained from subjects who died of other causes. Five molecules (FLIP, AQP3, Tryptase, CD15, IL-15) (17, 21, 24) resulted to be statistically significant (**Figure 2**).

The comparison between AVIDIN, CD1A, and MHC Class II did not produce relevant results (22). Another interesting result—showing a good significance—emerges from FLIP immunostaining, when comparing between ligature mark produced ante-mortem and vital skin wounds (17).

The present review article gives an updated overview of molecular and immunohistochemical assessment of ligature marks in hanging. The research focused on three key points: ligature mark dehydration, immunological response to mechanical injury, apoptosis induction as a direct consequence of previous points.

Actually, in ligature marks (due to physical and chemical processes involved) an increase of apoptosis is observed—as evidenced by FLIP depletion.

Immunohistochemical detection of AQP3 (15) and increase in the concentration of different electrolytes (16) (Fe and Zn; Fe/Ca ratio; Fe/Mg ratio) strictly relies on ligature mark dehydration as a response to intracellular water depletion.

The inflammatory response in the ligature mark is linked to the presence of neutrophils, macrophages, Langerhans cells, and dendritic cells, as revealed by the commonest immunohistochemical and molecular biology tests.

With the improvements of molecular biology, the analysis of time-dependent degradation of nucleic acids (especially RNA, miRNAs) has become a crucial point in forensic practice. In the future, miRNAs could become reliable forensic biomarkers for the diagnosis of vitality in ligature marks (23, 31–33).

Although the present study does not offer an alternative diagnostic process in differentiation between suicidal hanging and simulated hanging, a modification of the actual protocol (14) on ligature mark vitality study is proposed: along with crime scene investigation, external examination of the body, classical histology sampling, an immunohistochemical analysis should be added as routine use in the diagnostic process (both as a first-line test, and as a confirmation one), as demonstrated by the great reliability of identified markers.

All presented molecular markers, even though promising, need a stronger validation with standardized, multi-centric studies, conducted on larger populations. It is also preferable to perform a confrontation between cases of simulated hanging and same-features samples.

The forensic investigation in hanging should rely on modern and validated markers, even to the combination of different markers, to guarantee high reliability in judicial trials.

AUTHOR CONTRIBUTIONS

VF and AM: conceptualization. PF: methodology and funding acquisition. RL: validation. AD: resources. PS and LP: writing—original draft preparation. FD and AM: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Bone Marrow-Derived Cells and Wound Age Estimation

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Appropriate technology as well as specific target cells and molecules are key factors for determination of wound vitality or wound age in forensic practice. Wound examination is one of the most important tasks for forensic pathologists and is indispensable to distinguish antemortem wounds from postmortem damage. For vital wounds, estimating the age of the wound is also essential in determining how the wound is associated with the cause of death. We investigated bone marrow-derived cells as promising markers and their potential usefulness in forensic applications. Although examination of a single marker cannot provide high reliability and objectivity in estimating wound age, evaluating the appearance combination of bone marrow-derived cells and the other markers may allow for a more objective and accurate estimation of wound age.

Keywords: bone marrow-derived cells, hematopoietic stem cells, mesenchymal stem cells, skin wound, wound healing, wound age

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INTRODUCTION

Wound healing is a dynamic process in which numerous cells and extracellular matrix structures are involved. These cellular and molecular events are highly regulated. Wound healing is an ordered and controlled progression that matures through artificially defined phases of hemostasis (coagulation), inflammation (infiltration of granulocytes and mononuclear cells), proliferation (epithelization, fibroplasia, and angiogenesis), and maturation (collagen deposition and formation of scarring tissue) (1–5) (**Table 1**).

In the first step, platelet activation and the coagulation cascade play a major role, with fibrin strands adhering in the first few seconds, subsequently forming blood clots and trapping platelets in the wound area. The inflammatory phase is triggered by the recruitment of inflammatory cells to the wound site and attempts to eliminate damaged cells. Leukocyte recruitment is a hallmark of the inflammatory phase. In the first event, neutrophils infiltrate the wound site for the sterilization, followed by the accumulation of monocytes and lymphocytes. These leukocytes secrete various bioactive molecules, such as cytokines, chemokines, enzymes, and growth factors (6). Cytokines and chemokines are involved in the wound healing process by recruiting leukocytes. To date, several candidates, including IL-1, IL-6, IL-8, and TNF-α, have been identified for wound age determination in the early phase after injury (7–9).

The main objective of the proliferative phase is to cover and fill the wound. The margins of the wound start contacting with fibroblasts that are activated and differentiate into myofibroblasts. Thereafter, the re-epithelialization process also begins. This stage mainly results from extracellular matrix (ECM) deposition of collagen (10–12). Finally, during maturation, collagen fibers are reorganized from collagen type III to type I, tissue is restructured, and strength and flexibility are gained by promoting epithelialization and angiogenesis (13–15). In forensic pathology, growth factors capable of stimulating cell proliferation and cellular differentiation, such as TGF-α and TGF-β1, and some types of collagens have been shown to be available for wound age estimation (16, 17).

TABLE 1 | Wound healing phases.

Wound healing phases	Main events
Homeostasis	When blood vessels constrict, platelets are activated by contact with exposed collagen, releasing their granules, which further leads to platelet activation and aggregation. Along with activation of the coagulation cascade which results in the deposition of a temporary fibrin matrix within the wound (1, 2).
Inflammation	Numerous cytokines are secreted to promote neutrophil and macrophage chemotaxis, leading to the onset of the inflammatory phase (2, 3). Neutrophils are one of the first cells to appear acutely. Macrophages aid in phagocytosis and produce more cytokines and growth factors that promote fibroblast proliferation, angiogenesis, and keratinocyte migration.
Proliferation	Fibroblasts recruit to the wound transform into myofibroblasts under the influence of several cytokines, causing increased collagen production and eventual wound contraction (4, 5). Modeling and establishment of new blood vessels are important in wound healing and occurs simultaneously at all stages of the repair process (2).
Maturation	Granulation tissue is replaced by permanent scar (2).

Recently, several lines of accumulating studies have shown that bone marrow (BM)-derived cells (BMDCs) may contribute to tissue repair and/or regeneration of damaged tissue including the skin (18–21). After tissue injury, hematopoietic and multipotent progenitor cells are mobilized from the BM into a pool of circulating cells, which migrate to the site of injury and regulate the proliferation and migration of epithelial and dermal mesenchymal cells in the early inflammatory phase (22). The contribution of BMDCs to inflammatory cells in the acute response to injury is well-established, and the long-term role of BMDCs in the healing of skin wounds is being elucidated.

In this review, we assess the characteristics and key functions of BMDCs at each step of the wound healing process and whether they can be useful markers for forensic diagnosis of wound age.

HISTORY OF WOUND AGE ESTIMATION

Raekallio first introduced the application of a new method of enzymatic histochemistry and presented some new data for estimating wound age (23). A few years later, an important biochemical technique was reported that involved the detection of serotonin and histamine at the wound edge (24, 25). Over the next decade, significant progress has been made in the scientific research on immunology and immunohistochemistry. The application of immunohistochemical techniques has paved the way for a new field of wound age research by forensic pathologists (26). In the following decade, knowledge of basic immunological principles and application of immunohistochemical methods have led to significant scientific development (7, 8, 17, 27–44). The history of clinical medicine has been correlated with advances in basic research. Since forensic medicine is applied medicine, it is always necessary to apply the latest basic research knowledge to practice forensic medicine.

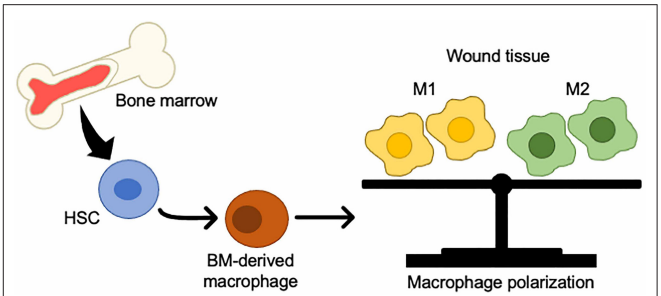

FIGURE 1 | Changes in BMDCs to macrophages in wound tissue. BM-HSCs toward M1/M2 macrophages in injured tissue.

BM-DERIVED HEMATOTOIETIC STEM CELLS

BM-Derived Hematopoietic Stem Cells (BM-HSCs) in Wound Healing

Hematopoietic stem cells (HSCs) constitute a relatively large fraction of BM mononuclear cells (45). Differentiation of HSCs into macrophages is one of the most important events during wound healing (46). There exist two major sources of wound macrophages: resident and BM; the latter accounts for a larger proportion and plays a dominant role in wound healing (47–49). When attracted to the wound, monocytes differentiate into macrophages, which can engage in multiple activities with many possible phenotypes (50, 51). Early in the healing process, macrophages produce multiple cytokines and chemokines that stimulate the inflammatory response (52, 53). Wound macrophages actively phagocytose, removing microbes, dying cells, and necrotic material (54). Several studies have suggested that macrophage phagocytosis of senescent neutrophils causes a switch from a pro-inflammatory to a growth-promoting phenotype (55).

BM-derived monocytes from the circulation are classified as either inflammatory monocytes, which are $CD14^{+}CD16^{-}$ that can differentiate into M1 macrophages, or anti-inflammatory monocytes, which are $CD14^{low}CD16^{+}$ that give rise to M2 macrophages (56). In a mouse model of wound healing, circulating monocytes can also be divided into two groups: $CX3CR1^{low}CCR2^{+}Ly6C^{+}$, which produces inflammatory cytokines and enters the wound first, and $CX3CR1^{high}CCR2^{-}Ly6C^{-}$ which enters later (57). M1 macrophages play an important role in protection from pathogens by producing high levels of iNOS and inflammatory cytokines such as TNF-α, IL-1b, IL-6, and IL-12, and initiate a Th1 immune response (58). M2 macrophages have anti-inflammatory properties and are characterized by high IL-10 secretion and high arginase-1 expression (58, 59) (**Figure 1**).

The potential of BM-HSCs in skin regeneration is derived from their high plasticity and involvement in the angiogenesis

(60, 61). In addition, they also affect ECM during wound healing by secreting collagen and downregulating MMP expression (62). Moreover, they stimulate the proliferation of keratinocytes and fibroblasts, significantly accelerating wound closure (63).

Macrophages in Wound Age Estimation

Macrophages are mononuclear phagocytes that are recruited from the BM under inflammatory conditions, such as tissue repair (64). Macrophages are involved in host defense, the initiation and resolution of inflammation, growth factor production, phagocytosis, and tissue restoration in wounds (65). During inflammation, macrophages are recruited to the wound site to develop classical and alternative activation phenotypic polarization mediated by cytokines, oxidants, lipids, and growth factors released by macrophages (57, 64, 66). These cells regulate the response to changing wound environments and participate in multiple overlapping wound healing phases.

During early wound healing, macrophages help to clear the wound of contaminating microbes and apoptotic neutrophils and debris via phagocytosis (67–69). In addition, macrophages regulate the activity of other wound cells through the production and release of cytokines, chemokines, and growth factors. Early after injury, macrophages release numerous inflammatory cytokines and chemokines, including IL-1β, IL-6, TNF-α, and CCL2, to amplify the inflammatory response (70). Topical application of CCL2 could promote skin wound healing in diabetic mice, and these effects may be mediated by the action of CCL2 on macrophages (71). Indeed, immunohistochemical studies on the time-dependent expression of chemokines in human skin wounds have shown that inflammatory macrophages are positive for anti-CCL2 antibodies; moreover, a positive rate of $>$ 30% for CCL2 indicates a wound age of at least 1 day (8).

Cyclooxygenase (COX) is an enzyme that is responsible for the formation of prostanoids, including thromboxane and prostaglandins such as prostacyclin, from arachidonic acid (72). COX-1 is constitutively expressed under physiological conditions, and COX-2 is expressed for increased production of prostanoids that occur at the site of disease and inflammation. Therefore, COX-2 may be involved in the inflammatory phase of wound healing. In human skin wound specimens, neutrophils are the main COX-2 expressing cells; however some macrophages also express COX-2 (73). In addition, the number of MMP-2^+ and MMP-9^+ macrophages significantly increase with wound age (74). These observations indicate that immunohistochemical detection of increased number of MMP-2^+ and MMP-9^+ macrophages in skin wounds, in combination with other markers such as COX-2, further enhances the reliability of wound age estimation.

Wound macrophages are also an important source of growth factors such as VEGF, which is important for angiogenesis (75, 76). Moreover, macrophages have been shown to be involved in collagen degradation during the tissue remodeling phase of wound healing (77, 78). In human wound specimens with wound ages of $>$7 days, granulation tissue and angiogenesis were observed with the migration of VEGF$^+$ macrophages (79).

Mast Cells in Wound Age Estimation

Mast cells (MCs), that are one of the immune cells involved in allergy and anaphylaxis, play pivotal roles in skin wound healing thorough the release of chemical mediators such as histamine and the production of cytokines and chemokines (80). From the aspects of wound age estimation, there are several immunohistochemical studies on the dynamics of MCs with focusing on triptase and chimase (81, 82). The number of MCs immediately increased after wounding, eventually reaching a peak at 1–3 h later followed by decreasing within 6 h (81, 82). The post-mortem release of proteins from MCs is known as an influence factor on the data interpretation, which should be taken into consideration in the forensic practices (83). In order to avoid the influences of the postmortem release from MCs, the stem cell factor (SCF) and the Kit receptor, which involved in the survival, growth, migration, and activation of MCs, are investigated. Actually, SCF$^+$ cells rapidly increased in the dermis by day 1 after injury, whereas the Kit receptor elevated more gradually, with a peak on day 14 (84). On the contrary, Oehmichen et al. (85) investigated the loss of MC enzymatic activity at the wound margin, and found the loss of naphtol AS-D chloroacetate esterase (NASDCAE) activity at wound margins in injuries of $<$ 60 min (85).

Dendritic Cells (DCs) in Wound Age Estimation

DCs are mononuclear and antigen presenting immune cells. DCs have an ultimate origin in HSCs from the BM (86). Intermediate precursors of DCs lack a lineage-specific marker (lin$^-$) and can be sought among BM cells that have not yet expressed DC markers such as CD11c and surface MHC class II molecules. Later, DC precursors can be found in BM cells that already express the DC marker CD11c, but that still lack cell surface expression of MHC class II molecules (86).

Several studies have indicated that dermal DC recruitment may be involved in the repair process of damaged tissue (87–90). CD11c and HLA-DR are considered specific markers for dermal DCs (91). Kuninaka et al. performed a double-color immunofluorescence analyses with anti-CD11c and anti-HLA-DRα antibodies to detect DCs in human skin wounds from autopsies (92). DCs were rarely detected in wounds aged $<$1 day, whereas DC accumulation increased over time in wounds aged 3–14 days. These findings suggest that DCs could be a useful cellular marker for determining wound age.

There is a specific DC population in the human epidermis, and those epidermal DCs express CD1a and CD207/langerin, and is called Langerhans cells (93). However, there is only one forensic study exploring the dynamics of dermal DCs after wounding. Bacci et al. (94) investigated the behavior of epidermal DCs/Langerhans cells in relation with wound ages. Both MHC-II$^+$ cells and CD1a$^+$ cells rapidly increased in number within the first hour after injury. Especially, CD1a$^+$ cells, as well-differentiated Langerhans cells, increased earlier and for a shorter time period than MHC-II$^+$ cells. These observations implied that the behavior of epidermal DCs/Langerhans could give a

useful information to differentiate antemortem skin lesions from postmortem damage especially in neck compression cases.

BM-DERIVED MESENCHYMAL STEM CELLS

History of BM-Derived Mesenchymal Stem Cells (BM-MSCs)

BM contains HSCs and MSCs. MSCs were first observed in the BM by Cohnheim in 1867 (95). Cohnheim discovered that these cells could be a source of fibroblasts involved in wound repair. Subsequently, these cells were isolated and cultured by Friedenstein (96). While culturing cells from rat BM, Friedenstein discovered that these cells were a population of non-hematopoietic cells that were morphologically similar to fibroblasts attached to the plastic of the culture flask. The term "mesenchymal stem cells" was presented by Caplan in 1991 after conducting human BM studies (97). To date, it is a hot topic of research that is being explored for multiple purposes.

BM-MSCs in Wound Healing

With the expansion of MSC research, its potential role in skin wound healing has been elucidated. BM-MSCs can accelerate wound healing by regulating the function of inflammatory cells such as neutrophils, macrophages and lymphocytes to provoke an anti-inflammatory response (98). In addition, BM-MSCs can be directed to differentiate into multiple skin cell lineages, including keratinocytes and endothelial cells, and secrete various cytokines to promote wound re-epithelialization and limit excessive scarring (98–103). In addition, BM-MSCs can be recruited to the wound site to induce neovascularization and to increase cell migration and proliferation (104, 105).

Several studies have revealed the underlying mechanisms of BM-MSC recruitment to the wounds. BM-MSCs express CCR7, a receptor of CCL21, which was found to be the main factor responsible for enhanced BM-MSC migration to the wounds in mice (106). Intradermal injection of CCL21 increased the recruitment of BM-MSCs to the wound, resulting in accelerated repair (106). Moreover, serum levels of HMGB1 are increased by skin grafting, and intravenously administered HMGB1 augment the accumulation of PDGFRα^+ MSCs in the skin graft by enhancing the expression of the SDF-1 receptor CXCR4 in these cells (107, 108).

The inflammatory phase is important for the wound healing process because it leads to the recruitment of immune cells to remove pathogens and clear the wound. MSCs can suppress the inflammatory responses in several ways. It is generally recognized that infiltrative M2 macrophages play an important role in the progression of wound healing, the promotion of angiogenesis, and the suppression of inflammation (109–111). MSCs promote macrophage polarization to the M2-like functional phenotype, which reduces inflammation and immunosuppressive function (112). Zhao et al. revealed that IL1RA from BM-derived MCSs inhibits the production and activity of IL-1 and TNF-α (113). These studies suggest that MSCs exhibit anti-inflammatory potential through the regulation of macrophage polarization and expression of anti-inflammatory cytokines.

In the proliferative phase, macrophages release growth factors such as EGF and TGF-α to stimulate keratinocyte migration and proliferation (114). Smith et al. revealed that BM-MSCs are a source of soluble signals that regulate dermal fibroblast migration and proliferation (115). MSCs can also contribute to angiogenesis at the wound site. In the wound area, MSCs secrete growth factors such as VEGF, PDGF, bFGF, and angiopoietin-1 to promote angiogenesis and wound healing (116, 117). In addition, SDF-1 secreted by MSCs induces endothelial cell survival, vascular branching, and pericyte recruitment (118). These paracrine mechanisms of MSCs play important roles in angiogenesis. The wounds treated with MSC-seeded hydrogels showed a significant enhancement of angiogenesis, which was associated with elevated VEGF levels within the wound (119). Qiu et al. demonstrated that educated MSC exosomes significantly increase wound healing by inducing angiogenesis (120).

MSCs have also been shown to contribute to the production and remodeling of ECM during the wound healing process. BM-MSCs secrete high levels of TIMPs, which stabilize vessels and protect the vascular basement membrane, forming MMP-induced degradation (121). This ECM production and remodeling function of MSCs may be associated with the promotion of angiogenesis and the formation of granulation tissue.

BM-MSCs may be involved in the regeneration of mesenchymal and other embryonic tissues, including the skin (106). In animal models of wound healing, intravenously transplanted MSCs can differentiate into cells of resident tissue, including fibroblasts, myofibroblasts, vascular endothelial cells, pericytes, and keratinocytes in the wound area (106, 116). In addition, MSCs injected into mouse wounds transdifferentiate into keratin-14$^+$ keratinocytes *in vivo* (106, 116). Labeled MSCs were observed in the hair follicles, sebaceous glands, and blood vessels in full-thickness wounds in an animal model (122). BM-MSC-engineered skin (EGF loaded) has been found to repair sweat glands and improve skin wound healing (123).

These studies indicate that BM-derived MSCs can differentiate into tissue-specific cells, secrete a wide range of paracrine factors, and regulate the immune response and the local tissue microenvironment (**Figure 2**).

Fibrocytes in Wound Age Estimation

In 1994, a distinct population of blood-borne fibroblast-like cells that rapidly entered sites of tissue injury was described (124). These cells, named "fibrocytes," comprise 0.1–0.5% of the non-erythrocyte cells in the peripheral blood and show an adherent, spindle-shaped morphology when cultured *in vitro*. Cultured fibrocytes express the fibroblast products including type I collagen I (Col I), type III collagen (Col III), and fibronectin, CD45RO, CD13, and CD34. Additionally, fibrocytes express MHC class II and costimulatory molecules (CD80 and CD86) and can present antigens *in vitro* and *in vivo* (125, 126). Fibrocytes differ from monocytes/macrophages, dendritic cells, and other antigen-presenting cells in their morphology, growth properties, and cell surface markers. In addition, fibrocytes isolated from

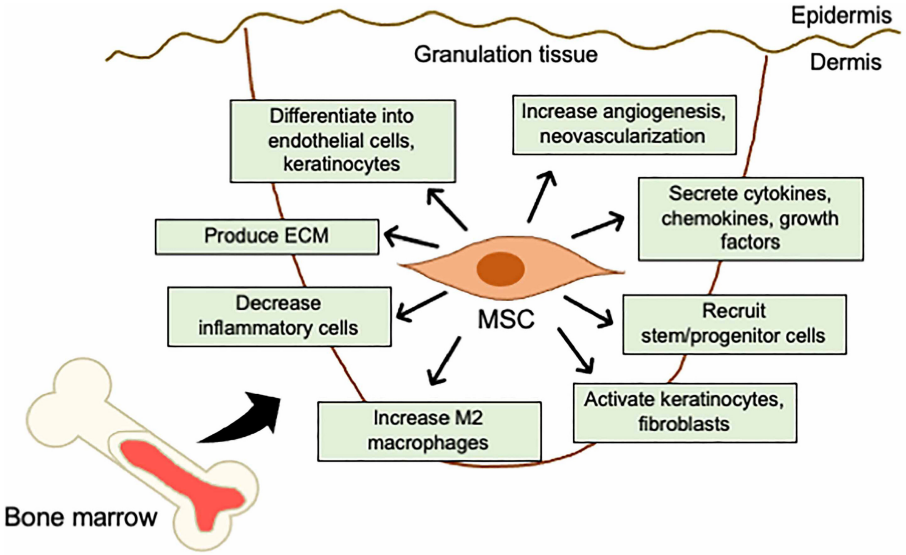

FIGURE 2 | Mechanistic roles of MSCs in the skin wound healing. Mechanisms of acceleration of wound healing by MSCs; (i) activation of keratinocytes and fibroblasts, (ii) increase in angiogenesis and neovascularization, (iii) increase in M2 macrophages infiltration, (iv) recruitment of stem/progenitor cells, (v) secretion of cytokines and growth factors, (vi) production of ECM, (vii) decrease in inflammatory cytokine levels by immunosuppressive effects, and (viii) differentiation into endothelial cells, fibroblasts, and keratinocytes.

peripheral blood and cultured *ex vivo* secrete cytokines, growth factors, and chemokines (127). TGF-β functions as a fibrocyte maturation factor during differentiation (128, 129).

There is an increasing evidence that fibrocytes contribute to new fibroblast and myofibroblast populations during wound healing. Prior to differentiation, immature fibrocytes secrete ECM-degrading enzymes, including MMP-2, -7, -8, and -9 which promote the migration of fibrocytes into granulation tissue and endothelial cell invasion (130, 131). CCL21 acts as a potent stimulus for fibrocyte chemotaxis *in vitro* and for the migration of injected fibrocytes to sites of skin wound site *in vivo* (128). In addition, exogenous TGF-β1 stimulates *in vitro* differentiation and synthetic activity of cultured human fibrocytes into mature fibroblasts or myofibroblasts (128). Moreover, fibrocyte differentiation can occur in conditions where serum amyloid P (SAP) and aggregated IgG levels are low, such as during the resolution phase of inflammation (132, 133). The main fibrocyte secreting cytokines include TGF-β1 and CTGF (134). Moreover, fibrocytes indirectly regulate resident fibroblast activity during wound healing (132).

Although the role of fibrocytes in wound healing has been postulated based on their accumulation at the wound sites (124), the molecular signals that mediate the migration of fibrocytes to the wounds have not been investigated. Abe et al. demonstrated that fibrocytes express several chemokine receptors, such as CCR3, CCR5, CCR7, and CXCR4 (128). Furthermore, Ishida et al. showed that $Ccl3^{-/-}$ and $Ccr5^{-/-}$ mice exhibit reduced bleomycin (BLM)-induced fibrosis and the number of $CCR5^{+}$ fibrocytes in the lungs compared to wild-type mice (135). This finding indicates that the CCL3-CCR5 axis can mediate the migration of BLM-induced fibrocyte to the lungs. In addition, fibrocytes also express CX3CR1, and their population increases in the lungs of mice with BLM-induced pulmonary fibrosis (136). These findings suggests that the CX3CL1-CX3CR1 axis is essential for the development of BLM-induced pulmonary fibrosis by regulating fibrocytes capable of exerting fibrosis-promoting activity. Therefore, some chemokine systems may be involved in the migration of fibrocytes to damaged and fibrotic tissues.

Ishida et al. performed a double-color immunofluorescence analyses using anti-CD45 and anti-Col I antibodies to examine the time-dependent appearance of fibrocytes in human skin wounds of different age groups (137). The appearance of fibrocytes in human skin wounds occurs at least a 4-days post infliction; therefore, detection of fibrocytes could be a useful marker for wound age determination.

Endothelial Progenitor Cells (EPCs) in Wound Age Estimation

EPCs are cells that act as endothelial precursors and help promote angiogenesis to improve tissue perfusion. EPCs were first described in 1997 as a population of postnatal mononuclear blood cells that have been shown to promote angiogenesis following recruitment from the BM (138, 139). EPCs are positive for the following cell surface markers: CD31, CD45, CD14, CD105, CD146, VEGFR-2, CD144, and von Willebrand factor (vWF). Morphologically they appear spindle-shaped (140, 141), and the presence of CD14 and CD45 on these cells indicates that they are hematopoietic rather than of endothelial origin. In addition, markers such as CD31, CD144, VEGFR-2, vWF, and eNOS are not necessarily endothelium-specific (142). There is no single marker that defines EPCs, and a combination of

TABLE 2 | Summary of related studies on the appearance and effects of BMDCs on the skin wound healing process.

Cell types	Markers	Methods	Model source used	Functions, effects/findings	Regular detection	Time frame	References
Macrophages	$F4/80^+CD115^+$ $CD11b^+$	Create a full-thickness wound on the back skin of WT mice, and transplant WT or db/db HSCs	Mouse	FACS analysis show that type 2 diabetes impairs monocytes/macrophages infiltration and ultimately impairs wound healing	3–14 d	3–14 d	(46)
Macrophages	CX3CR1-GFP	Create full-thickness wounds on the back skin	Mouse	FACS analysis show the GFP^{hi} population increases after injury	4–7 d	0–7 d	(47)
$CD34^+$ stem cells	$CD34^+$	Create full-thickness wounds on the back skin and transplant nanofiber-expanded human umbilical cord blood-derived (NEHUCB) $CD34^+$ cells	Mouse	GFP-NEHUCB $CD34^+$ cells home to wound area and accelerate wound healing	3 h−7 d	3 h to 7 d	(62)
Macrophages	Macrophage morphology	A subcutaneously implanted polyvinyl alcohol (PVA) sponge wound model (7 cm skin incision on the back)	Rat	Phagocytosis of wound macrophages on wound neutrophils	5–10 d	1–10 d	(67)
Macrophages	$F4/80^+$	Create full-thickness wounds on the back skin	Mouse	Immunohistochemistry staining of wounds shows that there is no difference in macrophage recruitment to the wounds of WT and $PPAR\gamma^{-/-}$ mice	3–5 d	3–5 d	(69)
Macrophages	$F4/80^+$	Create full-thickness wounds on the back skin	Mouse	Western blotting analysis shows that diabetic mice exhibit reduced infiltration of macrophages into wounds, and ultimately impaired healing	Uninjured and days 1–10	Uninjured and days 1–10	(71)
$MMP\text{-}2^+$ macrophages	$CD68^+MMP\text{-}2^+$	Double-color immunofluorescent staining using skin wounds	Human	$MMP\text{-}2^+$ macrophages on skin wounds are useful markers for determining the age of wounds	9–12 d	Uninjured and 12 h to 21 d	(74)
Macrophages	$F4/80^+CD11b^+$	Create full-thickness wounds on the back skin	Mouse	FACS analysis shows that CCR2 deficiency reduces macrophage infiltration into the skin wounds	2–7 d	2–14 d	(76)
DCs	$FXIIIa^+$	Immunostaining burn specimens	Human	Need further studies to clarify the significance of FXIIIa expression by dermal cells	Uninjured and days 5–30		(87)
Plasmacytoid DCs (pDCs)	$PDCA1^+B220^+$	Measure pDCs in tape stripped skin by flow cytometry	Mouse	Immunohistochemistry for Siglec-H, pDC-specific marker, shows lymphocytic cells in injured skin	24 h	24–48 h	(89)
pDCs	$BDCA2^+$	Immunostaining tape stripped skin	Human	Injury induces pDC infiltration and expression of IFN-α	24 h		(89)
DCs	$CD11C^+MHC\text{-}II^+Ly6G^-$	Measure DCs in burned skin by flow cytometry	Mouse	Wound closure in DC-deficient mice is delayed	4 d		(90)
DCs	$CD11c^+HLA\text{-}DR\alpha^+$	Double-color immunofluorescent staining using skin wounds	Human	The appearance of DC in human skin wounds provides information to help determine the age of the wound	4–14 d	3–21 d	(92)
MSCs	GFP^+	Create a full-thickness excisional skin wound and transplant with GFP^+ MSCs	Mouse	FACS analysis show that about 10% of total cells in day 7 wounds are GFP^+ BM-MSC, and MSCs enhance wound healing	7–14 d	7–28 d	(116)
Fibrocytes	$Col\ I^+CD34^+$	Implant the wound chamber	Mouse	10–15% of the cells present in wound chamber fluid are fibrocytes	Rapidly	Over 10 d	(124)
Fibrocytes	$Col\ I^+CD11b^+$	Inject cultured murine fibrocytes into the tail vein and create a full-thickness skin wound	Mouse	Chemokine SLC acts as a potent stimulus for homing of fibrocytes to the site of tissue injury	4 d		(128)
Fibrocytes	$Col\ I^+$	Culturing peripheral blood mononuclear cells (PBMC) in burn patients	Human	Fibrocyte development is systemically increased in burn patients	7 d to 12 m	7 d to 12 m	(129)

(Continued)

TABLE 2 | Continued

Cell types	Markers	Methods	Model source used	Functions, effects/findings	Regular detection	Time frame	References
Fibrocytes	$CD45^+$Col I^+	Double-color immunofluorescent staining using skin wounds	Human	Fibrocytes are involved in wound healing in human skin, and detection of fibrocytes is a useful marker for wound age determination	9–14 d	4 d to 21 d	(137)
Human EPCs	$acLDL^+$ulex-$lectin^+$	EPC transplantation into a dermal excisional wound model	Mouse	EPC transplantation increases neovascularization and ultimately accelerates wound re-epithelialization			(1)
Mouse EPCs	c-$Kit^+$$Tie\text{-}2^+$	Create full-thickness wounds on the back skin	Mouse	FACS analysis shows that the absence of CCR5 reduce vascular EPC accumulation, and ultimately delay skin wound healing	2–4 d	2–4 d	(156)
Human EPCs	$CD34^+$$Flk\text{-}1^+$	Double-color immunofluorescent staining using skin wounds	Human	EPC detection helps determine wound age	7–12 d	2–21 d	(159)

markers has been used to identify them within a heterogeneous population. EPCs are mobilized from the BM through a complex process involving enzymes, growth factors and cell surface receptors. The first step in the EPC mobilization is MMP-9 activation (143). VEGF plays an important role in the activation of MMP-9 and can increase the recruitment of EPCs from the BM (144). Interestingly, fibrocytes can produce angiogenic factors, including MMP-9 and VEGF, as demonstrated *in vivo* (131).

EPCs are known to be sensitive to hypoxia because they respond to HIF-1-induced SDF-1 under conditions of oxygen deprivation (145). They contribute to angiogenesis and are promising targets for the treatment of chronic wounds such as diabetic ulcers (146–148). Transplanted BM-MSCs induce the recruitment of endogenous EPCs to the wound site from the BM or circulation via growth factors such as VEGF, PDGF, HGF, and insulin-like growth factor and the SDF-1-CXCR4 axis (149–153). Transplantation of human EPCs into a mouse skin wound model has been shown to accelerate wound closure and increase angiogenesis (154). EPC transplantation accelerated wound re-epithelialization in a mouse skin excision wound model compared to that in control mice (155). EPCs produce several chemoattractants of monocytes and macrophages which are known to play important roles in the early stages of wound healing. In addition, EPCs migrate to the wound and are incorporated directly into the newly formed capillaries in the granulation tissue (155). Thus, EPCs have the potential to differente into the endothelium, recruit other cells to the wound site, and secrete growth factors and cytokines; these factors explain the effects on wound healing.

Ishida et al. demonstrated that topical application of CCL2, a potent macrophage chemoattractant, can promote neovascularization, collagen accumulation, and eventual cutaneous wound healing in mice with diabetes (71). The effects of CCL2 may be mediated by EPCs and macrophages, which are critically involved in angiogenesis and collagen production, respectively, and their effects on steps essential to the wound healing process. In addition, the CCL5-CCR5 axis is essential for EPC recruitment (156). In a mouse model of skin wounds, gene expression of the *Ccl5* and *Ccr5* genes was upregulated at the wound sites and CCR5 protein was detected in endothelial cells. $Ccr5^{-/-}$ mice showed delayed wound healing with diminished neovascularization. The $CCR5^+$ EPCs were directly incorporated into the vasculature at the wound sites. Moreover, EPCs produce growth factors such as TGF-β and VEGF, which are important for skin wound healing (157, 158). These observations suggest that EPCs may contribute to skin wound healing as a source of endothelial cell origin and growth factors.

The accumulation of EPCs in wound sites increases over time after injury; this finding indicates that EPC accumulation may help estimate wound age (156, 159). In forensic practice, examining only a single marker does not provide forensic safety; therefore, some markers need to be investigated in wound samples for a more accurate estimation of wound age. For example, detection of both EPCs and VEGF (79) provides more reliable information for estimating wound age, especially during the proliferative phase, as their collaboration synergistically promotes angiogenesis.

CONCLUSION AND FUTURE PERSPECTIVES

Over the last few decades, numerous studies have elucidated the role of BMDCs in skin wound healing (**Table 2**). It is clear that BMDCs have great potential for skin tissue regeneration as they not only regenerate lost tissue, but also promote wound repair in a paracrine manner. Several cell types, including HSCs and MSCs, are currently being investigated. Recent data on BM cell therapy in skin repair show great promise as therapeutic agents in clinical practice. Further investigation into experimental and clinical applications is required to identify the most effective cell migration system for BM cells at the wound sites. However, it is evident that BMDCs contribute to skin wound healing; therefore, these cells can serve as candidates for wound age estimation

in forensic practice. New molecular biomarkers and innovative devices and technologies are constantly being sought to correctly diagnose the cause of death, postmortem interval, wound age, and more.

Recent studies have stimulated us to recognize the importance of BMDCs in the skin; however, many questions remain. For example, BMDCs contribute not only to inflammatory and mesenchymal cells of the dermis, but also to keratinocytes of the epidermis. In addition, it is not yet known whether BM-derived cells are essential to contribute to the cells that make up the normal skin. Furthermore, the specific types of BMDCs playing a role in these processes are unidentified. We believe that answers to these questions will help us understand skin homeostasis and the wound healing process as well as develop new techniques for future skin wound age estimation in future.

Finally, there is a limitation of wound age estimation as the forensic evidence. From the aspects of forensic pathology, the purpose of wound age estimation is to present the objective evidence in court. Cell types, enzymes and chemical mediators are experimentally and practically applied to wound age estimation as the marker (160). Actually, when only a single marker is investigated, contradictory results are often obtained, eventually making confusion the interpretation of data. It is needless to say that various populations of BMCs and BMC-derived enzymes as well as chemical mediators should be investigated. Moreover, among multiple forensic institutes, the accumulation of practical evidence using different types and sizes of wound samples with known post-injured intervals is necessary.

AUTHOR CONTRIBUTIONS

YI and TK contributed to conception and design of the review, drafted the original manuscript and drew the figures, and provided the main funding for the manuscript. MN edited various versions of the manuscript. All authors read and approved the final version of the review.

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Forensic Application of Epidermal Ubiquitin Expression to Determination of Wound Vitality in Human Compressed Neck Skin

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Ubiquitin is a member of the heat shock protein family and is rapidly induced by various types of stimuli, including ischemic and mechanical stress. However, its significance in determining wound vitality of neck compression skin in forensic pathology remains unclear. We immunohistochemically examined the expression of ubiquitin in the neck skin samples to understand its forensic applicability in determining wound vitality. Skin samples were obtained from 53 cases of neck compression (hanging, 42 cases; strangulation, 11 cases) during forensic autopsies. Intact skin from the same individual was used as the control. Ubiquitin expression was detected in 73.9% of keratinocytes in intact skin samples, but only in 21.2% of keratinocytes in the compression regions, with statistical differences between the control and compression groups. This depletion in the case of neck compression may be caused by the impaired conversion of conjugated to free ubiquitin and failure of *de novo* ubiquitin synthesis. From a forensic pathological perspective, immunohistochemical examination of ubiquitin expression in the skin of the neck can be regarded as a valuable marker for diagnosing traces of antemortem compression.

Keywords: ubiquitin, compression, neck skin, immunohistochemistry, forensic pathology

INTRODUCTION

In forensic practices, differentiating antemortem injury from postmortem damage is one of the important issues, which is a classical but still modern topic (1–3). Thus, there are numerous forensic studies exploring available markers for the determination of wound vitality using biochemical, histochemical, immunohistochemical and molecular biological techniques (4–6). Moreover, there are several recent studies focusing on omics sciences and micro RNA (7, 8).

Fatal asphyxia due to neck compression is often encountered during forensic autopsies. Neck ligature marks can result from various lesions, including manual strangulation, ligature strangulation, strangulation, direct striking, armlocks, and cord entanglement (9, 10). Ligature mark is the most important finding on the neck for forensic pathologists. The mark is in the

form of a furrow or groove in the tissue, pale in color and may later change from tan to dark brown. The dryness and desiccation of the abraded skin makes the mark hard and parchment-like (11). In contrast, if a soft material, such as a towel, is used, or if a beard or a part of the cloth is between the ligature and the skin, the ligature mark may be faint or inconspicuous. Microscopy of the thyroid and salivary glands usually reveals focal interstitial hemorrhages, and the lymph gland shows congestion, supporting the antemortem nature of neck compression (12). However, only few studies have focused on the relationship between the ligature compression and neck tissue, and very few markers have been investigated as an evidence for neck compression (13–15).

Ubiquitin is an 8.5 kDa protein containing 76 amino acids and is commonly found in eukaryotic cells (16). Ubiquitin has seven lysine and methionine sites at its N-terminus, which tend to get self-ubiquitinated and extend to form different types of polyubiquitin chains (17). Ubiquitination is closely associated with many cellular processes such as cell cycle regulation, immune response, inflammatory response, and apoptosis. Major chronic degenerative diseases in humans, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, are primarily associated with the nervous system, and ubiquitin is incorporated into many inclusion bodies that characterize these neurodegenerative diseases (18). Additionally, ubiquitin is involved in the response to acute cell injury. Transcription of ubiquitin mRNA in mammalian cells is induced by heat shock and other stresses (19, 20). In contrast, short-term ischemia causes depletion of free ubiquitin in gerbil hippocampal neurons, which are the most vulnerable to ischemic injury (21). Moreover, ubiquitin gene expression after ischemia/reperfusion has been studied in the rat brains, where it was observed to initially decrease after reperfusion; however, the expression increased after the blood flow was restored (22), suggesting that ubiquitin expression may serve as an indicator of the ischemic stress.

In the field of forensic pathology, there are several studies have investigated whether ubiquitin expression can be used as an indicator for forensic diagnosis. An immunohistochemical study of ubiquitin in the human locus coeruleus has shown that the number of neurons with ubiquitin expression is significantly higher in the case of long-term death struggles (23). In human kidney tissues, ubiquitin-immunopositive tubular epithelial cells are higher than the other groups involving subjects who died due to fire, blunt injury, sharp injury, and fatal hypothermia, suggesting that ubiquitin positivity is a characteristic of death due to injury and hypothermia (24, 25). In addition, the ubiquitin immune responsiveness of pigmented substantia nigra neurons in the midbrain has been suggested to be triggered by severe, deadly stress from asphyxia, drowning, and fire (26, 27). We also have previously reported that ubiquitin would be one of the markers for wound age estimation (28). However, there have been no immunohistochemical or forensic diagnostic studies pertaining to ubiquitin using compressed neck skin. In this study, we investigated the immunohistochemical expression of ubiquitin in neck skin specimens from autopsy cases and discussed whether it can be a useful marker for the forensic diagnosis of compression.

MATERIALS AND METHODS

Antibodies

The following polyclonal antibodies (pAbs) were used for immunohistochemical analysis in the present study: rabbit anti-ubiquitin pAbs (UBA52 Ab, AF0289, Affinity Biosciences, Jiangsu, China).

Human Ligature Marks

A total of 53 ligature marks (hanging, 42 cases; strangulation, 11 cases) with a postmortem interval of <96 h were obtained from forensic autopsies at our institute. In each case, the cause of death was carefully determined based on autopsy, histopathological findings and toxicological data. Intact skin from the same individual was used as the control. The detailed profiles of all cases (sex, age, and postmortem intervals) are shown in **Table 1**.

Immunohistochemical Analysis

Skin specimens were fixed in 4% formaldehyde solution buffered with PBS for 4–7 days, embedded in paraffin, and sectioned at a thickness of 4 μm. Briefly, deparaffinized sections were incubated with PBS containing 1% normal goat serum and 1% bovine serum albumin (BSA) to reduce non-specific reactions. Thereafter, the sections were further incubated with anti-ubiquitin pAbs (dilution 1:100) for 12–17 h at 4°C. After incubation with biotinylated secondary antibodies, immune complexes were visualized using Catalyzed Signal Amplification System (Dako, Kyoto, Japan) according to the manufacturer's instructions. As a negative control, sections were incubated with normal rabbit serum instead of the primary antibodies; and no positive signal was detected in this case, thus indicating the specificity of the antibodies.

Morphometrical Analysis

To evaluate ubiquitin expression in the skin, the ratio of ubiquitin-positive keratinocytes to the total number of corresponding keratinocytes wase calculated in five randomly selected high-power fields (×400). The average values were evaluated as an indicator for ubiquitin expression. Morphometric evaluation was blindly performed by two investigators without the prior knowledge of the samples.

Statistical Analysis

The mean and standard error of the means (SEM) were calculated. Statistical analysis was performed using analysis of variance or Mann-Whitney *U*-test. Statistical significance was set at $P < 0.05$. Correlation analysis was performed using the non-parametric Spearman's correlation coefficient. Statistical significance was set at $P < 0.05$.

TABLE 1 | Cases profile.

Number	Male/Female	Age (y)		Postmortem interval (h)	
		Range	Mean	Range	Mean
53	32/21	16–90	59.2	10–84	35.8

Ethical Approval

This study was approved by the Research Ethics Committee of Wakayama Medical University (No. 3313). All the procedures were performed in accordance with the Declaration of Helsinki Principles. Moreover, this study was conducted using autopsy records from the past, and we could not obtain informed consent from the bereaved family for the use of these records. Therefore, in accordance with the "Ethical Guidelines for Medical Research Involving Human Subjects (enacted by the Ministry of Health, Labor, and Welfare in Japan), section 12–1 (2) (a) (c)." Since this was a de-identified retrospective study of archived autopsy-derived tissue, the review board of the Research Ethics Committee of Wakayama Medical University waived the need for written informed consent from the relatives of the individuals studied.

RESULTS

Immunohistochemical Analysis of Ubiquitin in Autopsy Samples

We examined the distribution of ubiquitin in the skin samples. Consistent with previous observations (28), ubiquitin-positive signals were observed predominantly in predominantly keratinocytes of uninjured skin samples (**Figure 1A**). However, in most ligature marks, ubiquitin was not detected in the keratinocytes (**Figure 1B**).

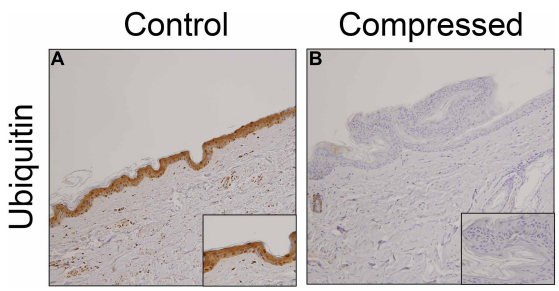

FIGURE 1 | Immunohistochemical analysis. Immunohistochemical analysis were performed by using anti-ubiquitin pAb in the human skin samples. **(A)** control; **(B)** compressed neck skin. Original magnification, × 200; inset, × 400.

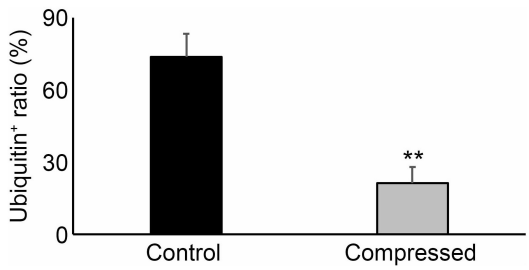

FIGURE 2 | The ratio of ubiquitin positives in the corresponding keratinocytes in the skin sample. $^{**}P < 0.01$.

The Expression of Ubiquitin Was Lower in Neck Compression Cases

As shown in **Figure 2**, the ratio of ubiquitin expression in keratinocytes was significantly suppressed in compressed skin samples, compared to that in the control samples. There were no significant differences among sex, age, and postmortem intervals in terms of ubiquitin expressions (**Figure 3**).

DISCUSSION

It is an essential work to determine wound vitality and wound age in forensic autopsy cases. To achieve the purpose, advanced biological techniques are applied to forensic pathology (4–8). In forensic medicine, the compression mark on the neck is one of the most important criteria for determining whether the

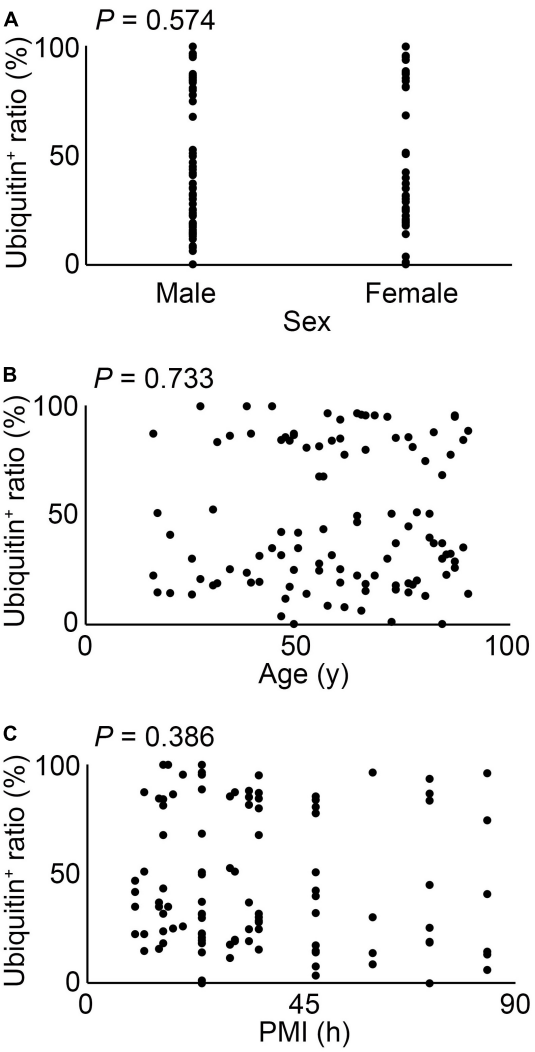

FIGURE 3 | The relation between sex **(A)**, age **(B)** or postmortem intervals (PMI) **(C)** and ubiquitin expression in all cases. These results were obtained with Spearman's correlation coefficient by rank test.

neck has been affected by the contractile force. Asphyxiation in the forensic context is associated with mechanical asphyxia through various mechanisms such as strangulation, hanging, smothering, choking and aspiration. Direct causes of death include systemic hypoxia due to airway obstruction, as well as possible neurological effects of cerebral ischemia and neck pressure. In general, it is not difficult to observe such marks upon gross examination. However, in certain situations, such as when the force is weak or the vital reaction is uncertain, it may be necessary to confirm the presence of the compression mark using a staining method during the histological examination of the neck skin. Immunohistochemical analyses may provide reliable information for the estimation of vitality using compression marks (2, 3, 15).

There are lot of studies on the determination of wound vitality of ligature marks (15). Turillazzi et al. have investigated the immunohistochemical expression of various cytokines in skin specimens from autopsy cases of death by hanging (29). Previous studies have reported that high expression of IL-15 in the skin of the neck may be a reliable marker of ligature mark vitality. IL-15 is known to activate neutrophils, which are major players in inflammation of damaged tissues. It has been reported that neutrophils were first observed in human skin wounds aged about 20–30 min (30). On the other hand, Kondo et al. have demonstrated that neutrophils were observed primarily at wound sites aged approximately 4–12 h, and were a source of cytokines and chemokines such as IL-1α, IL-8, CCL2, and CCL3 (31, 32). In an experimental study of cytokine expression during skin wound healing in mice, infiltration of numerous neutrophils producing IL-1α, IL-1β, IL-6, and TNF-α was observed at the wound sites 3 and 6 h after injury (33). Therefore, it has been suggested that inflammatory cells and cytokines may act as markers for the determination of wound age and vitality (34–36). Actually, several lines of accumulating evidence implied that IL-1β, one of the representative inflammatory cytokines, would be a candidate molecule for the discrimination between antemortem-postmortem hangings in both animal experiments and human samples. Grellner could confirm that IL-1β immunostaining of epidermal cells was a useful tool to discriminate antemortem and post-mortem hanging (37, 38).

Additionally, Maiese et al. demonstrated that intracytoplasmic depletion of FLIP playing as an inhibitor of apoptosis was evident in the epidermal layers of antemortem neck compression (39). Pérez et al. (40) reported a higher frequency of cells positive to cathepsin D and P-selectin was found in subcutaneous injured skin. Tryptase and CD15 were also useful for the determination of wound vitality in ligature marks (29). Focardi et al. (41, 42) focused on Langerhans cells determined by both antigens of MHC-II and CD1A and revealed that Langerhans cells and their-derived iNOS was significantly higher in ligature marks with vitality. Alternatively, De Matteis et al. (43) examined neck muscle tissues not neck skin samples, and evaluated the use of Troponin I—fast skeletal muscle (TNNI2) to perform differential diagnoses about vitality in suicide by hanging and simulated hanging.

Prangenberg et al. (4) showed heat shock protein was widely applied to forensic pathology such as fire-related death, hypothermia, cardiac death, drowning, excited delirium, trauma and SIDS. Ubiquitin is known as a heat-shock protein induced by several stress conditions (18). Therefore, ubiquitin may contribute to the degradation of denatured proteins produced under various stress conditions. Several lines of accumulating evidence demonstrated the availability of ubiquitin in the postmortem forensic diagnosis of pathophysiology such as asphyxia, drowning, fire, and hypothermia (24–27). Moreover, only our group examined the expression of ubiquitin in human mechanical skin wounds such as stab wounds, cut wounds, surgical wounds and lacerations (28). Subsequently, we have observed an increase of ubiquitin expression in antemortem skin wounds, and demonstrated that a significant ubiquitin positivity rate of above 30% in human skin wounds may indicate the wound age to be of 7–14 days (28). These observations prompt us to examine the expression of ubiquitin in compressed neck skin samples with a hypothesis that ubiquitin expression might be enhanced after antemortem compression. Unexpectedly, we have found the suppression of ubiquitin expression in compressed neck skin samples, compared with intact skin samples. Actually, in the present study, we found that 73.9% of the keratinocytes in the control specimens were ubiquitin-positive, whereas only 21.2% of the keratinocytes in the compressed specimens were ubiquitin-positive with a statistical difference. These observations were similar to those of Maiese's study that intracytoplasmic depletion of FLIP was found in the epidermal layers of antemortem ligature marks (39).

Ubiquitin gene expression after ischemia/reperfusion has been studied in the rat brains, wherein it was observed to initially decrease after reperfusion, but increased before the blood flow was restored to normal levels (22). These results suggest that ubiquitin may act as a useful marker of ischemic stress. In addition, Morimoto et al. have demonstrated that short-term ischemia causes depletion of free ubiquitin in gerbil hippocampal neurons, which are the most vulnerable to ischemic injury (21). Thus, the discrepancy would result from the difference of wound type between previous (28) and present studies. Although our previous study examined open skin wounds such as cutting and stabbing (28), we employed compressed skin samples indicating closed skin wounds in the present study. Compression to the neck skin can cause more severe local ischemia, eventually resulting in the decrease of ubiquitin expression at the compressed skin area including ligature marks. In other words, a substantial reduction in ubiquitin expression in the skin of the neck may be characteristic of the compression.

From a forensic safety standpoint, it is not sufficient to make a diagnosis using a single marker. For example, immunohistochemical detection of aquaporin-3 (AQP3) in the skin of the neck can be considered a valuable marker for diagnosing traces of antemortem compression (14). Thus, it is emphasized that several different markers reported previously should be examined in forensic practices in order to prevent over-diagnosis or missing of wound vitality. In addition to examining the skin tissue of the neck, markers of neck compression of lung tissue were examined in another study. There were partial differences in the level of immunohistochemical staining for AQP5 among the causes of death such as choking, choking, and

sudden cardiac death (44). Moreover, increased thyroglobulin, total T3, and free T3 levels in postmortem blood samples may suggest neck compression (45–47). Recently, Neri et al. showed an increase in the expression of miRNAs recognized as regulators of the inflammatory response in skin lesions such as miR125a-5p and miR125b-5p, implying that regulation of miRNAs as new tool for cutaneous vitality lesions demonstration in ligature marks (48).

CONCLUSION

We have showed that the detection of ubiquitin in the neck skin is possible with the accuracy required for forensic purposes. This fact is especially true for soft-marks, which are particularly difficult to assess based on gross examination and conventional histological analysis using HE staining.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of Wakayama Medical University (No. 3313). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SZ, YI, and TK formulated the hypothesis and designed the project. SZ, MN, YK, and AK performed the main experiments. AI, SH, and HiY provided technical assistance and discussion. YH, JM, and HaY helped with some experimental procedures. YI and TK oversaw the experiments and provided the main funding for the project. YI, FF, and TK participated in writing the manuscript. All authors contributed to the article and approved the submitted version.

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Myeloperoxydase and CD15 With Glycophorin C Double Staining in the Evaluation of Skin Wound Vitality in Forensic Practice

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Background: The determination of skin wound vitality based on tissue sections is a challenge for the forensic pathologist. Histology is still the gold standard, despite its low sensitivity. Immunohistochemistry could allow to obtain a higher sensitivity. Upon the candidate markers, CD15 and myeloperoxidase (MPO) may allow to early detect polymorphonuclear neutrophils (PMN). The aim of this study was to evaluate the sensitivity and the specificity of CD15 and MPO, with glycophorin C co-staining, compared to standard histology, in a series of medicolegal autopsies, and in a human model of recent wounds.

Methods: Twenty-four deceased individuals with at least one recent open skin wound were included. For each corpse, a post-mortem wound was performed in an uninjured skin area. At autopsy, a skin sample from the margins of each wound and skin controls were collected (n = 72). Additionally, the cutaneous surgical margins of abdominoplasty specimens were sampled as a model of early intravital stab wound injury (scalpel blade), associated with post-devascularization wounds (n = 39). MPO/glycophorin C and CD15/glycophorin C immunohistochemical double staining was performed. The number of MPO and CD15 positive cells per 10 high power fields (HPF) was evaluated, excluding glycophorin C—positive areas.

Results: With a threshold of at least 4 PMN/10 high power fields, the sensitivity and specificity of the PMN count for the diagnostic of vitality were 16 and 100%, respectively. With MPO/glycophorin C as well as CD15/glycophorin C IHC, the number of positive cells was significantly higher in vital than in non-vital wounds ($p < 0.001$). With a threshold of at least 4 positive cells/10 HPF, the sensitivity and specificity of CD15 immunohistochemistry were 53 and 100%, respectively; with the same threshold, MPO sensitivity and specificity were 28 and 95%.

Conclusion: We showed that combined MPO or CD15/glycophorin C double staining is an interesting and original method to detect early vital reaction. CD15 allowed to obtain a higher, albeit still limited, sensitivity, with a high specificity. Confirmation studies in independent and larger cohorts are still needed to confirm its accuracy in forensic pathology.

Keywords: CD15, myeloperoxidase (MPO), glycophorin, vitality, wound datation, histology, immunohistochemistry, forensic

INTRODUCTION

The determination of skin wound vitality is a challenge for the forensic pathologist. The detection of an inflammatory infiltration based on histology is to date the gold standard, being highly specific but showing a very low sensitivity in recent wounds. Most notably, in the first minutes or hours after the infliction of a wound, standard histological examination may not determine whether the wound was inflicted in pre- or post-mortem period. Indeed, the delay before the detection of first polymorphonuclear neutrophils (PMN) infiltration may vary from 10 min to 6 h (1, 2). Immunohistochemistry (IHC) is a cost effective and easy-to-use method that could allow to obtain a higher sensitivity (3). Upon the candidate markers, CD15 and myeloperoxidase (MPO) may allow to early detect PMN (4–7). However, one potential pitfall on IHC slide is the difficulty to differentiate passive extravasation of PMN in hemorrhagic infiltration from true active diapedesis. The association with a red blood cells marker, like glycophorin C, could allow to increase specificity, by avoiding the count of inflammatory cells in hemorrhagic areas.

The aim of this study was to evaluate the sensitivity and the specificity of CD15 and MPO, with glycophorin C co-staining, in comparison with standard histology, in a series of recent medicolegal wounds and post-mortem controls, and in a prospective human experimental surgical model.

MATERIALS AND METHODS

Study Population and Sample Collection

Medicolegal Wounds

Twenty-four individuals (20 men, 4 women, mean age = 51.0 ± 24.3 years) with at least one recent open skin wound were included at the mortuary of the University Hospital of Montpellier. Skin wounds consisted in 20 lacerations from polytrauma cases (traffic accidents, falls from high height) and 4 gunshot wounds. They were mostly located on the lower limbs and the torso. The time interval between trauma and death (survival time) was determined with medical records and police reports, including testimony from witnesses. It varied from a few seconds to 180 min (mean = 47 min). Bodies displaying putrefactive changes were excluded from the study, as well as individuals with severe malnutrition, known immunodeficiencies and immunotherapy.

For each corpse, a post-mortem 2 cm-incision was performed with a scalpel in an uninjured skin area contralateral to the ante-mortem wound, shortly after arrival at the mortuary and before refrigeration. The elapsed time between death and the infliction of the post-mortem wound was comprised between 0 and 180 min (median: 40 min). In patients with multiple ante-mortem wounds, the wound of interest was selected based on its location (no skin sample was collected from the head or hands, for ethical reasons) and on its size (large wounds were preferred to small ones).

At autopsy, a skin sample from the margins of each wound (ante- and post-mortem) and from an uninjured skin area located on the midline incision line (control samples) were collected on each corpse and immediately placed for fixation in 10% buffered formalin solution. A total of 72 skin samples were removed, including 24 samples from each of the conditions: ante-mortem wounds, post-mortem wounds, and healthy skin (control samples). The average post-mortem interval at the time of sampling was 66.3 ± 28.3 h (24–117 h).

Surgical Wounds

As a model of recent vital stab wound injury, the cutaneous surgical margins of abdominoplasty specimens were prospectively collected at the Department of Maxillofacial and Plastic Surgery of the University Hospital of Nancy, France. The precise time interval between incision and devascularization was recorded for each margin, ranging from 0 to 61 min (median: 24 min). As a model of early post-mortem wounding, a wound was inflicted with a sterile scalpel in the center of the specimens, 5 min. after devascularization. In the Pathology Department, tissue sampling was performed perpendicularly to the skin margins, on fresh tissue, before fixation in buffered formalin solution. Thirty-nine samples (26 pre-devascularization and 13 post-devascularization) were obtained from 13 patients.

Standard Histology

After formalin fixation and paraffin embedding, 5-μm sections were stained with hematoxylin, eosin, and saffron (HES), and a blind histological examination of the vital and post-mortem wounds was performed, taking into account the presence or absence of hemorrhagic infiltration and counting the number of PMN in 10 consecutive high-power fields (HPF) (×400 magnification; 0.237 mm^2).

Immunohistochemistry

Paraffin 5-μm sections were immersed in a 10 mM sodium citrate buffer (pH6) for 20 min at 97°C for dewaxing and antigen retrieval. The following primary antibodies were used

(30 min incubation): CD15 (mouse monoclonal, ready-to-use, DakoCytomation Agilent, Glostrup, Denmark); myeloperoxidase (MPO) (rabbit polyclonal, ready-to-use, Dako); glycophorin C (mouse monoclonal, 1/40, Diagnostic BioSystems). Immunohistochemistry was performed with Dako Autostainer Plus (Dako) using Flex Envision revelation system (Dako) for CD15 or MPO, followed by Envision Magenta (Dako) for glycophorin C. Appropriate positive and negative controls were used throughout the experiment.

A quantitative evaluation of staining for CD15 and MPO was counted in 10 consecutive HPF (0.237 m^2/field) on one representative slide per case, in the immediate vicinity of the wound margin, from the superficial dermis to the deep subcutaneous adipose tissue, taking into account all interstitial leucocytes showing stained cytoplasm, excluding intravascular cells and those within hemorrhagic areas, these latter being underlined by the anti-glycophorin C antibody.

Statistical Analyses

The presence of absence of interstitial hemorrhage was considered as a qualitative variable, and the numbers of PMN, positive MPO and positive CD15 cells as quantitative variables. Statistical analysis was performed with IBM SPSS Statistics version 27.0. software. To compare the different groups (ante-mortem vs. post-mortem; pre-devascularization vs. post-devascularization), the Fisher exact test was used for qualitative variables and the Mann-Whitney Wilcoxon test for quantitative variables. The correlation between quantitative variables was evaluated with the Spearman's coefficient correlation. A p-value lesser than 0.05 was considered as statistically significant.

Ante-mortem and pre-devascularization wounds were considered as vital wounds, whereas post-mortem wounds, control samples, and post-devascularization wounds were defined as non-vital wounds. For the evaluation of sensitivity and specificity, true positivity was defined by a cell count number equal or greater to the defined threshold in vital skin wounds; false positivity by a number equal or greater to the defined threshold in non-vital samples; true negativity by a number lesser to the threshold in non-vital samples; false negativity by a number lesser to the threshold in vital wounds. Receiving operating characteristic (ROC) curves were performed for each marker, in order to screen for the most optimal threshold.

RESULTS

Evaluation of Inflammation With Standard Histology

In the medicolegal wounds, no significant inflammatory reaction was seen on standard histology slides (**Figure 1A**). In the surgical wounds, a significant inflammatory reaction was found in 2 cases, showing a PMN infiltration (7 and 31 PMN/10 HPF). PMN evaluation showed a median number of 1 PMN/10 HPF (min.-max.: 0–31) in vital wounds and 1 PMN/10 HPF (min.-max.: 0–3) in non-vital wounds (**Table 1**). No significant difference was found between vital and non-vital wounds, in both autopsy cases and surgical wounds ($p = 0.557$ and $p = 0.294$, respectively).

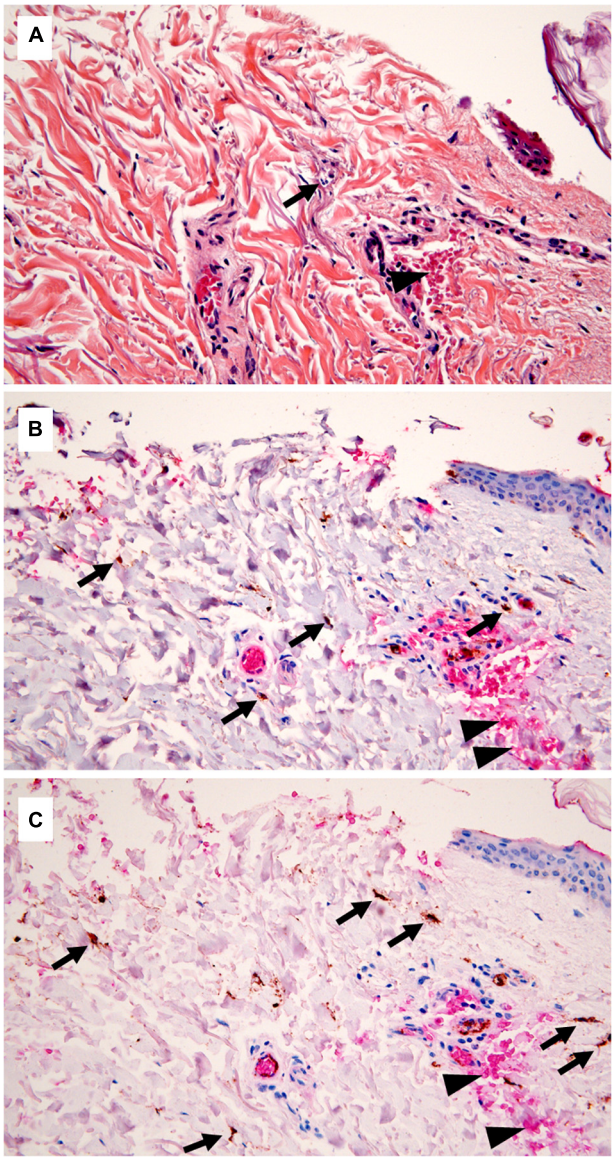

FIGURE 1 | Standard histology and immunohistochemistry (IHC) in a medicolegal wound. **(A)** Standard histology showing only one polymorphonuclear (PMN) (arrow) close to the wound margin, and hemorrhagic infiltration (arrowhead) (hematoxylin, eosin, and saffron, × 400 magnification). **(B)** Double staining for myeloperoxidase (MPO) and glycophorin C, underlining the presence of several inflammatory cells (arrows), evaluated outside the hemorrhagic areas (glycophorin C—positive, arrowheads) to avoid the count of passively extravasated leucocytes (IHC, ×400). **(C)** CD15/glycophorin C double staining, showing a greater number of positive cells, comparing with MPO (IHC, ×400).

A significant correlation between the number of PMN/10 HPF and the survival/pre-devascularization time was found in surgical wounds (rho = 0.424; $p = 0.031$), but not in autopsy wounds (rho = 0.135; $p = 0.511$).

With a threshold of at least 4 PMN/10 HPF, the sensitivity and specificity of the PMN count for the diagnostic of vitality were 16 and 100%, respectively.

TABLE 1 | Evaluation of the median number of polymorphonuclears neutrophils (PMN) on standard histology and results of anti- myeloperoxydase (MPO) and CD15 immunohistochemistry, in the autopsy cohort and in the surgical model [median (min.-max.); * statistically significant, $p < 0.05$; Mann-Whitney Wilcoxon test].

Samples	Vital wounds	Non-vital	*p*-value	Total
PMN				
Autopsy	1 [0–3]	0 [0–2]	0.557	1 [0–3]
Surgery	1 [0–31]	0 [0–3]	0.294	1 [0–31]
Total	1 [0–31]	0 [0–3]	0.106	1 [0–31]
MPO				
Autopsy	1 [0–12]	0 [0–7]	0.015*	1 [0–12]
Surgery	2 [0–20]	0 [0–3]	0.014*	2 [0–20]
Total	2 [0–20]	0 [0–7]	<0.001*	1.5 [0–20]
CD15				
Autopsy	3 [0–24]	0 [0–3]	<0.001*	1 [0–24]
Surgery	5 [0–24]	1 [0–3]	0.004*	5 [0–24]
Total	4 [0–24]	0 [0–3]	<0.001*	4 [0–24]

Interstitial Hemorrhage

With standard histology, a significant interstitial hemorrhage was noticed in 88% of ante-mortem wounds (**Figure 1A**) vs. 44% of post-mortem wounds and 17% of control skin samples. Using the anti-glycophorin C antibody, an interstitial hemorrhage was noticed in 96% of ante-mortem wounds vs. 56% of post-mortem wounds and 25% of control skin samples. In the surgical model, standard histology and anti-glycophorin C antibody showed an interstitial hemorrhage in, respectively, 88 and 96% of pre-devascularization wounds vs. 54 and 84% of post-devascularization wounds.

For the diagnosis of vitality, the sensitivity and specificity of the identification of interstitial hemorrhage on HES slides were 88 and 66%, respectively. With the anti-glycophorin C antibody, sensitivity raised to 96%, with a specificity of 50%.

Myeloperoxidase and CD15 Immunohistochemistry

With MPO/glycophorin C IHC (**Figure 1B**), the number of positive cells was significantly higher in vital than in non-vital wounds [$p < 0.001$; median (min.-max.): 2 (0–20) vs. 0 (0–7)] (**Table 1**). This difference was still significant in the autopsy and surgery subgroups ($p = 0.015$ and $p = 0.014$, respectively). Similarly, with CD15/glycophorin C IHC (**Figure 1C**), the number of positive cells was significantly higher in vital than in non-vital wounds [$p < 0.001$; median (min.-max.): 4 (0–24) vs. 0 (0–3)]. This difference was still significant when considering the autopsy ($p < 0.001$) and surgery ($p = 0.004$) subgroups.

The ROC curve for the diagnosis of vitality showed that the area under the curve was higher for CD15 (0.78) than MPO (0.69) and standard PMN count (0.58) (**Figure 2**). With a threshold of at least 4 positive cells/10 HPF, the sensitivity and specificity of CD15 immunohistochemistry were 53 and 100%, respectively; with the same threshold, MPO sensitivity and specificity were 28 and 95%. With a threshold of at least 2 positive cells/10 HPF, the sensitivity of CD15 reached 65%, but with a lower specificity (81%). For MPO, sensitivity and specificity were 51 and 81%.

The numbers of MPO and CD15 positive cells were significantly correlated with the standard histological count for PMN (rho = 0.339, $p < 0.001$; and rho = 0.333, $p < 0.001$, respectively).

In the surgical model, the numbers of MPO and CD15 positive cells were significantly correlated with survival/pre-devascularization time (rho = 0.400, $p = 0.043$; and rho = 0.559, $p = 0.003$, respectively), but not in autopsy wounds ($p > 0.50$).

DISCUSSION

In the first minutes or hours, the standard histological examination may not be able to determine whether the wound was inflicted in the pre- or post-mortem period. While hemorrhagic infiltration was classically considered as a sign of vital reaction, several studies have shown that the extravasation of blood cells can also occur after death and does not represent a reliable marker in wound vitality diagnosis (8–10). Various methods can be used to detect markers of vitality, such as study of mRNAs or microRNAs (RT-PCR, *in situ* hybridization) and proteins (ELISA technique, Western blot, immunofluorescence, immunohistochemistry), focusing on the different phases of inflammation and wound healing (3). Most notably, in skin lesions, several studies about cell adhesion molecules (ICAM-1, VCAM-1, P-selectin, E-selectin, fibronectin, . . .) were published, reporting for some markers a good sensitivity in recent wounds, but limited by a significant risk of post-mortem false positivity (3, 11–16). More recently, the use of microRNAs in forensic science has been proposed in various applications, including wound vitality, showing for few microRNAs interesting but still preliminary results (17–19).

In this study, we propose an original method for the detection of early inflammation, based on an immunohistochemical double staining of leucocytes and red blood cells. We tested two markers of PMN: MPO and CD15. CD15 showed a higher sensitivity than MPO, which may be explained by its ability to also detect activated monocytes (20), in addition to PMN. Staining for CD15 was previously reported as a marker of early vital reaction, focusing in most of studies on brain trauma or other organs (7, 21, 22). However, as the timing and the intensity of inflammation may be influenced by the type of the trauma and the nature of the lesioned tissue (1, 23), these studies cannot be applicable to skin injuries. In skin, we previously studied this marker in surgical and medicolegal wounds (5), and it was also reported to be an interesting marker in ligature marks (6, 24) or for the assessment of the vitality in corpse dismemberment (25) and in decomposed bodies (26).

Comparing with other methods, IHC staining of inflammatory cells allows the pathologist to have a morphological control of the signal, i.e., the recognition of leucocyte shape and the precise localization within the sample. In addition to anti-CD15 or MPO antibodies, we performed a double staining with the anti-glycophorin C antibody. Glycophorin C, like glycophorin A and D, is a sialylated glycoprotein

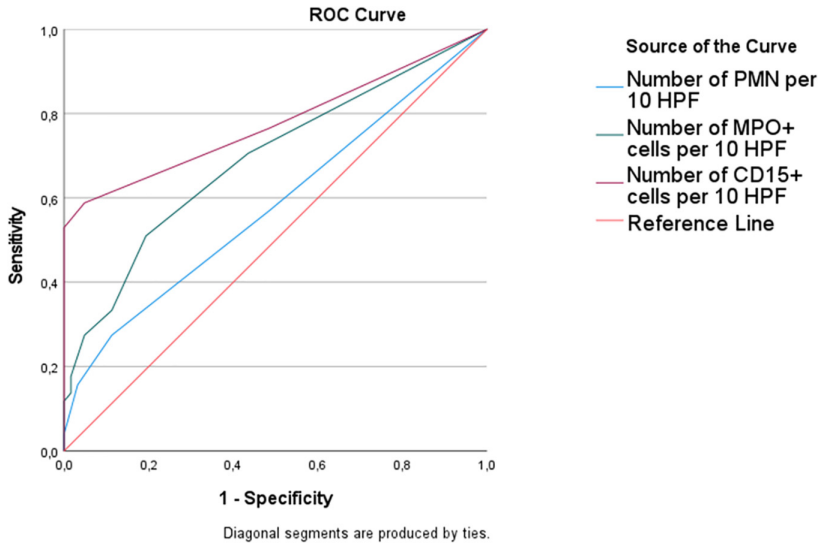

FIGURE 2 | Receiver operating characteristic (ROC) curve for the diagnosis of wound vitality. The area under curve was higher for CD15 (0.78) than MPO (0.69), followed by standard histological count (0.58).

in human erythrocytes membranes. Anti-glycophorin immunohistochemistry has been proposed in various studies for the identification of the hemorrhagic infiltration, most notably in decomposed bodies or in specific conditions, such as Amussat's sign or retinal hemorrhage (27–31). In our study, the association with the anti-glycophorin C antibody limits the risk of counting leukocytes originating from a passive extravasation of PMN in hemorrhagic infiltration, because red blood cells are more difficult to detect on IHC slides. It may also allow to detect more easily the wound margins.

The limit of this method is a relatively low sensitivity in very recent wounds, albeit higher than standard histology. The sensitivity is closely related to the type of wound or experimental model. We included in the present study only recent wounds, with a survival or pre-devascularization time of few seconds or minutes in a significant number of cases, which may explain the low sensitivity, whatever the method. In the same series of medicolegal wound, we previously found a sensitivity of 21% for the evaluation of IL8 staining, which reached 46% when using IL8 in a multiplex immunoassay, normalized on healthy skin levels (32, 33). Hence, we can conclude that IHC is less sensitive that immunoassay, but the latter has the disadvantage of needing fresh frozen tissue and to be a method which is not as largely developed as IHC, requiring training sets and data normalization, without morphological control.

Measures of test accuracy such as sensitivity and specificity depend crucially on the selected threshold, and the optimal value of this threshold is a key question for forensic practice. Given the fact that a 100% sensitivity is probably unreachable in very recent wound, we aimed to obtain a theatrical 100% specificity, to strictly avoid false positivity and obtain a high positive predictive value for vitality assessment. A threshold of 4 CD15-positive per 10 HPF cells allowed to obtain a 100% specificity and would be probably more relevant that a lower threshold, which exposes to false positivity, albeit with better sensitivity. In a previous study in which we compared CD15, FVIIIra and tryptase in medicolegal stab wounds showing inflammation and in surgical specimens (breast reductions), we found similar results for CD15, with the same threshold of 4 cells per 10 HPF (sensitivity: 47%; specificity: 100%) (5). CD15 had also the advantage to show a very good inter-observer reproducibility (0.90) (5).

CONCLUSION

In conclusion, based on a series of recent medicolegal wounds associated with post-mortem controls and an experimental human model of surgical wounds, we showed that combined CD15/glycophorin C double IHC staining is an interesting and original method to detect early vital reaction. In comparison with standard histology and MPO staining, CD15 allowed to obtain a significantly higher, albeit still limited, sensitivity, with a high specificity. Confirmation studies in independent and larger cohorts are still needed in order to confirm its accuracy in forensic pathology.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The post-mortem study protocol was approved by the French Agency of Biomedicine, Nr. PFS15-003. The surgical model protocol was approved by the review board of the Direction of Research and Innovation, CHRU of Nancy, France (CPRC2013, DRCI, CHRU Nancy) and a written consent was obtained from patients for using surgical specimens (study promoter:

CHRU Nancy, CPRC2012; sample collection DC2008-459). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GG and P-AP designed the study, collected the samples, analyzed the data, and wrote the manuscript. AB collected the samples and the data and analyzed the histological preparations. SC, MB, and ES collected the samples. LM, EL, and PC participated to the elaboration of study design. All authors agree to be accountable for the content of the work.

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