A systematic review of novel biomarkers for the measurement of pain in animals

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Executive Summary

In order to ensure the welfare of animals it is essential to minimise pain following human interventions associated with routine husbandry practices. The International Association for the Study of Pain (IASP) defines pain as "'(A)n unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." The emotional or subjective component associated with the experience of pain complicates our understanding of how pain is perceived by an individual. Whilst it can be difficult to quantify pain in humans who are able to effectively communicate what they are feeling, the difficulty of measuring pain in animals poses an even greater challenge. As the general public become more aware and concerned about the painful husbandry procedures that affect livestock, the ability to effectively measure pain experienced by animals becomes essential to aid in the assessment of analgesia techniques. Current methods associated with measuring pain in animals include measurement of physiology parameters and behaviour, however these methods are relatively crude and not always definitive. The discovery of new biological markers of pain that can be measured in biological samples such as plasma may hold promise for improving the measurement of pain in the future.

Therefore the aims of the current study were to scope for and then perform a systematic review of novel pain biomarkers in animal studies. Even with exclusion criteria to limit the numbers of studies resulting from the literature search, almost 7,000 studies were found in the initial search, highlighting the enormous literature using animal models of pain. With these numbers it is not surprising researchers studying pain in livestock species, a parallel but much smaller field of research compared to animal models of human pain, are not as familiar with this literature. Thus the current study represents an unprecedented access to novel biomarkers of pain for research in pigs and other livestock species.

A total of 2,205 biomarker entries were extracted from the 379 Included Studies. These biomarkers were measured in over 100 different tissues and bodily fluids, with the spinal cord and dorsal rood ganglia (n=671), the sciatic nerve (n=203) and blood serum (n=183) the most common tissues used. A total of approximately 600 individual biomarkers were used, a plethora contrasting with the usual limited number of biomarkers used in studies of pain in livestock (e.g. cortisol, β-endorphin, C-reactive protein). Biomarkers used extensively in the Included Studies are known to be important in transduction and transmission of pain, such as Interleukin-10 (n=82), Interleukin 1β (n=193), Interleukin 6 (n=116) and Tumor necrosis factor α (n=196). The majority of studies utilised rats or mice, and nerve injury models, representing neuropathic pain, were the most commonly performed.

Mechanical allodynia[1] and thermal hyperalgesia[2] were the most used methods to elicit pain in the Included Studies.

Limiting the list of biomarkers to tissues and bodily fluids that are likely to be more accessible in livestock species (blood plasma and serum, blood vessels, cerebrospinal fluid, endoneurial macrophages, lymph nodes, lymphocytes, blood mononuclear cells and peritoneal fluid) resulted in an extensive list, including many known mediators of pain and inflammation. Others listed such as leptin, which is usually associated with obesity and satiety signalling, are not commonly associated with pain,. However, leptin has been associated with the progression of osteoarthritis, and appears likely to have a role in inflammation, which is linked to pain.

The extensive list of biomarkers resulting from our systematic review are of interest, as our current understanding of pain and its mechanisms is rapidly expanding, with a paradigm shift away from solely neuronal hypotheses to the holistic neuroimmune mechanisms of pain. Importantly, the neuroimmune view of pain has expanded the potential cellular contributors to pain processing within the brain and spinal cord, to include peripheral blood factors. Given that pain is an adaptive response of an organism to avoid harmful stimuli and survive, there is a greater appreciation of the crosstalk between the pain (or nociceptive) and immune systems in host defences to injury and disease. Thus, while not all of the biomarkers listed in this review are accepted measures of pain, with further study some may represent novel biomarkers. This review represents a body of information that can be used by future researchers as a basis for new research.

The systematic review conducted represents an unprecedented s analysis of pain biomarkers and is of substantial relevance to the field. Whilst there is great promise, this analysis has revealed the bias in the past pain research community of an extremely reductionist and targeted approach in the research to explore pain mechanisms. What this has created are artificial silos in the literature which do not account for the systems nature of pain. This in turn has not enabled a networks approach to capture pain in a single measure. As such, when searching for biomarkers the assumption that a single target will "measure pain" is clearly fraught with inaccuracies. It is clear that there are no single available measures than can be applied today to solve the production animal pain problem. This does not make inaction appropriate. Rather it says timely action is needed. Hence, the multiplexed approach will be needed to create a more robust test that is transferable across pain states. For this to be cost effective, existing biologics based assessments will be cost prohibitive. Novel approaches will be needed.

[1] Allodynia means a painful response to a stimulus that is not normally painful, e.g. when you are sunburnt wearing a shirt can be painful although it is not normally.

[2] Hyperalgesia means an increased painful response to a stimulus that is normally painful e.g. when you are sunburnt, if somebody slaps you on the back it will be more painful than it usually is.

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1. Background to Research

In order to protect the welfare of animals, adequate analgesia to prevent, or at least minimize, pain following human interventions is necessary. The International Association for the Study of Pain (IASP) defines pain as "'(A)n unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." As this definition suggests, pain includes a subjective or emotional component which complicates our understanding of how pain is perceived by an individual. It can be difficult to quantify pain in humans who are able to tell us what they are feeling; the difficulty in measuring pain in animals is an even greater challenge. Despite this, we must make our best attempt, as the general public are becoming more aware and concerned with husbandry procedures that cause pain in livestock.

Measurement of pain will never involve a single physiological or behavioural measure, as no one measure will suffice. A 'bigger picture' measure will come closest to approximating the painful experience of an animal, including both physiological and behavioural measures. Acute and severe pain results in reliable physiological (e.g. cortisol) and behavioural (often species specific) changes (Fisher 2011, O'Connor, Anthony et al. 2014). Measures are needed that can be used reliably to assess the binary presence of pain (i.e. is an intervention painful, or not?) or the spectral degree of pain in larger populations in the field. This is particularly relevant for circumstances in which chronic pain may occur, such as in lameness, or following routine husbandry procedures, such as uncomplicated castration.

Pain research is a highly active area. Pain can be described in terms of severity (mild, moderate, severe), duration (acute or chronic), and type (nociceptive, inflammatory and neuropathic). Nociceptive pain is a normal acute pain sensation to an injury or trauma, involving mechanical, thermal or chemical stimulation. When this quickly resolves then the pain disappears, for example following routine castration or tooth clipping in piglets. Inflammatory and neuropathic pain types are more likely to involve chronic pain, for example if a castration wound becomes chronically infected. Inflammatory pain begins through an insult, or injury, to tissues resulting in an inflammatory state. This includes pain, heat, redness, swelling and lack of function. The inflammatory process once commenced results in **hyperalgesia** (increased pain response to a painful stimulus). An example in husbandry would be tail biting in pigs (Scollo, Contiero et al. 2015), in which the surrounding area can become very red and inflamed, or leg ulcers in a sow due to lying on concrete. Neuropathic pain is defined by the IASP as "Pain initiated or caused by a primary lesion or dysfunction in the nervous system." Neuropathic pain includes not only hyperalgesia, but also **allodynia**, (a painful response to a stimulus that would not normally be painful). Neuropathic pain may occur in chronic lameness caused by osteoarthritis (Meijer, van Nes et al. 2015). Critically, neuropathic pain serves no beneficial purpose to the sufferer and cannot be explained or quantified in proportion to the stimulus that elicits the painful response.

The pathways involved in pain transmission, transduction and perception are incredibly complex, and have been elucidated largely through the use of rodent models (Mogil 2009). Through research our understanding of pain and its mechanisms are currently rapidly expanding, with paradigm shifts away from solely neuronal hypotheses to the holistic neuroimmune mechanisms of pain. The field has embraced the critical role of the "other brain" or the other 90% of cells within the central nervous system. These immune-like cells, termed glia, provide the critical missing link mechanistically changing nociceptive processing during both acute and chronic painful behaviours. Importantly, this neuroimmune view of pain has expanded the potential cellular contributors to pain processing within the brain and spinal cord, to include peripheral blood factors. Given that pain is an adaptive response of an organism to avoid harmful stimuli and survive, there is a greater appreciation of the crosstalk

between the pain (or nociceptive) and immune systems in host defences to injury and disease (McMahon, La Russa et al. 2015). It is therefore timely with this shift in our understanding and with the increasing body of pain research to re-assess if there are novel approaches that can be used to objectively quantify pain in animals, perhaps through these unique neuroimmune windows into the experience of pain.

2. Objectives of the Research Project

The objectives of the research project were to:

- Scope current pain studies in animal models to determine the criteria for a systematic review of novel pain biomarkers
- Perform a systematic review to extract novel pain biomarkers to use in future studies of pain in animals used in agriculture.
- Provide recommendations to Industry on how and where the novel pain targets/markers might be used in future assessment of animal pain

3. Introductory Technical Information

Physiological measures of pain used in livestock research have included a relatively small number of biomarkers, including measures such as neutrophil to lymphocyte ratio, cortisol, beta-endorphin and haptoglobin levels (Colditz, Lloyd et al. 2009, O'Connor, Anthony et al. 2014). A recent study of pain in castrated piglets did not find any better measures of pain (Lonardi, Scollo et al. 2015). The numbers of studies performed on agricultural animals to determine mechanisms and treatment of pain, however, is an order of magnitude less than the studies published using animal models of human disease. In 2008 over 1000 studies were published using rats and mice as models for pain research (Mogil 2009) and an even higher number would be expected today. This is ironic, since one could argue animals are better models for pain in other animals, than for pain in humans. In fact, these two areas run parallel to each other, with researchers involved in animal research of human pain not always interacting with those studying pain in livestock. The current study aims to open up the results from studies using animal models of human pain, and to make them available to those working with agricultural species. The goal is to find some novel pain biomarkers that can then be tested in future research.

Systematic reviews are a methodical approach to extracting information from the literature. They have been used widely in human research, and their use is now increasing in the veterinary field. For example, a recent review of pain management in the neonatal piglet during routine management procedures used a systematic review (O'Connor, Anthony et al. 2014). Once a research question and protocol is defined, a systematic approach means all data fitting the inclusion criteria will be included, without the bias that can arise if specific studies are 'cherry picked'. Thus the approach used in this study was to systematically review the research involving pain models in animals, and to derive a list of biomarkers used in the measurement of pain.

4. Research Methodology

4.1 Scoping Study

The initial phase was a scoping study to determine the potential areas in which novel targets could be found (e.g. hormonal assays, immune markers, thermal imaging or combinations of these). Following the scoping study a protocol was developed and agreed upon by the research team.

4.2 Systematic Review Protocol

Inclusion criteria

- Any type of pain
- Any controlled initiator for the pain
- Animal studies

Exclusion

- Natural models
- Cancer models (Excluded to keep numbers of studies down and relevance to industry)
- Viral infection models (as for cancer e.g. HIV)
- Human studies
- Immunohistochemical measures (Excluded owing to their poor practical translation to industry)

Types of outcomes

This review will consider studies that include the following outcome measures performed in a blinded fashion to the animals:

- At least one validated measure of pain, which may include behaviour (e.g. cortisol, heart rate)
- May include quantitative and semi-quantitative measures

Types of studies

- Blinded studies (the person scoring for pain must not know the treatment of the animal)
- N=6 or more per group for multi-group studies
- N=12 for correlational studies

In order to gain the maximum number of the available literatures, three different search fields were identified:

1. Pain descriptive:

The key words related to different definitions of pain in the literature.

(Allodyni*[TIAB] OR Hyperalgesi*[TIAB] OR Pain*[TIAB] OR Nocicepti*[TIAB] OR Nocifensive[TIAB] OR Hyperaesthesia*[TIAB] OR Hyperesthesia*[TIAB] OR Dysesthesia*[TIAB] OR Dysaesthesia*[TIAB] OR Paresthesia*[TIAB] OR Paraesthesia*[TIAB] OR Ache*[TIAB])

N.B: [TIAB] is a PubMed tag for limiting the search to the title and abstract.

2. Procedures:

The selected procedures designed to create or measure pain were identified and applied in the search term as the second category:

("Hot plate test"[TIAB] OR "Tail Pinch"[TIAB] OR "Von frey "[TIAB] OR Capsaicin[TIAB] OR Formalin[TIAB] OR Formaldehyde[TIAB] OR "Mustard Oil"[TIAB] OR Carrageenan[TIAB] OR "Complete Freund's Adjuvant" [TIAB] OR "Acetic Acid" [TIAB] OR "Spared Nerve Injury"[TIAB] OR "Spinal Nerve ligation"[TIAB] OR Peripheral nerve Injury[TIAB] OR "Chronic constriction injury"[TIAB] OR "Peripheral nerve demyelination"[TIAB] OR "Sciatic nerve transaction"[TIAB] OR "Partial sciatic nerve ligation"[TIAB] OR "Sciatic inflammatory neuropathy"[TIAB] OR "Drug-induced neuropathy"[TIAB] OR burn*[TIAB] OR Ischemia[TIAB] OR Pancreatitis[TIAB] OR Prostatitis [TIAB] OR Osteoarthritis[TIAB] OR "Mono iodoacetate"[TIAB] OR Migraine[TIAB] OR Dysmenorrhea[TIAB] OR Diabete*[TIAB] OR "Adjuvant arthritis"[TIAB] OR "Escape test"[TIAB] OR Dolognawmeter[TIAB] OR Incision[TIAB] OR Fracture*[TIAB] OR Lame*[TIAB] OR Infect*[TIAB] OR Infarction[TIAB] OR Sciatica[TIAB] OR Neuropathy[TIAB] OR Inflammat*[TIAB] OR Colic[TIAB] OR Myalgia[TIAB] OR Mastodynia[TIAB] OR Causalgia[TIAB] OR Arthralgia[TIAB] OR "Tissue damage"[TIAB] OR Hernia [TIAB] OR Trauma[TIAB] OR Ulcer[TIAB] OR Edema[TIAB]

OR Arthritis[TIAB] OR "nerve injury"[TIAB] OR Myopathy[TIAB] OR Mastitis[TIAB] OR Caesarean[TIAB])

3. Biomarkers:

The selected biomarkers or the tissue that the biomarkers are sampled from are shaped the third category of the search term,

Biomarker*[TIAB] OR Biological Markers[MH] OR Blood[TIAB] OR Serum[TIAB] OR Plasma[TIAB] OR CSF[TIAB] OR "Cerebrospinal Fluids"[TIAB] OR Saliva[TIAB] OR Urine[TIAB] OR Cytokine*[TIAB] OR Chemokine*[TIAB] OR Tears[TIAB] OR Breath[TIAB] OR Lymph[TIAB] OR Sweat[TIAB] OR Biopsy[TIAB] OR Smear*[TIAB] OR Cortisol[TIAB] OR glucocorticoid*[TIAB] OR Corticosterone[TIAB] OR Diagnostic*[TIAB] OR Hormone*[TIAB]).

The previously mentioned three parts formed the main section of the search terms; however, filters such as English and Journal article were activated as well.

Finally, since the "Other animal" filter limits is a MeSH all the "As supplied by publisher" and "In process" citations will be lost, a set of keywords which was employed consisting of the different animal species were included in the search term.

Assessment of methodological quality

Quantitative papers selected for retrieval were assessed by two independent reviewers for methodological validity prior to inclusion in the review using standardized critical appraisal instruments in an Excel spreadsheet (e.g. blinded and N=6 or more per group for multi-group studies). Any disagreements that arose between the reviewers were resolved through discussion, or with a third reviewer.

Data collection

Quantitative data was extracted from papers included in the review using a standardized data extraction tool in an Excel spreadsheet. The data extracted included specific details about the interventions, participants, study methods and outcomes of significance to the review question and specific objectives.

4.2.1 Search results

Using the designed search term in PubMed a CSV file was extracted and the following items for inclusion and exclusion criteria were applied for each article.

A. First filter was the relevancy of the article to the designed search term; completely irrelevant papers were excluded at this stage. Case reports, observational studies, and human studies were also excluded at the first step.

B. If the paper was relevant, availability of the PDF file was recorded.

C. Pain initiators, which are the different categories of elements of different natures which have been applied to create pain in a designed study, were identified and recorded by the relevant sub category.

D. Identifying the tool or the method of assessing the created pain was the next step.

E. Different types of created and assessed pain were recorded.

F. The type of the pain model was recorded

G. If the pain was not evaluated by a classic method and instead a physiological or behavioural approach was adopted, it was also recorded.

H. Whether or not the behavioural analysis was done by a blind observer and the sample number which should be over 6 per group was noted and recorded.

The initial criteria A-H were extracted from the studies in a first step, followed by extraction of the final category (I) with the biomarkers recorded.

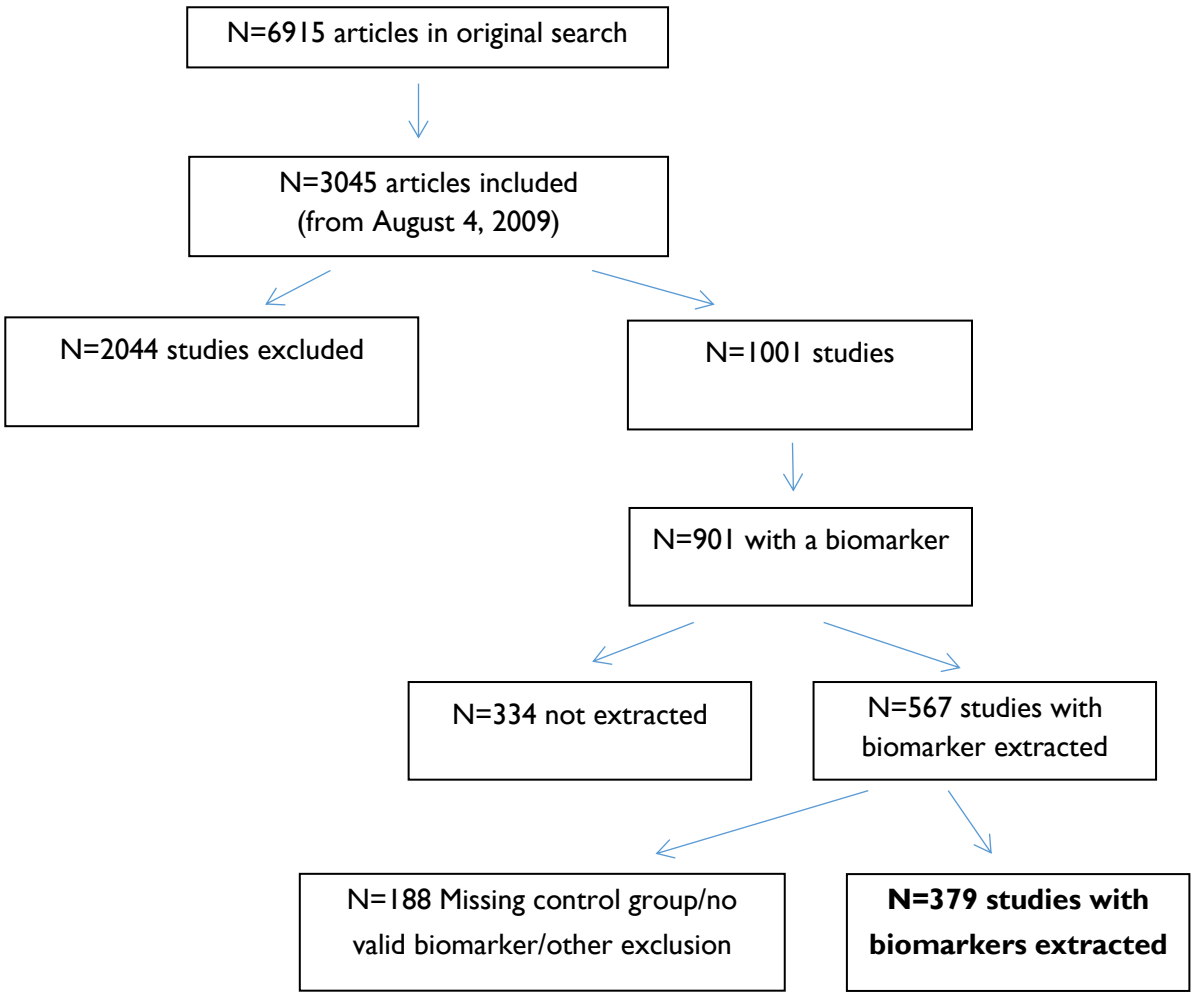
Following these steps, a second person extracted the biomarkers from the included studies.

5. Results

5.1 Data Extraction

With no date restrictions the original search resulted in a total of 6915 individual articles. Due to the time consuming nature of data extraction, data was extracted from 3045 of these articles, dating from August 4, 2009 (see Figure 1 for a schematic of included studies). Of these 3045 studies, 2044 (67%) had exclusion criteria (see list above) and 1001 studies were in the initial inclusion list. These studies were then scrutinised for extraction of biomarkers, and 130 (13%) were found not to include valid biomarkers. This left 901 included studies with a biomarker, of which the time allocation for this grant allowed full extraction of 567 studies. The studies included were the most recent. **There were 379 studies included in the final analysis, with biomarkers recorded.** This represents an unprecedented systematic analysis of pain biomarkers and is of substantial relevance to the field.

Figure 1: Breakdown of the included studies in the systematic review



5.2 Biomarkers

A total of 2,205 biomarker entries from the Included 372 studies were further analysed. A breakdown of the tissues from which these biomarkers were analysed is shown in Table 5.1. The numbers represent the numbers of individual biomarkers measured from each tissue- a single study could measure multiple biomarkers from the same tissue. Tissues with the highest numbers of biomarkers measured included the spinal cord (n=440), the dorsal root ganglia (n=231), the sciatic nerve (n=203) and blood serum (n=183). These tissue represent likely targets along the pain pathway (the spinal cord and dorsal root ganglia are involved in the transmission of pain signals to the brain) and also the most common pain models used (sciatic nerve injury and sciatic nerve compression were two common models in the Included Studies, see Table 5.5).

A total of 1676 biomarkers were measured as a protein, with 739 measured as mRNA levels.

Table 5.1 Tissues from which biomarkers were analysed in the Included Studies

	N
MUSCULOSKELETAL	
Hind paw	66
Articular cartilage/tissue	16
Synovial fluid	15
Gastrocnemius muscle	14
Knee joint/tissue	10
Synoviocytes	10
Synovial tissue	9
Ankle joint/tissue	7
Muscle	7
Temporomandibular joint	6
Soleus muscle	5
Tendon	5
Masseter muscle	4
Bone	3
Flexor digitorum muscle	3
Equine hoof lamellae	3
Periarticular tissue	3
Hind limb	2
Radius-ulna	2
VASCULAR RELATED	
Serum	183
Plasma	71
Blood	13
Cerebrospinal fluid	10
Lymph node	7
Peripheral blood	6
Blood vessels	4
Myocardium	4
Spinal microvessels	4
Lymphocytes	1
Blood mononuclear cells	1

Endoneurial macrophages	
BRAIN	**N**
Prefrontal cortex	24
Hippocampus	23
Frontal cortex	20
Somatosensory cortex	15
Nucleus acumbens	12
Thalamus	11
Amygdala	10
Hypothalamus	10
Periaquaductal gray (PAG)	9
Cuneate nucleus	8
Ventral tegmental area	6
Forebrain cortex	5
Cerebral microvessels	4
Red nucleus	4
Satellite glial cells	4
Ventrolateral orbital cortex	4
Brain	3
Rostral ventromedial medulla	3
Caudate/putamen	2
Cerebral cortex	2
Meninges	2
Ventromedial medulla	2
Dorsal raphe nucleus	1
Nucleus ceruleus	1
Olfactory bulb	1
Paraventricular nucleus	1
Piriform cortex	1
SPINAL CORD	**N**
Spinal cord	440
Dorsal root ganglia	231
Lumbar dorsal horn	172
Dorsal horn	136
Dorsal spinal cord	41
Nucleus pulposus	10
Dorsal horn laminae	7
Spinal microglia	3
Ventral horn	3
Lumbar tissue	2
Neck dorsal horn	1
Nucleus proprius	1
Spine	1
Thoracic spinal cord	1
NERVE RELATED	**N**
Sciatic nerve	203
Trigeminal nerve	27

Neurons	3
Saphenous nerve	2
Stellate ganglia	2
Schwann cells	1
SKIN RELATED	**N**
Paw skin	20
Skin	4
Epidermis	4
Incised paw skin	3
Epidermal keratinocytes	1
OTHER	**N**
Paw tissue	71
Bladder	26
Liver	15
Plantar tissue	12
Spleen	8
Air pouch exudate	7
Ear tissue	7
Edematous tissue	6
Colon	5
Deep paw tissue	3
Kidney	3
Labiar tissue	3
Ovary	3
Pancreas	3
Peritoneal fluid	3
Pleural wash	3
Urothelium	3
Uterus	3
Detrusor smooth muscle	2
Front paw tissue	2
Popliteal lymph node	2
Duodenum	1
Faeces	1
Interstitial fluid	1
Lungs	1
Stomach	1
Tight junction	1

In the 372 Included Studies there were approximately 606 individual biomarkers used. The complete list of biomarkers and the numbers of times they were measured in the Included Studies is shown in Appendix 1. Biomarkers were sometimes measured more than once in the same study in different tissues. Not surprisingly, the most commonly measured biomarkers are known to be important in pain pathways and inflammation: GFAP (Glial fibrillary acidic protein; n=72), IL-10 (Interleukin-10; n=82), IL-1β (Interleukin 1 β; n=193), IL-6 (Interleukin 6; n=116) and TNFα (Tumor necrosis factor α; n=196). However, the number of biomarkers that have been measured only a few times is surprising, and some of these may represent novel biomarkers for future studies. For example, A2AAR (Adenosine A2a receptor), ATF3 (Activating transcription factor 3) and Beclin-1 were only measured once.

5.3 Pain Models Used in the Included Studies

A range of pain models were used in the 372 Included Studies. Overall, nerve injury models were the most common model used, with chronic constriction injury being the most common of these nerve injury models (Table 5.2). Nerve injuries are models of neuropathic pain (Jaggi, Jain et al. 2011). Agents and chemicals were the next most common model used, with Complete Freunds Adjuvant the most used agent.

Table 5.2 Types of Pain Models used in the Included Studies in the Systematic Review

	No of studies
Nerve Injury	
Chronic Constriction Injury (CCI)	56
Spinal Nerve Ligation(SNL)	30
Partial Sciatic Nerve ligation	20
Spared nerve Injury	16
Spinal nerve Transaction(SNT)	4
Nucleus pulposus Injection (NP)	4
Spinal Cord injury	3
Sciatic Nerve Injury	2
Sciatic Nerve Compression	2
Spinal nerve L5 transection	2
intra spinal acid injection	1
Spinal Cord transaction	1
Nerve damage(Doxoroubincon)	1
Spinal cord I/R mode	1
DRG compression	1
Traumatic brain injury	1
Tetanic stimulation of the sciatic nerve (TSS)	1
Spinal contusion injury	1
Sciatic nerve crush (SNC)	1
DRG Inflammation	1
Chronic compression of the dorsal root ganglia (CCD)	1
Median and ulnar nerves transaction	1
C7 nerve root transient compression	1

Partial ligation of infraorbital nerve (pIONL)	1
TOTAL	**153**
Arthritis Osteoporosis	
Other types of induced arthritis	9
Collagen Induced arthritis	5
Surgery induced arthritis or OA	4
Adjuvant induced Arthritis(AIA)	3
K/B N serum, transfer	3
TOTAL	**24**
Inflammatory complications	
Cystitis	3
Prostatitis	1
Endometritis	1
Dermatitis	1
Gout attack	1
Encephalomyelitis (EAE)	1
Pancreatitis	1
TOTAL	**9**
Agents and chemicals	
Complete Freunds Adjuvant	45
Formalin	25
Carrageenan	21
Acetic Acid	9
Capscaicin	3
Monosodium Urate (MSU)	2
Cyclophosphamide	2
NTG-induced hyperalgesia	1
Venom	1
Reserpine	1
Tamoxifen (Adenomyosis)	1
TNBS (2,4,6 trinitrobenzenesulphonic acid	1
5-hydroxytryptamine (5-HT)	1
black walnut extract (BWE)	1
TOTAL	**114**
Diabetes	
STZ	25
Other types	4
Zucker diabetic fatty (ZDF)	1
TOTAL	**30**
Surgery	
Incision	15
Fracture	4
Husbandry interventions	4
Surgical interventions	3
Ischemia	2
Plantar incision surgery	2
Chung method	1
Disc herniation	1
Cold injury model	1

Trauma	1
Disc degeneration model	1
Facet joint compression	1
Burn	1
Myocardial infarction	1
Vagal dysfunction	1
TOTAL	**39**
Other agents	
LPS	4
Nerve growth factor (NGF)-induced pain	2
Parasitological infestation	2
UPK3A 65-84 cictitis	1
TOTAL	**9**
Physical	
Swimming	2
Fatigue	1
Treadmill training	1
High repetition high force (HRHF)	1
TOTAL	**7**

To measure pain in the different models, a variety of methods were used. The methods of pain measurement stated in the studies are shown in Table 5.3. Mechanical **allodynia** (a response to a stimulus that would not normally be painful) and thermal **hyperalgesia** (an increased painful response to a painful stimulus) were the most used methods to elicit pain. An example of a method used to measure mechanical allodynia is using von Frey fibres, thin fibres used to touch the skin. The fibres are calibrated to a specific force (measured in g) and placed on the skin until they bow lightly, for a specific period of time. Thus the minimum force that elicits a withdrawal indicates the level of allodynia present in that area. In a pig model of postoperative pain, von Frey fibres were used on pig skin (Castel, Willentz et al. 2014). Thermal hyperalgesia uses a high intensity lamp beam focussed onto a painful area, and the time to withdrawal of the affected region is measured.

Table 5.3 Measured types of pain in the Included Studies

Measured type of Pain	**No of studies**
Mechanical allodynia	99
Thermal hyperalgesia	88
Mechanical Hyperalgesia	44
Paw withdrawal threshold	36
Mechanical hypersensitivity and sensitivity	25
Paw thermal withdrawal latency	24
Tactile allodynia	22
Quantifiable behaviours (eg. licking, jumping, flinches)	20
Cold allodynia	14
Is not applied	12
Hot plate latency	11
Hind paw withdrawal response	7
Mechanical Withdrawal Threshold	6
Thermal Allodynia	6
Thermal sensitivity	6
Weight distribution/bearing	4

Thermonociceptive threshold	3
Cold sensitivity	3
Tail flick latency	3
Allodynia	2
Tail withdrawal latency	2
Pain threshold	1
Cold hyperalgesia	1
Thermal nociception	1
Gait Analysis	1
Not identified	5

Pain models used in the 372 Included Studies mostly represented chronic types of pain, including neuropathic and inflammatory pain (Table 5.4). However, there were a range of other pain models used overall.

Table 5.4 Pain Models used in the Included Studies

Pain Model	**No of Studies**
Neuropathic Pain	149
Inflammatory Pain	49
Postincisional/Incisional/Postoperative pain	13
Chronic pain	9
Musculoskeletal pain:	4
Visceral pain	3
Cancer pain	2
Diabetes	1
Diabetic hyperalgesia	1
Not identified	115

5.4 Further Information on Biomarkers from more Relevant Tissues in the Included Studies

Further analysis was performed on the biomarkers measured in the tissues most likely to be easily accessible in agricultural animals (Table 5.5). From the complete list of tissues in Table 5.1, the tissues selected were the blood (including plasma and serum), blood vessels, cerebrospinal fluid, endoneurial macrophages, lymph node, lymphocytes, peritoneal fluid and blood mononuclear cells. Of these, the cerebrospinal fluid, endoneurial macrophages, lymph nodes and peritoneal fluid are likely to be more difficult to access.

Many of the biomarkers identified in Table 5.5 below are known mediators of pain. For example, following tissue injury and inflammation, vasoactive mediators are released, including histamine, substance P, serotonin, nitric oxide prostaglandins and bradykinin (Das 2015). These activate the nociceptors, the initial sensory receptor involved in transduction of pain (Millan 2002). Following initial stimulation of the nociceptor, release of pronociceptive neurotransmitters occurs, such as substance P, calcitonin gene-related peptide, dynorphin, neurokinin A, glutamate, adenosine triphosphate, nitric oxide, prostaglandins and neurotrophins such as brain-derived neurotropic factor (Willis and Besch Jr

1995, Millan 2002, Dubner 2004, Yaksh 2006). Others are not as commonly associated with pain, for example leptin is known as a regulator of hunger, but has also been associated with progression of osteoarthritis (Scotece and Mobasheri 2015) and appears likely to play a role in inflammation.

Table 5.5 Further information, including biological function, on a selection of biomarkers identified in the systematic review and likely to be easily measured (Blood, Blood vessels, Cerebrospinal fluid, Endoneurial macrophages, Lymph node, Lymphocytes, Myocardium, Peripheral blood, Blood mononuclear cells, Plasma, Serum).

	Row Labels	**Full name**	**Biological function**
	Blood	**11**	
Blood	CD3+	CD3 (cluster of differentiation 3) T-cell co-receptor is a protein complex composed of four distinct chains.	Involved in signalling to T lymphocytes; present at all stages of T lymphocytes
Blood	CD4+	CD4 (cluster of differentiation 4) is a glycoprotein found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells.	Involved in immune function
Blood	Glucose	Glucose	Main energy source for the body. Increases due to high cortisol secretion.
Blood	IL-10	Interleukin-10	Anti-inflammatory cytokine
Blood	IL-17	Interleukin-17	A potent mediator in delayed-type reactions - increases chemokine production in various tissues to recruit monocytes and neutrophils to the site of inflammation.
Blood	IL-6	Interleukin-6	Pro-inflammatory cytokine
Blood	α1-AR	α 1-Adrenoceptors	Perform different functions in the physiological responses of the sympathetic nervous system
	Cerebrospinal fluid (CSF)	8	
CSF	Adrenomedullin	Adrenomedullin	Results in dilation of the blood vessels
CSF	Aspartate	Aspartate	Negatively charged amino acid
CSF	Glutamate	Glutamate	Negatively charged amino acid

CSF	MIF	Macrophage migration inhibitory factor	Important regulator of innate immunity
CSF	PGE2	Prostaglandin E2	A number of functions, including inducing fever and stimulating bone cells to resorb bone.
CSF	ROS	Reactive oxygen species	Chemically reactive molecules containing oxygen
CSF	TNF-α	Tumor necrosis factor α	A cell signalling protein (cytokine) involved in systemic inflammation - one of the cytokines that make up the acute phase reaction. Mostly secreted by activated macrophages.
	Cerebrospinal fluid cells	2	
CSF cells	IL-10	Interleukin-10	Anti-inflammatory cytokine
CSF cells	TNF-α	Tumor necrosis factor α	A cell signalling protein (cytokine) involved in systemic inflammation - one of the cytokines that make up the acute phase reaction. Mostly secreted by activated macrophages.
	Endoneurial macrophages	**1**	
Endoneurial macrophages	GFAP	Glial fibrillary acidic protein	Protein expressed by numerous cell types of the central nervous system. Thought to maintain astrocyte mechanical strength, but function not well understood.
	Faeces	**1**	
Faeces	Corticosterone	Corticosterone	Steroid hormone of the adrenal cortex, secreted in response to stress.
	Lymph node	**7**	
Lymph node	CINC-1	Cytokine-induced neutrophil chemoattractant 1	Results in increased pain
Lymph node	CINC-2	Cytokine-induced neutrophil chemoattractant 2	Important role in neutrophil recruitment
Lymph node	CNTF	Ciliary neurotrophic factor	A nerve growth factor

Lymph node	Fractalkine	Also known as chemokine (C-X3-C motif) ligand 1	Soluble CX3CL1 attracts T cells and monocytes; cell-bound it promotes adhesion of leukocytes to activated endothelial cells
Lymph node	GM-CSF	Granulocyte-macrophage colony-stimulating factor	A white blood cell growth factor secreted by macrophages, T cells, mast cells, NK cells, endothelial cells and fibroblasts.
Lymph node	IFN-γ	Interferon gamma	A cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections
Lymph node	NGF-β	Nerve growth factor β	Involved in regulation of growth, maintenance, proliferation, and survival of neurons.
	Lymphocytes	**1**	
Lymphocytes	A2aAR	Adenosine A2a receptor	Plays a role in regulating myocardial oxygen consumption and coronary blood flow. Can also negatively regulate overreactive immune cells, protecting tissues from collateral inflammatory damage.
	Peripheral blood (membrane/soluble)	**3**	
Peripheral blood (membrane/soluble)	ABP	Androgen binding protein	Binds specifically to testosterone (T), dihydrotestosterone (DHT), and 17-beta-estradiol.
Peripheral blood (membrane/soluble)	APN	Aminopeptidase N	Has a role in the final digestion of peptides generated from hydrolysis of proteins by gastric and pancreatic proteases.
Peripheral blood (membrane/soluble)	DPPIV	Dipeptidyl peptidase-4	An enzyme expressed on the surface of most cell types associated with immune regulation, signal transduction and apoptosis.

	Peripheral blood mononuclear cells	**1**	
Peripheral blood mononuclear cells	TNF-α	Tumor necrosis factor α	A cell signalling protein (cytokine) involved in systemic inflammation - one of the cytokines that make up the acute phase reaction. Mostly secreted by activated macrophages.
	Peritoneal fluid	**3**	
Peritoneal fluid	IL-6	Interleukin-6	Pro-inflammatory cytokine
Peritoneal fluid	PGE2	Prostaglandin E 2	A pro-inflammatory prostanoid that causes increased blood flow and triggers hypernociception.
Peritoneal fluid	TNF-α	Tumor necrosis factor α	A cell signalling protein (cytokine) involved in systemic inflammation - one of the cytokines that make up the acute phase reaction. Mostly secreted by activated macrophages.
	Plasma	**65**	
Plasma	5-HT	5-hydroxytryptamine or serotonin	A monoamine neurotransmitter derived from tryptophan, primarily found in the gastrointestinal tract (GI tract), blood platelets, and the central nervous system (CNS) of animals. Thought to be a contributor to feelings of well-being and happiness.
Plasma	ABP	Androgen binding protein	Binds specifically to testosterone (T), dihydrotestosterone (DHT), and 17-beta-estradiol.
Plasma	ACCII	Anti-type II collagen antibodies	Antibodies to collagen type II
Plasma	ALT	Alanine transaminase	Mostly found in the liver, it catalyzes the two parts of the alanine cycle.

Plasma	APN	Aminopeptidase N	Has a role in the final digestion of peptides generated from hydrolysis of proteins by gastric and pancreatic proteases.
Plasma	AST	Aspartate aminotransferase	Catalyzes the reversible transfer of an α - amino group between aspartate and glutamate - an important enzyme in amino acid metabolism.
Plasma	CGRP	Calcitonin gene related peptide	Produced in both peripheral and central neurons and is a potent vasodilator. It can function in the transmission of pain
Plasma	CK	Creatine kinase	Present in the mitochondrial intermembrane space- it regenerates phosphocreatine (PCr) from mitochondrially generated ATP and creatine (Cr) imported from the cytosol.
Plasma	Corticosterone	Corticosterone	Steroid hormone of the adrenal cortex, secreted in response to stress.
Plasma	Cortisol	Cortisol	Steroid hormone of the adrenal cortex, secreted in response to stress.
Plasma	C-Reactive Protein	C-Reactive Protein	A protein found in blood plasma, levels increase due to inflammation. Known as an acute phase protein.
Plasma	DPPIV	Dipeptidyl peptidase-4	An enzyme expressed on the surface of most cell types associated with immune regulation, signal transduction and apoptosis.
Plasma	ET1	Endothelin-1	Constricts blood vessels and increases blood pressure. Also thought to have a role in pain mediation.
Plasma	Glucose	Glucose	Main energy source for the body. Increases due to high cortisol secretion.

Plasma	GSH	Glutathione	An antioxidant preventing damage to important cellular components caused by reactive oxygen species.
Plasma	H2S	Hydrogen sulfide	Results from the bacterial breakdown of organic matter in the absence of oxygen.
Plasma	HbA1C%	Glycated haemoglobin percentage	A form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods
Plasma	HDL cholesterol	High-density lipoprotein cholesterol	Thought to be the 'good' cholesterol.
Plasma	IFN-γ	Interferon gamma	A cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections
Plasma	IL-10	Interleukin-10	Anti-inflammatory cytokine
Plasma	IL-1β	Interleukin-1 β	An important mediator of the inflammatory response.
Plasma	IL-2	Interleukin-2	Part of the body's natural response to microbial infection, and in discriminating between foreign ("non-self") and "self".
Plasma	IL-4	Interleukin-4	Key regulator in humoral and adaptive immunity.
Plasma	IL-6	Interleukin-6	Pro-inflammatory cytokine
Plasma	Insulin	Insulin	Secreted by the pancreas and critical for regulation of blood glucose levels.
Plasma	Iron	Iron	Has an important role in biology, forming complexes with molecular oxygen in hemoglobin and myoglobin and in other enzymes.
Plasma	KYN	Kynurenic acid	A product of the normal metabolism of amino acid L-tryptophan. It has neuroactive

			activity, acting as an antiexcitotoxic and anticonvulsant, most likely as an antagonist at excitatory amino acid receptors. May influence important neurophysiological and neuropathological processes.
Plasma	KYN/TRP	Ratio of kynurenic acid to trypophan.	May indicate increased disease progression.
Plasma	Lactate	Lactate or lactic acid	Constantly produced from pyruvate via the enzyme lactate dehydrogenase (LDH) during normal metabolism and exercise.
Plasma	LDH	Lactate dehydrogenase	Catalyzes the conversion of lactate to pyruvate - released during tissue damage.
Plasma	MDA	Malondialdehyde	Occurs naturally and is a marker of oxidative stress.
Plasma	MIF	Macrophage migration inhibitory factor	An important regulator of innate immunity.
Plasma	Motilin	Motilin	Secreted by endocrine M cells in crypts of the small intestine, especially in the duodenum and jejunum. Released into the general circulation and controls the inter-digestive migrating contractions; and stimulates endogenous release of the endocrine pancreas.
Plasma	NE	Norepinephrine I	A mediator of the sympathetic nervous system, and also involved in neurotransmission in the CNS.
Plasma	Nitric oxide	Nitric oxide	A free radical important in cellular signaling and involved in many physiological and pathological processes, including causing vasodilation.
Plasma	Nitrite	Nitrite	A source of nitric oxide in the body.

Plasma	Peroxide	Peroxide	A compound containing an oxygen–oxygen single bond or the peroxide anion, $O2^{-2}$. Highly unstable in the body.
Plasma	SOD	Superoxide Dismutase	Catalyses the partitioning of the superoxide radical into either ordinary molecular oxygen or hydrogen peroxide
Plasma	TAC	Total antioxidant capacity	Total antioxidant activity of tissue.
Plasma	TGF-β	Transforming growth factor beta	Controls proliferation, cellular differentiation, and other functions in most cells.
Plasma	TNF-α	Tumor necrosis factor α	A cell signalling protein (cytokine) involved in systemic inflammation - one of the cytokines that make up the acute phase reaction. Mostly secreted by activated macrophages.
Plasma	Triglyceride	1	
Plasma	β-endorphin	β-endorphin	An endogenous opioid neuropeptide found in the neurons of both the central and peripheral nervous system.
Plasma	ACTH	Adrenocorticotropic hormone	Secreted by the pituitary gland and causes secretion of cortisol/corticosterone from the adrenal cortex in response to stress.
	Serum	178	
Serum	5-HT	5-hydroxytryptamine or serotonin	A monoamine neurotransmitter derived from tryptophan, primarily found in the gastrointestinal tract (GI tract), blood platelets, and the central nervous system (CNS) of animals. Thought to be a contributor to feelings of well-being and happiness.
Serum	HbA1c	Glycated haemoglobin	A form of hemoglobin that is measured primarily to identify the average plasma

			glucose concentration over prolonged periods
Serum	Adiponectin	I	
Serum	Aldose reductase	I	
Serum	ALP	Alkaline Phosphatase	An enzyme that removes phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids.
Serum	ALT	Alanine transaminase	Mostly found in the liver, it catalyzes the two parts of the alanine cycle.
Serum	AST	Aspartate aminotransferase	Catalyzes the reversible transfer of an α - amino group between aspartate and glutamate - an important enzyme in amino acid metabolism.
Serum	BDNF	Brain-derived neurotrophic factor	Helps to support neurons and encourage growth of new neurons.
Serum	C5a	Complement component 5a	A protein fragment released from complement- -acts as a highly inflammatory peptide.
Serum	Calcium	Calcium	A chemical element critical to normal biology, involved in bone metabolism and cell signalling.
Serum	CAT	Catalase	A common enzyme found in nearly all living organisms - catalyses the decomposition of hydrogen peroxide to water and oxygen.
Serum	CBG	Corticosteroid binding globulin/Transcortin	Major protein binding glucocorticoids and progestins in the blood.
Serum	CCP	Cytochrome c peroxidase	An enzyme of the peroxidase family that reduces hydrogen peroxide to water:

Serum	Cholesterol	Cholesterol	An essential structural component of all animal cell membranes, required to maintain membrane structural integrity and fluidity.
Serum	Corticosterone	Corticosterone	Steroid hormone of the adrenal cortex, secreted in response to stress.
Serum	Cortisol	Cortisol	Steroid hormone of the adrenal cortex, secreted in response to stress.
Serum	COX-1	Cyclooxygenase-1	An enzyme responsible for formation of prostanoids, including prostaglandins such as prostacyclin and thromboxane.
Serum	COX-2	Cyclooxygenase-2	An enzyme responsible for formation of prostanoids, including prostaglandins such as prostacyclin and thromboxane.
Serum	CK	Creatine kinase	Present in the mitochondrial intermembrane space- it regenerates phosphocreatine (PCr) from mitochondrially generated ATP and creatine (Cr) imported from the cytosol.
Serum	Creatinine	Creatinine	A breakdown product of creatine phosphate in muscle, usually produced at a constant rate by the body.
Serum	CRP	C-Reactive Protein	A protein found in blood plasma, levels increase due to inflammation. Known as an acute phase protein.
Serum	CXCL1	(C-X-C motif) ligand 1	Expressed by macrophages, neutrophils and epithelial cells. and is a neutrophil chemoattractant. Plays a role in spinal cord development and is involved in angiogenesis, inflammation, wound healing, and tumorigenesis.
Serum	Estradiol	Estradiol	The primary female sex hormone.

Serum	GAG	Glycosaminoglycans	Long unbranched polysaccharides useful in the body as lubricants and shock absorbers.
Serum	GGT	Gamma-glutamyl transferase	An enzyme that transfers gamma-glutamyl groups, it has regulatory effects in cellular signal transduction and cellular pathophysiology.
Serum	GLP-1	Glucagon-like peptide-1	A neuropeptide and potent antihyperglycemic hormone.
Serum	Glucose	Glucose	Main energy source for the body. Increases due to high cortisol secretion.
Serum	HbA1c	Glycated haemoglobin	A form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods
Serum	GSH	Glutathione	An antioxidant preventing damage to important cellular components caused by reactive oxygen species.
Serum	HbA1c	Glycated haemoglobin	A form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods
Serum	hs-CRP	High-Sensitivity C-Reactive Protein	A marker of chronic low grade inflammation.
Serum	ICAM-1	Intercellular Adhesion Molecule 1	A type of intercellular adhesion molecule present in low concentrations in the membranes of leukocytes and endothelial cells.
Serum	IFN-γ	Interferon gamma	A cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections

Serum	IgG	Immunoglobulin G	The most common type of antibody present in the circulation, produced by plasma B cells.
Serum	IL-10	Interleukin-10	Anti-inflammatory cytokine
Serum	IL-1α	Interleukin-1 alpha	Pro-inflammatory, and promotes fever and sepsis.
Serum	IL-1β	Interleukin-1 β	An important mediator of the inflammatory response.
Serum	IL-2	Interleukin-2	Part of the body's natural response to microbial infection, and in discriminating between foreign ("non-self") and "self".
Serum	IL-4	Interleukin-4	Key regulator in humoral and adaptive immunity.
Serum	IL-6	Interleukin-6	Pro-inflammatory cytokine
Serum	iNOS	Inducible nitric oxide synthase	Catalyses the production of nitric oxide (NO) from L-arginine, and expressed mostly by immune cells.
Serum	Insulin	Insulin	Secreted by the pancreas and critical for regulation of blood glucose levels.
Serum	LDH	Lactate dehydrogenase	Catalyzes the conversion of lactate to pyruvate - released during tissue damage.
Serum	Leptin	Leptin	A hormone made by adipose cells that helps to regulate energy balance by inhibiting hunger.
Serum	LPO	Lactoperoxidase	A peroxidase enzyme secreted from mammary, salivary, and other mucosal glands that functions as a natural antibacterial agent.
Serum	MCP-1	Monocyte Chemoattractant Protein-1	One of the key chemokines that regulate migration and infiltration of monocytes/macrophages.

Serum	MDA	Malondialdehyde	A reactive species that occurs naturally and is a marker of oxidative stress.
Serum	MIP-3	Macrophage Inflammatory Protein 3 Alpha	Strongly chemotactic for lymphocytes and weakly attracts neutrophils.
Serum	mMCPT1	Mast Cell Protease-1	A mast cell protease mainly expressed in intestinal mucosal mast cells where it promotes mucosal permeability in intestinal allergic hypersensitivity reactions.
Serum	MPO	Myeloperoxidase	Expressed mostly in neutrophils and helps them to carry out their antimicrobial activity.
Serum	Na+K+ATPase	Sodium-potassium adenosine triphosphatase	Present in all cell membranes, a solute pump that pumps sodium out of cells while pumping potassium into cells.
Serum	NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells	Controls transcription of DNA, cytokine production and cell survival.
Serum	NGF	Nerve growth factor	Involved in the regulation of growth, maintenance, proliferation, and survival of certain target neurons.
Serum	Nitrite	Nitrite	A source of nitric oxide in the body.
Serum	Nitric oxide	Nitric oxide	A free radical important in cellular signaling and involved in many physiological and pathological processes, including causing vasodilation.
Serum	NPY	Neuropeptide Y	A neurotransmitter in the brain and in the autonomic nervous system.
Serum	Osteocalcin	Osteocalcin	A protein found in bone and dentin, secreted by osteoblasts.
Serum	PGE2	Prostaglandin E2	Important roles in labour, bone, and ultimately results in fever.
Serum	Phosphorus	Phosphorus	A chemical element essential for life.

Serum	Prolactin	Prolactin	Essential for lactation and also plays a role in metabolism and regulation of the immune system
Serum	SALP	Sulfated alkaline phosphatase	Sulfated form of alkaline phosphatase
Serum	SOD	Superoxide dismutase	Catalyses the partitioning of the superoxide radical into either ordinary molecular oxygen or hydrogen peroxide.
Serum	SP	Substance P	Substance P is an important element in pain perception
Serum	TBARS	Thiobarbituric acid reactive substances	Formed as a byproduct of lipid peroxidation and markers of oxidative stress.
Serum	Testosterone	Testosterone	The principal male reproductive hormone.
Serum	TGF-β	Transforming growth factor beta	Controls proliferation, cellular differentiation, and other functions in most cells.
Serum	GSH	Glutathione	An antioxidant preventing damage to important cellular components caused by reactive oxygen species.
Serum	TNF-α	Tumor necrosis factor α	A cell signalling protein (cytokine) involved in systemic inflammation - one of the cytokines that make up the acute phase reaction. Mostly secreted by activated macrophages.
Serum	Cholesterol	Cholesterol	An essential structural component of all animal cell membranes, required to maintain membrane structural integrity and fluidity.
Serum	Total Protein	Total protein levels in the serum	A marker of nutritional status.
Serum	Triglyceride	Triglyceride	Help to enable the bidirectional transference of adipose fat and blood glucose from the liver.
Serum	Urea	Urea	Has an important role in the metabolism of nitrogen-containing compounds.

Serum	Uric acid	Uric acid	A product of the metabolic breakdown of purine nucleotides, high levels occur in diabetes and gout.
Serum	α-amylase	α-amylase	A protein enzyme that hydrolyses large polysaccharides, such as starch and glycogen, to give glucose and maltose.
Serum	β-endorphin	β-endorphin	An endogenous opioid neuropeptide found in the neurons of both the central and peripheral nervous system.
Serum	T3	Triiodothyronine	A hormone secreted by the thyroid gland, critical for normal metabolism.
Serum	T4	Thyroxine1	A hormone secreted by the thyroid gland, critical for normal metabolism- the main thyroid hormone in the circulation.

Some of the protein biomarkers measured increased in response to pain and others were decreased (see Table 5.6). Most of the proteins measured at increased levels during pain were in blood plasma and serum, with 27 proteins increased in plasma and 55 proteins measured in the serum. Some biomarkers, such as cortisol, were measured at both decreased and increased levels during pain. This highlights the risk of relying on a single biomarker, as during acute pain cortisol levels will rise, but following prolonged and severe pain there may be adrenal exhaustion, resulting in decreased secretion of cortisol (McEwen and Kalia 2010).

Table 5.6 There were increased or decreased levels of the following protein biomarkers in the included studies, from the selected list of tissues expected to be more practical for measuring in future studies.

Tissue/Biomarker	Increased	Decreased
Blood		
	CD3+CD4+	
	Glucose	
	IL-17	
	IL-6	
Blood vessels		
	α1-AR	
Cerebrospinal fluid		
	Adrenomedullin	
	Aspartate	
	Glutamate	
	MIF	
	PGE2	
	ROS	
	TNF-α	
Endoneurial macrophages		
	GFAP	
Lymphocytes		
	A2AAR	
Peripheral blood (membrane)		
		ABP
	APN	APN
	DPPIV	DPPIV
Peritoneal fluid		
	IL-6	
	PGE2	
	TNF-α	
Plasma		
	5-HT	
	ACCII	
	CGRP	
	CK	

	Corticosterone/Cortisol	Cortisol
	CPK	
	C-Reactive Protein	
	ET1	
	Glucose	
		GSH
	H2S	
	HbA1C%	
	HDL cholesterol	
	IFN-γ	
		IL-4
	IL-10	IL-10
	IL-1β	
	IL-6	
		Insulin
		Iron
	Kynurenic acid	
	Lactate	
	Malondialdehyde	
	MDA	
		Motilin
	NE	
	Nitric oxide	
	Nitrite	
	NO2	
	Peroxide	
		SOD
		TAC
		TGF-β
	TNF-α	
	β-endorphin	
Serum		
	5-HT	
	A1c	
	Adiponectin	
	Aldose reductase	
	ALP (Alkaline phosphatase)	ALP (Alkaline phosphatase)
	ALT	ALT
		BDNF
	C5a	
	Calcium	
	CAT	CAT
		CBG
	CCP	
	Cholesterol	

	Corticosterone	Corticosterone
	Cortisol	
	COX-1	
	COX-2	
	CPK	
	C-RP	
	Estradiol	Estradiol
	GAG	
	Glucose	
		GLP-1
	Glutamic oxaloacetic transaminase (AST)	
		GSH
	HbA1c (Glycated Hb)	
	hs-CRP	
	ICAM-1	
		IFN-y
	IgG	
	IL-10	IL-10
		IL-1α
	IL-1β	
		IL-2
		IL-4
	IL-6	IL-6
	iNOS	
	Insulin	Insulin
	Leptin	
	LPO	
	MCP-1	
	MDA	
	MIP-2	
	MIP-3	
	MPO	
		NA+K+ATPase
	NF-κB	
	NGF	
	Nitrite	
	NO	
	Osteocalcin	
	PGE2	
		Prolactin
	SALP	
	SGPT	
	SOD	SOD
	SP	

		TBARS
	Testosterone	Testosterone
	TGF-β	
	TNF-α	TNF-α
	Total Cholesterol	
		Total protein
	Triglyceride	
		T3
		T4
	Uric acid	
	α-amylase	
	β-endorphin	

6. Discussion

The most important finding of this systematic review is the enormous number of studies, mostly using rodent models, targeted at understanding and treatment of human pain. Owing to the constraints of the project the recent and hence most developed elements of the field of literature were included in the systematic analysis work program. This dataset represent a range of noxious injuries that were employed to create and research the exaggerated pain state. As such, these data are relevant across a range of injury and disease states that are associated with increased amounts of pain. Finally, the enormous list of biomarkers versus the relatively restricted number of biomarkers commonly used in pain studies in livestock, means the quantification of these factors in readily accessible biological fluids and tissues provides an exciting opportunity to integrate this information into a translatable test of pain.

While the higher number of pain studies directed at human pain versus pain in livestock is understandable given the multi-billion dollar market for analgesics in human medicine, one can argue that the animal models are a better model of pain in other animal species than in humans. It is interesting that even when larger animal models are set up they may still be used as a model of human pain. For example, in a pig incisional model of postoperative pain (Castel, Willentz et al. 2014) the stated aim was to validate new topical and localised treatments for postoperative pain in humans. In a more recent publication, a model of peripheral neuritis trauma, a type of neuropathic pain, has been developed in pigs and seems to mimic human disease more closely than rodent models (Castel, Sabbag et al. 2015). The literature involving animal models of human pain can be used to inform research on pain in livestock, an approach used in this study.

The dominance of immune factors, and the increase in predominantly proinflammatory factors in the range of biologics associated with pain in the analysis confirms the underlying hypothesis of the neuroimmune system involvement in pain. Both nociceptors and immune cells have important roles in protecting the host from potential threats to homeostasis, and the bidirectional signaling that occurs between these two systems is still being elucidated (McMahon et al. 2015). For example, proinflammatory cytokines, such as IL-6 and TNF-α, act directly on the nociceptive system, significantly contributing to hyperalgesia (Schaible 2014). Moreover, the prevalence of examples of these factors being measured in accessible biological samples empowers the search for readily available pain biomarkers. Importantly, unlike past assessments of factors, such as cortisol, that are associated with

and triggered following a painful state, here we see the abundance of neuroimmune factors causally contributing to the enhanced nociception. Such a biomarker range is less likely to be susceptible to false positives and negative results.

Although it is valid to search for additional biomarkers to use in the measurement of pain, no single biomarker is likely to be useful. To gain a better understanding of the response of the animal, both physiological and behavioural measures are necessary (Mogil 2009). Behavioural measures were not the focus of this review, although a range of behavioural assessments were employed in the Included Studies, providing transferable measures between species and hence of relevance to more than just to the human setting. Our focus was on physiological measures, in the form of biomarkers. In a recent review of using physiological measures to objectively measure pain, Cowen et al. (2015) discuss five different methods. These include:
1) monitoring changes in the autonomic nervous system (e.g. measuring blood pressure and heart rate);
2) biopotentials, or electrical signals used to transfer information across living cells (e.g. electrocardiography (ECG), electro-encephalography (EEG) or electromyography (EMG))
3) neuroimaging (e.g. CAT or MRI scan);
4) use of biomarkers- defined as *"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention"* (Treister, Kliger et al. 2012); and
5) composite algorithms (multiple independent measures combined to a composite score),

The use of composite algorithms is particularly interesting and relevant for the enormous list of biomarkers resulting from the current systematic review. It is obvious that individual studies used multiple biomarkers, with around 600 individual biomarkers used in the 372 Included Studies. Individual measures are unlikely to be validated markers of pain alone, or all studies would have used a single biomarker. The way forward is likely to involve multiple biomarkers and other physiological and behavioural measures, combined to give the best estimate of the experience of pain to an animal. Such algorithms have already been conducted in human patients with relatively homogeneous forms of pain and have shown promise (Seitsonen, Korhonen et al. 2005, Treister, Kliger et al. 2012). There is great potential in using this approach in animal studies. Given the systematic review was conducted using the available data, it is unclear if compiling all the identified biologics into a single test would result in greater precision and sensitivity than a subset of targets. As such, future prospective studies should consider a principle components analysis of the collected data to facilitate identification of the most informative biologics. This approach will enable the creation of a more cost effective testing procedure.

A systematic review is a type of literature review to address a specific research question, and seeks to identify, select, apprise and synthesise all relevant information for that question. The structured process followed to complete a systematic review, and the inclusion of all studies based on inclusion and exclusion criteria devised prior to the systematic review commencing, make it an unbiased method to review previous studies (de Vries et al. 2014). It is also reproducible provided others follow the same steps. In contrast, narrative reviews may aim to provide expert opinion on a research topic, with included studies based on the authors' expert knowledge, which may be subjective. Increased interest in the use of systematic reviews in animal research is illustrated by new organisations such as SYRCLE (SYstematic Review Centre for Laboratory animal Experimentation; https://www.radboudumc.nl/Research/Organisationofresearch/Departments/cdl/SYRCLE/Pages/defaul

t.aspx) and CAMARADES (Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies, http://www.dcn.ed.ac.uk/camarades/)

Biomarkers were extracted from studies using models of nociceptive pain (e.g. Postincisional/Incisional/Postoperative Pain, n=13), inflammatory (n=49) and neuropathic pain (n=149). Chronic pain is the major problem in human medicine, and this was reflected by models using inflammatory and neuropathic pain being more common. In intensively and extensively farmed animals, common types of pain would include each of these categories. In uncomplicated husbandry procedures where wounds heal without infection (e.g. castration, tail docking, teeth clipping) acute nociceptive pain is likely to be resolved within days. Inflammation is a normal response of the body to tissue injury, but if there is unresolved healing and/or infection then chronic inflammatory pain may result. Examples include if infection occurred following the normal husbandry procedures listed above, or in chronic laminitis in dairy cows. Neuropathic pain is associated with injury or disease of neural tissue. For example, traditional methods of beak trimming of layer hens using a hot blade were more likely to result in neuroma formation, likely to be associated with chronic pain (Breward and Gentle 1985). Finally, using rings for castration is likely to involve ischaemic pain; in this review only two included studies used an ischaemic surgical model.

It is likely a panel of biomarkers, rather than an individual marker, will be needed to more accurately predict and monitor pain in animals. Each potential biomarker should be assessed in relation to:

- Ease of sample collection. This report has concentrated on body tissues (e.g. blood) which may be more readily sampled.
- Ease of detection and testing. Newer techniques, such as those using biophotonics, allowing non-invasive measurement and microscopic amounts to be analysed will extend the list of potential biomarkers.
- Lack of ambiguity- ideally some biomarkers will be increased and others decreased by pain. Data analysis techniques may allow a 'fingerprint' of pain to be identified using several markers, meaning it is not simply an increase or decrease in single biomarkers to be diagnostic, but the overall pattern.
- Timely and low cost- new technologies are reducing the potential time needed to collect and analyse tissue samples. To be feasible in farming systems, this will be critical.

The full literature search found 901 studies including a biomarker, with only 63% of these (n=567) fully extracted. Extraction occurred in two steps, initially extracting all of the information relating to the study (e.g.) into an Excel spreadsheet, and then looking at only the studies that were possibly identified as including biomarkers to extract the biomarkers used and the tissue from which they were sampled. Once the full text was interrogated more fully during the process of extracting the biomarkers, only 379 of the 567 studies (67%) included a valid biomarker. Although not all of the studies were extracted for this research project due to the time intensive nature of the search and extraction processes, the studies were taken in sequence beginning with the most recent papers, meaning it was unlikely any bias was introduced in relation to the biomarkers found. Due to time constraints, it was not possible within this project to list the biomarkers in relation to the specific type of pain model. However, with continued work on the dataset, which is available to all funding bodies. the aim will be to obtain lists of potential biomarkers in relation to specific types of pain.

7. Implications & Recommendations

There will be ongoing changes in the expectations and standards for food production, both due to shifting community ideals and ongoing research in animal welfare science. The current project represents a pro-active approach to learn more about pain and facilitate markers for future research into analgesics for pork and other livestock production, in order to optimize welfare of animals. This approach may even prevent future emergencies in public trust in animal industries due to concerns relating to animal welfare by staying one step ahead, but at the very least will place the industry in a strong position to respond rapidly to future community concerns.

Specific industries may use the data set to determine biomarkers most valid in their own farming systems. For example, if husbandry procedures such as castration or tail docking are most problematic in terms of public opinion, then the incisional and post-surgical models may be examined to find potential biomarkers. The potential biomarkers will then need to be reviewed to ensure there are existing laboratory methods to measure them. Many of the studies included involved rodent models, and there are more monoclonal antibodies and commercial assays available for rodent proteins than for similar proteins in livestock species. Hence it is possible part of this process will be in validating new methods of measurement. Once several potential biomarkers are identified, the next step will be to determine if they can be used to detect pain in experimental studies, for example with animals castrated versus handled without castration. These experimental models may use multiple body tissues, with the aim to determine the optimal site and time of measurement in relation to pain. Techniques such as biophotonics are moving ahead very quickly, and it will be necessary for experts in animal handling and biology to collaborate with experts in this and similar innovative technologies.

The systematic review conducted represents an unprecedented systematic analysis of pain biomarkers and is of substantial relevance to the field. Whilst there is great promise, this analysis has revealed the bias in the past pain research community of an extremely reductionist and targeted approach in the research to explore pain mechanisms. What this has created are artificial silos in the literature which do not account for the systems nature of pain. This in turn has not enabled a networks approach to capture pain in a single measure. As such, when searching for biomarkers the assumption that a single target will "measure pain" is clearly fraught with inaccuracies. It is clear that there are no single available measures than can be applied today to solve the production animal pain problem. This does not make inaction appropriate. Rather it says timely action is needed. Hence, the multiplexed approach will be needed to create a more robust test that is transferable across pain states. For this to be cost effective, existing biologics based assessments will be cost prohibitive. Novel approaches will be needed.

8. Intellectual Property

Since all of the studies have previously been published, there is no new intellectual property arising from the present study.

9. Technical Summary

Systematic review protocols are well validated, and no new technical developments were used in this research.

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11. Publications Arising

There have been no publications arising from this work to date. However, there are plans to publish in a high impact scientific journal in the next months.

Appendix 1

Table A.1 Full list of biomarkers used to evaluate pain in included studies in all tissues.

Biomarker	**No/times measured**
μOR	2
1- and 2-AG	5
11βHSD1	2
3-nitrotyrosine	1
3-NT	1
4-HNE	2
5-HIAA	3
5-HT	6
5HT2A	4
5-HT3	1
8-OHG	1
A1c	1
A2AAR	1
ABP	6
ABTS	1
ACCII	1
Acetyl H3	1
ACTH	2
ADAMTS5	2
Adiponectin	1
Adrenomedullin	1
AEA	5
AGE	1
AIF	2
Aldose reductase	1
Alkaline phosphatase	2
ALP	3
ALT	5
ALX	1
AP-1	1
APN	7
Apobec3b	3
Arg1	1
Aspartate	1
AST	6
AT1R	2
AT2R	2
ATF3	16
B7-H1	1
Bax/Bcl2	1
BDNF	11

Beclin-1	1
BKα1	1
Bv8	2
C%a	1
C1q	2
C3	1
C5a	2
Calcium	3
Camk2d	1
CaMKII	1
CamKIII	1
CaMKIIIα	1
Cardiac troponin-I	1
Caspase-1	2
Caspase-12	1
Caspase-3	3
Caspase-4	1
Caspase-8	1
CAT	12
Caveolin-1	2
CB1	1
CB2	2
CBG	1
CCL1	2
CCL11	1
CCL12	1
CCL19	1
CCL2	17
CCL20	1
CCL21	1
CCL3	6
CCL4	1
CCL5	3
CCP	1
CCR1	4
CCR2	8
CCR5	5
CCR8	2
CD11+CD86+DCs	1
CD11b	11
CD11b/c	1
CD11c	1
CD14	1
CD206	1
CD25	1

CD3+CD4+	1
CD4	1
CD4+CD25+	1
CD40	3
CD68	1
CD74	2
CD86	1
CDK4	1
c-fos	5
CGRP	16
Cholesterol	4
CINC	1
CINC1	1
CINC-1	1
CINC-2	2
CINC3	1
CK	1
Cleaved Caspase-3	1
Cleaved Caspase-9	1
Cleaved PARP	1
Cleaved SPECTRIN	1
Clec7a	3
CNPase	1
CNTF	2
Col2	1
Collagen	1
Corticosterone	9
Cortisol	9
COX-1	5
COX-2	21
CPK	2
Creatinine	2
C-Reactive Protein	3
CREB	2
CTGF	1
CX3CL1/fraktalkine	4
CX3CR1	2
CXCL1	10
CXCL1/KC	2
CXCL10	5
CXCL11	2
CXCL13	3
CXCL14	2
CXCL16	2
CXCL19	1

CXCL2	5
CXCL5	1
CXCL9	3
CXCR1	1
CXCR2	4
CXCR3	2
CXCR4	4
cyclin D1	1
cyclin E	1
DA	1
DAPI	2
D-hair fibres	1
DOPAC	2
Dopamine	2
DP1	1
DP2	1
DPPIV	5
Dynorphin	5
EAAC1	1
ED1	3
ED1+CD45+	1
EM-2	1
EMR1 F4/80	2
Enkephalin	4
eNOS	1
EØP	2
EP 24.15/24.16	1
EP 24.15/24.17	1
EP 24.15/24.18	1
EP 24.15/24.19	1
EP 24.15/24.20	1
ERK	3
ERK1	1
ERK-1	1
ERK1/2	1
ERK-2	2
Estradiol	3
ET1	2
F4/80	1
F4/80+CD206+	1
F4/80+iNOS+	1
FAAH	6
Fam22f	3
FcεR1α	1
FGF	1

FGF1	2
FGF2	2
Fibronectin	1
FKN	2
Fos	3
Fos+ cells	3
FosB/ΔFosB	1
FRAP	1
Gabrα6	4
GAG	2
Gal3	1
Galanin	1
Gamma glutamyl	1
GAP43	1
G-CSF	2
GDNF	7
GDNFRa-1	1
GFAP	72
Gimap5	3
GITR	1
GITRL	1
GLAST	1
GLP-1	1
GLP-1R	2
GLT-1	2
GluA1	3
GluA2	2
Glucose	11
GluR1	3
GluR2	2
GLUT-3	1
Glutamate	2
Glutamic pyruvic transminase	1
Glycated Hb	1
GM-CSF	2
GPx	4
GR	2
GRK6	2
GSH	12
GSH-PX	1
H2O2	1
H2S	5
HbA1c	2
HDAC1	1
HDL cholesterol	1

HIF-2α	1
HMBG1	1
HMG-B1	2
HMG-B2	1
HMG-B3	1
HMG-B4	1
HO-1	6
HO-2	2
H-PGDS	1
hs-CRP	1
HSP70	1
HSP72	3
HSP90	1
Iba-1	32
Iba-2	3
ICAM-1	2
Ido1	4
Ido2	1
IENF	1
IFN-γ	20
IGF1	2
IgG	3
IKKβ	2
IL-1	2
IL-10	82
IL-12	1
IL-12p40p70	1
IL-12p70	1
IL-13	8
IL-15	2
IL-16	2
IL-17	13
IL-17RA	4
IL-18	2
IL-1ra	11
IL-1rn	1
IL-1α	11
IL-1β	193
IL-2	6
IL-23	1
IL-23R	1
IL-3	2
IL-4	14
IL-4Rα	2
IL-6	116

IL-8	8
IL-9	1
iNOS	17
Insulin	6
IP-10	2
IR	2
Iron	1
ITGAM	6
IκB	1
IκBα	2
JNK	2
JNK-1	1
JNK-2	1
Kalirin	3
KC	6
KC/CXCL1	1
KCC2	1
KOR	14
KYN	1
KYN/TRP	1
Kynurenic acid	2
Lactate	1
LCN2	2
LDH	2
Leptin	1
Leu-enkephalin	1
Leukocytes	2
LIF	6
LIF-R	5
Lipid peroxide	1
LIX	1
L-PGDS	1
LPO	5
L-selectin	1
Ly6G	1
Lymphocytes	1
MAC-1	1
MAGL	2
Malondialdehyde	2
MAP kp38	1
Mapk14	1
MBP	3
MCP-1	13
MCP-3	2
M-CSF	1

MDA	12
Met-enkephalin	1
MFI	1
Mfn2	1
mGluR5	1
MHC-I	1
MHC-II	4
MIF	4
MIG	1
MIP-1α	5
MIP-2	4
MIP-2α	1
MIP-3	1
mMCPT1	1
MMP	1
MMP-1	3
MMP-13	6
MMP-2	7
MMP2 (Active)	1
MMP2 (Pro)	1
MMP-3	3
MMP8	1
MMP-9	7
Monocytes	1
MOR	4
Motilin	4
MPO	36
MPZ	1
MRC2	1
MrgC	1
Mrp4	1
mTOR	1
Na+K+ATPase	2
NAG	1
NAPE-PLD	1
NaV1.7	1
NDRG2	1
NE	2
NEP	1
Neuropeptide Y	4
Neutrophil:Leukocyte	1
Neutrophils	1
NF200	5
NFATc1	2
NFATc2	2

NFATc3	2
NFATc4	4
NF-κB	26
NF-κB p65	2
NGF	11
NGF-β	4
NITEGE	1
Nitric oxide	1
Nitrite	8
Nitrotyrosine	1
NK1	2
NK1r	1
NMDA1r	1
NMDAR1	2
nNOS	4
NO	4
NO2	1
Noradrenaline	2
NOS1	1
NOS-2	2
Nox	1
NOX-2	1
NR1	1
NR2B	2
NRG1-β1	1
NT-3	3
Occludin	3
OEA	3
Osteocalcin	2
OX-42	25
P2X3	7
P2X4	6
P2X7	4
p38	7
p38 MAPK	4
p42 MAPK	1
p44 MAPK	1
PAFR	2
p-AKT	1
PAR-2	2
pCaMKII	1
PCNA	1
PDYN	16
PEA	3
Penk	1

pERK	3
p-ERK	6
pERK-1	3
p-ERK1/2	1
pERK-2	3
Peroxide	1
PGE-2	5
PGEM	1
p-GluA1 (ser831)	1
p-GluA1 (ser845)	1
pGluR1 (Ser 831)	1
P-gp	1
PGP+	2
PGP9.5	1
p-GSK3β	1
Phosphorus	1
P-IKKβ	2
p-IR	1
p-IκBα	2
pJAK2	1
pJNK-1	1
pJNK-2	1
PK2	3
PKCα	1
PKCαβγ	1
PKCβ I	1
PKCβ II	1
PKC-γ	4
PKCε	1
PKC-σ	1
PKR1	3
PKR2	2
PLF	2
p-NF-κB	1
pNR1	3
P-NR1	4
p-NR2B	3
POMC	1
POP	5
p-p38	21
p-p38 MAPK	3
p-p44/p42	1
p-p65	2
PPARα	2
PPARγ	3

pPKC	1
pPKCγ	2
ppt-A	1
Prkaca	1
Prkcg	1
Prodynorphin	1
PROK2	2
Prolactin	4
proMMP-2	1
proMMP-9	1
Protein carbonyl	1
PSD-95	1
pSGK1	1
p-STAT3	3
Ptgs2	1
PY14Cav-1	1
RA-Aβ fibres	1
Rab4	1
Rab7	2
RANKL	1
RANTES	2
ROS	1
Rwdd3	3
S100β	2
S1P	1
SA-Aβ fibres	1
SALP	1
SCOCS3	1
SDF1	4
SERT	2
SGK1 (total)	1
SGOT	1
SGPT	1
Slc7a7	3
sICAM-1	1
Smase	1
SOCS1	1
SOCS2	1
SOCS3	3
SOD	19
SOM	4
SOM-LI	1
SP	6
SphK1	1
SPT	1

SSeCKS	2
SST	2
sstr2	2
sTNF RI	1
sTNF RII	1
Substance P	9
Synaptophysin	1
T3	2
T4	2
TAC	1
T-AOC	1
TBARS	6
Tenascin-C	1
Testosterone (Female)	1
Testosterone (Male)	1
TGF-β	4
TGF-β1	2
TGF-β-R1	2
t-GSH	2
Thymus chemokine	1
TIMP1	2
Timp-1	2
TIMP2	2
TLR2	1
TLR4	10
TNFR1	3
TNFR2	1
TNF-α	196
tNR2B	1
Total p44/42	1
Total Protein	1
Total WBC	1
TP	1
TPH-1	2
Trem	1
Triglyceride	5
TRPA1	3
TRPM2	1
TRPM8	1
TRPV1	7
TRPV1/PTH2	1
TRPV-4	2
T-SOD	1
TSPO	1
TUJ1	2

TXAS	1
Urea	1
Uric acid	2
VCAM	1
VEGF	2
VEGF120	1
VEGF164	1
VEGF188	1
VEGF-A	1
Vimentin	1
VR1	1
ZO-1	1
α,β me-ATP	1
α1-AR	8
α2/δ1	2
α-amylase	1
αCD206	1
β-actin	1
β-endorphin	13
β-EP	1
δ-ALA-D	2
δOR	1