Forensic Evaluation of Bruises in Pigs

Barington, Kristiane

Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Barington, K. (2018). *Forensic Evaluation of Bruises in Pigs*. Copenhagen.

UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

FORENSIC EVALUATION OF BRUISES IN PIGS

Kristiane Barington

Doctoral Dissertation

September 2018

FACULTY OF HEALTH AND MEDICAL SCIENCES
DEPARTMENT OF VETERINARY AND ANIMAL SCIENCES
UNIVERSITY OF COPENHAGEN

DOCTORAL DISSERTATION 2018
ISBN 978-87-7209-260-7

KRISTIANE BARINGTON

FORENSIC EVALUATION OF BRUISES IN PIGS

SL grafik

FORENSIC EVALUATION OF BRUISES IN PIGS

Doctoral Dissertation by

Kristiane Barington

September 2018

This doctoral dissertation has been submitted to the Faculty of Health and Medical Sciences, University of Copenhagen

The Faculty of Health and Medical Sciences at the University of Copenhagen has accepted this dissertation, which consists of the already published dissertations listed below, for public defence for the doctoral degree in Veterinary Science. Copenhagen, 3rd of April 2019.

Ulla Wewer, Head of Faculty

Public defence will be held in auditorium A1-05.01, Dyrlægevej 100, Frederiksberg Campus, University of Copenhagen, Monday the 17th of June 2019 at 1 p.m.

Kristiane Barington
Department of Veterinary and Animal Sciences
Faculty of Health and Medical Sciences
University of Copenhagen
Ridebanevej 3
DK-1870 Frederiksberg C
Denmark
Krisb@sund.ku.dk

ISBN 978-87-7209-260-7
Printed by SL grafik, Frederiksberg, Denmark (www.slgrafik.dk)

This dissertation is based on the following 10 original articles, which are referred to in the text using Roman numerals (I–X):

I. Barington K, Jensen HE. Forensic cases of bruises in pigs. Vet Rec. 2013;173:526.

II. Barington K, Jensen HE. Experimental animal models of bruises in forensic medicine – a review. Scand J Lab Animal Sci. 2015;41:14.

III. Barington K, Jensen HE. A novel, comprehensive, and reproducible porcine model for determining the timing of bruises in forensic pathology. Forensic Sci Med Pathol. 2016;12:58–67.

IV. Barington K, Jensen HE. The impact of force on the timing of bruises evaluated in a porcine model. J Forensic Leg Med. 2016;40:61–66.

V. Barington K, Agger JFG, Nielsen SS, Dich-Jørgensen K, Jensen HE. Gross and histopathological evaluation of human inflicted bruises in Danish slaughter pigs. BMC Vet Res. 2016;12:247.

VI. Barington K, Jensen HE. Forensic aspects of incised wounds and bruises in pigs established post-mortem. Res Vet Sci. 2017;112:42–45.

VII. Barington K, Jensen HE, Skovgaard K. Forensic aspects of gene expression signatures for age determination in bruises as evaluated in an experimental porcine model. Forensic Sci Med Pathol. 2017;13:151–160.

VIII. Barington K, Skovgaard K, Henriksen NL, Johansen ASB, Jensen HE. The intensity of the inflammatory response in experimental porcine bruises depends on time, anatomical location and sampling site. J Forensic Leg Med. 2018;58:130–139.

IX. Barington K, Skovgaard K, Henriksen NL, Johansen ASB, Jensen HE. Histological evaluation of experimental porcine bruises. Data in Brief. 2018;20:1166-1176.

X. Barington K, Jensen HE, Skovgaard K. Forensic age determination of human inflicted porcine bruises inflicted within 10 h prior to slaughter by application of gene expression signatures. Res Vet Sci. 2018;120:47–53.

Contents

Preface

In 2012, I was employed as a Research Assistant at Department of Veterinary and Animal Sciences, University of Copenhagen. This was the beginning of my research in forensic veterinary pathology. During my time as a Research Assistant, I was trained to perform animal necropsies submitted for forensic investigation. Determining the age of lesions was central to the majority of these forensic veterinary investigations, especially in cases involving porcine bruises. However, establishing when the bruises were caused was challenging because lesions were inflicted in the hours before slaughter, and studies on porcine bruises were limited. This led to the planning of a research project concerning porcine skin lesions in forensic veterinary pathology. I have selected 10 publications focusing on forensic evaluation of bruises in pigs. A porcine model for bruises inflicted by humans was successfully developed, characterised and compared to non-experimental bruises in slaughter pigs. This model has increased our understanding of porcine bruises and provided a basis for developing and implementing new methods to assess the ages of bruises. This dissertation summarises the results of my research and provides an updated review of experimental bruises in animals and humans.

During the project, I have collaborated with many people to whom I would like to express my thanks. First of all, I would like to express my sincere gratitude to Henrik Elvang Jensen who introduced me to veterinary pathology and gave me the opportunity to perform this research. Thank you for your inspiration and guidance and for believing in me throughout the whole process. I could not have wished for a better mentor.

A special thanks to Kerstin Skovgaard and Karin Tarp for teaching me how to perform qPCR analysis. Thank you for your enthusiasm, patience and help during the evaluation of hundreds of samples. I would like to thank Jens Frederik Agger for his genuine interest in my work and his advice on statistical analyses. I would also like to thank Gyda Lolk Ottesen and Steffen Heegaard for inspirational discussions.

I would like to convey my sincere thanks to: Elisabeth Wairimu Petersen and Betina Gjedsted Andersen for their excellent work in the histo-laboratory; Frederik Andersen and Dennis Brok for their technical assistance during the experimental work and image preparation; Kristine Dich-Jørgensen, Nicole Lind Henriksen and Anne Sofie Boyum Johansen for their assistance during the experimental studies; Benedicte Kragh for rapid but careful proofreading of my manuscripts; and the veterinarians and animal technicians at the Laboratory Animal Unit at Frederiksberg Campus, University of Copenhagen for their excellent care of the pigs and help during the experimental procedures.

To all my colleagues at the Veterinary Pathology Unit. Thank you for your support, the many happy moments during work, and the social activities. Moreover, a special thanks to Julie and Godelind for being the best office mates I could wish for.

Last, but certainly not least, I would like to thank my friends and family for their support and Jakob for his endless patience and trust in me.

Kristiane Barington

Copenhagen, September 2018

Abbreviations

AM = ante mortem

APOA1 = apolipoprotein A1

BW = body weight

CCL2 = C-C motif chemokine ligand 2

CFD = complement factor D

DVFA = Danish Veterinary and Food Administration

E = kinetic energy

FOS = c-Fos proto-oncogene

HE = haematoxylin and eosin

ICAM-1 = intercellular adhesion molecule 1

IFNA1 = interferon α 1

IL6 = interleukin 6

m = mass

NFKB1 = nuclear factor κ β subunit 1

PCA = principal component analysis

PC#1 = principal component number 1

PC#2 = principal component number 2

PLAT = plasminogen activator

PLS = partial least-squares regression analysis

PM = post mortem

PTGS2 = prostaglandin-endoperoxide synthase 2

qPCR = quantitative polymerase chain reaction

SELE = selectin E

SELP = selectin P

TNFAIP3 = tumour necrosis factor α-induced protein 3

v = velocity

Summary

Porcine bruises inflicted by humans are painful for the animals and illegal according to the Danish Animal Protection Act. Annually, a mean of 17 cases of bruises in pigs are submitted to the University of Copenhagen for forensic investigation. More than 90% of these bruises are assumed to be inflicted within 8 h prior to slaughter. During this time period, the pigs are usually handled by several different people. Veterinary pathologists are asked to determine the age of these bruises, and this information can potentially identify the perpetrators in judicial proceedings. The ages of bruises are determined by histological evaluation of the inflammatory response in the skin and underlying muscle tissue. However, although the course of inflammation is predictable, variations in the inflammatory response within and between bruises, and between pigs, can make it difficult to accurately determine the ages of bruises.

A porcine model of bruises was developed and characterized. Time dependent changes in the inflammatory response in experimental bruises were studied. The results were used to estimate the age of non-experimental bruises in slaughter pigs. A mechanical device capable of inflicting blunt trauma with a force and type of impact equivalent to a man striking was developed. Gross examinations showed that the experimental bruises developed a tramline pattern identical to non-experimental bruises in both pigs and humans. Experimental bruises were inflicted on pigs to evaluate how bruise age, pig age, anatomical location, sampling site and force, speed and mass of the impacting object influenced the intensity of the inflammatory response. The extent of tissue damage and the intensity of the inflammatory response were affected by anatomical location, the sampling site within the bruise, and the force, speed and mass of the impacting object. Therefore, in forensic cases, skin and underlying muscle tissue should be systematically sampled from bruises at various anatomical locations and sampling sites within a bruise to ensure that inflammation is evaluated at its maximum intensity.

Time-dependent histological changes in the inflammatory response were observed in skin and muscle tissue sampled from experimental bruises in small (30 kg) but to lesser extent in large (100 kg) pigs. In small pigs, a combination of selected histological parameters could determine whether bruises were greater or less than 4 h old. However, in large pigs, the time-dependent increase in the intensity of the inflammatory response was probably obscured by variation among individuals. In addition, an mRNA signature from four genes expressed in the subcutaneous fat tissue could determine the age of experimental bruises in large pigs with a precision of less than ±2h. Moreover, this gene expression signature resulted in realistic age estimates for 95% of non-experimental bruises in slaughter pigs. Therefore, bruise age estimates based on selected histological changes

should be accompanied by a second method e.g., measuring the expression of selected genes for optimal age assessment.

This dissertation provides a thorough description of forensic cases of bruises in Danish pigs, focusing on the handling of cases and gross and histological evaluations of lesions in bruised skin and muscle tissue. Moreover, general descriptions of haemostasis and acute inflammation are included, followed by a short introduction to bruises in humans. A review of experimental bruises in animals and humans with a focus on porcine models is provided. In addition, experimental animal studies of vital reactions in experimental (pseudo) bruises inflicted post mortem are reviewed. Finally, the applicability of histological parameters and gene expression signatures for determining the ages of bruises inflicted by humans on slaughter pigs are discussed.

Sammendrag

Menneskepåførte blå mærker efter stump traumatisering (slaglæsioner) hos svin betragtes som smertefulde og er en overtrædelse af den danske dyreværnslov. Hvert år indsendes gennemsnitligt 17 sager omhandlende stump vold mod svin til en forensisk, patoanatomisk undersøgelse på Københavns Universitet. Lidt over 90% af slaglæsionerne regnes for at være påført inden for 8 timer før aflivningstidspunktet ved slagtning. I dette tidsinterval håndteres grisene af flere personer i forbindelse med transporten til slagteriet. Veterinære patologer skal på anmodning fra politiet estimere læsionernes alder i forhold til aflivningstidspunktet. Denne aldersbedømmelse kan spille en afgørende rolle i tilfælde af, at sagen kommer for domstolene. Aldersbedømmelsen er udelukkende baseret på den histopatologiske undersøgelse af inflammationsresponset i huden og den underliggende muskulatur. Til trods for forudsigeligheden i det inflammatoriske respons findes der individvariation og variation mellem slaglæsioner på samme dyr.

Med det formål at studere inflammationsresponset over tid, med henblik på tidsfastsættelse af slaglæsioner, er der udviklet en eksperimentel grisemodel for menneskepåførte slaglæsioner. Modellen består af en stålkonstruktion der er i stand til at påføre slag sammenlignelige med slag påført af en voksen mand. De eksperimentelle slaglæsioner har togskinnekonfiguration og er identiske med slaglæsioner hos svin og mennesker påført med aflange genstande. I de eksperimentelle studier er det undersøgt, hvordan faktorerne tid, kraft, masse og hastighed af slagredskabet, anatomisk placering og selve stedet for udtagningen langs slaglæsionen påvirker intensiteten af det inflammatoriske respons i slaglæsionerne hos svin. Resultaterne fra de eksperimentelle slaglæsioner er senere anvendt til aldersdatering af ikke-eksperimentelle slaglæsioner hos slagtesvin. Graden af vævsødelæggelse og inflammation afhænger af slaglæsionernes anatomiske placering, hvor langs slagmærket vævet bliver udtaget samt kraften, massen og hastigheden af redskabet hvormed slaglæsionerne blev påført.

Det konkluderes, at i forensiske sager bør hud og underliggende muskulatur udtages systematisk således, at der udtages væv flere steder fra slaglæsionerne og fra slaglæsioner med forskellig anatomisk placering. Således det sikres, at aldersbedømmelsen baseres på det maksimale inflammatoriske respons.

Ved histologisk evaluering af de eksperimentelle slaglæsioner er der fundet en tidafhængig udvikling i det inflammatoriske respons hos små (30 kg) og i nogen grad hos store grise (100 kg). I små grise kunne alderen bestemmes som værende over eller under 4 timer på baggrund af en kombination af udvalgte histologiske parametre. I de store grise var det tidsafhængige inflammationsrespons sløret af variation mellem individerne. En mRNA -ekspressionssignatur med fire specifikke gener i det subkutane fedtvæv fandtes at være i

stand til at bestemme alderen af eksperimentelle slaglæsioner med en præcision på under ±2 timer. Samme ekspressionssignatur blev afprøvet på non-eksperimentelle slaglæsioner hos slagtesvin og resulterede i sandsynlige aldersbedømmelser for 95% af læsionerne. Dette betyder, at en optimal aldersbedømmelse vil kunne opnås ved kombination af udvalgte histologiske parametre og bestemmelse af mRNA niveauet af udvalgte gener.

Afhandlingen giver en udførlig beskrivelse af forensiske sager omhandlende danske svin med menneskepåførte slaglæsioner. Der fokuseres på håndteringen af de forensiske sager samt de makroskopiske og histologiske læsioner i huden og den underliggende muskulatur. Endvidere gives en generel beskrivelse af hæmostase og akut inflammation efterfulgt af en kort introduktion til blå mærker hos mennesker. Afhandlingen består yderligere af et review af eksperimentelle slaglæsioner hos dyr og mennesker med særlig vægt på grisen som model, samt en beskrivelse af histologiske forandringerne i slaglæsioner påført efter dyrets død. Endvidere er anvendelsen af resultaterne fra eksperimentelle slaglæsioner hos grise til aldersdatering af ikke-eksperimentelle slaglæsioner hos slagtesvin præsenteret og diskuteret.

Chapter 1

Introduction

Inflicting bruises on pigs is a violation of the Danish Animal Protection Act, as stated in legal proceedings in a district court and in the Eastern High Court (1, 2). In Denmark, all pigs with bruises suspected of being inflicted by humans are registered at meat inspection (3). A fraction of these cases are reported to the police, who might request a forensic necropsy to be carried out at the University of Copenhagen (I, 4, 5). At the University of Copenhagen, veterinary pathologists are asked to assess the age of the bruises and to determine whether they were inflicted by humans (I). The latter is determined by assessing the uniformity, pattern and anatomical location of the bruises (I, V). Assessing the ages of bruises is much more complicated, although it is of crucial importance for determining who inflicted the lesions (I, V, X). In Denmark, approximately 90% of these bruises in pigs are estimated to be 8 h or less old (I). Moreover, in more than 90% of these cases, the time span between collection of the pigs at a farm and slaughter is less than 8 h (I). During this time period, pigs are usually handled by several different people, e.g., farmers, drivers and employees at the slaughterhouse (I) The age estimates of bruises provided by veterinary pathologists can be presented in judicial proceedings to identify perpetrators (I, V, X). Therefore, to ensure that the civil rights of the accused are not violated, it is of the utmost importance that bruise age estimates are correct.

In Denmark, all forensic necropsies concerning porcine bruises are carried out by veterinary pathologists at the University of Copenhagen (4). The age assessment of bruises is based on histological evaluation of skin and underlying muscle tissue sampled from a proportion of the bruises (I). Experimental models of bruises have been developed since 1957, but there was only one porcine model until 2016 (II, 6–9). However, in that model, there were few descriptions of histological changes over time because the model focused on reflectance spectroscopic evaluation of bruises (6–9). Time-dependent changes in bruises have been described in other species (II). However, caution must be exercised when extrapolating histological results from one animal species to another (II). Consequently, a porcine model resembling forensic cases of bruises was considered necessary. In particular, the hypotheses were:

Hypotheses

- A porcine model of bruises comparable to bruises inflicted by humans on pigs can be developed and used to obtain objective methods for determining the ages of porcine bruises.

- Histological evaluation of selected parameters in the inflammatory response within skin and underlying muscle tissue can be used to assess the ages of porcine bruises.

- Gene expression signatures, i.e., measuring mRNA expression of specific genes in bruised tissue can be used to assess the ages of porcine bruises.

- The age and/or body weight (BW) of pigs do not influence the timing of bruises based on selected histological changes and mRNA expression signatures.

- The force, speed and mass of the impacting object affect the extent of tissue damage and the intensity of the inflammatory response.

- The anatomical location of bruises and the sampling site within bruises reflect the intensity of the inflammatory response.

- Blunt trauma inflicted post mortem (PM) cannot induce injuries (pseudo bruises) comparable to bruises inflicted ante mortem (AM).

Overview of papers

This dissertation is based on ten papers. Paper I describes cases of bruises in pigs submitted for forensic investigation to the University of Copenhagen with respect to typical gross and histological evaluations of bruises, the number of cases per year and the challenges associated with estimating the ages of bruises. Paper II is a review of experimental animal models of bruises from a forensic standpoint, published from 1957 to 2013.

A highly reproducible model of bruises in pigs was developed and presented in papers III, IV, VII, VIII and IX. Paper III, describes the characteristics of this model and gives a detailed histological description of the time-dependent changes in the inflammatory response in the skin and underlying muscle tissue from bruises inflicted on pigs with a BW of approximately 30 kg. In paper IV, additional experiments were carried out to study the

impact of force on the timing of bruises in pigs (BW = 30 kg). In paper VII, mRNA expression signatures were evaluated in subcutaneous fat and underlying muscle tissue to determine the ages of experimental bruises in pigs (BW = 30 kg). In papers VIII and IX, experimental bruises were inflicted on pigs with a BW of approximately 100 kg. Histology and mRNA expression signatures were used to evaluate how the intensity of the inflammatory response was affected by time, the anatomical location of the bruises, the sampling site within the bruises and the mass and speed of the object that inflicted the bruises.

Additionally, experimental blunt traumas were inflicted on pigs within 3 min after cardiac arrest. PM injuries were inflicted to examine whether signs of vitality could be observed in pseudo bruises (and incised wounds) inflicted on pigs shortly after death. These results are presented in paper VI.

Two studies were performed to assess the applicability of histological parameters and gene expression signatures for determining the age of forensic bruises in slaughter pigs (V, X). The tissue evaluated in the two studies originated from the same pigs. However, when the histological evaluations were carried out, the prediction models based on mRNA expression had not yet been developed. Therefore, the results were presented in two separate papers (V, X).

Overview of dissertation

This dissertation consists of a description of forensic cases of bruises in Danish pigs and focuses on the handling of cases, the gross and histological morphology of lesions and the timing of bruises (Chapter 2). Moreover, a general description of haemostasis and acute inflammation is included, followed by a short introduction to bruises in humans for comparative purposes (Chapter 2). A review of experimental bruises in animals and humans, particularly focusing on porcine models, is provided (Chapter 3). In addition, experimental animal studies of vital reactions in experimental pseudo bruises (inflicted PM) are reviewed (Chapter 4). The applicability of histological parameters and gene expression signatures for determining the age of forensic bruises in slaughter pigs is described (Chapter 5). Finally, all chapters are discussed with an emphasis on the results from the 10 publications on which this dissertation is based (Chapter 6). This led to the formulation of 11 bullet points that summarise the overall conclusions (Chapter 6).

Chapter 2

Porcine bruises inflicted by humans

Persons inflicting bruises on pigs are violating the Danish Animal Protection Act, as demonstrated by legal proceedings in a district court and in the Eastern High Court (Figs. 1, 2) (1, 2). From 2010 to 2013 the prevalence of pigs with bruises slaughtered at the 12 largest Danish slaughterhouses was 0.011% of a total of 65,504,021 slaughtered pigs (5). Although the number of affected pigs is relatively small, each case is a violation of the Danish Animal Protection Act, which is designed to protect animals from suffering, regardless of the numbers involved (1).

Fig. 1: (Left) Several slaughter pigs with bruises inflicted by humans.

Fig. 2: (Right) Bruises inflicted by humans on the back of a slaughter pig.

Bruises detected at meat inspection

In April 2010, a new system for recording skin lesions in slaughter pigs was introduced by the Danish Veterinary and Food Administration (DVFA) (Table 1) (3, 5). At PM meat inspection, skin lesions are recorded as human-inflicted lesions (registered as code 904) or as skin lesions not inflicted by humans or below the limit of triviality (registered as code 901) (Table 1) (3). Whether bruises are below the limit of triviality is determined by the veterinarians carrying out meat inspections (4, 5). The assessments depend on the number of affected animals, the number of bruises, the anatomical locations and the depth of the bruises (10). If bruises are located in sensitive areas, e.g., the head or genitals, they are considered more serious than if located in less sensitive areas, e.g., the back or sides (10). Moreover, the presence of haemorrhage in the muscle tissue underlying the bruises is considered an aggravating circumstance (10). In addition, excessive tattooing (more than two tattoos per pig) is also registered as code 904 (5). Two tattoos, one on each hind quarter, are permitted to identify a pig (11). However, because tattooing inflicts penetrating skin lesions they are not included in this dissertation.

Before 2010, all bruises were registered at meat inspection together with bite marks using the same code (12). However, an increase in the number of slaughter pigs with bruises inflicted by humans registered at Danish slaughterhouses, combined with an increase in public interest for the welfare of pigs, led to the establishment of the more specific registration codes (codes 904 and 901) for skin lesions in slaughter pigs (5, 12–14). Between the implementation of these specific codes in 2010 and 2017, 9,607 of 128,913,163 slaughtered pigs (0.007%) have been registered with code 904 (15). During the same time period 0.74% of pigs slaughtered were registered with code 901(15). In 2010, the number of code 904 registrations was at its highest, reaching nearly 0.02% (Fig. 3). However, from 2014 to 2017 the proportion of pigs registered with code 904 has been approximately 0.004 to 0.005% (Fig. 3). Moreover, between 2010 and 2017 a slight decrease in the proportion of pigs registered with code 901 has occurred (Fig. 4).

In a retrospective study performed from 2010 to 2013, the proportion of pigs at the 12 largest Danish slaughterhouses registered with code 904 ranged from 0.002% to 0.055% (5). The proportions of code 904 pigs were greater at small slaughterhouses than at medium-sized and large slaughterhouses (5). Moreover, the number of pigs registered with code 904 from a single delivery (a batch of pigs from a specific herd owner delivered on a specific day) ranged from 1 to 102 pigs (5). The pigs were delivered from a total of 10,796 pig producers of which 21% and 9% had a minimum of one delivery or more than one delivery of pigs registered with code 904, respectively (5).

TABLE 1: Criteria for recording bruises at post mortem meat inspection at Danish slaughterhouses.

	Code 904	Code 901
Definition (3)	Blunt trauma, fresh contusions, suspicion of violation of animal welfare	Blunt trauma, fresh contusions, not inflicted by humans or below the limit of triviality
Number of lesions (10)	Several bruises	Often single bruises/lesions
Pattern (10)	Uniform, distinct outline, tramline, pattern reflecting a tattoo hammer or other tools	Non-uniform, no distinct outline
Anatomical location (10)	Head, back and sides	Non-specific

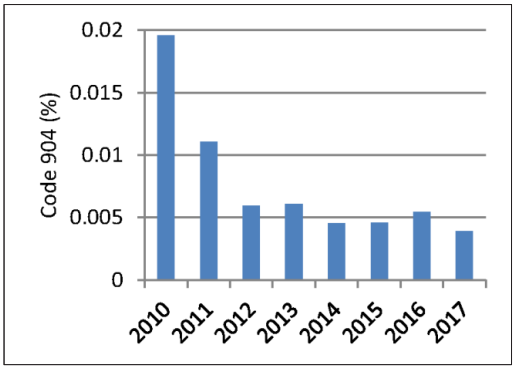

Fig. 3: Prevalence of pigs registered with code 904 at Danish slaughterhouses (15).

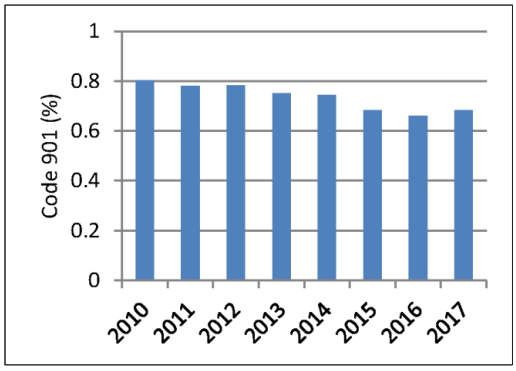

Fig.4: Prevalence of pigs registered with code 901 at Danish slaughterhouses (15).

Risk factors

Bruises are typically inflicted around the time when pigs are driven onto trucks for transport to the slaughterhouse (12, 16). In a prospective study of pigs slaughtered at two major Danish slaughterhouses from November 2013 to May 2014, risk factors for porcine bruises inflicted by humans were identified (16). Factors increasing the risk of bruises inflicted by humans included: identification of pigs by tattooing, large shipments (162 to 245 pigs in one shipment) and staff herding the pigs using plastic paddles and herding boards (16). Moreover, the general health and sex of the pigs also apparently affected the risk of bruises being inflicted by humans (16). In the same study, pig producers who were interviewed identified human factors (including temper, stress, time pressure and educational level), the physical environment (space and lighting), identification of pigs by tattooing, and loose objects in the housing system as risk factors for inflicting bruises on pigs (16).

Handling of forensic cases

Since 2004, a mean of 17 cases of bruises in pigs have been submitted annually for forensic investigation to the University of Copenhagen (Fig. 5). Skin bruises are initially recorded by technicians carrying out routine PM meat inspections at Danish slaughterhouses (Fig. 6) (4, 5). If code 904 is registered for a carcass, a veterinarian employed by the DVFA determines whether any bruises were probably inflicted by humans and are above the limit of triviality (Table 1, Fig. 6) (5). If the bruises are registered with code 904 then an injunction is sent to the pig producer, driver and contractor or the case is reported to the police (Fig. 6). When a case is reported to the police, skin and muscle tissue are stored at −18°C until they are sent to the University of Copenhagen for a forensic necropsy (Fig. 6). From 2004 to 2017 almost 1,700 animals were evaluated by forensic veterinary pathologists at the University of Copenhagen (4). Among these, 79% were pigs and of these, 49% were pigs with bruises that were identified and reported to the police by veterinarians employed at Danish slaughterhouses (Figs. 7, 8) (4).

The police can request that a forensic necropsy is carried out by a veterinary pathologist to determine whether bruises were inflicted by humans and to estimate their age (I, 4). The procedure for handling forensic cases of porcine bruises submitted to the University of Copenhagen is described in Table 2. In Denmark, approximately 90% of bruises in pigs are estimated to be 8 h old or less (I). Moreover, in more than 90% of these cases the time span from collection of the pigs at a farm until slaughter was also less than 8 h (I). During this time period, pigs are usually handled by several different people, e.g.,

farmers, drivers and employees at the slaughterhouse (I). The age estimates of bruises provided by veterinary pathologists can be presented in judicial proceedings to identify perpetrators (I, V, X). Therefore, to ensure that the civil rights of the accused are not violated, it is of the utmost importance that bruise age estimates are correct.

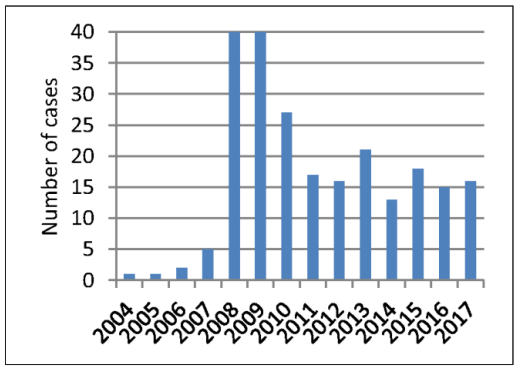

Fig. 5: The annual number of forensic cases of pig bruises submitted to the University of Copenhagen. Each case involved a minimum of 1 pig and a maximum of 42 pigs (I).

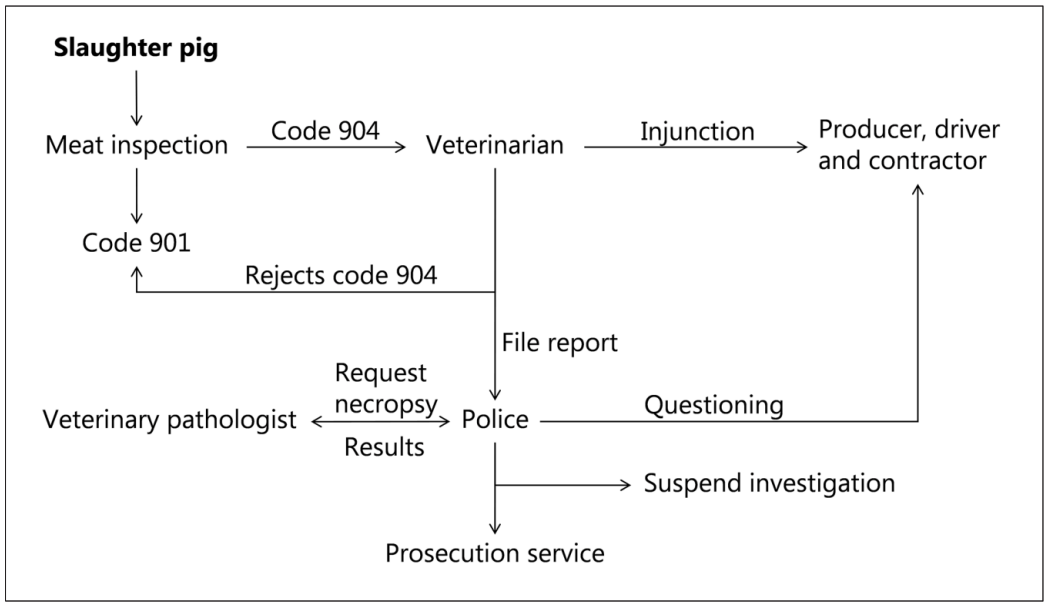

Fig. 6: Skin bruises are initially recorded by technicians carrying out the post mortem meat inspection. If code 904 is recorded for a carcass, a veterinarian determines whether there is a suspicion that bruises were inflicted by humans and whether they are above the limit of triviality. If the bruises are suspicious then either an injunction is sent to the pig producer, driver and contractor or the case is reported to the police. In the latter case, the police carry out a criminal investigation and might request that a veterinary pathologist performs a forensic necropsy. Provided that a sufficient amount of evidence is gathered, the case is submitted to the Prosecution service.

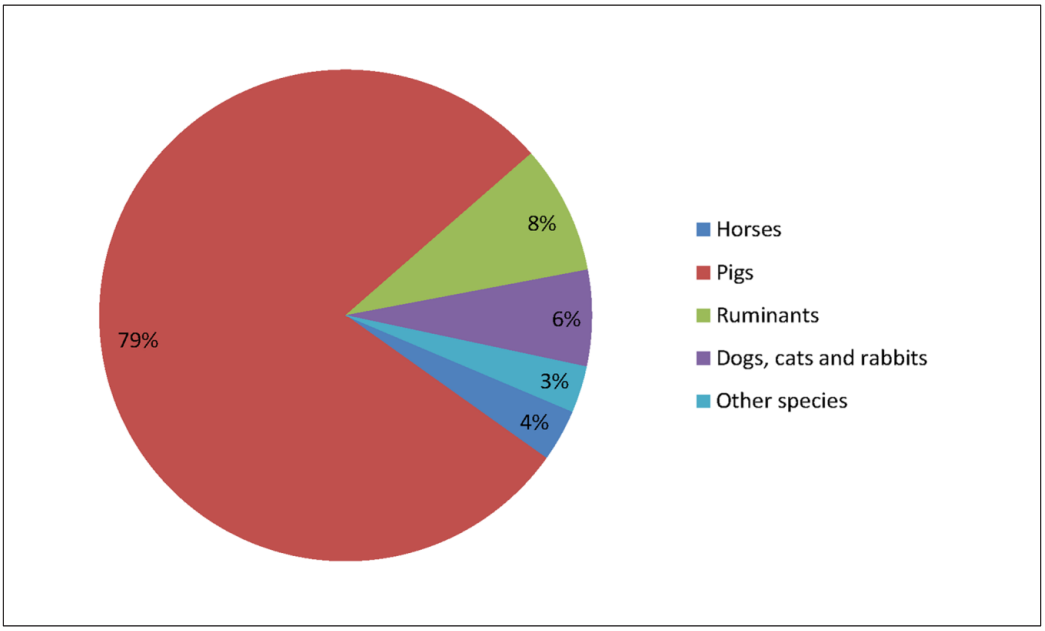

Fig. 7: The proportions of pigs, horses, ruminants, dogs, cats and rabbits, and other species as a percentage of the total number of animals sent to the University of Copenhagen for forensic investigation from 2004 to 2017. In total, 1,682 animals (entire animals or selected body parts) were submitted (4).

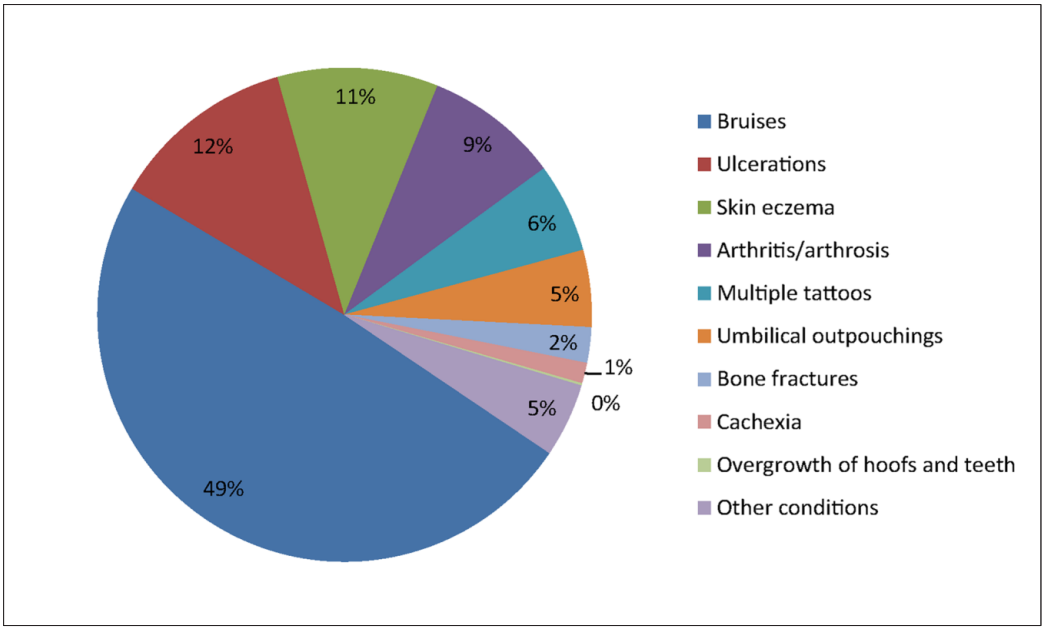

Fig. 8: The percentage of pigs with bruises, ulcerations, skin eczema, arthritis/arthrosis, multiple tattoos, umbilical outpouchings, bone fractures, cachexia, overgrowth of hoofs and teeth and other conditions. In total, 1,332 pigs (entire animals or selected body parts) were submitted from 2004 to 2017 (4).

TABLE 2: The procedure for handling of forensic cases of porcine bruises submitted to the University of Copenhagen (4).

1. Information submitted to the veterinary pathologist
- Case numbers and contact information for the agencies involved (slaughterhouse and police).
- Pig identification information.
- Reason for submission and specific questions.
- Photo documentation of bruises.
- Detailed crime scene report including any examination of the pig conducted by the veterinarian at the slaughterhouse.
- Date of evidence material collection and how the material has been handled and stored.

2. Submission of forensic evidence material
- Skin and underlying muscle tissue containing multiple bruises from one or several pigs.
- Packaging is marked with identifying information.
- Identifying information from the pigs, e.g., ear tags must not be removed.

3. Presentation of evidence material
- The date of arrival and date of forensic necropsy is recorded.
- How the material is received, e.g., packaging, quantity, frozen/thawed is recorded.
- Tags, labels or accompanying paperwork are photographed or added to the case file.
- Consistency between the material and the information received prior to submission is checked.
- The material is assigned a unique local case number.

4. Animal identification
- Tattoos, tags or clippings are recorded.
- Physical characteristics, such as status after processing of the carcass during slaughter are recorded.
- Previous signs of forensic necropsy examination, e.g., cut lines, are recorded.

5. Photos
- Overview photos of the material, including identifying information, e.g., case number and a scale bar.
- Detailed photos of lesions found at forensic necropsy are recorded.
- Photos are stored electronically.

6. Necropsy
- Detailed drawings of the bruises on the surface of the skin, including measurements of the dimensions of the bruises and entire pieces of skin and muscle tissue.
- Cross sectioning of bruises. Haemorrhage in the subcutaneous fat tissue and/or underlying muscle tissue is recorded.
- All associated paperwork is labelled with identifying information, e.g., case numbers, and stored.
- Skin and underlying muscle tissue from 5 to 10 bruises are sampled and fixed in neutral buffered formalin.
- Evidence material is destroyed.

7. Report
- All findings are presented and interpreted in a final report.

Definition of a bruise

A bruise is an accumulation of extravasated erythrocytes in the subcutis and surrounding tissue that can be seen from the skin surface in pigs and humans (I, 17). An impact from a blunt object tears the walls of vessels in the dermis and subcutis causing haemorrhage in the tissue without disrupting the continuity of the epidermis (Figs. 9, 10) (17, 18). Moreover, haemorrhage can also occur in muscle tissue underlying the site of impact (Fig. 10) (I). Descriptions of the type of vessels that are torn, e.g., arteries, veins and capillaries, may be contradictory. For example, Langlois and Gresham (19) defined a bruise as being caused by haemorrhage from capillaries and small veins, whereas Saukko and Knight (17) stated that haemorrhaging from capillaries is not easily visible. However, in experimental porcine bruises haemorrhages were found near capillaries and also surrounding larger vessels, suggesting that both capillaries and larger vessels were torn (III).

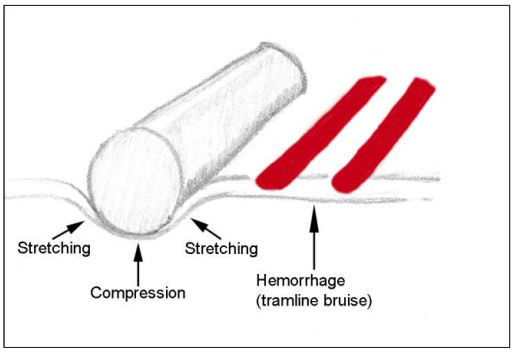

Fig. 9: Impact from a blunt object compresses the underlying tissue and stretches the tissue on both sides of the object. The traction tears the blood vessel, whereas compression causes little or no damage to the vessels, resulting in the characteristic tramline pattern. Modified after (17).

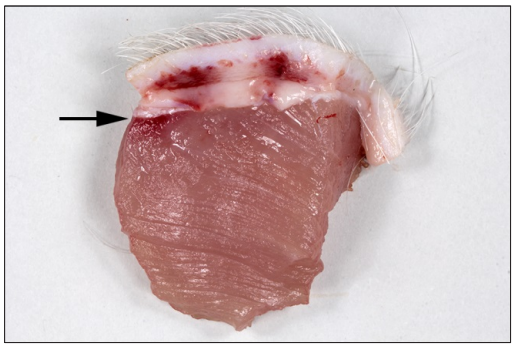

Fig. 10: Cross-section of bruised skin and muscle tissue. Two areas of haemorrhage are visible in the subcutaneous tissue. Moreover, haemorrhage is present in the underlying muscle tissue (arrow) (III).

Gross appearance of bruises

In live pigs, bruises on the skin are difficult to see because of the thick epidermis (I). However, the epidermal layer is removed during slaughter, and bruises are easier to see at the PM inspection of the carcass (Figs. 1, 2, 11, 12) (I). Bruises inflicted by humans are typically located on the back or upper sides of the pigs (Figs. 1, 2) (I, V). Usually, there are several such bruises and they have a uniform pattern that often reflects the object used for striking, e.g., a chain, tattoo-hammer or the handle of a plastic paddle (which is a handling device normally used for herding pigs) (Figs. 12, 13) (I, V). Frequently, bruises have a tramline pattern characterised by two parallel lines of haemorrhage separated by apparently normal skin (Figs. 1, 2, 11) (I, V). This pattern is caused by impact from a cylindrical or rectangular object (III, 17). When the object hits the skin, traction is applied to the tissue on both sides of the object, whereas tissue is compressed in the middle (Fig. 9) (8, 17). The traction tears the blood vessels, whereas the compression does little or no damage to the vessels resulting in the characteristic tramline pattern (Figs. 9–11) (8, 17). Tramline bruises are also observed in humans beaten with police batons or similar rods (17, 20).

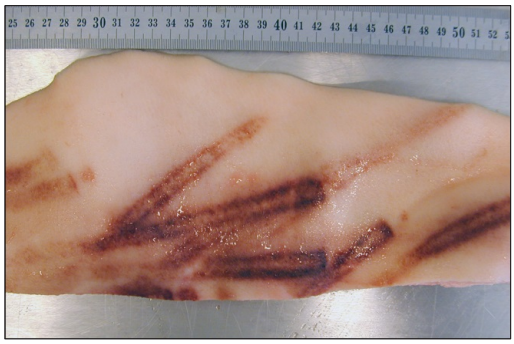

Fig. 11: Porcine skin showing bruises with a tramline pattern.

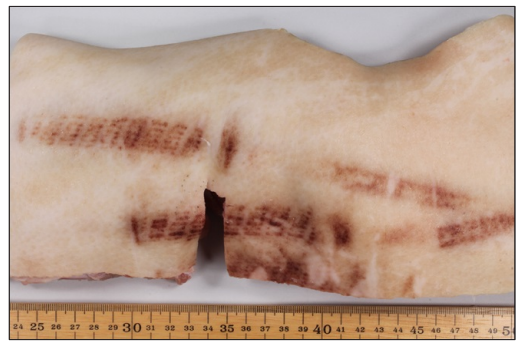

Fig. 12: Porcine skin showing bruises caused by the handle of a plastic paddle (Fig. 13) (V).

Fig. 13: Handle of a plastic paddle and the entire plastic paddle (inset) (V).

Histological appearance of bruises

Histologically, bruises are characterised by haemorrhage, necrosis and inflammation (I, III). In the subcutis, haemorrhage is present between adipocytes and along the fibrous connective tissue, and depending on the timing, infiltration of neutrophils and macrophages may be present (Fig. 14) (I, III).

In the underlying muscle tissue, haemorrhage and necrotic muscle fibres may be present (I, III). Muscle necrosis is a focal monophasic reaction because it results from a single mechanical injury (21). The affected myofibres are either isolated or they appear as clusters of necrotic muscle tissue surrounded by normal myofibres (Fig. 15) (I). Necrotic muscle fibres have a swollen, fragmented, pale and homogenous cytoplasm with a loss of striation, and depending on the timing, infiltration of intracellular and interstitial neutrophils and macrophages may be present (Fig. 15) (I).

During the slaughter process, carcasses are scalded, singed and scraped. This removes the epidermal layer and transforms the dermis into a homogenous mass when viewed microscopically (I). Moreover, to avoid decomposition, tissue is stored at approximately –18°C for several months before being sent for forensic investigation (I, X). However, freezing and thawing reduces the quality of the tissue for histological evaluation (Fig. 16) (I). Erythrocytes burst and appear either as ghost cells or as a homogenous and eosinophilic mass when stained with haematoxylin and eosin (HE) (Fig. 16) (I).

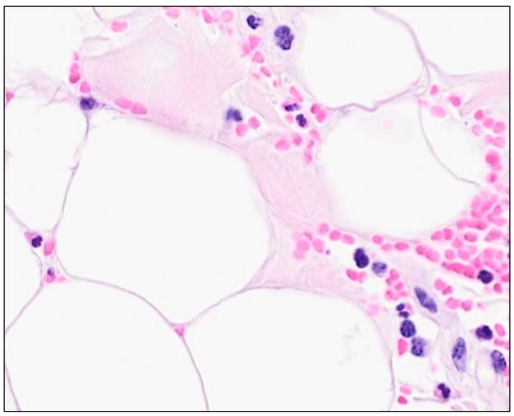

Fig. 14: Skin sampled from an experimental bruise in a pig. The bruise is 5 h old. Haemorrhage and neutrophils are visible in the subcutaneous fat tissue, HE.

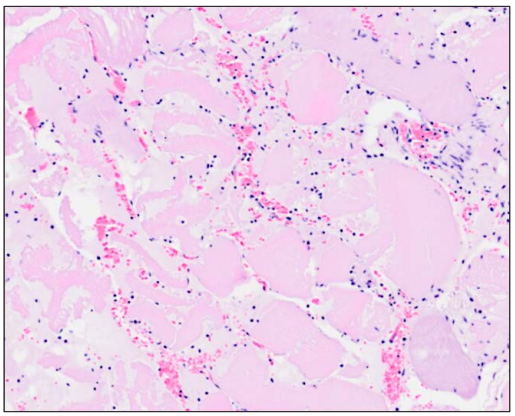

Fig. 15: Muscle tissue sampled beneath an experimental bruise in a pig. The bruise is 8 h old. Haemorrhage, necrotic myofibres and infiltration of inflammatory cells are visible in muscle tissue, HE.

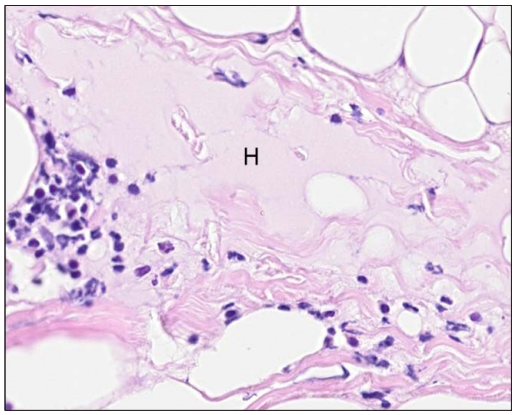

Fig. 16: Skin sampled from a forensic case of bruises inflicted by humans on a slaughter pig. Haemorrhage (H) and neutrophils are visible in the subcutaneous fat tissue. The erythrocytes have been lysed and appear homogenous and eosinophilic, HE.

Age determination of porcine bruises

In Denmark, porcine bruise age determination is based solely on a histological evaluation of the inflammatory response (I). Gross assessment of bruises is considered unreliable, and no other methods for determining the ages of bruises have been developed for direct application (II).

To determine the ages of bruises, the veterinary pathologist compares the inflammatory response in the skin and underlying muscle tissue to those described in studies reporting the healing of bruises and wounds (I). Although the course of inflammation is considered predictable, variations in the inflammatory response within and between pigs complicates bruise age determination (V). Moreover, occasionally bruises that were not inflicted recently but show no inflammatory infiltration are observed (I). Therefore, experimental porcine bruise models are urgently required to study time-dependent changes in bruises and to improve the accuracy of bruise age determination in forensic cases.

Haemostasis and acute inflammation in bruised tissue

Blunt trauma results in the tearing of blood vessels, haemorrhage in the dermis and subcutis, and laceration of the underlying muscle tissue which initiates haemostasis and inflammation (III, 17, 18). Haemorrhage is stopped by transient vasoconstriction and platelets adhering to the exposed subendothelial extracellular matrix via von Willebrand's factor (22). As the platelets are activated, they release adenosine diphosphate and thromboxane A_2, which increases platelet aggregation and forms the primary haemostatic plug (22). Primary haemostasis is followed by the coagulation cascade (secondary haemostasis) that through the intrinsic, extrinsic and common pathway leads to the formation of a meshwork of fibrin surrounding the aggregated platelets (22). Simultaneously, plasminogen is converted to plasmin by plasminogen activator (PLAT) (Table 3).

Cell membrane injury is induced by direct mechanical trauma and by hypoxia as a consequence of the disrupted blood supply (22). In general, cell membrane injury causes an influx of extracellular calcium, leading to irreversible cell injury and necrosis (22). Cell membrane injury is observed in muscle tissue as individual, or clusters of, necrotic myofibres, whereas in the subcutaneous fat tissue adipocytes are apparently unaffected (I, III). However, in an *in vitro* study, compression of human adipocytes increased the secretion of the pro-inflammatory cytokines colony stimulating factor 3, interleukin (IL)6, IL8 and C-C motif chemokine ligand (CCL)5 (23). Therefore, the mechanical compression of subcutaneous fat tissue in bruises probably also contributes to initiate inflammation.

In addition to the coagulation cascade and the fibrinolytic system, tissue injury activates the complement cascade, the kinin cascade and the cyclooxygenase and lipooxygenase pathways (Table 3). Traumatic injury induces the synthesis and release of several inflammatory mediators, such as histamine, bradykinin, tumour necrosis factor α, cytokines, degradative enzymes, chemokines, interferons, prostaglandins, leukotrienes and platelet activating factor (22). Many inflammatory mediators are preformed or synthesised in the liver and in neutrophils, basophils, macrophages/monocytes, platelets, mast cells, endothelial cells, fibroblasts and epithelial cells. These generally bind to receptors on target cells to amplify or suppress the secretion of additional inflammatory mediators (22).

Transient vasoconstriction during haemostasis is followed by vasodilation of arterioles and capillaries, which is caused by the release of histamine from inflammatory cells and platelets. This leads to an increase in blood flow to the injured site, resulting in erythema and an increase in temperature. Histamine and other preformed inflammatory proteins (e.g., serotonin, bradykinin, and substance P) induce an increased vascular permeability that may persist for several minutes or hours (22, 24).

Vasodilation slows down the blood flow and together with changes in the junctional complexes between endothelial cells (initiated by several inflammatory mediators) this allows time for plasma and plasma proteins to leak out into the extracellular space causing swelling and pain (22, 24). Chemoattractants such as complement factors, leuktrienes, chemokines and cytokines activate receptors and molecules on endothelial cells (e.g., selectin E (SELE), selectin P (SELP) and intercellular adhesion molecule 1 (ICAM-1)) and leukocytes (e.g., selectin L, sialyl Lewis X-modified glycoprotein, β_2 integrins (CD111/CD18) and chemokine receptors) to initiate and mediate the leukocyte adhesion cascade (22, 24). The leukocyte adhesion cascade consists of margination, rolling, adhesion and transendothelial cell migration of leukocytes (Fig. 17). Neutrophils are the first type of leukocyte to leave the vessels and migrate into tissue towards chemoattractant molecules released by the host cells (24). The neutrophils degrade necrotic tissue by phagocytosis and release granules that enhance the inflammatory response (22). Later, monocytes migrate into the injured tissue and differentiate into macrophages that phagocytise and degrade necrotic tissue and erythrocytes. Over a period of days, inflammation subsides and is replaced by repair and regeneration of normal structures (e.g., blood vessels, fibroblasts, adipocytes and myofibres) (25). However, in more than 90% of forensic cases, porcine bruises are less than 8 h old and these regenerative changes have not been initiated (I).

TABLE 3: Overview of cascades and systems activating chemical mediators responsible for the acute inflammatory response. Tissue injury initiates the coagulation cascade, the fibrinolytic system, the kinin cascade, the complement cascade and formation of prostaglandins, leukotrienes and lipoxins (arachidonic acid metabolites) (22, 24).

Cascade/system	Pathway	End product	Function
Coagulation cascade	Intrinsic, extrinsic and common pathways	Fibrin	Coagulation
Fibrinolytic system	Plasminogen → Plasmin	Fibrin split products	Fibrinolytic
Kinin cascade	Plasma and tissue kinin pathway	Bradykinin	Vasodilation, vascular permeability
Complement cascade	Classical pathway, mannose-binding lectin pathway and alternative pathway	Activated complement factor proteins (C3a, C5a)	Chemotaxis and leukocyte activation
Arachidonic acid metabolites	Cyclooxygenase and lipoxygenase pathways	Prostaglandins, leukotrienes and lipoxins	Multiple functions mediating the acute inflammatory response

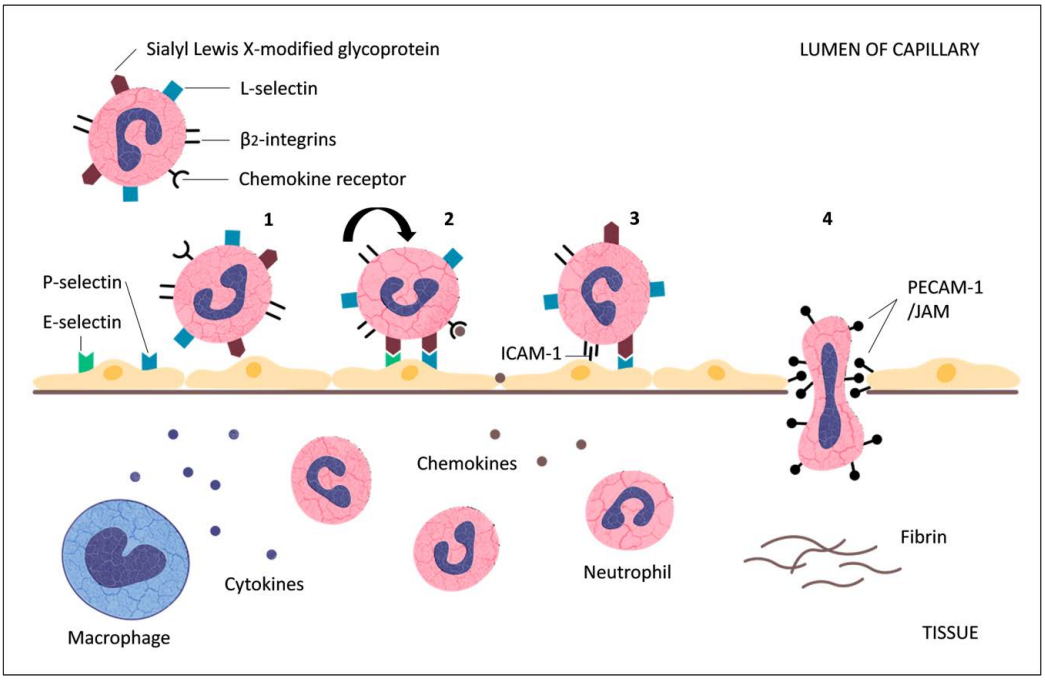

Fig. 17: Leukocyte adhesion cascade. Neutrophil migration from the vessels into the tissue can be divided into four phases: 1) Margination of neutrophils from the centre towards the periphery of the vascular lumen. 2) Rolling of neutrophils on the endothelial cells. Weak binding between selectins and their receptors slows down the neutrophils and brings them close to the endothelial cells. 3) Adhesion of activated neutrophils to endothelial cells. Neutrophils and endothelial cells are activated by cytokines such as interleukin 1, interleukin 6, tumour necrosis factor, complement factors (C5a), platelet activating factor, platelet derived growth factor, chemokines and other inflammatory mediators. Stable adhesion is reached when β_2-integrins on the neutrophils bind to intercellular adhesion molecule 1 (ICAM-1) receptors on endothelial cells. 4) Transendothelial cell migration. Neutrophils and other leukocytes migrate between endothelial cells. Selectins, platelet endothelial cell adhesion molecule-1 (PECAM-1) and junctional adhesion molecules (JAM) mediate this migration. In the tissue, neutrophils migrate towards chemoattractant molecules released by the host cells. Modified after (22).

Bruises in humans

Estimating the ages of bruises is central to the forensic investigation of bruises in humans (26, 27). In a medico-legal context, information on the timing of a bruise may assist in determining when an accident or crime took place (17). In the latter case, the ages of the bruises can be used to identify potential perpetrators (28, 29). For example, non-accidental bruises in children and injuries allegedly inflicted on people while in police custody (20, 30, 31). Furthermore, the gross appearance of a bruise might help to determine the shape of an impacting object, the degree of force used to cause the injuries and the minimum number of blows sustained (20, 30, 31).

Assessing bruises in living subjects

In contrast to forensic cases of bruises in pigs, a clinical forensic medical examiner may be asked to estimate the ages of bruises in people who are still alive (27, 31, 32). In living subjects, the assessment of bruises is limited to their gross external appearance and the history provided by the victim and witnesses (27, 31). Previously, forensic textbooks suggested that bruises undergo a defined course of colour changes as they age (17, 33). Extravasated blood is dark red. However, when viewed through the skin it can appear purple or almost black (17). As extravasated erythrocytes lyse, red haemoglobin is released into the tissue and is phagocytised by macrophages that enzymatically degrade haemoglobin into iron and biliverdin, which is green (34). Subsequently, billiverdin is degraded into bilirubin, which is responsible for the yellow colour in bruises (34). Over time, some of the iron released from haemoglobin becomes oxidised and degraded into haemosiderin, a golden-brown pigment that is stored in macrophages (34). The perceived colour of a bruise depends on the oxidative state of haemoglobin, the depth and area affected by extravasation, the age and constitution of the victim and the skin's melanin pigmentation (34–36). Moreover, an individual's ability to perceive the colour yellow in a bruise declines with age (37). Gross evaluation and digital image analysis of photographs are generally unsuitable for determining the age of accidental human bruises (19, 28, 32, 33, 38, 39).

Reflectance spectroscopic techniques, including reflectance spectrophotometry and hyperspectral imaging, have been used to try to measure changes in bruise colour concentrations objectively (35, 40–46). Reflectance spectroscopy is a non-invasive technique that measures the wavelength of light reflected from the skin to quantify colours and the degradation products of haemoglobin (35, 40). Reflectance spectra have

shown that time-dependent changes occur in the colour and concentrations of chromophores (e.g., haemoglobin, biliverdin, bilirubin and haemosiderin) in bruises of living human subjects (35, 40, 41, 43, 46). The bruises were between 1 h and 47 days old and accidentally inflicted on male and female subjects, with different skin colours, aged from 0–86 years old (35, 40, 41, 43, 46). However, reflectance spectra of bruises are affected by the size, shape, depth and anatomical location of the bruise, as well as by the subjects' age, skin thickness and skin type (36, 45). Therefore, in a number of studies, reflectance spectra of human bruises have been compared to and combined with computer simulated bruises that are able to include information regarding these variables (42, 44, 45). The depth and area affected by haemorrhage can also be assessed using ultrasonography (47, 48). Recently, pulsed photothermal radiometry was used to measure the depth of bruises (49). In addition, this technique was used to quantify haemoglobin and its degradation products and could potentially be used to assess the ages of bruises (49, 50). However, although reflectance spectroscopy and pulsed photothermal radiometry have shown promising results, these techniques are not yet available as diagnostic tools.

Assessing bruises at necropsy

PM, the assessment of bruises in humans can be assisted by histological evaluation of the tissue, and the predictable course of the inflammatory response forms the basis for bruise age determination (27, 30, 34). Time-dependent changes in the immunohistochemical identification of macrophages expressing haeme-oxygenase-1 were observed in human bruises (51). The infiltration of macrophages expressing haeme-oxygenase-1 was absent in bruises less than 2 h old, increased in bruises that were 3 h to 4 days old, and subsequently decreased in bruises that were 5 to 10 days old (51). Moreover, apoptotic cells were identified by DNA end labelling (i.e., the TUNEL method) in bruises from 20 human corpses, and a time-dependent change in the number of apoptotic cells present was observed in the epidermis and dermis (52). However, optimal staining was achieved in only 20% of the samples and the number of apoptotic cells could not be reliably determined when the PM interval exceeded 6 days (52).

In other case studies, variations in the intensity of the inflammatory response between and within human subjects have been reported (26, 30). In three children with bruises known to be at least 30 h old, leukocytes were absent in some bruises and present in others (30). Moreover, in an autopsy study of two adult males with bruises that were 19 h and 5 days old, respectively, an inflammatory response was absent in all but a single tissue sample from the bruises (26). The lack of inflammatory response in these cases might reflect variations between individuals and/or variations in the inflammatory response between sampling sites, e.g., the periphery versus the centre of bruises (26).

Chapter 3

Experimental animal models of bruises

A review of the literature concerning experimental animal and human models of bruises from a forensic viewpoint was carried out. The keywords listed in Table 4 were combined and used to search PubMed, MEDLINE, Web of Science and Google Scholar. Animal and human models of skin bruises that were specifically linked to forensic evaluations and were written in English were included. Studies of experimental bruises generated by blunt trauma that left the skin surface intact were included, whereas studies of experimental lesions caused by surgical incisions or injection of blood into the skin were excluded. In total, 35 studies were included in the review. Tables 5 to 10 summarise the design of the studies with regards to number of animals or human subjects, age/weight, sex, bruise age and evaluation methods.

TABLE 4: List of keywords.

Topic	Keywords
Bruise	Bruise, blunt trauma, blunt force, contusion(s)
Species	Animal model, pig, porcine, swine, rat(s), mouse, mice, rodent(s), rabbit(s), horse(s), equine, sheep, ovine, cow(s), bovine, chicken(s), bird(s), human(s), child, children, infant(s), men, man, woman, women
Forensic	Forensic, age, time
Tissue	Skin, cutis, subcutis, subcutaneous, muscle

TABLE 5: Studies of experimental models of bruises inflicted on Danish and Norwegian Landrace crossbred female pigs.

Year	No. of pigs	BW	Bruise age	Evaluation method
2007 (6)	4	25–40 kg	Up to 5 h	Gross evaluation, histochemistry, reflectance spectroscopy, hyperspectral imaging
2007 (7)	1	42 kg	0–1 h	Hyperspectral imaging, high-speed video, gross evaluation, histochemistry
2007 (8)	1	34 kg	0–10 min	Hyperspectral imaging, reflectance spectroscopy, gross evaluation, histochemistry, computer simulation
2010 (9)	4	25–40 kg	NA	Hyperspectral imaging, image analysis, computer simulation, histochemistry
2016 (III)	10	25–34 kg	1–10 h	Gross evaluation, histochemistry
2016 (IV)	12*	23–38 kg	2–8 h	Gross evaluation, histochemistry, immunohistochemistry, image analyses
2017 (VII)	18**	23–38 kg	1–10 h	Real-time qPCR
2018 (VIII)	12	91–115 kg	2–8 h	Gross evaluation, histochemistry, real-time qPCR
2018 (IX)	12***	91–115 kg	2–8 h	Histochemistry

NA = not available; BW = body weight; qPCR = quantitative polymerase chain reaction. *Data from 4 pigs from paper III were included. **Tissue samples from skin and underlying muscle tissue from 10 pigs from paper III and 8 pigs from paper IV were included. ***Data from 12 pigs from paper VIII were included.

TABLE 6: Studies of experimental models of bruises inflicted on Sprague–Dawley rats.

Year	No. of rats	Age	BW	Sex	Age of bruises	Evaluation method
2010 (54)	48	84 d	250–300 g	M	0.5–36 h	Real-time qPCR
2010 (59)	45	NA	280–320 g	M	3 h–14 d	Immunohistochemistry, western blot, RT-qPCR
2011 (61)	24	NA	140–170 g	NA	1–6 h	Electric impedance spectroscopy
2013 (53)	72	70–84 d	250–300 g	M	4–48 h	Real-time qPCR
2014 (57)	45	NA	280–320 g	M	3–14 d	Immunohistochemistry, western blot, real-time qPCR
2016 (58)	40	NA	300–320 g	M	1–21 d	Histochemistry, immunohistochemistry, western blot, real-time qPCR
2016 (56)	72	70–84 d	250–300 g	M	4–48 h	Real-time qPCR
2016 (60)	50	NA	250–280 g	M	6–21 d	Immunohistochemistry, real-time qPCR
2017 (55)	111	70–84 d	200–250 g	M	4–48 h	Histochemistry, real-time qPCR

NA = not available; BW = body weight; M = male; d = days; qPCR = quantitative polymerase chain reaction; RT-qPCR = reverse transcriptase quantitative polymerase chain reaction.

TABLE 7: Studies of experimental models of bruises inflicted on mice.

Year	No. of mice	Age	BW	Type	Sex	Age of bruises	Evaluation method
2005 (62)	35	42 d	30–35 g	DDY	M	1 h–10 d	Immunohistochemistry, real-time qPCR, in situ hybridisation
2018 (63)	8	49–63 d	22–28 g	BALB/c	M	0.5 h	RNA sequencing, real-time qPCR
2018 (64)	24	NA	NA	NA	NA	0.5 h	Western blot, real-time qPCR

NA = not available; BW = body weight; M = male; d = days; qPCR = quantitative polymerase chain reaction.

TABLE 8: Studies of experimental models of bruises inflicted on ruminants and other species.

Year	Species	No. of animals	Age	Sex	Age of bruises	Evaluation method
1957 (67)	Cattle	55	NA	NA	15 min–9 d	Gross evaluation, chemical analysis
1957 (68)	Cattle	Several	NA	NA	15 min–8 d	Chemical analysis, conductivity measurements
1957 (69)	Rabbits	>146	2–8 months	NA	46 h–8 d	Gross evaluation, chemical analysis
	Cattle*	NA		NA	Up to 72 h	
	Sheep*	NA		NA	Up to 72 h	
	Pigs*	NA		NA	Up to 72 h	
1978 (66)	Calves	20	10–14 d	F, M	0–48 h	Gross evaluation, histochemistry, enzyme histochemistry
	Lambs	20	5–6 months			
1986 (65)	Lambs	50	3–12 months	F, M	1–72 h	Histochemistry

NA = not available; F = female; M = male; d = days. *Details were absent.

TABLE 9: Studies of experimental models of bruises inflicted on chickens.

Year	No. of chickens	Age	Sex	Age of bruises	Evaluation method
1961 (70)	780	56–70 d	NA	2 min–5 d	Gross evaluation, chemical analysis
1961 (71)	1,024	56–70 d	NA	0–4 d	Gross evaluation, chemical analysis
2000 (72)	36	41 d	M	0–24 h	Gross evaluation, histochemistry, colourimetry

NA = not available; M = male; d = days.

TABLE 10: Studies of experimental models of bruises inflicted on humans.

Year	No. of humans	Age (years)	Ethnicity	Sex	Age of bruises	Evaluation method
2006 (73)	11	21–44	Japanese	M	1 h–14 d	Reflectance spectroscopy, colourimetry
2010 (74)	11	NA	Caucasian	NA	0 h–9 d	Photographs, gross evaluation
2011 (29)	13	NA	Caucasian	NA	0 h–9 d	Photographs, gross evaluation and image analysis
2012 (75)	15	18–55	South Indian, Sri-Lankan, Caucasian	F, M	10 min–10 d	Reflectance spectroscopy, colourimetry
2013 (39)	12	23–40	European descent Indian descent	F	3–7 d	Photographs, gross evaluation
2013 (76)	103	18–45	Mixed	F, M	0 h–4 d	Colourimetry

NA = not available; F = female; M = male; d = days.

Models of blunt trauma

Pigs

Experimental models of porcine bruises have been developed and validated by two research groups located at the University of Copenhagen in Denmark and at the Norwegian University of Science and Technology and Trondheim University Hospital in Norway (Table 5). In total, nine studies concerning experimental bruises inflicted on female Danish or Norwegian Landrace crossbred white pigs were reviewed (Table 5).

The methods of inducing trauma reflected the purpose for which the models were developed. The method developed and validated by the Danish research group closely resembled how porcine bruises are inflicted in forensic cases (I, III–V, VII–IX). The Danish research group constructed a mechanical device capable of delivering a single blow with a force of impact similar to a man striking (Fig. 18) (III). The mechanical device consisted of a spring fixed to a rotatable wheel that was mounted between two steel pillars (Fig. 18) (III). Hollow tubes of plastic or iron could be attached to the wheel (III, VIII). As the wheel was turned, the spring tightened and when released, the energy stored in the spring turned the wheel back to its original position while the tube struck the skin with some force (III). Depending on how much the wheel was turned, trauma could be inflicted with differing degrees of force, speed and kinetic energy (Table 11) (III, IV, VII–IX). The mechanical device had a maximum force of 6.5 N/mm^2 which is comparable to a force of 7.3 N/mm^2 applied by a man using the same type of plastic tube (III). On each pig, four bruises were inflicted along the right m. longissimus dorsi from the area caudal to the scapula to the lumbar region using a plastic tube or an iron bar. The pigs were euthanized between 1 and 10 h after trauma was inflicted (III, IV, VII–IX). The method inflicted reproducible bruises with a tramline pattern which was identical to forensic cases of bruises in pigs beaten with sticks or similar objects (Fig. 19) (I, III, V, VIII). Experimental bruises were inflicted on a total of 30 pigs using five different settings (Table 11). In summary, bruises were inflicted on 18 pigs with a mean BW of 30 kg (23 to 38 kg) using a plastic tube and a force of 6.5, 4.2 or 2.1 N/mm^2 (settings no. 1 to 3) (III, IV, VII). Moreover, bruises were inflicted on 12 pigs with a mean BW of 100 kg (91 to 115 kg) using a plastic tube or an iron bar and a force of 6.5 N/mm^2 (settings no. 4 and 5) (VIII, IX).

By contrast, the Norwegian research group developed methods for inflicting porcine bruises comparable to bruises in humans (6–9). The Norwegian research group developed two experimental methods (A and B) for trauma induction (Table 12). These methods were intended to inflict an injury comparable to a punch from a boxer (A) or a blow from a cane (B) (6). Method A: The device consisted of a rig with a pendulum that could be released from different angles (6, 7, 9). Attached to the pendulum was an exchangeable weight of 2.5 kg with a rounded tip or a square shape (6, 7, 9). Method B:

Trauma was inflicted by a 3.5 g paint-filled paint ball (d = 1.71 cm) propelled by compressed air from a distance of 0.5 m (6–9). The skin was covered by a thin plastic film to avoid paint marking the skin (6–9). Blunt traumas were inflicted at the shoulder and hip regions and the pigs were kept under anaesthesia for up to 5 h before being euthanized (6–9). The experimental bruises inflicted by paint balls resembled human bruises that were inflicted by paint balls (Fig. 20) (6, 9). Bruises inflicted by the pendulum did not inflict bruises visible on the skin surface (6). Instead, haemorrhage was observed in the muscle tissue underlying the site of impact (6).

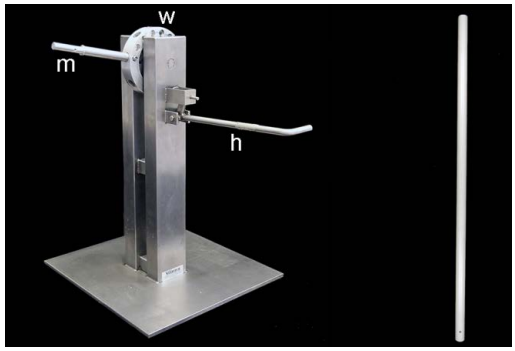

Fig. 18: Mechanical device used to inflict blunt trauma. When the wheel (w) is turned, the spring inside the wheel tightens. The wheel is locked and unlocked using the handle (h). When unlocked, the spring turns the wheel back to its initial position. Tubes of plastic can be attached to the metal stick (m). In one study (VIII) (setting 2), the metal stick (m) was removed and an iron bar was mounted on the wheel (III).

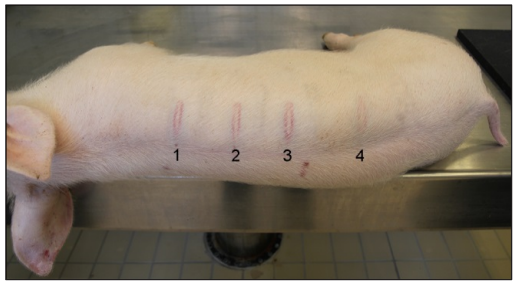

Fig. 19: Four experimental bruises inflicted using a plastic tube on areas of impact No. 1 to 4. Bruises were inflicted with a force of 6.5 N/mm^2 (setting 1) (VII).

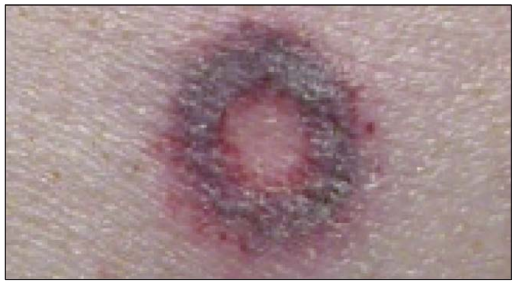

Fig. 20: Experimental bruise inflicted by a paint ball (Method B) (6).

TABLE 11: The Danish research group inflicted experimental bruises on a total of 30 pigs using a plastic tube or an iron bar fixed to a mechanical device made from steel. Five different experimental settings were used. The type of object, mass, impact speed, force, and total energy for each of the experimental settings are presented. The force/mm^2, energy/m^2, and the number and body weights (BWs) of pigs are also presented. Force and energy were calculated using the assumption that these were dissipated equally over an area of impact of 1,200 mm^2.

	Setting 1 (III, IV, VII)	Setting 2 (IV, VII)	Setting 3 (IV, VII)	Setting 4 (VIII, IX)	Setting 5 (VIII, IX)
Object	Plastic tube	Plastic tube	Plastic tube	Plastic tube	Iron bar
Mass	0.047 kg	0.047 kg	0.047 kg	0.047 kg	0.4 kg
Impact speed	47.4 m/s	40.4 m/s	21.5 m/s	47.4 m/s	19.7 m/s
Force	7,800 N	5,000 N	2,500 N	7,800 N	7,800 N
Force/mm^2	6.5 N/mm^2	4.2 N/mm^2	2.1 N/mm^2	6.5 N/mm^2	6.5 N/mm^2
Total energy	26 J	20 J	5 J	26 J	26 J
Energy/m^2	2.2×10^4 J/m^2	1.7×10^4 J/m^2	0.4×10^4 J/m^2	2.2×10^4 J/m^2	2.2×10^4 J/m^2
No. of pigs	10	4	4	6	6
BW	25–34 kg	23–38 kg	23–38 kg	91–115 kg	91–115 kg

TABLE 12: The Norwegian research group applied two experimental methods (A and B) to induce bruises. In method A, bruises were inflicted using a rig with a pendulum that could be released from different angles. Attached to the pendulum was an exchangeable weight with a rounded or square edge. In method B, bruises were inflicted by a paint filled paint ball (d = 1.71 cm) propelled by compressed air. The type of object, mass, impact speed, force, energy/m^2, and the number and body weights (BWs) of the pigs used are presented.

	Method A (6, 7, 9)	Method B (6–9)
Object	Pendulum	Paint ball
Mass	2.5 kg	0.0035 kg
Impact speed	4.1 m/s	104 m/s
Force	500 N	600 N
Energy/ m^2	4.5×10^4 J/m^2	6.1×10^4 J/m^2
No. of pigs	9	10
BW	25–42 kg	25–42 kg

Other animal species and humans

In rats, experimental bruises have been inflicted by dropping weights (m = 0.1 to 0.25 kg) onto the right posterior limb through clear Lucite guide tubes (150 to 200 cm) (53–56) or by using a mechanical weight drop device (m = 0.5 kg, V = 3 m/s, E = 2.25 J, area of impact = 1.127 cm^2) designed by Yu *et al* (57–60). In another model, rats were struck vertically on the right buttock by an iron hammer dropped from a height of 0.1 m (m = 0.5 kg, diameter = 3 cm) (61). In mice, bruises have been inflicted either using a flat mouth tong or by compressing the dorsal skin to a width of 0.1 cm for 30 s between two metal plates with holes 0.3 cm in diameter (62–64).

Three different methods have been used to study experimental bruises in ruminants: 1) Similar to the models in rats, experimental bruises in lambs have been inflicted by dropping a lead weight (1.5 kg) through a tube (100 cm) with perforated sides onto the skin (area of impact = 0.8 cm^2) at different anatomical locations (65). 2) In another study on calves and lambs, bruises were inflicted on the right hind limb midway between the femorotibial joint and the ischium by single blows from captive bolt pistols with modified striking surfaces (66). 3) In cattle, bruises were inflicted using two blows from a seven pound sledgehammer travelling a distance of 3 feet in 0.5 to 1 s (67–69).

In rabbit models, bruises have been inflicted using ten blows from rubber pressure tubing one-half inch thick, travelling 2 feet in 0.5 s (69).

Experimental bruises have been inflicted on the breast, wings and thighs of chickens using an instrument delivering blows of 4.1 kg and driven by a spring (70–72). This instrument was originally designed by Hamdy *et al*. in 1961 and used again several years later by Northcutt *et al*. in 2000 (71, 72).

In humans, experimental bruises have been inflicted by four methods: 1) dropping a lead weight (0.455 kg) through a tube (100 cm) onto the participants' left biceps brachii (area of impact 1.77 cm^2) (39); 2) pinching the skin on the inside of the arm (73); 3) by applying suction to the arm using a 30 mL or 60 mL syringe barrel and a suction pump (29, 74, 75); and 4) striking the subject's arm with a paint ball pellet (diameter: 1.7 cm; mass: 3.2 g) with an impact speed of 67 m/s (76).

Anaesthesia

Pigs

Experimental pigs were kept under anaesthesia to avoid pain, in accordance with international and national legislation (III, IV, VII–IX, 6–9). Drugs used for premedication, induction and maintenance of anaesthesia are listed in Table 13. In the experiments carried out by both the Danish and Norwegian research groups, dissociative anaesthetics (i.e., tiletamine and ketamine) and benzodiazepines (i.e., zolazepam and diazepam) were used for premedication and induction of anaesthesia. Moreover, both groups used a combination of opioids (i.e., fentanyl), benzodiazepine (i.e., midazolam) and isoflurane gas to maintain pigs under anaesthesia. In all experiments, pigs were kept under anaesthesia until being euthanized by an overdose of pentobarbital (III, IV, VII–IX, 6–9). The immunomodulatory effects (mostly anti-inflammatory and some pro-inflammatory) of these anaesthetics have been described and might have influenced the intensity of inflammation in the experimental bruises (77–79). Moreover, the experimental pigs did not experience anxiety when being beaten, as a conscious pig would have. Acute stress leads to the release of catecholamines, which induce leukocytosis and enhance the immune response (80, 81). However, the gross and histological appearance of the experimental bruises was very similar to that of bruises inflicted on pigs from forensic cases (I, III–V, VIII). Moreover, in a number of previous studies investigating the inflammatory reaction in porcine models of osteomyelitis, endocarditis and pyaemia, similar anaesthetic protocols have been used (82–85). In addition, the course of inflammation during these studies was similar to that observed in spontaneous cases of osteomyelitis, endocarditis and pyaemia (82–85).

TABLE 13: Drugs used for premedication, induction and maintenance of anaesthesia and for euthanasia of experimental pigs.

	Danish research group (III, IV, VII–IX)	Norwegian research group (6–9)
Premedication and induction	Tiletamine, zolazepam, xylaxin and butorphanoltartrat	Azaperone, diazepam, ketamine and thipenthon
Maintenance	Fentanyl, midazolam and isoflurane gas	Fentanyl, midazolam and isoflurane gas
Euthanasia	Pentobarbital	Pentobarbital

Other animal species and humans

In the experimental studies performed from 1957 to 1986 using other animal species, no anaesthetic or analgesic treatment was recorded (65–71). Subsequently, chickens were anaesthetised with ketamine and mice and rats with 10% chloral hydrate, 2% sodium pentobarbital, ether or isoflurane inhalation prior to bruises being inflicted (53–64, 72). After trauma had been inflicted, these animals recovered from the anaesthesia without analgesic treatment (53–64, 72). However, because animal experiments require monitoring for pain, analgesic treatment may have been unnecessary or could have been provided without being recorded in the reports (II). In human studies, bruises were inflicted on conscious subjects and no analgesic treatment was provided (29, 39, 73–76).

Analysis

All animal species and humans

Various methods have been used to study time-dependent changes in experimental bruises (Tables 5–10, Fig. 21). Gross evaluation of bruises has been carried out in pigs, ruminants, chickens, rabbits and humans (III, IV, VIII, 6–8, 29, 39, 66, 67, 69–72, 74). Moreover, bruises in pigs, humans and chickens have been studied using colourimetry, reflectance spectroscopy and hyperspectral imaging (6–9, 72, 73, 75, 76). A colourimeter uses a white light source and three receptors tuned to the red, green and blue regions of the visible spectrum to objectively measure colour (34, 72). Reflectance spectroscopy and hyperspectral imaging measure multiple points within the visual spectrum to evaluate colour, haemoglobin oxygenation, dermal blood volume fraction, erythema index and melanin index in bruises (6–9, 34, 73, 75, 76). In the studies on pigs, reflectance spectra were recorded over the 400 to 1000 nm wavelength range using an integrating sphere setup and a hyperspectral camera system (6–9).

Histochemistry and immunohistochemistry have been applied to study the infiltration of neutrophils and macrophages into tissue using light microscopy, fluorescence microscopy and confocal laser scanning microscopy (III, IV, VIII, IX, 6–9, 55, 57–60, 62).

Western blotting has been used to evaluate the timing of changes in the quantities of specific proteins present in experimental bruises in rats and mice (57–59, 64).

Real-time quantitative polymerase chain reaction (real-time qPCR) and conventional reverse transcription quantitative polymerase chain reaction (RT-qPCR) have been used to measure the mRNA expression of specific genes involved in inflammatory responses and tissue repair in several experimental bruise models in pigs, mice and rats (VII, VIII, 53–60, 62–64). In response to a particular stimulus, mRNA is transcribed in the nucleus of a eukaryotic cell, transported to the cytoplasm and translated by ribosomes into protein (86). Measuring mRNA expression can reveal the cell's 'intention' even before a protein is produced. This might be crucial in determining the age of a recently inflicted bruise.

Other methods include measuring the electric properties of traumatised muscle tissue using electric impedance spectroscopy and other techniques (61, 68). Fouché's reagent (trichloroacetic acid, 10% ferric chloride and distilled water) has been used to assess colour reactions and detect bilirubin in tissue sampled from bruises in cattle, rabbits, pigs, sheep and chickens (68–71). Spectrophotometric measurements of haemoglobin and bilirubin extracted from bruises in cattle have also been carried out (67). In addition, porcine bruises have been studied by recording high speed videos of the skin at the moment of impact (7).

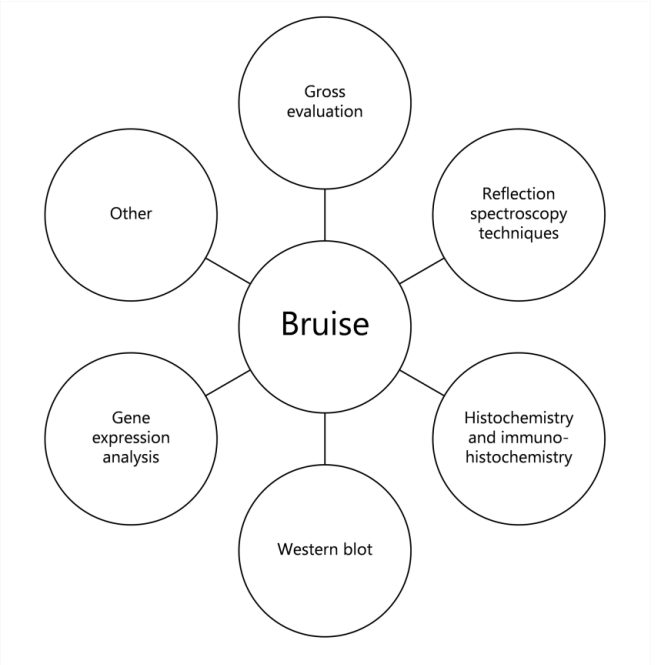

Fig. 21: Overview of the methods used to analyse experimental bruises in animals and humans. Other techniques included electric impedance spectroscopy, Fouché's reagent (trichloroacetic acid, 10% ferric chloride and distilled water) for assessing colour reactions and detecting bilirubin, spectrophotometric measurements of haemoglobin and bilirubin extracted from bruises, and high speed videos of the skin at the moment of impact.

Factors

Determining the ages of bruises is the central focus of forensic cases. Therefore, several experimental models have focused solely on changes in bruises as they age. However, time is not the only factor that affects the intensity of the inflammatory response in a bruise (II). Some experimental studies have investigated the influence of other factors, such as the type of impact (force, mass and speed of the object used for striking), age of the animal, anatomical location and sampling site within a bruise (Fig. 22). In the following each of these factors is addressed.

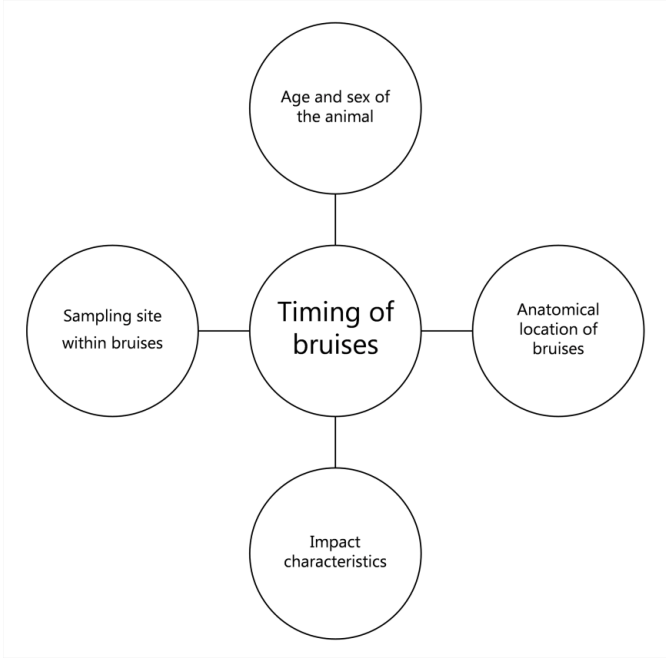

Fig. 22: Factors affecting the degree of tissue damage and the intensity of the inflammatory response within bruises.

Timing of bruises

Pigs

Gross evaluation

At gross evaluation of bruises inflicted using a plastic tube, iron bar and paint ball these develop in a similar way (III, IV, VIII, 6, 7). Within 5 to 20 s of impact a pronounced erythema with a central area of white skin becomes visible (Fig. 23) (III, IV, VIII, 6–8). The erythema is followed by a haemorrhage in the skin that appears 1 to 4 min after trauma either as two curved lines or a ring, depending on the shape of the impacting object (i.e., a tube or paint ball) (Fig. 23) (III, IV, VIII, 6, 7). During the first 5 to 15 min the erythema fades and the haemorrhage develops fully over the first hour (Figs. 20, 24) (III, IV, VIII, 6–8). Blunt trauma inflicted by a weight attached to a pendulum created a wheal and flare reaction that developed within 20 to 30 seconds and disappeared within minutes (6, 7).

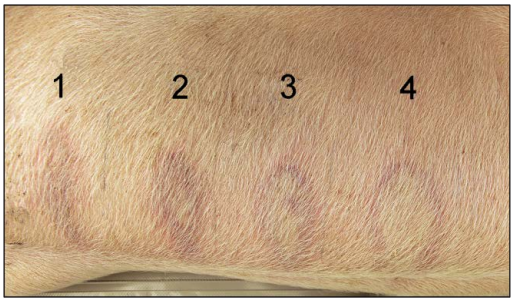

Fig. 23: Four experimental bruises inflicted on pig skin over a period of 3 min. Bruise Nos. 1, 2, 3 and 4 are 3, 2, 1 min and 5 s old, respectively (III).

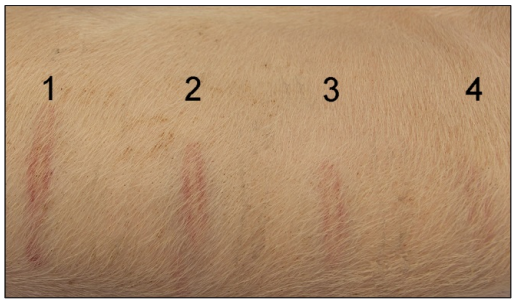

Fig. 24: Four experimental bruises inflicted on pig skin. The bruises are approximately 1 h old (III).

Reflectance spectroscopy and hyperspectral imaging

Reflectance spectra have been collected from bruises inflicted by a paint ball or a pendulum within the first hour after injury (6–9). The deep haemorrhage in the muscle tissue inflicted by the pendulum (Method A) could not be predicted by reflectance spectroscopy and hyperspectral imaging (6, 7, 9). Only a temporary depletion of haemoglobin oxygenation and blood volume, lasting a few minutes, was detected in the upper skin layers (6, 7, 9). The reflection spectra showed almost no changes in haemoglobin oxygenation, dermal blood volume fraction and erythema index during the first hour after impact (6, 7). In bruises inflicted by a paint ball (Method B), haemoglobin oxygenation decreased and the dermal blood volume and erythema index increased immediately after impact before slowly returning to a normal level during the first hour after impact (6, 7).

Histology

The experimental bruises were characterized by haemorrhage, myofiber necrosis and infiltration of neutrophils and macrophages (Figs. 25, 26). Both the Danish and Norwegian research groups found that neutrophils were the first type of leukocyte to infiltrate the traumatised tissue (III, IV, 6). Moreover, the Danish group demonstrated a time-dependent increase in the number of neutrophils in the dermis and subcutaneous fat tissue sampled from bruises inflicted on 10 pigs with a mean BW of 30 kg (setting 1) (Figs. 27, 28) (III). In the underlying muscle tissue, a time-dependent increase in the number of macrophages was also observed (Fig. 29). Moreover, as the age of the bruises increased, leukocytes were more frequently found within necrotic muscle fibres rather than in the interstitial space (III). By combining the histological parameters, bruises that were less than 4 h old could be differentiated from those that were between 4 and 10 h old (Table 14) (III). However, the number of neutrophils and macrophages overlapped during these time intervals, reflecting variations in the intensity of the inflammatory response in bruises within and between pigs (Table 14) (III). When this experimental setup was repeated using pigs with a BW of 100 kg (setting 4 and 5), no or only vague time-dependent changes were observed in the individual histological parameters (VIII, IX). However, when the histological parameters (i.e., neutrophils and haemorrhage in the dermis; neutrophils, macrophages and haemorrhage in the subcutaneous tissue; and neutrophils, macrophages, haemorrhage and necrotic muscle fibres in the underlying muscle tissue) were combined with mRNA expression data from 13 selected genes (Table 15) in a partial least-squares regression analysis (PLS), the ages of bruises could be determined with a precision of ± 2.04 h (Table 16) (VIII).

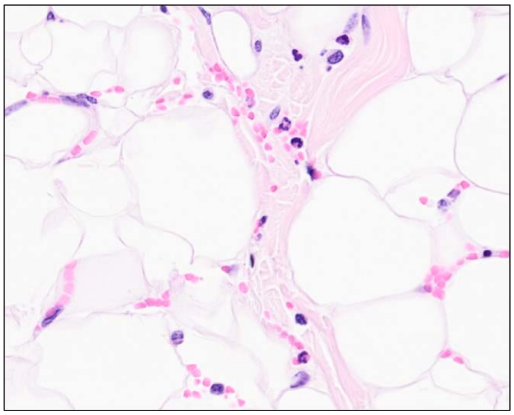

Fig. 25: Skin sampled from an experimental bruise in a pig (setting 5). The bruise is 5 h old. Haemorrhage and neutrophils are visible in the subcutaneous fat tissue, HE.

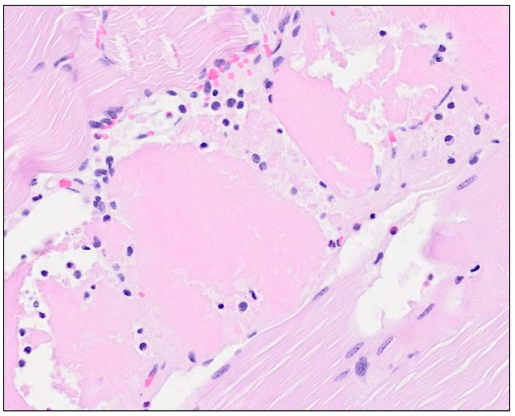

Fig. 26: Muscle tissue sampled from an experimental bruise in a pig (setting 5). The bruise is 5 h old. Haemorrhage, necrotic myofibres and infiltration of inflammatory cells are visible in muscle tissue, HE.

TABLE 14: Timing of histological parameters in experimental bruises inflicted on 10 pigs with a mean body weight of 30 kg (setting 1) (III). The blunt traumas were inflicted using a plastic tube and a force of 6.5 N/mm^2, which was equivalent to striking by a human (III). In each bruise, the numbers of neutrophils and macrophages in a single high-power viewing field (400×, HE stained tissue sections) were counted in the area with the highest cell density (III) and are shown in parenthesis.

Time	Dermis	Subcutis	Muscle
1–3 h	• Haemorrhage • Few neutrophils (1–30+) • Macrophages (0–10)	• Haemorrhage • Few neutrophils (1–30) • Few or no macrophages (0–5)	• Haemorrhage • Varying numbers of neutrophils (0–30+) • From 2 h: macrophages (0–30) • Necrotic muscle fibres • Leukocytes predominantly located in the interstitial space
4–10 h	• Haemorrhage • 7–10 h: more neutrophils (11–30+) • Macrophages (0–10)	• Haemorrhage • More neutrophils (11–30+) • Few or no macrophages (0–5)	• Haemorrhage • Varying numbers of neutrophils (0–30+) • Increasing numbers of macrophages (0–30+) • Necrotic muscle fibres • Leukocytes predominantly located within necrotic muscle fibres

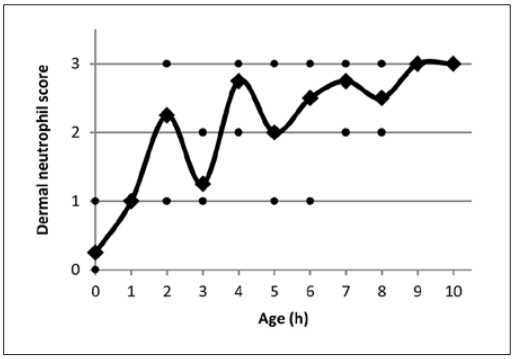

Fig. 27: Scoring of neutrophils in the dermis. The large connected symbols represent the mean scores from four bruises. The small dots represent the range among individual bruises. Control pig data are shown at 0 h (III).

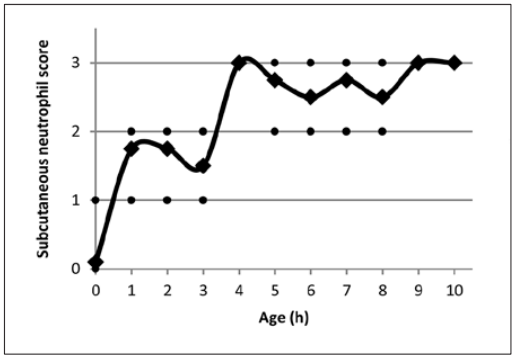

Fig. 28: Scoring of neutrophils in the subcutaneous fat tissue. The large connected symbols represent the mean scores from four bruises. The small dots represent the range among individual bruises. Control pig data are shown at 0 h (III).

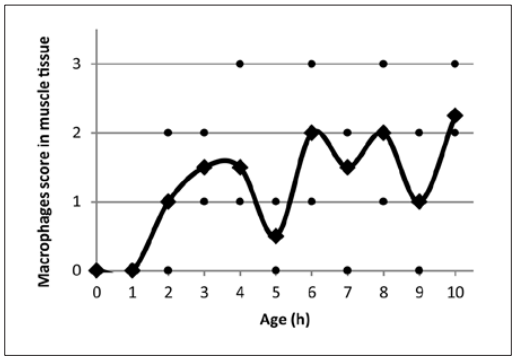

Fig. 29: Scoring of macrophages in the muscle tissue. The large connected symbols represent the mean scores from four bruises. The small dots represent the range among individual bruises. Control pig data are shown at 0 h (III).

Figs. 27–29: Experimental bruises were inflicted with a force of 6.5 N/mm^2 in 10 pigs with a mean body weight of 30 kg (setting 1). Neutrophils and macrophages in the dermis, subcutaneous tissue and the underlying muscle tissue were scored on a semi-quantitative scale: 0 = no neutrophils or macrophages; 1 = 1–10 neutrophils or macrophages; 2 = 11–30 neutrophils or macrophages; 3 = >30 neutrophils or macrophages. The scoring was carried out on HE stained tissue sections in a single high-power viewing field (400×) in the area with the highest cell density (III).

Gene expression

Gene expression signatures that include genes involved in inflammation and tissue modelling in the subcutaneous fat reflect the age of porcine bruises (VII, VIII). High throughput real-time qPCR was used to measure the mRNA expression of 42 genes in subcutaneous fat tissue sampled from bruises in pigs with a mean BW of 30 kg (setting 1) (VII). The 42 genes (listed in Appendix Table I) were involved in haemostasis, inflammation, tissue damage and repair, and were selected based on descriptions of wound healing and acute inflammation (87–91). Principal component analysis (PCA) of the gene expression data grouped bruises into three age intervals: 1 to 3 h, 4 to 6 h and 7 to 10 h (Fig. 30) (VII). These groupings were mainly explained by the expression pattern of 13 specific genes (Table 15). PLS analysis of the expression data from these 13 selected genes resulted in a prediction model (prediction model no. 1). The statistical model specified the linear relationship between the ages of bruises and the 13 gene expression variables in subcutaneous fat from bruises between 1 and 10 h old. The prediction model had a root mean square error (RMSE) of 1.06, meaning that in 95% of bruises, the age could be determined with a precision of ±2.12 h (Table 16) (VII).

The experimental setup was repeated on 12 pigs with a mean BW of 100 kg (settings 4 and 5). The mRNA expression levels of the 13 genes (Table 15) were evaluated and again, bruises were grouped according to age (VIII). Table 17 presents the mean expression of genes relative to their mean expression in control tissue scaled to one according to the ages of bruises and the BW of the pigs. Gene expression data (13 genes) were combined with histological data and subjected to a PLS analysis (prediction model no. 2A) (Table 16). However, the grouping of bruises according to age was mainly defined by IL6, nuclear factor κ β subunit 1 (NFKB1), SELE and SELP (VIII). Therefore, the expression data from these four genes were used to create a third model to predict the ages of bruises using PLS analysis with full cross-validation (prediction model no. 2B) (VIII). A PLS analysis of expression data from these four genes resulted in a prediction model with a RMSE of 0.92, meaning that in 95% of bruises, the age could be determined with a precision of ±1.84 h (no. 2B) (Table 16) (VIII).

In addition, the expression of 43 genes (listed in Appendix Table II) was evaluated in the muscle tissue underlying the site of impact in pigs with a mean BW of 30 kg (setting 1) (VII). However, gene expression profiles in the muscle tissue did not reflect the age of the bruises. This was probably due to variations in the overlying subcutaneous fat tissue (VII).

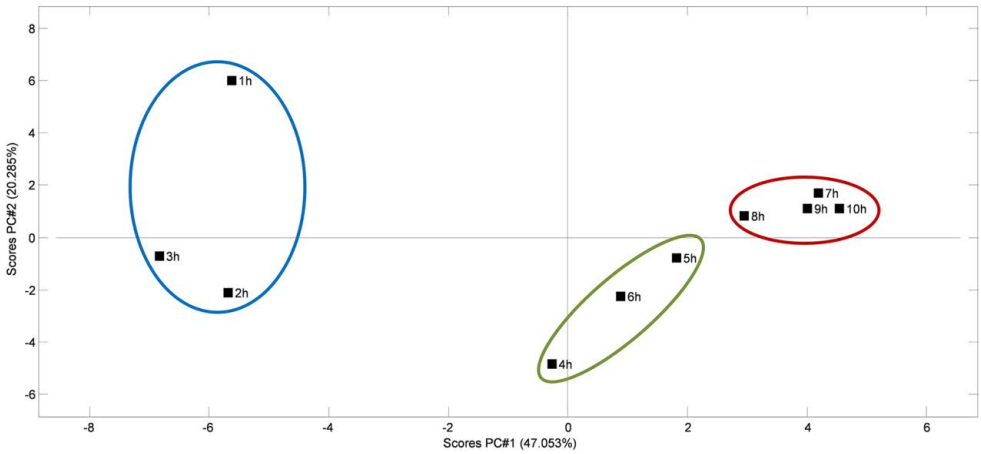

Fig. 30: Principal component analysis of gene expression data from 42 genes expressed in the subcutaneous fat of 10 pigs (setting 1). Tissue was sampled from experimental bruises inflicted with a force of 6.5 N/mm^2. Each square represents the mean of four bruises on the back of a pig. Bruises were separated according to age into three intervals: 1 to 3 h (blue circle), 4 to 6 h (green circle), 7 to 10 h (red circle). The percentages in parentheses denote variations in the data explained by principal components 1 and 2 (PC#1 and PC#2). The groupings according to bruise age were attributable to the mRNA expression patterns of the following 13 genes: Apolipoprotein A1, C-C motif chemokine ligand 2, complement factor D, c-Fos proto-oncogene, intercellular adhesion molecule 1, interferon α 1, interleukin 6, nuclear factor κ β subunit 1, plasminogen activator, prostaglandin-endoperoxide synthase 2, selectin E, selectin P, tumour necrosis factor α-induced protein 3 (VII).

TABLE 15: List of genes included in the prediction models. Gene symbols, names and selected functions are presented. Forward and reverse primer sequences are shown in Appendix Table I. Prediction model no. 1 was based on the mRNA expression of the 13 genes in the subcutaneous fat tissue sampled from experimental bruises in pigs with a body weight (BW) of 30 kg (setting 1) (VII). Prediction model no. 2A was based on the mRNA expression of the 13 genes in the subcutaneous fat tissue and histological data from the skin and underlying muscle tissue sampled from experimental bruises in pigs with a BW of 100 kg (settings 4 and 5) (VIII). Prediction model no. 2B was based on the mRNA expression of IL6, NFKB1, SELE and SELP in the subcutaneous fat tissue sampled from experimental bruises in pigs with a BW of 100 kg (settings 4 and 5) (VIII).

Gene symbol	Gene name	Function of gene (22, 105, 106)
APOA1	Apolipoprotein A1	Component of high density lipoprotein, anti-thrombotic
CCL2	C-C motif chemokine ligand 2	Chemotactic (monocytes)
CFD	Complement factor D	Complement cascade
FOS	c-Fos proto-oncogene	Cell proliferation and differentiation
ICAM-1	Intercellular adhesion molecule 1	Leukocyte adhesion cascade
IFNA1	Interferon α1	Anti-viral activity
IL6	Interleukin 6	Inflammatory mediator
NFKB1	Nuclear factor κ β subunit 1	Transcription regulator of immunological proteins
PLAT	Plasminogen activator	Fibrinolytic system
PTGS2	Prostaglandin-endoperoxide synthase 2	Prostaglandin synthesis
SELE	Selectin E	Leukocyte adhesion cascade
SELP	Selectin P	Leukocyte adhesion cascade
TNFAIP3	Tumour necrosis factor α-induced protein 3	Inflammatory mediator

TABLE 16: Overview of prediction models no. 1, 2A and 2B. Parameters shown include: precision, root mean square error (RMSE), the standard error of precision (SEP), bias, correlation coefficient (r^2), explained variance in X and Y (Exp.var.X and Exp.var.Y), genes of interest, histological data (neutrophils and haemorrhage in the dermis, subcutaneous fat tissue and muscle tissue; macrophages in the subcutaneous fat tissue and muscle tissue; and necrotic muscle fibres in the underlying muscle tissue), experimental bruise age, number and body weight (BW) of pigs. All experimental bruises were inflicted using a plastic tube or an iron bar with a force of 6.5 N/mm^2 (settings 1, 4 and 5) (VII, VIII).

	Prediction model no. 1 (VII)	Prediction model no. 2A (VIII)	Prediction model no. 2B (VIII)
Precision	±2.12 h	±2.04 h	±1.84 h
RMSE	1.06	1.02	0.92
SEP	1.11	1.03	0.93
Bias	0.09	-0.05	0.002
r^2	0.87	0.82	0.86
Exp.var.Y	90%	83%	87%
Exp.var.X	78%	42%	83%
Genes	APOA1, CCL2, CFD, FOS, ICAM-1, IFNA1, IL6, NFKB1, PLAT, PTGS2, SELE, SELP, TNFAIP3	APOA1, CCL2, CFD, FOS, ICAM-1, IFNA1, IL6, NFKB1, PLAT, PTGS2, SELE, SELP, TNFAIP3	IL6, NFKB1, SELE, SELP
Histological data	No	Yes	No
Bruise age	1–10 h	2–8 h	2–8 h
No. of pigs	10	12	12
BW	30 kg	100 kg	100 kg

APOA1 = Apolipoprotein A1; CCL2 = C-C motif chemokine ligand 2; CFD = Complement factor D; FOS = c-Fos proto-oncogene; ICAM-1 = Intercellular adhesion molecule 1; IFNA1 = Interferon α 1; IL6 = Interleukin 6; NFKB1 = Nuclear factor κ β subunit 1; PLAT = Plasminogen activator; PTGS2 = Prostaglandin-endoperoxide synthase 2; SELE = Selectin E; SELP = Selectin P; TNFAIP3 = Tumour necrosis factor α-induced protein 3.

TABLE 17: The mean expression of genes relative to mean expression in control tissue scaled to one is presented according to the ages of bruises and the body weight (BW) of the pigs (VII, VIII).

		Control		1–3 h		4–6 h		7–10 h	
Gene symbol	BW	RE	SEM	RE	SEM	RE	SEM	RE	SEM
APOA1	30	1.00	0.04	0.86	0.06	0.64	0.08	0.43	0.02
	100	1.00	0.13	0.61	0.07	0.51	0.10	0.37	0.03
CCL2	30	1.00	0.00	21.26	2.50	17.80	3.97	9.81	2.11
	100	1.00	0.18	7.59	0.89	6.66	1.56	2.81	0.54
CFD	30	1.00	0.02	0.85	0.11	0.50	0.02	0.40	0.03
	100	1.00	0.08	0.51	0.04	0.39	0.03	0.32	0.03
FOS	30	1.00	0.37	10.94	6.68	1.08	0.08	0.61	0.10
	100	1.00	0.39	3.73	0.49	1.85	0.48	1.08	0.18
ICAM-1	30	1.00	0.06	8.71	2.38	1.67	0.27	1.13	0.10
	100	1.00	0.16	3.72	0.70	0.85	0.17	0.60	0.07
IFNA1	30	1.00	0.01	0.46	0.01	0.32	0.01	0.23	0.02
	100	1.00	0.13	0.43	0.08	0.34	0.06	0.24	0.04
IL6	30	1.00	0.31	45.40	16.44	12.67	1.76	5.50	1.21
	100	1.00	0.46	6.12	0.69	1.78	0.44	0.61	0.08
NFKB1	30	1.00	0.07	1.49	0.19	0.97	0.04	0.77	0.00
	100	1.00	0.07	1.90	0.10	0.93	0.10	0.71	0.05
PLAT	30	1.00	0.12	1.47	0.07	0.60	0.06	0.58	0.07
	100	1.00	0.09	0.71	0.01	0.38	0.06	0.36	0.06
PTGS2	30	1.00	0.02	9.25	1.99	2.07	0.34	1.08	0.32
	100	1.00	0.08	4.78	0.27	2.77	0.43	1.67	0.17
SELE	30	1.00	0.34	76.59	52.36	7.31	4.39	2.79	2.01
	100	1.00	0.23	4.18	1.01	0.72	0.28	0.21	0.11
SELP	30	1.00	0.19	5.91	0.62	1.82	0.44	0.99	0.30
	100	1.00	0.12	3.68	0.71	1.10	0.12	0.53	0.19
TNFAIP3	30	1.00	0.02	1.37	0.28	0.77	0.04	0.50	0.04
	100	1.00	0.16	1.05	0.22	0.76	0.12	0.79	0.14

APOA1 = Apolipoprotein A1; CCL2 = C-C motif chemokine ligand 2; CFD = Complement factor D; FOS = c-Fos proto-oncogene; ICAM-1 = Intercellular adhesion molecule 1; IFNA1 = Interferon α 1; IL6 = Interleukin 6; NFKB1 = Nuclear factor κ β subunit 1; PLAT = Plasminogen activator; PTGS2 = Prostaglandin-endoperoxide synthase 2; SELE = Selectin E; SELP = Selectin P; TNFAIP3 = Tumour necrosis factor α-induced protein 3.

Other animal species and humans

Gross evaluation

Colour changes in the skin following bruising have been studied in humans, calves, lamb and chickens (29, 39, 66, 67, 70, 72, 74). Initially, bruises appeared red, then gradually changed to purple, green and yellow (29, 39, 66, 67, 70, 72, 74). Although the sequence of colours was more or less consistent, the timing of the changes differed across the experimental studies. As in humans, gross evaluation of bruises in animals is probably affected by the oxidative state of haemoglobin, the depth of the bruise, the skin's melanin pigmentation and the observer's ability to perceive colours (34–37). Moreover, in animals the thickness of the epidermis and type of fur coat may also affect the visibility of bruises (I, 18). Therefore, gross assessment is unsuitable for estimating the ages of bruises in animals and humans.

Reflectance spectroscopy

Spectrophotometric and colourimetric measurements showed that human bruises change colour over time (73, 75, 76). Bruises that were less than 2 days old were generally dark red and blue due to decreased reflectance of light in the green, yellow and orange wavelength. Bruises that were more than 2 days old gradually became more yellow as the reflectance of indigo and blue light wavelengths decreased (73, 75, 76). However, variations in timing were observed within and between studies due to differences in skin colour, bruise size and individual differences among human subjects (73, 75, 76).

Histochemistry and immunohistochemistry

The infiltration of neutrophils and macrophages has been evaluated in skin and underlying muscle tissue sampled from bruises in calves, lambs, mice and rats (Tables 18, 19). Variations were observed across studies for the infiltration of skin and muscle tissue by neutrophils and macrophages over time (Tables 18 and 19). These variations were probably produced by the different sampling intervals used in each study (Tables 18, 19). However, differences among species, tissues and the methods of inducing trauma may also influence the pattern of leukocyte infiltration over time. Thornton and Jolly (65), carried out a mathematical evaluation of the histological data from bruises in lambs using a Bayesian probability model. They found that the model was able to separate bruises aged 1 to 20 h from those aged 24 to 72 h (65).

The timing of regenerative changes has been studied in experimental bruises in rats (Table 20). In summary, fibroblasts were observed 1.5 days after bruising at the earliest (55). Centro- and multinucleated regenerating myotubes were observed at 3 to 14 days, after which the myotube nuclei gradually became marginated (54, 57, 58, 60). Moreover, in rats, double-direct and indirect immunofluorescence showed time-dependent changes occurring in the expression of specific proteins in macrophages, satellite cells and myofibroblasts within traumatised muscle tissue (Table 21).

TABLE 18: Timing of infiltration of neutrophils into the skin or underlying muscle tissue of experimental bruises in calves, lambs, mice and rats. The data shown include the species, tissue, study period, and timing of neutrophil appearance (earliest and increased).

Species	Tissue	Study period	Earliest appearance	Increased appearance
Calves and lamb (66)	Skin	4 h–2 d	NA	8–24 h
Mice (62)	Skin	1 h–10 d	1 h	8–72 h
Rats (57)	Muscle	3 h–14 d	3 h	NA
Rats (58)	Muscle	1–21 d	1 d	NA
Rats (59)	Muscle	3 h–14 d	3 h	12 h–1 d
Rats (60)	Muscle	6 h–21 d	6 h	NA

d = days; NA = not available.

TABLE 19: Timing of infiltration of macrophages into the skin or underlying muscle tissue of experimental bruises in calves, lambs, mice and rats. The data shown include the species, tissue, study period, and timing of macrophage appearance (earliest and increased). Moreover, the time when macrophages outnumbered neutrophils is included.

Species	Tissue	Study period	Earliest appearance	Increased appearance	Dominating cell type
Calves and lamb (66)	Skin	4 h–2 d	8 h	24–48 h	48 h
Mice (62)	Skin	1 h–10 d	3 h	8–144 h	144 h
Rats (57)	Muscle	3 h–14 d	3 h	1–3 d	NA
Rats (58)	Muscle	1–21 d	1 d	3 d	NA
Rats (59)	Muscle	3 h–14 d	NA	12 h–7 d	NA
Rats (60)	Muscle	6 h–21 d	NA	NA	NA

d = days; NA = not available.

TABLE 20: Histological evaluation of the timing of regenerative changes in muscle tissue sampled from experimental bruises in rats. The study periods and the timing of fibroblast/myofibroblasts, centro- and multinucleated myotubes and the margination of myotube nuclei are presented.

Study period	Fibroblast/ myofibroblast	Centro- and multi-nucleated myotubes	Margination of myotube nuclei
3 h–14 d (54)	3–14 d	3–14 d	NA
4 h–2 d (55)	1.5–2 d	NA	NA
3 h–14 d (57)	5–14 d	5 d	NA
1–21 d (58)	3–21 d	5–9 d	13–21 d
6 h–21 d (60)	NA	3 d	NA

d = days; NA = not available.

TABLE 21: Double direct or indirect immunofluorescence was used to study specific proteins expressed by macrophages, satellite cells and myofibroblasts in traumatised muscle tissue from rodents. Proteins, cell types, study periods and the timing of protein expression (earliest change, peak and decrease) are presented.

Protein	Cell type	Study period	Earliest change	Peak	Decrease
α7 nicotine acetylcholine receptor (57)	Macrophages	3 h–14 d	3 h	1–3 d	5–14 d
α7 nicotine acetylcholine receptor (57)	Myofibroblasts	3 h–14 d	3 d	5–10 d	14 d
Paired-box transcription factor 7 (58)	Satellite cells	1–21 d	3 d	5 d	7–21 d
Myoblast determination protein (58)	Satellite cells	1–21 d	3 d	5 d	7–21 d
Cannabinoid receptor type 2 (59)	Macrophages	3 h–14 d	NA	1 d	3–14 d
Matrix metalloproteinase 2 (60)	Macrophages	6 h–21 d	NA	24 h	1–21 d
Tissue inhibitor of metalloproteinase 2 (60)	Macrophages	6 h–21 d	NA	24 h	1–21 d

NA = not available; d = days.

Western blot and gene expression analysis

In experimental bruises inflicted on rats and mice, the expression of a number of proteins and the mRNA expression of several genes have been evaluated using western blotting analysis and qPCR, respectively. Time-dependent changes were observed in the concentrations of specific proteins and the mRNA expression levels of the corresponding genes (Tables 22 and 23). In the muscle tissue, the greatest time-dependent changes were observed in the quantities of protein and/or mRNA of matrix metalloproteinase 2, tissue inhibitor of metalloproteinase 2, paired-box transcription factor 7 and myoblast determination protein (58, 60). Expression data from these proteins/genes could likely be combined to determine the ages of bruises that are several days old. Moreover, mRNA expression of tissue type plasminogen activator in the skin could be used to estimate the ages of bruises that are a few hours old as expression peaked after 1 h and returned to normal after 8 h (Table 23) (62).

Other techniques

Other techniques, such as electric impedance spectroscopy and chemical analysis have also identified time-dependent changes that could potentially be used to assess the ages of bruises (61, 67–72). In the earliest animal models, chemical analyses were used to quantify haemoglobin and bilirubin in bruises (67–71). In cattle, the concentrations of iron, haemoglobin and bilirubin increased during the first 4 to 5 days and decreased to normal or near normal levels within 7 to 9 days after blunt trauma (67). Moreover, time-dependent colour changes were observed when bruised tissues from cattle and chickens were immersed in Fouché's reagent, reflecting the amount of bilirubin in the tissue (68, 70).

TABLE 22: Western blotting analysis was used to measure the amounts of specific proteins in traumatised muscle tissue from rodents. Proteins, study periods and the timing of protein appearance (earliest, peaks and return to normal levels) are presented.

Protein	Study period	Earliest change	Peak	Normal level
α7 nicotine acetylcholine receptor (57)	3 h–14 d	12 h	7 d	-
Paired-box transcription factor 7 (58)	1–21 d	1 d	5 d	21 d
Myoblast determination protein (58)	1–21 d	3 d	3–7 d	21 d
Cannabinoid receptor type 2 (59)	3 h–14 d	3 h	5–7 d	-

- = no return to normal levels during the study period; d = days; NA = not available.

TABLE 23: Quantitative polymerase chain reaction was used to measure the mRNA expression of specific genes in traumatised muscle tissue from rodents. Genes, study periods and the timing of expression (earliest, peaks and return to normal levels) are presented.

Gene	Study period	Earliest change	Peak	Normal level
Sodium-coupled neutral amino acid transporter 2 (53)	4–48 h	4 h	4–24 h	28–48 h
Skeletal troponin I (54)	0.5–36 h	0.5 h	-	-
Pumilio 2 (55)	4–48 h	8 h	-	44–48 h
Transforming growth factor-β activated kinase 1 binding protein 2 (55)	4–48 h	12 h	-	48 h
Gap junction protein gamma 1(55)	4–48 h	4 h	-	48 h
Nicotinic cholinergic receptor α 1(55)	4–48 h	16 h	44 h	-
Frizzled-related protein 5 (56)	4–48 h	4 h	-	-
Frizzled class receptor 4 (56)	4–48 h	8 h	40 h	16–32 h, 44–48 h
Fos-like antigen 1(56)	4–48 h	4 h	12 h	28–44 h
α7 nicotine acetylcholine receptor (57)	3 h–14 d	6 h	7 d	-
Paired-box transcription factor 7 (58)	1–21 d	3 d	3–7 d	9–21 d
Myoblast determination protein (58)	1–21 d	1 d	3 d	9–21 d
Cannabinoid receptor type 2 (59)	3 h–14 d	3 h	7 d	-
Matrix metalloproteinase 2 (60)	6 h–21 d	3 d	3 d	14–21 d
Tissue inhibitor of metalloproteinase 2 (60)	6 h–21 d	6 h	3 d	10 d
Tissue-type plasminogen activator (62)	1 h–10 d	1 h	1 h	8–24 h

- = no peak or no return to normal levels during study period; d = days; NA = not available.

Age and sex of the animal

Pigs

Experimental bruises have been inflicted on pigs with BWs varying from 23 to 42 kg (mean = 30 kg) and from 91 to 115 kg (mean = 100 kg) (III, IV, VII–IX). The 30 kg pigs were approximately 3 months old, whereas the 100 kg pigs were approximately 5 to 6 months old. Gross tissue evaluations showed that haemorrhages in the muscle tissue underlying the area of impact occurred more frequently in pigs with a mean BW of 30 kg than in those with a mean BW of 100 kg (III, VIII). Moreover, the tramline pattern of bruises was more often visible from the skin surface in the smaller than in the larger pigs (III, VIII). These differences could be due to changes in the volume and maturation of supporting tissue that overlies the blood vessels, the amount of fibrous tissue and differences in the density of blood vessels during growth (17).

In experimental bruises, time-dependent changes were observed in individually selected histological parameters in pigs with a BW of 30 kg but to lesser extent in pigs with a BW of 100 kg (III, VIII). However, the regulation of specific genes was apparently unaffected by the BW/age of the pigs (Table 17) (VII, VIII). However, in small pigs (BW of 30 kg), several genes showed higher fold change differences than in large pigs (BW of 100 kg) (Table 17) (VII, VIII). These differences could be due to age related alterations of the inflammatory gene expression response to trauma or to differences between small and large pigs in the thickness of the subcutaneous fat tissue (VIII, 92).

In pigs with a BW of 30 kg (setting 1) and 100 kg (settings 4 and 5) bruises had a mean length of 5.8 and 7.5 cm, respectively (III, VIII). This indicates that the area of impact was generally bigger in the pigs weighing 100 kg compared to the pigs weighing 30 kg. When force and energy are distributed over a larger area, less tissue damage will occur (18). Therefore, the differences in the size of the area struck by the plastic tube or iron bar might also have contributed to the differences observed between small and large pigs.

All experiments were carried out in female pigs and it has not been investigated whether sex affects the inflammatory response in porcine bruises (Table 5). However, in mini pigs, the expression levels of some acute phase proteins varied between males and females after sexual maturation (93). Before sexual maturation, no differences were observed between the sexes (93). Slaughter pigs are slaughtered when they are approximately 6 months old, just before reaching sexual maturity at 6 to 8 months old. Because the majority of bruises are inflicted on slaughter pigs, the immune response is probably not significantly affected by the sex of the pigs.

Other animal species and humans

Experimental animal models of bruises have been developed in young and mature animals, in male and females, and in rats, mice, cows, sheep, rabbits and chickens (Tables 6 to 9) (II). No differences among species were observed in gross changes and in the timing of the formation of bilirubin occurring in experimental bruises in cattle, hogs, sheep and rabbits (66, 69). Moreover, in calves and lambs, no differences due to sex or castration were found (66). In addition the timing of histological changes was similar in calves and lambs (66).

The quantity of damaged tissue was greater in older rabbits (5 to 8 months) compared to young rabbits (2 to 5 months) after bruises were inflicted (69). In addition, degradation of haemoglobin was detected earlier in bruises in young rabbits compared to bruises in older rabbits (69). Similar results were obtained for experimental bruises in chickens and these were explained as age-dependent differences in metabolic activity (69, 71).

In humans, experimental bruises have been inflicted on men and women aged from 18 to 55 years (Table 10). More severe haemorrhages were observed in bruises inflicted on men aged from 30 to 50 years old than in those aged from 20 to 30 years old (73). The bruise colour results obtained by colourimetry did not differ between the sexes (76).

Anatomical location of bruises

Pigs

The thickness of the subcutaneous fat and muscle tissue and the presence of bone underlying the area of impact both influence the amount of tissue damage and the intensity of the inflammatory response (VIII, IX, 6, 7). The Norwegian research group inflicted bruises on the shoulders and hips of pigs using a pendulum (Method A) and found that more extensive haemorrhaging occurred at sites with a thin muscular layer (< 3 cm) over underlying bone compared to sites with a thick (> 3 cm) muscular layer (6, 7). In addition, bruises inflicted by paint balls (Method B) in areas with a thin muscle layer (e.g., the abdomen) had a different reflectance spectrum from bruises inflicted on areas with a thick layer of muscle tissue and underlying bone (e.g., the shoulder and hip regions) (6).

The Danish research group inflicted four bruises on the back of each pig (III, IV, VII, VII–IX). The first bruise was always inflicted caudal to the scapula and the subsequent bruises were inflicted along the back approximately 6 to 7 cm distal to the most recent bruise (III, VIII). The subcutaneous tissue underlying the areas of impact was significantly thinner in areas no. 3 and 4 compared to area no. 1 in pigs with a mean BW of 100 kg (Fig. 19) (VIII). Histologically, the amount of haemorrhage, necrosis and infiltration of leukocytes in the muscle tissue were increased in bruises inflicted on areas no. 3 and 4 compared to areas no. 1 and 2 (Figs. 31, 32) (VIII). Histological and gene expression data from bruises inflicted on pigs with a mean BW of 100 kg (settings 4 and 5) were combined in a PCA (VIII). The score plot in Fig. 31 shows that bruises inflicted in areas of impact no. 3 and 4 tended to group in the upper and lower left quadrants along the negative axis of principal component number (PC#)2 (horizontal axis). Fig. 32 shows that bruises located along the negative axis of PC#2 are characterised by high scores for most of the histological parameters. Moreover, many of the gene expression variables did not contribute to the grouping according to anatomical location as these were close to zero along PC#2 in the PCA loading plot (Fig. 32). These differences were probably due to the protection afforded by a thicker layer of subcutaneous fat overlying the muscle tissue in areas no. 1 and 2. However, the possibility that vasodilation and the release of inflammatory mediators after the first blunt trauma might have intensified the inflammation and tissue damage in the subsequent bruises cannot be excluded (VIII).

The mRNA expression of troponin C1, troponin T1, troponin I1 and c-Fos proto-oncogene (FOS) in muscle tissue differed among the four areas of impact in pigs with a mean BW of 30 kg (VII). Troponins, which are involved in the contraction of skeletal muscle, were significantly upregulated in the first bruise but not in subsequent bruises (Fig. 33) (VII). Therefore, the first blunt trauma may have saturated upstream receptors or transcription factors and exhausted the capacity of the muscle tissue to express troponins

(VII). However, this would not explain the expression of FOS, which increased as the number of bruises increased (Fig. 33) (VII). The mRNA expression patterns were probably not attributable to area specific variations in myofibres as no variations were observed among the same four areas in control pigs (VII). Although we did not investigate this, the thickness of the subcutaneous tissue may have varied at the different areas of impact, as it did in pigs with a mean BW of 100 kg. If so, this might have influenced mRNA expression patterns in muscle tissue. However, when evaluating the mRNA expression of the 42 and 38 other genes in the skin and muscle tissue, respectively, no differences were found among the four areas of impact in individual pigs (Appendix Tables I and II) (VII).

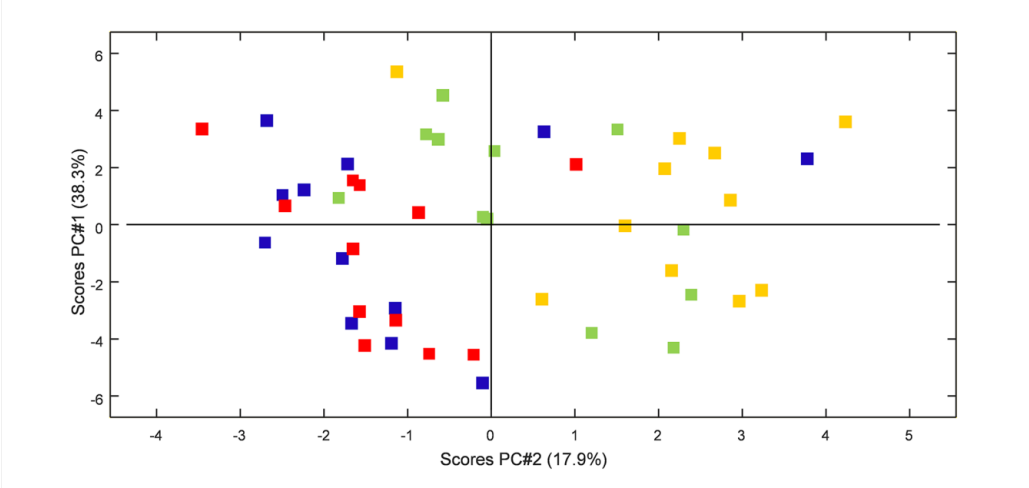

Fig. 31: Principal component analysis scores based on histological evaluations of neutrophils, macrophages, haemorrhage and necrotic myofibres in bruised skin and muscle tissue and the mRNA expression of 13 genes (apolipoprotein A1, C-C motif chemokine ligand 2, complement factor D, c-Fos proto-oncogene, intercellular adhesion molecule 1, interferon α 1, interleukin 6, nuclear factor κ β subunit 1, plasminogen activator, prostaglandin-endoperoxide synthase 2, selectin E, selectin P, tumour necrosis factor α-induced protein 3) in the subcutaneous fat tissue. Bruises were inflicted on areas no. 1 (yellow), no. 2 (green), no. 3 (blue) and no. 4 (red) on the backs of pigs with a mean body weight of 100 kg (settings 4 and 5) (Fig. 19). The percentages in parentheses denote variations in the data explained by principal components 1 and 2 (PC#1 and PC#2). Bruises inflicted on areas no. 3 and 4 were mostly located in the upper and lower left quadrants (VIII).

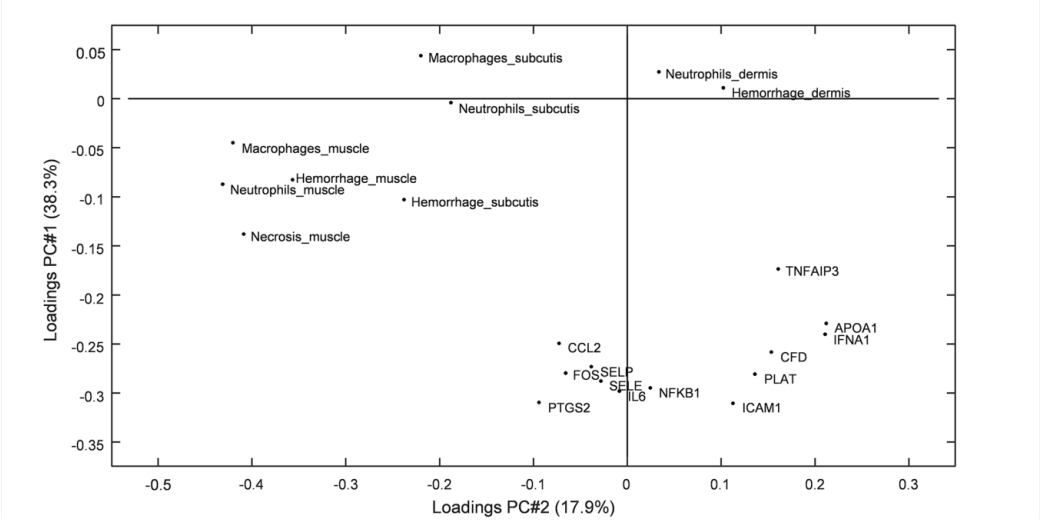

Fig. 32: Loadings from the principal component analysis (Fig. 31). The percentages in parentheses denote variations in the data explained by principal components 1 and 2 (PC#1 and PC#2). Neutrophils, macrophages, haemorrhage and necrosis in the muscle tissue were more pronounced in bruises inflicted on areas no. 3 and 4 compared to areas no. 1 and 2 (VIII).

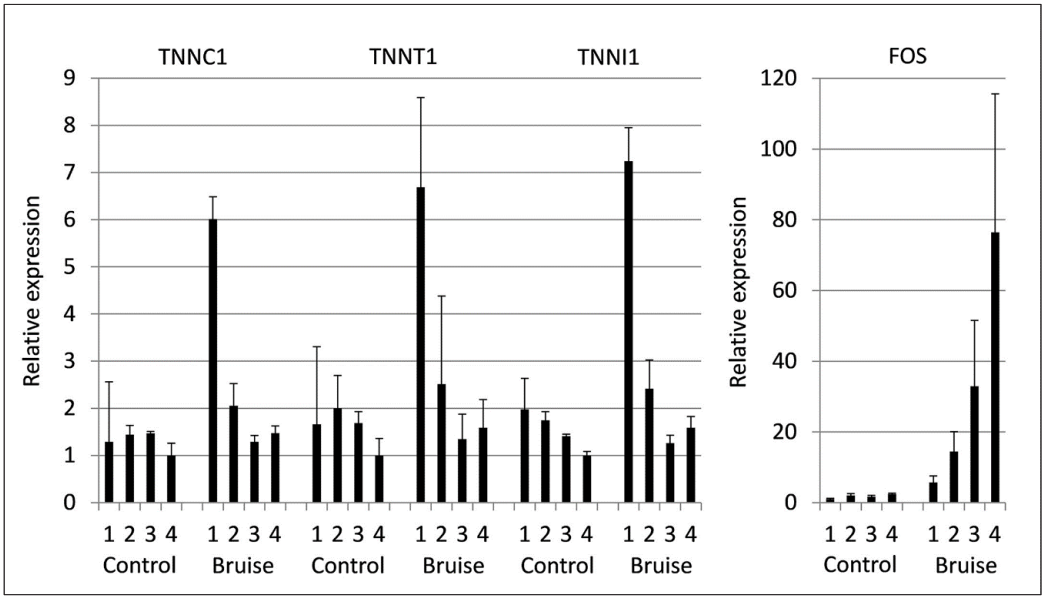

Fig. 33: The relative expression of troponin C1 (TNNC1), troponin T1 (TNNT1), troponin I1 (TNNI1) and c-Fos proto-oncogene (FOS) in muscle tissue. Muscle tissue was sampled from four bruises in areas no. 1 to 4 (Fig. 19) on 18 experimental pigs with a mean body weight of 30 kg. Uninjured muscle tissue was sampled from two control pigs. The expression of each gene in each area was calculated relative to the expression in area no. 4 in the control pigs scaled to 1 (VII).

Other species

The anatomical location of a bruise can influence its colour and severity (69, 71–73). In rabbits, cows and chickens, more severe haemorrhages are observed in bruises inflicted close to the bones (69, 71). Moreover, in chickens, histopathological changes are more severe in bruises inflicted on the thighs compared to the breast and wings (72). In humans, more severe haemorrhages are observed in bruises on the upper arm compared to the forearm (73).

The influence of one or two previous bruises on the healing of a second or third bruise has been evaluated in rabbits and chickens (69, 71). In two studies, 1, 2 or 3 bruises were inflicted 2 to 3 days apart and evaluated grossly and also by quantifying bilirubin (69, 71). In both species, the presence of bilirubin was detected earlier in bruises inflicted subsequent to initial bruises compared to a single isolated bruise (69, 71). In addition, a gross evaluation of subsequently inflicted bruises showed these healed faster than single isolated bruises (69, 71). The accelerated healing and degradation of haemoglobin into bilirubin may indicate that the first blunt trauma induce an upregulation of inflammatory cell mediators that accelerate the processes of inflammation and regeneration in subsequent bruises. The anatomical locations of each bruise was not reported but might also have influenced the rate of healing.

Impact characteristics

Bruises are caused by blunt trauma and inflicted by objects without sharp edges that cause non-penetrating injuries when they strike the skin (18). The severity and depth of lesions depend on a combination of the force, impact time, surface area, kinetic energy, speed and mass of the impacting object (IV, VII, VII–IX, 6). According to Newton's second law, the total force (F) on an object is equal to the mass (m) multiplied by the acceleration (a) of the object (94):

1) $F = m \times a$

Moreover, the kinetic energy (E) is defined as the energy that an object needs to accelerate from a resting position to a given velocity (V), assuming the object is not rotating (94):

2) $E = ½ \times m \times V^2$

The kinetic energy of an object attached to a rotating centre, i.e., the method applied by the Danish research group, can be calculated based on the angular velocity (ω) and mass of the object (94):

3) $E= ⅓ \times m \times \omega^2$

Different objects delivering the same amount of energy and force do not necessarily inflict the same amount of tissue damage (VII, IX, 6, 7). The severity of an injury also depends on the weight and velocity of the object and the surface area over which the force and energy is delivered (VIII, 6, 7, 17). Generally, the smaller the area over which the kinetic energy is transferred, the more tissue is damaged (17, 18).

In the experimental situation, the animal is static and the severity of the bruise depends on the impacting object's capacity to displace tissue, the impact time and the plasticity of the tissue (18). The higher the plasticity of the tissue, the less damage is induced (18). The plasticity of bone is much lower than that of skin and muscle tissue. This is why more severe injuries occur when bone underlies the site of impact (6, 7, 69, 71). Moreover, a long impact time allows the tissue to adapt compared to when impact occurs within a fraction of a second.

In reality, pigs are not static when being beaten by humans. Relative movement between the body and the object used for striking could theoretically affect the total kinetic energy and force involved. However, if the impacting object is the tip of a tube with a velocity of nearly 50 m/s, then the movement of the pig is several times slower than this and should not noticeably alter the force and kinetic energy parameters.

Pigs

Force and kinetic energy

Specifications of mass, impact speed, force and kinetic energy for the experimental settings are provided in Tables 11 and 12. The force applied by the Danish research group was up to 15 times greater than that applied by the Norwegian group. However, the energy/m^2 applied by the Norwegian research group was up to 15 times greater than the energy applied by the Danish research group. This can be explained by differences in the size of the area of impact. The energy from the paintball or pendulum was delivered to a smaller area compared to the energy from the tube (III, IV, VIII, 6–9).

The Danish research group compared porcine bruises inflicted using three different levels of force and found that grossly and histologically haemorrhage, necrosis and inflammation were most pronounced in bruises inflicted using the greatest level of force (settings 1 to 3) (IV). The number of neutrophils was scored on a semi-quantitative scale in HE stained tissue sections of subcutaneous fat from the bruises (Fig. 34) (IV). More neutrophils were observed in bruises inflicted with a high level of force compared to a medium or low level (Fig. 34) (IV). This was confirmed by immunohistochemical staining of neutrophils, monocytes and macrophages using monoclonal antibodies to MCA874G/MAC387 on selected tissue sections (Fig. 35) (IV). Immunostained tissue sections were scanned and evaluated using image analysis computer software able to count positively stained cells (Fig. 36). More immunostained cells were counted in bruises inflicted with a high level of force compared to a medium or low level (Fig. 37) (IV).

In addition, expression data from 42 genes expressed in the subcutaneous fat of bruises inflicted using high, medium and low levels of force (settings 1 to 3) were analysed using PCA (VII). The genes examined were involved in haemostasis, inflammation, tissue damage and repair and are listed in Appendix 1 Table I. The gene expression data were able to group bruises according to the force of impact (Fig. 38) (VII). These groupings were mainly defined by higher mRNA expression of IL1β, IL1 receptor antagonist, IL6, IL8, IL18, CCL2, complement factor B, haeme oxygenase 1, and transforming growth factor β in bruises inflicted with high compared to medium and low levels of force and control tissue (VII). The Danish research group varied the level of force used to inflict bruises by tightening the spring on the mechanical device (Fig. 18) (IV, VII). The more the spring was tightened, the higher the acceleration, impact speed, force and kinetic energy of the tube. Therefore, not only force but all of these parameters in combination probably explain the observations made by the Danish research group.

The Norwegian research group found that tissue damage generated by the pendulum was more extensive than that produced by the paintball although the force and kinetic energy applied was comparable in both methods (6).

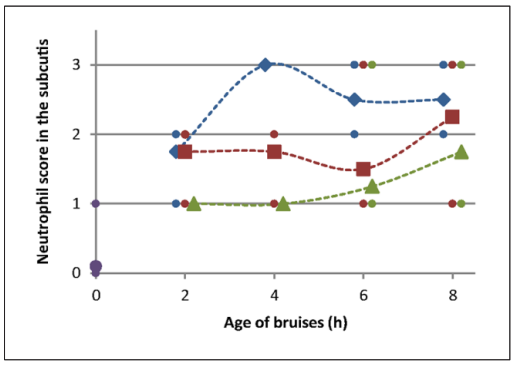

Fig. 34: Neutrophil score in the subcutaneous fat tissue in bruises inflicted with a force of 6.5 N/mm^2 (blue), 4.2 N/mm^2 (red) and 2.1 N/mm^2 (green).

Fig. 34 continued: At each force setting, four bruises were inflicted on 4 pigs weighing approximately 30 kg each (settings 1, 2 and 3). Neutrophils in the subcutaneous tissue were scored on a semi-quantitative scale: 0 = no neutrophils; 1 = 1–10 neutrophils; 2 = 11–30 neutrophils; 3 = >30 neutrophils. The scoring was carried out on HE stained tissue sections in a single high-power viewing field (400×) in the area with the highest cell density. The large connected symbols represent the mean scores from four bruises. The small dots represent the range among individual bruises. Control pig data are shown at 0 h (III).

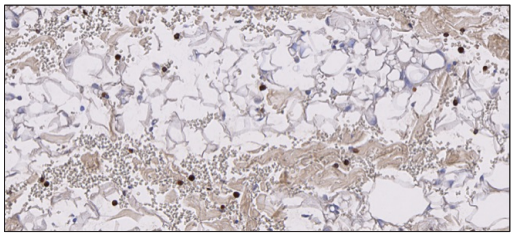

Fig. 35: Subcutaneous fat from a bruise in a pig with an approximate body weight of 30 kg. The tissue was immunostained against neutrophils, monocytes and macrophages (monoclonal antibodies to MCA874G/MAC387). Positive cells are stained dark brown (IV).

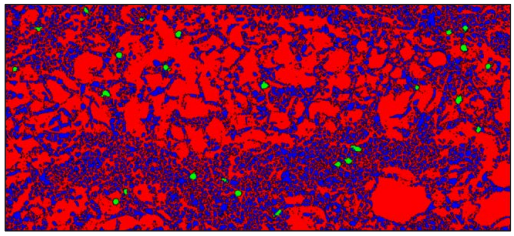

Fig. 36: Image analysis of the subcutaneous fat shown in Fig. 35 using Visiopharm Integrator System software. Immunostained cells are shown in green, whereas adipocytes, fibrous tissue and erythrocytes are shown in red and blue (IV).

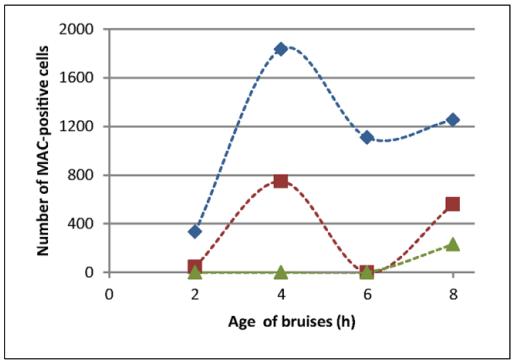

Fig. 37: Number of immunostained cells (Figs. 35 and 36) in the subcutaneous fat tissue in bruises inflicted with a force of 6.5 N/mm^2 (blue), 4.2 N/mm^2 (red) and 2.1 N/mm^2 (green). Cell counting was performed using Visiopharm digital image analysis software and immunostained tissue slides (monoclonal antibodies to MCA874G/MAC387). Each symbol represents a single bruise inflicted on area no. 1 (Fig. 19) (IV).

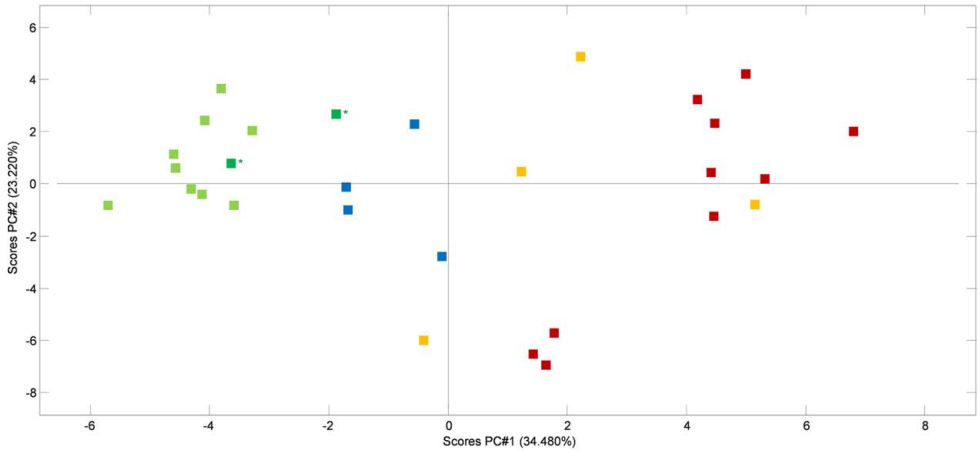

Fig. 38: Principal component analysis of gene expression data from 42 genes expressed in subcutaneous fat. Tissue was sampled from 18 experimental pigs and 2 control pigs: uninjured tissue from the thighs of 9 experimental pigs (light green), tissue from the backs of two uninjured control pigs (green*), tissue from bruises inflicted on 4 pigs with a force of 2.1 N/mm^2 (blue; setting 3), tissue from bruises inflicted on 4 pigs with a force of 4.2 N/mm^2 (yellow; setting 2), tissue from bruises inflicted on 10 pigs with a force of 6.5 N/mm^2 (red; setting 1). Each square represents the mean of four bruises on the back of a pig, with the exception of the light green squares that represent single samples of normal subcutaneous fat from the thighs of experimental pigs. The percentages in parentheses denote variations in the data explained by principal components 1 and 2 (PC#1 and PC#2) (VII).

Speed and mass of the impacting object

The Norwegian research group found that a paintball (low mass, high speed; Method B) caused haemorrhage in the dermis, whereas a weight attached to a pendulum (high mass, low speed; Method A) resulted in haemorrhage in the muscle tissue underlying the site of impact (6–9). Similarly, the Danish research group found that haemorrhage in the muscle tissue was more frequently observed in bruises inflicted using an iron bar (high mass, low speed; setting 5) than a plastic tube (low mass, high speed; setting 4) (VIII). Moreover, a tramline pattern was more frequently visible on the skin surface in bruises inflicted using a plastic tube (setting 4) (VIII). However, tissue cross-sections showed that two areas of haemorrhage were present in the subcutaneous tissue regardless of the type of object used to inflict the bruise (VIII). Histologically, the odds of a haemorrhage in the muscle tissue were five times greater when a bruise was inflicted using an iron bar compared to a plastic tube (VIII, IX). In addition, the odds of significant neutrophil

infiltration in the dermis were five times greater when a bruise was inflicted using a plastic tube compared to an iron bar (VIII, IX). Besides speed and mass the plasticity of the objects probably also affected the depth of the injuries. The plasticity of the plastic tube was much higher than that of the iron bar. When the plastic tube hit the skin, the deformation of the tube probably increased the contact area between the tube and the pig. However, the iron bar would have deformed much less and energy would have been transferred to the pig over a smaller impact area, penetrating deeper into the tissue.

Other factors affecting the amount of tissue damage

Clearly, the presence of gross haemorrhage in the skin and muscle tissue underlying the site of impact depends on the mass and speed of the object used for striking (VIII, IX, 6–9). However, the shape of the impacting object and the size of the area of impact also affect the amount of tissue damage inflicted. The published photographs shows that haemorrhaging in the muscle in bruises inflicted using a weight attached to a pendulum was more extensive than in those inflicted using a tube (VII, VIII, 6). This difference was probably due to differences in the size of the impact area and the mass of the impacting object. The weight attached to the pendulum had a higher mass and a smaller surface area compared to the tubes (Tables 11, 12). Moreover, attaching square-shaped weights to the pendulum created more haemorrhaging in muscle tissue than did attaching hemispherical weights (6).

Other animal species

Studies on cattle, rabbits, and chickens have shown that the extent and severity of bruises is directly affected by the force, speed and mass of the impacting objects (69, 71). In cattle and rabbits, objects with a high mass and low speed caused minimal injury (69). Moreover, objects with a low mass and high speed inflicted small, discrete but severe bruises (69). When both the mass and speed of the impacting object were increased the area and the depth of tissue damage increased (69).

Sampling site within bruises

Pigs

Bruises inflicted by paint balls and tubes had white skin in the centre surrounded by either a circular line (paint ball) or two parallel lines of haemorrhage (tube) (III, IV, VII, VIII, 6–9). In bruises inflicted by paint balls, the level of haemoglobin oxygenation, calculated from reflectance spectra, between the central region and the edge of each bruise varied (6). Histologically, the inflammatory response in the white central region was absent or far less pronounced than in the haemorrhagic areas in bruises inflicted by paint balls or tubes (III, 6, 7). In addition, the Danish research group found that the intensity of the inflammatory response varied across the bruise and was most pronounced at the site where the plastic tube or iron bar first struck the skin, i.e., the optimal site for sampling (Figs. 39–41) (VIII, IX). Skin and muscle tissue were sampled from the centre (B) and the dorsal (A) and ventral (C) ends of experimental bruises and evaluated histologically (i.e., neutrophils and haemorrhage in the dermis; neutrophils, macrophages and haemorrhage in the subcutaneous fat; and neutrophils, macrophages, haemorrhage and necrosis in the muscle tissue) (Fig. 39). The histological data were subjected to a PCA and samples tended to group according to sample sites (Fig. 40) (VIII). Samples from the centre were mainly located in the upper quadrants and in the lower right quadrant of the PCA score plot (Fig. 40). On the PCA loading plot all histological variables were located in the upper or lower quadrants on the positive axis of PC#1, meaning that the samples from the centre were generally characterised by higher scores for the histological variables (Fig. 41). In contrast, samples from the ventral end (C) were mainly located in the lower left quadrant of the PCA score plot and, therefore, characterised by low scores for the histological variables (Figs. 40, 41). Because the pig's skin has a concave shape when struck, most of the energy is transferred to the centre, and this is where most tissue damage occurs (VIII, IX). In forensic cases of pig bruises, the initial site of impact is often unknown and several tissue samples should be taken along the bruise to identify the location where the intensity of inflammation is greatest. Moreover, samples should include both skin and underlying muscle tissue so that deeper injuries are not overlooked.

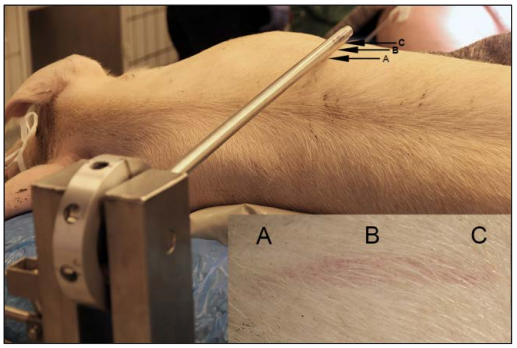

Fig. 39: Experimental bruises inflicted on pigs with a mean body weight of 100 kg (settings 4 and 5). Skin and muscle tissues were sampled from the centre (B), the dorsal end (A) and the ventral end (C) of the bruises (VIII).

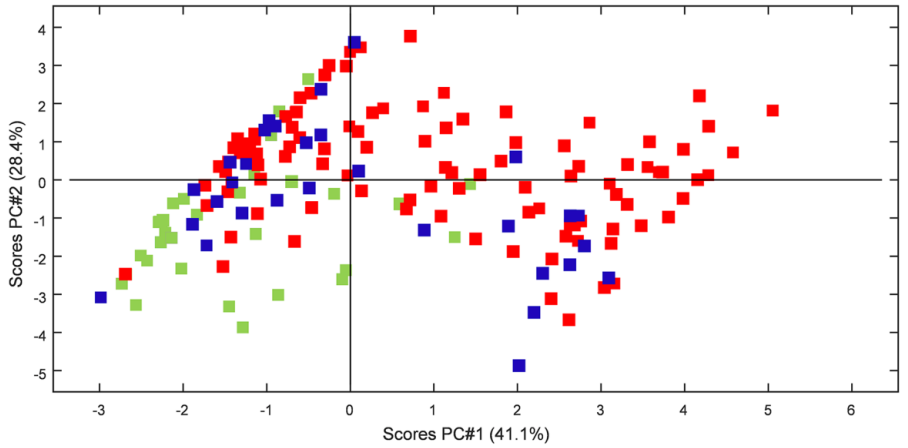

Fig. 40: Principal component analysis scores based on histological evaluations of bruised skin and muscle tissue sampled from the centre (B, red), the dorsal end (A, blue) and the ventral end (C, green) of the bruises. Bruises were inflicted on pigs with a mean body weight of 100 kg using a plastic tube or an iron bar (settings 4 and 5). The percentages in parentheses denote variations in the data explained by principal components 1 and 2 (PC#1 and PC#2). Samples from the dorsal end (A, blue) are scattered among all four quadrants; samples from the centre (B, red) are mainly located in the upper quadrants and in the lower right quadrant; and samples from the ventral end (C, green) are mainly located in the lower left quadrant (VIII).

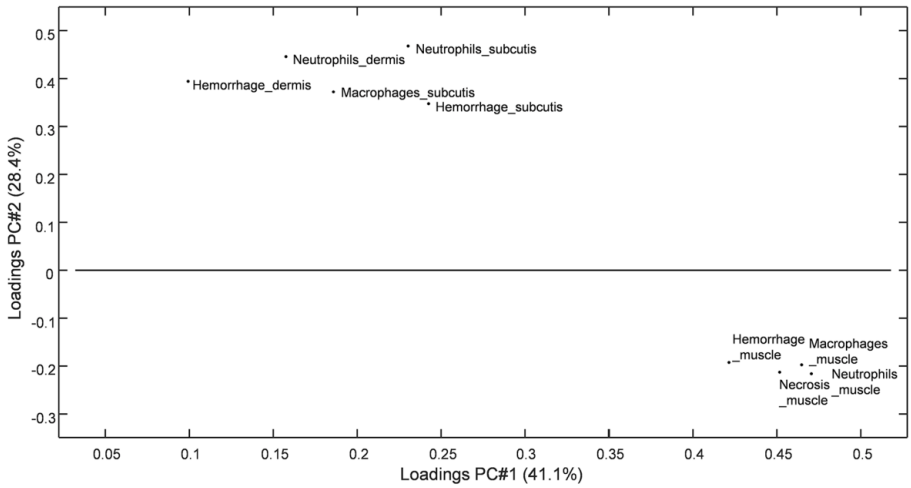

Fig. 41: Loadings from the principal component analysis (Fig. 40) based on histological data from sampling sites A, B and C and bruises inflicted using a plastic tube or an iron bar on pigs with a mean body weight of 100 kg (settings 4 and 5). The percentages in parentheses denote variations in the data explained by principal components 1 and 2 (PC#1 and PC#2). The histological variables are located in the upper and lower right quadrants, i.e., samples from the centre (B) are generally characterized by higher histological parameter scores compared to samples from the ventral end (C) (VIII).

Other animal species and humans

In humans, one third of the experimental bruises (37/104) inflicted by paint ball impacts were characterised by a central region of white skin surrounded by a circular haemorrhage (76). The central clear region affected colourimeter measurements and complicated bruise age estimation that were based on colour changes (76). The effect of sampling site has not been investigated in experimental bruise models that use other animal species.

Chapter 4

Vital reactions in bruises

To ascertain whether a bruise was inflicted AM or PM, its vitality must be determined (I, VI). However, the vitality of a lesion can be difficult to determine if the lesion was generated shortly before or after death (VI, 95–97). Active infiltration of leukocytes from outside an area of haemorrhage is regarded as a vital reaction and has been observed in experimental porcine wounds after 1 h (VI, 25). In porcine wounds generated in the agonal period (30 to 60 s prior to cardiac arrest) no vital reactions are observed (95). However, migration of leukocytes PM is a controversial subject. When blood circulation ceases, tissue survives for a variable period of time under ischaemia, called the supravital period (98). Leukocytes have reportedly survived and been motile for more than 12 h after cardiac arrest (17). Moreover, migration of leukocytes has been observed in rodents when chemotactic substances are injected subcutaneously after circulatory arrest (99, 100). However, when the same chemotactic substances were injected into pigs, there was no reaction (100). Moreover, in studies of porcine wounds generated within 5 min after circulatory arrest and monitored from 1 to 14 h PM, no cell infiltration was observed (VI, 101). By contrast, an inflammatory response was reportedly observed in porcine wounds generated 10 min PM (102). In six studies, blunt traumas were inflicted on mice, rats and pigs within minutes after cardiac arrest and until 12 h PM (Fig. 42, Table 24).

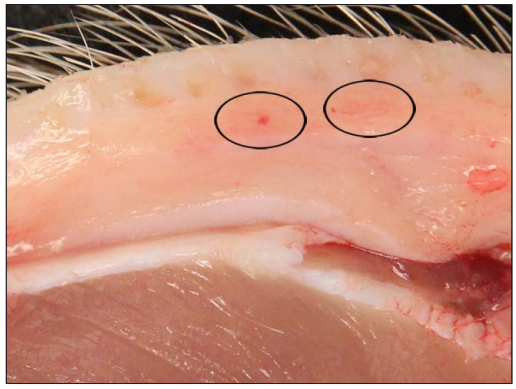

Fig. 42: Cross section of skin from a pig with a pseudo bruise inflicted post mortem. Indistinct haemorrhages (encircled) are visible in the subcutaneous tissue. The bruises were inflicted within 3 min after cardiac arrest (VI).

TABLE 24: Experimental pseudo bruises inflicted on rats, mice and pigs after cardiac arrest. The data shown include the year, species, sex, number of animals, post mortem (PM) infliction time (time from cardiac arrest to trauma), sampling time after injury and evaluation method.

Year	Species	Sex	No. of animals	PM infliction time	Sampling time after injury	Evaluation method
2010 (53)	Rats	M	24	0.5 h	6–24 h	Real-time qPCR
2010 (54)	Rats	M	9	0.5 h	0.5–6 h	Real-time qPCR
2011 (61)	Rats	NA	8	0.5 h	6–48 h	Electric impedance spectroscopy
2016 (60)	Rats	M	15	3–12 h	0.5 h	Real-time qPCR
2017 (VI)	Pigs	F	8	1–3 min	1–7 h	Histochemistry
2018 (64)	Mice	NA	3	0.5 h	0 h	Western blot, Real-time qPCR

M = male; F = female; NA = not available; qPCR = quantitative polymerase chain reaction.

Pigs

In eight pigs with BWs of 20 to 40 kg, four blunt traumas were inflicted within 1–3 min of cardiac arrest (VI). The pseudo bruises were inflicted on the back with an iron tube and settings similar to setting 5 in Table 11 using the mechanical device developed by the Danish research group (III, VI). The areas subjected to blunt trauma were sampled from 1 to 7 h PM (VI). Gross evaluations revealed indistinct red areas on the skin surface of five of the eight pigs (VI). Cross sections revealed minor haemorrhages in the subcutaneous tissue of three pigs (Fig. 42) (VI). However, compared to the bruises analysed in forensic cases and experimental bruises inflicted AM, the changes were vanishingly small (Fig. 42). Histologically, extravasated erythrocytes were found in 84% of the pseudo bruises but with no active infiltration of neutrophils (Fig. 43) (VI). Disrupted myofibres and lacerated tissue were observed in the underlying muscle tissue (Fig. 44) (VI). The disrupted myofibres appeared necrotic, i.e., they were fragmented and vacuolated and had lost their homogenous, striated appearance. However, none of them had been infiltrated by leukocytes (Fig. 44) (VI). Histologically, the changes inflicted PM were very similar to those observed in forensic cases of bruises believed to be inflicted AM in which leukocyte infiltration was absent (I, VI).

Chronic infiltration of lymphocytes and macrophages has been observed in the subcutaneous and muscle tissues of experimental and control pigs (Fig. 45) (III, VI).

When assessing the vitality of a bruise, the veterinary pathologist must be aware of these chronic cell infiltrations that occur incidentally in pig skin and muscle tissues (III, VI).

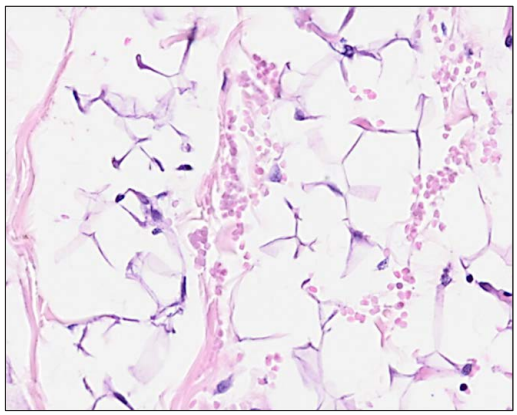

Fig. 43: Subcutaneous fat tissue sampled from an experimental pseudo bruise in a pig. The bruise was inflicted within 3 min after cardiac arrest. Extravasated erythrocytes are visible between adipocytes and along the fibrous tissue, HE.

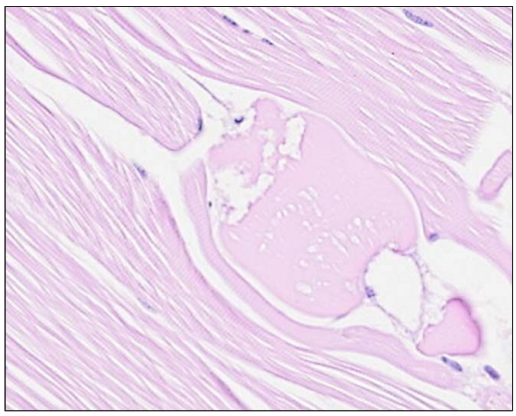

Fig. 44: Muscle tissue sampled from an experimental pseudo bruise in a pig. The bruise was inflicted within 3 min after cardiac arrest. A disrupted myofibre with a necrotic appearance is surrounded by normal myofibres, HE.

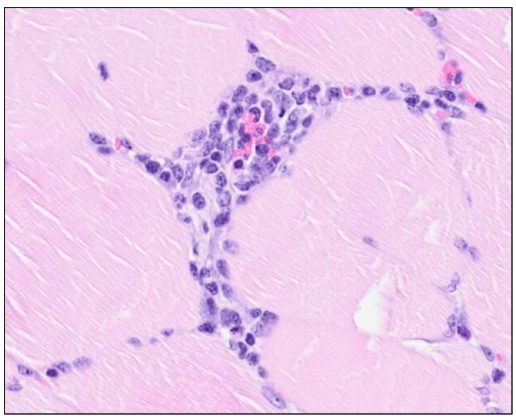

Fig. 45: Muscle tissue sampled from a normal pig. Infiltration of mononuclear cells in the interstitial tissue surrounded by normal myofibres is visible. The accumulation of cells is an incidental finding in pigs and is not caused by a recent, blunt trauma, HE.

Other animal species

The mRNA expression of a number of genes was assessed in pseudo bruises in rodents (Table 25). The mRNA expression of sodium-coupled neutral amino acid transporter 2, matrix metalloproteinase 2, C-X-C motif chemokine ligand 1 and C-X-C motif chemokine receptor 2 may be used to evaluate the vitality of bruises inflicted around the time of death and sampled in the following hours (53, 60, 64). For these genes, the mRNA levels measured in the earliest AM bruises (0.5 to 6 h old) and those measured in the PM injuries (inflicted at 0.5 h PM and sampled at 0.5 to 24 h) differed significantly (53, 60, 64). However, as the age of the AM bruises increased, an overlap between AM and PM injuries was observed in the levels of mRNA expression of sodium-coupled neutral amino acid transporter 2 (53). Moreover, as the PM interval increased (i.e., the time between death and sampling) mRNA encoding C-X-C motif chemokine ligand 1 and C-X-C motif chemokine receptor 2 were degraded and could not be used to differentiate between AM and PM injuries (64).

In one study, electric impedance spectroscopy was able to differentiate between bruises inflicted AM and tissue subjected to blunt trauma PM (61). The tissue composition in injuries inflicted PM was similar to control tissue but different from that in bruises inflicted AM (61).

TABLE 25: Level of mRNA expression (normal, increased, decreased) for different genes in bruises inflicted ante mortem (AM) and pseudo bruises inflicted post mortem (PM). The ages of the bruises inflicted AM and the times the PM bruises were inflicted are shown in parentheses.

Gene name	mRNA level in AM bruises (bruise age)	mRNA level in PM pseudo bruises (PM infliction time)
Sodium-coupled neutral amino acid transporter 2 (53)	Increased (4 h)	Normal (0.5 h PM)
Skeletal troponin I (54)	Decreased (0.5 h)	Decreased (0.5 h PM)
Matrix metalloproteinase 2 (60)	Normal (6 h)	Decreased (3, 6, 12 h PM)
Tissue inhibitor of metalloproteinase 2 (60)	Decreased (6 h)	Decreased (3, 6, 12 h PM)
C-X-C motif chemokine ligand 1 (64)	Increased (0.5 h)	Normal (0.5 h PM)
C-X-C motif chemokine receptor 2 (64)	Increased (0.5 h)	Normal (0.5 h PM)

Chapter 5

Application of experimental models in the evaluation of forensic cases

Models of experimental bruises have been developed in a number of animal species and human subjects (Tables 5–10) (II). Clearly, porcine models are the most comparable to forensic cases concerning pigs. In fact, the method by the Danish research group was developed to imitate forensic cases of porcine bruises as closely as possible (III, IV, VII–IX). An adult man with a plastic tube was able to strike with a mean force of 7.3 N/mm^2 (III). Therefore, experimental bruises inflicted with a force of 6.5 N/mm^2 were comparable to forensic cases (III, IV, VII–IX). The experimental bruises showed a tramline pattern that was identical to forensic cases of bruises in pigs that were beaten with sticks or similar objects (I, III, V, VIII, IX). Moreover, four bruises were inflicted on the back of each experimental pig to imitate multiple bruises observed in forensic cases (I, III, V, VIII–IX).

To assess the applicability of histological parameters and gene expression profiles for determining the age of forensic bruises in slaughter pigs, two studies were performed (V, X). The tissue evaluated in the two studies originated from the same pigs. However, when the histological evaluations were carried out, the prediction models based on mRNA expression levels had not yet been developed. Therefore, the results were presented in two separate papers, V and X.

Slaughter pigs and sampling procedure

In studies V and X, 5 to 6 month-old pigs with bruises inflicted by humans were identified during the routine meat inspection at two major Danish slaughterhouses (16). In total, 101 pigs were included, and two bruises were designated A and B on each of these pigs (Fig. 46) (V, X). Sampling of skin and muscle tissue was carried out by veterinarians at the slaughterhouses and the samples were fixed in 10% neutral buffered formalin (V). Simultaneously, subcutaneous fat tissue was sampled from bruises A and B and stabilised in RNAlater (X). Sampling was carried out within 6 h after slaughter. The skin and muscle tissue, including the remaining bruises, were excised, frozen and submitted to the University of Copenhagen together with the fixed and stabilised tissue samples (V, X).

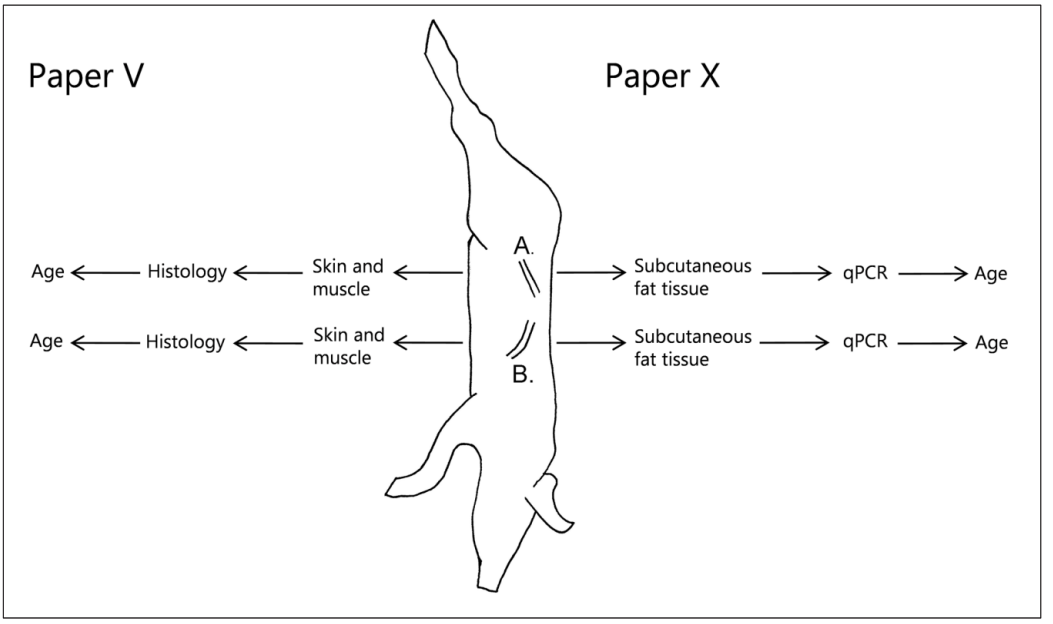

Fig. 46: Overview of sampling, evaluation and age assessment of bruises in slaughter pigs presented in papers V and X. The tissue evaluated in the two studies originated from 101 slaughter pigs. On each pig, two bruises were designated A and B, respectively. For paper V, skin and muscle tissues were sampled from bruises A and B for histological evaluation. The ages of bruises were determined based on the histological evaluations of neutrophils and macrophages. For paper X, subcutaneous fat tissue was sampled from bruises A and B. The level of mRNA expression from 13 selected genes was measured using high throughput real-time quantitative polymerase chain reaction and used to assess the age of the bruises. Modified from paper V.

Gross evaluation of forensic bruises on slaughter pigs

In all 101 pigs, the patterns and uniform appearance of the bruises were consistent with their being inflicted by humans (V). The exact anatomical locations of bruises A and B and the sampling sites within the bruises (e.g., ends or centre) could not be determined. Moreover, the true ages of the bruises were unknown. However, due to the uniform bruise patterns observed on each of the pigs, it was assumed that bruises A and B were inflicted minutes apart at most (V, X).

Histological evaluation of forensic bruises on slaughter pigs

Skin and muscle tissue sampled from the bruises (i.e., A and B) were available for histological evaluation in 81 of the 101 pigs (Fig. 47). The remaining 20 pigs were excluded due to missing samples or putrefaction of samples caused by using an insufficient volume of formalin (V). Fixed skin and muscle tissues were processed through ethanol and xylene and embedded in paraffin wax. Thereafter, 4 to 5 µm tissue sections were cut and stained with HE to evaluate the histological parameters shown in Table 26. The agreement between the histological parameters observed in bruises A and B was none to moderate (Cohen's kappa values: 0 to 0.58) (V). Moreover, the age of each bruise was determined as a time interval based on the infiltration of neutrophils and macrophages into the subcutaneous and underlying muscle tissue, respectively. The age of each bruise was determined using the results from experimental bruises in pigs with a mean BW of 30 kg (Table 14) (III). Bruises were assigned to one of four age categories: 1) inconclusive; 2) less than 4 h old; 3) 4 to 10 h old; 4) age interval overlapping 4 h. A detailed description of the method is presented in paper V. Similar age categories were obtained for 39 of 81 paired bruises (48%) (Table 27). Therefore, evaluation of only two samples using only two histological parameters is insufficient to determine the ages of bruises (V).

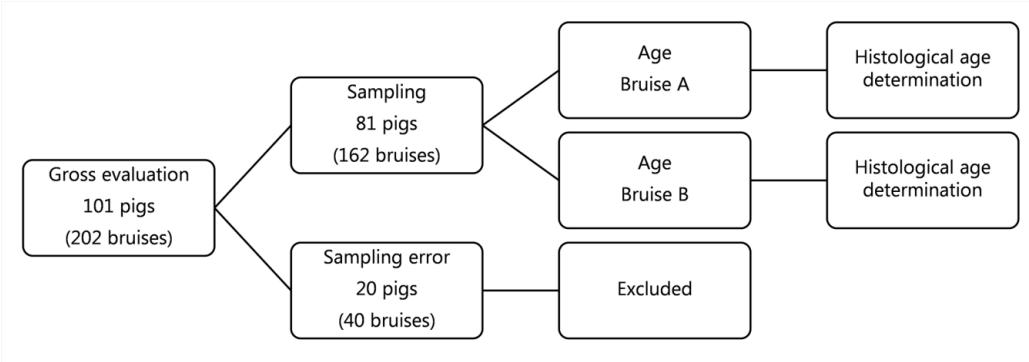

Fig. 47: The total number of pigs and bruises (A and B) subjected to a histological evaluation or excluded due to missing or decomposed samples (V).

TABLE 26: Histological parameters evaluated in the dermis, subcutaneous fat tissue and underlying muscle tissue in bruises A and B (V).

Dermis	Subcutaneous tissue	Muscle tissue
• Leukocytes (neutrophils and macrophages) • Hyper-leukocytosis • Haemorrhage	• Neutrophils • Macrophages • Hyper-leukocytosis • Haemorrhage	• Neutrophils • Macrophages • Hyper-leukocytosis • Necrosis • Interstitial or intracellular location of leukocytes • Haemorrhage

TABLE 27: Agreement and differences in the age estimates of bruises A and B on each of the 81 pigs based on histological evaluation of neutrophils and macrophages in the subcutaneous fat tissue and underlying muscle tissue, respectively (V).

		Bruise A			
		Inconclusive	< 4 h	> 4 h	Overlapping 4 h
Bruise B	Inconclusive	1	3	0	3
	< 4 h	1	20	3	12
	> 4 h	0	3	6	4
	Overlapping 4 h	3	4	6	12

mRNA expression signatures in forensic bruises on slaughter pigs

Subcutaneous fat tissue was sampled from the bruises (A and B) in 71 of the 101 pigs (142 bruises in total) (Fig. 48). The remaining 30 pigs were excluded because samples were missing or had putrified due to an insufficient volume of RNAlater (X). High throughput real-time qPCR was performed to measure the expression of 13 selected genes in subcutaneous fat from the bruises (Table 15). Reproducible mRNA expression data were obtained for both bruises (A and B) in 19 pigs (Fig. 48). Moreover, reproducible mRNA expression data were obtained for an additional 37 single bruises (A or B) in 37 pigs (Fig. 48). In total, reproducible expression data were obtained for 75 of 142 bruises. The remaining 67 bruises were excluded due to RNA degradation (Fig. 48) (X).

The expression data were combined with experimental expression data in a PLS analysis to estimate the ages of bruises (X). Two prediction models were applied. The first prediction model (no. 1) was based on 13 genes expressed in subcutaneous fat from experimental bruises in younger pigs (3 months old, BW = 30 kg; Table 16) (VII). The second prediction model (no. 2B) was based on 4 genes expressed in subcutaneous fat from experimental bruises in large pigs (5–6 months old, BW = 100 kg; Table 16) (VIII). For a detailed description of the method, the reader is referred to paper X.

Prediction model no. 1 was unsuitable for determining the ages of bruises because it calculated ages of ≤ 0 h for 40% of the bruises (Table 28). In contrast, prediction model no. 2B calculated ages of ≤ 0 h for only 5.3% of the bruises (Table 28). The negative age estimates generated by prediction model no. 1 may reflect age-related alterations in the innate immune response between experimental pigs (3 months old) and slaughter pigs (5 to 6 months old). Negative age estimates could also be generated for bruises that were inflicted with greater force than the experimental bruises (used as reference data for prediction models). However, the experimental bruises were all inflicted with a force

comparable to a man striking; therefore, this is a less likely explanation for the negative age estimates (X).

When age estimates were made using mRNA expression data for the genes SELE, SELP, IL6 and NFKB1 (prediction model no. 2B), the differences in age between bruises A and B were less than 4 h for more than 90% of the paired bruises. Moreover, the mean difference in age between paired bruises (A and B) was 1.5 h (Table 28) (X).

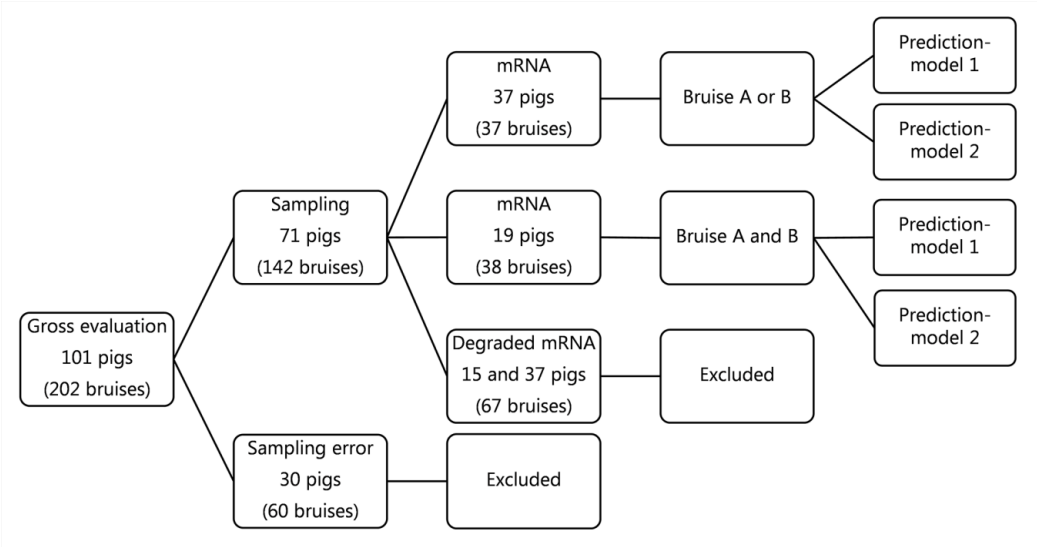

Fig. 48: Subcutaneous fat tissue was successfully sampled from 71 of 101 pigs and stabilized in RNAlater. Reproducible mRNA expression data were obtained from 75 of 142 bruises. Among these 75 bruises, 38 were paired (bruises A and B from 19 pigs) and 37 were single bruises (A or B from 37 pigs). The remaining 67 bruises were excluded due to degradation of mRNA (X).

TABLE 28: The mean predicted ages of bruises (n = 75), the minimum (min) to maximum (max) predicted ages of bruises, and the number of bruises predicted as ≤ 0 h old based on prediction models no. 1 and 2B are shown. The mean difference in predicted age between paired bruises (A and B) on the same pig is also presented (X).

	Prediction model no. 1	Prediction model no. 2B
Mean age	0.9 h	2.7 h
Min to max	–4 to 9.5 h	–1.7 to 6.3 h
Bruises ≤ 0 h	30 (40.0%)	4 (5.3%)
Mean age difference between bruise A and B (min to max)	2.3 h (0 to 4 h)	1.5 h (0 to 4.3 h)

Quality and degradation of mRNA

It is important to know the time interval between death and sampling because a decrease in mRNA caused by degradation could be misinterpreted when determining the ages of bruises. In mice and rats, the mRNA level for specific genes decreased as the time interval between death and sampling increased (54, 60, 64).

In forensic cases of porcine bruises, pieces of skin and underlying muscle tissue are typically stored at –18°C for up to several months prior to forensic investigation (I, X). Therefore, an additional 40 slaughter pigs from forensic cases were included in paper X to evaluate whether the current procedure was suitable for implementing gene expression signatures. Skin with bruises inflicted by humans had been excised and frozen for up to several months prior to sampling and stabilisation of subcutaneous fat in RNAlater (X). In more than 90% of these bruises, the mRNA was too degraded to be quantified. Therefore, to determine the ages of bruises using mRNA expression data, sampling must be done prior to freezing the tissue (IX).

Gene expression profiles can help to generate age estimates for bruises inflicted by humans on slaughter pigs (VII, VIII, X). However, if mRNA expression signatures are to be implemented in the forensic investigation of porcine bruises, subcutaneous fat tissue must be sampled and stabilised in RNA stabilising solution within a few hours of slaughter (X). Reproducible expression data were obtained from only half of the bruises (75 of 142) sampled within 6 h of slaughter and before freezing (Fig. 48) (X). The RNA was partially degraded and the RNA integrity numbers (RIN) were between 4.9 and 8.3 (X). In contrast, highly reproducible gene expression data were obtained in 98% of experimental bruises sampled within 1 h PM; in these cases, RIN were between 6.7 and 9.2 (VIII). A comprehensive training programme for veterinarians handling forensic cases could increase the number of samples with high-quality mRNA. Sampling and stabilisation of tissue in RNA stabilising solution, e.g., RNAlater, must be done using clean instruments and gloves to protect the tissue from ribonucleases on the skin and used instruments. In addition, samples should not be more than 0.5 mm thick to ensure the RNA stabilising solution can penetrate the tissue completely (X).

Evaluation and age determination of forensic bruises on pigs

Gene expression signatures cannot distinguish between bruises inflicted by humans and accidental bruises, e.g., inflicted by other pigs (X). Before determining the ages of bruises, gross evaluation of the skin is crucial to determine whether bruises were inflicted by humans (Fig. 49). Using mRNA expression signatures of selected genes

together with histological evaluation of tissue will improve bruise age estimates (Fig. 49) (X). Prediction model no. 2B was based on the mRNA expression levels of IL6, NFKB1, SELE and SELP in subcutaneous fat from experimental bruises in pigs with a mean BW of 100 kg (VIII). All four genes were upregulated 2 h after bruises were inflicted and downregulated to, or below, normal levels within 5 to 8 h (Table 17) (VIII, X). In some forensic cases, bruises may be inflicted more than 8 h prior to slaughter, and this could result in false predictions. Therefore, bruise age estimates should never rely solely on gene expression patterns (X). Histological evaluation of the bruised tissue is extremely important as it ensures that tissue morphology and the inflammatory response is consistent (i.e., acute inflammation and no regenerative changes) with age estimates based on mRNA expression patterns (X).

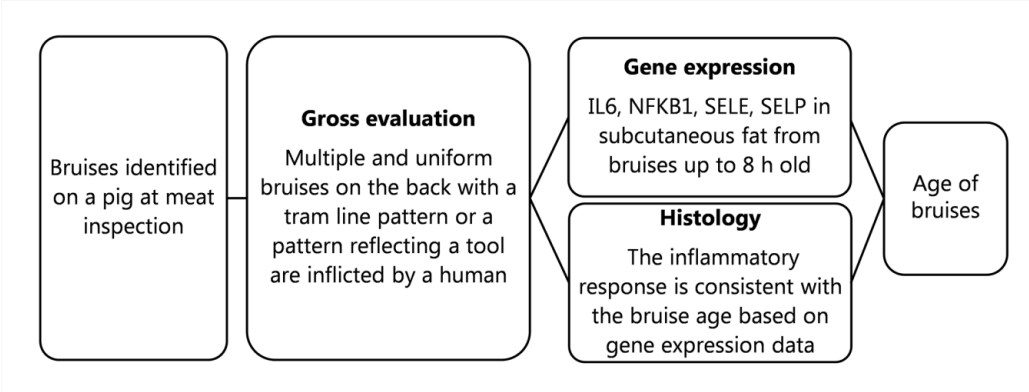

Fig. 49: Handling of forensic cases. All bruises should be subjected to a gross evaluation to determine whether they were probably inflicted by humans. Combining the mRNA expression data from the interleukin 6 (IL6), nuclear factor κ β subunit 1 (NFKB1), selectin E (SELE) and selectin P (SELP) genes provides an estimate of the ages of bruises that are up to 8 h old using partial least squares regression analysis. However, the timing of bruises should never rely solely on gene expression data. Histological evaluation of the bruised tissue is crucial to ensure that the inflammatory response at the cellular level is consistent with the bruise age based on the mRNA expression pattern (X).

Chapter 6

Discussion

In Denmark, porcine bruises inflicted by humans have regularly been the subject of public and political attention, and several scientific studies have been published (I–X, 5, 12–14, 16). Studies from other countries have also investigated bruises in pigs; however, the majority of these lesions were not inflicted by humans and the studies focused on risk factors during transportation and not on the age of the bruises (103, 104).

Bruises in Danish pigs are typically detected during the PM meat inspection at slaughterhouses and registered with code 904 (inflicted by humans and above the trivial limit) or 901 (not inflicted by humans or below the trivial limit) (I, 3, 5). Since 2014, the prevalence of pigs registered with code 904 and the number of forensic cases submitted to the University of Copenhagen have remained stable (Figs. 3, 5). Prior to a forensic necropsy of bruises inflicted by humans, veterinarians employed by the DVFA sample skin and muscle tissue from the carcass, store the tissue at –18°C and ensure that the tissue is submitted to the University of Copenhagen, if this is requested by the police (I, X). Currently, no guidelines for the sampling procedure exist. Therefore, tissue dimensions, the number of tissue layers (i.e., skin and muscle) and the number of full-length bruises preserved vary significantly among the forensic cases that are submitted (I). Moreover, although photographs of the entire carcass are almost always included, the exact anatomical location of the submitted tissue cannot always be determined. From experimental bruises, we know that the intensity and the extent of tissue damage and inflammation depend on the thickness of subcutaneous fat tissue and muscle tissue and the presence of bone underlying the site of impact (VIII, 6, 69, 71). In addition, the inflammatory response may vary within a bruise if the amount of energy is unevenly distributed over the area of impact (VIII, IX). The optimal sampling site within a bruise is where the greatest amount of energy was transferred because this should be where there is most tissue damage (VIII, IX). Moreover, freezing the tissue reduces its quality for histological evaluation (I). Therefore, to carry out an optimal forensic investigation that includes both gross and histological evaluations, whole or mid-sectioned carcasses should be stored at 5°C and submitted for forensic investigation as fast as possible after slaughter.

When a forensic necropsy is requested, the veterinary pathologist should determine whether the bruises were inflicted by humans and estimate when the bruises were inflicted relative to the time of death (I, V). In Denmark, more than 90% of bruises are assumed to be inflicted within 8 h prior to slaughter (I). During this time period, pigs are

usually handled by several different people, e.g., farmers, drivers and employees at the slaughterhouse, and bruise age estimates by veterinary pathologists may be presented at judicial proceedings to identify the perpetrator (I). Therefore, it is of utmost importance that bruise age estimates are correct.

Currently, bruise ages are estimated histologically based on the intensity and progression of the inflammatory response in the bruised tissue (I). However, the amount of tissue damage and the intensity of the inflammatory response are affected by factors other than time. These factors include the force, kinetic energy, mass and speed of the impacting object, the size of the impact area, the shape of the object, the anatomical location, the sampling site, and the BW/age of the animal (III, IV, VII-IX, 6–9, 69, 71). Factors such as anatomical location and sampling site can be taken into account by systematically sampling the skin and underlying muscle tissue at different anatomical locations and sites along the bruises (VIII, IX). However, the object and method used to inflict the bruise are typically unknown. Therefore, cross sectioning of the tissue at several sites should be carried out to assess tissue damage in the underlying muscle tissue, which is not visible from the skin surface.

Histological evaluation is simple and inexpensive, and it can be applied to tissue that has been frozen for months or years (I). However, studies of experimental bruises and bruises in forensic cases imply that histological assessment of selected changes alone may be insufficient to determine the ages of bruises (V, VIII, IX, 26, 30). Time-dependent changes were observed in selected histological parameters for bruises in small pigs (BW = 30 kg) but to lesser extent in large pigs (BW = 100 kg) (III, VIII). This difference was probably due to variations among pigs. In pigs, neutrophils were present in the dermis, subcutaneous tissue and underlying muscle tissue of bruises after 1 h (III). This is consistent with results from experimental bruises in mice and rats, which showed that neutrophils were present in the skin and muscle tissue within 1 to 3 h after bruises were inflicted (57, 59, 62). In porcine bruises, the number of neutrophils in the dermis and subcutaneous fat tissue increased from 4 h (III). In contrast, an increase in the number of neutrophils in the skin was not observed before 8 h in calves, lambs or mice (62, 66). In porcine bruises, macrophages were observed in the underlying muscle tissue from 2 h after the bruises were inflicted, and the number of macrophages present increased as the bruises became older (III). Similarly, macrophages have been observed in lacerated rat muscle tissue after 3 h (57). Experimental models have focused on single histological parameters and, in particular, changes in the numbers of inflammatory cells over time. However, to assess the ages of bruises in forensic cases, veterinary pathologists consider several additional parameters, including hyper-leukocytosis, the activation of endothelial cells, the relationship between haemorrhage area and the number of inflammatory cells, and the location/migration of inflammatory cells.

There is a subjective element to histological evaluation because observational and interpretational errors may be introduced by the observer based on their experience. To

avoid observational errors, immunohistochemical detection of neutrophils and macrophages in the subcutaneous tissue of porcine bruises followed by quantification using image analysis software was carried out (IV). A time- and force-dependent increase in the number of immunostained cells was registered. However, this method was most suitable for bruises inflicted with great force (6.5 N/mm^2) but less suitable for bruises inflicted with low (2.1 N/mm^2) or moderate (4.2 N/mm^2) force (IV). Moreover, when tissue sections from bruises were stained with HE and evaluated histologically, a time- and force-dependent increase in neutrophils was observed (III, IV). This was probably because porcine neutrophils are easily recognised by their pink cytoplasm when stained with HE. Moreover, good and very good agreement was achieved between two observers evaluating skin and underlying muscle tissue from bruises for several histological variables (VIII, IX). To avoid interpretational errors, Thornton and Jolly (65) suggested using a statistical model based on calculated probabilities for a number of histological variables in ovine bruises. The model was able to differentiate between bruises that were 1 to 20 h and 24 to 72 h old (65). However, these time intervals are too wide to be relevant for forensic cases concerning pigs.

Forensic porcine bruises with no inflammatory response are occasionally encountered (I, V). In comparison, bruises in three children and two adults, which were inflicted several hours before death but had no leukocyte infiltration, have been described (26, 30). This was suggested to be due to differences in individual responses or sampling sites within the bruises (26). Porcine bruises with no leukocyte infiltration should not be interpreted as inflicted PM or in the agonal period. Instead, the age should be stated as unknown. Moreover, the veterinary pathologist should be aware that injuries inflicted PM can resemble bruises inflicted AM (VI). However, PM pseudo bruises appear vague and would probably not be registered at slaughterhouse meat inspections. Histologically, extravasated erythrocytes and muscle fibres with a necrotic appearance can be found in PM inflicted pseudo bruises (VI). However, if bruises are inflicted PM, no vital reactions are observed, i.e., no infiltration of leukocytes (VI). In addition, the veterinary pathologist should be aware that chronic infiltration of mononuclear cells occasionally occurs in the skin and often occurs in the muscle tissue of pigs (III, VI).

Several methods have been used to investigate changes in bruises over time (Fig. 21, Tables 5 to 10). Gross assessment is unreliable for determining the ages of bruises in humans and animals (II, 28, 32, 33, 39). As an alternative, reflectance spectra of human and porcine bruises showed time-dependent changes in bruises that were several hours to several days old (6, 40, 44, 45). Reflectance spectrophotometry is a non-invasive technique, and this is advantageous for the evaluation of living subjects (35, 40). However, porcine bruises are not usually recorded AM (I). Moreover, haemorrhages in porcine muscle tissue could not be detected by reflectance spectroscopy (6). Therefore, the value of using reflectance spectroscopy in forensic cases of porcine bruises seems to be limited.

Another method for determining the ages of bruises in forensic cases that has been evaluated involves measuring the mRNA expression of selected genes in the subcutaneous fat tissue. Expression profiles of genes involved in inflammation and tissue modelling in subcutaneous fat were used to calculate statistical models (i.e., prediction models) that could determine the ages of experimental porcine bruises with a precision of approximately ±2 h in small and large pigs (VII, VIII). In forensic cases, when one of the prediction models (2B) was applied to paired bruises (i.e., two bruises, A and B, sampled from each individual pig), the mean difference between the estimated ages of the bruises was 1.5 h (X). In contrast, histological evaluations of the paired bruises resulted in similar age estimates for only 48% of the pigs (V). The results from these studies imply that the mRNA expression signature is better for determining the ages of bruises than histological evaluations of two parameters (V, X).

Although mRNA expression signatures can be used to predict the ages of bruises, this method cannot currently be applied to subcutaneous tissue from forensic cases (X). This is because mRNA is easily degraded, and the time interval between slaughter and sampling, when the tissue is stabilised, is often several months (X). In both pigs and other species, mRNA can be degraded to such an extent that the ages of bruises cannot be determined (X, 54, 60, 64). However, if bruises are sampled and immersed in RNA stabilising solution within the first hour after slaughter, mRNA expression levels could contribute to precise age assessments (X). If this method was implemented in forensic cases, sampling could be carried out by trained veterinarians at the slaughterhouses (X).

Even if gene expression profiles were used to determine the ages of bruises, gross and histological evaluations would still be indispensable. Gross evaluations are necessary to confirm that bruises were inflicted by humans. Histological evaluations are necessary to identify sites of maximum inflammation within the bruises, i.e., optimal sampling sites. Histological evaluations are also required to see that changes in the inflammatory response are consistent with age estimates based on gene expression.

Conclusions

- A mechanical device capable of inflicting blunt trauma with a force and type of impact equivalent to a man striking was developed. Experimental bruises developed a tramline pattern identical to forensic cases of bruises in pigs and humans (III).

- Time-dependent changes in selected histological parameters were observed in skin and underlying muscle tissue sampled from experimental bruises in small pigs and to a lesser extent in large pigs (III, IV, VIII, IX).

- In small pigs, a combination of selected histological parameters can determine whether a bruise is less than 4 h or between 4 and 10 h old (III).

- Bruise age estimates based on selected histological changes should be verified by a second method, e.g., measuring the expression of selected genes in subcutaneous fat (VII, VIII, X).

- mRNA expression signatures can determine the ages of bruises with a precision of ±2 h in small and large experimental pigs (VII, VIII).

- The age/BW of pigs does influence the mRNA expression of genes in the subcutaneous fat tissue from bruises.

- The mRNA expression signature that included four genes (SELE, SELP, IL6 and NFKB1) in bruises inflicted on large experimental pigs resulted in realistic age estimates for 95% of non-experimental porcine bruises. The mRNA expression signature that included 13 genes in bruises inflicted on small experimental pigs could not determine the age of non-experimental porcine bruises (X).

- The extent of tissue damage and the intensity of the inflammatory response depend on the thickness of the subcutaneous fat tissue, the sampling site within a bruise, and the force, speed and mass of the impacting object (III, IV, VII–IX).

- The optimal sampling site within a bruise is where the greatest energy transfer occurred, causing the most tissue damage and resulting in the most pronounced inflammatory response (VIII, IX).

- In forensic cases, skin and underlying muscle tissue should be sampled systematically from bruises in different anatomical locations and from different sampling sites within each bruise to ensure that regions with maximum inflammation and tissue damage are

evaluated (VIII, IX). Sampling two bruises from a pig and evaluating two histological parameters are insufficient to determine the ages of bruises (V).

- Blunt trauma inflicted PM can induce injuries comparable to bruises inflicted AM. However, vital reactions are absent in injuries inflicted PM (VI).

References

1. Bekendtgørelse af dyreværnsloven. LBK nr 20 af 11/01/2018, Miljø- og Fødevareministeriet. Available at: https://www.retsinformation.dk/Forms/r0710.aspx?id=197059

2. Retspraksis, Landsretterne. Udskrift af Østre Landsrets dombog. AM2012.06.14Ø. Available at: https://vidensbasen.anklagemyndigheden.dk/h/6dfa19d8-18cc-47d6-b4c4-3bd07bc15ec0/VB/1a9cd241-a5d2-4644-aaf5-0a1d8e87d705

3. Vejledning om udøvelse af kødkontrol.VEJ nr 9767 af 12/09/2018, Miljø- og Fødevareministeriet. Available at: https://www.retsinformation.dk/Forms/R0710.aspx?id=203009

4. Barington K, Johansen ASB, Jensen HE. Forensisk Veterinærpatologi. Dansk Veterinærtidsskrift. 2018;04:28-37.

5. Nielsen SS, Michelsen AM, Jensen HE, Barington K, Opstrup KV, Agger JF. The apparent prevalence of skin lesions suspected to be human-inflicted in Danish finishing pigs at slaughter. Prev Vet Med. 2014;117:200-206.

6. Randeberg LL, Winnem AM, Langlois NE, Larsen EL, Haaverstad R, Skallerud B, et al. Skin changes following minor trauma. Lasers Surg Med. 2007;39:403-413.

7. Randeberg LL, Winnem AM, Larsen ELP, Haaverstad R, Haugen OA, Svaasand LO. In vivo hyperspectral imaging of traumatic skin injuries in a porcine model. Proc SPIE. 2007;6424; doi: 10.1117/12.699380.

8. Winnem AM, Randeberg LL, Larsen ELP, Lilledahl MB, Haaverstad R, Haugen OA, et al. Biomechanical characterization of soft tissue injuries. Proc SPIE. 2007;6424: doi: 10.1117/12.700205.

9. Randeberg LL, Larsen EL, Svaasand LO. Characterization of vascular structures and skin bruises using hyperspectral imaging, image analysis and diffusion theory. J Biophoton. 2010;3;53-65.

10. Personal communication. Danish Veterinary and Food Administration.

11. Det Veterinære Sundhedsråd. Udtalelse om overdreven anvendelse af tatoveringshammer. 12/5/2009. Available at: https://detvetsund.dk/generelle-udtalelser/2009/udtalelse-om-overdreven-anvendelse-af-tatoveringshammer/

12. Jensen HE, Dahl-Pedersen K, Krarup C, Selmer O, Elvestad K, Kristensen SS, et al. Skader efter stump vold hos svin – patologiske og forensiske aspekter. Dansk Veterinær Tidskrift. 2009;22:1-3.

13. Attrup L. Landmænd slår grise med kæder. Jyllands-Posten. 30/11/2009. Available at: https://jyllands-posten.dk/protected/premium/erhverv/article4278377.ece

14. Lindqvist A. Danske svin bliver banket gule og blå. Politiken. 26/7/2010. Available at: https://politiken.dk/indland/art4977605/Danske-svin-bliver-banket-gule-og-bl%C3%A5

15. Personal communication. Jesper Valentin Petersen. Danish Agriculture and Food Council.

16. Agger JF, Jensen HE, Barington K, Nielsen SS. Prospektivt studium af slagmærker hos svin: Typer, forekomst, sammenhænge og årsager. University of Copenhagen. 23/3/2015. Available at: https://www.foedevarestyrelsen.dk/SiteCollectionDocuments/Dyresundhed/Oms%C3%A6tnings-gruppen/Rapport%20om%20prospektivt%20studium%20af%20slagm%C3%A6rker%20hos%20svin%20JF%20Agger%20et%20al%20%2023-03-2015%20(3).pdf

17. Saukko P, Knight B. The pathology of wounds. In Knight's Forensic Pathology. Boca Ranton, Florida, CRC Press, 2016.

18. Ressel L, Hetzel U, Ricci E. Blunt force trauma in veterinary forensic pathology. Vet Pathol. 2016;53:941-961.

19. Langlois NE, Gresham GA. The ageing of bruises: a review and study of the colour changes with time. Forensic Sci Int. 1991;50:227-238.

20. Armstrong E. Distinctive patterned injuries caused by an expandable baton. Am J Forensic Med Pathol. 2005;26:186-188.

21. Cooper BJ, Valentine BA. Muscle and tendon. In: Jubb, Kennedy, and Palmer's pathology of domestic animals. Maxie MG, editor. St. Louis, Missouri: Elsevier, 2016.

22. Ackermann MR, Newkirk KM, Brannick EM, Kusewitt DF. Inflammation and healing; Neoplasia and tumor biology. In: Pathologic basis of veterinary disease. Zachary JF, editor. St. Louis, Missouri: Elsevier, 2017.

23. Pellegrinelli V, Heuvingh J, du Roure O, Rouault C. Human adipocyte function is impacted by mechanical cues. J Pathol. 2014;233:183-195.

24. Cheville NF. Inflammation. In: Introduction to veterinary pathology. USA: Blackwell Publishing, 2006.

25. Barington K, Dich-Jørgensen K, Jensen HE. A porcine model for pathomorphological age assessment of surgically excised skin wounds. Acta Vet Scand. 2018;60:33

26. Ross CG, Langlois NEI, Heath K, Byard W. Further evidence for a lack of reliability in the histological ageing of bruises – an autopsy study. Aust J Forensic Sci. 2014;47:224-229.

27. Vanezis P. Interpreting bruises at necropsy. J Clin Pathol. 2001;54:348-355.

28. Maguire S, Mann MK, Sibert J, Kemp A. Can you age bruises accurately in children? A systematic review. Arch Dis Child. 2005;90:187-189.

29. Grossman SE, Johnston A, Vanezis P, Perrett D. Can we assess the age of bruises? An attempt to develop an objective technique. Med Sci Law. 2011;51:170-176.

30. Byard RW, Wick R, Gilbert JD, Donald T. Histologic dating of bruises in moribund infants and young children. Forensic Sci Med Pathol. 2008;4:187-192.

31. Fersini F, Govi A, Tsokos M, Etzold S, Tattoli L. Examples of tramline bruises in clinical forensic medicine. Forensic Sci Med Pathol. 2017;13:508-510.

32. Bariciak ED, Plint AC, Gaboury I, Bennett S. Dating of bruises in children: an assessment of physician accuracy. Pediatrics. 2003;112:804-807.

33. Stephenson T, Bialas Y. Estimation of the age of bruising. Arch Dis Child. 1996;74:53-55.

34. Langlois NE. The science behind the quest to determine the age of bruises – a review of the English language literature. Forensic Sci Med Pathol. 2007;3:241-251.

35. Hughes VK, Langlois NE. Use of reflectance spectrophotometry and colorimetry in a general linear model for the determination of the age of bruises. Forensic Sci Med Pathol. 2010;6:275-281.

36. Bohnert M, Baumgartner R, Pollak S. Spectrophotometric evaluation of the colour of intra- and subcutaneous bruises. Int J Legal Med 2000;113:343-348.

37. Hughes VK, Ellis PS, Langlois NE. The perception of yellow in bruises. J Clin Forensic Med. 2004;11:257-259.

38. Hughes VK, Ellis PS, Langlois NE. Alternative light source (polilight) illumination with digital image analysis does not assist in determining the age of bruises. Forensic Sci Int. 2006;158:104-107.

39. Lecomte MM, Holmes T, Kay DP, Simons JL, Vintiner SK. The use of photographs to record variation in bruising response in humans. Forensic Sci Int. 2013;231:213-218.

40. Hughes VK, Ellis PS, Burt T, Langlois NE. The practical application of reflectance spectrophotometry for the demonstration of haemoglobin and its degradation in bruises. J Clin Pathol. 2004;57:355-359.

41. Duckworth MG, Caspall JJ, Mappus RL, Kong L, Yi D, Sprigle SH. Bruise chromophore concentrations over time. Proc SPIE. 2008; 6915:doi: 10.1117/12.770399.

42. Kim O, McMurdy J, Lines C, Duffy S,Crawford G, Alber M. Reflectance spectrometry of normal and bruised human skins: experiments and modeling. Physiol Meas. 2012;33:159-175.

43. Mimasaka S, Ohtani M, Kuroda N, Tsunenari S. Spectrophotometric evaluation of the age of bruises in children: measuring changes in bruise color as an indicator of child physical abuse. Tohoku J Exp Med. 2010;220:171-175.

44. Randeberg LL, Winnem AM; Blindheim S, Haugen OA, Svaasand LO. Optical classification of bruises. Proc SPIE. 2004;5312:54-64.

45. Stam B, van Gemert MJ, van Leeuwen TG, Teeuw AH, van der Wal AC, Aalders MC. Can color inhomogeneity of bruises be used to establish their age? J Biophotonics. 2011;4:759-767.

46. Sprigle S, Yi D, Caspall J, Linden M, Kong L, Duckworth M. Multispectral image analysis of bruise age. Proc SPIE. 2007;6514:doi:10.1117/12.709930.

47. Helm T, Bir C, Chilstrom M, Claudius I. Ultrasound characteristics of bruises and their correlation to cutaneous appearance. Forensic Sci Int. 2016;266:160-163.

48. Mimasaka S, Oshima T, Ohtani M. Characterization of bruises using ultrasonography for potential application in diagnosis of child abuse. Leg Med (Tokyo). 2012;14:6-10.

49. Vidovič L, Milanič M, Majaron B. Objective characterization of bruise evolution using photothermal depth profiling and Monte Carlo modeling. J Biomed Opt. 2015; 20:doi: 10.1117/1.JBO.20.1.017001.

50. Vidovič L, Milanič M, Majaron B. Quantitative analysis of hemodynamics in bruised skin using photothermal depth profiling . Int J Thermophys. 2015;36:849-856.

51. Nakajima T, Hayakawa M, Yajima D, Montani-Sitoh H, Sato Y, Kiuchi M, et al. Time-course changes in the expression of heme oxygenase-1 in human subcutaneous hemorrhage. Forensic Sci Int. 2006;158:157-163.

52. Sawaguchi T, Jasani B, Kobayashi M, Knight B. Post-mortem analysis of apoptotic changes associated with human skin bruises. Forensic Sci Int. 2000;108:187-203.

53. Du QX, Sun JH, Zhang LY, Liang XH, Guo XJ, Gao CR, et al. Time-dependent expression of SNAT2 mRNA in contused skeletal muscle of rats: a possible marker for wound age estimation. Forensic Sci Med Pathol. 2013;9:528-533.

54. Sun JH, Wang YY, Zhang L, Gao CR. Time-dependent expression of skeletal muscle troponin I mRNA in the contused skeletal muscle of rats: a possible marker for wound age estimation. Int J Legal Med. 2010;124:27-33.

55. Sun JH, Zhu XY, Dong TN, Zhang XH, Liu QQ, Li SQ, et al. An ”up, no change or down” system: Time-dependent expression of mRNAs in contused skeletal muscle of rats used for wound age estimation. Forensic Sci Int. 2017;272:104-110.

56. Zhu X, Du Q, Li S, Sun JH. Comparison of the homogeneity of mRNAs encoding SFRP5, FZD4, and Fosl1 in post-injury intervals: subcellular localization of markers may influence wound age estimation. J Forensic Leg Med. 2016;43:90-96.

57. Fan YY, Zhang ST, Yu LS, Ye GH, Lin KZ, Wu SZ, et al. The time-dependent expression of α7nAChR during skeletal muscle wound healing in rats. Int J Legal Med. 2014;128:779-786.

58. Tian ZL, Jiang SK, Zhang M, Wang M, Li JY, Zhao R, et al. Detection of satellite cells during skeletal muscle wound healing in rats: time-dependent expressions of Pax7 and MyoD in relation to wound age. Int J Legal Med. 2016;130:163-172.

59. Yu TS, Cheng ZH, Li LQ, Zhao R, Fan YY, Du Y, et al. The cannabinoid receptor type 2 is time-dependently expressed during skeletal muscle wound healing in rats. Int J Legal Med. 2010;124:397-404.

60. Yu T, Li Z, Zhao R, Zhang Y, Zhang ZH, Guan DW. Time-dependent expression of MMP-2 and TIMP-2 after rats skeletal muscle contusion and their application to determine wound age. J Forensic Sci. 2016;61:527-533.

61. Mao S, Fu F, Dong X, Wang Z. Supplementary pathway for vitality of wounds and wound age estimation in bruises using the electric impedance spectroscopy technique. J Forensic Sci. 2011;56: 925-929.

62. Takamiya M, Saigusa K, Kumagai R, Nakayashiki N, Aoki Y. Studies on mRNA expression of tissue-type plasminogen activator in bruises for wound age estimation. Int J Legal Med. 2005;119:16-21.

63. Xu J, Zhao R, Xue Y, Xiao H, Sheng Y, Zhao D, et al. RNAseq profiling reveals differentially expressed genes as potential markers for vital reaction in skin contusion: a pilot study. Forensic Sciences Research. 2018;3:153-160.

64. He JT, Huang HY, Qu D, Xue Y, Zhang KK, Xie XL, et al. CXCL1 and CXCR2 as potential markers for vital reactions in skin contusions. Forensic Sci Med and Pathol. 2018;14:174-179.

65. Thornton RN, Jolly RD. The objective interpretation of histopathological data: an application to the aging of ovine bruises. Forensic Sci Int. 1986;31:225-239.

66. McCausland P, Dougherty R. Histological ageing of bruises in lambs and calves. Aust Vet J. 1978;54:525-527.

67. Hamdy MK, Deatherage FE, Shinowara GY. Bruised tissue I. Biochemical changes resulting from blunt injury. Proc Soc Exp Biol Med. 1957;95:255-258.

68. Hamdy MK, Kunkle LE, Deatherage FE. Bruised tissue II. Determination of the age of a bruise. J Anim Sci. 1957;16:490-495.

69. Hamdy MK, Kunkle LE, Rheins MS, Deatherage FE. Bruised tissue III. Some factors affecting experimental bruises. J Anim Sci. 1957;16:496-501.

70. Hamdy MK, May KN, Flanagan WP, Powers JJ. Determination of the age of bruises in chicken broilers. Poult Sci. 1961;40:787-789.

71. Hamdy MK, May KN, Powers JJ. Some physical and physiological factors affecting poultry bruises. Poult Sci. 1961;40:790-795.

72. Northcutt JK, Buhr RJ, Rowland GN. Relationship of broiler bruise age to appearance and tissue histological characteristics. J Appl Poult Res. 2000;9:13-20.

73. Yajima Y, Funayama M. Spectrophotometric and tristimulus analysis of the colors of subcutaneous bleeding in living persons. Forensic Sci Int 2006;156:131-137.

74. Pilling ML, Vanezis P, Perrett D, Johnston A. Visual assessment of the timing of bruising by forensic experts. J Forensic Leg Med. 2010;17:143-149.

75. Thavarajah D, Vanezis P, Perrett D. Assessment of bruise age on dark-skinned individuals using tristimulus colorimetry. Med Sci Law. 2012;52:6-11.

76. Scafide KR, Sheridan DJ, Cambell J, Deleon VB,Hayat MJ. Evaluating change in bruise colorimetry and the effect of subject characteristics over time. Forensic Sci Med Pathol. 2013;9:367-376.

77. Kock MD, Loix S, Lavand'homme P. Ketamine and peripheral inflammation. CNS Neurosci Ther. 2013;19:403-410.

78. Kurosawa S, Kato M. Anesthetics, immune cells, and immune responses. J Anesth. 2008;22:263-277.

79. Zavala F. Benzodiazepines, anxiety and immunity. Pharmacol Ther. 1997;75:199-216.

80. Benschop RJ, Rodriguez-Feuerhahn M, Schedlowski M. Catecholamine-induced leukocytosis: early observations, current research and future directions. Brain Behav Immun. 1996;10:77-91.

81. Dhabhar FS. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. Neuroimmunomodulation. 2009;16:300-317.

82. Christiansen JG, Jensen HE, Johansen LK, Koch J, Koch J, Aalbaek B, et al. Porcine models of non-bacterial thrombotic endocarditis (NBTE) and infective endocarditis (IE) caused by Staphylococcus aureus: a preliminary study. J Heart Valve Dis. 2013;22:368-376.

83. Johansen LK, Frees D, Aalbæk B, Koch J, Iburg T, Nielsen OL, et al. A porcine model of acute, haematogenous, localized osteomyelitis due to Staphylococcus aureus: a pathomorphological study. APMIS 2011;119:111-118.

84. Johansen LK, Koch J, Frees D, Aalbæk B, Nielsen OL, Leifsson PS, et al. Pathology and biofilm formation in a porcine model of staphylococcal osteomyelitis. J Comp Path. 2012;147:343-353.

85. Nielsen OL, Iburg T, Aalbaek B, Leifsson PS, Agerholm JS, Heegaard P, et al. A pig model of acute Staphylococcus aureus induced pyemia. Acta Vet Scand. 2009;51:14.

86. Alberts B, Bray D, Hopkin K, Johnson A, Lewis J, Raff M, et al. From DNA to protein: how cells read the genome. In: Essential cell biology. New York, USA, Garland Science, 2004.

87. Ackermann MR. Acute inflammation. In: Pathologic basis of veterinary disease. McGavin MD, Zachary JF, editors. St. Louis, Missouri, USA: Mosby Elsevier, 2007.

88. Cecchi R. Estimating wound age: looking into the future. Int J Legal Med. 2010;124:523-536.

89. Dressler J, Bachmann L, Koch R, Müller E. Enhanced expression of selectins in human skin wounds. Int J Legal Med. 1998;112:39-44

90. Dressler J, Bachmann L, Streje P, Koch R, Müller E. Expression of adhesion molecules in skin wounds: diagnostic value in legal medicine. Forensic Sci Int. 2000;113:173-176.

91. Kondo T, Ohshima T. The dynamics of inflammatory cytokines in the healing process of mouse skin wound: a preliminary study for possible wound age determination. Int J Legal Med. 1996;108:231-236.

92. Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. J Pathol. 2007;211:144-156.

93. Christoffersen BØ, Jensen SJ, Ludvigsen TP, Nilsson SK, Grossi AB, Heegaard PM. Age- and sex-associated effects on acute-phase proteins in Göttingen Minipigs. Comp Med. 2015;65:333-341.

94. Alonso M, Finn EJ. Work and Energy; Dynamics of a rigid body. In: Physics. USA, Addison Wesley Publishing Company, 1972.

95. Barington K, Jensen HE. Forensic relevance of agonal wounds in slaughter pigs. J Comp Path. 2018;158:108.

96. Betz P. Histological and enzyme histochemical parameters for the age estimation of human skin wounds. Int J Legal Med. 1994;107:60-68.

97. Grellner W, Madea B. Demands on scientific studies: vitality of wounds and wound age estimation. Forensic Sci Int. 2007;165:150-154.

98. Madea B. Importance of supravitality in forensic medicine. Forensic Sci Int. 1994;69:221-241.

99. Ali TT. The role of white blood cells in post-mortem wounds. Med Sci Law. 1988;28;100-106.

100. Grellner W, Madea B, Kruppenbacher JP, Dimmeler S. Interleukin-1α (IL-1α) and N-formyl-methionyl-leucyl-phenylalanine as potential inducers of supravital chemotaxis. Int J Legal Med. 1996;109:130-133.

101. Grellner W, Dimmeler S, Madea B. Immunohistochemical detection of fibronectin in postmortem incised wounds of porcine skin. Forensic Sci Int. 1998;97:109-116.

102. Hernández-Cueto C, Luna A, Lorente JA, Villanueva E. Study of cathepsin A, B and D activities in skin wound edges. Its application to the differential diagnosis between vital and postmortem wounds. Forensic Sci Int. 1987;35:51-60.

103. Dalla Costa OA, Faucitano L, Coldebella A, Ludke JV, Peloso JV, dalla Roza D, et al. Effects of the season of the year, truck type and location on truck on skin bruises and meat quality in pigs. Livest Sci. 2007;107:29-36.

104. Scheeren MB, Gonyou HW, Brown J, Weschenfelder AV, Faucitano L. Effects of transport time and location within truck on skin bruises and meat quality of market weight pigs in two seasons. Can J Anim Sci. 2014;94:71-78.

105. Agger R, Andersen V, Leslie G, Aasted B. Immunologi. Frederiksberg, Denmark: Biofolia, 2005.

106. Yui Y, Aoyama T, Morishita H, Takahashi M, Takatsu Y, Kawai C. Serum prostacyclin stabilizing factor is identical to apolipoprotein A-I (Apo A-I). A novel function of Apo A-I. J Clin Invest. 1988;82:803-807.

Appendix

Table I: List of 42 genes evaluated in the subcutaneous fat tissue from bruises in pigs with a mean body weight of 30 kg (VII). Gene symbol, name, and primer sequences (F = forward and R = reverse).

Gene symbol	Gene name	Sequence (5' to 3')
APOA1	Apolipoprotein A1	F:GTTCTGGGACAACCTGGAAA
		R: GCTGCACCTTCTTCTTCACC
C3	Complement component 3	F:ATCAAATCAGGCTCCGATGA
		R: GGGCTTCTCTGCATTTGATG
CASP1	Caspase 1	F:GAAGGACAAACCCAAGGTGA
		R: TGGGCTTTCTTAATGGCATC
CCL2	C-C Motif chemokine ligand 2	F:CTTCTGCACCCAGGTCCTT
		R: CGCTGCATCGAGATCTTCTT
CCL3	C-C Motif chemokine ligand 3	F:CCAGGTCTTCTCTGCACCAC
		R: GCTACGAATTTGCGAGGAAG
CCL5	C-C Motif chemokine ligand 5	F:CTCCATGGCAGCAGTCGT
		R: AAGGCTTCCTCCATCCTAGC
CD163	CD163	F:CACATGTGCCAACAAAATAAGAC
		R: CACCACCTGAGCATCTTCAA
CFB	Complement factor B	F:TGATGGAGCGGGGTACTG
		R: TCGGCTGCAGTAGTAGGTGA
CFD	Complement factor D	F:CCTCGGAGCAGCTGTATGT
		R: ATGCCATGTAGGGTCTCTCG
CLDN1	Claudin 1	F:TGATGAGGTGCAGAAGATGC
		R: CCATGCTGTGGCAACTAAGA
CXCL10	Chemokine (C-X-C Motif) ligand 10	F:CCCACATGTTGAGATCATTGC
		R: GCTTCTCTCTGTGTTCGAGGA
FGF2	Fibroblast growth factor 2 (basic)	F:TACTCCAGTTGGTATGTGGCACT
		R: TCTGCCCAGGTCCTGTTTT
FN1	Fibronectin 1	F:AAGTGTGATCCCCATGAAGC
		R: TGGCACCGAGATATTCCTTC
FOS	c-Fos proto-oncogene	F:CTCCAAGCGGAGACAGACC
		R: CTTCTCCTTCAGCAGGTTGG
HMOX1	Haeme oxygenase 1	F:TCCACTCCCGTGACAACC
		R: GCCACCAGAAAGCTGAGTGT
ICAM-1	Intercellular adhesion molecule 1	F:AAGCTTCTCCTGCTCTGCTG
		R: GGGGTCCATACAGGACACTG
IFNA1	Interferon α 1	F:ATCGTCAGGGCAGAAGTCAT
		R: CCAGGTGTCTGTCACTCCTTC

IL18	Interleukin 18	F:CTGCTGAACCGGAAGACAAT R: TCCGATTCCAGGTCTTCATC
IL1B	Interleukin 1 β	F:TCTCTCACCCCTTCTCCTCA R: GACCCTAGTGTGCCATGGTT
IL1RN	Interleukin 1 receptor antagonist	F:TGCCTGTCCTGTGTCAAGTC R: GTCCTGCTCGCTGTTCTTTC
IL33	Interleukin 33	F:GTAAACCTGAGCCCCACAAA R: CTGTTCTGGCAGTGGGTTTT
IL6	Interleukin 6	F: TGGGTTCAATCAGGAGACCT R: CAGCCTCGACATTTCCCTTA
IL8	Interleukin 8	F: GAAGAGAACTGAGAAGCAACAACA R: TTGTGTTGGCATCTTTACTGAGA
MAPK9	Mitogen-activated protein kinase 9	F:CAACCTTCAGATGCAGCAGT R: GTCTGCTCAGTGGACATGGA
MMP2	Matrix metallopeptidase 2	F:GTGTTCTTTGCAGGGAATGAGTA R: GGACATCAGGCGGAAGC
NFKB1	Nuclear factor κ β subunit 1	F:CTCGCACAAGGAGACATGAA R: GGGTAGCCCAGTTTTTGTCA
NFKBIA	Nuclear factor κ β subunit 1 inhibitor α	F:GAGGATGAGCTGCCCTATGAC R: CCATGGTCTTTTAGACACTTTCC
OCLN	Occludin	F:GACGAGCTGGAGGAAGACTG R: GTACTCCTGCAGGCCACTGT
PLAT	Plasminogen activator, tissue	F:TGCTTCCAGGAGAGGTTCC R: CTCTCCAGGGACCAGCCTAT
PGS2	Prostaglandin-endoperoxide synthase 2	F:GAACTTACAGGAGAGAAGGAAATGG R: TTTCTACCAGAAGGGCAGGA
SELE	Selectin E	F:GGATGCTGCCTACTTGTGAAG R: CAGGAGCCAGAGGAGAAATG
SELP	Selectin P	F:CCTAGCAGGGCCATTGAC R: CCCACCCATCACTAAACCTG
TF	Transferrin	F:CTCAACCTCAAAACTCCTGGAA R: CCGTCTCCATCAGGTGGTA
TGFB	Transforming growth factor β 1	F:GCAAGGTCCTGGCTCTGTA R: TAGTACACGATGGGCAGTGG
TJP1	Tight junction protein 1	F:ATGACTCCTGACGGTTGGTC R: TGCCAGGTTTTAGGATCACC
TNF	Tumour necrosis factor	F:CACGTTGTAGCCAATGTCAAAG R: GAGGTACAGCCCATCTGTCG
TNFAIP3	Tumour necrosis factor α-induced protein 3	F:CCCAGCTTTCTCTCATGGAC R: TTGGTTCTTCTGCCGTCTCT
TNNC2	Troponin C type 2 (fast)	F:ATCTTCGACAGGAACATGGAC R: ATCAGGGATTCGAGCTCCTC
TNNI2	Troponin I type 2 (skeletal, fast)	F:CCTGAAGCAGGTCAAGAAGG R: CCGACTTCTCCTCGATGTTC

VCAM1	Vascular cell adhesion molecule 1	F:CTTGACGTGAAAGGAAGAGAAAG R: GGATGCACAATAGAGCACGA
VEGFA	Vascular endothelial growth factor A	F:AGTTCGAGGAAAGGGAAAGG R: CAGGGTTTATACCGGGATTTC
VEGFB	Vascular endothelial growth factor B	F:CTCTGGCCACCAAAAGAAAG R: TCCATGGTTAGAGGCACCAC

Table II: List of 43 genes evaluated in the muscle tissue from bruises in pigs with a mean body weight of 30 kg (VII). Gene symbol, name, and primer sequences (F = forward and R = reverse) are presented.

Gene symbol	Gene name	Sequence (5' to 3')
APOA1	Apolipoprotein A-I	F:GTTCTGGGACAACCTGGAAA R: GCTGCACCTTCTTCTTCACC
C3	Complement component 3	F:ATCAAATCAGGCTCCGATGA R: GGGCTTCTCTGCATTTGATG
CASP1	Caspase 1	F:GAAGGACAAACCCAAGGTGA R: TGGGCTTTCTTAATGGCATC
CCL2	C-C Motif chemokine ligand 2	F:CTTCTGCACCCAGGTCCTT R: CGCTGCATCGAGATCTTCTT
CCL3	C-C Motif chemokine ligand 3	F:CCAGGTCTTCTCTGCACCAC R: GCTACGAATTTGCGAGGAAG
CCL5	C-C Motif chemokine ligand 5	F:CTCCATGGCAGCAGTCGT R: AAGGCTTCCTCCATCCTAGC
CD163	CD163	F:CACATGTGCCAACAAAATAAGAC R: CACCACCTGAGCATCTTCAA
CFB	Complement factor B	F:TGATGGAGCGGGGTACTG R: TCGGCTGCAGTAGTAGGTGA
CFD	Complement factor D	F:CCTCGGAGCAGCTGTATGT R: ATGCCATGTAGGGTCTCTCG
CLDN1	Claudin 1	F:TGATGAGGTGCAGAAGATGC R: CCATGCTGTGGCAACTAAGA
CXCL10	Chemokine (C-X-C Motif) ligand 10	F:CCCACATGTTGAGATCATTGC R: GCTTCTCTCTGTGTTCGAGGA
FGF2	Fibroblast growth factor 2 (basic)	F:TACTCCAGTTGGTATGTGGCACT R: TCTGCCCAGGTCCTGTTTT
FN1	Fibronectin 1	F:AAGTGTGATCCCCATGAAGC R: TGGCACCGAGATATTCCTTC
FOS	c-Fos proto-oncogene	F:CTCCAAGCGGAGACAGACC R: CTTCTCCTTCAGCAGGTTGG
HMOX1	Haeme oxygenase 1	F:TCCACTCCCGTGACAACC R: GCCACCAGAAAGCTGAGTGT
ICAM-1	Intercellular adhesion molecule 1	F:GCCCAATTGAAGCTGAATGT

		R: CACCTGGGTCTGGTTCTTGT
IFNA1	Interferon α 1	F:TACTCAGCTGCAATGCCATC
		R: CTCCTCATTTGTGCCAGGAG
IL18	Interleukin 18	F:CTGCTGAACCGGAAGACAAT
		R: TCCGATTCCAGGTCTTCATC
IL1B	Interleukin 1 β	F:TCTCTCACCCCTTCTCCTCA
		R: GACCCTAGTGTGCCATGGTT
IL1RN	Interleukin 1 receptor antagonist	F:TGCCTGTCCTGTGTCAAGTC
		R: GTCCTGCTCGCTGTTCTTTC
IL33	Interleukin 33	F:GTAAACCTGAGCCCCACAAA
		R: CTGTTCTGGCAGTGGGTTTT
IL6	Interleukin 6	F: TGGGTTCAATCAGGAGACCT
		R: CAGCCTCGACATTTCCCTTA
IL8	Interleukin 8	F: GAAGAGAACTGAGAAGCAACAACA
		R: TTGTGTTGGCATCTTTACTGAGA
MAPK9	Mitogen-activated protein kinase 9	F:CAACCTTCAGATGCAGCAGT
		R: GTCTGCTCAGTGGACATGGA
MMP2	Matrix metallopeptidase 2	F:GTGTTCTTTGCAGGGAATGAGTA
		R: GGACATCAGGCGGAAGC
NFKB1	Nuclear factor κ β subunit 1	F:CTCGCACAAGGAGACATGAA
		R: GGGTAGCCCAGTTTTTGTCA
NFKBIA	Nuclear factor κ β subunit 1 inhibitor α	F:GAGGATGAGCTGCCCTATGAC
		R: CCATGGTCTTTTAGACACTTTCC
OCLN	Occludin	F:GACGAGCTGGAGGAAGACTG
		R: GTACTCCTGCAGGCCACTGT
PLAT	Plasminogen activator, tissue	F:CATGAAGCCTCTTCTCCTTTCT
		R: TTATTAAACAAATGTTTTGAAGTGCAG
PTGS2	Prostaglandin-endoperoxide synthase 2	F:GAACTTACAGGAGAGAAGGAAATGG
		R: TTTCTACCAGAAGGGCAGGA
SELP	Selectin P	F:CCTAGCAGGGCCATTGAC
		R: CCCACCCATCACTAAACCTG
TGFB	Transforming growth factor β 1	F:GCAAGGTCCTGGCTCTGTA
		R: TAGTACACGATGGGCAGTGG
TJP1	Tight junction protein 1	F:ATGACTCCTGACGGTTGGTC
		R: TGCCAGGTTTTAGGATCACC
TNF	Tumour necrosis factor	F:CACGTTGTAGCCAATGTCAAAG
		R: GAGGTACAGCCCATCTGTCG
TNFAIP3	Tumour necrosis factor α-induced protein 3	F:CCCAGCTTTCTCTCATGGAC
		R: TTGGTTCTTCTGCCGTCTCT
TNNC1	Troponin C type 1 (slow)	F:TGTTTGACAAAAACGCTGATG
		R: TCATGAGCTCCTCGATGTCA
TNNC2	Troponin C type 2 (fast)	F:ACGAGGATGGCAGTGGAA
		R: AGCCAGCTCCTCCTCACTCT
TNNI1	Troponin I type 1 (skeletal, slow)	F:CCAAATGCCTGCACAACA

		R: GAGGGCGCTTGAACTTCC
TNNI2	Troponin I type 2 (skeletal, fast)	F:CCTGAAGCAGGTCAAGAAGG
		R: CCGACTTCTCCTCGATGTTC
TNNT1	Troponin T type 1 (skeletal, slow)	F:CCTGGTCAAGGCAGAACAG
		R: TCCCATGTGGTCGATATTCA
VCAM1	Vascular cell adhesion molecule 1	F:CTTGACGTGAAAGGAAGAGAAAG
		R: GGATGCACAATAGAGCACGA
VEGFA	Vascular endothelial growth factor A	F:AGTTCGAGGAAAGGGAAAGG
		R: CAGGGTTTATACCGGGATTTC
VEGFB	Vascular endothelial growth factor B	F:CTCTGGCCACCAAAAGAAAG
		R: TCCATGGTTAGAGGCACCAC