DOI: 10.1093/jas/skaa153

Original Article

Salivary miRNAs as pain biomarkers in piglets

Salivary miRNAs are potential biomarkers for the accurate and precise identification of inflammatory response after tail docking and castration in piglets

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Abstract

The present study aimed to investigate whether acute pain associated with castration and tail docking of male piglets may modulate the expression of salivary miRNAs and to explore their potential use as biomarkers. Thirty-six healthy piglets (Hermitage x Duroc) 4 days old were randomly assigned to three groups: the first group (12 piglets) has been pre-treated with anesthetic and anti-inflammatory drugs (ANA) and then castrated and tail docked; the second one (12 piglets) has been castrated and tail docked without any drugs (CONV); the third one (12 piglets) has been only handled (SHAM). Saliva was collected 10 minutes before (control group) and 30-45 minutes after procedures. Salivary cortisol has been quantified. The expression concentrations of seven miRNAs, namely miR-19b, miR-27b-3p, miR-215, miR-22-3p, miR-155-5p, hsa-miR-365-5p and hsa-miR-204, were measured and assessed as potential biomarkers of pain by qPCR using TaqMan® probes. The area under the receiver operating curve (AUC) was used to evaluate the diagnostic performance of miRNAs.

The concentration of salivary cortisol increased after treatment in CONV and ANA, while no significant variation was observed in the SHAM group. The comparative analysis demonstrated that the concentrations of salivary miR-19b ($P = 0.001$), miR-27b ($P = 0.042$) and miR-365 ($P < 0.0001$) were significantly greater in CONV as compared to pre-treatment. The Area Under the Curve (AUC) of pre-treatment *vs* CONV and CONV *vs* ANA were excellent for miR-19b and miR-365 and fair for miR-27b. Combining two miRNAs, namely miR-19b and miR-365, in a panel increased the efficiency of distinguishing between pre- and post-treatment groups. No differences have been identified between SHAM and ANA groups. mRNA potential targets of DE-miRNA were investigated, and genes related to pain and inflammation were identified: miR-19b potentially modulates TGF-beta and focal adhesion pathways, miR-365 regulates cytokines expression (i.e. IL-1, TNF-alpha and IL-8 cytokine) and miR-27b macrophage inflammatory protein pathways (i.e. MIP1-beta). In conclusion, we

demonstrated that the abundance of miR-19b, miR-27b and miR-365 increases in the saliva of piglets castrated and tail docked without the administration of pain-relieving drugs. Further studies are needed to assess their potential during routine husbandry procedures, and to extend their assessment in other stressful events, such as weaning or chronic pain.

Keywords **:** microRNA, pain, non-invasive biomarkers, piglets

Abbreviations

ANA: piglets castrated and tail docked with pre-treatment with anesthetic and anti-inflammatory drugs

CONV: piglets castrated and tail docked without any drugs

SHAM: piglets only handled

miRNA: microRNA

DE-miRNA: differentially expressed microRNA

Introduction

Tail docking and castration of male piglets are routine husbandry procedures, which aim to reduce the damage caused by tail biting and the undesirable sexual behaviour, making males less aggressive and easier to manage. In addition, castration avoids the boar taint in typical products, such as Italian Protected Designation of Origin hams, which is produced with heavy mature pigs. These procedures are traditionally performed without anaesthesia or analgesia, which are expensive and time consuming. Tail docking and castration have become a significant animal welfare concern in recent years causing stress, pain and discomfort for the piglets. The European Declaration on the Alternatives to Pig Castration (European Declaration, 2010) (https://ec.europa.eu/food/animals/welfare/practice/farm/pigs/castration_alternatives_en), establishing that from 1 January 2012 surgical castration of pigs should be performed with prolonged analgesia and/or anesthesia was voluntarily signed by many organizations. A wide range of pain assessment measures has been investigated in pigs, such as behavioural indicators, including posture change (Edwards et al., 2009), behaviour score, Piglet Grimace Scale (di Giminiani et al., 2016) and physiological parameters, such as cortisol/adrenocorticotrophic hormone (Prunier et al., 2005). Multidisciplinary approaches based on the simultaneous measuring of both behavioural and physiological indicators are the most promising and wider procedures used to assess the impact of the painful condition in piglets (Ison et al., 2016).

The microRNAs (miRNAs) collected from body fluids, such as saliva, have potential as diagnostic molecules (Wang et al., 2017). MiRNAs play a pivotal role in the orchestration of cellular homeostasis and their dysregulation was also associated with increased stress and pain (Lecchi et al., 2016; 2018). Several studies have been carried out on blood miRNA in pigs, to study the pathogenesis of infectious diseases (Huang et al., 2011; Hansen et al., 2016; Fleming and Miller, 2018; Bao et al., 2015) or stressful events, such as weaning for example (Tao et al., 2016; Zhang et al., 2019) for investigating pathogenesis or in pigs, targeting infectious diseases

The knowledge about the expression of salivary miRNAs in livestock saliva is limited. Saliva is a complex fluid, the composition of which is not constant and changes follow the daily cycle, diet and health status. Saliva is considered the mirror of the body, since it is a biofluid that filters and processes itself from the vasculature, nourishing the salivary glands into the oral cavity and reflecting virtually the entire spectrum of normal and disease states (Lee and Wong, 2009). Approximately 40% of biomarkers suggested for diseases, such as cancers, cardiovascular disease and stroke are found in the whole saliva as well (Loo et al., 2010). Collecting saliva is a less invasive procedure than blood collection. Therefore, exploring the diagnostic potential of saliva should be a priority to improve pain mitigation strategies, thus improving animal welfare . To the best of authors' knowledge, no information about microRNA presence in pig saliva is available.

Considering the diagnostic potential of miRNAs and the evidence of their identification in the porcine genome (Martini et al., 2014), tissues (Hou et al., 2016; Q. Wang et al., 2017) and serum (Hansen et al., 2016; 2018), the aims of the present study were to a) ascertain whether acute pain associated with castration and tail docking may modulate the expression of salivary miRNAs; b) investigate the potential use of differentially expressed (DE)-miRNAs as biomarkers to measure pain in pigs aiming to provide parameters capable to measure as finely as possible the pain that this procedure causes to the animals; and c) integrate miRNAs to their target genes and their relative biological processes.

Materials and methods

Animals husbandry and procedures

The procedures were carried out during routine surgical castration and tail docking. The protocol for care, handling, and sampling of animals defined in the present

114 study was reviewed and approved by the Università degli Studi di Milano Animal
115 Care and Use Committee (protocol no. 97/18).

116 The study was carried out at a sow farrow commercial pig farm, following a batch management
117 production system. Thirty-six piglets (Hermitage x Duroc) 4 days old, clinically healthy and with
118 homogeneous weight were enrolled. Piglets were randomly assigned to three experimental groups
119 (Table 1): group ANA including 12 piglets castrated and tail docked with pre-treatment with anesthetic
120 and anti-inflammatory drugs; group CONV including 12 piglets castrated and tail docked without any
121 drugs; group SHAM including 12 piglets only handled. The experimental design included a control
122 group, consisting of all thirty-six piglets before performing the procedures. The anaesthetic approach
123 has been selected based on previous studies (Keita et al., 2010; Hansson et al., 2011; Kluivers-Poodt et
124 al., 2013; Bonastre et al., 2016). Anesthetic procaine hydrochloride (40.0 mg and adrenaline tartrate
125 0.036 mg/mL) and meloxicam (0.4 mg/kg) was administrated according to the information sheets.
126 Briefly, meloxicam was administered intra-muscular (IM) in the neck, while the anesthetic was
127 administered with an insulin syringe in each testicle (0.125 ml/testicle). Piglets assigned to
128 SHAM group were handled in the same way than others, without castration and tail docking.

129 Castration was performed according to the farm practice. Briefly, the piglet was grabbed
130 by the back legs and hold in an upside-down position. Holding the testicle, the scrotum was
131 incised with a scalpel and the testicle was pushed out through the incision, then the testicles and
132 attached cord were pulled and discarded. The operation was repeated for the other testicle. The
133 incision was left open and sprayed with a disinfectant (oxytetracycline hydrochloride spray, 74-
134 148 mg/capo/die) (Allen D. Leman, 1992). Tail docking was performed at 12-18 mm from the
135 base of the tail with a gas-heated iron for tail docking to seal the wound as soon as possible.

136

137 *Saliva collection*

138 Saliva was collected between 8 am and 12 am, 10 minutes before and 30-45 minutes
139 after castration and tail docking (Table 1). Sampling was carried out in the farrowing room to

allow the piglets an olfactory and vocal contact with their mother. Saliva was collected using specialized salivary tubes (Salivette®, Sarstedt, Nümbrecht, Germany) keeping the animals in standing position and promoting salivation with 2-3 drops of 5% citric acid with a plastic Pasteur pipette (Gallagher et al., 2002). The cotton roll was kept in the piglet mouth allowing the animal to chew it for 2-3 minutes followed by centrifugation of sponge-containing Salivette® tubes at 1000 x g for 15 min to collect saliva. If during collection the sponge was spat out, a new one was used. Samples were kept at 4-8°C and delivered to the laboratory within 8 hours. Saliva was then transferred to cryovials, frozen in liquid nitrogen and then stored at -80°C.

Salivary cortisol quantification

The saliva's cortisol concentration was measured using a solid-phase, competitive chemiluminescent enzyme immunoassay, the Immulite 1000 Cortisol (catalog number LKC01, Medical System, Genova, Italy), validated for determinations in porcine saliva (Escribano et al., 2012). An increase in concentration was interpreted as a positive stress response (Hellhammer et al., 2009).

MiRNAs extraction and real-time quantitative PCR

Total RNA was extracted from saliva using miRNeasy Serum/Plasma Kit (Qiagen, catalogue number 217184, Milan, Italy). An aliquot of 50 µl per sample was transferred to a new tube and 1 ml of Qiazol (Qiagen) was added. The *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen, catalogue number 219610) was used as synthetic spike-in control because of a lack of sequence homology to swine miRNAs. After incubation at room temperature for 5 min, 3.75 µl (25 fmol final concentration) of spike-in control was added and the samples were vortexed to ensure complete mixing. The RNA extraction was then carried out according to the

manufacturer's instruction. The reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, catalogue number 4366596, Monza, Italy) using miRNA-specific stem-loop RT primers, according to manufacturer's instructions. Reverse transcription reactions were performed in 15 µl volume reactions containing 1.5 µl 10X miRNA RT buffer, 1 µl MultiScribe reverse transcriptase (50 U/µl), 0.30 µl 100 mM dNTP mix, 0.19 µl RNase Inhibitor (20 U/µl), 6 µl of custom RT primer pool and 3.01 µl of nuclease-free water. The custom RT primer pool was prepared by combining 10 µl of each 5X RT primer in a final volume of 1000 µl; the final concentration of each primer in the RT primer pool was 0.05X each. Three µl saliva RNA was added to each RT reaction. Every RT reaction mixture was incubated on ice for 5 minutes, 16°C for 30 minutes, 42°C for 30 minutes and then 85°C for 5 minutes.

The qPCR experiments were designed following the MIQE guidelines. Small RNA TaqMan assays were performed according to the manufacturer's instruction. The selection of miRNAs was based on previous publications in which these miRNAs were found to be related to pain in humans and mice (Heyn et al., 2016; Wang et al., 2016; Pan et al., 2016; Sakai et al., 2017; Wu et al., 2018). The selected primer/probe assays (Life Technologies, Monza, Italy) included cel-miR-39-3p (assay ID000200), hsa-miR-19b (assay ID000396), hsa-miR-27b-3p (assay ID000409), hsa-miR-215 (assayID000518), has-miR-22-3p (assay ID000398), has-miR-155-5p (assay ID000479), has-miR-204 (assay ID000508), has-miR-365-3p (assay ID001020). Quantitative reactions were performed in duplicate in scaled-down (12 µl) reaction volumes using 6 µl TaqMan 2X Universal Master Mix II (Applied Biosystems, catalogue number 4440044), 0.6 µl miRNA specific TaqMan Assay 20X and 1µl of the RT product per reaction on Eco Real-Time PCR detection system (Illumina, San Diego, CA, USA). The standard cycling program was 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Data were normalized relative to the expression of cel-miR-39. MiRNA

expression concentrations are presented in terms of fold change normalized to cel-miR-39 expression using the formula $2^{-\Delta\Delta Cq}$. The targets of the significant miRNA were determined from the TargetScan database (http://www.targetscan.org/vert_71/), functional enrichment of the mRNA was performed using the DAVID bioinformatics resources (https://david.ncifcrf.gov/) and biological pathways in the KEGG were examined for enrichment (http://www.genome.jp/kegg/).

Statistical analysis

Statistical analysis was performed using XLStat software (Addinsoft SARL, Paris, France). Statistical significance was accepted at $P \leq 0.05$. Data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene test, respectively. As data were not normally distributed, non-parametric statistical tests were applied. The nonparametric Wilcoxon signed-rank test for paired samples and Kruskal-Wallis test were used to assess differences in cortisol and miRNA concentrations between groups, respectively. Match paired Wilcoxon test was run to compare miRNA concentrations pre- and post-treatment. Receiver Operating Characteristic (ROC) analysis was performed to determine the diagnostic accuracy of targets. The diagnostic values were calculated for miR-19b, miR-27 and miR-365 alone and in combination. The ROC analysis was carried out by plotting the true positive (sensitivity) versus the false positive (1-specificity). The definitions of the relationship between the area under the curve and diagnostic accuracy were as previously reported (Šimundić, 2009).

Results

Salivary cortisol increased in CONV and ANA groups.

Large variability in cortisol concentration between individual piglets was measured (Figure 1). The cortisol concentration in the saliva of piglets increased after treatment in all

groups. In detail, the median values of CONV (1.312 μg/dL; *P*= 0.01) and ANA (0.972 μg/dL; *P*= 0.043) groups were significantly higher than median value of pre-treated group (0.629 μg/dL). No significant variation was observed in SHAM group (0.651 μg/dL; *P*= 0.83) (Figure 1).

Castration and tail docking without drugs alters expression amounts of miR-19b, miR-27b, and miR-365 in the saliva of piglets

Small RNA was extracted from all collected samples, except for one saliva sample of the CONV group, the volume of which was not sufficient to carry out the extraction. MiRNA were normalized to the concentration of the artificial spike-in cel-miR-39, which was used as internal control and as reference miRNA. Four miRNAs, namely miRNA miR-215, miR-22-3p, miR-155-5p, and miR-204 were not found in saliva and thus were excluded from further analysis. Three miRNA, namely miR-19b, miR365p and miR-27b, were detected in all samples and then subjected to further analysis. The comparative analysis demonstrated that 3 salivary miRNAs, namely miR-19b, miR-27b and miR-365 had a significant differential expression (DE) in the saliva of piglets (Figure 2). CONV expressed a higher level of miR-19b, miR-27b and miR-365 than pre-procedures. The amounts of miR-19b and miR-365 were also higher in CONV, as compared with ANA. In details, the abundance of in CONV saliva miR-19b was increased 10.7 (*P*= 0.001) and 19.9 (*P*= 0.002) folds more than controls and ANA, respectively. The abundance of miR-365 was increased to 3.7 (*P*<0.001) and 6.7 (*P*= 0.033) folds as compared to controls and ANA, respectively. Finally, miR-27b was increased in the CONV group compared to controls (6.9 folds; *P*=0.042).

Diagnostic performance of miR-19b and miR-365 alone or in combination discriminated between groups with different treatments.

To analyze the diagnostic value of DE-miRNAs in saliva, ROC curve analysis was performed and the associated area under the curve (AUC) was used to confirm the diagnostic potency of each miRNA. Cut-off points were set to maximize the sum of sensitivity and specificity (Table 2).

The ability of miRNAs to separate the tested samples into those with different treatments is defined diagnostic accuracy and is measured by the area under the curve (AUC), where an area of 1 represents a perfect test; an area of 0.5 represents a worthless test. The ability to discriminate control group and CONV was excellent for miR-19b (AUC: 0.925; 95% CI 0.769-0.989; $P< 0.0001$) and miR-365 (AUC:0.919; 95% CI 0.760-0.987; $P< 0.0001$), and good for miR-27 (AUC:0.770; 95% CI 0.581-0.903; $P= 0.0149$) (Table 2; Figure 3). Thus, miR-19b and miR-365 can well discriminate between animals castrated without analgesics and pre-castrated group. The AUC of CONV *versus* ANA was excellent for miR-19b (AUC:0.946; 95% CI 0.699-0.999; $P< 0.0001$) and miR-365 (AUC:0.968; 95% CI 0.742-1; $P< 0.0001$), and good for miR-27b (AUC: 0.750; 95% CI 0.466-0.931; $P= 0.0639$) (Table 2; Figure 3). Thus, miR-19b and miR-365 can well discriminate between animals castrated without and with analgesics. The ability to discriminate SHAM group and ANA was good or sufficient for all DE-miRNAs (Table 2).

Further statistical analysis was performed considering the average relative quantification (RQ) values of DE-miRNAs with excellent AUC, namely miR-19b and miR-365. The AUCs of control group *versus* CONV and CONV *versus* ANA were excellent, 0.919 (95% CI 0.760-0.987; $P< 0.0001$; sensitivity 85.71% and specificity 82.61%, cut-off 0.012) and 0.964 (95% CI 0.735-1; $P< 0.0001$; sensitivity 100% and specificity 87.5%, cut-off 0.0063), respectively (Figure 3).

DE-miRNAs potential modulate TGF-beta signalling pathway.

Predicted gene targets of the significant DE-miRNAs were computationally retrieved from the TargetScan database. Only gene targets with a cumulative weighted context score (CWCS), that measures the target thresholds, of < -0.4 were included in the miRNA-gene interaction network. The mRNA enrichment was performed using the DAVID bioinformatics resources, to explore the function of these candidate biomarkers. The Gene Ontology analysis was carried out using DAVID at three different levels: Molecular Function, Cellular Component and Biological Process. Figure 4A illustrates the top 10 items that were significantly enriched by target genes for each of the above Gene Ontology levels. The enriched Gene Ontology terms in Molecular Function mainly included genes involved in binding of DNA and RNA, zinc ion and clathrin. The Cellular Component items were associated with the hallmarks of a cell: nucleoplasm, cytoplasm, Golgi apparatus, nucleus and apical part of the cell. Most Gene Ontology Biological Process items converged on the modulation of protein shelf life, gene expression and axon guidance. The KEGG pathway analysis was performed on the whole targets of DE-miRNAs and Figure 4B outlines the significantly enriched pathways, among which TGF-beta signalling pathway, focal adhesion and platelet activation were at the top.

Discussion

To the best of the knowledge of the authors, the presence of microRNA in pig saliva had not been detected. The present study demonstrated for the first time the relationship between the abundance of salivary miRNA and the insurgency of pain and stress related to surgical castration and tail docking in piglets. We found that the concentration of miR-19b, miR-365 and miR-27b is significantly upregulated in castrated and dock tailed piglets. These effects are mitigated by the pre-emptive administration of anaesthetic drugs. The results reported in the present study suggest that salivary concentrations of miR-19b and miR-365 were

effective in accurately differentiating piglets affected by acute pain from both controls and animals treated with local anesthetics (ANA group), as shown by ROC analysis. The KEGG analysis demonstrated that DE-miRNAs are involved in inflammation and pain development by directly targeting mRNAs coding for proteins involved in TGFβ pathways and focal adhesion. To assess stress responses in piglets, salivary cortisol, which reflects only the free active fraction of cortisol, has been quantified as well. Salivary cortisol concentration showed a large variability between piglets, as previously reported (Ott et al., 2014; Martínez-Miró et al., 2016). Our results are in line with cortisol level quantified using different stressor models (Escribano et al., 2012; Contreras-Aguilar et al., 2019), indicating that castration is a stressful event for piglets compared with handling alone and that analgesia/anesthesia did not affect cortisol level, as previously reported on blood cortisol concentration (Numberger et al., 2016).

Practices routinely carried out on piglets, such as castration and tail docking, are regarded as painful. Although many of European stakeholders committed themselves to stop surgical castration by 2018, however, 75% of male pigs are still surgically castrated in the EU (De Briyne et al., 2016). Moreover, the stakeholders of some countries, including Denmark and Eastern Europe countries (De Briyne et al., 2016), envisage that the surgical castration of male pigs without anesthesia or analgesia is not an issue (Bonneau and Weiler, 2019). The efficacy of current pain mitigation procedures is poorly understood and depends on an accurate measurement of pain, which has become a significant animal welfare concern in husbandry management. Surgical castration using analgesia and anesthesia includes all the advantages of surgical castration and also improved welfare, meaning no or less pain during and after surgery. Some disadvantages include lower feed efficiency, higher environmental impact and increased costs associated with the application of pain relief (De Briyne et al., 2016). Saliva has been identified as a potential source of miRNAs, being not invasive and reflecting the physio-pathological condition of the individual. By finding that microRNAs are differently abundant

315 in animals subjected to stress related to castration and dock tailing, we provide information that
316 will allow for a more accurate pain assessment during routine husbandry procedures. These
317 results provide also the background for the use of miRNAs to assess the mitigation effect of
318 pain management procedures and drugs in pigs, an issue that is still debated (Dzikamunhenga
319 et al., 2014).

320 The finding that miR-19b, miR-365 and miR-27b were more abundant in animals where
321 the pain related to surgery was not mitigated by the use of anaesthetic is consistent with their
322 physiological roles, as shown by the pathway enrichment of their predicted mRNA targets. The
323 results provided evidence that differentially expressed miRNA target the expression of genes
324 coding for proteins involved in pain-regulatory pathways and inflammation. MiR-19b is a
325 member of the miR-17-92 cluster, whose role is to act as a powerful modulator of the TGFβ
326 pathway during the immune response (Jiang et al., 2011).

327 Tumor Growth Factor β belongs to a superfamily with over 30 member proteins that are
328 released after injury within the inflamed area and amplifies peripheral nociceptor transduction,
329 evoking functional plasticity and increasing the excitability of nociceptors, aiming to protect
330 the injured area by increasing pain sensitivity (Schaible et al., 2011). Tumor Growth Factor β
331 family members exert a protective role against nerve-injury-induced neuropathic pain
332 (Echeverry et al., 2009) and maintain the integrity of the blood-brain barrier, preventing the
333 development of pathological pain following peripheral inflammatory (Ronaldson et al., 2009)
334 or neural injuries (Echeverry et al., 2011). Tumor Growth Factor β interacts with their cell
335 targets through two receptors, Tumor Growth Factor β receptor (TGFβR) I and TGFβRII.
336 TGFβR activation recruits SMAD proteins, that are also involved in the miRNAs biogenesis at
337 both transcriptional and post-transcriptional level (Blahna and Hata, 2012). Remarkably,
338 SMAD 3 transcriptionally regulates miR-17-92 cluster, to which miR-19b belongs (Luo et al.,
339 2014). The authors may, therefore, speculate that miR-19b increased abundance induced by

castration and tail docking in piglets may be driven by TGFβ upregulation, which in turn may be feedback regulated by miR-19b.

MicroRNA-365 is involved in pathways involved in the regulation of inflammation. An analogue investigation carried out in a rat model of morphine analgesic tolerance (Wu et al., 2018) demonstrated that up-regulation of miR-365 was related to morphine tolerance by targeting the mRNA expressions of β-arrestin2, ERK and CREB and decreasing the contents of IL-1β, TNF-α and IL-18. Similarly, to what demonstrated for TGFβ, also the expression of miR-365 is upregulated by pro-inflammatory cytokines, such as IL1β, providing a further example of the feedback-regulatory loop (Yang et al., 2016). Therefore, also miR-365 may be involved in a complex regularity network linking pain to inflammation, contributing to explain at the molecular level the decrease of inflammatory defences during statuses where animals are stressed. The last miRNA whose abundance was found to be increased in animal subjected to dock tailing and castration without anaesthesia was miR-27b. Finally, as miR-19b and miR-365, miR-27b as well is related to the dampening of inflammation, which is carried out by targeting the pro-inflammatory protein MIP1-β (Li et al., 2017).

Conclusions

In conclusion, the present study demonstrated that salivary miRNAs were found to be more abundant in animals exposed to surgical removal of tail docking and castration. This effect is reduced by the use of anaesthesia. The finding of these miRNAs in saliva and their good diagnostic values, as demonstrated by ROC analysis, suggests the use of miR-19b and miR-365 as potential non-invasive biomarkers to assess pain and stress in pigs, although validation on a larger number of animals, and comparison with other physiological and behavioural parameters are required. Measurements of miRNAs might also be applied to identify potential sex differences in pain sensitivity. No significant differences in behaviour, facial grimacing or

emitted vocalisations between male and female piglets were found in a model of pain caused by tail-docking (Viscardi and Turner, 2019). Given the background that miRNA measurement identified sex differences in pig adipose tissue (Mentzel et al., 2015), miRNA measure may address the issue of measuring pain in husbandry procedure in sows, providing information useful to the development of targeted pain mitigation strategies.

Conflict of interest statement

The authors declare there are no competing interests.

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564

565 **Table 1** Procedures and samples collection.

Time **Nr of sampling**	**10 min before** **1st**	**0 min**	**30-45 min after** **2nd**
ANA[1]	Sampling and then drugs administration[4] and [5]	Castration, tail docking, iron and antibiotic	Sampling
CONV[2]	Sampling	Castration, tail docking, iron and antibiotic	Sampling
SHAM[3]	Sampling	Handling	Sampling

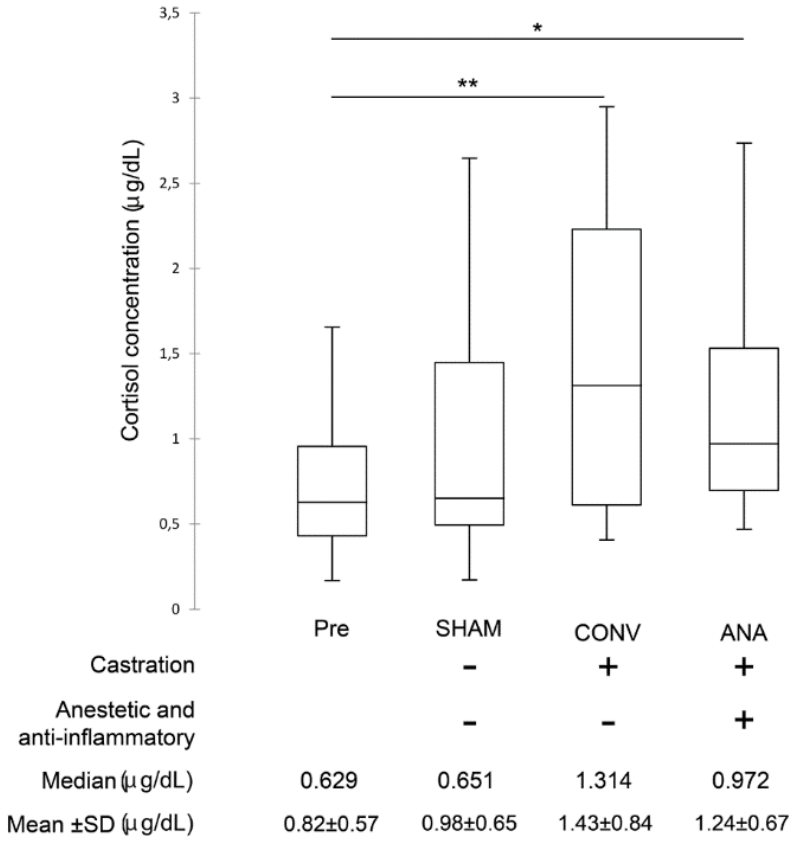
566 [1]piglets treated with drugs; [2]piglets treated without any drugs; [3]SHAM castration and dock tailing; [4]

567 aesthetic: procaine and adrenaline; [5] anti-inflammatory: meloxicam.

568

Table 2 Area under the curve (AUC), sensitivity and specificity of DE-miRNAs in piglet's saliva.

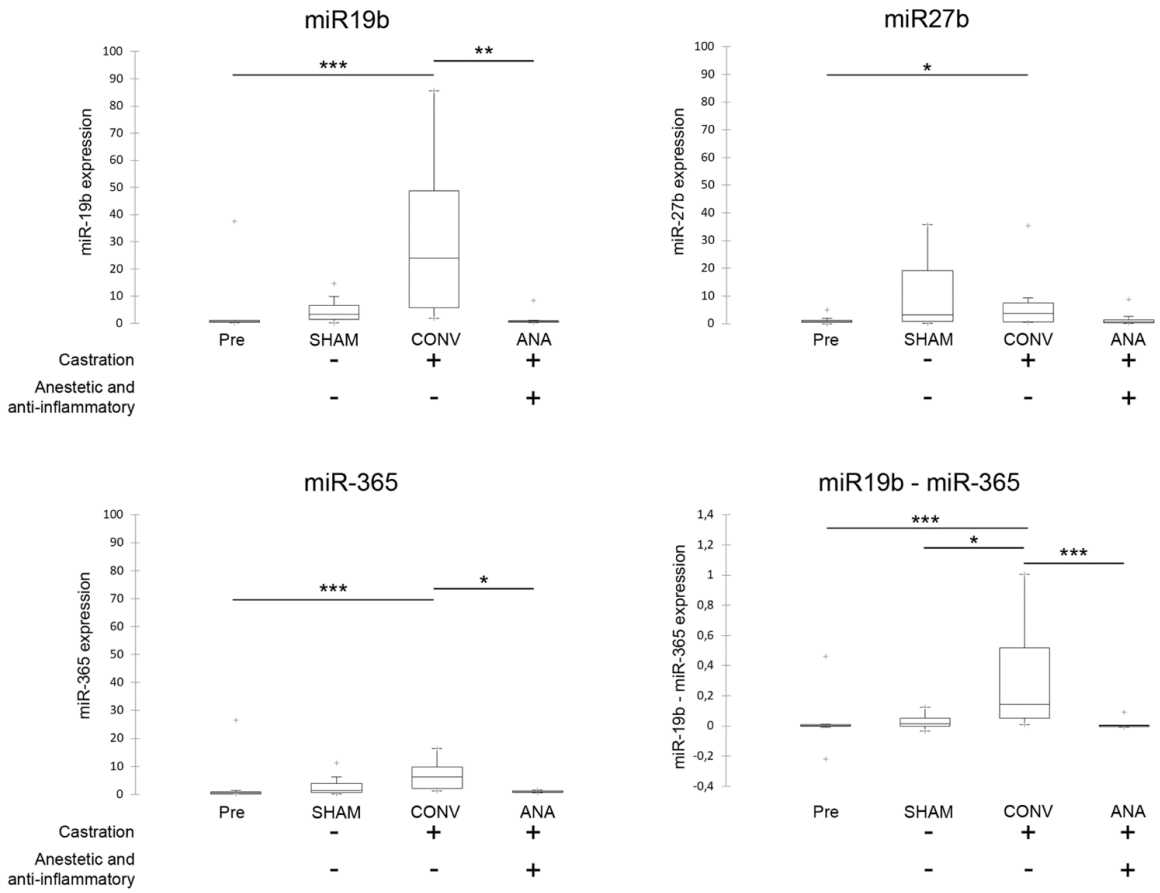
		AUC	95% CI	*P* value	Cut off	Sensitivity-specificity
miR-19b	Pre-CONV	0.925	0.769-0.989	<0.0001	1.07	100-78.26
	Pre-ANA	0.543	0.356-0.723	0.7261		
	Pre-SHAM	0.709	0.519-0.857	0.0904		
	CONV -ANA	0.946	0.699-0.999	<0.0001	1.07	100-87.5
	CONV -SHAM	0.804	0.523-0.958	0.0121	5.59	71.4-75
	ANA -SHAM	0.742	0.468-0.923	0.0841		
miR-27b	Pre-CONV	0.770	0.581-0.903	0.0149	0.83	71.43-60.87
	Pre-ANA	0.511	0.326-0.694	0.9353		
	Pre-SHAM	0.723	0.533-0.868	0.1017		
	CONV -ANA	0.750	0.466-0.931	0.0639		
	CONV -SHAM	0.500	0.239-0.761	1		
	ANA -SHAM	0.734	0.460-0.918	0.0836		
miR-365	Pre-CONV	0.919	0.760-0.987	<0.0001	1.43	85.7-91.3
	Pre-ANA	0.698	0.508-0.849	0.0366	0.51	100-52.17
	Pre-SHAM	0.723	0.533-0.868	0.039	0.51	87.5-52.17
	CONV -ANA	0.968	0.742-1	<0.0001	1.54	85.71-100
	CONV -SHAM	0.750	0.466-0.931	0.0584		
	ANA -SHAM	0.594	0.326-0.826	0.5765		
miR19b -miR-365	Pre-CONV	0.919	0.760-0.987	<0.0001	0.012	85.71-82.61
	Pre-ANA	0.592	0.402-0.764	0.4554		
	Pre-SHAM	0.609	0.418-0.778	0.4474		
	CONV -ANA	0.964	0.735-1	<0.0001	0.0063	100-87.5
	CONV -SHAM	0.786	0.504-0.950	0.0275	0.0396	71.43-75
	ANA -SHAM	0.778	0.506-0.942	0.0309	0.0063	100-66.67



578

579 Fig. 1. Salivary cortisol concentrations in piglets change after castration and tail docking. The

580 black lines inside the boxes mark the medians. * $P < 0.05$; ** $P < 0.01$.

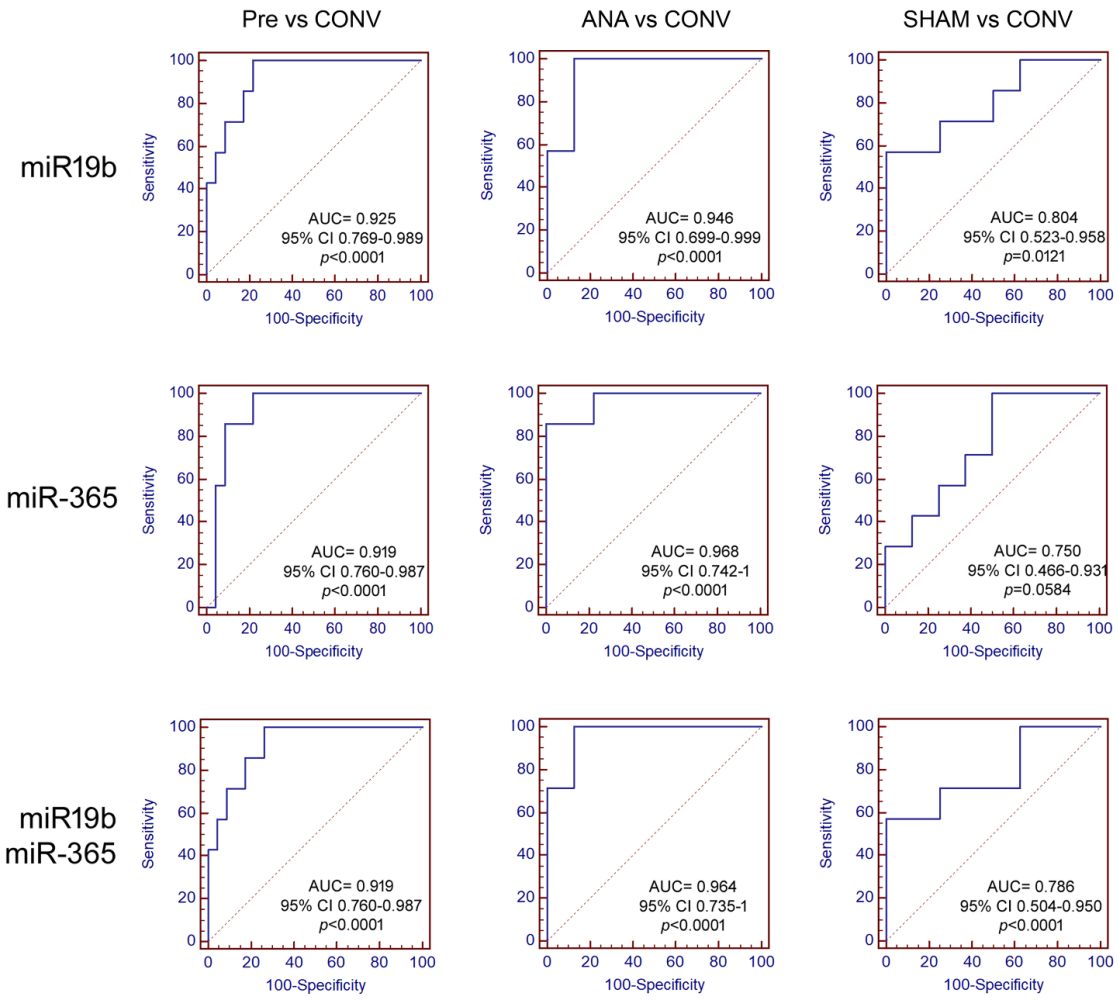


581

582 Fig. 2. Distribution charts of salivary miRNA concentrations.

583 Saliva was collected from piglets 30-45 minutes after castration and tail docking or handling;
584 samples were analysed for the presence of pain-related miRNAs. Distribution charts of (A)
585 miR-19b, (B) miR-27b, (C) miR-365, (D) combination of miR-19b and miR-365 amounts. The
586 black lines inside the boxes mark the medians. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

587

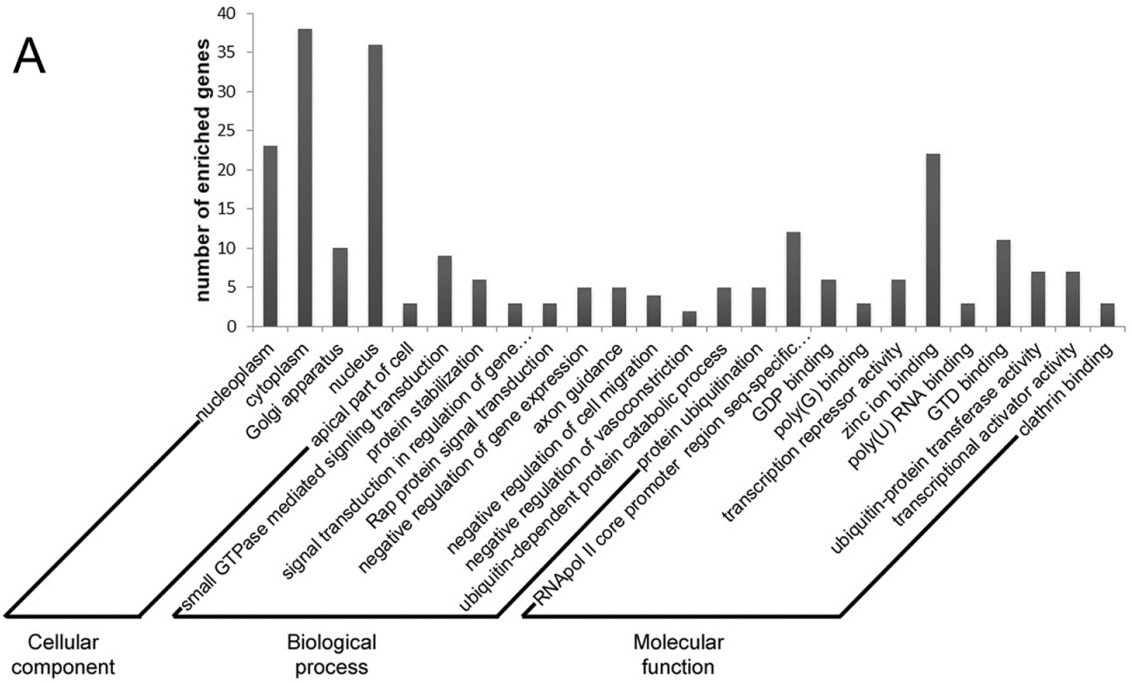


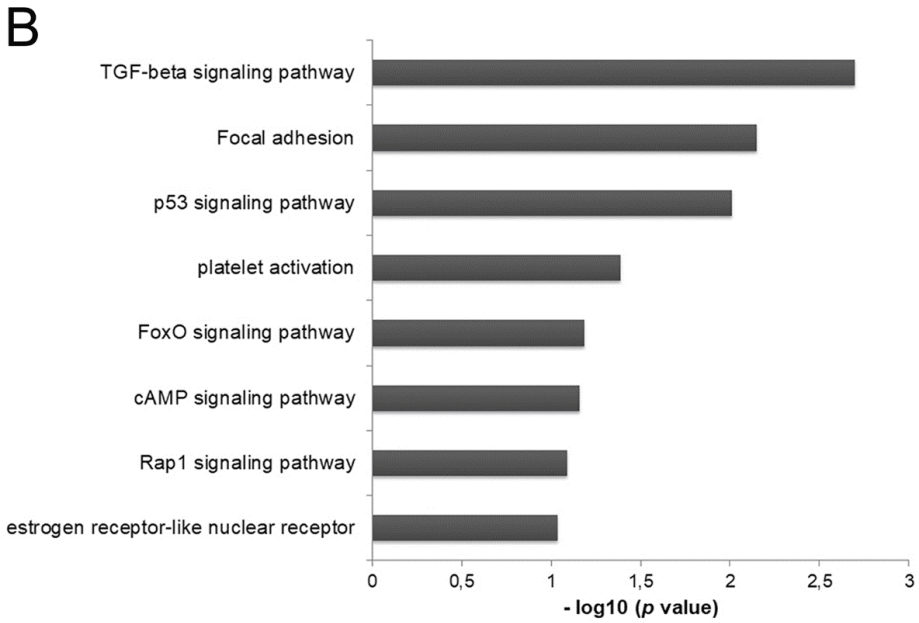
588

589 Fig. 3. Receiver-operator characteristics (ROC) curve analysis of candidate pain-related

590 miRNA. AUC, area under the curve; CI, confidence interval.

591





592

593 Fig. 4. miRNA target prediction and pathway enrichment.

594 (A) GO annotation of genes regulated by identified pain-related miRNAs. The targeted genes

595 were annotated by DAVID tools at three levels, including Cellular Component, Biological

596 Process and Molecular Function.

(B) Pathway enrichment for genes regulated by pain-related miRNAs. Genes were retrieved and enriched in KEGG pathway with DAVID tools. The statistical significance level (*P* value) was negative 10-based log transformed.