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Improving the histotechnical quality of skin and liver samples from deceased pigs and humans for use in teaching and research on post-mortem interval

Paula Teixeira

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Improving the histotechnical quality of skin and liver samples from deceased pigs and humans for use in teaching and research on post-mortem interval

Paula Cristina Araujo Teixeira

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

PAULA CRISTINA ARAÚJO TEIXEIRA

IMPROVING THE HISTOTECHNICAL QUALITY OF SKIN AND LIVER SAMPLES FROM DECEASED PIGS AND HUMANS FOR USE IN TEACHING AND RESEARCH ON POST-MORTEM INTERVAL

Dissertation application for the Master degree in Forensic Medicine, submitted to the ICBAS – School of Medicine and Biomedical Sciences – University of Porto.

Supervisor – Eduardo Jorge Sousa da Rocha

Category – Full Professor

Affiliation – ICBAS – School of Medicine and Biomedical Sciences – University of Porto

Co-supervisor – Ana Silvia Pires Luís

Category – Hospital Assistant and Invited Auxiliary Professor

Affiliation – Centro Hospitalar de Vila Nova de Gaia/Espinho and ICBAS – School of Medicine and Biomedical Sciences – University of Porto

“Every man's life ends the same way. It is only the details of how he lived and how he died that distinguish one man from another.” **— Ernest Hemingway**

“If you can see, look. If you can look, observe.” **— José Saramago, book Blindness**

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Finally, I must acknowledge the two most significant people in my life, my parents, to whom I dedicate this thesis and express my gratitude for shaping me into the person I am today.

To everyone, my heartfelt gratitude!

Declaration of honour

I declare that the present dissertation is my original work and has not been used in previous courses or curricular units at this or any other institution. References to other authors (statements, ideas, thoughts) strictly adhere to the attribution requirements and are appropriately noted in the text and references, in accordance with the referencing guidelines. I am aware that plagiarism and self-plagiarism are academic offences.

Declaração de honra

Declaro que a presente dissertação é da minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

Paula Cristina Araújo Teixeira

Abstract

The post-mortem interval is one of the primary goals in the investigation of human and animal death, in a medico-legal context, and consists of the period of time elapsed from death to the moment of discovery of the corpse or carcass. Its precise determination remains one of the greatest challenges of forensic medicine since it is influenced by several factors, such as temperature, humidity, animal activity, level of decomposition and cause of death, which justifies the variability of results obtained in the different methodologies described in the literature.

Histological analysis is already used to determine the cause and mechanism of death and/or injury, being considered an essential complementary exam for the conclusion of the autopsy. Likewise, histology has a high potential for determining PMI, being opportune to carry out further studies aimed at evaluating PMI in organs that are routinely evaluated at autopsy.

The skin is considered a gold standard tissue in forensic investigation, as it provides information about injuries and pathologies and the PMI, with the advantage of easy collection. However, when the autopsy is performed, the skin may present a considerable state of putrefaction. Some studies aim to reverse some of these changes to recover the histological architecture.

The liver is one of the organs commonly collected in the autopsy and due to its great activity associated with anabolic and catabolic reactions, it undergoes biochemical changes after death that cause histological changes that are used in the determination of PMI.

The purpose of this research is to improve the histotechnical quality of skin and liver samples obtained from carcasses or cadavers, as well as analyse added values in the estimation of the post-mortem interval and the generation of teaching material.

The study included 20 domestic pig necropsies and 20 human autopsies with known or expected post-mortem intervals, from which skin and liver samples were collected. The embedding in paraffin and methacrylate, as well as the HE, PAS, and Goldner's Masson trichrome staining were all completed.

We conclude that skin and liver samples obtained from necropsies or autopsies have great potential as good teaching material and a potential indicator of PMI, even though more cases are needed in this study to draw more significant conclusions about the variables in the study.

Keywords: Post-mortem interval; histology; skin; liver; teaching material.

Resumo

O intervalo *post-mortem* é um dos objetivos primordiais da investigação da morte humana e animal, em contexto médico-legal, e consiste no período de tempo decorrido desde a morte até ao momento da descoberta do cadáver ou carcaça. A sua determinação precisa continua a ser um dos maiores desafios da medicina legal, uma vez que é influenciado por diversos fatores, tais como a temperatura, humidade, atividade animal, nível de decomposição e causa de morte, o que justifica a variabilidade de resultados obtidos nas diversas metodologias descritas na literatura.

A análise histológica é já utilizada na determinação da causa e mecanismo da morte e/ou lesão, sendo considerada um exame complementar essencial para a conclusão da autopsia. De igual modo, a histologia apresenta uma alta potencialidade para a determinação do PMI, sendo oportuno a realização de novos estudos que visam a avaliação do PMI em órgãos que são por rotina avaliados na autópsia.

A pele é considerada um tecido *gold standard* em investigação forense, uma vez que permite obter informações alusivas a lesões, patologias e PMI, tendo ainda como vantagem a sua fácil colheita. Contudo, aquando a realização da autópsia, a pele pode apresentar-se num estado severo de putrefação. Existem estudos que visam reverter algumas dessas alterações, com a finalidade de recuperação da arquitetura histológica.

O fígado é um dos órgãos comumente colhidos na autópsia e devido à sua grande atividade associada a reações anabólicas e catabólicas, sofre alterações bioquímicas após a morte que causam alterações histológicas que são usadas na determinação do PMI.

O objetivo deste trabalho é a melhoria da qualidade histotécnica de amostras de pele e fígado obtidas em cadáveres, bem como a análise de mais-valias na estimativa do intervalo pós-morte e geração de material didático.

O estudo incluiu 20 casos de necropsia de porco doméstico e 20 casos de autopsias humanas, com PMI conhecido ou previsto, do qual foram colhidas amostras de pele e fígado. Foram realizados o processamento com impregnação em parafina e metacrilato, bem como as colorações de HE, PAS e tricrómio de Goldner.

Concluímos que as amostras de pele e fígado colhidas em necropsias e autópsias apresentam um grande potencial como material de ensino e como indicador de PMI, apesar de ser necessário um aprofundamento do estudo com o aumento do número de casos, para obter conclusões mais significativas das vaiáveis em estudo.

Palavras-chave: Intervalo post-mortem; histologia; pele; fígado; material didático.

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1. Introduction

1.1. Definition of post-mortem Interval and its importance in forensic medicine

A forensic autopsy is an examination conducted to address medico-legal issues such as determining the deceased's identity, determining the cause of death, confirming or refuting the manner of death, and estimating the post-mortem interval (PMI) (Kotabagi, Charati et al. 2005, Menezes and Monteiro 2022). By definition, the PMI consists of the period elapsed from death time to the moment of discovering the corpse (Lee Goff 2009, Madea 2016, Dell'Aquila, De Matteis et al. 2021, Noshy 2021). By proxy, in a controlled study, we may establish the PMI as the period between the animal or human death and the moment of the sample collection.

To know the PMI significantly impacts the investigation of human and animal death in medico-legal and forensic veterinary context (Munro and Munro 2013, Brooks 2016, Ehrenfellner, Zissler et al. 2017, Yahia, El-Amir et al. 2018), and veterinarians and biologists when studying wildlife (Cooper 2012). Considering the human case, PMI has relevance in criminal and civil matters (Kaliszan, Hauser et al. 2009, Salam, Shaat et al. 2012, S, Menéndez et al. 2014, Dell'Aquila, De Matteis et al. 2021). Determination of the time of death has an essential role, particularly in cases of violent death, such as homicide and suicide, accidental deaths (Madea 2016, Zissler, Stoiber et al. 2020). In such cases, it is necessary to obtain the most accurate estimate of the PMI, since, at the reconstruction of the mode and circumstances of death, that information permits connecting or not the suspect to the crime scene(s) or victim and establishing the veracity of the information provided by witnesses (Kaliszan, Hauser et al. 2009, Salam, Shaat et al. 2012, Munro and Munro 2013, Dell'Aquila, De Matteis et al. 2021). In civil proceedings, PMI can be a key aspect in situations involving inheritance, such as multiple killings or accidents concerning several individuals, when the death order may conflict with the inherence determination (Kaliszan, Hauser et al. 2009).

Referring to the animal death investigation, the PMI is not only used to corroborate the witness testimony as in human cases (Brooks 2016) but is usually employed in cases of the out-of-season shooting of game animals, poaching, animal deaths during transportation, and neglect or willful cruelty of companion animals (Munro and Munro 2013, Brooks 2016). Furthermore, the PMI can still be used to determine if the incident was a one-time occurrence or a continuing problem (Erlandsson and Munro 2007, Brooks 2016, Wilson, Neilsen et al. 2020), which can influence the legal penalty. We can also emphasise the importance of PMI in the investigation of fraudulent claims, particularly those involving

animal insurance, which is becoming more popular but often requires checking if the identity and stated circumstances of death are consistent with the PMI indicated by the veterinary pathologist (Brooks 2016).

1.2. Methods for determination of PMI and potential substitutes

When a body enters the Medical-Legal Institute, it is common to be accompanied by a police report, which may include witness testimony as to when the deceased was last seen or known to be alive based on circumstantial evidence provided in the information, which may help to determine the PMI (Lee Goff 2009, Zissler, Stoiber et al. 2020, Ceciliason, Andersson et al. 2021). However, in the case of witness evidence, such information might not be detailed enough or can even be fake.

The determination of PMI is essentially done by the study of physical processes (body cooling and post-mortem lividity), physicochemical processes (rigour mortis and supravital rigidity of skeletal muscle), bacterial processes (putrefaction), metabolic processes (autolysis) and entomology (Madea 2005, S, Menéndez et al. 2014, Madea 2016, Ali, Ibrahim et al. 2017, Zissler, Stoiber et al. 2020, Dell'Aquila, De Matteis et al. 2021). Some of these strategies will now be mentioned.

The algor mortis, or body cooling, consists of cooling the body after death, which results in four different activities (convection, conduction, radiation and evaporation). Estimating the PMI with algor mortis requires a mathematical equation that considers the ambient circumstances, posture, body weight and clothes (Madea 2005, Brooks 2016, Madea 2016), with the nomogram approach created by Henssge (1988) being the most reliable at the moment (Madea 2016, Schweitzer and Thali 2019). To use the nomogram, a relationship between rectal body temperature (measured during the crime scene investigation) and ambient temperature must be established and connected by a straight line (Figure 1).

The livor mortis is the purple-red staining of soft tissues caused by post-mortem gravity-dependent blood sedimentation towards the lowest levels or parts of the body, starting to appear 20 to 30 minutes after death (Brooks 2016, Madea 2016). It is worth noting that the livor mortis is nonfixed at approximately 8 hours, blanching when subjected to digital pressure, with potential livor mortis disposable variations produced by body position shift. That scenario changes after 8 to 12 hours of death, when the livor mortis becomes permanent, with no pattern when pressure, and no changes independent of body position (Lee Goff 2009, Brooks 2016). This information is also significant since a difference

between the site of the livor mortis and body position can indicate body manipulation (Brooks 2016).

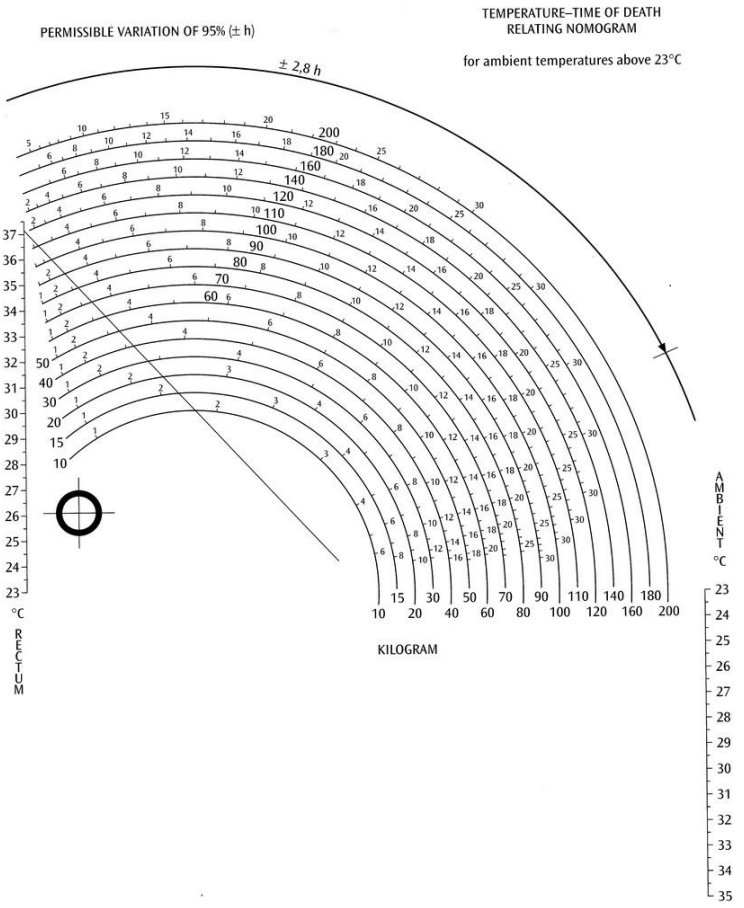

Figure 1. Henssge nomogram for ambient temperatures higher than 23 °C. *Reproduced from ref. (Madea, Ortmann et al. 2019) with permission from Springer Nature*.

Rigour mortis is characterised by cadaveric rigidity caused by a drop in adenosine triphosphate (ATP) levels, which causes prolonged binding of actin and myosin muscle filaments, resulting in muscle contraction (Martins, Ferreira et al. 2015), and has multiple phases. It usually starts about 3 to 6 hours after death and peaks between 6 to 12 hours, with full rigour lasting 18 to 36 hours; after wards, stiffness gradually dissipates, causing muscles to become flaccid again (Brooks 2016, Madea 2016).

The putrefaction consists of the body changes associated with a bacterial process predominantly influenced by environmental factors, such as temperature (Table 1) (Brooks 2016, Madea 2016).

The autolysis effect can also be determined by observing an increased potassium level or a decrease in sodium and chloride concentration in the extracellular fluid (Madea 2005, Madea 2016). For the study of metabolic processes, the most common biological fluid

used is vitreous humour since less susceptible to degradation and contamination (Madea 2005, Dell'Aquila, De Matteis et al. 2021). The vitreous humour approach implies calculating of potassium concentration, whose value is proportional to the PMI. However, that value is not liable in case of metabolic disturbances before death (James, Hoadley et al. 1997, Madea 2005, Madea 2016, Ave, Ordóñez-Mayán et al. 2021).

Forensic entomology studies insects and others arthropods found at crime scenes to determine the PMI and cause of death (Joseph, Mathew et al. 2011, Pacini, Santos et al. 2015, Brooks 2016, Harvey, Gasz et al. 2016, Matuszewski 2021). It can be determined both by the study of the life phases of insects (Joseph, Mathew et al. 2011) and by analysing chemical components, such as pharmaceuticals or drugs, taken by the victim and passed to the animal analysed (entomotoxicology) (Pacini, Santos et al. 2015).

Although it is not one of the most commonly used methodologies mentioned in the literature, observing the degree of food digestion in the stomach or intestine and the amount of urine in the bladder can also aid in PMI assessment, despiting involving significant subjective description and evaluation. (Madea 2005, Babu, Gayathri et al. 2016, Madea 2016).

At the moment, the temperature approach devised by Henssge (1988) based on the two exponential formulas of body cooling by Marshall and Hoare (1962), coupled with data on supravital responses, post-mortem lividity, and rigour mortis, is the gold standard in for early PMI (Madea, Ortmann et al. 2019, Schweitzer and Thali 2019).

Considering the importance of PMI determination, many studies have sought new and more precise methods. The approaches vary in tools, such as recurring to the analysis of the degeneration of mRNA, DNA and proteins (Pittner, Monticelli et al. 2016, Li, Ma et al. 2017, Scrivano, Sanavio et al. 2019, Pittner, Gotsmy et al. 2020, Tozzo, Scrivano et al. 2020, Dell'Aquila, De Matteis et al. 2021, Noshy 2021, Sangwan, Singh et al. 2021), and changes in metabolites (metabolomics) and lipids (lipidomics) (Li, Ma et al. 2017). Histological tools have proved their role, such as using the immunohistochemical detection of insulin, thyroglobulin and calcitonin (Madea 2005). More technicality and precision may imply higher costs per analysis, which is one disadvantage to enforcing routine applications.

1.3. Problems associated with PMI determination

There is no doubt that the PMI is one of the most significant tasks in medical-legal inquiry, but several factors influence its estimation. The methods used to estimate PMI, including the ones previously mentioned, are inherently biased both by intrinsic factors such

as age, gender, physiological and pathological conditions, and extrinsic factors such as temperature, humidity, animal activity, stage of decomposition, and cause of death, which explains the disparity in results obtained by different techniques and the need to investigate new methods or improve the previous ones (Ali, Ibrahim et al. 2017).

Also, the more common methods are based on the observation of transformative cadaveric phenomena, which opens the possibility of increasing inter-individual variability assessment (and therefore of the PMI estimation), being the forensic pathologist's sensitivity and experience an important role (Dell'Aquila, De Matteis et al. 2021).

The measurement of PMI in animals is far more variable, owing to the variety of study methodology and species examined, making it impossible for researchers, to this date at least, to draw broad conclusions about the relatedness of animal study findings. However, studies determined that some of the procedures used in human studies to estimate PMI might apply to animal subjects, but further research is needed, particularly to validate the use of these approaches in court for animal offences (Brooks 2016).

Even though research on new or enhanced PMI detection methodologies has been conducted for more than 60 years, further studies are required. In detail, it is necessary to search for procedures that allow for more exact, dependable, and timely results that meet the requirements of medico-legal practises (Madea 2005).

1.4 Histology's potential (viz. using liver and skin) as a PMI estimating tool

Histological analysis is already used to investigate the causes and mechanisms of injury and death. It is considered an essential complementary exam for the autopsy's conclusion (Roulson, Benbow et al. 2005, Molina, Wood et al. 2007, Singh, Tiwari et al. 2022) and even as quality control (Bernardi, Saldiva et al. 2005). Likewise, histology has a high potential for helping determine the PMI, making it opportune to introduce it in studies aimed at evaluating that parameter in samples from organs that are routinely assessed at autopsy.

The skin is considered a gold standard organ in forensic investigation, as it provides information about injuries and pathologies and, in parallel, is used for estimating the PMI (Kovarik, Stewart et al. 2005, Bardale, Tumram et al. 2012, El-Nahass, Moselhy et al. 2017), with the advantage of easy collection. However, when performing autopsies, the skin may often display a severe state of putrefaction (Boracchi, Andreola et al. 2016). Some studies

aim to reverse some of these changes to recover the histological architecture (Collini, Andreola et al. 2014, Boracchi, Andreola et al. 2016).

The liver is one of the organs commonly collected in the autopsy (Fumeaux, Scarpelli et al. 2018). Due to its great activity associated with anabolic and catabolic reactions, it undergoes biochemical changes after death that cause histological changes that can be used to determine PMI (Yahia, El-Amir et al. 2018, Ceciliason, Andersson et al. 2021).

1.5. Use of animal models for post-mortem studies: the domestic pig

Animal models are widely used in scientific research due to ethical issues regarding the limits of experimentation and the practical challenges of getting fresh and healthy human samples. The domestic pig (*Sus domesticus*) is commonly used as a large-animal model, having remarkable morphofunctional similarities with humans (Eberlová, Liskac et al. 2017, Matuszewski, Hall et al. 2020).

Regarding skin, despite small mammals have been the most commonly used animal model for studies tackling human-relevant issues, they are poor surrogates of the human skin (Avon and Wood 2005). In contrast, the domestic pig is anatomic and physiologically comparable to humans and became a significant model for dermatologic and wounds studies (Avon and Wood 2005, Summerfield, Meurens et al. 2015, Yang, Galang et al. 2021), including histological approaches (Debeer, Le Luduec et al. 2013, Khiao In, Richardson et al. 2019). Despite its similarities to the human skin, that of pigs has some histological differences, including poor vascularisation of the cutaneous glands and, to a lesser extent, of the subepidermal capillary plexus region, also the possible extensive fat deposition below the subcutis, the smaller holocrine sebaceous glands, and the absence of eccrine sweat glands (Avon and Wood 2005, Summerfield, Meurens et al. 2015).

When it comes to the human liver, there is no doubt that the porcine liver is the best documented and the most suitable surrogate (Eberlová, Liskac et al. 2017, Junatas, Tonar et al. 2017). The organ size, gross anatomy, microscopic morphology and physiology can validate this idea (Eberlová, Liskac et al. 2017, Junatas, Tonar et al. 2017). Concerning the histological aspects, the main difference between porcine and human livers is that pigs have liver lobules physically demarcated by interlobular connective tissue septa that separate the lobules sharply while continuing to connect with the portal areas (Junatas, Tonar et al. 2017).

The pig has already established itself as a scientifically acceptable animal model for forensic entomology and taphonomy (Matuszewski, Hall et al. 2020, Probst, Gethmann et al. 2020), including PMI research, namely concerned with temperature effects on the body (Kaliszan, Hauser et al. 2005), entomology related aspects (Pittner, Bugelli et al. 2020), histological alterations related to different PMIs in different organs, including skin and liver (Khiao In, Richardson et al. 2019, Ahmed Alaa El-Din, Mohamed Ahmed et al. 2021) and recent studies associates with degradation of proteins (Pittner, Monticelli et al. 2016, Pittner, Bugelli et al. 2020).

We may conclude that using a pig as a model for PMI research into new methods or enhancements to existing ones is a perfect answer since it allows for easier replication of experiences, faster availability in large numbers at a cheap cost, added to the possibility of improved control of experience variables (Matuszewski, Hall et al. 2020).

1.6. Human and animal biological samples for histology teaching

The study of histology and associated histopathology continues to significantly impact human medicine training and practice and other areas (Wood, Whiten et al. 2015). (Wood, Whiten et al. 2015). For example, at the ICBAS – School of Medicine and Biomedical Sciences of the University of Porto (U.Porto), complete histology courses are offered to students of Aquatic Sciences, Biochemistry, Dental Medicine and Veterinary Medicine. In the practical classes, students observe a wide range of histology slides which imply the previous acquisition of the human or animal biological samples necessary to make such didactic material.

Observation of the slides can be made with a microscope or with a computer after slide scanning. The use of virtual microscopy increased in popularity since it permits better self-learning by the students and the sparse material used permits the observation of slides in any place and, potentially, by an unlimited number of students (Wood, Whiten et al. 2015, Al Khader, Odeh et al. 2021). However, it also requires initially physical histological slides to create their digitalization for observation on computer screen. The biological material's structural and tintorial qualities are essential for teaching purpose.

Except for the COVID-19 pandemic, a teaching mix of virtual microscopy and slide observation under a microscope has been employed in histology lectures given by the Laboratory of Histology and Embryology, Department of Microscopy, ICBAS-U.Porto.

COVID-19 was declared a worldwide health emergency on January 30, 2020, by the World Health Organization. Consequently, regulatory and social-distancing measures were

imposed across the world (Natsis, Lazaridis et al. 2022, Saverino, Marcenaro et al. 2022). One of the measures was the elimination of face-to-face lessons in all schools, including universities, emphasising the significance of innovative teaching approaches that allow for distant instruction (Natsis, Lazaridis et al. 2022, Saverino, Marcenaro et al. 2022), namely in pathology via virtual lessons (Darici, Reissner et al. 2021, Iwanaga, Loukas et al. 2021). That need was also amply demonstrated in histology and anatomy courses (Saverino, Marcenaro et al. 2022), where manual engagement with cadavers (Iwanaga, Loukas et al. 2021) or observation of histological slides in a microscope is still regarded as the most effective teaching approaches.

Cultural, ethical, bureaucratic and casual circumstances often make the obtention of human cadavers and biological samples for teaching challenging. The COVID pandemic may have aggravated the situation, and the evidence points to a decrease in the number of donated bodies in many medical schools (Iwanaga, Loukas et al. 2021). Overall, there is a need to take advantage of the previous biological samples retrieved from humans, including from cadavers, potentiating the quality of the histological preparation.

1.7. Inclusion media for histological processing: paraffin vs methacrylate

The current study seeks to employ samples from pig corpses and human cadavers, which may pose certain difficulties, such as the usual observation of spontaneous disruption of normal organ histology due to cell death and matrix degradation. As a result, artefacts are more likely to develop during histological processing, which we believe can be minimised by employing acrylic resins.

According to the literature, sections of tissues embedded in methacrylate-based resins offer significantly higher resolution than those from paraffin embedding because they allow the easy routine generation of thinner sections (1-3 µm thick) than with paraffin (3-6 µm thick), leading to a more accurate assessment of the tissue morphology (Woodruff and Greenfield 1979, Gerrits and Horobin 1996). Furthermore, methacrylate avoids deformation of tissue elements occurring when pieces are embedded in paraffin, and because it does not need to be removed from sections before staining (contrarily to paraffin), it results in minimal dimensional alterations (Gerrits and Horobin 1996).

We found one study on PMI determination using a resin with several properties of methacrylate, such as minimal structural distortion and shrinkage. The authors used epoxy resin embedding in samples from the kidney, pancreas, liver, heart, and skeletal muscle of

Wistar rats, which were compared to paraffin-embedded ones (Tomita, Nihira et al. 1999). They concluded that the epoxy outperformed the paraffin in determining the time of death.

1.8. Aims

Given the context presented above and within the scope of the Master Degree in Forensic Medicine of the ICBAs – U.Porto, the current dissertation had the following aims:

a) to assess eventual gains of using a methacrylate-based resin (versus paraffin) in the quality of histological sections of skin and liver from human cadavers and pig carcasses, in parallel to test refinements of the routine processing that would promote improvements;

b) to conclude about the suitability of the sections, under several types of stainings, for use in histology classes, and, if so, what would be the acceptable PMI for sampling;

c) to devise and implement scoring systems for staging the degree of decomposition of the skin and liver in the two species, aiming to correlate the worst deterioration with greater PMI;

d) to conclude if the processing improvements would translate into a more easy or discriminative evaluation of the histological decay after death and/or better correlation with the known (or estimated) time of death.

If achieved, the desired improvements in the sections' histological quality would be useful in histology teaching and forensic estimation of the PMI and serve for more accurate histopathological analyses associated with autopsies and necropsies and encourage the development of educational resources in forensic and general histopathology.

2. Materials and methods

This research occurred in the Laboratory of Histology and Embryology, Department of Microscopy, ICBAS-U.Porto. It was divided into several sections, including using biological materials from carcasses of the animal model (the pig) and human cadavers, improving the histotechnical quality of skin and liver samples, and identifying post-mortem histological changes to tentatively relating them with the known or estimated time of death.

2.1. Use of the animal model to improve the histotechnical quality of skin and liver samples

2.1.1. Animals and sampling procedures

Using samples from domestic pig carcasses in the current study was approved by the Responsible Animal Welfare Organ (ORBEA) from ICBAS-U.Porto.

Given the difficulty in obtaining human samples, particularly during the COVID-19 pandemic, the first part of the experiment used the pig as an animal model. Thus, between 16th December 2020 and 15th January 2021, twenty carcasses of Large White (a British breed) domestic pigs were selected from necropsies performed during Pathologic Anatomy I lectures for the Integrated Master of Veterinary Medicine at ICBAS-U.Porto. For every case, the liver and a piece of skin (about 10 cm long by 5 cm wide) located in the chest area of each carcass were retrieved. The chest area was chosen for skin sampling because it is non-articular, has a thinner subcutaneous layer, and is less likely to be affected by cumulative exposure, friction, trauma or obesity (Wei, Michu et al. 2020). In addition, information about the animals was gathered, including gender, age, weight, time of death, place of death (city) and cause of death (Table 1).

Due to the fact these animals came from pig farms, some variables such as age, the time of death and temperature at death time were carefully analysed to the possible extent. Regarding age, a graph and table that associate pig weight with its age (http://www.hendersons.co.uk/pigequip/Pig_growth_rate.html) was used to estimate the animals' ages, except for pig number four, whose weight was lower than usual, being the age reported by the owner.

Concerning the time of death, there was no documented animal sacrifice, and it was informed the animals had passed away at night. So, it was vital to consider the time delay between the animal's latest sighting alive and the time it was found dead. As a result, the

moment of death was standardized as half that delay time. The time of death at necropsy corresponds to the interval between the estimated death moment and necropsy (Table 1).

The temperature at the time and place of the animal's death was estimated using the free resource Weather Spark (https://weatherspark.com/) (Table 1). It should be noted that the carcasses were placed at 4 °C from the moment they arrived at the university building until the necropsy.

After gathering the samples and most of the case-specific information (for instance, it took days after the necropsy to determine the cause of death), it was necessary to move forward with the sample storage.

After necropsy, the skin and liver samples were laid flat in a refrigerated set to 4 °C. At specific times after necropsy (0, 24, 48, 72 and 96 hours), two fragments of the harvested skin piece (about 2,5 cm long, 2,5 cm wide by 0,5 cm height, each) and two liver fragments (about 2,5 cm long, 2,5 cm wide by 1 cm height, each) were taken and fixed in 10% neutral buffered formalin (Thermo Scientific, USA) at room temperature during three days, and then in 70% ethanol, at room temperature for at least three days, until further processing for histological studies.

Table 1. Data concerning the pigs used in this research.

Case	Sex	Weight (Kg)	Age (weeks)	Temperature at Time of Death (°C)	Death Time at Necropsy (days)	Cause of Death
1	Female	1.4	2	7.5	1.5	Not determined
2	Female	1.5	2	7.5	1.5	Crushing trauma
3	Male	8.6	8 – 12	7.5	1.5	Pneumonia
4	Female	80.0	36 – 40	7.5	1.3	Fibrous peritonites
5	Male	7.9	4	8.5	1.3	Fibrinous polyserositis
6	Male	5.6	3	8.5	1.3	Strangulated scrotal hernia
7	Male	7.1	4	7.3	0.5	Suppurative polyarthritis
8	Female	7.0	4	7.3	0.5	Pulmonary haemorrhage
9	Male	1.0	2	7.3	0.5	Starvation
10	Male	1.6	2	7.3	0.5	Fibrinous polyserositis
11	Female	1.2	2	7.3	0.5	Not determined
12	Male	5.0	3	7.2	5.5	Fibrinous polyserositis
13	Male	1.5	2	7.2	5.5	Starvation
14	Female	2.3	2	7.2	5.5	Pneumonia and fibrinous polyserositis
15	Female	3.2	3	7.2	5.5	Pneumonia and fibrinous polyserositis
16	Female	4.8	3	7.2	7.3	Renal pathology
17	Female	0.7	1	7.2	8.3	Crushing trauma
18	Male	1.5	2	7.2	8.3	Starvation
19	Male	1.7	2	7.2	8.3	Crushing trauma
20	Male	30.0	10	7.2	8.3	Fibrinous pericarditis

2.1.2. Histological procedures in the pig samples

One of the goals of the present research was to determine whether a different embedding solution would improve the appearance of histologic features, particularly in situations where putrefaction is a concern. Thus, for each specific time of tissue collection after necropsy, two samples were taken – one for paraffin embedding and one for methacrylate embedding.

2.1.2.1. Paraffin procedures in the pig samples

From the samples stored in 70% ethanol, one fragment of skin and liver, corresponding to the times 0, 48 and 96 hours after the necropsy, were submitted to a long routine protocol in an automatic processor (Leica TP 1020, Germany) (Table 2), and embedded in paraffin (Thermo Scientific Histoplast, UK) in an embedding workstation (Leica EG 1140C, Germany). The formed paraffin blocks were sectioned at 3 and 4 µm in a fully automatic microtome (Leica RM2255, Germany) to make six sections for each thickness per block, two sections for slide. Slides were kept at 60 °C for 1 hour and then at 37 °C overnight, and stained by Hematoxylin-Eosin (HE), Periodic Acid-Schiff (PAS) and Goldner's Masson trichrome as described below.

Table 2. Detailed steps of the long histological processing protocols used in this research.

Histological Processing Stage	Long Processing (Hours)
70% Ethanol	-
90% Ethanol	2
96% Ethanol	2
1st Absolut ethanol	2
2nd Absolut ethanol	3
Absolut Ethanol/Xylene (1:1)	2
1st Xylene	2
2nd Xylene II	3
1st Paraffin	3
2nd Paraffin	3

HE staining in paraffin sections protocol

1. Dewax sections with two xylenes (VWR, France) passages lasting 10 minutes each.

2. Rehydrate through descending ethanol concentrations from absolute, 96%, 70% to 50% (Valente e Ribeiro, Lda, Portugal), lasting 5 minutes each.
3. Rinse thoroughly for 5 minutes under running tap water.
4. Stain for 2 minutes with Mayer's hematoxylin (Merck, Germany).
5. Rinse thoroughly for 5 minutes under running tap water.
6. Stain with 1% eosin Y solution, aqueous for 5 minutes.
7. Quickly rinse with tap water.
8. Dehydrate by a quick dip three times indifferent absolute ethanol (Valente e Ribeiro, Lda, Portugal).
9. Quick dipping in two different xylenes (VWR, France).
10. Mount with Coverquick 2000 mounting media (VWR, France).

Solutions preparations:

Eosin solution was prepared in house by diluting 10 g of eosin Y (yellowish) C.I. 45380 (Merck, Germany) in 1 L of distilled water to make 1 L of 1% eosin Y solution, aqueous. It was advised to add 0.5 mL to the prior solution to intensify staining.

PAS staining in paraffin sections protocol

1. Dewax sections with two xylenes (VWR, France) passages lasting 10 minutes each.
2. Rehydrate through descending ethanol concentrations from absolute, 96%, 70% to 50% (Valente e Ribeiro, Lda, Portugal), lasting 5 minutes each.
3. Rinse thoroughly for 5 minutes under running tap water.
4. Treat with 0.5% periodic acid for 15 minutes.
5. Rinse thoroughly for 5 minutes under running tap water.
6. Stain in Schiff's reagent (Sigma-Aldrich®, USA) for 20 minutes.
7. Rinse thoroughly for 5 minutes under running tap water.
8. Counterstain nuclei with Mayer's hematoxylin (Merck, Germany) for 1 minute.
9. Dehydrate by a quick dip three times in different absolute ethanol (Valente e Ribeiro, Lda, Portugal).
10. Quick dipping in two different xylenes (VWR, France).
11. Mount with Coverquick 2000 mounting media (VWR, France).

Solutions preparations:

The 0.5% periodic acid solution was prepared by adding 0.5 g of periodic acid (Fluka® Analytical, China) to 100 mL of distilled water.

Goldner's Masson trichrome staining in paraffin sections protocol

1. Dewax sections with two xylenes (VWR, France) passages lasting 10 minutes each.
2. Rehydrate through descending ethanol concentrations from absolute, 96%, 70% to 50% (Valente e Ribeiro, Lda, Portugal), lasting 5 minutes each.
3. Rinse thoroughly for 5 minutes under running tap water.
4. Treat with Bouin's solution (VWR, Belgium) for 1 hour, at 60 °C.
5. Rinse thoroughly for 5 minutes under running tap water.
6. Stain for 2 minutes with celestine blue solution.
7. Rinse thoroughly for 5 minutes under running tap water.
8. Stain for 2 minutes with Mayer's hematoxylin (Merck, Germany).
9. Rinse thoroughly for 5 minutes under running tap water.
10. Stain with a mixture of ponceau xylidine and acid fuchsin for 5 minutes.
11. Rinse in 1% acetic acid.
12. Treat with a mixture of phosphomolybdic acid and orange G solution for 5-10 minutes.
13. Rinse in 1% acetic acid.
14. Stain with 0,2% light green for 5-10 minutes.
15. Treat with 1% acetic acid for 1-5 minutes.
16. Dehydrate by a quick dip in three different absolute ethanol (Valente e Ribeiro, Lda, Portugal).
17. Quick dipping in two different xylenes (VWR, France).
18. Mount with Coverquick 2000 mounting media (VWR, France).

Solutions preparations:

For Goldner's Masson trichrome Staining in Paraffin Sections, the following solutions were prepared in house:

-Celestine blue solution (final volume 500 mL): 25 g of ferric ammonium sulphate (Merck, Germany) was dissolved in cold distilled water while being stirred, and 2.5 g of celestine blue (Aldrich®, USA) was added. The mixture was then brought to boiled for a

few minutes. After cooling the stain was filtered and 70 mL of glycerin (Panreac, Barcelona) was added.

-Mixture of ponceau xylidine and acid fuchsin: two parts of 1% of ponceau xylidine in 1% acetic acid and one part of 1% of acid fuchsin in 1% acetic acid were combined. For each 100 mL of 1% of ponceau xylidine in 1% acetic acid was dissolved 1 g of ponceau xylidine (Sigma®, India) in 100 mL of water distilled and added 1 mL of acetic acid glacial 100% (Merck, Germany). Relatively to the 1% of acid fuchsin, in 1% acetic acid solution, it was dissolved 1 g of acid fuchsin (Merck, Germany) in 100 ml of water distilled and 1 ml of acetic acid glacial 100% (Merck, Germany) was then added.

-Mixture of phosphomolybdic acid and orange G: 4 g of phosphomolybdic acid hydrate (VWR, EC) and 2 g of orange G (Merck, Germany) were dissolved in 100 mL of distilled water.

-1% acetic acid: 1 mL of acetic acid glacial 100% (Merck, Germany) was mixed with 100 mL of distilled water.

-0.2% light green: 0,2 g of light green SF Yellowish (Fluka® Analytical, India) were dissolved in 100 mL of distilled water, and then 2 ml of acetic acid glacial 100% (Merck, Germany) was added.

2.1.2.2. Methacrylate procedures in the pig samples

Skin and liver samples collected at the times of 0, 48 and 96 hours after necropsy, were split into two sections, and each component experienced a brief and long histological processing using the Technovit® 7100 embedding kit (Kulzer, Romania) (Table 3).

To summarise the steps in the methacrylate embedding process, the samples, after fixation, were dehydrated using ethanol in increasing concentrations, then underwent pre-infiltration, infiltration and embedding (Table 3).

The pre-infiltration was done by exposing the samples to a 1:1 mixture of infiltration solution and absolute ethanol (Valente e Ribeiro, Lda, Portugal) (pre-infiltration solution).

The process of infiltration entails switching out the pre-infiltration solution for an infiltration solution. In order to make the infiltration solution, 100 mL of base liquid Technovit® 7100 and 1 g of hardener I were combined and stirred for 10 minutes.

In the inclusion, the infiltration solution was swapped out for a polymerisation solution (15 ml of infiltration solution to 1 ml of hardener II) using the moulds that the manufacturer advised. The moulds containing the samples were left at room temperature for the first hour before being heated to 37 °C overnight to produce the block.

The blocks were cut into sections at 3 and 4 µm, using a tungsten-carbide knife and a microtome (Leica RM2155, Germany), along with the adapters to the knife. Each section yielded 2 sections for 3 different slides, making a total of 6 sections of each thickness per block. In a heated water bath (30 °C), the section extension was carried out. The slides were heated to 60 °C until they were dehydrated, and kept at 37 °C overnight. HE and an improved PAS staining protocol were performed, and each procedure is described below.

We attempted to optimise the Goldner's Masson Trichrome protocol for methacrylate sections, with various factors such as temperature, solutions times and pH being evaluated, but unfortunately it did not present consistent results.

Table 3. Detailed steps of the short and long methacrylate processing used in this research.

Histological Processing Stage		**Short Processing**	**Long Processing**
Dehydration	90% Ethanol	1 hour	2 days
	96% Ethanol	1 hour	3 days
	1st Absolute ethanol	1 hour	2 days
	2nd Absolut eethanol	2 hours	2 days
Pre-infiltration		4 hours	3 days
Infiltration		12 hours	7 days
Embedding		1 hour at room temperature and overnight at 37 °C	1 hour at room temperature and overnight at 37 °C

HE staining for Technovit® 7100 slides

1. Rinse thoroughly for 5 minutes under running tap water.
2. Stain for 30 minutes with Mayer's hematoxylin (Merck, Germany) at 36 °C.
3. Rinse thoroughly for 5 minutes under running tap water.
4. Stain with 1% eosin Y solution, aqueous for 5 minutes at 36 °C.
5. Quickly rinse with distilled water.
6. Dehydrate by dipping in three different absolute ethanol (Valente e Ribeiro, Lda, Portugal) for 1 minute each.
7. Diaphanized in two different xylenes (VWR, France) for 2 minutes each.

8. Mount with Coverquick 2000 mounting media (VWR, France).

Solutions preparations:

The 1% eosin Y solution preparation was previously described in the paraffin procedure section.

PAS staining for Technovit® 7100 slides

1. Rinse thoroughly for 5 minutes under running tap water.
2. Treat with 0.4% periodic acid for 30 minutes at 60 °C.
3. Wash with a quick dip in three different distilled waters.
4. Stain in Schiff's reagent (Sigma-Aldrich®, USA) for 15 minutes at 36 °C.
5. Wash with a quick dip in three different distilled waters.
6. Stain for 15 minutes with Celestine blue solution, at 36 °C.
7. Rinse thoroughly for 5 minutes under running tap water.
8. Counterstain nuclei with Mayer's hematoxylin (Merck, Germany) for 20 minutes at 36 °C.
9. Rinse thoroughly for 10 minutes under running tap water.
10. Dehydrate by dipping in three different absolute ethanol (Valente e Ribeiro, Lda, Portugal), for 1 minute each.
11. Diaphanized in two different xylenes (VWR, France) for 2 minutes each.
12. Mount with Coverquick 2000 mounting media (VWR, France).

Solutions preparations:

The preparation of the 0,4% periodic acid solution required 0.4 g of periodic acid (Fluka® Analytical, China) in 100 mL of distilled water, for every 100 mL of solution.

Celestine blue solution preparation was described in the Goldner's Masson trichrome staining in the paraffin section.

2.2. Application of the improved histological processing protocol in human samples

2.2.1. Human sampling and its procedures

Human skin and liver samples were collected from autopsies performed at the National Institute of Legal Medicine and Forensic Sciences (INMLCF, I.P.), North Branch, Porto. The procedure and inclusion of the material here in were approved by the Ethics Committee CHUP/ICBAS (CE CHUP/ICBAS) and the Ethics Committee of INMLCF, I.P.

Twenty Caucasian cadavers were selected between March 28th and August 3rd, 2022, based on the criterion of age greater than 12 years and a known or approximative PMI. From each case, the forensic pathologist responsible for the autopsy collected 2 samples of skin (about 2,5 cm long by 2,5 cm wide each) from the midline of the anterior thoracic wall over the sternal region, approximately 18 cm below the suprasternal notch; and 2 samples of the liver (about 2,5 cm long, 2,5 cm wide by 2,5 cm height, each). The collected samples were placed in a plastic container with 10% neutral buffered formalin (Thermo Scientific, USA).

In addition, it was also collected information on the cadavers' gender, age, weight, time of death, information about the last time they were seen and the time it was reported, location of death (city), whether it happened indoors or outdoors, clothing (if the zone of skin collecting was cover or not), the period spent in the INMLCF, I.P. refrigerator, stage of decomposition, the period spent collecting samples and cause of death (Table 4).

2.2.2. Histological procedures of the human samples

All the samples were subjected to the long protocols of paraffin and methacrylate histological processing. The sections were taken at 3 µm thickness and stained with HE, PAS, and Goldner's Masson trichrome if they were paraffin embedded samples, and HE and PAS if concerning methacrylate embedded pieces, as described previously (2.1).

Note that these specific protocols were selected after the evaluation of the slides corresponding to the pig samples by optical microscopy. From such observations, it was concluded that the long protocols for both paraffin and methacrylate embedding were the best, and 3 µm sections were considered the optimal thickness.

Table 4. Data concerning the human cadavers used in this research.

Case	Sex	Weight (Kg)	Age (Years)	Clothing	Place of death	Decomposition	Temperature at Time of Death (°C)	Death Time at Necropsy (days)	Cause of Death
1	Female	61	70	Fully dressed	Indoor	Absent	11.0	3	Airway obstruction by food bolus
2	Male	70	62	Fully dressed	Outdoor	Absent	15.5	3	Possible respiratory infection
3	Male	85	42	Fully dressed	Indoor	Coloring phase	16.0	4	Carbon monoxide poisoning
4	Male	70	87	Partially Dressed	Hospital	Absent	9.0	1	Burn injuries
5	Female	90	69	Fully dressed	Indoor	Absent	12.5	1	Probable acute myocardial infarction
6	Male	85	63	Fully dressed	Indoor	Absent	11.5	2	Probable acute myocardial infarction
7	Female	58	70	Fully dressed	Outdoor	Coloring phase	12.0	4	Suspected abdominal lymphoma
8	Female	92	69	Fully dressed	Indoor	Absent	14.0	1	Probable acute myocardial infarction
9	Female	122	56	Fully dressed	Indoor	Absent	12.0	1	Arrhythmia due to cardiac disease (amyloidosis)
10	Male	84	50	Partially Dressed	Outdoor	Absent	11.4	3	Neck extrinsic compression asphyxia (hanging)
11	Male	94	70	Fully dressed	Indoor	Absent	13.6	3	Neck extrinsic compression asphyxia (strangulation)
12	Male	56	52	Fully dressed	Indoor	Coloring phase	13.4	3	Probable acute myocardial infarction
13	Male	89	47	Partially Dressed	Indoor	Coloring phase	20.5	3	Acute myocardial infarction
14	Male	86	42	Partially Dressed	Hospital	Absent	21.0	2	Traumatic thoracic injuries
15	Male	62	62	Fully dressed	Indoor	Absent	22.2	2	Airway obstruction
16	Male	56	79	Partially Dressed	Hospital	Coloring phase	18.5	2	Traumatic brain injury
17	Male	90	88	Partially Dressed	Hospital	Absent	20.3	1	Traumatic brain injury
18	Male	78	18	Partially Dressed	Hospital	Absent	23.0	2	Polytraumatism
19	Male	70	67	Fully dressed	Indoor	Coloring phase	16.3	1	Acute myocardial infarction
20	Male	43	64	Fully dressed	Outdoor	Coloring phase	17.0	2	Pneumonia

2.3 Construction of a Score System

All slides were evaluated by optical microscopy. Histological changes at different times after death were noted and used to define a scoring system for each organ (skin and liver) and species (human and pig). Thus, four score systems were constructed: pig skin, human skin, pig liver and human liver.

For skin samples, the scoring system consisted of three grades in both species, depicted in Tables 5 and 6. These grades considered changes in the epidermis, dermis, sweat and sebaceous glands and vascular structures, for instance.

The scoring system for the liver samples consisted of five grades for pig samples (Table 7) and three for humans (Table 8). The main criteria were changes in architecture, hepatocytes and portal areas.

Table 5. Skin decomposition score system proposed for pig samples.

Score	Histological Criteria		
	Epidermis	**Dermis**	**Other Structures**
1	Epithelial layers well preserved and easily recognizable; Mostly well-preserved cells, however mild keratinocyte degeneration might be seen; Keratohyalin granules present and mostly well preserved.	Dermo-epidermal interface mostly maintained or with mild focal separation; Collagen bundles mostly well preserved.	Sweat glands: mild cytoplasmic vacuolation in luminal cells, discontinuous myoepithelial cell layers; Mild cytoplasmic vacuolation of hair follicle cells; Mild sebaceous glands disintegration; Nerves fascicles mostly without alterations.
2	Moderate loss of the normal epithelium morphology and keratinocyte degeneration; Keratohyalin granules present but faint.	Focal dermo-epidermal separation; Collagen bundles with mild fragmentation, mostly in reticular dermis.	Sweat glands: moderate cytoplasmic vacuolation in luminal cells, discontinuous myoepithelial cell layer; Mild to moderate cytoplasmic vacuolation of hair follicle cells; Nerves fascicles with mild decrease of fibre undulation and mild fading of nuclear contours.
3	Prominent loss of the normal epithelium morphology; Keratinocytes degeneration – cytoplasm vacuolization; karyolysis might be seen; Keratohyalin granules mostly inexistent.	Multifocal dermo-epidermal separation; Collagen bundles with moderate fragmentation, mostly in reticular dermis; Fragmentation of the dermis.	Sweat glands: moderate to prominent cytoplasmic vacuolation in luminal cells, discontinuous myoepithelial cell layer; Moderate cytoplasmic vacuolation of hair follicle cells; Nerves fascicles with mild to a moderate decrease of fibre undulation and mild to moderate fading of nuclear contour.

Table 6. Skin decomposition score system proposed for human samples.

Score	Histological Criteria		
	Epidermis	**Dermis**	**Other Structures**
1	Epithelium mostly normal; Some peri-nuclear vacuolization present in keratinocytes.	Dermo-epidermal interface maintained; Collagen bundles with mild or absent fragmentation.	Sweat glands: mild cytoplasmic vacuolation in luminal cells, discontinuous myoepithelial cell layers.
2	Epithelium mostly normal; Considerable peri-nuclear vacuolization present in keratinocytes.	Dermo-epidermal interface might present focal separation; Collagen bundles with mild fragmentation.	Sweat glands: moderate cytoplasmic vacuolation in luminal cells, discontinuous myoepithelial cell layer.
3	Epithelium with some alterations; Sharp peri-nuclear vacuolization present in keratinocytes.	Dermo-epidermal interface with present multifocal separation; Collagen bundles with mild fragmentation more noticeable in papillary dermis.	Sweat glands: moderate to prominent cytoplasmic vacuolation in luminal cells, discontinuous myoepithelial cell layer.

Table 7. Liver decomposition score system proposed for pig samples.

Score	Histological Criteria		
	Architecture	**Hepatocytes**	**Portal Areas**
1	Hepatic lobules present well-defined boundaries; Hepatic cords maintained, without rupture; Hepatic sinusoids well-defined, intact erythrocytes.	Without alterations - euchromatic with evident nucleoli.	Bile duct and blood vessels well defined; Distinct nuclei.
2	Hepatic lobule architecture mostly maintained; Hepatic cords less defined than in grade 1; Hepatic sinusoids with intact erythrocytes.	Mild hepatocyte cytoplasmic vacuolization may be seen; Pyknosis may be seen.	Bile duct and blood vessels mostly distinguishable; Arterioles with perinuclear vacuolization.
3	Hepatic lobule architecture sometimes maintained; Hepatic cords with mild rupture/fragmentation; Hepatic cords and sinusoids are still distinguishable; Hepatic sinusoids with atrophy and dilation, with intact erythrocytes.	Moderate hepatocyte cytoplasmic vacuolization and/or cell atrophy; Pyknosis and karyorrhexis observed.	Bile duct and blood vessels distinguishable; Bile duct – loss of limits, nuclear pseudostratification and vacuolization; Blood vessels wall with moderate degeneration.
4	Moderate loss of hepatic lobule architecture; Hepatic cords with prominent fragmentation, individual hepatocytes may be present; Hepatic cords and sinusoids still observed, however most of spaces consisted in extravascular erythrocytes.	Moderate hepatocyte cytoplasmic vacuolization and ballonization; Pyknosis and karyorrhexis observed; Evident nucleoli still observed.	Bile duct and blood vessels less frequently distinguishable; Bile duct – nuclear pseudostratification moderate cell vacuolization.
5	Total loss of hepatic lobule architecture; Extensive hepatic cords fragmentation, with frequent individual hepatocytes; Hepatic sinusoids not distinguishable; Erythrocyte disintegration.	Moderate to prominent hepatocyte cytoplasmic vacuolization; Karyolysis.	Some portal space structures might be observed; Bile duct epithelium and vascular wall disintegration.

Table 8. Liver decomposition score system proposed for human samples.

Score	Histological Criteria	
	Architecture	**Portal Areas**
1	Hepatic cords maintained, without rupture; Good distinction between hepatic cords and sinusoids; Dilated hepatic sinusoids; Intact erythrocytes.	Bile duct and blood vessels well defined; Epithelium fragmentation and basal membrane detachment (bile duct).
2	Hepatic cords with mild to moderate rupture, created by cell atrophy and consequent stretching effect; Moderate distinction between hepatic cords and sinusoids; Partial fragmentation of hepatic sinusoids.	Bile duct and blood vessels less frequently distinguishable; Moderate bile duct alterations – pyknosis and epithelium fragmentation; Arteriole hyalinization; Possible cell vacuolization.
3	Hepatic cords with prominent rupture, frequent individual hepatocytes; Prominent fragmentation of hepatic sinusoids, with extravasated erythrocytes.	At least some bile duct and blood vessels disintegrated.

3. Results

3.1. Improving the histotechnical quality of pig skin and liver samples

Pig histological slides matching the cases with higher PMI time were analysed using optical microscopy to determine the optimal methodology for each type of embedding. The assumption was that if we could successfully and optimally preserve the supposedly more deteriorated specimens, it would better serve the overall study. Some histological features, notably those mentioned in prior studies (post-mortem) that should be examined in the target organs, were analysed to conclude about the best outcome. The epidermis, dermis, sweat glands, sebaceous glands and hair follicles were all scrutinized in the skin. In the liver, portal areas, hepatocytes, and hepatic lobules were inspected. Other components were the overall tissue quality, thickness and staining quality.

Taking into account the routine procedures, the first method analysed was based on paraffin embedding, being decided to perform the “short” and “long” protocols used in the Laboratory where this took place (Table 2). Sectioning paraffin blocks was flawless, yielding quality tissue sections with minimal troubleshooting related to processing. Through microscopic observation of paraffin sections, we noticed good preservation of the overall structure and the recognisability of the detailed architectural (Figures 2 and 3).

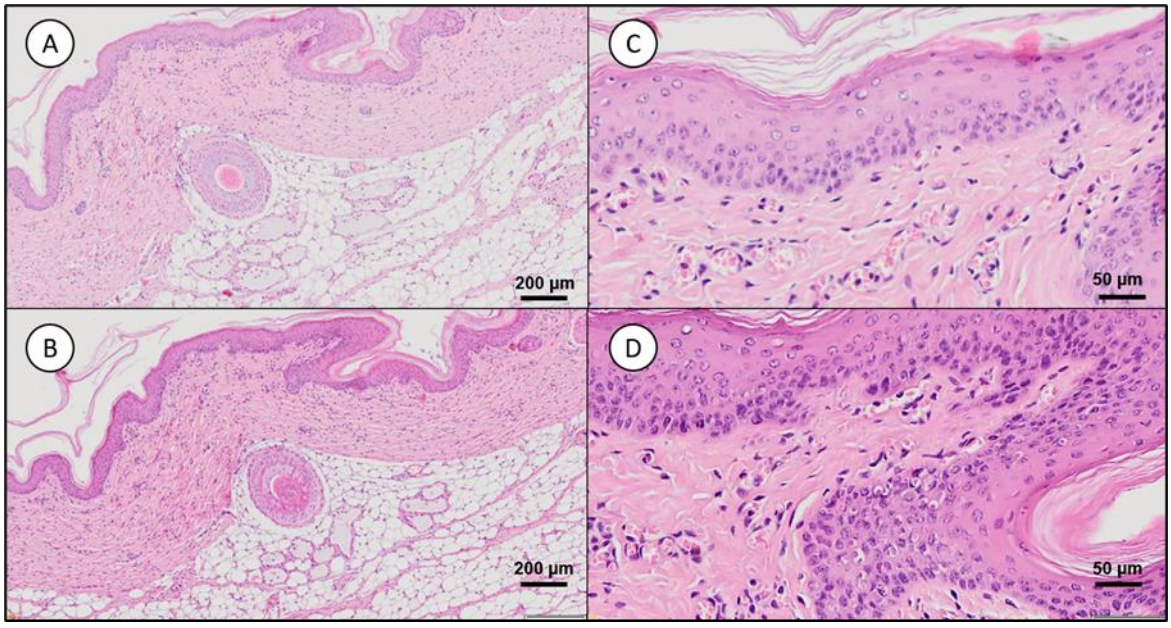

Figure 2. Skin of **pig** (sample no. 15), processed using the **long protocol** of histological processing for **paraffin**. **A** – General observation from a 3 µm section, HE. **B** - General overview from a 4 µm section, HE. In A and B the epidermis and dermis are observed above the hypodermis (at lower-right of the image). **C** – Detail of the epidermis (top) and dermis, sectioned at 3 µm, HE. **D** – Detail of the epidermis (top) and dermis, sectioned at 4 µm, HE

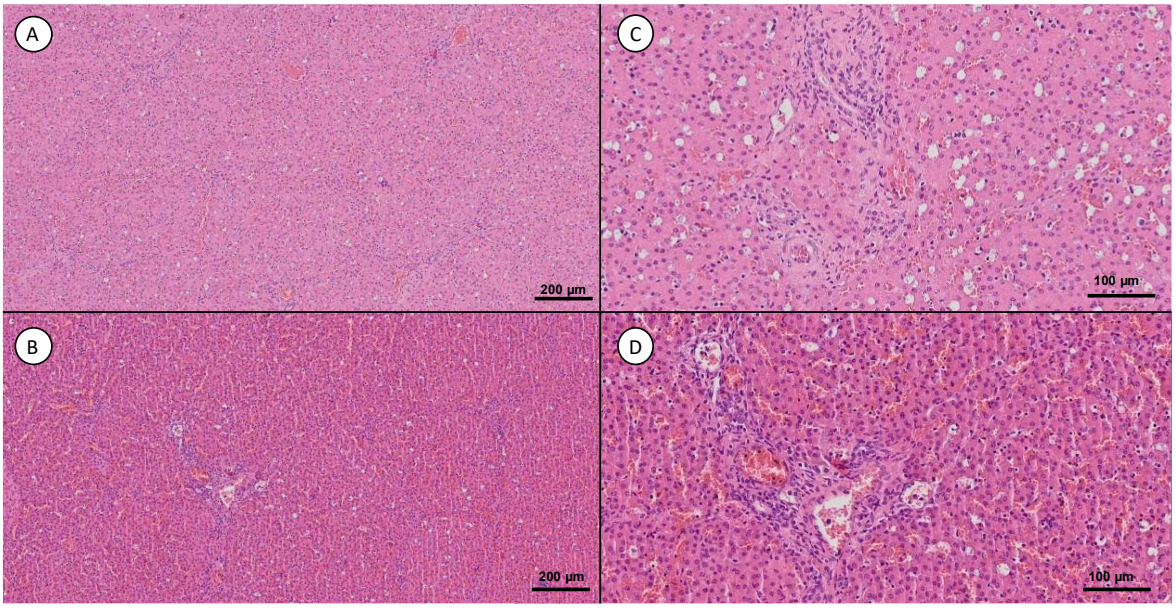

Figure 3. Liver of **pig** (sample no. 15), processed using the **long protocol** of histological processing for **paraffin**. **A** - General view allowing perceiving the hepatic lobules, sectioned at 3 µm, HE. **B** – Overview depicting the hepatic lobulation, sectioned at 4 µm, HE. **C** – Image with a (centrally located) portal area, surrounded by parenchyma, sectioned at 3 µm, HE. **D** - Same kind of field as in C, with the portal area (centre-left), sectioned at 4 µm, HE.

In contrast, the lack of substantial experience with methacrylate-based embedding, associated with the huge disparity in processing times (Table 3), made us consider it pertinent to perform both (short and long) methacrylate processing protocols.

When implementing the short processing protocol, excellent sections from the methacrylate block was challenging because the tissue sue shrank back into the block. There was also a significant need of troubleshooting associated with the poor processing, such as the emergence of incomplete sections, including the formation of tissue holes (Figure 4), a higher risk of folds, and sections expanding or disintegrating on water, all of which had a significant impact on the quality of microscopic observation. Anyway, despite not being perfect, when viewed in isolation, good observations could still be made (Figures 5 and 6).

In contrast, the long methacrylate processing scheme resulted in a significant improvement, namely an easy sectioning, and an astounding reduction in troubleshooting, particularly those issues mentioned previously. An enhancement in tinctorial quality, without staining protocol modifications, linked with overall higher physical section quality, including at the cell level, improved the microscopic observation in both types of tissues (Figures 7 to 9).

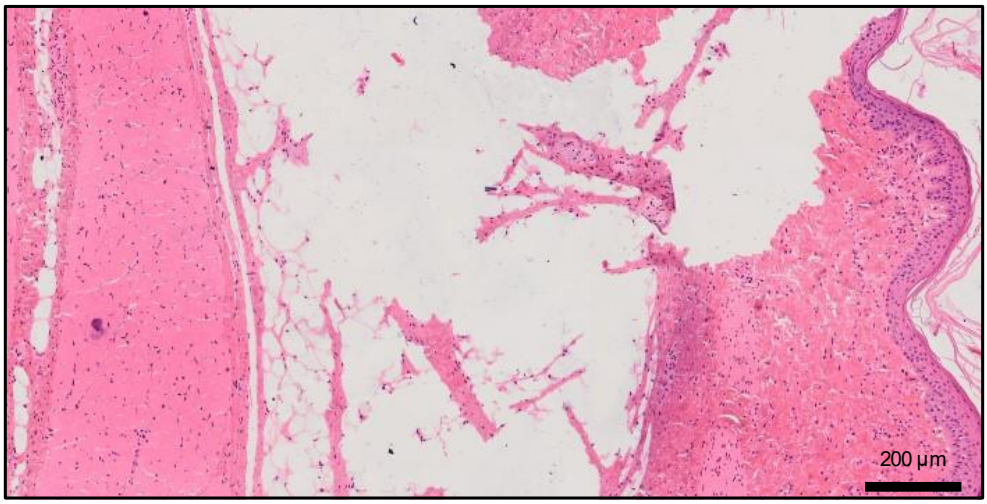

Figure 4. Skin of **pig** (sample no. 15, 96 hours after necropsy), processed using the **short protocol** of histological processing for **methacrylate**. Sectioned at 3 µm, HE. It noticed the formation of a tissue hole, which resulted in the loss of the majority of the tissue.

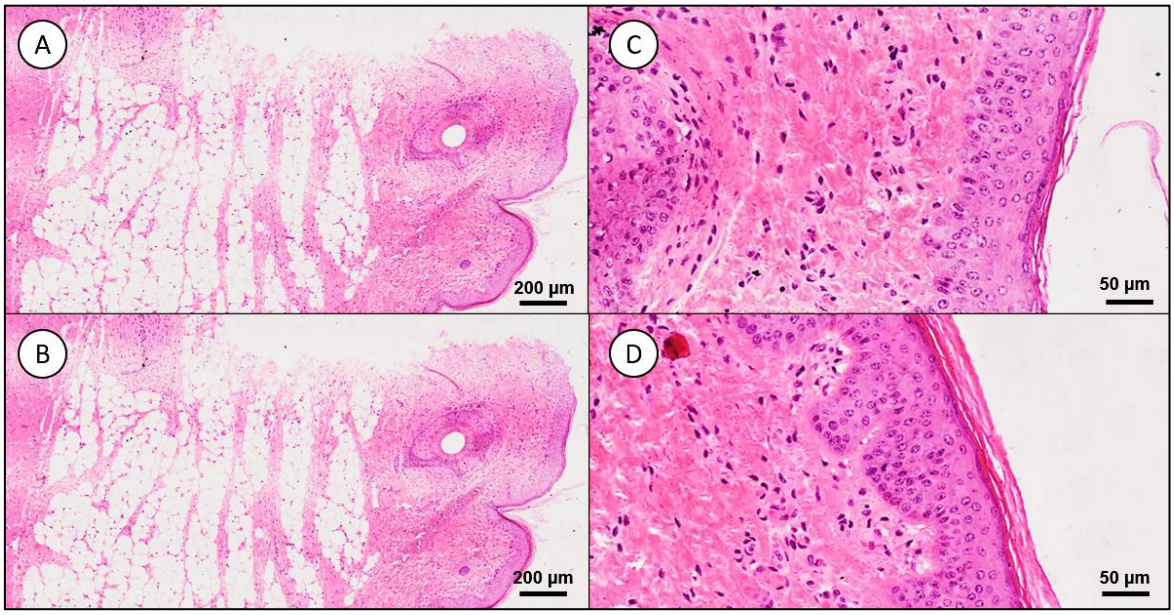

Figure 5. Skin of **pig** (sample no. 15), processed using the **short protocol** of histological processing for **methacrylate**. **A –** General observation from a 3 µm section, HE. **B -** General observation from a 4 µm section, HE. In A and B, epidermis and dermis are at the right and hypodermis at the left. **C –** Detail of the epidermis (right) and dermis, sectioned at 3 µm, HE. **D –** Detail of the epidermis (right) and dermis, sectioned at 4 µm, HE.

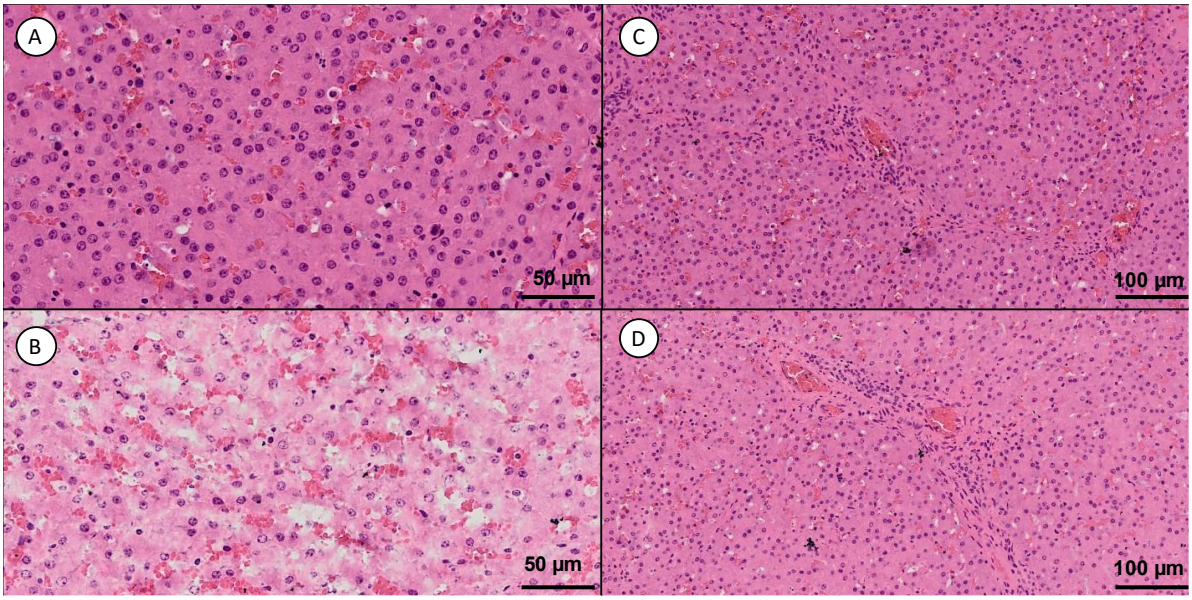

Figure 6. Liver of **pig** (sample no. 15), processed using the **short protocol** of histological processing for **methacrylate**. **A** – Area of parenchyma where many roundish basophilic nuclei of hepatocytes stand out against eosinophilic cytoplasm, sectioned at 4 µm, HE. **B** – Similar area as seen in A, sectioned at 3 µm, HE. **C** – Hepatic area with a portal space (at the image centre), surrounded by parenchyma, sectioned at 4 µm, HE. **D** – Similar area as observed in C, also with a portal area (upper centre of the image), sectioned at 3 µm, HE.

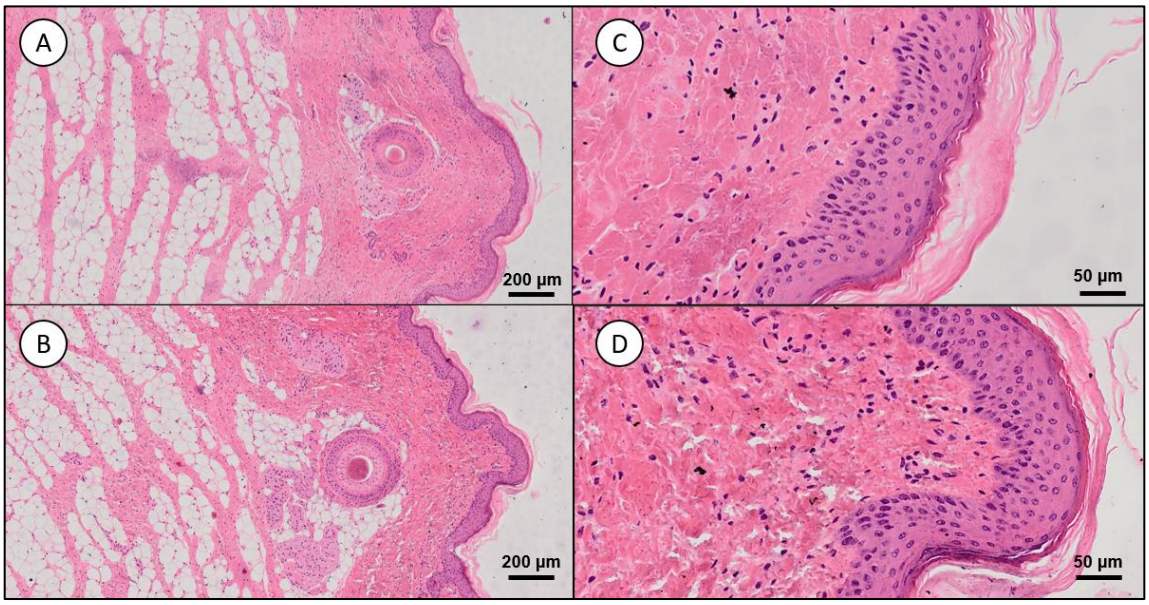

Figure 7. Skin of **pig** (sample no. 15), processed using the **long protocol** of histological processing for **methacrylate**. **A** – General observation from a 3 µm section, HE. **B** - General overview from a 4 µm section, HE. In A and B, the epidermis and dermis are seen on the right and the hypodermis on the left. **C** – Detail of epidermis (right) and dermis, sectioned at 3 µm, HE. **D** – Detail of epidermis (right) and dermis, sectioned at 4 µm, HE

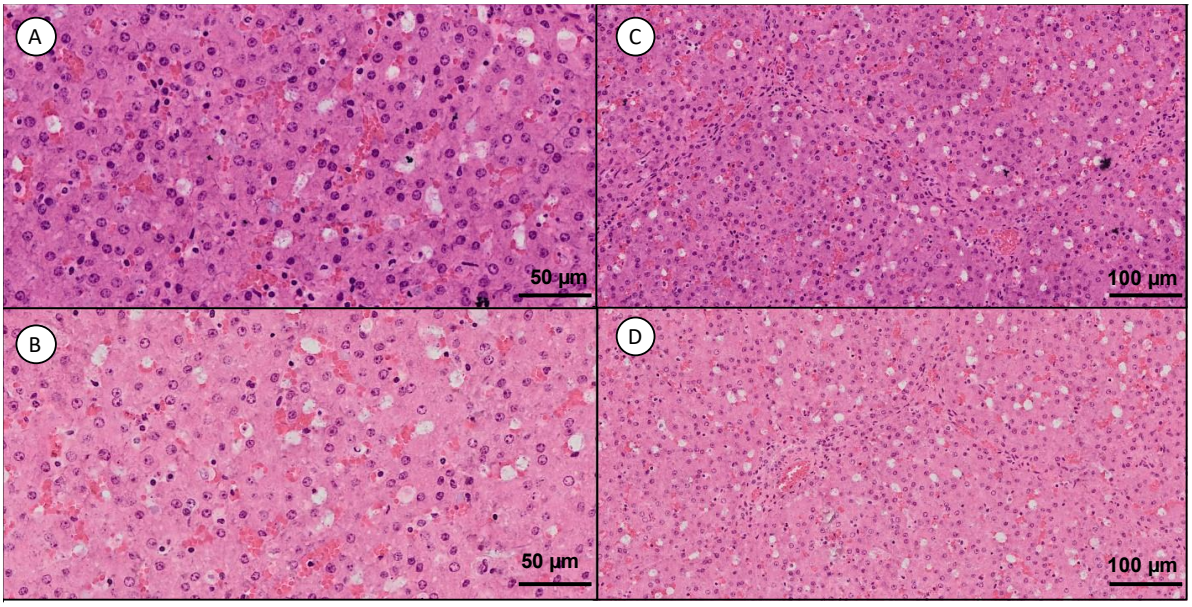

Figure 8. Liver of **pig** (sample no. 15), processed using the **long protocol** of histological processing for **methacrylate**. **A –** Area of parenchyma where many roundish basophilic nuclei of hepatocytes stand out against eosinophilic cytoplasm, sectioned at 4 µm, HE. **B –** Similar area as seen in A, sectioned at 3 µm, HE. **C –** Hepatic area with a portal space (at the image centre), surrounded by parenchyma, sectioned at 4 µm, HE. **D –** Similar area as observed in C, also with a portal area (upper centre of the image), sectioned at 3 µm, HE.

Another element considered was tissue thickness, specifically 3 and 4 µm. For both paraffin-embedding and methacrylate-embedding, microscopic observation of the main histological features of skin and liver were globally similar, however 3 µm thickness allowed us to distinguish some histological components, such the nuclei (Figures 2 to 9) and sinusoids (Figures 3, 6 and 8). Both appeared with a slightly more generous definition, making it easier to detect the slightest histological alterations required for this investigation. Also, technically easier and overall better sections were obtained at 3 µm thickness for methacrylate-embedding blocks (derived from the long protocol).

Because two embedding media were tested, it was considered informative to duplicate the staining methods (HE, PAS and Goldner's Masson trichrome) in each medium.

With the paraffin embedding protocol, this study's host laboratory routine HE, PAS, and Goldner's Masson trichrome protocols (Materials and Methods, section 2.11.2.1) were used, providing reliable results in paraffin sections, as observed in Figure 10.

For methacrylate sections, no reliable results were obtained with those routine protocols, and some improvements were implemented. First, HE application were lengthened, but the support resin also stained, particularly with eosin, which lowered the quality of the microscopic observation. Then, the temperature variable was tested. Good

retention of the stains in the tissue submitted to 36 °C was observed, for the same time as the initial protocol, and the stain in the support resin was easily removed, optimising the HE staining protocol (Materials and Methods, section 2.1.2.2,) (Figure 11A).

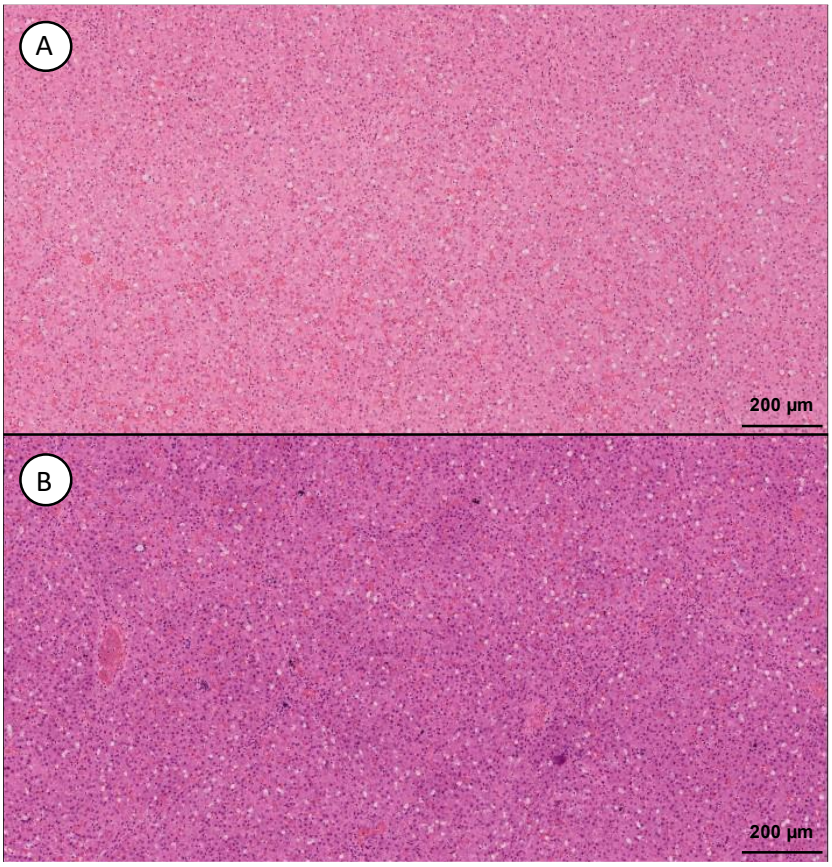

Figure 9. Liver of **pig** (sample no. 15), processed using the **long protocol** of histological processing for **methacrylate**. **A** – Sectioned at 3 µm, HE. **B** – Sectioned at 4 µm, HE. Both low magnification images, where the hepatic lobules are already perceptible, denote a good homogeneous staining and suggest overall good histological quality.

In the PAS staining, by performing the protocol available from the Technovit® producer company (Kulzer, Romania), it was experienced a lack of nuclear resolution and observed that the PAS-positive structures were pale. Firstly, the conjugation of celestine blue solution with Mayer's hematoxylin was attempt, which resulting in a significant improvement in the nuclear definition. To enhance the stain, the temperature variable was examined by exposing the tissue sections to 36 °C during staining, which allowed not only an increase in stain intensity of the PAS-positive structures, but also an improvement in nuclear definition (Figure 11B).

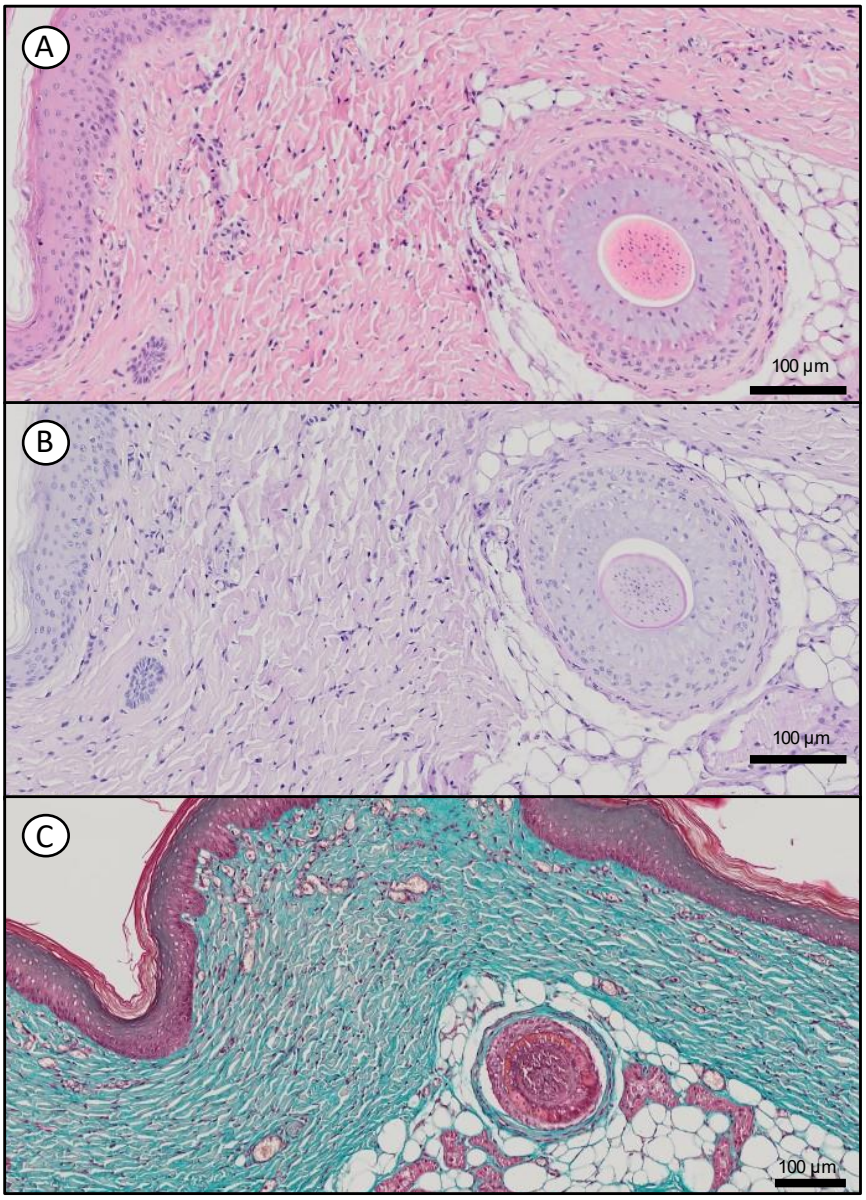

Figure 10. Skin of **pig** (sample no. 15, 0 hours after necropsy), processed using the **long protocol** of histological processing for **paraffin**. Sectioned at 3 µm. Observation of good quality staining, with good structures distinction. **A -** HE. **B -** PAS. **C -** Goldner` Masson trichrome.

Goldner's Masson trichrome protocol for methacrylate sections was one of the biggest histotechnical challenges in this work. Many protocols cited in the literature were tried, especially ones referring to methacrylate embedding, such as that of Gruber (1992), but the findings were extremely unstable. Modifications tested included the temperature factor (submitting sections to 36 °C), increasing the solutions' (ponceau xylidine and acid fuchsin, mixture of phosphomolybdic acid and orange G, light green and acetic acid) application times, using a different mixtures in terms of concentration (light green and acetic acid) or preparation (ponceau xylidine and acid fuchsin and mixture of phosphomolybdic

acid and orange G), and others, but none of them permitted stability between structures staining, resulting in inability to distinguish the histological structures. This problem was intensified by the staining of the resin surrounding the tissue by the same tone as the tissue, resulting in background staining and consequently worst contrast for the observer (Figure 12). Thus, it was decided to perform after that, in all pig and human samples, the long processing protocol in both embedding media, generating 3 µm thick sections. As to staining, HE, PAS, and Goldner's Masson trichrome were chosen for paraffin sections, and HE and PAS were implemented on methacrylate sections.

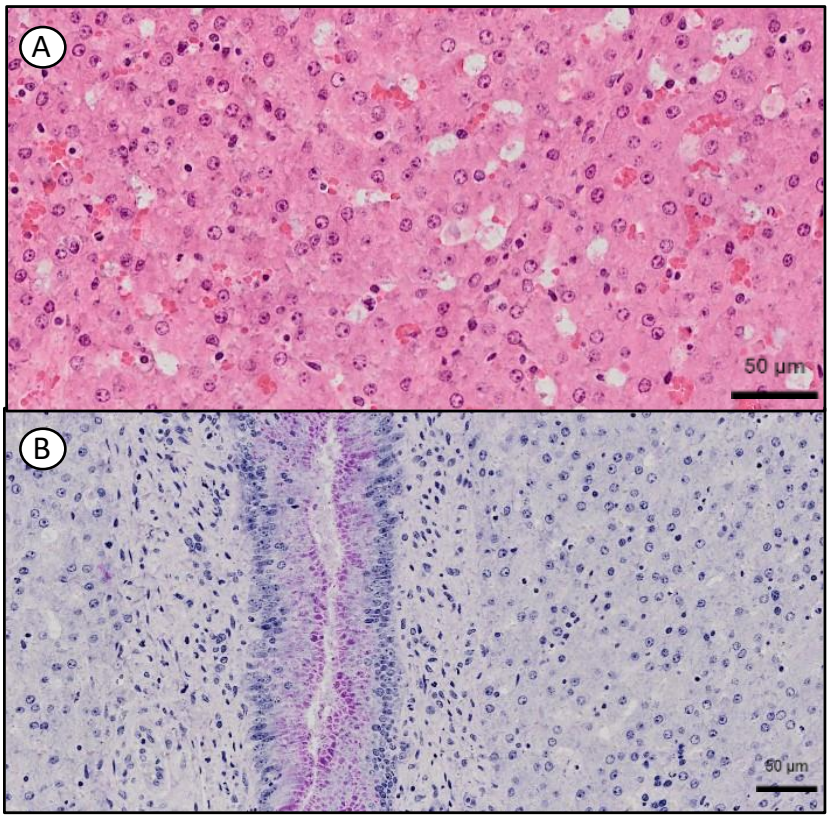

Figure 11. Liver of **pig** (sample no. 15, 0 hours after necropsy), processed using the **long protocol** of histological processing for **methacrylate**. Sectioned at 3 µm. Observation of good staining quality, permitting a good structure distinction. **A -** HE. **B -** PAS.

After reviewing the data, we decided which procedures produced the highest tissue quality, thickness, and staining quality, doing them in the remaining pig samples and in all the human ones, which consistently presented the same results as the above referred.

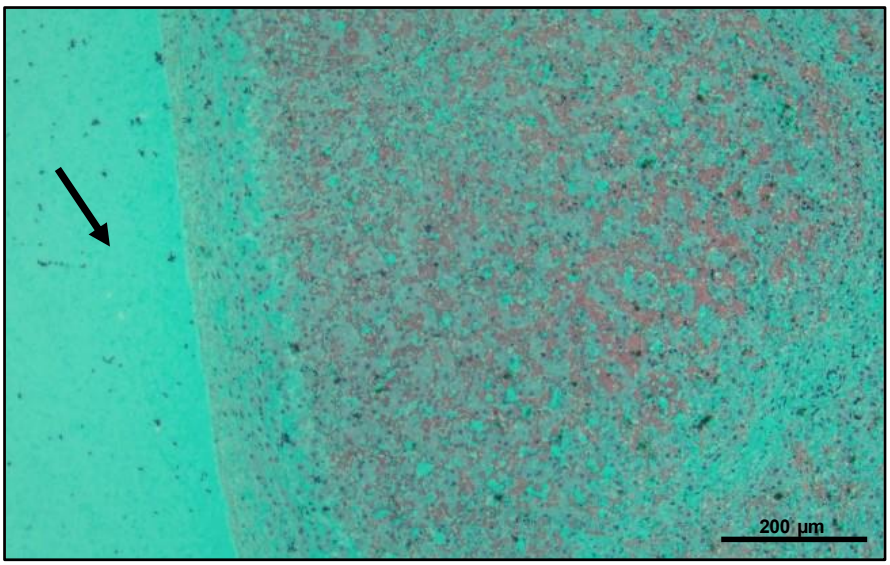

Figure 12. Liver of **pig** (sample no. 15, 0 hours after necropsy), processed using the **long protocol** of histological processing for **methacrylate.** Sectioned at 3 µm, Goldner` Masson trichrome**.** Observation of historesin staining (black arrow), which significantly reduces staining quality, reducing the possibility of histological structures distinction.

3.2. Comparison and choice of the best samples of post-mortem samples for histology and histopathology teaching

Optimal slides prepared using the best protocols described in the previous section were analysed to detect all possible histological changes in skin and liver in both species, obtain as much information as possible, and pass to the next step (development of Scores Systems for evaluation of post-mortem changes).

The histological features observed in this study slides were compared to those presented as typical of the normal skin and liver histology, given in well-known and prestigious histology textbooks covering either human (Pawlina and Ross 2018) and animal (including pig) histology (Bacha and Bacha 2012, Eurell and Frappier 2013); such literature imagery is in line with our experience from viewing the slide collections for the teaching offered by the laboratory that hosted this dissertation.

Significant variations in the stages of degradation of histological characteristics were observed, but they were not homogeneous for all the tissues. For example, it was simpler to compare the liver samples, particularly in the pig. There was a greater deterioration disparity, particularly in hepatocytes and sinusoids (including their link) (Figure 13) and hepatic lobule architecture. However, in terms of hepatic lobule architecture, that

experience was (expectedly) more complex in human samples. However, in the latter, it was facilitated by observation of the slides stained by Goldner's Masson trichrome, as the connective tissue in portal areas and particular limits between lobules evidently stained green, permitting a better contrast and, as a result, a better perception of the lobular architecture. The liver samples with the lowest levels of deterioration had traits comparable to those seen in the histology books.

The degradation was less noticeable in the skin, compared to the liver. In addition, a better delineation of histological features was evident in human skin than in pig skin. Considering all analysed conditions, skin samples with a low grade of histological degradation were also quite comparable to those described and illustrated in histology books.

When looking at paraffin sections, the observer perceived a more straightforward observation and recognition of some histological features, for instance, a better distinction between hepatocytes and sinusoids (even if artefactually retracted), creating a more pleasant, familiar, microscopic evaluation. Conversely, we confirmed that the histological features were better preserved in the methacrylate sections.

3.3. Score Systems for evaluating the post-mortem changes

The information gathered from slide observation was organized in Score Systems, specific for the organs and species, as there were variations in the decay of the microanatomical integrity when comparing the pig with human samples and the skin with liver tissue.

3.3.1. The pig model

Elaborating scores for pig skin was the most challenging by the minor morphological alterations found, despite progressive. It was possible to divide all pig skin slides into three groups based on observation of the epidermis, dermis, and other skin appendages (hair follicles, sweat glands, and sebaceous glands) (Table 9). In the first group, it was visualized superior preservation of structures, particularly in the epidermis, where the layers were well-preserved and easily identifiable, with largely well-preserved cells. However, some deterioration was also seen, such as separation of the dermo-epidermal interface, minor cytoplasmic vacuolization on hair follicle cells and luminal cells of sweat glands (Table 5 – Score 1) (Figure 14A).

The intermediate group presented a considerable loss of the epidermis morphology, with degeneration of keratinocytes, increased dermo-epidermal separation and cytoplasmic vacuolization of hair follicle cells and luminal cells of sweat glands (Table 5 – Score 2) (Figure 14B).

The last group showed the worst degradation, from all skin samples, with a prominent loss of normal epidermal morphology, cytoplasmic vacuolization not only of hair follicle cells and luminal cells from sweat glands but also of keratinocytes, together with other alterations (Table 5 – Score 3) (Figure 14C).

It was also possible to include another histological feature, the nerve fascicles, which decreased the fibre normally undulated outline and increased in fading of nuclear contour along the three scores (Table 5) (Figure 15).

By contrast, the grouping of liver pig specimens was the least challenging by the striking morphological alterations found. The finding allowed the scoring of all pig liver slides in five groups based on observation of the hepatic lobules' architecture, hepatocytes and portal areas (Table 9).

The hepatic lobules architecture progressively decreases, paralleled by prominent hepatocyte cytoplasmic vacuolization and nuclear dissolution, with the disintegration of the portal areas and bile ducts in particular. The pig liver cases with the highest score showed the worst results in tissue degradation, observed in total loss of hepatic lobule architecture.

Analysing the skin and liver samples taken at the necropsy time, 48 and 96 hours, from the same case, an overall comparable histological morphology was noted, resulting in the same or a higher grade in the sample of 96 hours. However, in some cases, such as in the skin of animals 14 and 20, some relevant structures were not observed (sweat glands and nerves), which may have contributed to grading with smaller scores than 96 hours.

Significant histological alterations were unnoticed in the slides stained with PAS and Goldner's Masson trichrome.

3.3.2 Humans

Grouping human skin cases in the defined scores was easier than in the pig, despite the division considered also three categories and was based on observing the same structures (epidermis, dermis, hair follicles, sweat glands, and sebaceous glands) (Table 10).

To summarise, the epidermis was well-preserved overall, irrespective of the grade, despite suffering some significant alterations in cases rated with the highest score. Early histological changes, such as peri-nuclear vacuolization in keratinocytes, cytoplasmic vacuolation in luminal secretory cells of sweat glands, and fragmentation of collagen bundles in the dermis, were also noted, increasing in severity from lowest to the highest scores (Table 6) (Figure 16).

Scoring the human liver was easier than with skin, because the noted morphological alterations were more evident and frequent. In the end it resulted in scoring of all human liver samples in three grades (Table 10), essentially based on the observation of the hepatic cords' architecture (Figure 17) and portal areas.

With decidual deterioration, and among other aspects, there was an increasing rupture of hepatic cords associated with fragmentation of hepatic sinusoids in parallel with the augmenting epithelium fragmentation in bile ducts (Table 8). Some samples presented steatosis, but it seemed neither to impact in the adopted grades nor to induce score variability. With PAS staining we noted no changes that could be relevant to be incorporated in scoring.

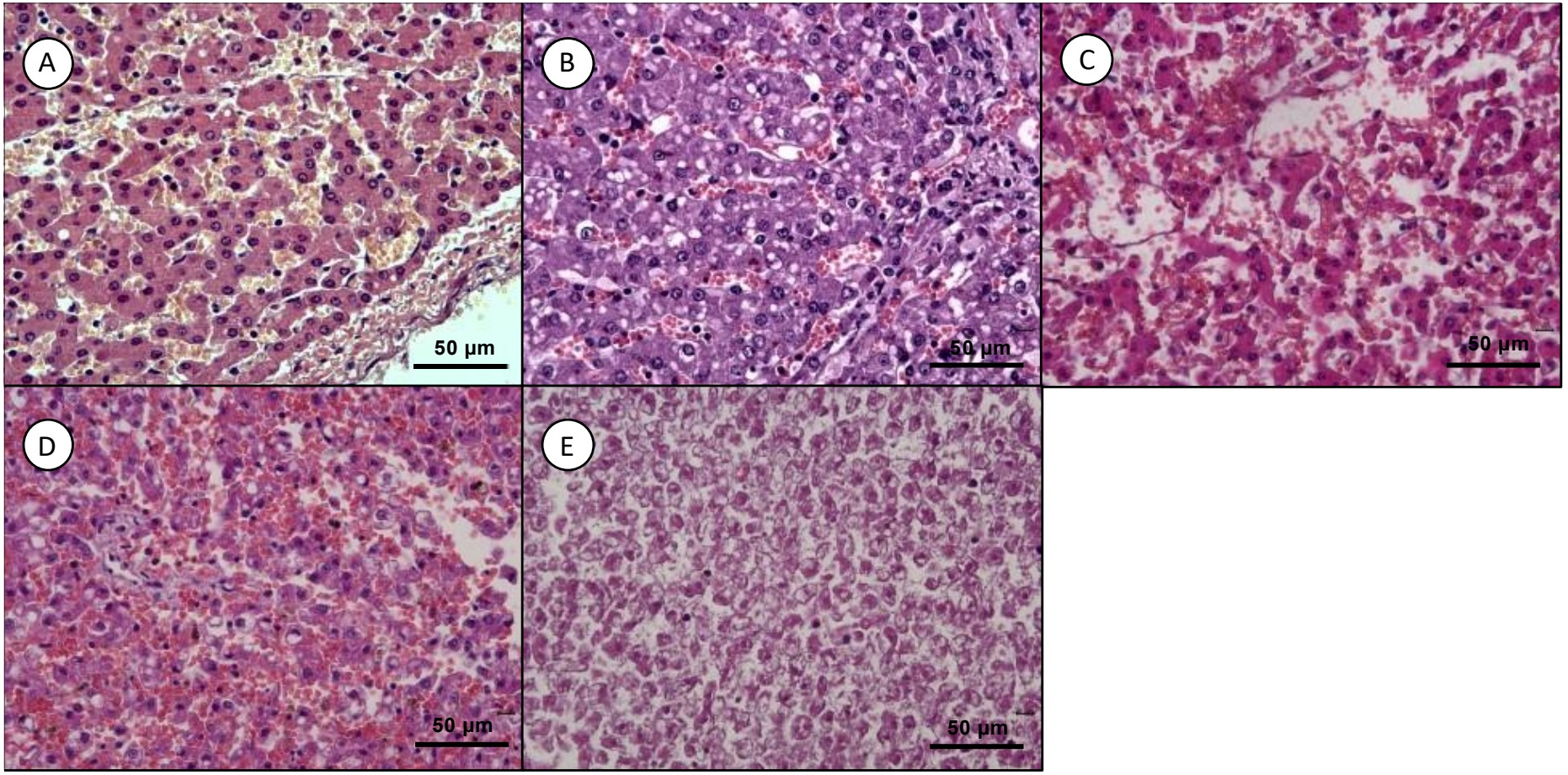

Figure 13. Liver of pig, processed using the **long protocol** of histological processing for **paraffin**. Observation of the progressive hepatocyte degradation and loss of distinguishability hepatic sinusoids, between the scores. **A –** Good preservation of hepatic cords and well-defined hepatic sinusoids (**Grade 1**). Sample no. 6 sectioned at 3 µm, HE. **B –** It is noted a loss of hepatic cords definition, but those cords (and erythrocytes) are still intact (**Grade 2**). Sample no. 8 sectioned at 3 µm, HE. **C –** Despite the hepatic cords rupture and there is sinusoidal dilation, those structures are still distinguishable (**Grade 3).** Sample no. 5 sectioned at 3 µm, HE. **D –** Hepatic cords with prominent fragmentation, but it is still possible to observe the sinusoids (Grade 4). Sample no. 19 sectioned at 3 µm, HE. **E –** In this case the sinusoids are not distinguishable and there are many individualized hepatocytes (**Grade 5**). Sample no. 1 sectioned at 3 µm, HE.

Table 9. Grades attributed to the skin and liver of each pig.

Case	Sex	Skin Score			Liver Score		
		0 hours after necropsy	48 hours after necropsy	96 hours after necropsy	0 hours after necropsy	48 hours after necropsy	96 hours after necropsy
1	Female	3	3	3	5	5	No sample
2	Female	1	1	1	4	4	4
3	Male	1	1	1	3	3	3
4	Female	2	2	2	3	3	3
5	Male	2	2	2	3	3	3
6	Male	1	No sample	No sample	1	1	1
7	Male	1	1	1	2	3	3
8	Female	2	2	2	2	3	3
9	Male	1	1	1	2	2	No sample
10	Male	2	2	2	1	1	1
11	Female	2	2	2	3	3	No sample
12	Male	1	2	2	2	2	2
13	Male	2	3	3	5	5	No sample
14	Female	2	2	1	2	2	2
15	Female	1	2	2	2	2	2
16	Female	2	2	2	3	3	4
17	Female	2	2	2	No sample	No sample	No sample
18	Male	2	2	3	3	4	4
19	Male	2	2	3	4	4	4
20	Male	1	2	1	3	3	3

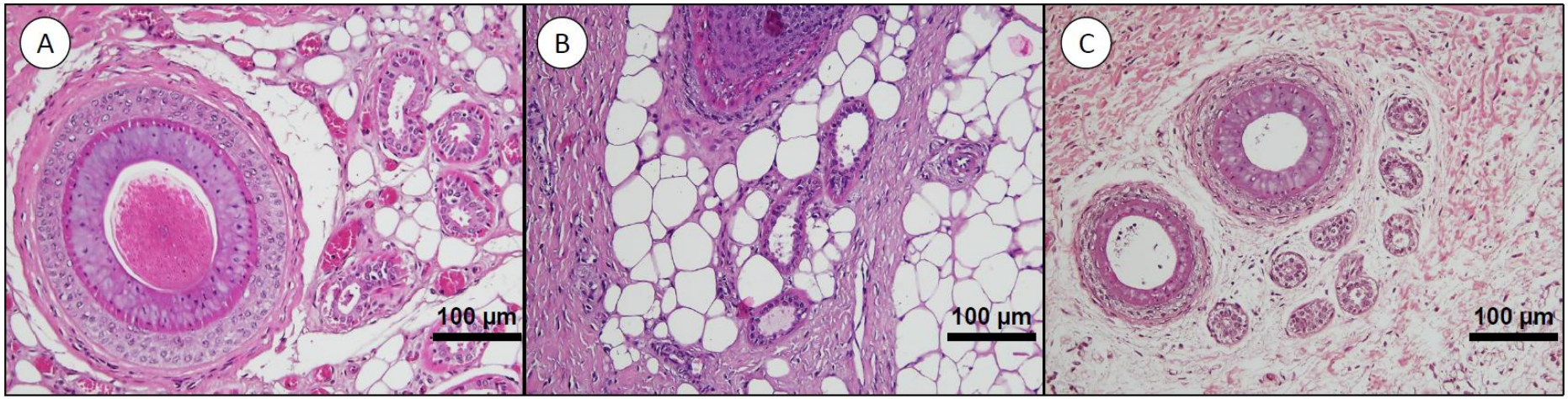

Figure 14. Skin of **pig**, processed using the **long protocol** of histological processing for **paraffin**. Observation of the progressive alterations of sweat and sebaceous glands between the different grades. **A-** Mild cytoplasmic vacuolation in luminal cells from sweat glands and mild sebaceous glands disintegration. (**Grade 1**) Sample no. 3 sectioned at 3 µm, HE. **B-** Moderate cytoplasmic vacuolation in luminal cells from sweat glands and mild sebaceous glands disintegration. (**Grade 2**) Sample no. 16 sectioned at 3 µm, HE**. C-** Moderate to prominent cytoplasmic vacuolation in luminal cells from sweat glands and mild to moderate sebaceous glands disintegration. (**Grade 3**) Sample no. 1 sectioned at 3 µm, HE**.**

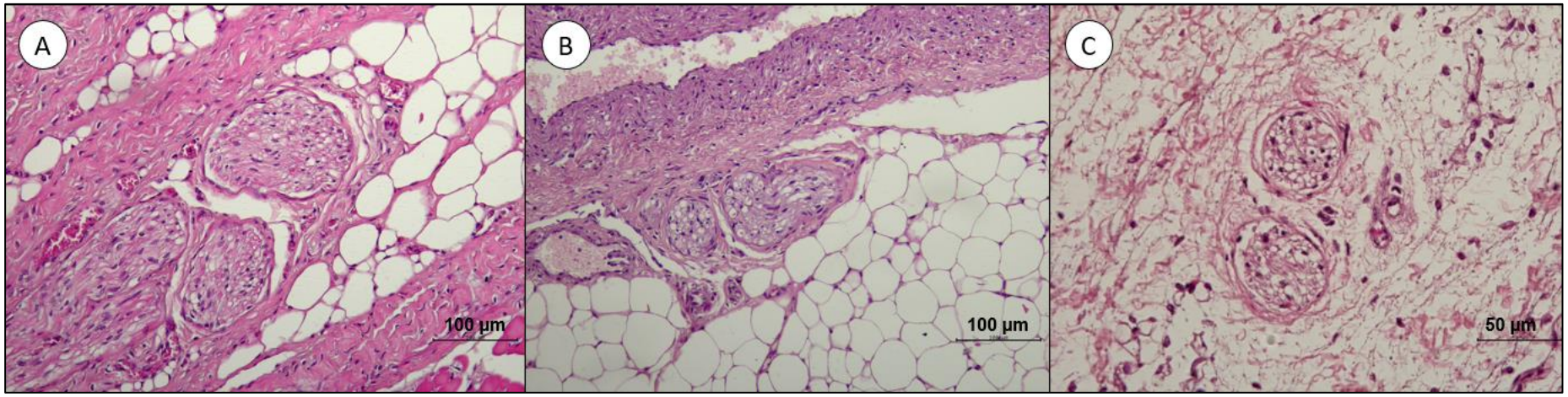

Figure 15. Skin of **pig**, processed using the **long protocol** of histological processing for **paraffin**. Observation of the progressive alterations of nerve fascicles between the different grades. **A –** Nerve fascicles mostly without structural alterations. (**Grade 1**). Sample no. 3 sectioned at 3 µm, HE. **B –** Nerve fascicles with mild decrease of fibre undulation and mild effacement of nuclear contours (**Grade 2**). Sample no. 16 sectioned at 3 µm, HE. **C –** Nerve fascicles (that at this have mild to moderate decrease of fibre undulation – not shown) and mild to moderate fading of nuclear contour (**Grade 3).** Sample no. 1 sectioned at 3 µm, HE.

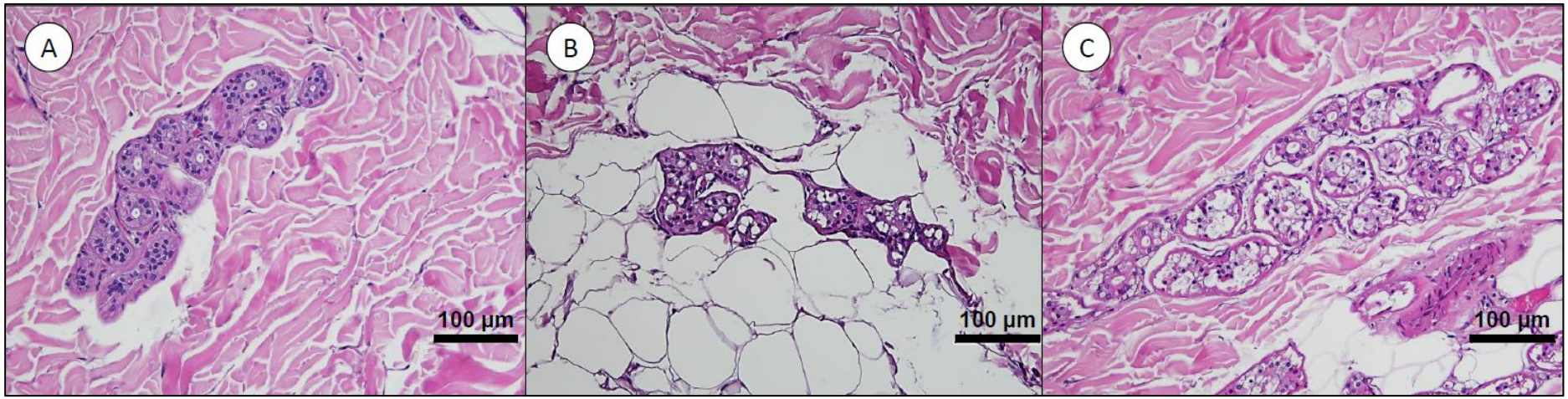

Figure 16. Skin of **human**, processed using the **long protocol** of histological processing for **paraffin.** Observation of the progressive alterations of sweat glands between the different grades. **A-** Mild cytoplasmic vacuolation in luminal cells from sweat glands. (**Grade 1**) Sample no. 10 sectioned at 3 µm, HE. **B-** Moderate cytoplasmic vacuolation in luminal cells from sweat glands. (**Grade 2**) Sample no. 5 sectioned at 3 µm, HE**. C-** Moderate to prominent cytoplasmic vacuolation in luminal cells from sweat glands. (**Grade 3**) Sample no. 13 sectioned at 3 µm, HE**.**

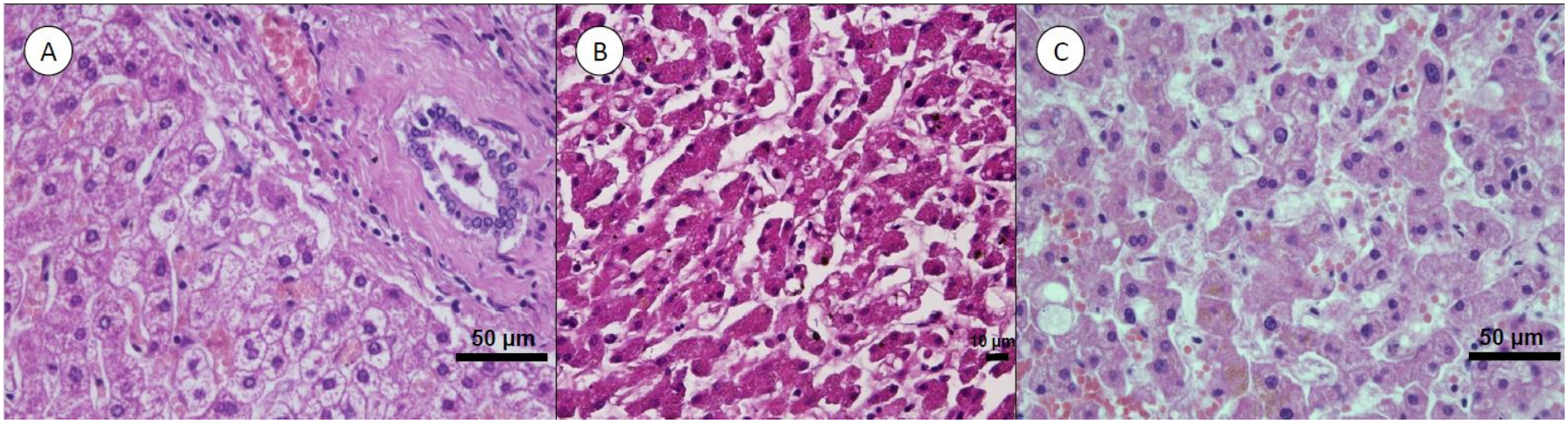

Figure 17. Liver of **human**, processed using the **long protocol** of histological processing for **paraffin**. Observation of the progressive hepatocyte degradation and loss of distinguishability hepatic sinusoids, between the scores. **A-** Good preservation of hepatic cords and well-defined hepatic sinusoids. (**Grade 1**) Sample no. 5 sectioned at 3 µm, HE. **B-** Hepatic cords with mild to moderate rupture, by cell atrophy and consequent stretching effect, with partial fragmentation of hepatic sinusoids. However, it is still possible the distinction between hepatic cords and sinusoids. (**Grade 2**) Sample no. 20 sectioned at 3 µm, HE**. C-** Prominent fragmentation/rupture of hepatic cords and hepatic sinusoids. (**Grade 3**) Sample no. 3 sectioned at 3 µm, HE**.**

As a final remark, when looking at the skin and liver score side by side (Table 10), it is obvious that they do not follow the same trend consistent for the same case. There are cases where lower scores perfectly agree for both organs (e.g., cases 6 and 16), but other totally disagree (e.g., cases 1 and 2). Also, neither pattern of grades, i.e., for skin and liver, is perfectly aligned with the PMI values shown in the Table 4 (see Material and Methods).

Table 10. Grades attributed to the pig skin and liver samples.

Case	Sex	Skin Score	Liver Score
1	Female	3	1
2	Male	3	1
3	Male	3	3
4	Male	1	3
5	Female	2	1
6	Male	1	1
7	Female	2	1
8	Female	2	1
9	Female	2	2
10	Male	1	2
11	Male	2	3
12	Male	3	2
13	Male	3	3
14	Male	1	2
15	Male	1	2
16	Male	1	1
17	Male	1	2
18	Male	1	2
19	Male	2	2
20	Male	3	2

4. Discussion

4.1. Improving the histotechnical processing of post-mortem skin and liver fragments

The selection of the pig as an animal model was a critical initial step in the development of this study. As expected, it was complicated to collect human samples, especially at the time of the Covid-19 pandemic, which explains the discrepancy in the moments of collecting samples of pigs and humans (Eberlová, Liskac et al. 2017, Matuszewski, Hall et al. 2020).

Given that fact, the investigation has been done by using skin and liver samples acquired from pig carcasses. To save time and reduce sample loss, the histology techniques optimization done in this study was limited to just a few pig cases. Considering that the body experiences a series of irreversible and progressive physical and chemical changes after death (Lee Goff 2009, Brooks 2016), it was reasonable to assume that the best pig cases for optimization tests would be those with a higher PMI, as they have a higher probability of deterioration of histological features.

As previously stated, only a lengthy paraffin treatment had been tested. That was consistent with previous observations in works done in the Department of Microscopy of the ICBAS (DMIC), including the development of teaching materials utilised in classes; often, difficult to prepare biological samples greatly benefited from extended protocols. It is important to note that most of the material used in teaching comes from samples with minimal PMI, not to mention pieces retrieved from live donors (e.g., materials derived from surgical procedures). Comparing the histological learning slides with the ones produced in the current work, the new skin and liver slides seem pretty similar, if not better. In general, in this study, it was possible to distinguish, with excellent quality and definition, in the skin the epidermis, dermis, sweat and sebaceous glands, and hair follicles, and, in the liver, the portal regions (with all components), hepatocytes (plus sinusoids), and hepatic lobules; despite the observation of some degradation. In this approach, we concluded that the adopted paraffin processing as sufficiently optimised (Figures 2 and 3).

The same thing did not occur with methacrylate-based embedding. Although it was already used in this DMIC, it still requires a comparison between short and long protocols because it was not commonly utilised in this type of material, particularly in teaching.

The short protocol resulted in the worst outcomes across all thicknesses, with more artificial tissue injury. The failure of the brief protocol can be attributed to two factors: a) first, because the samples were from corpses, where there may have been a significant state of

putrefaction, which inevitably induced structural fragility in the samples, and thus artefacts are commonly generated (e.g. Vazzano, Sinclair et al. (2021)); b) proneness to histological artefacts being introduced during sample collection or throughout tissue processing (e.g. Venne, John-Baptiste et al. (2014)).

Fixation is the initial step in histological processing and is likely the most fundamental since it denatures proteins, making the tissue resistant to further autolysis (Singh, Bishen et al. 2019, Wolfe 2019). As time after death passes before fixation, there is a higher risk of insufficient incorporation of the processing solutions in tissues, especially the infiltration solution, which can justify the odds of tissue shrinking back into the block (Wolfe 2019). Given that the time passed until sample fixation was significantly in this study, it should have been meaningful to favour autolysis (Rastogi, Puri et al. 2013, Venne, John-Baptiste et al. 2014).

It is reasonable to assume that the appropriate fixation of tissues for histological examination is important to all histological procedures, as without this process, all tissues decompose and analysis is worthless; the fixation solution chosen is a vital factor. The most often used fixative in diagnostic pathology is 10% neutral formalin, which was used in the current study. However, a recent study indicates that 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH of 7.2) is a better fixative for methacrylate-based resins (Ferreira, Palhares et al. 2022), but the drawback is that pieces have to be tiny, otherwise, they will not be adequately fixed, due to the fixative's very slow penetration rate (Srinivasan, Sedmak et al. 2002).

In terms of thickness, 3 µm was determined to be the ideal thickness because it was observed to have a better definition of nuclear and overall structural limits (Figures 2, 3, 6-8). Although the methacrylate embedding permits sections with a smaller thickness, which is one of the vantages of this embedding method (Woodruff and Greenfield 1979, Gerrits and Horobin 1996), it was relevant here to use a similar thickness in both kinds of embedding, permitting a better comparison of the same condition.

In terms of staining, the methacrylate embedding required more optimisation. In the case of HE staining, methacrylate tends to provide a poor contrast (Woodruff and Greenfield 1979, Katbamna and Ralston 1994), however, this aspect was shown to be less influenced when the HE and PAS stain was applied at a higher temperature (36 °C).

This study tested several of Goldner's Masson-Trichrome variations presented in the literatures for methacrylate (e.g., Gruber (1992)). Unfortunately, despite all efforts to optimise the use of the trichrome, the results were disappointing. The persistence of the hydrophilic historesin in the slides, as mentioned in the literature, results in poor contrast,

necessitating an increase in the staining period of the aqueous stains or other reagents (Cerri and Sasso-Cerri 2003). Still, the findings were extremely unstable, even when increasing the times for staining and immersion in the various reagents and even the rise of temperature in the HE staining, making it impossible to achieve optimisation of trichrome in our material.

4.2. Suitability of post-mortem samples for histology and histopathology teaching

Histology and histopathology continue substantially impact human medicine training and practice, as well as other domains. (Wood, Whiten et al. 2015, Abuhaimed, Almulhim et al. 2020). Because of that, there is a need to get the best possible material for teaching. In theory, as long as they present non-diseased tissues/organs, Grade 1 cases from the skin and liver of both species analysed here will be the best for histology study. Our results backed this idea, as the mentioned scores and the diverse structures were still relatively well presented and compared relatively well with teaching material (namely that used at the MIC of the ICBAS) derived from live donors (surgical acts) or just euthanised animals. In this study, the mentioned selected cases offered overall histological quality and details compatible with the general descriptions of the skin and liver in well-known human and veterinary histology books (Bacha and Bacha 2012, Eurell and Frappier 2013, Pawlina and Ross 2018).

In addition to the uses for normal histology, post-mortem material can be useful for general histopathology teaching and forensic histopathology training. Therefore, taking advantage of cases with autolytic degradation signs or pathological signs would be valuable. For such purposes, our analysis indicate that it can be justified using sections rated with other (higher) Grades of skin and liver and for both species, such as Grade 2 for pig and human skin (especially for studying epithelia), Grades 2 and 3 for pig liver, and Grade 2 for human liver. That can be a solution to reduce the reported discourage of pathologists from pursuing histology, said to be justified by poor training (Matkowski and Benbow 2021). Note that all grades of deterioration defined here could help train the recognition of progressive post-mortem changes, as processing was successful in all cases and embedding media.

According to the literature, sections of tissue embedded in methacrylate have far higher resolution than those of similar tissue embedded in paraffin (Woodruff and Greenfield 1979, Gerrits and Horobin 1996). Moreover, the same literature indicates that semi-thin methacrylate sections are easily obtained, resulting in a more accurate interpretation of

tissue morphology. Also, methacrylate does not need to be removed from sections before staining, preventing the distortion of tissue elements that occur after deparaffination, such as less shrinkage of tissues and thus less dimensional changes (Gerrits and Horobin 1996). Because with methacrylate the tissue will have more resemblance with the in vivo condition, it should enable a more thorough knowledge of normal histology and, by proxy, of the histopathological signs of the disease.

However, in clinical and research facilities, the processing is currently done routinely to make a formalin-fixed and paraffin-embedded block of tissue (Abuhaimed, Almulhim et al. 2020), being necessary to train the students with the most common embedding method.

Here, while we confirmed that the methacrylate sections typically offered more structural refinement, there was also a possible downside when thinking about visualizations to be made by students. Precisely because there is much less retraction, the histological appearance may look different in several aspects when compared with those seen in paraffin (even at the same section thickness), and so also with most images displayed in histology books of reference (Bacha and Bacha 2012, Eurell and Frappier 2013, Pawlina and Ross 2018). As a result, it would be interesting to do a conjugation from both embedding methods as a teaching method for histology. That would allow to conjugating the advantages of both methods; for example, the possibility of a better resolution and a more similar histological aspect as in life provided by plastic resin embedding and the students' trainee for a more applicable real practice with the most commonly used embedding method (paraffin).

Another benefit is the possibility that tissue samples obtained by students during dissection in gross pathology classes being processed and used for histology and histopathology sessions. Such an integrated approach enables students to further connect with their "first patient", the corpse, from the gross to microanatomy findings. This strategy would allow students to hone their pre-clinical skills, better grasp the human (and animal) body, and further promote their introduction to the hospital setting (Abuhaimed, Almulhim et al. 2020). Here we showed that the histotechnical quality could be granted, even if more time is needed.

4.3. Developed score systems for evaluation of post-mortem changes

There is a requirement for a more rigorous evaluation of the tested tissue during a histological study to prove a group difference or to substantiate the initial inspection observations. A scoring tool can translate into a semi-quantitative scale a qualitive

impression of one or more features from biological systems, including tissues, for analysis and group comparisons (Gibson-Corley, Olivier et al. 2013). That factor is relevant, especially in cases for estimation of PMI, since there are a lot of confounding variables (intrinsic and extrinsic factors) (Kovarik, Stewart et al. 2005, Ali, Ibrahim et al. 2017), increasing the risk of inconsistent results between forensic pathologists. The development of Score Systems can offer more reproducible and relevant results from different pathologists or researchers, resulting in consistent results (Gibson-Corley, Olivier et al. 2013, Meyerholz, Tintle et al. 2019).

Scoring systems may benefit from histotechnical improvements, including for estimating the PMI, which was one more factor that led us to consider methacrylate embedding.

Tomita, Nihira et al. (1999) conducted a study on PMI determination using the epoxy resin embedding method in samples taken from the kidney, pancreas, liver, heart, and skeletal muscle of Wistar rats compared to paraffin-embedded pieces. The author concluded that the epoxy resin embedding method outperformed paraffin for determining the time of death (Tomita, Nihira et al. 1999). In contrast, in the present study, the observer (the master student) did not notice that methacrylate allowed better grading than when using paraffin. It may be the case that the student's inexperience in analysing samples embedded in methacrylate may have impaired to take advantage of the resin’s superior resolution. Eventually, weaker staining contrast in historesin sections (Woodruff and Greenfield 1979, Katbamna and Ralston 1994, Cerri and Sasso-Cerri 2003) also contributed to less perception by the observer. A second review of all the slides of this study by a second and more experienced observer can confirm the present conclusion, extending and strengthening the current data.

To our knowledge, there are no universally standardized systems for grading post-mortem histological changes in the skin and liver of humans and pigs. Nevertheless, the Scoring Systems devised here looked at features that have been reported in earlier works (Kushwaha, Yadav et al. 2010, Ceciliason, Andersson et al. 2021), along with others that seemed relevant to us after qualitative observations of selected cases (from best to the poorest preserved). Overall, the systems we proposed were easily applicable, despite a few especially challenging cases that were reclassified after a second (consensual) revision by the student and supervisor. The grading systems had to be adapted to each species. In this respected, it is recalled that there were significant differences in the times after death for collecting the pig and the human samples, with promoted more post-mortem damage in the

pig. This fact is most likely due to differing values of environmental elements, such as temperature (Ali, Ibrahim et al. 2017, Wei, Michu et al. 2020).

Without international standards, our grading systems are systematic and alternative approaches. They serve as a baseline for further refinement aiming for more discrimination.

4.4. Correlation of observed post-mortem changes with the likely date of death

There are already studies of PMI determination using histological alterations in the skin (Cingolani, Osculati et al. 1994, Kovarik, Stewart et al. 2005, Bardale, Tumram et al. 2012, Babu, Gayathri et al. 2016, Calderon, Denis-Rodríguez et al. 2016, El-Nahass, Moselhy et al. 2017, Wei, Michu et al. 2020, Ahmed Alaa El-Din, Mohamed Ahmed et al. 2021) and liver (Li, Elwell et al. 2003, Kushwaha, Yadav et al. 2010, Babu, Gayathri et al. 2016, Yahia, El-Amir et al. 2018, Ceciliason, Andersson et al. 2021). Nonetheless, they are still limited in number and scope, and typically recognise and emphasize the need of more studies with a bigger number of cadavers and carcasses and evaluation in different environments (Kovarik, Stewart et al. 2005).

4.4.1. The pig model

We studied twenty pig carcasses, covering males and females, aged from 2 to 38 weeks (mode = 2 weeks), whose death occurred in an environment with an estimated temperature ranging from 7.2 to 8.5 ºC. The necropsy took place from just a half a day to 8 days after death. In view of these circumstances, we expected to observe variable histological changes related to more advanced post-mortem decomposition (Wei, Michu et al. 2020).

For the skin, our literature search did not retrieve articles using pig as a model. However, some studies were done with that organ, such as that of Wei, Michu et al. (2020), with human samples, demonstrating skin changes over a long period (32 days). As shown in the pig skin Score System (Table 5), the criteria used have some similarities with those of Wei, Michu et al. (2020), but one variable was added here: the nerve structure. Indeed, we noticed consistent time-related modification in histological integrity of the skin nerves, and so these were viewed as relevant targets for grading.

In contrast with the skin, most of the studies using the pig were done with the liver (Kushwaha, Yadav et al. 2010, Babu, Gayathri et al. 2016). As shown in the pig liver Score

System (Table 7), the criteria we adopted have some parallels to those of the literature, with the definition of 5 grades, as in Babu, Gayathri et al. (2016), but with more detailed descriptions. In our limited experience, it seemed helpful to offer detail for the microscopist's decision. Anyway, (Kovarik, Stewart et al. 2005) proved that the conjugation between the histology of skin (epidermis and dermis) with that of its appendages is essential for the estimation of PMI. So, for a Score System to be reliable, it is vital to have both histological structures.

It was anticipated that the increase in Score System grade would be proportionate to PMI in both organs. However, this was not observed, as shown in Table 1. Despite the fact that a large number of carcasses were chosen, we did not depict a consistent pattern in which the lower scores would smoothly correspond to shorter times after death, and the greater grades would be seen in the opposite situation. For example, pig number 1 showed the highest grade in both organs, despite having a low PMI. Most likely, such lack of consistency derives from the diversity of intrinsic and extrinsic factors that can influence the assessment of PMI, including using histological analyses (Kovarik, Stewart et al. 2005, Ali, Ibrahim et al. 2017, Kinney, Kanakov et al. 2020).

Although we cannot compare the grades of the skin and liver score systems, it seems that we exposed a trend of better preservation of the skin samples, as we can see for example in case 2. That is consistent with the notion that the skin is an organ with more resistance to post-mortem changes (Wei, Michu et al. 2020). In contrast, because of its hundreds of metabolic functions, the liver undergoes a myriad of biochemical changes after death that cause histological alterations, which can be used to determine PMI (Yahia, El-Amir et al. 2018, Ceciliason, Andersson et al. 2021). The consequence is that when comparing skin with liver samples, the former presents less structural deterioration for the highest grades.

Experimentally, one aspect that was possible to analysed on pig samples, was the effected of the sample's preservation versus deterioration at 4 ºC. This approach was made by analyses of samples taken at the necropsy time, 48 and 96 hours later, by the same pig case. After grading, it was discovered that most received either the same grade or one score higher. The increase in score confirms that the devised Score System can reveal progressive decay. As to the sustenance of grade despite the time increase, the fact may not be so surprising, considering that Cocariu, Mageriu et al. (2016) concluded that autolytic progress at a considerably slower rate in a regulated cold environment. However, there were exceptions. In some cases, such as pigs fourteen and twenty, it was assigned a lower grade in the skin (96 hours post-necropsy). As there was no mistake in the labelling of the

samples, we conclude that other factors play a role, and there is a chance, at least for some specimens, of obtaining distinct grades for one animal when analysing different skin zones (even if physically close). This important aspect has not been discussed in the literature and deserves further investigation.

At last, although HE staining is the default to observe post-mortem histomorphology (Wei, Michu et al. 2020), it was performed PAS and Goldner's Masson trichrome to check other possible variables, such as concentration of collagen in the dermis or the integrity of the epidermis' basal membrane. However, the results were not found relevant for grading.

4.4.2. Humans

This study included twenty cadavers, males and females, adults (18 to 88 years old), whose death occurred at estimated temperatures from 9 to 22 ºC, that were dressed or undressed when found dead, and whose autopsy was made from 1 to 4 days after demise. Despite the variable conditions, we expected to observe histological changes that could be related to early post-mortem periods (Kovarik, Stewart et al. 2005, Wei, Michu et al. 2020).

To our best knowledge, the existing literature does not present any kind of skin score system for histologic changes that would help determining the PMI, and we found only one article with a grading score for the liver (Ceciliason, Andersson et al. 2021). Therefore, to develop the scores given in this study, we adapted published histological findings (but lacking integrated into a coherent, systematic grading system) the estimation of PMI from human skin (Cingolani, Osculati et al. 1994, Kovarik, Stewart et al. 2005, Bardale, Tumram et al. 2012, Babu, Gayathri et al. 2016, Calderon, Denis-Rodríguez et al. 2016, El-Nahass, Moselhy et al. 2017, Wei, Michu et al. 2020, Ahmed Alaa El-Din, Mohamed Ahmed et al. 2021) and pig and human livers (Li, Elwell et al. 2003, Kushwaha, Yadav et al. 2010, Babu, Gayathri et al. 2016, Yahia, El-Amir et al. 2018, Ceciliason, Andersson et al. 2021).

Because our proposed grading systems for the human skin and liver have the same number of grades, it is possible to somewhat "directly compare" the parallel post-mortem progress of the two organs. As it occurred in the pig, it seemed to us that the human corpses also had better structural preservation of the skin, especially in the smallest PMI.

When comparing the pig results with those from humans, we conclude that the porcine cases had a higher degradation level. Given that the body undergoes a series of irreversible and progressive physical and chemical changes after death (Lee Goff 2009, Brooks 2016), our findings are logical because all the pigs had higher PMI compared with humans. Despite this obvious explanation, the more important is that results support the

overall discriminative power of the used Score Systems when attributing higher scores to higher PMI.

As for pigs, the PAS and Goldner's Masson trichrome stainings did not offer essential added value for positive relations with the PMI, yet, the trichrome was helpful in rapidly identifying the portal areas and so more easily pinpointing the general structural references of the hepatic lobules, permitting an easier application of the score grade to the human liver.

Despite the logical scoring system adopted, the current data did not expose a clear correlational pattern of low scores for shorter PMI and high scores for longer PMI, neither for skin nor liver. Although several cases agree with the prospect, others do not. So, despite the scoring being applicable and discriminating between less versus worst degraded cases, there is no absolute consistency given the well-known or circumstantially estimated PMI. As for the pigs, the inconsistency is likely caused by the diversity of factors that can influence the progress of post-mortem changes, and that makes it difficult to stabilise a correlation between the histological data (even if graded) and PMI. Yet, it is crucial to refer that this is a preliminary study. So, there is a need for more detailed studies based on higher numbers (to surpass intrinsic and extrinsic factors), aiming for consistency in the correlative patterns and ample power for multiple regression analysis relating histological changes with PMI.

5. Conclusion

Taken into consideration the declared aims of this dissertation, we concluded that:

a) refinements of processing, namely using longer processing times, resulted in high quality histological sections of post-mortem samples of skin and liver, of pigs and humans, including after embedding in methacrylate. With the latter, there were benefits on resolution but drawbacks in HE contrast and impairment of using the Goldern's Masson trichrome;

b) overwhelmingly, the histological slides were suitable for several stainings and use in classes versing the normal and (by proxy) the abnormal microanatomy of the human and pig skin and liver, including the study of post-mortem changes (at least within the scope of studied times after death). Therefore, the materials seem adequate for studies on the PMI;

c) it was possible to devise and implement new simple systematic scoring systems that were able to stage the degree of decomposition of skin and liver in the two species, and consistently relate growingly deteriorating histological aspects with greater scores;

d) the processing improvements translated into easy and discriminative evaluation of the histological decay after death, but, along with the developed scoring systems failed to deliver an inter-individual pattern of accumulated post-mortem microanatomical changes that univocally and consistently related with either the known or the estimated time of death.

Because estimating the PMI is still the "Achilles' tendon" of forensic medicine, it is evident that further studies are needed, in particular, related to the observation of histological changes after death. In this vein, we intend to refine and expand the data presented here

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