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DOCTORAL THESIS

Study of immunohistochemical markers useful in forensic practice in determining vital response in skin lesions

PhD supervisor : **Prof. Univ. Dr. Mariana Aschie**

Doctorand: **Sorin Deacu**

Coupons

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INTRODUCTION

Violent death (most often due to the action of a traumatic agent on the body) is the subject of judicial investigations. It can occur as a result of accidents (road traffic, precipitation, failure to provide medical care), suicide or homicide. The first organ to absorb the shock of the traumatic agent is the skin.

Very often, investigators are placed in the position of assessing bodies where death occurred without witnesses, with multiple injuries, and it is necessary to differentiate between vital and non-vital injuries and also to determine their chronology.

Particularly problematic are lesions produced shortly before (agonal stage) or shortly after the time of death (supravital period). During this time, the histological appearance is not helpful, as changes cannot be revealed by the standard technique (haematoxylin-eosin).

Thus, the current diagnosis of vital reaction, in the above-mentioned timeframe, is speculative in nature, requiring a "cocktail" of many markers. This requires a significant financial effort on the part of forensic institutions as well as more time

The desire to identify a reliable factor in this area led me to choose this topic for my PhD thesis. Therefore, I propose to study new markers, markers that have proven their usefulness and involvement in inflammatory processes and tumor progression, but whose role in forensic practice has not yet been evaluated or has been evaluated too little.

These markers are Vascular adhesion protein-1 (-1 (VAP-1), high mobility group box 1 protein (HMGB1), receptor for advanced glycation end products (RAGE), Toll Like Receptor 4 (TLR4) and nuclear factor kappa b (NFkB). These were determined immunohistochemically in skin wounds of different ages with known time of production.

THE CURRENT STATE OF KNOWLEDGE

It was structured in four chapters that reviewed the main research directions in forensic science in general and in vital response in particular.

Chapter 1. Histology

In chap. 1 elements of cutaneous and vascular histology were reviewed.

Chapter 2 Wounds in forensic medicine

In chap. 2 we have addressed the mechanisms of skin wound production, classified mechanical trauma agents and discussed the main differences between intra- and postmortem wounds.

Intentionally inflicted post-mortem injuries are encountered in situations where, for example, in order to conceal a murder, the body may be burnt by setting fire to it, or placed on the railway track to simulate a rail traffic accident, or a suicide may be simulated by hanging a corpse, etc. These are just a few examples of the circumstances in which the vital nature of the injuries is of paramount importance in justice. [9]

The problem of differential diagnosis of ante- and postmortem lesions, especially wounds, requires interdisciplinary collaboration between the forensic pathologist and the pathologist, in that the forensic pathologist must ensure the collection of organ/tissue fragments with all the characteristics of the wound, as far as possible (edges, angles) and provide details to the pathologist on the investigation data.

There are accompanying changes of each injury produced intravitally such as haemorrhage-blood infiltration, clotting, inflammation and tissue retraction. Although these have been shown to contribute to the prediction of the range of occurrence of a traumatic injury, tissue shrinkage, which has been studied mainly in the wound situation, remains questionable.

Chapter 3 The wound healing process

Most of the information we have about this phenomenon overlaps with the normal healing process of skin lesions [10].

The healing process is very complex, dynamic and involves numerous cell, humoral and molecular mechanisms that start immediately after the injury [13].

There are four (three or five, according to some authors) distinct but largely overlapping phases: haemostasis, inflammation, new tissue formation and remodelling [14].

Haemostasis will result in the production of a temporary structure called a blood clot. This limits the amount of blood lost through haemorrhage, prevents wound infection and forms a temporary matrix where all the cells involved in the inflammation process will migrate [15].

The inflammatory phase can be subdivided into the early phase, that of the neutrophilic response, and the late phase, in which the predominant cells are macrophages [21].

The proliferative phase is characterized by the appearance of granulation tissue [25] . In this stage, fibroblasts are the most important cells due to their ability to produce collagen fibers, thus replacing the temporary matrix, mainly consisting of fibrin [24].

The maturation phase is characterized by the transformation of fibroblasts into myofibroblasts and the amount of collagen produced will gradually decrease. The density of capillaries in the wound decreases as well as the number of fibroblasts resulting in a relatively acellular mature scar [35].

Chapter 4 Vital Response and its Importance in Forensic Medicine

The vital reaction represents the totality of local (tissues, organs) and general (whole body) changes that occur in the living organism in response to the action of trauma (mechanical, physical, chemical, biological), allowing the differentiation of injuries produced intravitally from those produced postmortem (accidental or intentional) [40].

Given that the body triggers the healing process immediately after an injury has been sustained, the efforts of forensic pathologists have been focused on highlighting or not highlighting this process in the wounds investigated.

All stages of the normal healing process were investigated in the hope of identifying a component that would allow a clear differentiation between antemortem and postmortem wounds and the time interval from injury to death. Among the molecules investigated are: vasoactive peptides and enzymes [51], factors involved in haemostasis/coagulation [58, 61], adhesion molecules [63, 65], chemokines and cytokines [70, 71, 77], growth factors [78].

PERSONAL CONTRIBUTION

Chapter 5 General methodology

The aim of the present study is to identify the expression of immunohistochemical markers in skin lesions of forensic interest in order to determine their vitality and the relationships between the expression of these markers.

In order to achieve the proposed goal, we pursued several objectives, as follows:

5.1.1 Objectives:

1. Establish study groups and create a database of cases.
2. Demographic and macroscopic characterization of the study group.
3. Histopathological evaluation by Hematoxylin-Eosin, Van Gieson Verhoeff and Perls stains.
4. Assessment of VAP-1 expression in blood vessels in the vicinity of the wound and in control tegument fragments collected during autopsy.
5. Assessment of the HMBG1-RAGE-TLR4-NF-κB axis in blood vessels in the vicinity of the wound as well as in tegument-control fragments collected during autopsy.
6. Evaluation of HMBG1-RAGE-TLR4-NF-κB axis expression in wound keratinocytes as well as in tegument-control fragments collected during autopsy.
7. Establish a score for grading the positive expression of the five markers.
8. Statistical processing of information from immunohistochemical expression evaluation.
9. Drawing conclusions.

For this prospective study, the main study group included cases autopsied in the County Clinical Service of Forensic Medicine Constanta, during the period 01.01.2018 - 31.12.2021. Of the total 4404 cases autopsied during this period, 1353 were violent deaths, and of these, 690 had at least one traumatic skin lesion involved in the tanatogenic mechanism.

Of the 690 cases, 117 were selected. From each case, one to four skin fragments were harvested from wounds as well as a control fragment from incisions made during autopsy. In total, 167 skin fragments and 117 control skin fragments were harvested. The main batch was segregated into six secondary batches (LS) based on survival interval (SI).

To the six segregated batches of the main batch was added a control batch consisting of 10 skin fragments harvested from patients undergoing surgery for excision of skin tumours. The skin fragments came from section slices.

All fragments were subjected to histopathological processing and staining with Hematoxylin-Eosin, Perls (Prussian blue) and Van Gieson Verhoeff.

The following immunohistochemical staining was also performed:

Antigen	Clone	Host	Tip	Izotop	Company	Concentration
VAP-1	E-10	mouse	monoclonal	IgG_{2a}	Santa Cruz Biotechnology	1:50-1:500
P-selectin	AK-6	mouse	monoclonal	IgG_1	eBioscience	1:100-1:1000
HMBG1	GT348	mouse	monoclonal	IgG2a	Invitrogen	1:100-1:1000
RAGE	A-9	mouse	monoclonal	IgG_1	Santa Cruz Biotechnology	1:50-1:500
TLR4	25	sunny	monoclonal	IgG_1	Santa Cruz Biotechnology	1:50-1:500
NF-kB	F-6	mouse	monoclonal	IgG1	Santa Cruz BioT.	1:50-1:500

Quantification of markers was performed in the areas of interest, the vessels of the wound lip, the bleeding area and the periphery of the lesions (for all markers) respectively in the epithelium of the wound lip and its vicinity for HMGB1, RAGE, TLR4 and NFkB. Scores to grade marker expression were set between 0 and 3 or 0 and 4 (quantification in epithelium).

The preparations thus obtained were examined under the Accuscope EXC-500 light microscope and acquired on computer using an Excelis HD video camera.

Statistical analysis of the data was performed in R, version 3.1, R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. R uses a number of mean magnitudes (mean, median and mode) as well as indicators of dispersion (standard deviation and variance). The Gaussian character of the variables was assessed using the Shapiro-Wilk test.

Chapter 6 Study 1 Demographic, macroscopic and histopathological characteristics of the study group

Aim of the study.

The aim of the present study is to analyse the group described in Chapter 5 demographically, macroscopically and histopathologically.

Results:

The mean age of patients included in the study was 49.57 years+/-9.9, with a minimum age of 1 year and a maximum of 88 years. Seven patients were minors and 12 were over 75 years old.

Each individual wound was characterised in terms of how it was produced, the mechanism of production and the regions involved. Thus, it is observed that most wounds are contused, followed by lacerated and cut wounds. Another important category was represented by puncture-cut wounds (about 12%). At the opposite pole, shot and bitten wounds were the least represented groups, reaching a percentage of 4-5%.

In terms of how they were produced, it can be seen that 59 of them originate from road accidents. Of the modes of production, these have the highest proportion because road accidents are very frequent and present a high risk of fatality.

Assessing the regions from which the wounds originated, we find that more than 33% of them (56) are represented by the scalp, followed by the thorax (27) and abdomen (17). Thus, the fact that wounds in these areas predominate is not accidental, as they are the seat of vital organs.

Macroscopic aspects of traumatic injuries (wounds) included in the study :

Macroscopically, blood infiltrates of varying size and depth were found in all the traumatic injuries analysed, especially in the lips of the wounds. Even in postmortem wounds (especially in those produced within the first half hour after the person's death) blood infiltrates were observed, although of lower intensity.

Another macroscopic parameter assessed was tissue shrinkage. For a long time it has been considered a specific element of intravitally produced wounds. However, in our research, we found that in LS1, about 11% of wounds (8 wounds), although definitely intravitally produced, did not show tissue retraction. This may be confounded with postmortem wounds, which show reduced edge retraction.

Microscopic characterisation of study batches:

In all batches routine staining from the standard test battery did not yield much useful information. Haematoxylin-Eosin staining revealed areas of haemorrhage and inflammatory infiltrate (where present). In LS1, 15 fragments from LS2, 6 from LS3 and 1 from LS4 showed no infiltrates. In LS5 HE identified shrunken haematomas as well as the existence of granulation

tissue (in wounds with 24-72 hour survival), and scarring of the dermis in wounds with a survival of more than 8 days.

Perls staining was negative in all batches except LS 5 where it showed haemosiderin deposits on 18 of the 20 constituent fragments of the batch.

Van Gieson Verhoeff staining revealed elastic fibers in the dermis structure, fibers that were inconsistently altered (fragmented on 4 skin fragments in LS3) or moderately reduced in number at the lip of the wound in LS4. In LS5, it showed a decrease in the density of elastic threads to almost disappearance in areas with scar appearance.

Discussion:

Although in the majority of cases, macroscopic assessment is sufficient to affirm the vital/non-vital nature of an injury, as we have seen in the study above, there is a small percentage where there is uncertainty.

Scripcaru-Terbancea considers that in forensic practice there is an "uncertainty interval" of about 10 min antemortem and 5 min postmortem. [85] and Simonin considers it even 3 hours postmortem [86].

There are studies [87-91] showing that bleeding areas can occur even after the cardiac pump has stopped, in the so-called survival period.

Since macroscopic observations can be altered in various situations, it is necessary to complement them with a microscopic examination.

By far the best known and most widely used stain in general or forensic pathology, Hematoxylin-Eosin, is very useful in differentiating postmortem from intravital lesions on one condition: the inflammatory infiltrate must be present to be objectified. Of course, HE staining will also highlight areas of haemorrhage very well, but, as in the situations addressed in macroscopic examination, these may be of postmortem origin.

Inflammatory infiltrate is the only accepted element of certainty in the affirmation of vital reaction and an extremely important marker in assessing the age of the lesion [11].

Studies have shown that a minimum interval from which neutrophilic infiltration begins is 10-30 minutes and can reach a maximum of 12 hours. Most commonly, this neutrophilic infiltration occurs 1-2 hours after injury. [11, 60, 95]

As for the Perls stain (Prussian blue), its usefulness was rather limited, referring here to the batch of plagues with a survival age of more than 24 hours.

The Van Gieson Verhoeff stain assessed the presence, in particular, of elastic fibres. Their staining pattern did not allow estimation of the age of the lesion, except in a limited

number of cases in the group of wounds with a survival age of more than 24 hours, where reduction or disappearance of elastic fibres was observed in areas with scarring [10].

In general, routine staining is useful in lesions more than 1-2 hours old once inflammatory infiltrate is present at the bleeding area.

Conclusions:

1. The distribution of cases over the years 2018-2021 was affected by the overlap of the SARS Cov2 pandemic, with the period March 2020-July 2020 with very few cases incorporated into the study. Demographically, all age groups were male dominated with a sex ratio of 4.31.
2. In terms of wound type, most of the wounds were in the category of contusion wounds (45% of all injuries), mainly from road traffic accidents (35% of all wound types), and the most common location was on the scalp (30%).
3. Macroscopic examination allowed assessment of the vital-non vital character of a wound in about 80% of wounds using classical parameters (haemorrhagic infiltrate, retraction of wound margins, activation of coagulation, presence of inflammation).
4. Microscopic evaluation was superior to macroscopic evaluation by showing areas of haemorrhage and inflammatory infiltrate.
5. In the group with a survival time of less than 5 minutes, none of the wounds showed an inflammatory infiltrate, making it difficult to make a differential diagnosis with a postmortem wound.
6. Hematoxylin Eosin staining was useful in assessing the vitality of a wound after a time interval of about one hour.
7. Perls staining was of limited use, showing haemosiderin deposits more than 24 hours after lesions.
8. Van Gieson Verhoeff staining was also of limited use, as the elastic fibres revealed by it sometimes appeared fragmented or reduced in number, but without being able to assess the character of the lesion on this basis.

Chapter 7. Study 2 -- Comparative immunohistochemical study of VAP-1 with P-selectin in wounds with known survival time

Working hypothesis and purpose of the study

For this study, we started from the premise that the role that VAP-1 plays in the diapedesis process due to its function as an adhesion and oxidative molecule could have applications in forensic practice.

The aim of the study is to quantify the expression of this marker, quantification that was carried out simultaneously with the expression of P-selectin, a marker that, although it has its

limitations, still provides an important support in the assessment of vitality in newly produced wounds.

Material and method

For this immunohistochemical study, the immunohistochemical markers VAP-1 and P-selectin were used and staining was performed according to the protocol mentioned in Chapter 5.

Marker	Dilution	Antigen Retrieval	External control
VAP-1	1:150	EnVision^{TM} FLEX Target Retrievel Solution, high PH	Placenta
P-selectin	1:250	EnVision^{TM} FLEX Target Retrievel Solution, high PH	Tonsil

Results:

VAP-1 expression in intravitally produced wounds

In the area of haemorrhage, VAP-1 expression was accentuated, obtaining in more than 90% of cases scores 2 and 3 in LS1. Comparing VAP-1 expression in the haemorrhage area with that in the periphery and lip of the wound, we find statistically significant differences ($p<0.003$ vs periphery of the wound and $p<0.001$ vs lip of the wound). Comparing VAP-1 expression in the wound lip with that in the periphery, there were no statistical differences ($p=0.43$). Comparison with control fragments resulted in the same statistically significant expression across all three areas evaluated ($P<0.001$).

In LS2, VAP-1 expression in the bleeding area (Figure 83) was not different from that in the wound lip ($p=0.24$) or periphery ($p=0.5$). Comparison with control fragments was still statistically significant ($P<0.001$).

In LS3 the same trend as in LS2 was maintained, with no major differences in immunohistochemical expression between the three areas evaluated. The differences found with the control fragments were statistically significant in this batch as well.

LS4, had similar expressions in immunohistochemical evaluation of VAP-1 in the three target areas and a clear discrepancy ($p<0.001$) with control fragments.

In LS5 there was a reduction in VAP-1 expression in the area of hemorrhage, but no statistical difference with the other two areas (wound periphery expression $P=0.23$ and wound lip expression $p=0.84$).

VAP-1 expression in postmortem wounds

In post-mortem wounds, regardless of the area of expression assessment, scores of 0 and 1 were consistently found, with no statistically significant difference (p=0.37 vs. lesion periphery, respectively 0.79 wound lip)

Expression of VAP-1 in control fragments from decedents and live animals

The trend found in postmortem wounds was maintained in the fragments harvested during autopsy (Figure 90) as well as in LS7 (p=0.43).

The expression of P-selectin in LS1-LS5 was quite variable.

In LS1, score 1 predominated, with scores 2 and 3 occurring in about 40% of cases. Also, 12 of the 69 fragments in this group had score 0 in the bleeding area. A slight accentuation of expression was found at the periphery of the lesion, but without statistical evidence (p=0.73).

In LS2, there was an accentuation of expression in the area of hemorrhage (more than 70% of fragments registering scores 2 and 3) ($p<0.05$). In the periphery, the staining of vessels was similar to those in LS1 (p=0.2).

In LS3, expression was preserved markedly in the area of hemorrhage, with decreased stainability in the lesion periphery (P=0.07).

In LS4 the distribution across the 4 scores was approximately equal in the area of haemorrhage, whereas in the periphery of the lesion score 1 predominated (p=0.1).

In LS5, there were reduced scores in both the bleeding area and the lesion periphery, with no statistically significant differences (p=0.37).

LS6 and LS7 had similar characteristics as well as with control fragments harvested during autopsy ($p<0.05$).

Comparison of VAP 1 with P-selectin

Expression in the bleeding area in LS1 highlights the superiority of VAP-1, with it scoring significantly more 2s and 3s in the first five minutes after injury ($P<0.001$).

Expression at the periphery of the lesion was also more pronounced for the VAP-1 marker ($P<0.001$)

In the bleeding area of LS2, the expressions of the two markers are similar, with a small plus for P-selectin, but without statistical significance (p=0.43)

In LS3 to LS5, both in the area of hemorrhage and in the periphery of the wounds (Fig. 118), there were no notable differences between the two markers. (p=0.38)

In LS6 and in the control fragment batches, expression is similar, with most fragments having scores of 0 and 1, expression considered basal.

Discussion:

Under normal conditions, VAP-1 is expressed in three cell types: endothelial cells, smooth muscle cells (including pericytes [106]) and adipocytes [107]. In vessels, VAP-1 is found in its inactive form in cytoplasmic vesicles distinct from the Weidel-Palade corpuscles of endothelial cells [102].

In a study by a group of Finnish researchers, skin fragments from individuals without skin disease were found to have rare blood vessels with low or moderate expression of VAP-1. [110]. We scored this low or moderate expression with scores 0 and 1, respectively.

During inflammation, VAP-1 expression is induced in endothelial cells and the protein is translocated from cytoplasmic vesicles to the plasma membrane by a process similar to that of P-selectin [110]. This molecule mediates the diapedesis step and is also involved in rolling and firm adhesion [112].

The enzymatic activity of VAP-1 triggers synthesis and expression of endothelial adhesion molecules ICAM-1, MadCAM-1, E-selectin and P-selectin, induces secretion of chemokine CXCL8 and activates transcription factors such as NF-kB [115, 116]. As observed in our study, within the first minutes after injury there is an overexpression of VAP-1, followed by an increase in P-selectin positive vessels in study groups 2 and 3.

In the process of chemically induced cutaneous inflammation and intravenous administration of anti-VAP-1 antibody, binding of this antibody to endothelial cells was found in which VAP-1 expression was consecutive to its increased expression. In endothelial cells of skin vessels not exposed to chemical stimulation, binding of the antibody was not found [110]. This was evident in my study in control fragments, both those harvested during autopsy and in LS7.

In the same study it was shown that translocation of VAP-1 from the cytoplasm to the cell membrane occurs approximately 60 minutes after application of the chemical solution with a maximum reached at 8 hours. Then a decrease in the intensity of immunoreactivity was found within 24-48 hours post induction of the inflammatory stimulus [110].

In my study I found a decrease in the intensity of immunoreactivity in wounds with SI of more than 24 hours (LS5). However, comparing the immunohistochemical expression in LS1 (wounds with SI of less than 5 minutes) with the expression of the control fragments, I was able to show an overexpression in the former, with statistical significance ($p<0.001$), which contradicts the results of the study cited above.

P-selectin has been a very promising marker in attesting vital response and lesion tanatology.

However, as it has been studied more intensively, it has been found that its expression is not characteristic of intravital wounds but can also be positive in postmortem lesions.

This was not the case in our study, as P-selectin expression in these was low, with rare scores 2 encountered in the bleeding area.

In the study by Ortiz-Rey et al. it was demonstrated, using skin fragments from intravital wounds and postmortem fragments, that P-selectin did not allow their differentiation [64].

Conclusions:

1. Although the two markers show similar positivity pattern in vessels, VAP-1 expression is higher in LS1 (with IS less than 5 min) ($P<0.05$)

2. Examination in vessels in the area of haemorrhage gives better accuracy than examination at the wound lip or periphery.

3. VAP-1 expression in all batches containing intravitally produced wounds allowed their differentiation from postmortem wounds

4. No statistically significant differences were found between VAP-1 and P-selectin expression on LS2-LS5 batches.

5. A small percentage of the control fragments (10%) obtained scores 2 and 3 highlighting the existence of inter-individual variability and therefore it is necessary to keep these fragments in the harvest protocol, preferably from the region contralateral to the lesion.

Chapter 8 Study 3 Immunohistochemical exploration of the HMGB1-RAGE-TLR4-NFkB axis and its usefulness in attesting the vital response in wounds

8.1 Introduction

The way we adapt to the external environment is based on a long-standing but still highly effective process. Inflammation.

Many articles and books have been written about the pathophysiological basis of inflammation, about the mechanisms that trigger and maintain this process.

Among the countless molecules released during this process, there is one that has remained unchanged since life began, HMGB1. When this molecule is released from the

nucleus, the cells in the area know that there is danger and will start secreting various components needed to trigger the inflammation process.

RAGE and TLR4 are the receptors by which cells are alerted to changes in local homeostasis, and it is NFkB that will *translate* these signals and basically force the cells to sustain the inflammatory effort.

8.2 Working hypothesis/objectives

Given the data in the literature pointing to HMGB1 as a pivotal player in the inflammation process, we decided to test the expression of it and two of its receptors, along with the *ultimate target* of this activation, NFkB, in wounds of different ages.

The main objective is to evaluate these expressions and their potential applicability in forensic practice.

8.3. Material and method

The markers used were HMGB1, RAGE, TLR4 and NFkB and staining was performed according to the protocol described in Chapter 5 and Table XIX. External positive controls (placenta fragments) were used.

Marker	Dilution	Antigen Retrieval	External control
HMGB1	1:200	EnVision™ FLEX Target Retrievel Solution, high PH	Placenta
RAGE	1:100	EnVision™ FLEX Target Retrievel Solution, high PH	Placenta
TLR4	1:50	EnVision™ FLEX Target Retrievel Solution, high PH	Placenta
NFkB	1:50	EnVision™ FLEX Target Retrievel Solution, high PH	Placenta

Table XIX: *Dilution, epitope recovery solution and external control used for the four markers in this study*

The scoring used to assess marker expression in surface epithelium and blood vessels has been detailed in Chapter 5.

Results

8.4.1. HMGB1 expression: immunohistochemical microscopic aspects

In the case of HMGB1, a high expression in more than 90% of the nuclei can be observed in all fragments representing the external control and on the control skin fragments of batch 7, with moderate/low cytoplasmic expression

When we evaluated the epithelium of the wound lip, we noticed a very interesting aspect, namely that the expression of the marker is very low, even absent in the epithelium of the wound lip in wounds made before death (score 4). This aspect was found in the majority of

wounds belonging to groups LS1-LS5. The other fragments scored mostly 2 or 3. Note that no fragment had a score of 0.

A significant difference was observed with wounds made post-mortem ($P<0.05$) (LS6). In this group, the scores that predominated were 2 (9 fragments) and 1 (3 fragments). Note that there were two fragments that scored 4, even in this subgroup .

Control fragments collected during autopsy were scored 0 and 1 in more than 99% of cases. The same trend was found for skin fragments from LS7.

When assessing epithelial expression adjacent to the wound lip or remote from the wound, we observed in 45 cases a marked cytoplasmic expression or extracellular positivity of HMGB1 in LS1. This expression was scored as 3. Also, no 0 score was obtained in this epithelium in any of the five batches including intravitally produced wounds ($p<0.001$). No score 4 was recorded on any of the 155 antemortem produced wounds ($p<0.001$).

Examination of the vessels showed that nuclear expression tended to be lost in batches containing wounds produced during life ($p=0.061$). Most obviously, this becomes apparent in LS1 ($p<0.05$)

Cytoplasmic expression in vessels decreases as the time between wounding and death is longer, but without a statistically significant difference between intravital periods ($p=0.079$).

8.4.3 RAGE expression: immunohistochemical microscopic aspects

As in the case of HMGB1, also in the case of the RAGE marker, a reduced expression to disappearance was observed in the wound lip in the first 5 minutes, which may persist up to 24 hours, and was absent in post-mortem wounds ($p<0.05$). Moreover, staining decreases in intensity as the interval between the occurrence of the wound and the time of death increases in the spinous and granular layers, with no statistically significant difference between the various intervals studied ($p=0.84$).

What is noteworthy is that in post-mortem wounds, RAGE expression is limited to the basal layer and in very rare cases there is weak staining of the spinous and granular layers, these changes are also seen in skin fragments collected during autopsy ($p<0.05$).

At a distance from the wound the trend is similar, with expression accentuated at the membranous or cytoplasmic level, in the spinous and granular layers in the first 5 minutes.

For RAGE, cytoplasmic expression in vessels remained relatively constant, with no statistically significant difference between the different intravital periods ($p=0.91$).

8.4.5. TLR4 expression: immunohistochemical microscopic aspects

For the TLR4 marker, a reduced expression is observed, even absent in the wound lip in the first 5 minutes, which can persist up to 24 hours, and is absent in post-mortem wounds ($p<0.05$).

In skin fragments collected during autopsy, TLR 4 expression is limited to the basal layer and in very rare cases weak staining of the spinous and granular layers is observed ($p<0.05$). This observation was also made in control fragments.

In the epithelium located at a distance from the wound, scores 2 and 3 are observed in the majority of cases (60%). Also in the epithelium located at a distance from the wound, a very interesting aspect of nuclear positivity was observed. We scored with 0 the fragments that had less than 10% positive nuclei and with 1 the fragments that had more than 10% positive nuclei. In about 50% of the fragments in LS1 this positivity was found, with the proportion increasing for the next two categories, LS2 and LS3. The lowest expression was found in LS6 and in fragments collected during autopsy.

TLR4 expression in vessels for intravitally harvested fragments, no significant difference was found ($p=0.43$) In control wounds, difference is statistically significant, endothelial cells were weakly positive or absent ($p<0.05$)**.**

8.4.7 NFKB expression: immunohistochemical microscopic aspects

In the case of NFkB marker, little or no expression is observed in the wound lip in intravitally produced wounds ($p<0.05$) Nuclear expression (equivalent to score 3) is relatively rare in the epithelium of the wound lip.

In the epithelium located at a distance from the wound, nuclear positivity (Score 3) is observed in a proportion of about 25% in LS1. A similar proportion ($p=0.33$) is preserved on fragments from the other batches, except for control fragments and LS6.

8.4.8 Combined marker analysis

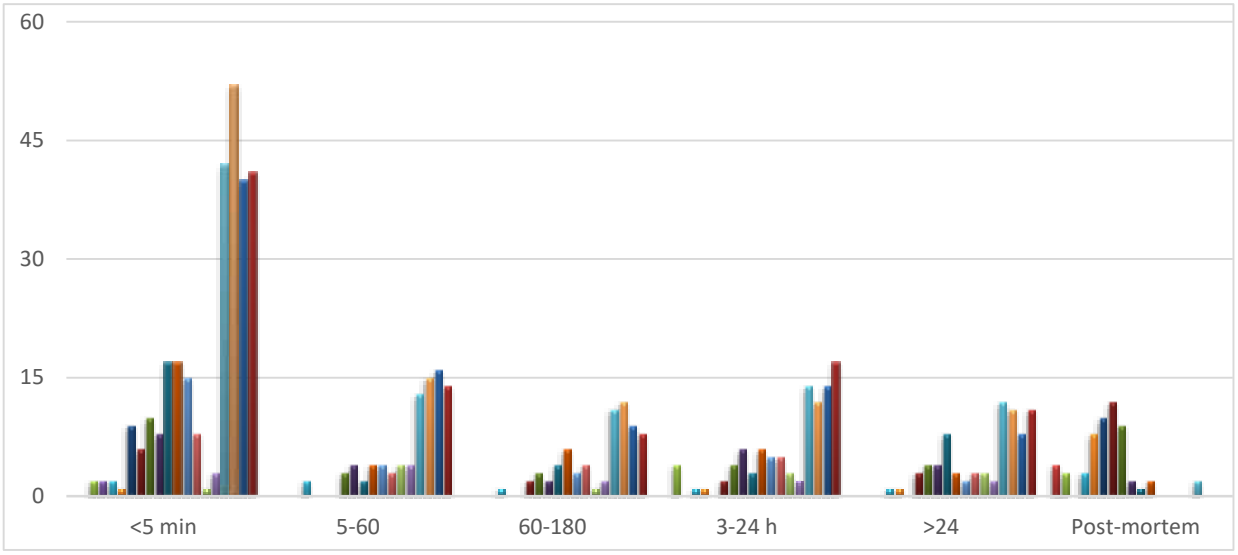

***Figure 1**: Scores of the 4 markers in the wound lip*

The predominance of 4 scores in the epithelium of the wound lip is easily observed in all batches containing vital wounds (Fig. 1). In LS1, the frequency of score 4 is more common, with HMGB1 and RAGE having the most pronounced expression.

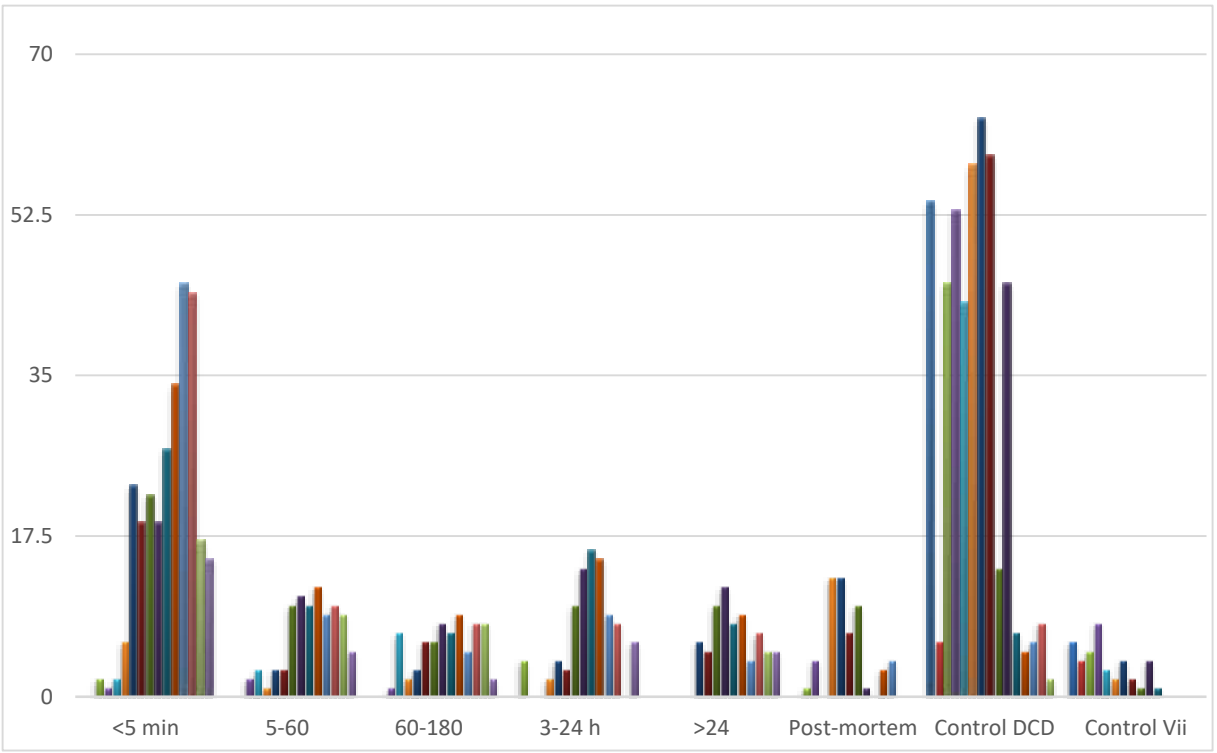

***Figure 2**: Scores obtained by the 4 markers in the epithelium adjacent to the wound lip*

In the epithelium away from the wound, a maximum score of 3 is observed in LS1 for most of the fragments stained with HMGB1 and RAGE (Fig. 2). Also, the predominance of 0 and 1 scores is observed in control as well as postmortem fragments.

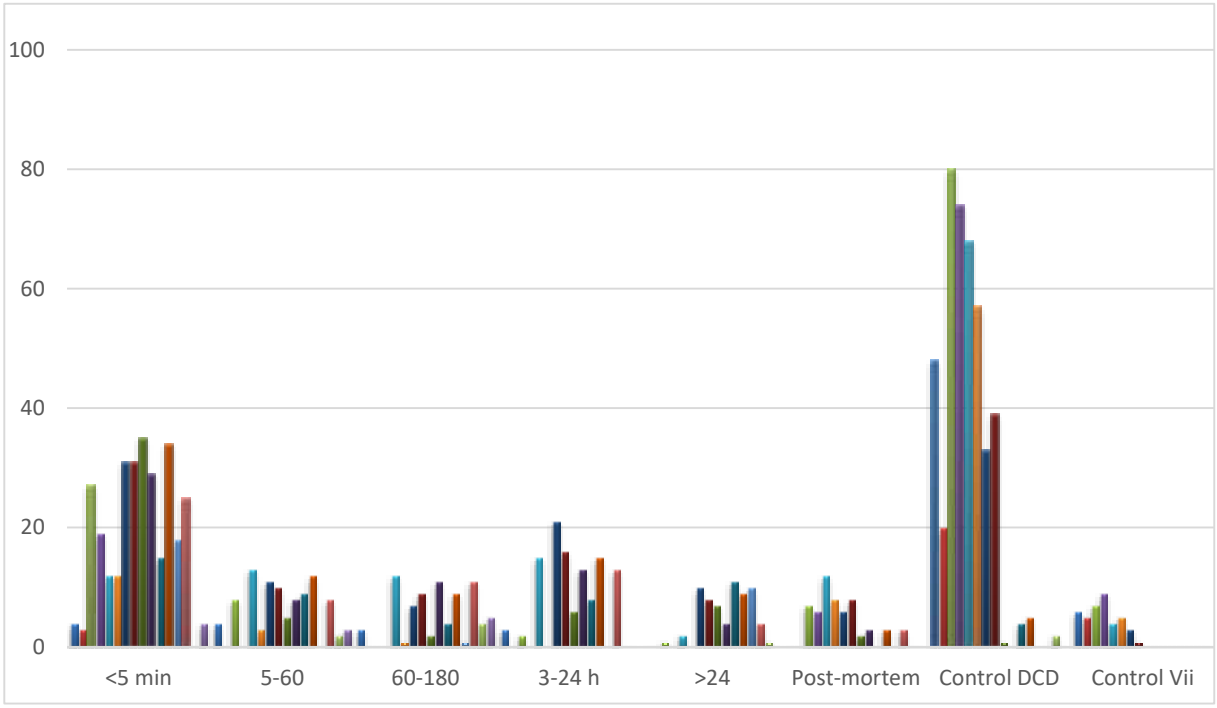

***Figure 3**: Scores obtained by the four markers in vessels.*

In the blood vessels, the expression of the 4 markers is approximately evenly distributed among the score categories, regardless of the category to which the fragments belong (Fig. 3).

A comparative table of the four markers in the different sampling areas was created. Based on the time elapsed between wound occurrence and death and using Bayesian statistics, the probabilities of a lesion falling into a particular category were calculated based on the score obtained:

a) In wound lip epithelium: in the first five minutes all four markers are significant. Of these, HMGB1 and RAGE, were most frequently scored 4, and a combination of these two markers would have a probability of classifying a wound as LS1 of over 70%. On the other hand, a score of 0 for these markers has a probability of over 90% of placing the lesion in the control or postmortem fragment group.

b) At wound distance: all four markers are significant, with an obvious plus for HMGB1 and RAGE. In terms of the score given, it can be seen that if the score is 2 or 3 for HMGB1 and RAGE markers, then the probability of the injury occurring less than 5 min before death is 70-80% compared to the other periods.

c) In vessels: no distribution on any of the markers was found to exceed more than 30%.

8.5. Discussions

In a very extensive review of vital reaction studies, Casse et al stress the very important role of immunohistochemistry in forensic research, although other research solutions such as RT-PCR, in situ hybridization, Elisa techniques or Western Blot are plausible [57].

The authors argue that immunohistochemistry remains the method of choice in forensic medicine because it is easy to implement on paraffin-embedded fixed tissues, has a relatively low cost (compared to other techniques) and allows localization of the component under investigation to the tissue or cell [57].

Although an element of certainty in attesting the vital reaction, the inflammatory infiltrate was not a defining element for the study, as we focused on wounds where it was not present (LS1, partial, LS2 and LS3, LS6, as well as control fragments).

8.5.1. Discussion of HMGB1 expression

HMGB1 is a non-histone, nuclear chromatin-associated protein, is expressed in all eukaryotic cells, and 99% of it has the same structure in all mammals [121].

HMGB1 increases the affinity of transcription factors such as p53, p73, nuclear factor-κB (NF-κB) and estrogen receptor. [123]

Normally, in physiologically resting cells, HMGB1 is localized in the nucleus and less in the cytoplasm (the ratio of nuclear to cytoplasmic HMGB1 is 30:1) [124]. In our study, we demonstrated that in both LS6 and LS7 in more than 90% of the cells the obvious nuclear expression was preserved, which corresponded in most cases to the 0 score and in a very small proportion to the 1 score. This applied across the entire thickness of the epithelium as well as the vessels.

As for the fragments collected during the autopsy, most of them fell into scores 0 and 1, with a small part of them being scored 2 and 3.

Because they seemed to be at odds with the general staining pattern, we decided to investigate the possible cause.

I consulted the article by El Din et al and found something very interesting. The authors, after evaluating batches of laboratory animals and skin fragments collected during autopsy, concluded that cytoplasmic expression occurs in fragments approximately 24 hours after death [125].

Following the same trajectory, we also re-evaluated cases that had a score of 2 or 3, and 18 of the 20 cases had autopsy time 48 hours after the time of death.

This finding raises the alarm about HMGB1 expression in the integument of deceased individuals.

When exposed extracellularly, HMGB1 becomes a DAMP, and not just any DAMP, but the best known of them [126].

HMGB1 is loosely bound to cell chromatin and rapidly reaches the extracellular if membrane integrity has been lost [127].

It is the most mobile nuclear protein, reaching the cytoplasm in 1-2 seconds [128]. This supports our findings that in LS1 (i.e. in the first minutes after the trauma) in the wound lip and in the epithelium at the periphery of the lesion there are changes in the dynamics of HMGB1 with its translocation from the nuclei (whereas cytoplasmic expression tends to become enhanced.

Also in the same LS1 batch, although not with the same intensity as in the epithelial lining, HMGB1 translocation from the nucleus to the cytoplasm was found in endothelial cells lining the vessels in the bleeding area.

HMGB1 reaches the extracellular environment either passively, from necrotic or destroyed cells, or by active mechanisms (secreted by cells of the immune system, monocytes, macrophages and dendritic cells (Fig. 213) [128]. Once released into the extracellular space, HMGB1 promotes inflammation, cell proliferation, migration and differentiation [129].

A very interesting aspect was the staining pattern in the epithelium of the wound lip. In a very high proportion of cases, HMGB1 expression was attenuated or disappeared at this level. In the literature we did not find a study addressing this issue in the integument. However, we hypothesize that in the wound lip keratinocyte necrosis occurs very soon after the lesion is made and by a passive secretion mechanism, encountered in cell necrosis, HMGB1 rapidly reaches the extracellular environment.

Another paper published by Gao et al evaluated HMGB1 and RAGE expression in traumatic brain injury, and the authors reached conclusions that are somewhat consistent with what we found in our study. This study was performed on brain fragments from laboratory animals and humans and revealed that HMGB1 expression disappeared from neurons in the contused area within about 30 minutes after the trauma occurred. Moreover, neurons surrounding the contusion area showed cytoplasmic translocation of HMGB1 approximately two hours after injury. [130]

Another study led by Qingjie et al found in a group of laboratory animals that suffered crush injury to the liver that cytoplasmic HMGB1 expression becomes enhanced approximately 6 hours after injury. [131]

These data should be interpreted with caution. In both studies, organs with a totally different structure from skin, brain and liver were examined.

Also, the traumatic agent did not act directly on these two organs, which are well protected in the cranial and abdominal cavities. Instead, all the injuries examined in our study groups were produced by direct, unmediated action of the traumatic agent on the skin. Therefore, it is possible that our recorded values (disappearance of HMGB1 expression in the epithelium of the wound lip or cytoplasmic overexpression of HMGB1 in the epithelium of the wound vicinity in the first minutes after injury) are directly proportional to the force of this aggressor.

8.5.2. Discussion of RAGE

RAGE belongs to a superfamily of immunoglobulins, similar in function to the Toll-like receptor (TLR) family [139].

Because it has a wide palette of ligands (exogenous and endogenous) RAGE is considered a pattern recognition receptor (PRR) [142].

In a 2005 study, Cheng et al, demonstrated that there are several staining patterns (four) of normal tissues with RAGE [152]. They assigned the skin pattern B, with small supranuclear granules confined to the basal layer. In the same study, endothelial cells had staining pattern A, with diffuse cytoplasmic expression [152].

In 2015, Iwamura et al, demonstrated immunohistochemically, that RAGE stains basal layer cells and rare spinous layer cells. They also found no link between RAGE expression in normal integument and the region from which it originated (sun-exposed or not sun-exposed integument) or the age of the person from whom it was harvested. [153] In our study we assigned this expression to the 0 score.

In the study by Gao et al, in addition to HMGB1 expression, RAGE expression in animal and human brain lesions was also determined. They observed that RAGE expression is accentuated starting at six hours in the area around the contusion zone [130].

In our study we identified a staining pattern in the epithelium of the wound lip that has not been reported before, namely the disappearance of basal RAGE expression. This expression was scored, exactly as in HMGB1, with a score of 4. In the epithelium adjacent to the wound, overexpression of RAGE was evident, especially membranous, from LS1 to LS5. This overexpression was overexpressed in cases where score 3 was recorded for HMGB1 expression.

Our hypothesis is that upon release of HMGB1, it binds to RAGE, this interaction facilitating the expression of adhesion molecules, proinflammatory cytokines, but, as mentioned above, also overexpression of RAGE.

RAGE expression in the vessels was constant regardless of the group to which the fragments belonged.

Although studies attesting to RAGE expression in the vital response in cutaneous wounds are conspicuously lacking, in the forensic field it has been tested in drowning cases and its expression in pneumocytes has allowed differentiation between submersion deaths and postmortem submersion or control fragments [154].

8.5.3 Discussion of TLR4 expression

In 1996, a transmembrane protein that mediates the antifungal response and embryonic development of the vinegar fly (Drosophila melanogaster) was discovered to be the expression of a gene called Toll. [158]

It is precisely because of this similarity that the name Toll-like Receptors (TLRs) was chosen and 10 such receptors have been identified in humans. [160]

They are considered to be part of the PRR, a very diverse group of molecules, with a role in intercepting pathogens (PAMPS) or endogenous molecules released by cells in distress (DAMPS) [161].

Kawai et al showed that TLR expression is reduced in keratinocyte cultures even after stimulation with LPS. However, moderate expression is present in normal skin at the basal layer. [178, 180]. This expression corresponded in our study to score 0.

In vivo and in vitro data suggest that TLR4 becomes upregulated in the first 24h after lesion occurrence, decreases to basal level by day 10 and is predominantly confined to keratinocytes. [182]

Cutaneous healing is impaired in laboratory mice lacking TLR4, with reduced neutrophilic or macrophage infiltration, and TLR4 is expressed in wound lips 6h to 3 days after wounding. Expression was more pronounced in the epidermis away from the wound margins. [182]. This confirms what we found in the studied batches, with TLR4 expression accentuated in the first 3 h in the epidermis close to the wound. Also, in the wound lip, staining pattern 4 was frequently encountered, with absence of basal TLR expression.

A particularly interesting aspect was found in our study regarding the nuclear expression of TLR4.

Although TLR receptors are receptors with membrane (as is the case with TLR4) or intracellular/cytoplasmic expression, studies have been reported in which nuclear expression has occurred, most of which are performed on tumor batches [186], [187], [188]. In a study coordinated by Huhta et al this nuclear positivity of TLR4 was demonstrated in intestinal

epithelium, and Janardhan et al demonstrated it in pneumocytes and bronchial cells stimulated with bacterial lipopolysaccharide. [189, 190]

Possible explanations for this nuclear staining pattern of the TLR could be as follows: Exposure of the integument to the sun, with damage by ultraviolet radiation. [191]

Although possible, this hypothesis was not confirmed in our study, as re-evaluation of the cases yielded conflicting data on the location of skin fragments, with no statistical significance between the different groups.

We can assume that the existence of a higher amount of existing ligand (in our case HMGB1) could favour TLR4 translocation from the cytoplasm to the nucleus.

The last, and probably most plausible, possibility is the existence of an alternative TLR4-mediated intracellular signalling pathway that allows TLR4 translocation into the nucleus. This potential alternative pathway remains to be addressed by further studies.

8.5.4 Discussion of nuclear factor kappa B (NF-κB) expression

NF-κB was discovered in 1986 by Baltimore et al. and was described as a transcription factor in the nucleus of B lymphocyte cells and is present in the cytoplasm of all cells, but in inactive form. When translocated into the nucleus, it becomes active. [192]

NF-κB (nuclear factor-κB) controls the expression of genes involved in inflammatory and oxidative stress response, differentiation, cell proliferation, cell adhesion and apoptosis. [196]

In response to PAMP and DAMP stimulation, macrophages rapidly activate and secrete a broad palette of cytokines and chemokines [203].

In the study published by Salles et al., NFkB activation in femoral fracture foci was shown in a group of laboratory animals in the first hours after injury, peaking at 6 h [210]. In our study we observed nuclear positivity (score 3) from LS1 to LS5 in the epithelium distant from the wound. This positivity was not found in any fragment belonging to LS6 or 7 or fragments collected during autopsy.

In forensic medicine, NFkB activation has been studied in traumatic brain injury in both laboratory animals and humans. Nonaka et al. as well as Tao et al. showed nuclear translocation of NFkB to the area at the periphery of the concussion zone approximately one hour after the trauma. The peak of this translocation was at 12-24 hours. [211, 212]

Conclusions:

1. From our studies, HMGB1 can be a reliable marker for affirming the vitality of a wound when it scores 4 in the epithelium of the wound lip, score 3 in the epithelium of the wound vicinity and score 3 in the vessels of the bleeding area.

2. Regarding the tanatology, we can state that HMGB1 can be considered positive from the first minutes after the lesion.

3. The expression of RAGE in the epidermis is useful in differentiating intra- and postmortem wounds, without being able to accurately objectify the time interval since injury. This interval can be estimated (at least for LS1) by combined HMGB1 +RAGE analysis leading to a probability of success of more than 70%.

4. The expression of RAGE in vessels is almost identical in batches containing intra- and postmortem wounds, making it useless in forensic practice.

5. TLR 4 allows the differentiation of intravital and postmortem wounds, and its nuclear expression should be further investigated as it can be a very important clue in forensic practice.

6. NFkB was a vitality certainty when graded with a score of 3, unfortunately, this expression occurred in a rather low proportion of about a quarter of all fragments examined.

7. Further studies are needed to evaluate the efficacy of these markers under conditions of tissue autolysis or in skin lesions without a continuation solution.

Limits of studies

One of the limitations of this study was the small number of skin fragments from postmortem lesions.

Another limit is the survival interval. Although the inclusion criteria were very strict (witnesses, 112 call, video surveillance cameras), there will always be a small delay between the time an accident/injury occurs and when it is reported. One option to overcome this limit is to test markers on a batch of laboratory animals, where the timing parameter is much easier to monitor.

Another limitation we identified is that VAP-1 also positivizes in smooth muscle fibers. Although their intensity is usually low, this positivity can usually interfere with the way VAP-1 is cleaved in arterioles and venules. For capillaries, this limitation is non-existent, as smooth muscle fibres are not part of their structure. Currently, I am working to overcome this limit by

testing a double staining by combining a marker that targets smooth muscle fibres (αSMA-Alpha Smooth Muscle Actin) with VAP-1.

A new limitation may be that the results have not been confirmed by another method, such as Western blot or RT-PCR.

A final limitation identified is that these markers have not been tested under autolysis/tissue repair conditions. In forensic medicine, physicians must frequently examine cadavers with a greater or lesser degree of autolysis and it is then imperative to test any new markers in these lesions from cadavers with longer exposure time to environmental conditions.

Chapter 9 General conclusions

1. Macroscopic assessment of wounds should always be complemented by microscopic examination, especially in lesions that occurred shortly before death or shortly after death.

2. HE staining is the cheapest and provides the most valuable information compared to Perls and Van Gieson Verhoeff, if inflammatory infiltrate is present.

3. The information provided by classical staining techniques is not sufficient to assess the vital-non vital character in wounds where inflammatory infiltrate is missing, therefore my proposal for these lesions is that microscopic diagnosis is limited to *wounds produced shortly before death or shortly after death or with uncertain vitality* .

4. After comparative evaluation of VAP-1 and P-selectin, we can conclude that VAP-1 expression in the bleeding area is a good variant for vitality affirmation in wounds with a survival interval of less than 24 hours.

5. Although we have searched for *The Holy Grail* of useful markers in the vital response, the recommendation to use the association of these markers remains valid. My proposal, following the completion of this research, is to use a marker that investigates expression in vessels (VAP-1) together with markers that assess the epithelium (HMGB1 +RAGE).

6. A combined score of the above markers (Score 3 in the haemorrhage area for VAP-1, Score 4 in the wound lip epithelium and 2 or 3 in the epithelium adjacent to the wound for HMGB1 and RAGE) provides approximately 95% classification of the lesion in LS1 and, in more than 99% of cases, differentiation with control or postmortem lesion fragments.

7. Control fragments are indispensable in assessing the expression of these markers, without them the confidence in the results obtained is diminished.

Chapter 10 Originality and innovative contributions of the thesis

Addressing new useful markers in vital response is a valuable addition to the forensic field. Immunohistochemistry is a very efficient method to highlight different tissue or cellular components. In addition to being very efficient, the costs of this method are quite low and the way it is implemented is fast.

The results are original, since:

1. Consulting the literature, we found that VAP-1 was first tested in forensic pathology nationally and internationally.

2. Following this study, immunohistochemical testing of HMGB1 was a first in skin lesions with a continuity solution.

3. Evaluation of the expression of the HMGB1 axis, the receptors of this protein, RAGE and TLR4, as well as the degree of NFkB activation, are novel elements in forensic medicine.

4. Following the studies described in this thesis we can state that among the markers tested, VAP-1 and HMGB1, together with RAGE, may be important markers in the affirmation of vitality in wounds produced shortly before death.

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