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Forensic age determination of human inflicted porcine bruises inflicted within 10 hours prior to slaughter by application of gene expression signatures

Kristiane Barington[1], Henrik E. Jensen[1], Kerstin Skovgaard[2]

[1] Faculty of Health and Medical Sciences, University of Copenhagen, Ridebanevej 3, DK-1870 Frederiksberg C, Denmark

[2] Department of Biotechnology and Biomedicine, Technical University of Denmark, Kemitorvet DK-2800 Kongens Lyngby, Denmark

Corresponding author: Kristiane Barington, Faculty of Health and Medical Sciences, University of Copenhagen, Ridebanevej 3, DK-1870 Frederiksberg C, Denmark.

krisb@sund.ku.dk

Abstract

Prediction-models based on gene expression profiles from experimental bruises are capable of determining the age of bruises with a precision of ±2 h. However, these models have not yet been applied on tissue from pigs in forensic cases requested by the police. We applied two prediction-models, based on mRNA expression of 13 (prediction-model no. 1) and 4 genes (prediction-model no. 2) involved in inflammation, on forensic cases of porcine bruises in order to determine if gene expression profiles can be used for age determination in forensic cases.

Subcutaneous fat tissue from bruises notified to the police was sampled: 1) within 6 h after slaughter (group no. I, n = 142), and 2) after freezing the skin for up to 1 year (group no. II, n = 40). qPCR of genes involved in inflammation was performed to predict the bruise age after partial least squares analysis.

mRNA expression data were obtained for 52.8% and 7.5% bruises in group nos. I and II, respectively. Prediction-model no. 2, based on the mRNA expression of Selectin E, Selectin P, Interleukin 6 and Nuclear Factor Kappa Beta Subunit1, was most suitable for predicting the age of bruises within 8 h prior to slaughter.

In conclusion, mRNA expression profiles can assist in estimating the age of bruises. However, when applying gene expression signatures in forensic cases the age estimate should be interpreted together with histological manifestations. Subcutaneous tissue must be stabilized hours after the bruises are detected in order to obtain mRNA of a sufficient quality.

Key words bruise; forensic pathology; mRNA; pig; qPCR; time factors

1 Introduction

Determining the age of bruises is of crucial importance in both human and veterinary forensic pathology (Byard et al., 2008; Gerdin and McDonough, 2013; Munro and Munro, 2013; Vanezis, 2001). In pigs, the age of human inflicted bruises is used to determine in whose custody the pig was when the lesions were inflicted in order to place guilt (Barington and Jensen, 2013). Approximately 90% of forensic cases of porcine bruises are assumed to have been inflicted within 8 h prior to slaughter (Barington and Jensen, 2013). Currently, age estimation is based on histological evaluation of the inflammatory response in skin and underlying muscle tissue. Based on the histological evaluation of neutrophils and macrophages, bruises can be determined to be more or less than 4 h old (Barington and Jensen, 2016).

Various methods, other than histological evaluation, for estimating the age of bruises have been investigated in humans and animals (Byard et al., 2008; Grossman et al., 2011; Hamdy et al., 1957; Hughes et al., 2006; Mao et al., 2011; Randeberg et al., 2007). Of these, mRNA expression profiles of selected genes involved in inflammation and tissue modeling seem promising for improving age estimation of bruises in pigs and rodents (Barington et al., 2017; Du et al., 2013, Sun et al., 2010; Takamiya et al., 2005). In experimental bruises between 1 and 10 h of age inflicted on pigs, gene expression signatures have been shown to determine the age of bruises with a precision of approximately ±2 h (Barington et al., 2017; Barington et al., 2018).

RNA is easily degraded by ribonucleases (RNases) in the tissue and environment, and low quality RNA may strongly compromise the acquisition of reliable qPCR data and thus gene expression profiles in forensic cases (Fleige et al., 2006). Skin bruises on pigs are usually not recognized before slaughter because of the thick epidermis, the hair layer and dirt (Barington and Jensen, 2013). At slaughter, the carcasses are scalded, singed and scraped

which removes the epidermis by which hemorrhage in the subcutaneous fat tissue becomes more apparent (Barington and Jensen, 2013). Moreover, the processing of the carcass removes part of the dermis and transforms the remaining part into a dense, homogenous mass of tissue when evaluated microscopically. Skin and underlying muscle tissue sampled at slaughterhouses for forensic pathological investigation, are kept frozen at -18°C, to avoid putrefaction, for up to several months prior to submission (Barington and Jensen, 2013).

The objective of the present study was to evaluate the use of mRNA expression data of selected genes expressed in the subcutaneous fat tissue in order to predict the age of human inflicted porcine bruises notified to the police. Bruises were sampled and stabilized in RNAlater within 6 h after slaughter (group no. I) or after long term storage at -18°C (group no. II) in order to evaluate the applicability of these procedures in forensic cases.

2 Materials and methods

2.1 Animals

Subcutaneous fat tissue was sampled from human inflicted bruises on slaughter pigs (age of 5 to 6 months). After slaughter, all pigs were examined for human inflicted bruises as part of the routine meat inspection at the slaughter line. Human inflicted bruises were defined as multiple bruises having a uniform pattern localized on the back or upper sides of the pigs (Barington et al., 2016). The slaughter pigs included in the study were divided into two groups. The slaughter pigs in group no. I (n = 71) had a mean carcass weight of 83.25 ± 6.58 kg and originated from a study requested by the Danish Veterinary and Food Administration including all slaughter pigs with human inflicted bruises detected at two major Danish slaughterhouses from November 2013 to May 2014 (Agger et al., 2015). The gross and histological evaluations of the bruises have been presented previously (Barington et al., 2016). The slaughter pigs in group

no. II (n = 40) were collected among the routine forensic cases submitted to the University of Copenhagen from slaughterhouses on request by the police during October 2013 to April 2017. The carcass weight was unknown.

The exact age of the bruises (group nos. I and II) could not be obtained which is the condition for forensic cases of bruises in pigs. In these cases there are no witnesses and the exact timing is not obtained from the perpetrators.

2.2 Sampling

From the pigs in group no. I, subcutaneous fat tissue (0.5 x 0.5 x 0.5 cm) from two separate bruises (a and b) on each pig (n=142 bruises) were sampled within 6 h after slaughter (Fig. 1). The sampling was done by veterinarians carrying out meat inspection, and each tissue sample was preserved in 4.5 mL RNAlater, stored at 5°C for 24 h and then at -20°C until RNA extraction. From the pigs in group no. II, skin with human inflicted bruises was removed after slaughter and stored at -18°C from 38 to 379 days before being submitted to the University of Copenhagen (Fig 1). On arrival at the University, the skin was thawed and subcutaneous fat tissue (0.5 x 0.5 x 0.5 cm) was sampled from the bruises (n=40) and preserved in 4.5 mL RNAlater stored at 5°C for 24 h and then at -20°C until RNA extraction.

2.3 RNA extraction, quantitation and quality assessment

Tissue samples of 100 mg were homogenized in QIAzol Lysis reagent (Qiagen, Hilden, Germany) using M-tubes (Miltenyi Biotec, Lund, Sweden) in a gentleMACS Dissociator (Miltenyi Biotec). RNA was extracted from the homogenized tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) and all samples were treated with the RNAase-Free DNAase set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The

purity of RNA was evaluated at optical density (OD) 260/280 and OD 260/230 ratios, and the concentration of RNA was quantified at OD 260 measured on a Nanodrop ND-1000 spectrophotometer (Saveen and Werner AB, Lindhamn, Sweden). The quality of RNA was assessed by measuring the RNA integrity number (RIN) on an Agilent Bioanalyzer (Agilent Technologies, Glostrup, Denmark) using the RNA 6000 Nano Kit (Agilent Technologies, Glostrup, Denmark). RNA was stored at -80°C until converted into cDNA.

2.4 cDNA synthesis

RNA was stored at -80°C until converted into cDNA. From each sample, duplicate cDNA syntheses were made from 100 ng extracted RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Non-reverse-transcriptase controls were made to assess potential DNA contamination. cDNA was diluted 1:10 in Low EDTA TE-Buffer (VWR-Bie & Berntsen) before pre-amplification.

2.4.1 Primers

Primers for the 13 genes of interest and 6 reference genes were selected based on recent studies of gene expression in experimental bruises (Table 1) (Barington et al., 2017 and 2018). Primers were synthesized at Sigma Aldrich.

2.5 Pre-amplification

Stocks of 200 nM primer pair mix containing equal amounts of all primers were prepared in low EDTA TE-Buffer. For pre-amplification 5 µL TaqMan PreAmp Master Mix (Applied Biosystems, CA, USA), 2.5 µL primer mix and 2.5 µL diluted cDNA were incubated at 95°C for 10 min, then 19 cycles of 95°C for 15 s and 60°C for 4 min. Pre-amplified cDNA was

incubated with 4 µL of 4 U/µL exonuclease (New England Biolabs) 30 min at 37°C followed by 15 min at 80°C before being diluted 1:10 in low EDTA TE-Buffer (VWR – Bie & Berntsen).

2.6 High throughput quantitative real time PCR (qPCR)

High throughput quantitative real time polymerase chain reaction (qPCR) was performed to evaluate mRNA expression of the 13 genes of interest, and the reference genes listed in Table 1. qPCR of the pre-amplified cDNA was performed in a 192.24 Dynamic Array (Fluidigm, San Francisco, CA, USA) combining 192 pre-amplified samples with 24 primer sets in 4608 individual and simultaneous qPCR reactions. Sample mix consisted of 3 µL TaqMan Gene Expression Master Mix (Applied Biosystems), 0.3 µL 20X DNA Binding Dye Sample Loading Reagent (Fluidigm), 0.3 µL 20X EvaGreen (Biotium, VWR – Bie & Berntsen), 0.9 µL low EDTA TE Buffer (VWR – Bie & Berntsen) and 1.5 µL pre-amplified cDNA. The primer mixes consisted of 3 µL 2X Assay loading reagent (Fluidigm) and 3 µL of 20 µM forward and reverse primers. Control line fluid was injected into the 192.24 Dynamic array according to the manufacturer's instructions (Fluidigm) and the array was primed in the HX IFC controller (Fluidigm). Thereafter, 4.9 µL sample mix and 4.9 µL primer mix was dispensed into the appropriate inlets in the 192.24 Dynamic array and then reloaded into the HX IFC controller (Fluidigm) combining each of the 192 samples with the 24 primers in 4608 separate reaction chambers. The 192.24 Dynamic array was subsequently placed in the BioMark qPCR instrument (Fluidigm) and the following cycling parameters were used: 2 min at 50°C, 30 min at 80°C for thermal mix, 2 min at 50°C, 10 min at 95°C, followed by 35 cycles with denaturing for 15 s at 95°C and annealing/elongation for 1 min at 60 °C. Melting curves were generated after each run (from 60°C to 95°C, increasing 1°C/3 s).

2.7 *Data analysis and statistics*

To compensate for variation between dynamic chips, three highly stable samples were used as interplate calibrators. Then, the data were corrected for PCR efficiency for each primer. GeNorm and GeNormFinder were used to identify the most stable reference genes (B2M, PPIA) and the geometric mean of these was used to normalize all genes in GenEx5 (MultiD Analyses AB Sweden) (Andersen et al., 2004; Vandesomple et al., 2002). Technical repeats of cDNA was compared, and if the deviation was more than 1.5 Cq for more than 15% of a specific cDNA replicate or gene of interest, data were excluded from further analysis. The average of the technical repeats of cDNA was calculated and missing values (3 out of 1500, 0.2%) were replaced by the highest Cq measured on a sample added with 1. Finally, the Cq values were transformed to a linear scale and $\log_2$ transformed prior to partial least squares regression analysis (PLS).

2.8 *Prediction of bruise age*

Previously, two models (prediction-model nos. 1 and 2) for determining the age of bruises has been published (Fig. 2, Table 2) (Barington et al., 2017 and 2018). Prediction-model 1 and 2 were based on the gene expression data of 13 and 4 genes, respectively, in the subcutaneous fat from experimental bruises in pigs (Fig. 2, Table 2). Genes selected for the two prediction-models were involved in hemostasis, inflammation, tissue damage and repair and were selected based on descriptions of genes involved in wound healing and inflammation (Ackermann, 2007; Cecchi, 2010; Dressler et al., 1998; Dressler et al., 2000; Kondo and Ohshima, 1996). The prediction-models were created by carrying out PLS analysis with full cross validation on the gene expression data.

In the present study both prediction-models were used to predict the age of bruises from forensic cases based on mRNA expression data. The age of the bruises (group no. I) was predicted by prediction-model nos. 1 and 2 by carrying out PLS in Latentix 2.13 (Frederiksberg, Denmark). Two age predictions per bruise were obtained (one for each of the prediction-model nos. 1 and 2, respectively) and analyzed separately (Fig. 3). The average, minimum and maximum age for bruises in group no. I and the number of bruises with a predicted age below zero were found. In addition, the mean age difference between paired bruises (a and b) on the same pig was calculated.

3 Results

3.1 RNA quality

Subcutaneous fat tissue was sampled from 142 human inflicted bruises within 6 h after slaughter (group no. I) and from another 40 human inflicted bruises after the skin had been frozen for a longer period (group no. II) (Figs. 1, 3 and 4). Mean, minimum and maximum RIN are listed in Figs. 3 and 4. The RNA integrity was found to be highly dependent on the sampling procedure applied. RNA isolated from group no. I had a mean RIN of 7.0, whereas RNA isolated from group no. II had a mean RIN of 3.8.

3.2 mRNA expression

Based on assay efficiency and cDNA reproducibility mRNA expression data were obtained for 52.8% (75 out of 142 bruises, (38 paired bruises (a and b) from 19 pigs and 37 none paired bruises from 37 pigs)) and 7.5% (3 out of 40 bruises) of bruises sampled within 6 h after slaughter (group no. I) and after the skin had been frozen (group no. II), respectively (Figs. 3

and 4). The remaining samples were excluded due to high variation between cDNA replicates. Bruises sampled after freezing and long term storage (group no. II) were further excluded due to the low number of high quality samples (n = 3 bruises).

3.3 Age prediction (group no. I)

The average, minimum and maximum predicted age for bruises (n = 75) and the number of bruises with a predicted age below zero are presented in Table 3. In addition, the mean age difference between paired bruises (a and b, n = 19 pairs) on the same pig are presented in Table 3. Expression data of all genes and predicted age of each bruise can be found in Supplementary material 1.

4 Discussion

Gene expression profiles can assist in estimating the age of human inflicted bruises in slaughter pigs. However, as RNA is easily degraded, subcutaneous fat tissue must be sampled and stabilized in RNA stabilizing solution, (e.g. RNAlater) as fast as possible after slaughter and prior to freezing, if mRNA expression signatures are to be implemented in the forensic investigation of porcine bruises. In 92.5% of bruises sampled after the skin had been frozen (group no. II) degradation of the RNA was so advanced that samples had to be excluded for further analysis. The degradation of RNA was probably due to the long storage period at -18°C and thawing of the tissue before it was stabilized in RNAlater. In half of the bruises sampled and stabilized in RNAlater within 6 h after slaughter (group no. I), highly reproducible expression data of the selected genes were obtained, even though RNA was partly degraded (RIN between 4.9 and 8.3). This is in agreement with a previous study of the impact of RNA

integrity on qPCR performance where RIN above 5 was recommended for reliable mRNA quantification using qPCR (Fleige et al., 2006). In comparison, sampling of experimental bruises within 1 h post-mortem (RIN between 6.7 and 9.2) resulted in highly reproducible gene expression data in 98% of the bruises (Barington et al., 2018). If gene expression profiles are to be implemented in a forensic setting, all samples need to be of sufficient RNA quality to allow valid quantification of the genes of interest. Therefore, human inflicted bruises on slaughter pigs should be sampled and stabilized as fast as possible within few hours after being detected during the routine meat inspection at the slaughter line. Moreover, additional training of veterinarians carrying out meat inspection for optimal sampling technique will improve the number of non-degraded samples. Sampling and stabilization of tissue should be done using RNase free instruments and gloves, in order to protect the tissue from degradation by RNases. Moreover, sample thickness should not exceed 0.5 mm in order to allow a quick diffusion of the stabilization reagent into the tissue.

In the present study, the age of human inflicted bruises was less or equal to 9.5 h and 6.3 h estimated by prediction-model nos. 1 and 2, respectively. In accordance, 90.6% of human inflicted bruises have previously been estimated to be inflicted within a timeframe of 8 h from the time of slaughter (Barington and Jensen, 2013). In those cases, age estimates were based on histological evaluation of skin and underlying muscle tissue of the bruises (Barington and Jensen, 2018).

Prediction-model nos. 1 and 2 were based on experimental bruises on pigs with an average bodyweight of 30 kg and 100 kg, respectively. In these experimental bruises, mRNA expression of the selected genes (CCL2, FOS, ICAM1, IL-6, NFKB1, PTGS2, SELE and SELP) were up-regulated during the first 1-3 h and then decreased to or below normal levels within 4 to 10 h (Barington et al., 2017; Barington et al., 2018). Other genes (APOA1,

CFD, IFNA1, PLAT and TNFAIP3) were down-regulated and did not return to normal values within the first 10 h (Barington et al., 2017; Barington et al., 2018). Prediction-model nos. 1 and 2 specify the linear relationship between the age of bruises and the gene expression variables in subcutaneous fat from bruises being 1 to 10 h old and 2 to 8 h old, respectively. In few cases bruises might be inflicted more than 10 h prior to slaughter which might result in false age predictions. Therefore, the timing of bruises should never solely rely on gene expression patterns. Histological evaluation of the bruised tissue is crucial in order to ensure that the inflammatory response on the cellular level is in accordance with the age based on the mRNA expression pattern i.e., acute inflammation and no regenerative changes. Moreover, the prediction-models cannot distinguish between bruises inflicted by humans and accidental bruises e.g., inflicted by other pigs. Therefore, gross evaluation of the skin is necessary in order to state that the bruises were actually inflicted by humans (Fig. 5).

Predicted values of less than zero were found for 40% of bruises by prediction-model no. 1. In experimental bruises inflicted with varying forces and amount of kinetic energy, data showed a positive correlation between IL6 and CCL2 and the force of impact/amount of kinetic energy (Barington et al., 2017). Consequently, bruises with predicted ages less than zero might be inflicted with a higher force compared to the reference values based on experimental bruises. However, the experimental bruises, from which the prediction-models were calculated, were inflicted using a mechanical device applying strikes equal to that maximally applied by a man (Barington et al., 2017). Therefore, it seems unlikely that 40% of the human inflicted bruises were applied by an even higher force. The negative age predictions found by prediction-model no. 1 likely reflect age-related alterations in the innate immune system between experimental pigs weighing 30 kg and slaughter pigs weighing around 100 kg (Gruver et al., 2007).

Prediction-model no. 2 resulted in only 5.3% of bruises predicted to be less than 0 h old, thus this model seemed to work better on sample material from slaughter pigs. This was probably because the reference data originated from experimental pigs with an age and weight similar to the pigs from forensic cases. Further, prediction-model no. 2 had the least predicted values below zero and the lowest difference in predicted age of paired bruises (a and b) sampled from the same pig. In addition, prediction-model no. 2 had the lowest root mean square error of 0.92, meaning that in 95% of bruises, the model was able to predict the age with a precision of ±1.84 h (Barington et al., 2018).

In forensic cases, multiple bruises on pigs are assumed to have been inflicted within minutes, and therefore, the predicted ages of two bruises on the same pig are expected to be almost similar (Barington and Jensen, 2013; Barington et al., 2016). For prediction-model no. 2, the difference in paired bruise age (a and b) was less than 4 h for more than 90% of the paired bruises (Supplementary material 1). In comparison, age estimation based on selected histological criteria of the exact same paired bruises (a and b) were less consistent and resulted in similar age estimates in only half of the pigs due to variation in the histological interpretation of the inflammatory response (Barington et al., 2016). When handling forensic cases the mRNA expression profile of four genes (prediction-model no. 2) can potentially assist in estimating the age of human inflicted bruises if subcutaneous tissue is sampled and stabilized in a RNA stabilizing solution within few hours after slaughter.

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Fig. 1: Overview of sampling. Slaughter pigs with human inflicted bruises belonging to group nos. I and II. Within 6 h after slaughter, subcutaneous tissue was sampled from two separate bruises (a and b) on each pig in group no. I. From pigs in group no. II, skin with bruises was removed and frozen for up to 1 year before being sampled and stabilized in RNAlater for subsequent gene expression analysis.

Fig. 2: The genes of interest were selected based on two recent studies of experimental bruises in pigs (Barington et al., 2017; Barington et al., 2018). In subcutaneous fat tissue sampled from experimental bruises in pigs (30 kg) mRNA expression pattern of 42 genes were evaluated. The 42 genes were involved in hemostasis, inflammation, tissue damage and repair and were selected based on studies of wound healing and acute inflammation. The expression patterns of the 42 genes were able to group bruises according to age using principal component analysis (PCA) and partial least squares regression analysis (PLS). The grouping was mainly due to 13 specific genes as these had higher regression coefficients than the remaining genes. The expression data of the 13 selected genes were used to create a model to predict the age of bruises using PLS analysis with full cross validation (prediction-model no. 1). The experimental setup was repeated on 12 pigs (100 kg). Subcutaneous fat tissue was sampled from experimental bruises and the mRNA expressions of the same 13 genes were measured. The expression patterns of the 13 genes were able to group bruises according to age using PCA and PLS. The grouping was mainly due to IL6, NFKB1, SELE and SELP as these had higher regression coefficients than the remaining genes. The expression data of the 4 genes were used to create a model to predict the age of bruises using PLS analysis with full cross validation (prediction-model no. 2).

Fig. 3: Porcine bruises sampled within 6 h after slaughter (group no. I). Information regarding the total number of bruises and the number of bruises from which highly reproducible

expression data were obtained (Included) or not (Excluded). Reproducible mRNA expression data were obtained for 75 bruises (38 paired bruises (a and b) from 19 pigs and 37 none paired bruises from 37 pigs). Based on the reproducible mRNA expression data, the age of bruises was predicted by prediction model nos. 1 and 2, i.e. two age predictions per bruise were calculated (one for each prediction-model) and analyzed separately. Moreover, the mean and minimum to maximum RNA integrity numbers (RIN) are presented.

Fig. 4: Porcine bruises sampled after freezing of the skin for up to 1 year (group no. II). Information regarding the total number of bruises and the number of bruises from which highly reproducible expression data were obtained (Included) or not (Excluded). Reproducible mRNA expression data were obtained for only 3 out of 40 bruises (7.5%) and no age-predictions were carried out. In addition, the mean and minimum to maximum RNA integrity numbers (RIN) are presented.

Fig. 5: Handling of forensic cases. All forensic cases regarding bruises in pigs should be subjected to a gross evaluation in order to determine if bruises are inflicted by humans. Bruises inflicted by humans are located on the back of the pigs and are often multiple with a uniform pattern reflecting the object used for infliction. Combining the mRNA expression of IL6, NFKB1, SELE and SELP is suitable for predicting the age of bruises being up to 8 h old using partial least squares regression analysis. Histological evaluation of the bruised tissue is crucial in order to ensure that the inflammatory response on the cellular level is in accordance with the age based on the mRNA expression pattern.

Supplementary file 1: Gene expression data (log_2-transformed values) and the age in hours of bruises predicted by prediction-model nos. 1 and 2 (Excel sheet nos. 1 and 2). The percentage

of paired bruises (a and b, n=19 pairs) with a difference in predicted age of less than 1, 2, 3, 4 and 5 h, for predictionmodel nos. 1 and 2 (Excel sheet no. 3).

Table 1: In total, 13 genes of interest and 6 reference genes were evaluated. Gene symbols, gene names, selected GO-terms, forward (F) and reverse (R) primer sequences and GenBank accession numbers are presented.

Gene symbol	Gene name	Selected GO-term	Sequence (5' to 3')	GenBank accession no.
APOA1	Apolipoprotein A1	Lipid metabolic process	F:GTTCTGGGACAACCTGGAAA R:GCTGCACCTTCTTCTTCACC	NM_214398.1
CCL2	C-C motif chemokine ligand 2	Chemotaxis	F:CTTCTGCACCCAGGTCCTT R:CGCTGCATCGAGATCTTCTT	NM_214214.1
CFD	Complement factor D	Complement activation	F:CCTCGGAGCAGCTGTATGT R:ATGCCATGTAGGGTCTCTCG	XM_013994801.1
FOS	C-fos proto-oncogene	Inflammatory response	F:CTCCAAGCGGAGACAGACC R:CTTCTCCTTCAGCAGGTTGG	NM_001123113.1
ICAM1	Intracellular cell adhesion molecule 1	Leukocyte cell-cell adhesion	F:AAGCTTCTCCTGCTCTGCTG R:GGGGTCCATACAGGACACTG	NM_213816.1
IFNA1	Interferon α1	Innate immune response	F:ATCGTCAGGGCAGAAGTCAT R:CAGGTGTCTGTCACTCCTTC	NM_214393.1
IL6	Interleukin 6	Inflammatory response	F:TGGGTTCAATCAGGAGACCT R:CAGCCTCGACATTTCCCTTA	NM_214399.1
NFKB1	Nuclear Factor Kappa β Subunit 1	Inflammatory response	F:CTCGCACAAGGAGACATGAA R:GGGTAGCCCAGTTTTTGTCA	NM_001048232.1
PLAT	Plasminogen activator	Blood coagulation	F:TGCTTCCAGGAGAGGTTCC R:CTCTCCAGGGACCAGCCTAT	NM_214054.1
PTGS2	Prostaglandin-Endoperoxide Synthase 2	Inflammatory response	F:GAACTTACAGGAGAGAAGGAAATGG R:TTTCTACCAGAAGGGCAGGA	NM_214321.1

SELE	Selectin E	Leukocyte cell-cell adhesion	F:GGATGCTGCCTACTTGTGAAG R:CAGGAGCCAGAGGAGAAATG	NM_214268.2
SELP	Selectin P	Leukocyte cell-cell adhesion	F:CCTAGCAGGGCCATTGAC R:CCCACCCATCACTAAACCTG	NM_214078.1
TNFAIP 3	TNF α Induced Protein 3	Inflammatory response	F:CCCAGCTTTCTCTCATGGAC R:TTGGTTCTTCTGCCGTCTCT	NM_001267890 .1
ACTB	Actin β	Reference gene	F:CTACGTCGCCCTGGACTTC R:GCAGCTCGTAGCTCTTCTCC	DQ452569.1
B2M	Beta-2-Microglobulin	Reference gene	F:TGAAGCACGTGACTCTCGAT R:CTCTGTGATGCCGGTTAGTG	NM_213978.1
HPRT1	Hypoxanthine phosphoribosyl-transferase I	Reference gene	F:ACACTGGCAAAACAATGCAA R:TGCAACCTTGACCATCTTTG	NM_001032376 .2
PPIA	Peptidylprolyl isomerase A (cyclophilin A)	Reference gene	F:CAAGACTGAGTGGTTGGATGG R:TGTCCACAGTCAGCAATGGT	NM_214353.1
RPL13A	Ribosomal protein L13a	Reference gene	F:ATTGTGGCCAAGCAGGTACT R:AATTGCCAGAAATGTTGATGC	XM_013998640 .2
TBP	TATA box binding protein	Reference gene	F:ACGTTCGGTTTAGGTTGCAG R:CAGGAACGCTCTGGAGTTCT	DQ178129.1

Table 2: Overview of prediction-model nos. 1 and 2 regarding the genes of interest, reference genes, 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), experimental bruise age, number and body weight (BW) of pigs. All experimental bruises were inflicted with a force or 6.52 N/mm^2. Prediction-model no. 1 had a RMSE of 1.06 h meaning that in 95% of bruises, the model no. 1 was able to determine the age with a precision of ±2.12 h.. Prediction-model no.2 had a RMSE of 0.92 h meaning that in 95% of bruises, the model was able to determine the age with a precision of ±1.84 h.

	Prediction-model no. 1 (Barington et al., 2017)	Prediction-model no. 2 (Barington et al., 2018)
Genes of interest	APOA1, CCL2, CFD, FOS, ICAM1, IFNA1, IL6, NFKB1, PLAT, PTGS2, SELE, SELP, TNFAIP3	IL6, NFKB1, SELE, SELP
Reference genes	ACTB, B2M, HPRT1, PPIA, RPL13A, TBP	B2M, HPRT1, PPIA
RMSE	1.06 h	0.92 h
SEP	1.11	0.93
Bias	0.09	0.002
r^2	0.87	0.86
Exp.var.Y	90%	87%
Exp.var.X	78%	83%
Bruise age	1 to 10 h	2 to 8 h
No. of pigs	10	12
BW	30 kg	100 kg

Table 3: The mean, minimum (min) to maximum (max) predicted age of bruises (n = 75) and bruises predicted to be ≤ 0h based on prediction-model nos. 1 and 2. The mean difference in predicted age between two paired bruises (a and b) sampled on the same pig is presented, too.

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

Highlights

- Age estimation of porcine bruises based on mRNA expression data was evaluated.
- Subcutaneous fat tissue was sampled from human inflicted bruises on pigs.
- mRNA expression of four genes resulted in plausible age estimates of bruises on pigs.
- Tissue should be stabilized within hours after slaughter to preserve mRNA.

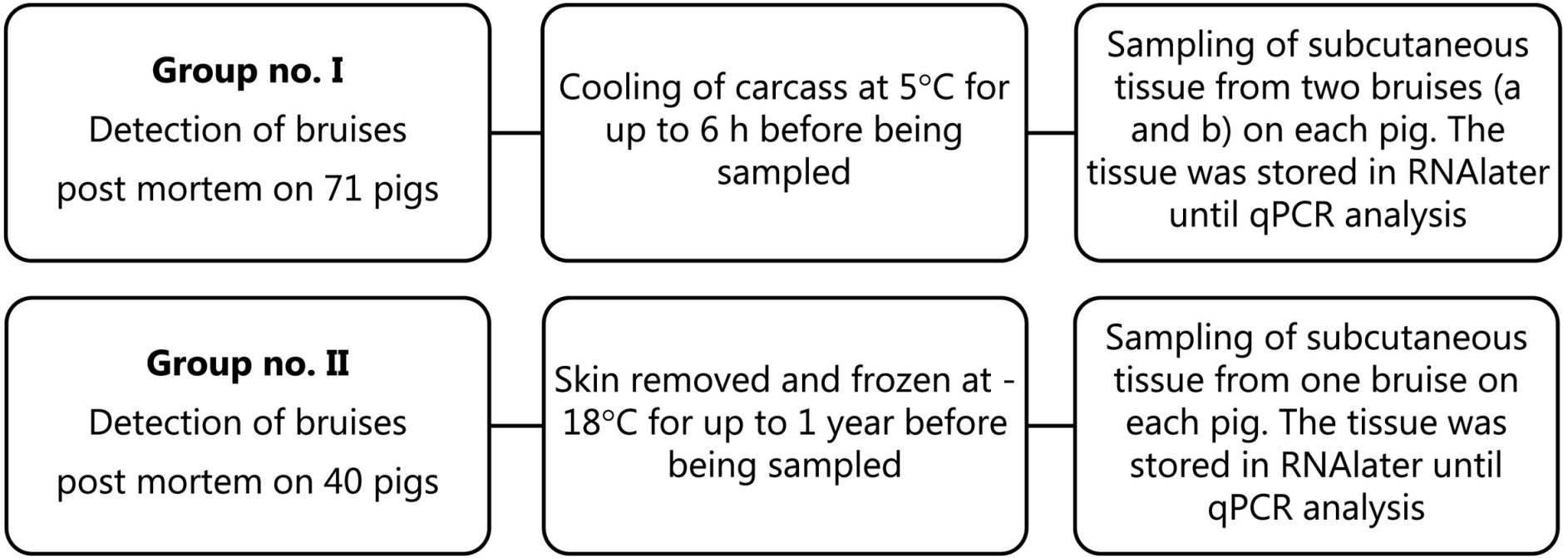
Group no. I
Detection of bruises post mortem on 71 pigs
Cooling of carcass at 5°C for up to 6 h before being sampled
Sampling of subcutaneous tissue from two bruises (a and b) on each pig. The tissue was stored in RNAlater until qPCR analysis
Group no. II
Detection of bruises post mortem on 40 pigs
Skin removed and frozen at -18°C for up to 1 year before being sampled
Sampling of subcutaneous tissue from one bruise on each pig. The tissue was stored in RNAlater until qPCR analysis

Figure 1

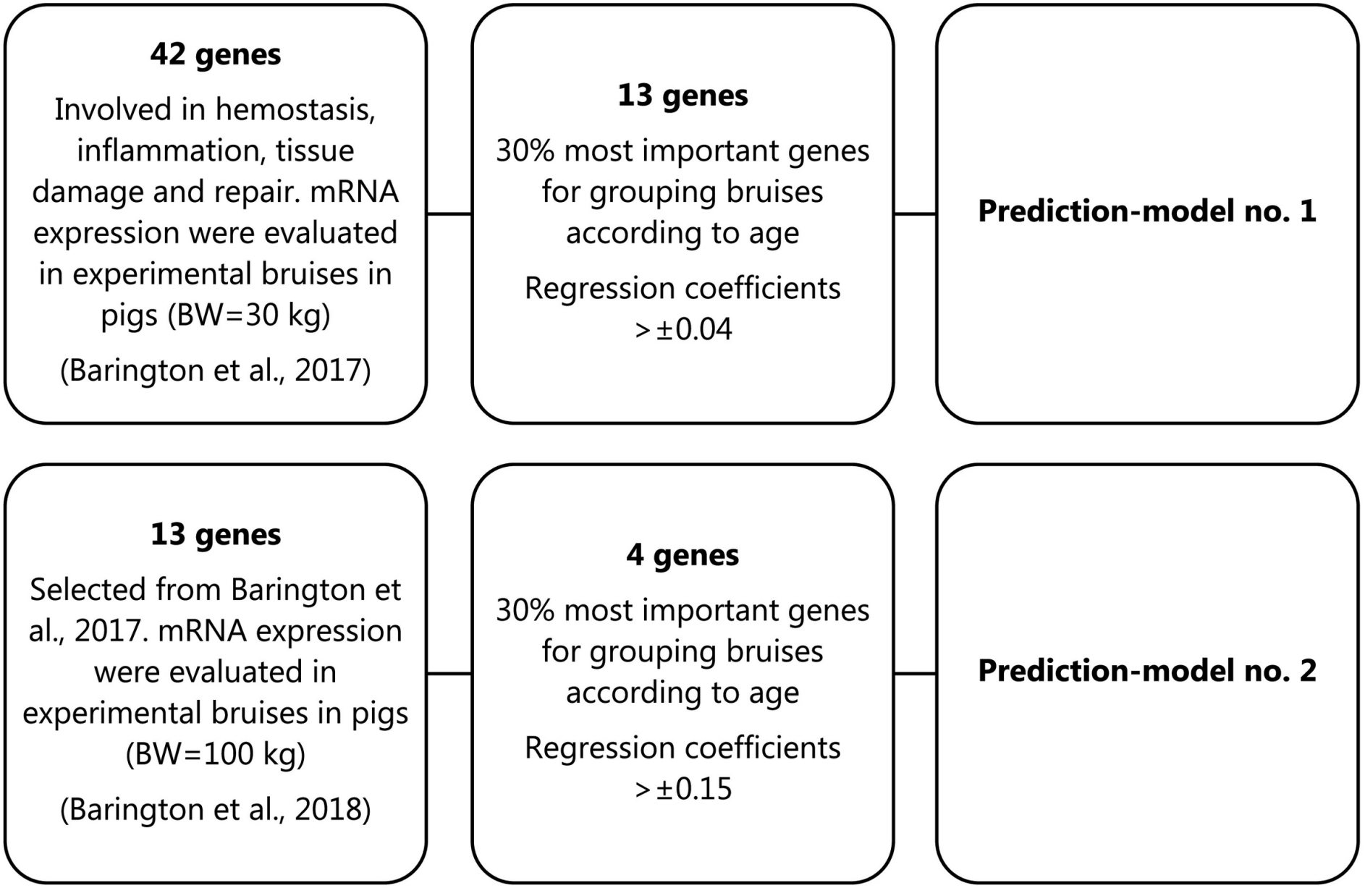
42 genes
Involved in hemostasis, inflammation, tissue damage and repair. mRNA expression were evaluated in experimental bruises in pigs (BW=30 kg)
(Barington et al., 2017)
13 genes
30% most important genes for grouping bruises according to age
Regression coefficients > ±0.04
Prediction-model no. 1
13 genes
Selected from Barington et al., 2017. mRNA expression were evaluated in experimental bruises in pigs (BW=100 kg)
(Barington et al., 2018)
4 genes
30% most important genes for grouping bruises according to age
Regression coefficients > ±0.15
Prediction-model no. 2

Figure 2

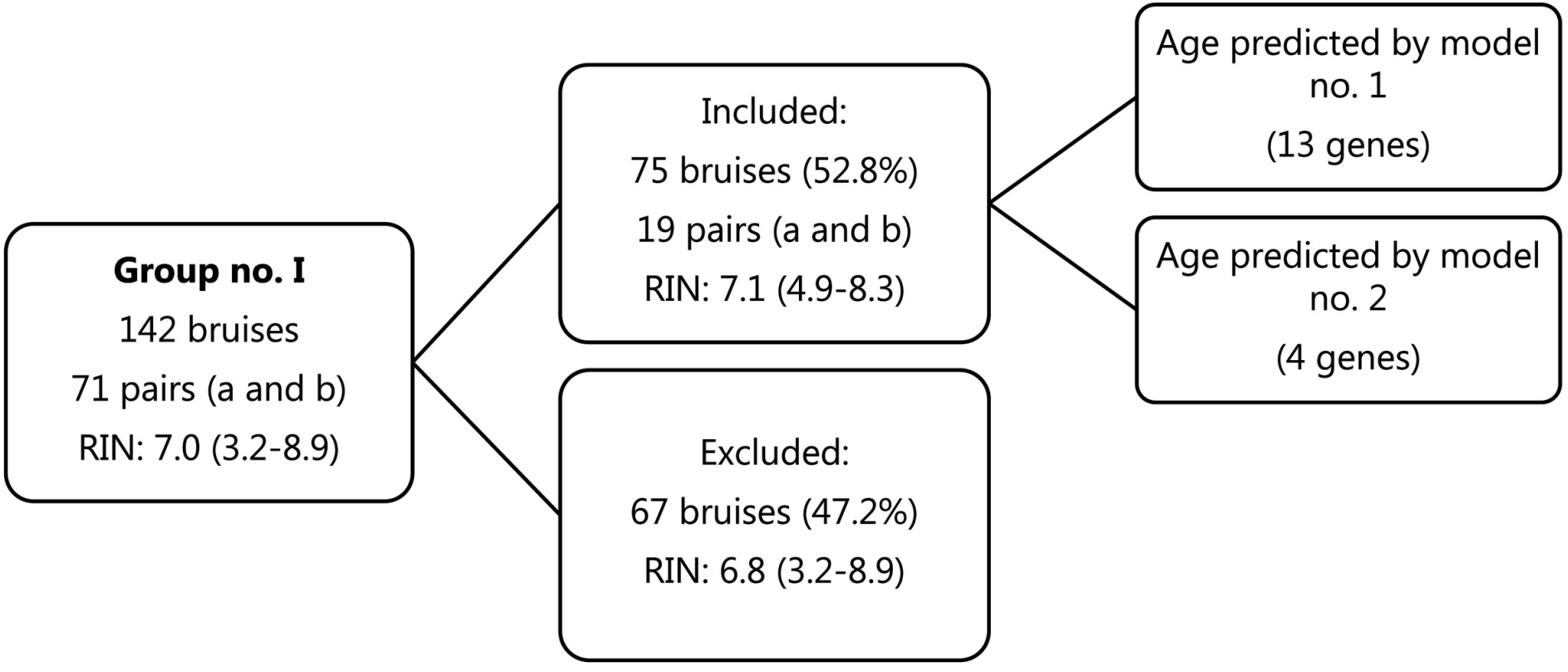
Group no. I
142 bruises
71 pairs (a and b)
RIN: 7.0 (3.2-8.9)
Included:
75 bruises (52.8%)
19 pairs (a and b)
RIN: 7.1 (4.9-8.3)
Excluded:
67 bruises (47.2%)
RIN: 6.8 (3.2-8.9)
Age predicted by model no. 1
(13 genes)
Age predicted by model no. 2
(4 genes)

Figure 3

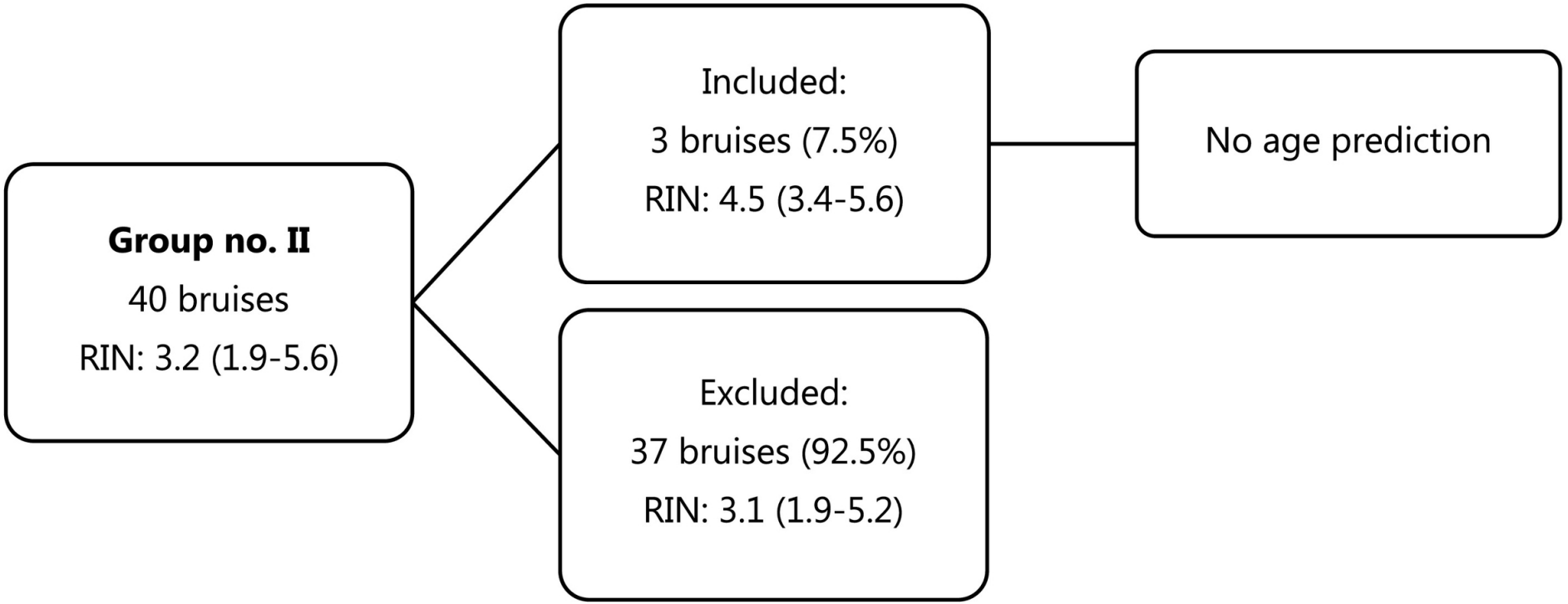
Group no. II
40 bruises
RIN: 3.2 (1.9-5.6)
Included:
3 bruises (7.5%)
RIN: 4.5 (3.4-5.6)
No age prediction
Excluded:
37 bruises (92.5%)
RIN: 3.1 (1.9-5.2)

Figure 4

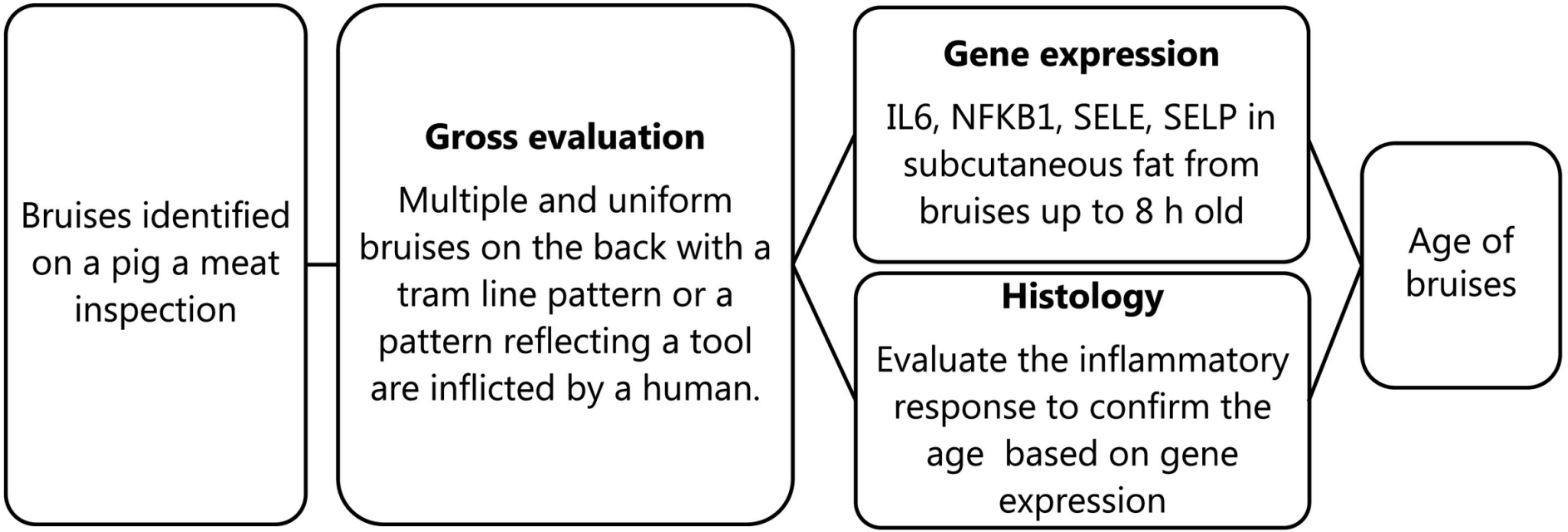
Bruises identified on a pig a meat inspection
Gross evaluation
Multiple and uniform bruises on the back with a tram line pattern or a pattern reflecting a tool are inflicted by a human.
Gene expression
IL6, NFKB1, SELE, SELP in subcutaneous fat from bruises up to 8 h old
Histology
Evaluate the inflammatory response to confirm the age based on gene expression
Age of bruises

Figure 5