Osteoarthritis: Translational animal research with rodents

Pharmacological and behavioral validation of Complete Freund's Adjuvant-induced ankle joint pain model in rats

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TIIVISTELMÄ

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Lääketieteellinen tiedekunta

ANNI SUOMINEN: Nivelrikko: translationaalinen eläintutkimus jyrsijöillä - Rotille Freundin täydellisellä adjuvantilla aiheutetun nilkkanivelkivun validointi farmakologisesti ja käyttäytymistestein

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Nivelrikko on yleisin toimintakyvyttömyyttä ja nivelkipua aiheuttava liikuntaelimistön vaiva, jonka syntymekanismit ovat vielä puutteellisesti tiedossa ja hoitomahdollisuudet rajalliset. Nivelkipu on suurin nivelrikkopotilaiden elämänlaatua heikentävä oire, jonka hoitamiseen tarvitaan uusia, tehokkaampia lääkkeitä ja tutkimukseen luotettavia, vertailukelpoisia eläinmalleja. Rotalle Freundin täydellisellä adjuvantilla aiheutettua nivelkipua käytetään mallintamaan ihmisen tulehduksellista nivelkipua. Tämän tutkimusprojektin tarkoituksena oli verrata kahden eri mittausmenetelmän, CatWalk ja Incapacitance tester, kykyä havaita tätä aiheutettua kipua.

Käytetty Freundin täydellinen adjuvantti eli CFA (engl. Complete Freund's Adjuvant) on mykobakteeria (*Mycobacterium tuberculosis*) sisältävä vesi-öljysuspensio, jota käytetään yleisesti aiheuttamaan tulehdus koe-eläimille. Tässä tutkimusprojektissa CFA ruiskutettiin rotan vasemman takajalan nilkkanivelen sisälle (= i.a. CFA rottamalli), jonka seurauksena rotille aiheutui niveltulehdus ja nivelrikkoa mallintava tila sekä siitä aiheutuvaa kipua. Tulehduskivun farmakologista lievittymistä mitattiin tunnettujen kipulääkkeiden, naproxenin ja pregabaliinin, avulla ja kivun spontaaniutta ja voimakkuutta arvioitiin käyttäytymistestein kävellessä (dynaaminen) ja seistessä (staattinen). Liikekipua mitattiin automatisoidulla CatWalk XT (Noldus, Alankomaat) laitteistolla, joka mittaa ja analysoi rotan kävelyä. Seistessä aiheutuvaa kipua arvioitiin Incapacitance tester -laitteella (Linton Instrumentation, Iso-Britannia), joka mittaa takajaloillaan seisovan rotan jalkojen välistä painonjakautumista. Farmakologista mallin validointia jatkettiin lisäksi testaamalla pre-kliinisesti kiinnostavia, kipua lievittäviä eri vaikutusmekanismein toimivia aineita.

CFA:lla aiheutettu toisen jalan niveltulehdus aikaansai epätasaisen painonjakautumisen takatassujen välillä, joka oli havaittavissa sekä kävellessä että seistessä ja osoitettavissa tassujen välisen painonjakautumissuhteen avulla (= weight bearing ratio). CatWalk laitteella niveltulehduksen vaikutukset kävelyyn pystyttiin lisäksi osoittamaan monien muidenkin parametrien avulla, joista kuvaavin oli "guarding index" eli kipeän tassun suojelua ja kompensointia muiden tassujen avulla kuvaava indeksi. Hoito naproxenilla (NSAID) auttoi osittain palauttamaan CFA:n aiheuttamat ongelmat, mutta neuropaattisen kivun lääkitsemiseen käytetty pregabaliini ei. Myöskään muut testatut farmakologiset yhdisteet eivät näyttäneet selviä parantavia vaikutuksia.

Tutkimusprojektin tulosten perusteella voidaan sanoa i.a. CFA rottamallin olevan tehokas nilkan tulehduksellisen nivelkivun mallintamisessa. Farmakologisesti se todistettiin osoittamalla tulehduskipulääkkeen toimivuus ja hermokipulääkkeen toimimattomuus, sekä toiminnallisesti objektiivisten ja hyvin toistettavissa olevien dynaamisen ja staattisen käyttäytymistestin avulla. Lisäksi malli on hyvin vertailukelpoinen kliiniseen potilastutkimukseen, sillä validoinnissa käytetyt lääkeaineet ovat kliinisessä käytössä ja kivun testaus rotilla tehtiin kuten potilailla: kipua mitattiin sekä seistessä että kävellessä.

Asiasanat: CFA, i.a. = intra-articular, käyttäytymistesti, farmakologinen aine, NSAID = non-steroidal anti-inflammatory drug

Content

Abbreviations

ACL	anterior cruciate ligament
ACR	American College of Rheumatology
ARRIVE	Animal Research: Reporting of *In Vivo* Experiments - guidelines
CB1R	cannabinoid 1 receptor
CB2R	cannabinoid 2 receptor
CFA	Complete Freund's adjuvant (=Freund's complete adjuvant)
COX1	cyclooxygenase 1 enzyme (which is a prostaglandin G/H synthase enzyme, EC 1.14.99.1)
COX2	cyclooxygenase 2 enzyme (which is a prostaglandin G/H synthase enzyme, EC 1.14.99.1)
DEPART	Design and Execution of Protocols for Animal Research and Treatment -guidelines
EMA	European Medicines Agency
EULAR	European League Against Rheumatism
FAAH	fatty acid amide hydrolase
FDA	US Food and Drug Administration
IL-1	interleukin-1
i.a.	intra-articular; administration route by injection of a compound within the cavity of a joint
i.p.	intraperitoneal; administration route by injection of a compound into intraperitoneal space
mAb	monoclonal antibody
MGL	monoacylglycerol lipase
MHIQ	McMaster Health Index Questionnaire
MIA	monosodium iodoacetate
MPQ	McGill Pain Questionnaire

NGF	neuronal growth factor
NSAID	nonsteroidal anti-inflammatory drug
NC3Rs	UK National Centre for the Replacement, Refinement and Reduction of animals in research
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
OMERACT	Outcome Measures in Rheumatology (an international, informally organized network initiated in 1992 aimed at improving outcome measurement in rheumatology)
PAM	pressure application measurement device
p.o.	per os/ peroral; oral administration route
SF-36	Short Form 36
sLA	spontaneous locomotor activity
SNRI	selective serotonin and noradrenaline reuptake inhibitor
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
TRPV 1	transient receptor potential vanilloid 1 receptor = capsaicin receptor
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index

LITERATURE REVIEW – Osteoarthritis from clinical aspects to pre-clinical studies and vise versa – the importance of animal research translatability

1 Introduction for the literature review

Osteoarthritis (OA) is a musculoskeletal disorder affecting people worldwide. A growing number of individuals suffer from it especially due to increasing age but also due to other predisposing risk factors such as the increasing prevalence of obesity (Dimitroulas *et al.*, 2014). It is a common disorder with elderly but occurs in younger individuals too, usually following injury or rigorous physical activity (Sharma, Kapoor and Issa, 2006). It is the most common musculoskeletal disorder causing functional disability and joint pain leading to limitations in everyday normal living and impaired quality of life. OA has also socioeconomic effects by elevated costs of national health systems (Sharma, Kapoor and Issa, 2006; Centers for Disease Control and Prevention (CDC), 2007), especially since pain is the major reason for seeking medical help. Due to vast prevalence, higher life expectancy of people, impacts on health care and the currently insufficient treatment options with limited analgesic medications and lack of disease modifying drugs, OA has a big unmet need for more efficacious therapies.

Besides clinical studies, pre-clinical experimental animal research is undoubtedly important, and at least to date, an inseparable part of both basic and applied sciences. Consequently drug development is not possible without animal models and experimental methods implemented *in vivo*. For investigating OA and OA-related pain many pre-clinical animal models and testing methods have been developed, of which this literature review concentrates on experimental models with mice and rats. Besides considering different ways to induce OA and surveying pain behavior measuring methods, the reliability and translational properties between pre-clinical models and measuring practices and clinical trials are also evaluated.

2 Osteoarthritis in patients

2.1 Phenotype of the disease

The definition of OA has developed and evolved considerably in time, and the consensus on the definition is currently the following:

> *"OA diseases are a result of both mechanical and biologic events that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix, and subchondral bone. Although they may be initiated by multiple factors, including genetic, developmental, metabolic, and traumatic, OA diseases involve all of the tissues of the diarthrodial joint. Ultimately, OA diseases are manifested by morphologic, biochemical, molecular, and biomechanical changes of both cells and matrix which lead to a softening, fibrillation, ulceration, loss of articular cartilage, sclerosis and eburnation of subchondral bone, osteophytes, and subchondral cysts. When clinically evident, OA diseases are characterized by joint pain, tenderness, limitation of movement, crepitus, occasional effusion, and variable degrees of inflammation without systemic effects."* (Kuettner and Goldberg, 1995) (Figure 1).

Consequently, OA is a disorder affecting joint tissues and can take place in almost any joint, but is the most common in large weight-bearing joints, knee and hip, and thereafter spine and hands. OA is a metabolically active repair process involving loss of cartilage and remodeling the underlying bone (Dimitroulas *et al.*, 2014) in which the repairing cannot compensate the degrading part because of reduced repairing potential or e.g. trauma. This results in continuing cartilage degradation, loss of tissue components and abnormal bone production (Jones, 2013). Besides the established definition and prevalence of OA, the etiology and pathophysiology of OA still remain partially unknown. Based on the increasing amount of knowledge from basic science OA can be taken as an umbrella term for a number of pathways leading to similar pain and structural outcomes (Jones, 2013).

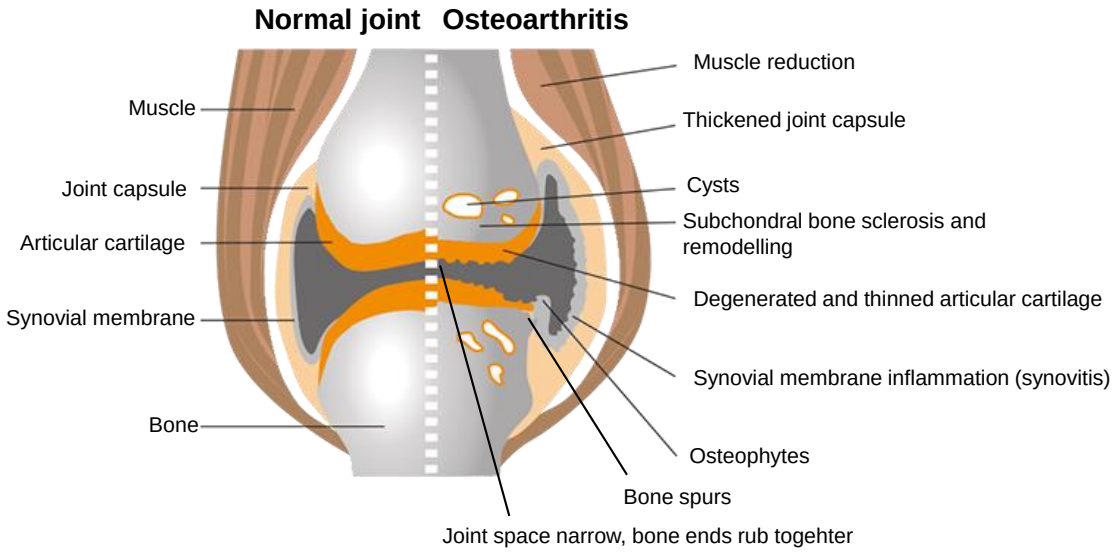

Figure 1. Anatomical and physiological changes in the joint and surrounding tissues due to osteoarthritis.
Figure modified from http://www.nivelopas.fi/nivelessatapahtuu.html

OA affects all structures within a joint. In addition to bone remodeling, a hyaline articular cartilage is lost, periarticular muscles become weaker, and besides arthritis also synovitis occur in some patients; ligaments become more loose involving laxity and lesions may develop in bone marrow inducing further trauma to the bone. OA affects the joint in a non-uniform and focal manner, with localized loss of cartilage even increasing the focal stress across the joint. When a sufficiently large area of cartilage is lost or once the bone has been remodeled, the joint starts to change its orientation and becomes tilted and develops malalignment. Malalignment is the most potent risk factor for structural weakening of the joint because it even further enhances focal loading in the joint leading to gradual deterioration of the joint and possibly eventually to failure of whole joint (Felson, 2006).

The traditional classification of OA describes the vast nature of its origin and many possible pathways leading to the disorder since OA can be determined to be primary (idiopathic), which means that the underlying reason is unknown, or it can be secondary due to joint trauma. OA can also origin from inborn or developmental abnormalities or it can develop due to other joint or bone diseases. Furthermore, even endocrine or systemic diseases can lead to OA development. When the underlying reason of OA is not known (idiopathic OA),

the disease can be limited to one joint or it can be localized affecting hands and at least one major weight bearing joint. When restricted to one joint, OA most commonly exists in the knee, hip, spine, hands or feet. In hands OA usually affects the middle or top joints in fingers (proximal and distal interphalangeals) or the bones in wrist (carpal bones) and palm (metacarpal bones), in the feet the joints between instep and toes (metatarsophalangeal joints and basal joints of toes) and the neck region of the spine (cervical) and the hip and lumbar region of the back (lumbosacral) (Sharma, Kapoor and Issa, 2006).

Even though a lot of work has been conducted to be able to create a standard definition of OA which would provide a concise description of the symptoms, disability and joint structural disease, it has turned out to be difficult. The challenge of establishing one definition is that even though some correlation has been shown between x-rays describing disease severity vs. symptoms and disability, the relationship is not that clear after all (Dieppe, 2004; Sharma, Kapoor and Issa, 2006). In addition, disease severity seen from x-rays, and the symptoms and degree of functional impairment and pain may not correspond; patient with severe OA revealed from x-ray may not have pain at all and patient with great pains may have mild OA when determined by x-ray (Figure 2) (Neogi *et al.*, 2009; Finan *et al.*, 2013).

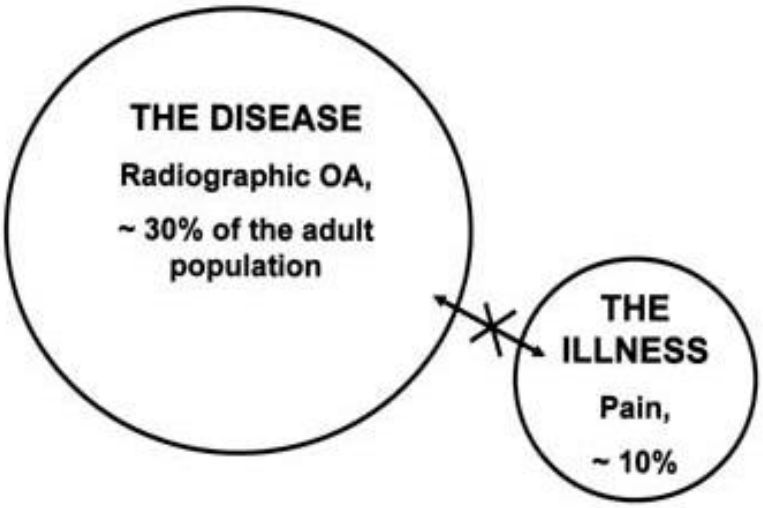

Figure 2. The disease, osteoarthritis, defined radiographically by x-ray has only a little correlation with the symptoms named as illness and characterized by pain.
Figure from (Dieppe, 2004)

2.1.1 Clinical assessment of structural severity of osteoarthritis

Structural severity of OA is most commonly determined by using the Kellgren-Lawrence grading system, a method determining radiographic severity based on the formation of osteophytes on the joint margins or in ligamentous attachments, narrowing of joint space associated with sclerosis of subchondral bone (thickness of the bone tissue right under the joint cartilage), small pseudocystic areas (fluid infiltration into tissue or fluid containing lesion) in the subchondral bone and altered shape of the bone ends (Kellgren and Lawrence, 1957; Spector and Cooper, 1993; Croft, 2005). The scoring system created by Kellgren and Lawrence was taking into use by the World Health Organization, and it has remained the predominant method for defining and grading OA since then, despite the availability of a number of competing grading systems. First the grading was divided into five grades: (0) None, (1) Doubtful, (2) Minimal, (3) Moderate and (4) Severe, where grade 0 indicating an absolute absence of x-ray changes of OA and grade 2 describing clear presence of OA with minimal severity (Kellgren and Lawrence, 1957; Spector and Cooper, 1993). Later this grading has been replaced by more accurate written definitions of each grade of OA on the Kellgren-Lawrence scale (Table 1). In this kind of scaling it is necessary that the previous grade is precursor for the next one, e.g. grade 2 need to be fulfilled when determining OA in grade 3 (Croft, 2005).

Table 1. Kellgren-Lawrence osteoarthritis structural severity grading scale

Grade	Definition
1	minute - osteophyte of doubtful significance the only feature
2	definite - osteophyte, joint space unimpaired
3	moderate - diminution of joint space
4	joint space greatly impaired, subchondral sclerosis

In epidemiologic studies, when trying to solve the incidence of OA and factors affecting it, magnetic resonance imaging (MRI) is commonly used in addition to radiography but criteria of OA based on MRI have not been established yet (Sharma, Kapoor and Issa, 2006).

2.1.2 Clinical assessment of osteoarthritic pain

Structural severity can be assessed from x-ray pictures by determining radiographic severity based on the Kellgren-Lawrence grading system as described above but it does not necessarily correlate with the symptoms an individual feels (Figure 3). Predictive validity of x-ray pictures and the use of them as a marker of clinical pain have a controversial status. This dilemma has been studied in numerous surveys and one conclusion has been that the central sensitization is especially apparent among patients reported with a high level of clinical pain in the absence of severe or even moderate pathologic changes of OA (Finan *et al.*, 2013).

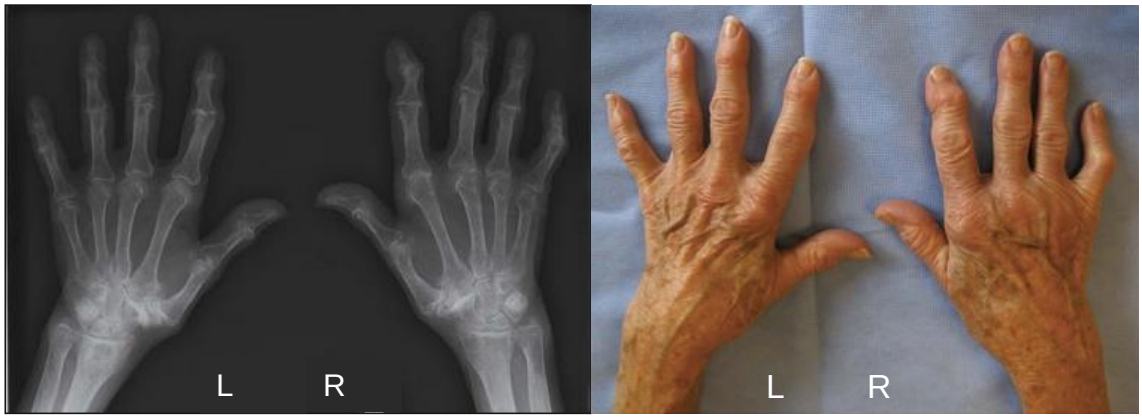

Figure 3. An example of radiographic (left) and clinical (right) features of osteoarthritis. Structural changes determination, severity assessment and their correlation is not always straightforward.
L - left; R - right
Figure modified from https://musculoskeletalkey.com/clinical-features-of-osteoarthritis/

Thus, the patient's experience of the severity of OA correlates only partially with the structural defects in the joint and the surrounding structures. The most eminent and crucial feature for patients is the pain, because it is the leading reason for impaired quality of life. Objective measuring of pain is challenging due to its subjective nature. Standardized questionnaires have been developed for evaluating individual pain as uniformly as possible. One of the most commonly used questionnaires to assess symptoms and physical and/or functional disability is Western Ontario and McMaster Universities Osteoarthritis Index WOMAC. The WOMAC index has gained growing acceptance among the OA research and clinical practice since it was introduced in 1986 (Salaffi *et al.*,

2003). In the early 1980s, the challenge among clinical pain research and practice was that there was no coherent, international way for measuring, reporting and defining the pain in different circumstances (Bellamy and Buchanan, 1984). Nicholas Bellamy, the initiator of the WOMAC index, has said:

> *"Prior to 1981, measurement procedures for quantifying pain, stiffness, and physical disability in hip and knee osteoarthritis (OA) in rheumatology were diverse and lacked standardization in content, format, and scaling. Further, health status questionnaires were available in very few languages, most often having been developed in English and translated into a few European languages. The challenge in 1981 was to build a standardized disease-specific patient-relevant self-reported health status questionnaire for hip and knee OA."* (Bellamy, 2002).

One intention for a new grading system was to create an assessment tool for clinically important patient-relevant changes in health status as a result of treatment interventions (Bellamy, 1995). At the end of 1980s, after creation of the WOMAC index, Bellamy and colleagues carried out validation experiments with OA patients receiving nonsteroidal anti-inflammatory drugs (NSAIDs) (Bellamy and Buchanan, 1986; Bellamy *et al.*, 1988b) and with OA patients undergoing total knee or hip arthroplasty (Bellamy *et al.*, 1988a) for providing evidence that reliability, validity and responsiveness, the important statements they defined themselves, were fulfilled. After these initial studies the WOMAC index has been inclusively validated in many additional studies; there are hundreds of references to the use of the WOMAC index in validation studies, comparative studies against other health status measures and in its application in various clinical research and clinical practice settings (Bellamy, 2002). Even though much has already been done and the WOMAC index has also been translated and linguistically validated in over 65 different language forms and is available in 5-point Likert (LK), 100mm visual analog (VA) and 11-point numerical rating (NR) scaling formats (Bellamy, 2005), establishing the measurement properties within any patient groups is an ongoing process.

The WOMAC index has undergone a great refinement since it was first developed from the original test form including five dimensions with 41 items

(Bellamy and Buchanan, 1986; Bellamy *et al.*, 1988b) to the current standardized three dimensional 24 items WOMAC LK3.1 and WOMAC VA3.1 versions which are particularly extensively used in assessing efficacy in pharmaceutical and biotechnology environments (Bellamy, 2002, 2005). From the original five dimensions test version pain, stiffness and physical function have been retained in the current 3.1 version WOMAC health status questionnaires which represent the core set for clinical domains in the OARSI and OMERACT (Osteoarthritis Research Society International-Outcome Measures in Rheumatoid Arthritis Clinical Trials) recommendations for clinical trials (Altman *et al.*, 1996; Bellamy *et al.*, 1997; Bellamy, 2005). Although emotional and social functions were excluded from the standard version, there are versions of the index that are either shorter or longer containing either a greater or lesser number of dimensions than the 24 items within the three dimensions of 3.1 WOMAC. Especially the emotional subscale has raised interest and is, for instance, one of the domains identified in the IMMPACT (the Initiative on Methods, Measurement and Pain Assessment in Clinical Trials) recommendations (Bellamy, 2005; Dworkin *et al.*, 2005).

The main differences between the versions of the WOMAC index are in the pain scaling type: WOMAC 3.1LK (Likert) scales the pain with 5 different adjective points (none, mild, moderate, severe, extreme), in the WOMAC 3.1VA (visual analog) version there is only adjective endpoints (e.g. no pain – extreme pain) in the 100 mm line where subjective feeling of pain is placed, and the 11-point numerical (NR) WOMAC, the primary variation of the previous ones, uses numerical rating from 0 to 11 (0 refers to no pain, 11 to extreme pain). The pain scales in general differ in their degree of responsiveness. The most used WOMAC versions, 3.1LK and 3.1VA, are more responsive than the more complex measuring methods (Bellamy, Campbell and Syrotuik, 1999; Litcher-Kelly *et al.*, 2007). The three dimensions of standard 3.1 WOMAC are pain, stiffness and physical function including five, two and seventeen questions, respectively (Appendix 1). The widespread use of the WOMAC index is presumably due to many factors, and one of the most important incentives is that patients were tightly involved with the developing process. The item content is focused on the aspects that OA patients feel relevant (Bellamy and

Buchanan, 1986). Furthermore, numerous studies evaluating the index properties the creators valued – reliability, validity and responsiveness – have been carried out. Also studies assessing and comparing the properties and variations between the different index versions as well as many development and linguistic validation studies have been conducted. All of these have made the WOMAC index well known and universally used. Continuing research and development of the content, problem solving attitude and recognition of the WOMAC index by respective groups like OARSI, OMERACT and IMMPACT and regulatory agencies like the FDA (US Food and Drug Administration) and EMA (European Medicines Agency), have created a reliable and appreciated impression of the WOMAC index (Bellamy, 2005). In Finland the working group of clinical guidelines has published a review study on the WOMAC index measuring properties stating them to be reliable and applicable for Finnish population (Arokoski, 2012) and the Finnish translation of WOMAC questionnaire to be alike with the original, valid and useful in Finnish osteoarthritis studies (Soininen *et al.*, 2008).

Besides the WOMAC index, there are also other pain measurement tools, like Doyle Index, Lequesne Index, Short Form 36 (SF-36), McGill Pain Questionnaire (MPQ) and McMaster Health Index Questionnaire (MHIQ). Some of those are OA specific like the WOMAC index, some are designed for more general pain detection (Table 2).

Table 2. Pain measurement questionnaires in addition to the WOMAC index

Questionnaire	Main features	Specificity of purpose of use	References
Doyle Index	- OA specific - unidimensional, simple (cf. WOMAC) - pain of different joints tested physically based on tenderness by firm pressure or by passive movement of the joint - findings graded 0 to 3: (0) no tenderness, (1) patient complains of pain, (2) patient complains of pain and winces and (3) patient complains of pain, winces and withdraw joint	- evaluate severity of the disease and monitor the effectiveness of therapeutic treatments over time	- Doyle *et al.*, 1981
Lequesne Index	- OA specific - originally developed as an interview format - don't separate symptom and disability grading (cf. WOMAC)	- evaluate symptoms and physical functional disability	- Lequesne *et al.*, 1987 - Stucki *et al.*, 1998
Short Form 36 (SF-36)	- not an OA specific • used combination with WOMAC - consists of 36 items divided into 8 domains	- a multi-purpose survey dealing with both physical and mental health - a general instrument for measuring the health related QoL	- Ware & Sherbourne, 1992 - SF-36® Health Survey Update - Creamer, 2000
McGill Pain Questionnaire (MPQ)	- not an OA specific - highly based on word descriptions and pain is explained with adjectives	- pain detection in 3 major classes: • evaluate the extreme and significance of the pain • monitor the pain over time • detect effectiveness of treatments	- Melzack, 1975
McMaster Health Index Questionnaire (MHIQ)	- not an OA specific - 3 dimensions consisting total 59 items; 24 physical, 25 social & 25 emotional function	- measurement of general health status and QoL - intended for diverse population	- Chambers, 1993

QoL – quality of life; OA – osteoarthritis; cf. – confer

2.2 Pain and its mechanisms in osteoarthritis

Pain is undeniably an important and dominant feature of OA but still a lot remains unknown about its nature, etiology and natural history. Regarding the causes of pain, we must remember that actually cartilage, the principal structure involved in OA, has only few pain-sensing fibers (Creamer, 2000). Therefore, there must be some other underlying factors responsible for the origin of pain causing the changes and weakening the cartilage as well. Potential sources inducing the pain can be osteophyte growth with simultaneous stretching of periosteum, perivascular and free nerve fibers within subchondral bone marrow and the marrow cavities of osteophytes, raised pressure inside the bone, microfractures, ligament damage, capsular tension, meniscal injury and synovitis (Creamer, 2000). General consensus refines OA pain as a heterogeneous condition; it can cause variable clinical conditions, it might be constant or intermittent, it can exist with or without neuropathic component

and/or central sensitization. Furthermore it is considered to be a prototypical nociceptive pain condition (Perrot, 2015).

OA pain is often described as deep aching in areas difficult to localize, with a duration of several years. Also typical for OA pain is that it may worsen with changes in weather, especially during storms or temperature falling, and with increased activity. Activity-related pain is one of the most typical forms of OA pain, usually starting immediately or shortly after the beginning of joint use and lasting for hours after the activity is finished. Along with prolonged pain, by the time when OA has progressed to more advanced stages the pain has typically become constant and chronic, e.g. disturbing sleep (Buckwalter and Martin, 2006).

Inflammatory mediators, matrix components and mechanical stress activate joint cells in OA which then imbalance the breakdown and repair process of joint tissue (Berenbaum, 2004) which in turn triggers the cartilage damage (Lajeunesse, 2004). One important component of OA pain is mechanical pain which can activate specific nociceptors (Heppelmann and McDougall, 2005). Cartilage damage can induce hyperpressure of the subchondral bone (Taljanovic *et al.*, 2008) affecting increased pain sensation. The attempt to recover the damaged cartilage then leads to a number of biochemical adaptions inside the osteoarthritic joint. Several mediators are present in the joint, both anabolic and catabolic, such as proteases, cytokines (Haringman, Ludikhuize and Tak, 2004), growth factors, radicals, neuropeptides and so on, competing in the anabolic – catabolic condition of joint (Lajeunesse, 2004). This continuously ongoing battle leads to inflammation in the osteoarthritic joint which in turn is directly linked to clinically detectable symptoms of OA, like joint swelling, stiffness, synovitis and inflammatory pain (Roach *et al.*, 2007). Besides the signs and symptoms of the disease, inflammation is also a major factor associated with the risk of progression of cartilage loss (Goldring and Otero, 2011). Also synovitis, the inflammation of the synovial membrane, has been proven to be an important effector in the pathogenesis of OA and cartilage loss (Sellam and Berenbaum, 2010). Inflamed synovium produces catabolic and pro-inflammatory mediators such as cytokines, nitric oxide, prostaglandin E_2 and neuropeptides which lead to excess production of proteolytic enzymes and

further, alter the balance of cartilage degradation and repair towards the breakdown (Sellam and Berenbaum, 2010). There is a vicious cycle in osteoarthritis and osteoarthritic pain; a disorganized situation inside the joint and subchondral bone cause pain and inflammation, and on the other hand, induced inflammation contributes to pain.

2.2.1 Development to neuropathic pain

OA pain is likely localized in the synovium, periosteum bone or tendons but not in cartilage, as already stated. In addition though, recently it has been proposed that the reason or the originator of the pain is the free axonal endings suggesting the neuronal component involved in the pain. OA pain is said to be a mixed phenomenon where there is both nociceptive and neuropathic mechanisms involved, in fact both at the local and central levels. Peripheral mechanisms are involved more in the early stage and central mechanisms more in the later and chronic phase of OA, but nevertheless, sensitization of them both has been suggested to be one of the underlying mechanisms of OA pain (Bajaj *et al.*, 2001; Imamura *et al.*, 2008; Arendt-Nielsen *et al.*, 2010). A nociceptive message involves neuromediators and regulating factors like neuronal growth factor (NGF) but also central modification of pain pathways (Perrot, 2015). Because OA pain is a result of complex changes both in the peripheral and central nervous system, there are confusing situations in patients with sensing neuronal impulses: nerves may be sensitized to respond even when the original stimulus is removed. This neuronal plasticity is also one reason for change of pain from acute to chronic in OA (Creamer, 2000).

Typical pathophysiological processes of neuropathic OA pain include four steps. First the energy from painful mechanical, chemical or thermal stimulus is converted to electrical signals by specific receptors and then transmitted from peripheral areas to central ones (spinal cord and brain) via specific pathways. This is followed by the comprehension of brain cortical zones and transformation of message by brain and spinal structures to inhibit and relive the sensation of pain (Perrot, 2015) (Figure 4). Research focusing on chronic

pain associated with OA has demonstrated the role of receptors on enhanced somatosensory modulation in OA. For example, the transient receptor potential vanilloid 1 receptor, TRPV1 receptor (also known as capsaicin receptor), is a member of the transient receptor potential (TRP) family of ion channels which plays an important role in neuropathic pain modulation and is found in many joint structures (Numazaki and Tominaga, 2004; Chu *et al.*, 2011). Opioid receptors have been found on nerve fibers and inflammatory cells of OA patients, and especially in enhanced and prolonged inflammation the immune and peripheral nervous systems upregulate sensory nerves to express opioid receptors (Mousa *et al.*, 2007). Also cannabinoid receptors, CB1R and especially CB2R, have an important role in chronic joint pain, and the endocannabinoid and endogenous opioid system has been proposed to have close relation and interaction in chronic joint pain modulation (La Porta *et al.*, 2013). However, defining osteoarthritic pain solely as neuropathic is controversial since the TRPV1 modulating agents and neuropathic pain medications, such as gabapentin and pregabalin, have been shown to be effective only in pre-clinical studies, but not in clinical trials in patients.

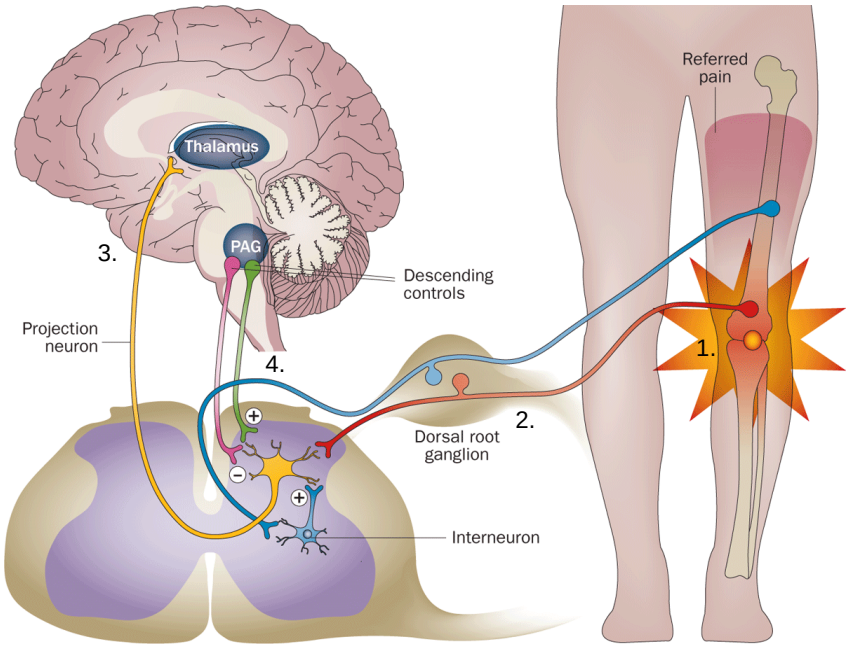

Figure 4. Pain in peripheral area (1.) is transmitted as electrical signals to spinal cord via specific neurons (2.). From the spinal cord ascending sensory messages pass onwards to the brain (thalamus) (3.) where information is modulated and descending neurons project back to spinal cord (from the PAG) transferring pain inhibition message.
PAG – periaqueductal grey
Figure modified from (Thakur, Dickenson and Baron, 2014)

2.3 Treatments

The development of OA depends greatly on various risk factors. Age, heredity, and lifestyle are considered to be the major ones because they are thought to be predisposing factors to other, more local joint harming effects, such as injuries, deformity, joint development disorders and physically hard work. The increasing load of risk factors often affects the onset of OA so the most important treatment is actually preventive actions (Polvi- ja lonkkanivelrikko: Käypähoito -suositus, 2014, engl. Knee and hip osteoarthritis: Current Care Guidelines Abstract, 2014). A healthy way of life, i.e. normal weight, healthy vegetable-rich diet and life-long exercise are the corner stones of OA prevention, just as in almost any health problem in general. Using and loading of joints with proper physical training actually enhances their health and mobility; one preventable and also post-operative remedial treatment way is appropriate physiotherapy and exercise training (Uusi-Rasi *et al.*, 2017). Knowing the importance of long-lasting exercise and physical activity programs, new self-management tool for improving the motivation and adherence of OA patients to exercise (Paterson *et al.*, 2016) and recommendations to guide health care practitioners (Roddy *et al.*, 2005) have been developed. Other non-pharmacological but widely accepted and proven action for relieving and delaying of OA onset and worsening, is weight control and weight loss (Toivanen *et al.*, 2010; Muthuri *et al.*, 2011; Jiang *et al.*, 2012).

Current pharmacological treatments can relieve the symptoms of OA but they cannot prevent or delay the progression of the disorder. Because the progression of OA to advanced and disabling stages is the leading reason for joint replacement (Sharma, Kapoor and Issa, 2006) there is a great need for new, disease modifying therapies. Knowledge gained from studies focused on disease progression or OA-related disability may help to find targets for development of disease modifying interventions (Sharma, Kapoor and Issa, 2006). Still, due to the important role of inflammation, the above mentioned neurotransmitter receptors and mediators of inflammation can act as putative targets for the drug development of analgesic therapies as could also targeting the synovium (Sellam and Berenbaum, 2010).

The pharmacological treatments in use for OA can be divided into topical, oral and intra-articular categories (Ringdahl and Pandit, 2011). Therapy typically consists of paracetamol, NSAIDs, opioids, intra-articular corticosteroid injections and intra-articular hyaluronic acid injections (Polvi- ja lonkkanivelrikko: Käypähoito -suositus, 2014, engl. Knee and hip osteoarthritis: Current Care Guidelines Abstract, 2014). Additionally, joint replacement surgery is considered to be an option when medical management is ineffective. The sequence of use (Figure 5) and many recommendations are decided based on consensus judgement of clinical experts from a wide range of disciplines and the best available evidence of benefit, safety and tolerability of pharmacologic interventions (Jordan *et al.*, 2003; Zhang *et al.*, 2005; Hochberg *et al.*, 2012).

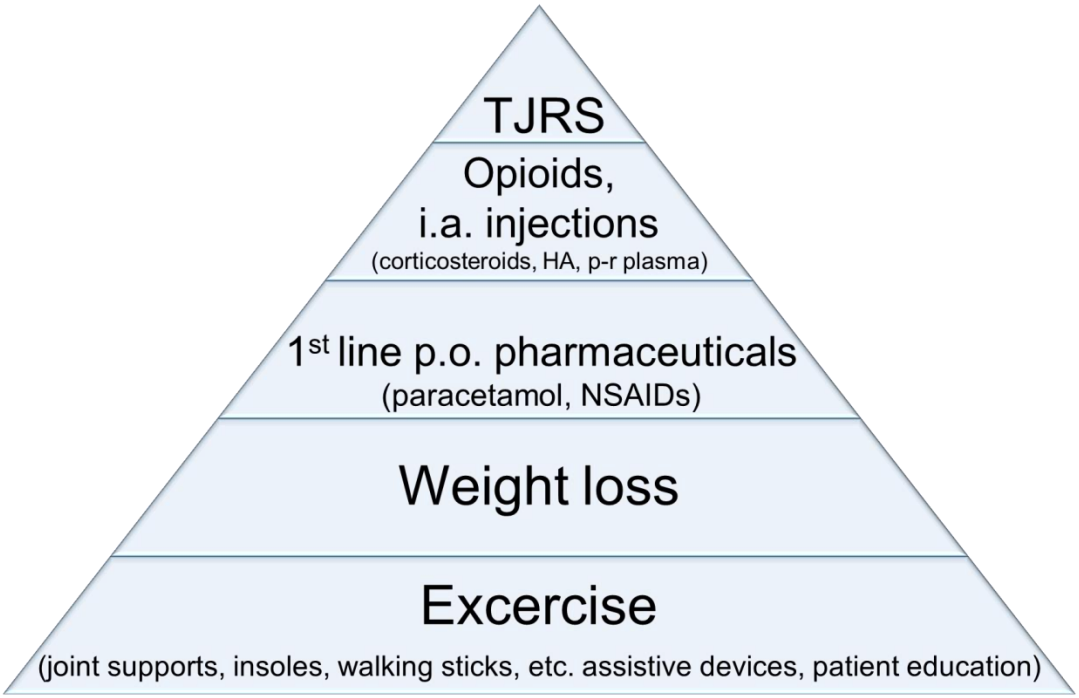

Figure 5. The sequence of osteoarthritis treatments.
NSAIDs – nonsteroidal anti-inflammatory drugs; p.o. – peroral administration route; i.a. – intra-articular; HA – hyaluronic acid; p-r plasma – platelet rich plasma; TJRS – total joint replacement surgery

2.3.1 Pharmacological treatment with paracetamol and NSAIDs

Paracetamol is considered as a primary medicine for OA due to its safety and efficacy profiles. It is a centrally affecting analgesic without anti-inflammatory component but its mechanism of action is still largely unknown (Moilanen and Kankaanranta, 2012). With therapeutic doses (up to 4 g/day) paracetamol is generally well tolerated and its efficacy in OA treatment is proven to be as good as NSAIDs or only a little weaker while causing less gastrointestinal events. Also, its renal and cardio effects as well as the effect on blood coagulation are less than NSAIDs due to its minor effect on prostanoid synthesis at peripheral tissues. Furthermore, paracetamol suits most patients suffering from acetylsalicylic acid sensitive asthma (Polvi- ja lonkkanivelrikko: Käypähoito -suositus, 2014, engl. Knee and hip osteoarthritis: Current Care Guidelines Abstract, 2014). Because of these factors paracetamol is the first line therapy for OA. If the patient does not have a satisfactory clinical response to full-dose paracetamol, then the use of oral or topical NSAID is recommended (Hochberg *et al.*, 2012).

Even though recommendations favor paracetamol as a first line analgesic for OA, its reputation is controversial nowadays. Many studies claim that paracetamol is not as effective as stated and that it has also more adverse effects than previously thought. Systematic reviews and meta-analyses confirm low-level effectiveness of paracetamol over placebo when comparing only these two, yet simultaneously highlighting increased risk of adverse events including GI adverse event (Bannuru, Dasi and McAlindon, 2010) and even multi-organ failure related to unintentional staggered paracetamol overdoses frequently taken to relieve pain (Craig *et al.*, 2012). OARSI has also followed the discussion and research done around the topic and has updated its own guidelines, most recently the guideline concerning knee OA, suggesting that there is a greater risk associated with paracetamol use than previously thought, especially when used for extended durations, and recommending cautious dosing and treatment duration (McAlindon *et al.*, 2014).

Comparison between paracetamol, placebo and different NSAIDs showed barely detectable effectiveness on pain symptoms at various doses of

paracetamol suggesting no role for single-agent paracetamol for the analgesic effects in patients with OA (da Costa *et al.*, 2016); on the contrary, it is even stated that “many patients could be suffering needlessly because of perceived NSAIDs risks and paracetamol benefits which might not be real” (Moore *et al.*, 2016). Paracetamol as a short-term analgesic for OA patients may still have some utility (Bannuru, Dasi and McAlindon, 2010), and it is presented as a first therapy in current recommendations and guidelines. Some changes may still take place over time, especially when the safety profile of paracetamol has recently been questioned and the continuously accumulating data refers to lack of efficacy. Although changes have already been made, the new meta-analysis and data from ongoing studies pushes clinical practice and treatment guidelines to be updated to reflect the evidence shown (Hunter and Ferreira, 2016).

The caution of NSAIDs usage relates to adverse events associated with them, the most important and most common of those being gastrointestinal adverse events. Furthermore, the dose-response relationship between NSAIDs and increasing incidence of mortality, cardiovascular and renal adverse events awake concerns and limit their use. Still e.g. the relatively new systematic literature review of observational studies demonstrated the consistent dose-response relationship between standard analgesic doses of paracetamol and occurrence of the same adverse events than with NSAIDs (Roberts *et al.*, 2016). In the context of such changing evidence, it is impossible to highlight one as the best and most suitable for all interventions of OA pain, and selecting the drug for pain relief in OA remains challenging. The risks versus benefits and efficacy versus tolerability will still have to be weighed in every therapeutic decision but moreover e.g. comorbidities, clinical context, polypharmacy and the modifiable risk factors that lead the progression of disease, compel to tailored treatment decisions.

Besides the known adverse events, NSAIDs are also noted to be very effective medications with anti-inflammatory, analgesic, antipyretic and antiplatelet effects. When OA pathophysiology is shown to be very complex with multiple driving forces and various roles of local and systemic inflammation (Berenbaum, 2013), it makes sense that NSAIDs relieve the discomfort caused by inflammation. These drugs reversibly inhibit the cyclooxygenase (COX) -1 and -

2 enzymes: the traditional NSAIDs are nonselective, each with varying degrees of COX1 and COX2 activity, while newer NSAIDs have been formulated to be COX2 selective (Loveless and Fry, 2016). Non-selective and COX2 selective NSAIDs are shown to be equally efficacious for the symptomatic relief of OA (Chen *et al.*, 2008) but the most common harm, the gastrointestinal adverse effects, are related to COX1 enzyme inhibition. For this reason traditional NSAIDs are recommended to be used with proton pump inhibitor, H_2-receptor blocker or misoprostol to reduce the gastrointestinal adverse events (Loveless and Fry, 2016). For the same reason COX2 selective NSAIDs are preferred even though the amount of evidence for this protective effect vary considerably across individual drugs (Chen *et al.*, 2008) and on the other hand they might cause more harmful cardiovascular effects; therefore drug selection is determined case-dependently.

A meta-analysis assessing the effectiveness of different compounds and doses of NSAIDs on improving OA pain and function suggests them to be the base and backbone of OA pain management. The comparison of different NSAIDs showed diclofenac (at dose 150 mg/day) to be the most potent NSAID treatment available at present when measured in terms of improving both pain and function. Still, also this study points out the safety risks of these drugs and the importance of taking into account all known risk factors, the individual clinical picture and the background when selecting the drug and dose for a single patient (da Costa *et al.*, 2016). Besides diclofenac, other well-known and largely used NSAIDs for OA treatment are naproxen and COX2 selective celecoxib.

Additional challenges to OA treatment are the character of OA as a chronic condition, and the fact that many of the medications currently used are not recommended for long-term use. Also, patients needing pharmacological treatment are often elderly whose tolerability for medication is lowered, and they experience adverse drug reactions more often. Individuals at age 75 or older are therefore recommended to have topical NSAIDs over oral ones as a first line therapy (Hochberg *et al.*, 2012). Even though topical NSAIDs are suggested to be safer and at least as effective as oral ones, they still seem to work only with more superficial joints such as hands and knees (Derry, Moore

and Rabbie, 2012). With multiple or deep arthritic joints oral NSAIDs are more efficacious and easier to use (Loveless and Fry, 2016).

2.3.2 Supplementary treatments

Risk of side effects of paracetamol and NSAIDs has led to a need for other alternative pain therapies. Several studies have focused on topical capsaicin, a substance found in chili peppers. It makes chili peppers taste hot and when topically applied to skin it binds to cutaneous C- and A-fiber nociceptors which are heat activated ion channels, e.g. capsaicin receptor VR1, in the pain pathway. The binding causes itching, vasodilation and burning sensation which is followed by a prolonged period of hypoalgesia that is usually referred to as desensitization or inactivation of TRPV 1 receptors and persistent reduced sensitivity after repeated applications. Desensitization produced by topical capsaicin has previously been thought to be caused by physiological desensitization rather than morphological alterations. However, when the most superficial nerve endings in the skin, those that are directly exposed to capsaicin, were studied, it was proposed that the functional effects of capsaicin are due to destruction of epidermal nerve fibers. This proposal was based on the morphology changes, the degeneration, seen of epidermal nerve fibers (Nolano *et al.*, 1999; Mason *et al.*, 2004). The safety of capsaicin is considered to be rather good since adverse effects are mainly irritations at the application site, but on the other hand, it has only moderate to poor efficacy in the treatment of chronic pain. Hence it is not an answer for OA pain treatment but may be useful as an adjunct or sole therapy for a small number of patients who are unresponsive to, or intolerant of, other treatments (Mason *et al.*, 2004). Since capsaicin is unlikely to cause systemic toxicity and drug interactions, topical capsaicin may be an option for polymedicated patients, like the elderly. It is also found that especially older patients are compliant to less effective treatments if there are fewer adverse effects (Fraenkel *et al.*, 2004).

The patient requirement for alternative or complementary medicine and desire to try natural approach besides so called traditional medicine have led to the

use of nutraceuticals, such as glucosamine and chondroitin as supplements in the management of OA. Glucosamine and chondroitin are essential components of the proteoglycan in normal cartilage which is the rationale for their use as supplements. Glucosamine is naturally produced in the human body and it is one of the principal substrates in the proteoglycan synthesis. Proteoglycans in turn are the water attracting complexes in the cartilage matrix that gives the cartilage its ability to withstand loading. Glucosamine is used as an agent to help relieve the symptoms and delay the progression of OA. Glucosamine is considered to stimulate the proteoglycan synthesis by chondrocytes and to act as a substrate for cartilage repair process. Chondroitin is a substance found in cartilage and connective tissue and it is one key factor for the structural and functional integrity of the joints. It is also said to help to maintain the viscosity in joints, stimulate cartilage repair and inhibit cartilage degrading enzymes. These properties can relieve OA pain and improve joint mobility. The use of glucosamine or chondroitin, or even better, their synergistic use, is suggested to have both symptomatic and preventive properties as they may maintain and rebuild cartilage, and further more relieve joint pain and retard progression of joint degradation (Clegg *et al.*, 2006; Huskisson, 2008).

2.3.3 Pharmacological treatment with opiates

In case of insufficient pain relief with paracetamol, NSAIDs and different supplements or their combinations, the prospective next alternative could be tramadol, an analgesic with weak affinity for μ-opioid receptors and capacity to block noradrenaline and serotonin reuptake. The main property of tramadol is opioid effect and the other one is non-opioid, parallel to tricyclic antidepressant effect, which enhances inhibitory effects on pain transmission in the spinal cord (Grond and Sablotzki, 2004; Kroenke, Krebs and Bair, 2009). Tramadol is considered to be a mild opioid with about 10 % analgesic potency compared to morphine when given parenterally. Due to only mild opiate nature of tramadol it has also less adverse effects than strong opioids, since it appears to produce less constipation and dependence than analogous doses of strong opioids (Grond and Sablotzki, 2004). Tramadol is a preferred alternative before strong

opioids if other options are contraindicated or insufficient. Tramadol can be used also together with non-opioid analgesics to improve analgesic efficacy.

Strong opioids are reserved only for patients in exceptional circumstances with extreme pain and are not suitable for other interventions. Long term prescribing of strong narcotics like morphine, oxycodone and hydromorphone for OA is not recommended but instead surgical treatment should be considered (Kennedy and Moran, 2010). Also OARSI, EULAR and the American College of Rheumatology (ACR) guidelines state that opioids should be avoided and used only for patients with symptomatic OA and who have not had an adequate response to either non-pharmacologic or pharmacologic modalities and are either unwilling to undergo or are not candidates for total joint arthroplasty (Jordan *et al.*, 2003; Roddy *et al.*, 2005; Zhang *et al.*, 2005; McAlindon *et al.*, 2014). Opioids should also always be started at low doses and titrated slowly, constantly monitoring side effects which are a significant reason for the recommended caution in prescribing opioid medication. Also, it is worth to consider for using other route than oral to administer opioids for creating better and more satisfactory results of treatment, e.g. transdermal buprenorphine has shown to be a worthy alternative (Conaghan *et al.*, 2016). Opioid use is specially challenging when used in patients who need long-term medication and get serious unwanted effects of opioids when using them beyond the acute period. Beside somnolence, nausea, vomiting and constipation, the risk of abuse and addiction increase and in prolonged use there is an increased risk to develop opioid induced hyperalgesia, a nociceptive sensitization, which causes an individual to become more sensitive to pain.

2.3.4 Intra-articular injections

Rather than opioids, intra-articular injections of corticosteroids can be tried to alleviate pain in OA. Corticosteroids have strong anti-inflammatory effects; they silence many inflammation- and inflammatory pain-related genes, and control cell and vascular responses typical for inflammation (Polvi- ja lonkkanivelrikko: Käypähoito -suositus, 2014, engl. Knee and hip osteoarthritis: Current Care

Guidelines Abstract, 2014). Triamcinolone, prednisolone and methylprednisolone are the most frequently used corticosteroids and OA of the knee joint is the usual site of intra-articular corticosteroid treatment although it has been evaluated for various other joints as well (Jüni *et al.*, 2015). Actually, this therapy has been used for knee OA for over 50 years but still there is controversy of its effectiveness and safety. It may provide a short-term symptomatic relief, typically for one to six weeks, with low risk of adverse effects but the duration of effectiveness decrease over time and is limited to a maximum of six months (Jüni *et al.*, 2015).

Other, more debatable intra-articular injections for therapeutic use to treat OA are hyaluronic acid and platelet-rich plasma injections. Hyaluronic acid or hyaluronate is a polysaccharide found in synovial fluid and cartilage. It lubricates and absorbs a shock in the joint. In case of degradation and inflammation in the joint and cartilage caused by OA the amount of hyaluronate is decreased. Adding extra hyaluronate by injection can improve joint lubrication, function and even production of hyaluronate in the joint. Beneficial anti-inflammatory and analgesic effects of hyaluronic acid injections may last even 24 weeks (Loveless and Fry, 2016). The place of hyaluronic acid injection among the available pharmacological treatments of OA is very controversial: recommendations regarding the use of it vary, with some guidelines recommending not to use it (Jevsevar, 2013; National Clinical Guideline Centre (UK), 2014) and many others supporting the use (Kon *et al.*, 2011; Chen *et al.*, 2016; Murphy *et al.*, 2017). The results of hyaluronic acid injections are often characterized by delayed onset but prolonged duration compared to, for example, corticosteroid injections (Bannuru *et al.*, 2009). The popularity of hyaluronic acid injection treatment is increasing, and it is considered to be an option especially for younger patients with moderate to mild OA of knee or ankle. Hyaluronic acid has been shown to improve analgesic effects and delay the need for surgery, supporting its usefulness as an additional treatment option (Altman *et al.*, 2015).

A novel study suggests hyaluronic acid injection to be combined with intra-articular platelet-rich plasma therapy to improve the results even though it is not widely used, basically due to lack of controlled clinical trials. Although based on

very limited patient populations, some of the studies have shown that hyaluronic acid treatment can alleviate pain, improve function, delay the need of surgery and give a chance for those who cannot undergo surgery at least in case of a severe knee OA (Chen *et al.*, 2016). Platelet-rich plasma is collected from patient's own blood and after collection centrifuged and separated to enrich the platelet count and the amount of growth factors to four to six times higher than the native. These kind of innovative clinical studies are trying to find approaches to stimulate the repair process or replace damaged cartilage, and growth factors seem to play a critical role in this. From this perspective platelet-rich plasma can serve as a simple, low-cost and minimally invasive treatment method (Kon *et al.*, 2011).

2.3.5 Novel treatment approaches and surgical option

Antidepressants have not generally been studied directly for the treatment of OA pain. However, they could be beneficial since studies have shown that depression co-occurring with arthritis can affect the pain intensity and arthritis severity felt (Katon, Lin and Kroenke, 2007). Furthermore, treatment of depression in arthritis patients can reduce, besides the depression, also pain (Lin *et al.*, 2003). For that reason, it is hypothesized that antidepressants could provide alleviation for chronic pain disorders as well. As one example, duloxetine is a centrally acting selective serotonin and noradrenaline reuptake inhibitor (SNRI) originally used to treat depression but more recently studied for OA pain indication and demonstrated efficacy and favorable adverse event profile in clinical trials (Chappell *et al.*, 2011; Myers *et al.*, 2014). In 2010 the FDA approved duloxetine for treatment of chronic pain due to OA, and updated recommendations of ACR for the medical management of OA followed in 2012, now including duloxetine as a therapy for patients who have inadequate response to conventional pharmacologic therapies (Hochberg *et al.*, 2012; Smelter and Hochberg, 2013).

There are several ongoing studies regarding new treatment strategies and ways to treat both the pain and function of OA. An option for the future could be the

development of symptomatic slow-acting drugs that possess structure-modifying properties. One strategy for this new approach could be strontium ranelate, better known for osteoporosis medication. It has been studied for the OA purpose since it has properties to modify the remodeling process of subchondral bone and change the imbalance from bone resorption to bone formation (Pelletier *et al.*, 2015; Han *et al.*, 2017). The endocannabinoid system has been a target of great interest of investigation for the discovery of novel therapeutic agents for inflammatory pain relief. Because the role of inflammation in OA, research around cannabinoid receptors 1 and 2 (CB1R and CB2R), endogenous ligands involved in their activation and enzymes degrading the ligands, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL), are also worth keeping in mind (Magrioti *et al.*, 2008).

One of the novel treatment approaches for OA is monoclonal antibodies (mAb) against NGF. Although the pain mechanisms of OA are still poorly understood, neuronal mechanisms are considered to be involved to some extent, because all structures of the joints, except for the cartilage, are innervated by nociceptors. Anti-NGF mAb treatments have raised great interest because affecting this neuronal pain mediator OA pain has been shown to be reduced (Eitner, Hofmann and Schaible, 2017). Besides its role as an important factor in nerve growth and neuronal pain mediator, NGF has been shown to contribute to the onset of inflammation and peripheral hyperalgesia. Therefore, blocking NGF actions has been hypothesized to attenuate OA pain. Efficacy of anti-NGF antibody treatments have been studied in mice and rats with monosodium iodoacetate (MIA) model of knee OA pain. Behavioral studies evaluating gait, asymmetry of static weight bearing and withdrawal of hind paw by mechanical stimulus (implemented with CatWalk, Incapacitance tester and monofilaments) have suggested that anti-NGF antibody treatments have potential and they might be valuable for therapeutic and also preventative treatments of OA (Xu *et al.*, 2016; Miyagi *et al.*, 2017). Anti-NGF antibodies, e.g. tanezumab, fasinumab and fulranumab, have been in active development and they have shown promising efficacy also in clinical trials. However, all clinical trials of NGF antibodies were put on hold by the FDA due to increased joint-related adverse events of rapidly destructive OA revealed during the clinical trials of tanezumab

(Smelter and Hochberg, 2013; Mayorga *et al.*, 2016; Miller, Malfait and Block, 2017). However, in 2017 the FDA granted the first NGF inhibitor-related Fast Track designation for tanezumab to expedite the development of new therapy to treat the serious condition and to fill the unmet medical need. Future role of anti-NGF antibodies in the treatment of OA patients will depend on the risk-benefit ratio to be clarified in future studies (Pfizer and Eli Lilly, 2017).

When pain and function limitations persist despite of different and various non-surgical therapy attempts or if controlling the symptoms requires high dose NSAIDs, paracetamol, long-term use of opioids or repeated intra-articular injections, the surgical options should be considered (Kennedy and Moran, 2010). However, total joint replacement is an irreversible intervention and should be considered carefully and only for patients with whom other treatment modalities have failed and the disease is really severe (Jordan *et al.*, 2003; Zhang *et al.*, 2005; Polvi- ja lonkkanivelrikko: Käypähoito -suositus, 2014, engl. Knee and hip osteoarthritis: Current Care Guidelines Abstract, 2014).

3 Pre-clinical investigation of osteoarthritis and related pain

Pre-clinical studies and thorough research first *in vitro* followed by *in vivo,* are crucial before studies in humans. Adequate evidence of safety and efficacy shown with different animal species and studies is essential for the continuation of drug development. It is vital that the pre-clinical properties observed *in vivo* remain in humans, and for that reason the development and validation of animal models and their continuous upgrading are needed to achieve translational models and testing methods. To get an understanding of efficacy of a new drug candidate of OA it has to be tested in animal models of pain. This is needed to give information to researchers which doses are to be tested in humans.

Mice and rats are usually the preferred laboratory animal species used for research due to their size, relatively short lifespan, quite inexpensive costs, appropriate time frame for study design, breeding for research purposes and availability also as genetically modified. In terms of investigating OA, it is also important to show pathogenesis similar to OA in humans. Many animal models of OA have been created (Figure 6), some by causing mechanical disturbances such as ligament transections and meniscectomies which advances in OA development, others by creating inflammation, pain and arthritis mimicking circumstances with injecting chemical agents into joints. Also models with no invasive actions have been established.

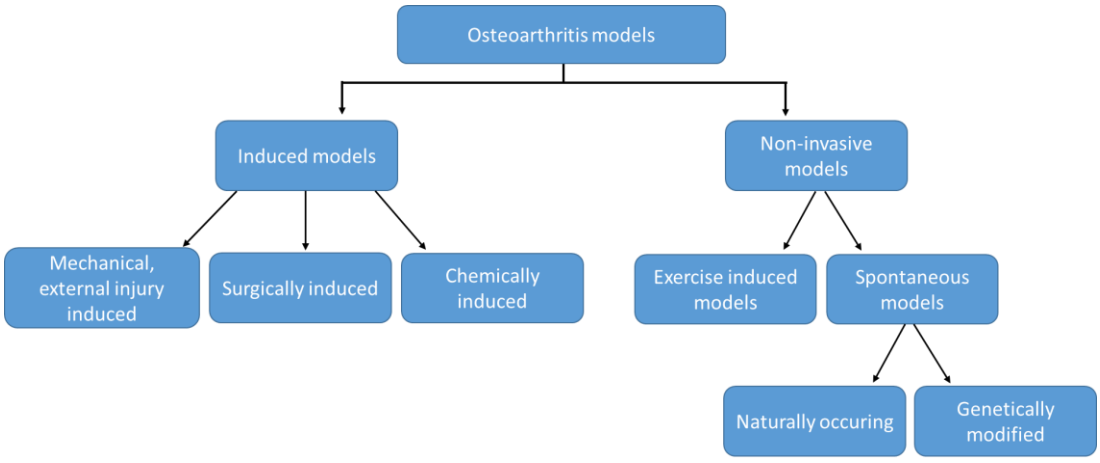

Figure 6. Classification of osteoarthritis mouse and rat models.

Animal models have proven to have an important role when clarifying mechanisms underlying joint damage in OA and providing proof of concept in the development of pharmacologic and biologic agents that may modify structural damage in the OA joint. However, the utility of animal models of OA for indicating analgesic effects of pharmacologic agents can be questioned (Brandt, 2002).

3.1 Induced models

3.1.1 External injury models

Inappropriate mechanical loading of joints, both over- and under-loading, have long been believed to be one of the main causes of OA (Saxby and Lloyd, 2017). Pre-clinical research with animals has demonstrated this with loading models which result in degenerative changes in the articular tissues. This type of models include, for example, acute loading and repetitive loading models. Acute loading causes joint injury in animals translatable to humans. One example of acute loading is the knee injury model in which a single hard pressure load into the mouse knee causes an injury in the knee joint comparable to post traumatic OA. Repetitive loading is comparable with acute one only with lighter pressure load and sequential loading times. Repetitive loading induces cartilage lesions that progress with time and lead to proteoglycan loss allowing to detect differentiation between lesion induction and progression. Pathological changes observed in joint ligaments include matrix component and cell shape changes (Christiansen *et al.*, 2012; Poulet, 2016).

Anterior cruciate ligament (ACL) is one of the most commonly injured structures within the human knee, resulting in increased risk of early onset of OA. Thereby a number of animal models have been developed to mimick ACL injury and investigate the post injury pathology and OA development as well as to evaluate potential therapies. These models include non-surgical, external injury models intended to closely mimic the clinical conditions (Maerz *et al.*, 2015). An example of an external ACL rupture model in small animals is similar to loading models since it is performed with single compressive load applied through the

tibia bone to the flexed knee in a way that ACL is damaged without additional macroscopic damage to other structures within the joint (Christiansen *et al.*, 2012, 2015).

One advantage of such injury models, i.e. externally mechanically induced joint injury, is avoiding surgical complications due to breaking the skin or disrupting the joint. Hence, external injury models can serve as a tool to investigate early adaptive processes initiated at the time of injury and the mechanical inducing manner can be more representative to human OA (Christiansen *et al.*, 2015).

3.1.2 Surgical models

So called surgical instability models in rodents include, among others, ligament transection, medial meniscus destabilization in mice, medial meniscal tear in rats and anterior cruciate rapture. One very common mouse model of OA used in research is the destabilization of the medial meniscus, DMM. This model reveals mild-to-moderate OA four weeks post-surgery in knee joint between femur and tibia bones and moderate OA after eight weeks measured with histological scoring (Glasson, Blanchet and Morris, 2007). Another relevant and similar model is the medial meniscal tear model. In this model, the medial meniscal tear is induced by cutting the meniscus and ligament apart while taking care not to harm the tibia bone. Rapidly progressive degenerative changes, characterized by e.g. chondrocyte and proteoglycan loss, osteophyte formation and fibrillation, occur after surgery and at 3 to 6 weeks post-operation tibia bone cartilage degeneration may be severe (Bendele, 2001). As stated above, ACL damage is often a risk factor for OA onset in humans. In addition to the external injury induced ACL rupture model, probably the most widely used and characterized model is surgical ACL transection (ACLT) in which, after surgical incision through skin and flesh, the joint capsule is revealed to allow cutting of the ACL (Blaker, Little and Clarke, 2016).

Surgical models are important, well characterized, reproducible, and have shown to provoke cartilage lesions and pathological changes comparable to human disease. Nonetheless, they do not faithfully mimic clinically relevant post

traumatic OA condition since surgical injury poses an invasive impact on the joint rather than the physiological impact typically occurring in human joint injuries. Surgical procedures may also present factors rising from surgery itself, like inflammatory and adaptive responses of joint elicited by e.g. the incision or sutures. These confounding factors may partially hide the actual biological responses caused by the injury (Christiansen *et al.*, 2012). Furthermore, even though surgical models are reproducible, their success depends on the surgeons' skills and experience. In addition, surgical models often require special equipment and are technically challenging to perform.

3.1.3 Chemically induced models

Currently used murine models of OA are often based on some kind of trauma caused to animal. These post traumatic OA models are important, but they do not faithfully mimic clinical conditions: a great number of OA patients have the disease without having any injury. For this reason, it is considered important to have models with differently induced disease as well.

Degenerative changes within the joints can be achieved with intra-articular injections of different degradative agents. There are many different toxic or inflammatory substances in use, including chemicals like monosodium iodoacetate (MIA), complete Freund's adjuvant (CFA), carrageenan and colchicine, proteolytic enzymes such as papain and collagenase and cytokines such as tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β) and interleukin-1 (IL-1). Different agents create pathological changes in the joint by different mechanisms which make these approaches applicable for studies of particular biological mechanisms (Christiansen *et al.*, 2015). Because each chemical model has a unique pathophysiology which does not correlate with the pathophysiology of post-traumatic OA, these models are mainly used to study the mechanisms of pain as well as the effects of drugs targeting inflammation and pain (Kuyinu *et al.*, 2016).

The MIA model has become the standard for modeling joint disruption and related pain in OA in both rats and mice as it generates reproducible, robust

and rapid pain-like responses in the injected limb. MIA is a glyceraldehyde-3-phosphatase dehydrogenase inhibitor which results in cellular glycolysis disruption and eventually cell death, neovascularization, and subchondral bone necrosis, collapse, and inflammation. These all predispose to cartilage lesions and subchondral bone alterations. Structural changes are partly reflective of patient pathology and lesions and functional impairment resemble OA and are analyzable and quantifiable. One of the advantages of the MIA model is that the level of pain can be controlled by altering the dose (Pitcher, Sousa-Valente and Malcangio, 2016).

Freund's adjuvants have been essential components of many experimental models of autoimmune diseases. These commonly used immune-adjuvants include incomplete (IFA) and complete (CFA) Freund's adjuvant. Both adjuvants are water-in-oil emulsions containing paraffin oil and aqueous suspension or solution of antigens with mannide mono-oleate as a surfactant. In addition, CFA contains heat-killed mycobacteria (*Mycobacterium tuberculosis*) which makes it more potent and adequate. The full mode of action of Freund's adjuvants is still unknown although they have been much used for decades, but the main action is to hyperimmunize the experimental animal and to induce various immune system responses which lead to autoimmune state and e.g. inflammation and pain. Treatment with IFA or CFA can cause arthritis in some strains of rats even without any joint-specific antigens. Arthritis induced with IFA is called oil-induced arthritis and it is an acute condition, whereas CFA-induced arthritis is a chronic disease, initially occurring with acute periarticular inflammation followed by alterations in bone. Both types of arthritis are T-cell-dependent and associated with an immune response to heat shock proteins, but especially pathogenesis of CFA-induced arthritis is assumed to be due to immune responses to mycobacterial heat shock protein antigens. Because strong and painful inflammatory reaction and immune responses are gained with CFA within days, it is often used in rodents' limbs (Billiau and Matthys, 2001). For OA studies, especially the pain component and behavioral changes caused by it have been under investigation.

Carrageenan is a sulphated mycopolysaccharide originated from the Irish moss Chondrus crispus or the red Scottish seaweed (Smith and Cook, 1953), and is

especially well known for its ability to induce local inflammation. Carrageenan has a variety of biological properties, and there are many mediators causing the inflammatory responses (Di Rosa, Giroud and Willoughby, 1971; Di Rosa, 1972) ranging from macrophage aggregation and fibroblastic proliferation to decrease in proteoglycan content in synovial fluid and synthesis in articular cartilage (Santer, Sriratana and Lowther, 1983). Single injection inside a knee or ankle joint of a rat produces a peak of symptoms after 3-4 hours lasting for 1-2 days (Ängeby Möller, Berge and Hamers, 2008). Due to the timeframe and symptoms shown to be sensitive to anti-inflammatory and anti-nociceptive treatments this model is feasible for pre-clinical research purposes targeting to investigate new pain-relieving agents.

Proteolytic agents injected in the joint cause destruction of joint architecture. Papain is a proteolytic enzyme which breaks down proteoglycans, the important components of cartilage that provide compressive resistance to it. This model is described with many species, and it enables investigation of different stages of OA progression by differentiating the dosage of papain since the low-dose injections do not completely block the joint repair processes. Still its use as an OA model is becoming rare (Lampropoulou-Adamidou *et al.*, 2014; Kuyinu *et al.*, 2016). Another proteolytic substance is collagenase which damages joint structures containing protein collagen I, like tendons and ligaments, resulting in decreased collagen matrix. Only one day after intra-articular injection of collagenase, mice develop patellar dislocation which initiate joint instability leading finally to OA, in a timeframe from lesions appearing at three weeks post-injection to 6 weeks after subchondral bone sclerosis is induced (Lampropoulou-Adamidou *et al.*, 2014).

Quinolone antibiotics have been demonstrated to have toxic effects on cartilage, tendons and bones of immature animals. They cause proteoglycan and chondrocyte loss and interfere with growth plate function resulting at least humerus and femur bones growth reduction in rats and initiating an inflammatory reaction in tendons. These mechanisms serve the use of quinolones in animals for causing lesions related to OA (Sendzik, Lode and Stahlmann, 2009). As stated before, aggrecan is one of the major components of cartilage providing compress resistance and resilience and its loss from the

cartilage matrix is an early feature of OA pathology. Furthermore, loss of aggrecan compromises cartilage function and leads also to loss of collagen, which has been demonstrated e.g. with cytokine interleukin-1 stimulation (Pratta *et al.*, 2003). The loss of aggrecan is a result of degradation by different enzymes such as aggrecanases and metalloproteinases. This situation is accomplished with intra-articular injection of tumor necrosis factor alpha in rat knee joint, since it has been shown to induce temporary aggrecan degradation and release of aggrecanase-generated aggrecan fragments from articular cartilage into the synovial fluid (Malfait *et al.*, 2009).

3.2 Non-invasive models

Besides injury models, also exercise-induced, naturally occurring and genetically modified models simulate OA with non-invasive methods. OA occurs spontaneously in various inbred strains of mice, *STR/ort* mice being one example, but not in rats. These mice develop OA spontaneously when aging, without any injury and because they are considered to reflect idiopathic, primary OA of humans (Christiansen *et al.*, 2015). Due to this property they are quite unique models, but still controversial since the underlying mechanisms may not be the same than those in humans. In addition, not all mice develop OA, so the incidence of osteoarthritic mice within the batch of animals in a specific study is not absolute, and the disease progression may also be variable and extended among animals (Little and Smith, 2008).

Exercise, especially unilateral, long-term, repeated or at elite level, is considered to be linked with increased risk of OA even though evidence is available both for and against of it. Anyway, exercise is a relevant method to use as a modulator of mechanical loads in weight bearing joints, like the knee, with rodents. They can be trained to run on wheels voluntarily throughout their life (Lapveteläinen *et al.*, 1995) or to use a treadmill for more controlled running exercises (Lapveteläinen *et al.*, 2002). Exercise-based methods need rather long time to show OA effects on joints, but on the other hand it is advantageous to represent directly a specific type of OA patients, like elite runners, and it can

be easily combined with other OA models, such as genetic or surgical ones, to create a more severe disease (Little and Smith, 2008).

There are several genetically modified mice models available for OA research purposes. A connective feature of these models seems to be the modification in genes affecting articular cartilage composition in a way that degeneration and progressiveness of the disease either worsen, speed up, or controversially ease. For example, often used mouse strains are the ones carrying a transgene which either disturbs procollagen II formation or causes targeted inactivation of type II, IX or XI collagen genes (Lapveteläinen *et al.*, 2002) and hence provoke cartilage loss. Another typical gene modification is related to proteolytic destruction of the major component of cartilage extracellular matrix, aggrecan, which provides compressive resistance to cartilage tissue. On the other hand, a mouse model in which catalytic domain of ADAMTS5 metalloprotease is deleted shows better resistance to cartilage degradation, and thereby shows protection or alleviation against OA by an effect of a single gene (Glasson *et al.*, 2005).

Studies on the interconnectedness of various joint tissues and factors and mediators affecting subchondral bone, osteophytes and pain signaling have revealed the genetic background and underlying reasons of pathology, disease progress and potential targets of therapies. The amount of mediators involved in the various joint tissues and different factors affecting the state of joint structures is huge, and reflect the need for future gene related research (Moon and Beier, 2015).

3.3 Testing methods with animal models

Like stated before, there exists no single ideal experimental model for studying OA. Because one model does not cover all aspects of OA it is relevant to consider the aims of the study and the resources and technical equipment available when selecting the most appropriate model. When primary OA and pathogenesis is the objective of studies, naturally occurring OA models should be used, while studies investigating molecular level mechanisms and even genetic background, the use of genetic models may be preferable. Surgical

models can provide appropriate approach for therapeutic studies and OA pain and pain mechanisms are the most valid targets to investigate with chemically induced models. In short, the objective of the study affects which animal model to use. Furthermore, not all testing methods are suitable to perform with all animal models, so the animal model chosen affects the choice of a testing method or on the other hand, the use of a certain testing method affects which animal model to prefer.

3.3.1 Pain at standing and walking

The biggest reason among OA patients for seeking medical help is pain. Also, the biggest socioeconomic burden caused by OA are therapies used to treat the pain. Discomfort and chronic pain have become the hallmarks of OA underlining the obvious importance of studying it and the need for developing new alleviating therapies. Since pain at standing and pain at walking, static and dynamic pain respectively, are the ways to survey pain in humans, it is considered as a translational way to study those features in animals too. Methods for such pain measurements are static weight bearing and dynamic weight bearing from gait assays.

For static weight bearing, a commonly used method is to measure weight distribution between hind paws of a rat. Incapacitance tester (Linton Instruments, UK) is a widely used apparatus for this purpose. It contains a plexiglass chamber where the rat is positioned so that its hind paws are on their respective force sensors, both front paws lean on the ramp or ladders, tail is outside the chamber and the animal is facing forward (Figure 9 in experimental phase, p.54) (Bove *et al.*, 2003). Since this kind of position is not natural for a rat who normally stands on four feet and further balances its position with a tail, well habituation to a restrainer and teaching to stand with two paws without leaning on the sides of the box is crucial (Malfait, Little and McDougall, 2013). When rats are comfortable with the restrainer box and willing to stand still the measurement procedure itself is very simple: three consecutive measurements each taking 1 to 5 seconds is carried out by pushing a button. Mean value from

the three recordings is used to calculate the difference in weight distribution of arthritic and contralateral control hind paw. This system offers a possibility for a rapid, reproducible and technically straightforward method for measuring OA related discomfort and is shown to predict effectiveness of pharmacological agents (Bove *et al.*, 2003; Malfait, Little and McDougall, 2013; Pitcher, Sousa-Valente and Malcangio, 2016).

Analyzing the gait is a valid method for evaluating the consequences of OA in both the pre-clinical OA models and OA patients (Allen *et al.*, 2012). Many attempts to record and analyze the imbalances and deformities of animals with induced OA have been invented and used with variable success. For the early attempts on determining what to measure and how, the crucial questions were which parameters are needed, relevant and informal to collect and analyze and how to achieve translatable, reliable and reproducible results and actually, even today these factors remain highly important.

One of the first tests regarding OA induced changes on gait was the test in which a rodent's paws were dipped into ink before it traversed a sheet of paper and drew a walking line. This was followed by a treadmill apparatus (Betts *et al.*, 1980) and a video camera exploiting several settings to provide an opportunity to view the recordings afterwards with careful judgement and detect paw positions and deformity as well as gait parameters. One conventional and actually the first method using recording was restricted movement analyzed by visual observation (Ängeby Möller *et al.*, 2012) which was followed by different gait analyzing apparatuses regarding the weight bearing and gait regularity during locomotion. These locomotion recording apparatuses differed slightly from each other but the underlying idea and the basic methods of function were still very similar.

For visual observation animals were placed individually in a plastic chamber with a glass floor and their movements and paw postures were recorded underneath. Afterwards the visual observation data were evaluated and scored by using a previously determined paw pressure visual rating scale which is mainly based on verbal description of the arthritic limb's contact area on the floor such as: "slightly reduced paw pressure, paw is completely on the floor but

toes are not spread", "moderately reduced paw pressure, paw curled with only some parts of the hind paw lightly touching the floor" and "severely reduced paw pressure, paw completely elevated" (Coderre and Wall, 1987; Ängeby Möller *et al.*, 2012). Rating of the visual observations during restricted movements allowed to detect OA-induced changes on weight bearing in animals. In addition, the visual observation method made it possible to compare changes between arthritic and non-affected paw within the same animal.

Early measurement techniques were mainly qualitative, such as visual observation of gait, progressing towards quantitative information of paw pressure distributions to full gait analyzing with definition of a wide range of detectible and measurable parameters. The first locomotion and foot pressure recording apparatuses utilized illuminated glass and plastic plates with an angled mirror underneath to reflect images from foot pressure force recorded with a video camera (Betts *et al.*, 1980; Clarke, 1992). From this kind of settings many slightly variable, tailored and custom made arrangements have been developed and independently used to record locomotion and evaluate dynamic footsteps of rodents. The PawPrint method is one of those allowing the recording of locomotion of monoarthritic rats and computer based software to automatically perform all analyses and calculations of different scores (Ängeby Möller *et al.*, 2012, 2015). A similar system to the PawPrint but commercially available and probably the most established one today is the CatWalk (Noldus, Netherlands). The CatWalk system consists of a closed corridor with a glass walkway with an open entrance at one end and a door to the goal cage at the other end (Figure 7 in experimental phase, p.52). Light from optic fibers is projected through one of the long edges of the glass floor of the walkway and it is entirely internally reflected except at the points where an object, e.g. the animal's paw, touches the glass and causes light to exit the floor and scatter at the paw, illuminating the contact area. The light intensity of each contact point reflects the pressure exerted at that point and the more pressure exerted, the larger the total area of skin-floor contact and thus the brighter the pixel. So, the light intensity reflects the weight load an animal is willing to put on each of its paws. Besides weight load, numerous parameters concerning weight bearing and gait pattern can be detected with this video-based and automated system.

Data acquisition and analysis is also almost entirely automatically handled with the system's own software (Ängeby Möller, Berge and Hamers, 2008).

Quantitative data on the dynamics of locomotion have not been so simple to produce. The CatWalk method, however, produce a large number of locomotion parameters such as inter- and intralimb coordination like swing and stance phase durations, degree of weight bearing, paw print areas, stride length, base of support and frequencies of normal step sequence occurrence, just to mention a few examples (Hamers *et al.*, 2001). As an objective study method with many measurement possibilities, automated system and featuring not only the individual paw parameters but also measurement of parameters related to inter limb coordination have made this method useful overall and also in the field of OA and pain behavior research especially since pain models have shown to change the CatWalk parameters (Ängeby Möller, Berge and Hamers, 2008). Besides enormous amount of quantitative data, the CatWalk produces also qualitative data such as print overviews, gait diagrams, gait formulas and opportunity to replay the walkway crossing which visualize the behavior of the animal: hesitations, stops, rearing, fluent movement, and a huge number of gait abnormalities (Hamers, Koopmans and Joosten, 2006).

3.3.2 Other methods assessing pain in osteoarthritis

The ideal situation and final objective is to find and develop interventions against both, OA emergence and disease progress. However, no disease modifying OA treatment is currently available or in a late development phase, therefore targeting the pain component is pivotal. Pain behavior is apparently easy to study even though behavioral studies with animals are generally open to interpretations. Besides recording pain at standing and walking, there are many more ways to measure pain. Evoked pain behavior and spontaneous pain behavior methods offer different approaches to investigate OA related pain. Since OA pain has multiple ways to manifest itself and all behavior measures have their own pros and cons it is valuable to carry out more than one test.

Evoked pain behavior methods are the most commonly used in laboratory conditions, since the majority of pain behavior tests use some sort of evoked response to an external stimulus. These stimuli can be mechanical, thermal or chemical. One of the most frequently used mechanical allodynia measuring method is von Frey filaments. Originally in 1896 Maximillian von Frey used real animal hairs from different origin but nowadays calibrated nylon filaments are used. Measurement is implemented by touching the hairless sole of a rodent hind paw with one filament at a time. These monofilaments are of various stiffnesses and consequently bend with a discrete force when pressed against the skin. Three different approaches to determine the threshold of mechanosensitivity have been developed. One starts with a mid-range von Frey filament to determine if it produces a real withdrawal response of the paw. If so then a thinner filament is chosen and again applied to the hind paw whereas if the animal does not respond, a thicker filament is chosen instead and in this manner the mechanical threshold is ascertained. The second approach uses an up-down scale originally described by Dixon (Dixon, 1980) and subsequently refined by Chaplan with colleagues (Chaplan *et al.*, 1994). In this regression analysis-based approach the mechanical threshold is inferred from response versus non-response observations. The third approach uses three filaments determined with either low, medium or high bending forces with which ten applications of each is performed and the number of positive responses is recorded. Despite the mechanism used to determine the threshold value in all approaches, a positive reaction to the mechanical stimulus can be notified from the rat behavior: a rapid paw withdrawal followed with possible licking of it (Malfait, Little and McDougall, 2013).

Vocalization and pressure application measurement (PAM) device are two different examples of test methods used to measure evoked pain behavior. Vocalization is a natural communication way of many animals to express their identity, mood and condition as well as physiological and psychological well-being. Actually, each vocalization has a distinct standard based on acoustic frequency, duration and sound pressure. With rodents nociceptive response to noxious stimuli is voiced besides audible squeaks also with ultrasonic chirps which actually reveal a more affective component of pain. Even though arthritic

animals have been found to emit both audible and ultrasonic sounds in response to irritation of the disrupted joint, its use for interpreting rodent pain is complicated due to the fact that ultrasonic vocalizations are highly context-specific since they are produced also e.g. following pairing, submission and the presence of a predator. PAM device in turn is a device consisting of a force sensor worn on the thumb of the experimenter who can press the device straight against the joint of interest and the peak force required to elicit a withdrawal response is indicative of mechanosensitivity. However, albeit this device has been used with rodent models of joint inflammation and it could well serve for OA pain research, it has not been tested on that indication (Malfait, Little and McDougall, 2013).

Even though evoked pain behavior methods are the most frequently used, the most plain or obvious method would be simply observing the OA animals and their behavior because animals in chronic pain tend to be withdrawn, diminish their locomotion, breath shallowly and become hypotensive. In addition, these spontaneous pain behaviors are thought to be more clinically relevant than the evoked ones (Malfait, Little and McDougall, 2013). Translation ability between current pre-clinical pain assays and real clinical pain has been considered as a concern because relatively few new analgesic treatment options have been developed. For this reason the current consent is that assays are needed utilizing spontaneous rather than evoked or reflexive measures to assess the global impact of pain beyond hypersensitivity (Vierck, Hansson and Yezierski, 2008).

Different recording and video documentation technologies have been used to document animal behavior for later evaluation. One example of this kind of non-reflexive measuring methods is spontaneous locomotor activity in which an automated monitoring system records animal behavior for the time interval decided in advance. A monitoring system encompasses an enclosed arena with sensory photobeams in two levels, one level elevated 3 cm from the arena floor and another 14 cm (when monitoring rats), for measuring the horizontal and vertical activity respectively, giving information by measuring parameters like ambulatory horizontal distance moved, rearing frequency and rearing time (Bryden *et al.*, 2015). With this kind of activity-based assessments it is possible

to calculate from the recordings the time OA animals have used for different activities and compare to the times with analogous times of naïve animals. Because exploring, rearing and climbing are naturally common activities for rodents, they can serve as parameters to detect. On the other hand, natural behaviors like licking, shaking, hanging and keeping the sore paw off the ground can be behaviors under surveillance. Still, using these behaviors as measures of chronic pain can also be questioned since many other situations, such as stress, illness and sedation, provoke the same behaviors in rodents, so it can be unclear whether they indicate pain or something else (Mogil, 2009).

One behavior innate for many rodent strains is burrowing. It is a normal behavior in which rodents have to use their paws to remove gravel. This behavior reflects rodents' well-being and in pain it is reduced. However, burrowing behavior can be reversed with analgesics and therefore it can act as a useful measure of non-evoked pain and testing novel drug candidates with animals of induced OA. A burrowing test, can be conducted in a tube (30 cm long and 10 cm wide) filled with 2.5 kilos of quartz sand positioned with the open end of the tube being elevated about 6 cm from the cage floor and then recording the amount of sand burrowed within a certain time, e.g. in 30 minutes (Bryden *et al.*, 2015).

3.3.3 Validation of testing methods and models

When developing drugs and using animal models to investigate the efficacy of new drugs, the goal is to find out if potential drug candidates will be effective for human treatment in the future. To reach the goal, it is very important that models and test methods used are validated and as translatable as possible. To ensure functionality every animal model and every testing method used must be validated for the research purpose of interest. Regarding the OA disease and pain related to it, the above mentioned animal models and testing methods are relevant and much used and their validation greatly depends on pharmacological validation with already clinically relevant compounds.

Paracetamol, non-selective and COX2-selective NSAIDs and opioids are drugs in clinical use to relieve pain in patients with joint disease. Compounds from these therapeutic classes are therefore used when proving utility of the testing methods and animal models for pre-clinical joint disease and joint pain research. For instance the CatWalk method, shown to be effective, predictive, objective and translatable way to investigate joint disease-related pain, was originally developed for studying rats with spinal cord injuries (Hamers *et al.*, 2001). When seen to produce relevant information of impaired locomotion and gait in spinal cord models, the method was thought to be useful for investigating other conditions affecting locomotor capabilities too. The CatWalk was then tried to assess gait changes and weight bearing with neuropathic pain model (Vrinten and Hamers, 2003) and arthritis-related inflammatory pain models (Ängeby Möller, Berge and Hamers, 2008; Ängeby Möller *et al.*, 2012). In case of studying pharmacological treatments and when trying to develop new treatments it is necessary to prove that besides the ability to produce relevant and quantitative data, the test method and system also captures changes and produces information comparable to clinical observations. For example, when well-known and clinically used analgesic naproxen is shown to alleviate pain in patients and improve their movements it is needed to see the same effects with pre-clinical pain models receiving the same medication with the measuring method in validation. The better the method's results correspond to different treatments currently in clinical use, the better are the validation, reliability and predictability properties of the method. From the reliability point of view, it is also important to remember to do the validation for each animal model used even when the test method itself stays the same and even if the two animal models would mimic the same condition. For instance, although both the injection of carrageenan into the knee joint and CFA into the ankle joint mimic the arthritis-related pain, they are different animal models and need to be validated separately. Also, even if the same testing method, such as the CatWalk, would be used for both animal models, the validation of the testing method must be performed for each animal model separately.

OA-related pain and disturbances in gait and weight bearing measured with automated video capture-based analyzing method (the PawPrint or the

CatWalk) have been validated at least with carrageenan and CFA-induced arthritis in the knee and ankle joints, respectively (Ängeby Möller, Berge and Hamers, 2008; Ängeby Möller *et al.*, 2012). Also the utility of the testing method for the pharmacological studies has been validated by using analgesics from many different classes, including at least diclofenac, ibuprofen, naproxen, oxycodone, paracetamol (Ängeby Möller *et al.*, 2012), morphine and rofecoxib (Ängeby Möller, Berge and Hamers, 2008).

4 Conclusion of the literature review

OA is traditionally thought to be a joint disease causing cartilage lesions and affecting elderly people. Moreover, it is proven to influence other joint structures and tissues besides cartilage, and complicate the function of the whole joint as well as disrupt the underlying bone and surrounding muscles. Although aging increases the risk of OA, it can occur also in younger individuals. Overweight, lack of physical activity or too intensive physical activity, especially unilateral physical loading of joints and nowadays lifestyle with extreme sports followed by injuries, such as meniscal tear or patella damage, promote outbreak of OA, if not immediately then later in life. All of this has made OA to become the most prevalent musculoskeletal disease worldwide and a major cost and burden for health care.

Since OA is a heterogeneous disease, every case is unique and assessing the severity of the disease or suitable treatment options is case-dependent. However, different recommendations have been developed as an attempt to help clinicians' work. Some guidelines to be highlighted are the Kellgren-Lawrence grading system for evaluating joint pathologic and structural changes from x-rays, and The WOMAC index which offers a standardized disease-specific patient-relevant self-reported health status questionnaire in support of assessing the pain severity.

Since the pain is a particular factor disturbing patients' everyday living and since it impairs the quality of live, the medical treatments concentrate on alleviating it, especially when there are no disease-modifying drugs available to cure physical impairments or stop the progression of the disease. The most important treatment options, however, are the preventive actions, including particularly weight loss, physical activity and avoiding accidents. Clearly there is a need for new treatment strategies and novel therapeutic agents with new mode of actions. This need drives translational OA research forward and underlines the importance of consistent and constant validation of animal models and testing methods. Many different models and OA-inducing methods as well as measuring options and devices are available, highlighting the importance of careful thinking of aims and purposes of studies.

When discussing the drug development, even in the early pre-clinical phase, the ultimate goal is always on the human patients and the objective is to improve their life quality. For that reason, the translation ability from pre-clinical findings implemented with experimental animals to proper, working treatment solutions for humans is crucial. Since the success rate of new OA drugs has been poor, it has brought up considerations of pre-clinical testing methods and models and their comparability to clinical measurement ways. Malfait and Little (2015) concluded the possible underlying reasons and important aspects of difficulties stating that pre-clinical testing is typically performed by treating prophylactically or early in induced models (mostly post-traumatic OA) in young and normal-weight animals, whereas clinical trials mostly focus on age/obesity associated, established/ late stage OA (Kellgren-Lawrence grade 2 to 3) leading to the OA target population and pre-clinical phenotype to be mismatched. Most pre-clinical studies reported are restricted to limited time points in one study, in one animal model, in one species and in one laboratory - that is to say, reproducibility is not tested. Additionally, the animal studies usually evaluate a limited set of outcome parameters, and these parameters typically interrogate the mode of action of the drug more than assessing the overall joint health and animal well-being. Despite the restrictions of animal characteristics, models and measurement ways, *in vivo* studies are an undeniable part of drug development. Conducting studies in accordance with mutual guidelines and collective goal can ensure better translation of pre-clinical research into successful treatment strategies in humans.

EXPERIMENTAL PHASE – Pharmacological and behavioral validation of Complete Freund's Adjuvant-induced ankle joint pain model in rats

5 Introduction for the experimental phase

Pain is a manifold sensation which is related to many different disorders, diseases and situations. By nature, it can be inflammatory or neuronal, or long-lasting inflammatory pain can also become chronic. In humans, for example, OA is a variable chronic disease in which pain is very often related to a various degree of strength. Also, the nature OA pain can vary from inflammatory to chronic pain between people and the state and phase of the disease. There is an unmet need for medical therapies to treat especially neuronal and chronic pain and hence it is important to develop and validate reliable pre-clinical animal models and testing methods for research.

Because the perception of pain in OA is often different in movement and in standing in humans, it is relevant to study it in both ways in animals too. For that reason, two different measuring methods were used when studied pain with rats: the CatWalk XT (Noldus, Netherlands) apparatus measured movement-related pain and the Incapacitance tester (Linton Instruments, UK) gave information of standing pain.

The intention of the experimental phase of this work was to validate the new CatWalk XT apparatus to be used in pain research to replace the previously used older CatWalk version which was not functionally sufficient anymore. Also, the results gained from the commercially available CatWalk XT system were compared and shown to be equal with the private PawPrint system used in similar studies (Ängeby Möller *et al.*, 2012, 2015). An equally important aspect was to study the pain relieving effects of two already clinically used pain therapeutics and three investigational pre-clinically relevant molecules. The aim was to see if the intra-articularly induced CFA monoarthritis rat model would work in this kind of pain research to test the efficacy of the candidate molecules.

The experimental phase consisted of five separately implemented experiments, albeit they all aimed to study the effects of the given drugs on inflammation, to the pain it caused, and gait and static weight bearing in rats. The drug used for the validation of the CatWalk XT apparatus and i.a. CFA monoarthritis rat model was naproxen, a well-known anti-inflammatory analgesic. Later naproxen was also used as a reference compound when novel drug candidates of interest, MGL (monoacylglycerol lipase) inhibitor, FAAH (fatty acid amide hydrolase) inhibitor and CB_1/CB_2 (cannabinoid receptor 1 and cannabinoid receptor 2) agonist were studied. Naproxen was also used as a reference in the study on the analgesic effects of pregabalin, an existing drug more familiar to treat neuropathic pain. Pregabalin was tested in a short five-day experiment but also when studying long-term effects of the i.a. CFA injection to see whether the inflammation pain caused by the CFA injection would have changed to neuronal or chronic pain or not. Preagabalin was convenient for the purpose since it is known that it does not affect inflammation pain but may relieve neuropathic pain and perhaps chronic pain.

6 Materials and methods

6.1 Animals and housing

In the first experiment male Wistar rats purchased from Harlan (RccHan:WIST, Harlan, The Netherlands) were used. At arrival the weight and age of the rats were 130 g and five weeks, respectively, and average experiment starting weights about 180-210 g. In the four other experiments the animals used were also Wistar rats from Harlan (RccHan:WIST, Harlan, United Kingdom) but they were purchased from United Kingdom due to delivery problems from the site in the Netherlands. Except for the breeder sites, the animals were similar in every experiment; stock and strain, arrival weight and arrival age and average experiment starting weights were same.

In every experiment, the rats were kept under a 12 h - 12 h dark-light cycle (illuminance in the daytime 300 ± 60 lux and at night 5 ± 4 lux). Dark time started at 5.30 p.m. and ended at 5.30 a.m. and there was a 30 minute dim period after the start and end of dark time during which a gradual changing in the light condition occurred to ensure proper acclimatization. In the testing room, there was a reading lamp over the static weight bearing apparatus but otherwise there was no lightning because testing with the CatWalk needed to be carried out in dark. There were no disruptive environmental sounds in the animal housing facilities except the ones caused by animal caretakers when checking the room and animals or changing cages. The rats were allowed free access of tap water and food (SDS RM1 (E) SQC, Special Diet Services Ltd, Witham, England). Conditions of animal housing rooms were standardized and continuously monitored and controlled: room temperature was 22 ± 2 °C, ventilation 12.5 ± 2.5 times/ hour and humidity 55 ± 15 %. The rats were housed four per cage in Makrolon IV-cages (1354G Eurostandard Type IV, TECNIPLAST). The cages were changed to clean ones twice a week and the water bottles were changed three times a week. Bedding material in the cages was aspen woodchips (TAPVEI®ASPEN BEDDING (Chips sizes 5x5x1 mm)) and animals had plastic cottage or tube made of red polycarbonate and wooden rod for environmental enrichment.

The procedures used in animal experiments were carefully documented. None of the animals had been used in previous procedures or used in any further experiments. The severity class of all experiments was moderate and after experiments the animals were sacrificed immediately after or no later than five days after the last administration of drugs and tests. The animals were acclimatized at least one week before starting the experiments. The studies were conducted in accordance with the Finnish law and the following guidelines: Act on Use of Animals for Experimental Purposes 497/2013, Decree on Use of Animals for Experimental Purposes 564/2013 and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purpose. All studies were approved by the Regional State Administrative Agency of Southern Finland (approval number ESAVI/7238/04.10.07/2014).

6.2 Drugs and solutions

Naproxen (naproxen sodium; Sigma-Aldrich) and pregabalin ((S)-Pregabalin; Toronto Research Chemicals Inc., Canada) were both dissolved in 0.9 % sodium chloride (NaCl; saline). Saline was used as a vehicle in all the other experiments except when studying FAAH inhibitor URB597 and CB_1/CB_2 receptor agonist WIN55,212-2 when there were vehicles consisting of Tween80/PEG400/saline and Tween80/saline, respectively. The doses of naproxen (2.5 and 7.6 mg/kg), pregabalin (10 and 30 mg/kg) and MGL inhibitor (2.5 and 10 mg/kg) were chosen based on knowledge from previous in-house experiments with different behavioral rat models, as well as pharmacokinetic studies. Scientific papers were used as a support for selection of effective doses of investigational substances URB597 (0.3 mg/kg) (Jayamanne *et al.*, 2006; Piomelli *et al.*, 2006; Manduca *et al.*, 2014) and WIN55,212-2 (1.0 mg/kg) (Schulz *et al.*, 2013; Fanarioti *et al.*, 2015).

Naproxen, pregabalin and MGL inhibitor were given orally via gavage (p.o.) in an administration volume of 3 ml/kg for naproxen and pregabalin and 5 ml/kg for MGL inhibitor. Naproxen was administered 4 hours before the CatWalk test and

it was dosed twice a day, the second dosing taking place 2 hours after the behavioral testing. Pregabalin was administered 2 hours and MGL inhibitor 1 hour before starting gait recordings in the CatWalk. In the experiment studying MGL inhibitor the saline vehicle was also administered 1 hour before the CatWalk, in another experiment pregabalin and the saline vehicle were administered concurrently and in the experiment studying only naproxen the saline vehicle and naproxen were concurrently administered. URB597 and WIN55,212-2 were both administered i.p. (intraperitoneal) route and the administration volume was 1 ml/kg for both substances. For URB597 and its vehicle administration time was 2 hours and for WIN55,212-2 and its vehicle half an hour before starting the CatWalk.

6.3 Induction of monoarthritis

50 µl of CFA (containing 1.0 mg/ml heat killed and dried *Mycobacterium tuberculosis*; Sigma-Aldrich) was injected with a 21-gauge needle into the left tibiotarsal joint from the dorsal side under deep isoflurane anesthesia (5 % isoflurane in breathing air). The injection was completed in less than a minute and the rats were left to recover in their home cages. The recovering from anesthesia occurred within a few minutes and depending on the experiment, the rats were first tested either four hours or one day after the injection. Naïve rats were used for comparison.

6.4 Randomization and blinding

The rats were randomly allocated into pharmacological treatment groups of 7-8 rats, before starting tests by using either the Latin square method or random scrambling. By randomization the idea was to create as homogeneous treatment groups as possible and to avoid the situation that cage mates of one cage would receive the same treatment.

The pharmacological treatments and end point measurements were blinded in every study. One person performed behavioral tests and the other drug dosing or if one person carried out both tasks, the formulation bottles were coded by someone else. Blinding of the animal model was impossible due to the swelling of the left hind paw after CFA-injection which made the concluding of naïve rats obvious.

6.5 Devices

6.5.1 The CatWalk

The CatWalk XT (Noldus, Netherlands) was used to test changes in gait caused by i.a. CFA-induced joint pain. The apparatus consisted of a walkway along which the rats walked towards their goal cage where other cage mates from the same home cage where waiting. Under the walkway, 70 cm below, there was a video camera recording the movements of animals and it was connected to a computer operating with the CatWalk XT software (Figure 7) by which the data was compressed, stored and analyzed.

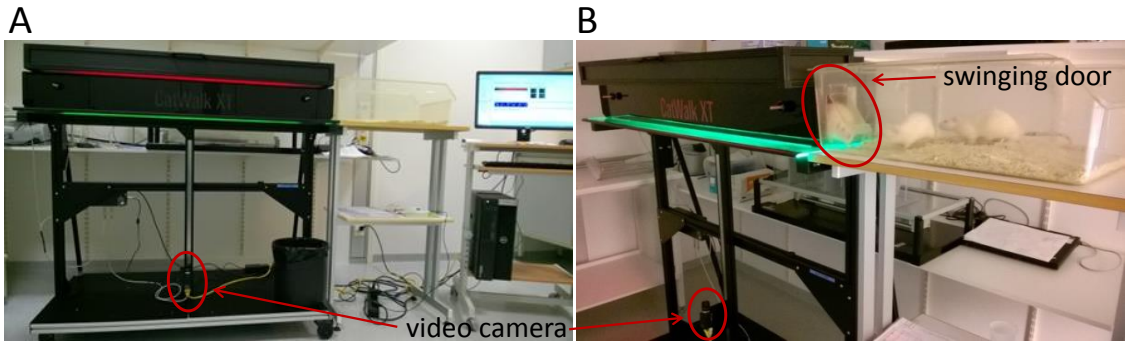

Figure 7. The whole CatWalk XT system shown: walkway attached with goal cage, video camera and computer (A) and a closer view of walkway with swinging door to goal cage and video camera beneath (B).

The floor of the walkway was a plate of 0.6 cm thick glass in which light traversed through the glass along one of the long edges. The roof reflected red light, the walls were black acryl about 10 cm apart from each other and the length of the walkway was 80 cm. The walkway had an entrance at the starting end and an exit which led to the goal cage through a swinging door.

The green led light emitted within the glass floor was internally reflected except when an object, for example an animal's paw, touched the surface of the floor causing the light to be scattered producing an illuminated picture. The degree of the contact and the pressure against the floor defined the intensity and clearance of the formed picture. The detection settings profile was defined for Camera gain 19.0 dB, Green intensity threshold 0.10, Red ceiling light 17.7 V and Green walkway light 16.0 V.

During experiments video camera recorded all the runs and acquired data were stored on the CatWalk XT software. Data acquisition, gait analysis and run recording started and ended automatically when the animal was inside the predetermined area and the predetermined algorithm of the CatWalk XT software recognized animal and its paws. After data acquisition, it was possible to classify the runs and paw prints automatically with the CatWalk XT software. Automatic classification worked well when an animal's run was regular and proceeded fluently ("normal", Figure 8A). On the other hand, the automatic classification was not always complete, for example, due to irregular gait (Figure 8B). For instance, rats often avoided using the injected paw (left hind paw) as a result of the pain caused by i.a. injection of CFA and the resultant inflammation. In these cases, it was possible to complete and correct paw prints manually.

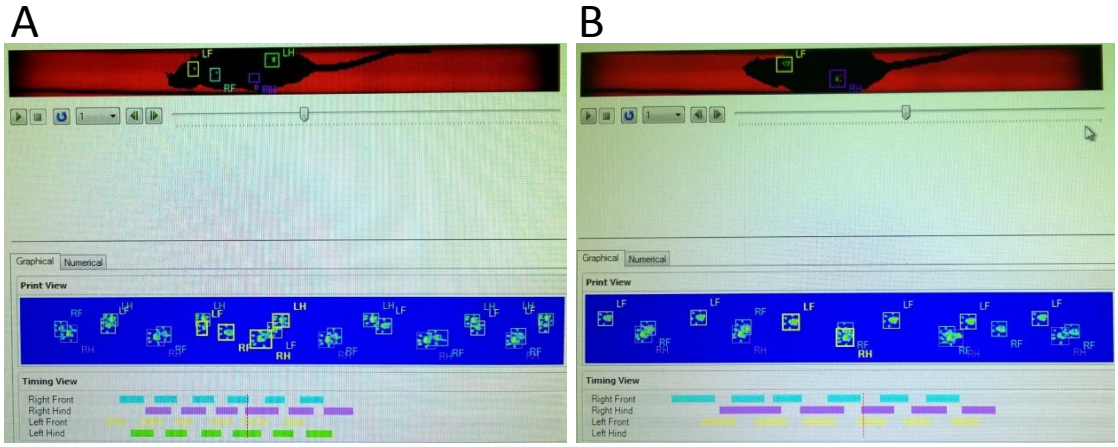

Figure 8. Normal, fluently and regularly proceeding run from a naïve rat (A) and a CFA-injected rat's run in which a left hind paw was not used at all, as illustrated by the lack of green line representing the left hind paw (B).

6.5.2 The Incapacitance tester

The restrainer was a transparent plastic box with an openable lid and a “ladder” on which a rat could place its’ front paws. A rat was placed in the restrainer in a way that it stood on top of the separate force sensors with its’ hind paws, tail lying outside the restrainer and front paws placed on the ladder. Sensors registered the weight of each hind paw (Figure 9).

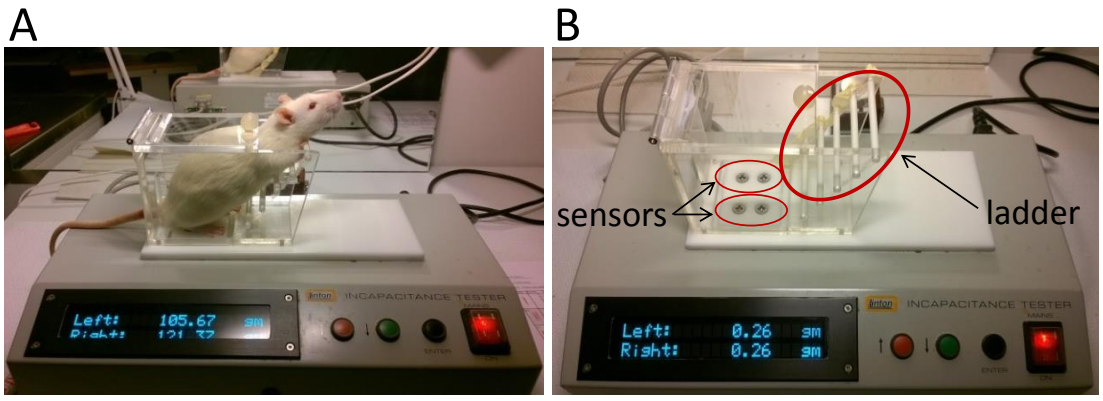

Figure 9. A rat in the restrainer of the Incapacitance tester (A) and the Incapacitance tester apparatus itself (B).

6.6 Data handling and statistical analysis

6.6.1 The CatWalk

The data received from the CatWalk experiments was first sorted out in Excel (Microsoft office 2010) and the parameters of interest were organized. Some derivative parameters were calculated in Excel in order to describe the gait and weight bearing during locomotion. These parameters were chosen based on previous knowledge and results from the CatWalk and the PawPrint experiments (Ängeby-Möller et al. 2008, Ängeby-Möller et al. 2012) and the most important or usable of those were found to be weight bearing (%) for each paw and guarding index. Parameters detected and their explanations are shown in Table 3 and described below.

To determine the weight bearing value, the mean value of all paw placements of one paw detected during walkway passage is first needed; the CatWalk software provided this automatically as a one value called

“MaxContactArea_(cm²)_Mean”. The CatWalk software also provided the light intensity value called “MaxContactMeanIntensity_Mean” for each paw placement of one paw taken during the walkway passage. The CatWalk software recognized intensities above a threshold value of 50 (intensity range 0-225 arbitrary units) as contact points and defined those as paw prints. These two values, max contact area and intensity, were then multiplied by each other and the given value was then divided with a sum total of all values of paw equivalents (max contact area * intensity). The received value was turned into a percentage value for evaluating relative contribution of each paw. This calculation was done separately for all four paws. Further, the guarding index was calculated by reducing the weight bearing value of injected (left) hind paw from the weight bearing value of non-injected (right) hind paw.

Other parameters were already calculated by the CatWalk XT software and were used in the analysis. The program used for statistical analyzes and visualization of the results was GraphPad Prism 5. As a statistical test, a one-way ANOVA was used at each measurement time point. If the p-values from ANOVA were significant ($p < 0.05$) then the Dunnett’s post hoc test was performed to compare the treatment effects against the CFA-injected vehicle treated group. If there were only two groups Student’s *t*-test was used to calculate significances.

Table 3. CatWalk XT gait parameters

Parameter	Explanation	Calculation
Dynamic weight bearing	Force a paw induce against the glass floor during walking, measured separately for all paws	Max area (mm^2) × Mean intensity (range 0−255; arbitrary units /mm^2)
Dynamic weight bearing (%)	Percent weight bearing of one paw in relation to all four paws during walking	$\left[\frac{\text{Dynamic weight bearing}_{\text{paw } x}}{\Sigma\ \text{Dynamic weight bearing}_{\text{all four paws}}}\right] \times 100\ \%$
Guarding index (%)	The shift in weight bearing (%) from the CFA-injected to the non-injected hind paw	$\text{Weight bearing (\%)}_{\text{non-injected hind paw}} - \text{Weight bearing (\%)}_{\text{CFA-injected hind paw}}$
Regularity index (%)	Proportional number of normal step sequence patterns (NSSP) relative to the total number of paw placements (PP)	$\left[\frac{\text{Number of NSSP} \times 4}{\text{Number of PP}}\right] \times 100\ \%$
Duty cycle (%)	Proportional duration a paw is in contact with the glass floor relative to entire step cycle (stance phase + swing phase)	$\left[\frac{\text{Duration}_{\text{stance}}}{\text{Duration}_{\text{stance}} + \text{Duration}_{\text{swing}}}\right] \times 100\ \%$

6.6.2 The Incapacitance tester

The data received from measurements of the Incapacitance tester were written down by hand during the experiments and afterwards entered to Excel (Microsoft office 2010). Data handling and calculations were done in Excel and statistical analyzes and visualization with GraphPad Prism 5. The parameter used to describe static weight bearing was weight bearing ratio. It was calculated by dividing the force induced by the injected (left) paw with the force induced by the non-injected (right) paw. The received values were turned into a percentage value (Table 4).

Table 4. Static weight bearing parameters

Parameter	Explanation	Calculation
Static weight bearing	Weight a paw induce against the force plate during standing, measured separately for each paw	
Static weight bearing ratio (%)	Weight of the injected paw divided by weight of the non-injected paw in percent	$\frac{\text{Weight bearing (g)}_{\text{CFA-injected paw}}}{\text{Weight bearing (g)}_{\text{non-injected paw}}} \times 100\,\%$

7 Experimental study design

7.1 Habituation

Before every experiment, the animals were identified by tail markings and their pre-experimental weights were recorded. Also, habituations to the CatWalk and the Incapacitance tester apparatuses as well as to the testing room and to experimenter took place before the testing phase.

During the habituation to the CatWalk, rats from one home cage were first let freely to get used to the goal cage for about five minutes. After that, one by one, all four rats from the same home cage were let to habituate to the walkway of the CatWak XT apparatus. One at a time, they were placed to the entrance of the walkway and left there for about five minutes to explore and walk back and forth along the walkway and also exit to the goal cage if wanted without disturbing. After free exploring, habituation continued with teaching the testing practice: one at a time, the animals were placed to the entrance in the starting end of the walkway and left there for so long that they voluntarily walked across the walkway. After the walk, they were helped to go through the swinging door to their goal cage without allowing them to walk back to the starting end anymore.

Rats are naturally very curious animals and in a stress-free environment they learn a lot and explore their surroundings willingly. This feature was exploited in the habituation and teaching process. With patient habituation, the cage mates waiting in the goal cage on the other end of the walkway the motivation was enough to make a rat willing to cross, and additional motivation, such as food, did not increase the learning or performance. Similarly, attempts to make a rat walk by making sounds or pushing it forward did not help but on the contrary, it only induced stress and reluctance to move. Habituation period for the CatWalk lasted for three to four days of which the first or the first two days, depending of the experiment schedule, comprised teaching as described above and the last two days establishing baseline measurements before CFA injection.

For the Incapacitance tester the habituation schedule was same as for the CatWalk: the first or the first two days of the habituation period were spent by teaching the testing practice and the last two days were used on baseline measurements simultaneously with habituation. In case of the Incapacitance tester, habituation started with a fairly short familiarizing to the static weight bearing restrainer box. The first time inside the restrainer was about one to two minutes because the restrainer and the situation were quite stressful due to the restricted space and position for the rat, in addition to the fact that the tail was positioned outside the box through a hole. At first the rats had to be held still by grasping the tail gently to teach them the way and orientation they needed to stand inside the restrainer. After the first habituation time the duration spend inside the restrainer could be extended, because the rats started to get used to it and stayed still quite calmly for a longer time.

7.2 Testing

All experiments were carried out during the light phase and the rats were habituated to the test room for a minimum of 30 minutes before each experiment. The rats were tested one cage at a time so that first all four rats were tested in the Cat Walk successively and after that they were tested in the same order in the Incapacitance tester. The studies were started with habituation and training of animals and consecutive baseline measurements as described above. The experiment phase began with induction of monoarthritis with intra-articular CFA injection which was followed by behavioral tests first for four hours and then one, two, three and four days after the induction to produce repeated measurements for assessing the pharmacological effects. The treatments were started on the day after the CFA injection and on the first treatment day behavioral tests were carried out both before and after dosing but on the last three days only after. One of the studies implemented was designed to detect the long-term constancy and stability of influence of CFA to monoarthritis and induced pain and therefore analogous behavioral testing and treatment administration period took place 21 days after the injection in that

study. Until that, the behavioral testing with the CatWalk XT and the Incapacitance tester were accomplished once a week.

Behavioral testing with the CatWalk was very objective since the rat was placed at the entrance of the walkway and let to behave and accomplish the run as it decided itself. The CatWalk apparatus automatically recorded the predetermined stretches within the runs: from the invisible starting line to the invisible finish line. After a successful run the walkway was wiped clean with a water and paper towel before testing the next rat. One proper run was enough for each animal and it was considered qualified when a rat walked across the walkway with at least three consecutive and continuous step cycles. In turn, if a rat stopped somewhere in the middle, was very slow or e.g. proceeded the walkway by sniffing, the test had to be repeated.

Static weight bearing could be implemented when a rat was well enough habituated so that it stayed still with two hind paws on the separate sensors without laying on either side of the restrainer or balancing itself with the tail. When the position was acceptable, five repeated measurements were recorded manually. The threshold time for the measurement was set to zero second so that the weight bearing values of each paw were detected every time without any delay. After each measurement, the values were written down and later transferred to a computer.

The objectives varied a little between the studies since at first the aim was to validate the animal model, testing methods and to decide the proper dose of naproxen for further use as a reference compound in future studies. After the validation, another commercially available drug, pregabalin, was tested to study its pain relieving efficacy against induced inflammatory pain. Then the study compounds under investigation with different mechanisms of action were tested to see whether they have any effect on monoarthritis and inflammatory pain indication. These compounds were MGL inhibitor, URB597 and WIN55,212-2.

8 Results

In all experiments, the number of rats was kept at 7-8 per each treatment group in order to get enough statistical power but simultaneously make the work reasonable in practice. All intra-articular CFA injections induced the desired outcome, the behavior of rats was not exceptional and none of the rats needed to be excluded from the results.

8.1 Development of weight

The weights of the rats were followed during the long-term study to ensure their overall wellbeing. The results showed no statistically relevant difference in weights when comparing not CFA-injected naïve rats and rats with intra-articular CFA-injection and vehicle treatment during the five-week testing period (Figure 10). Showing continuing and equal increase in weight between the groups proved the safety of the model.

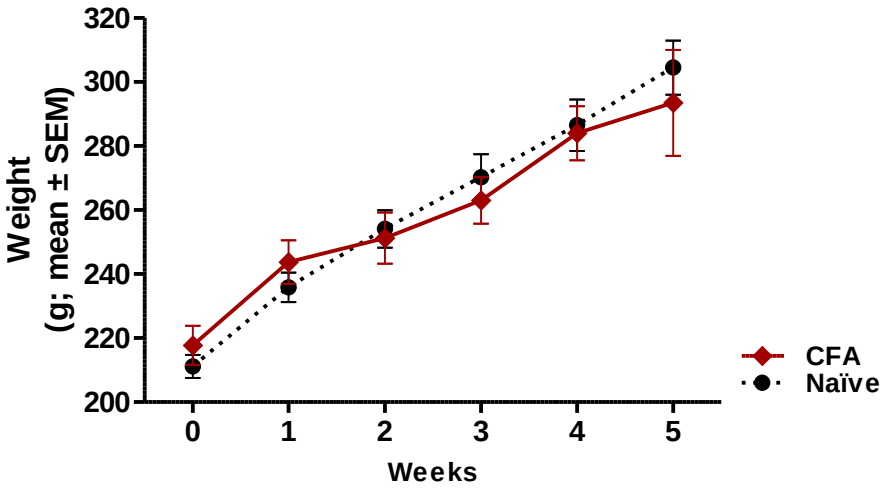

Figure 10. Comparison of development of body weight in naïve and CFA-induced monoarthritic rats with only vehicle treatment. Data are shown as mean ± SEM (n=8/group). No statistically significant differences were observed with unpaired Student's *t*-test.

8.2 Comparison of animals from two breeders of Harlan

CFA-induced monoarthritis was studied in male Wistar rats (RccHan:WIST) bred either in the United Kingdom or in the Netherlands. No significant differences between two breeding sites were observed in any of the end points (data of guarding index shown) or treatments (Figure 11).

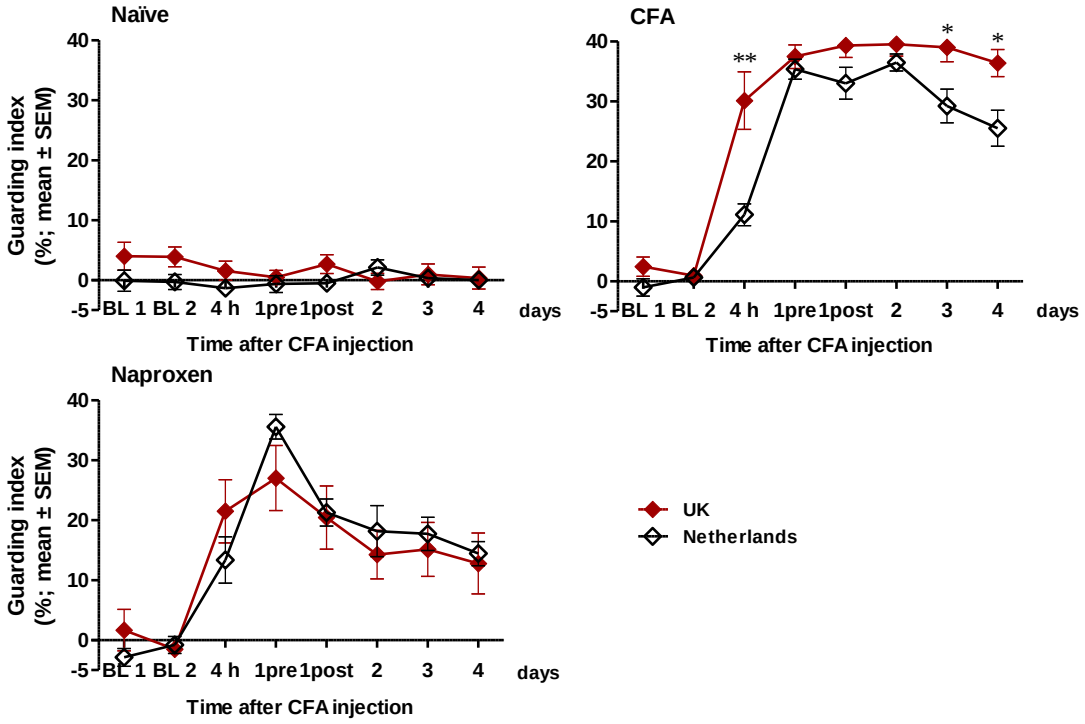

Figure 11. Wistar rats (RccHan:WIST) delivered from Harlan United Kingdom or Harlan Netherlands. Comparison of guarding index parameter of naïve, CFA-induced monoarthritic rats with only vehicle treatment and naproxen (7.6 mg/kg) treated monoarthritic rats. Data are shown as mean ± SEM (n=8/group). Unpaired Student's *t*-test was performed for each time point. *=p<0.05; **=p<0.01
BL – baseline measurement; 1pre – 1st day test before dosing; 1post – 1st day test after dosing

8.3 Gait analysis with naïve and monoarthritic rats

The data regarding naïve and CFA-injected vehicle-treated rats were similar in all studies. A representative image of an example rat from both groups visualizing qualitative information from gait and usage of paws is shown in Figure 8 (p.52). In addition to qualitative images of gait, a quantitative data

handling differences in usage of paws due to the CFA-induced monoarthritis and pain was gained (Figure 12).

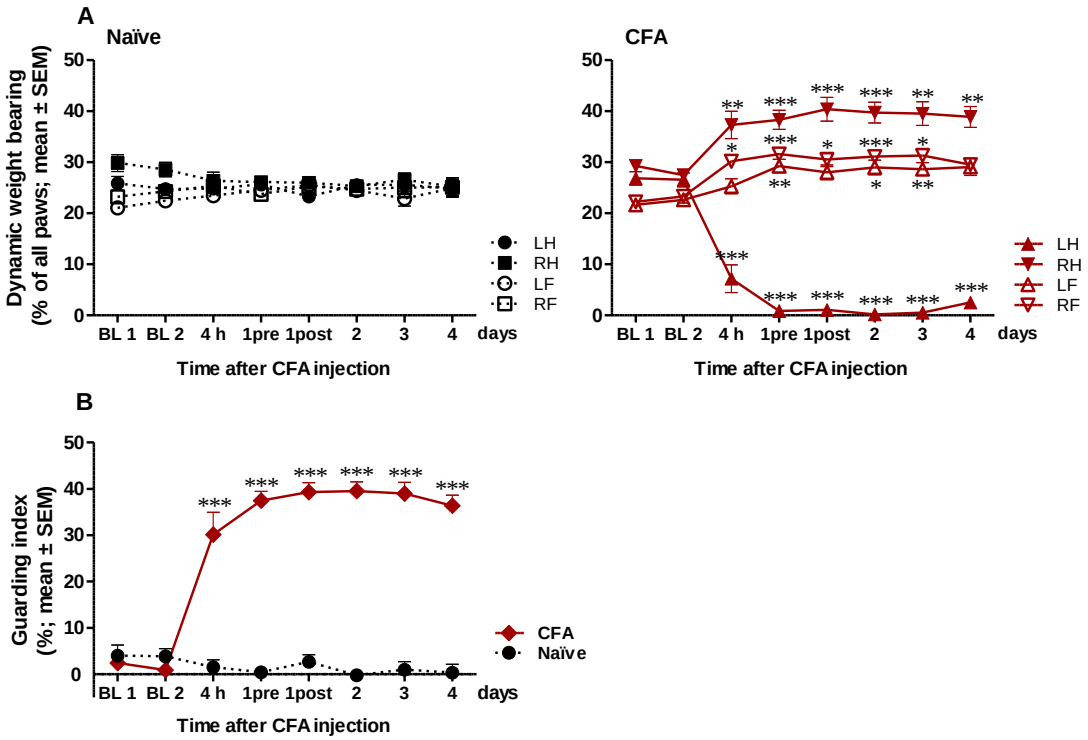

Figure 12. Changes in dynamic weight bearing shown as percentage for each paw in relation to all four paws in naïve and CFA-induced monoarthritic rats with only vehicle treatment (A) and the difference in the relative weight bearing between the two hind paws (guarding index) of naïve and CFA-induced monoarthritic rats with only vehicle treatment (B) are shown. Data are shown as mean ± SEM (n=8/group). Unpaired Student's *t*-test was performed for each time point versus the results from corresponding paws of naïve rats. *=p<0.05; **=p<0.01; ***=p<0.001
LH – left hind paw; RH – right hind paw; LF – left front paw; RF – right front paw; BL – baseline measurement; 1pre – 1st day test before dosing; 1post – 1st day test after dosing

A parameter describing overall willingness and capability to manage the walking test was regularity index. With naïve rats it was 100 % throughout the study (Figure 13A) showing that they crossed the CatWalk walkway with normal step sequence using all four legs equally with a coordinated fashion. Regularity index was 100 % also with monoarthritic rats during the baseline measurements but after the CFA-injection in the ankle of left hind paw it dropped by almost 10 % in mean value at its lowest level two days after the injection (Figure 13A).

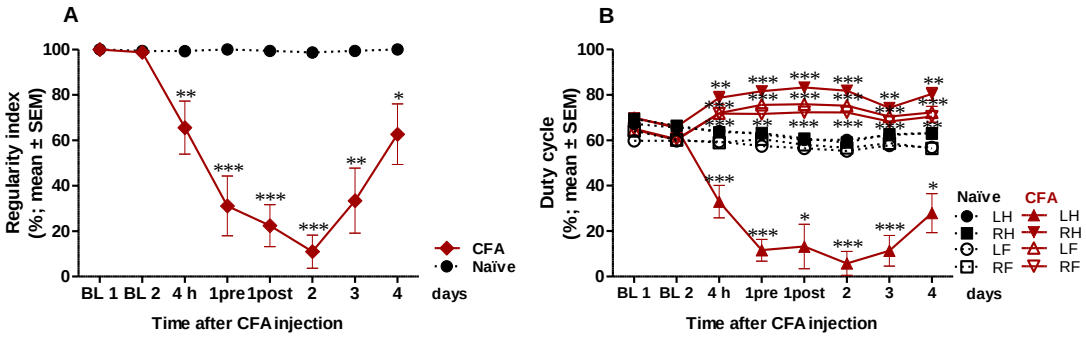

Figure 13. Changes in gait due to monoarthritis induced by CFA-injection into left hind paw shown as percentage of normal step sequences (regularity index) (A) and the relative time of paw placement compared to the entire step cycle (duty cycle) (B). Data are shown as mean ± SEM (n=8/group). Unpaired Student's *t*-test was performed for each time point versus the results from corresponding paws of naïve rats. *=p<0.05; **=p<0.01; ***=p<0.001
LH – left hind paw; RH – right hind paw; LF – left front paw; RF – right front paw; BL – baseline measurement; 1pre – 1st day test before dosing; 1post – 1st day test after dosing

Duty cycle, the willingness to put and keep paws on floor contact, was a little bit longer with hind paws than front paws with naïve rats but the situation stayed similar during the whole study (Figure 13B) hence representing the normal situation. On the contrary, with rats with CFA-injection into the ankle of the left hind paw the duty cycle decreased dramatically with simultaneous compensation, increased duty cycles in other paws (Figure 13B).

Even though dynamic weight bearing and guarding index were considered the most potential parameters for detecting arthritic pain felt and observed during locomotion in these studies with the CFA-induced rat model, there were numerous other parameters obtained from the CatWalk, such as swing speed, i.e. the time consumed for one paw to move from previous stand place to the next one, stride length, i.e. the distance between two consecutive paw placements of the same leg in millimeters and base of support (BOS), i.e. the distance between the paw placements of front legs or hind legs, respectively (Table 5).

Table 5. Effects of CFA-induced monoarthritis on the gait in rats with vehicle treatment. Effects are described with significances and measured with different parameters gained from the CatWalk tests. Data are shown as mean ± SEM (n = 8/group). 1-way ANOVA with Dunnett's multiple comparison test performed for each time point, comparisons made against naïve rats.

	Effects of CFA-induced monoarthritis on gait							
	Time points							
Parameters	**Baseline 1**	**Baseline 2**	**Day 0 (4 h after CFA)**	**Day 1 pre**	**Day 1 post**	**Day 2 post**	**Day 3 post**	**Day 4 post**
Weight bearing LH	ns	ns	***	***	***	***	***	***
Weight bearing RH	ns	ns	**	***	***	***	***	***
Weight bearing LF	ns	ns	ns	ns	ns	*	*	ns
Weight bearing RF	ns	ns	*	***	*	**	*	ns
Swing speed LH	ns	ns	***	***	***	***	***	***
Swing speed RH	ns	ns	**	ns	ns	ns	ns	ns
Swing speed LF	ns	ns	ns	ns	ns	ns	ns	ns
Swing speed RF	ns	ns	ns	ns	**	*	ns	ns
Stride length LH	ns	ns	ns	ns	***	***	ns	ns
Stride length RH	ns	ns	*	*	**	***	ns	*
Stride length LF	ns	ns	**	***	***	***	**	**
Stride length RF	ns	ns	**	***	***	***	*	**
BOS hind paws	ns	ns	ns	*	*	**	ns	ns
BOS front paws	ns	ns	ns	ns	ns	ns	ns	ns
	ns= non significant, *= p<0.05; **= p<0.01; ***= p<0.001							
	LH - left hind paw; RH - right hind paw; LF - left front paw; RF - right front paw; BOS - base of support							

8.4 Static weight bearing with naïve and monoarthritic rats

Static weight bearing in naïve rats was 100 % through the entire study (Figure 14) reflecting even weight distribution for both hind paws. With vehicle treated monoarthritic rats it was around 100 % also during baseline measurements but after the CFA-injection it fell down as low as mean value of about 25 % (Figure 14).

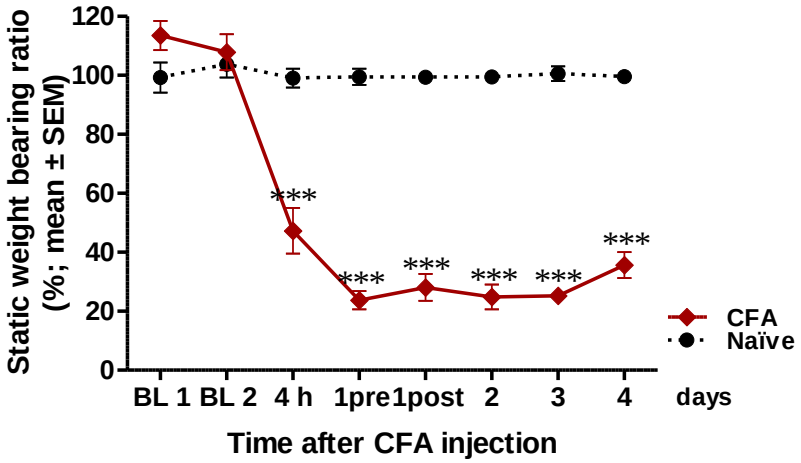

Figure 14. Changes of weight bearing during standing between the two hind paws in naïve and CFA-induced monoarthritic rats with only vehicle treatment. Data are shown as mean ± SEM (n=8/group). Unpaired Student's *t*-test was performed for each time point. ***=p<0.001
BL – baseline measurement; 1pre – 1st day test before dosing; 1post – 1st day test after dosing

8.5 Pharmacological effects in rats with monoarthritis induced by intra-articular ankle joint injection of CFA

Non-steroidal anti-inflammatory drug naproxen showed robust, reproducible and dose-dependent capability to restore the gait deficits (Figure 15) and also static weight bearing (Figure 16). Furthermore, other gait parameters showed similar effectiveness of naproxen to relieve the physical impairments induced with CFA (Figure 17) but instead, treatment with pregabalin could not affect either the gait deficits (Figure 17) or static weight bearing (Figure 19).

Investigational molecules, MGL inhibitor, URB597 and WIN55,212-2, could not relieve the deficits and pain induced by i.a. CFA-injection. They had no significant effects on any of the gait parameters measured (guarding index and regularity index shown in Figure 18) nor static weight bearing (Figure 19).

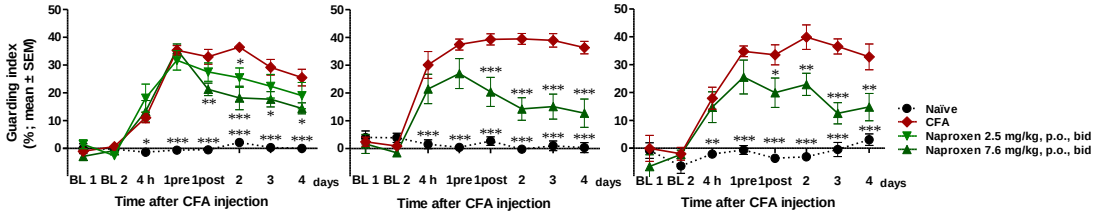

Figure 15. Effect of naproxen on gait in three independent studies. Data are shown as mean ± SEM (n = 8/group). 1-way ANOVA with Dunnett's multiple comparison test was performed for each time point, comparisons made against the CFA-injected vehicle treated group. *=p<0.05; **=p<0.01; ***=p<0.001
BL – baseline measurement; 1pre – 1st day test before dosing; 1post – 1st day test after dosing; p.o. – per oral; bid – twice a day

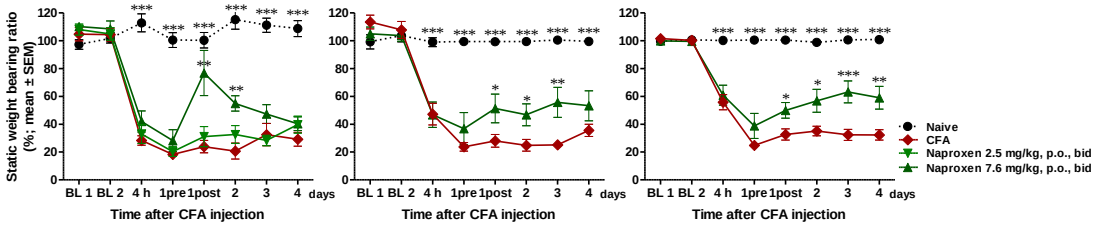

Figure 16: Effect of naproxen on static weight bearing in three independent studies. Data are shown as mean ± SEM (n = 8/group). 1-way ANOVA with Dunnett's multiple comparison test was performed for each time point, comparisons made against the CFA-injected vehicle treated group. *=p<0.05; **=p<0.01; ***=p<0.001
BL – baseline measurement; 1pre – 1st day test before dosing; 1post – 1st day test after dosing; p.o. – per oral; bid – twice a day

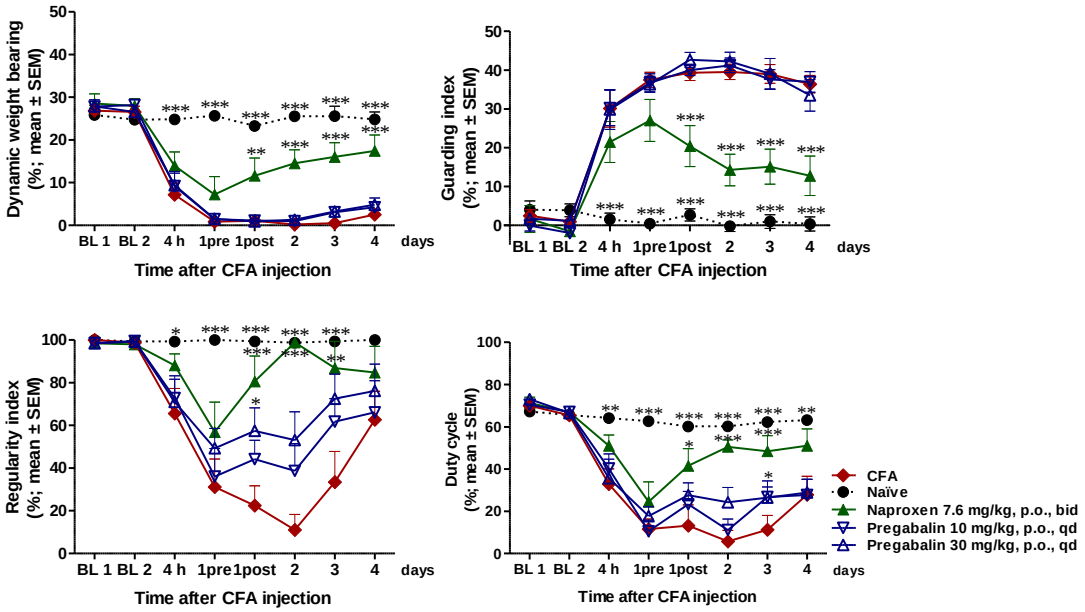

Figure 17: Effects of naproxen and pregabalin on gait. Dynamic weight bearing and duty cycle graphs are calculated from affected hind paws (data of other paws not shown). Data are shown as mean ± SEM (n = 8/group). 1-way ANOVA with Dunnett's multiple comparison test was performed for each time point, comparisons made against the CFA injected vehicle treated group. *=p<0.05; **=p<0.01; ***=p<0.001
BL – baseline measurement; 1pre – 1st day test before dosing; 1post – 1st day test after dosing; p.o. – per oral; qd – once a day; bid – twice a day

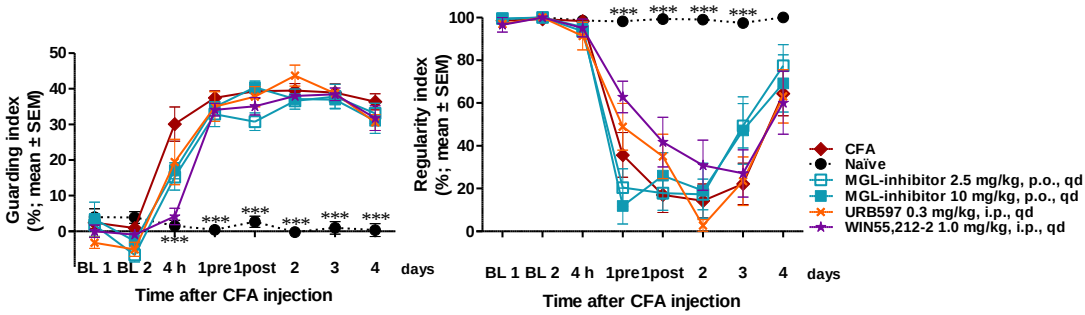

Figure 18: Effects of investigational test compounds on guarding index and regularity of gait. Data are shown as mean ± SEM (n = 8/group). 1-way ANOVA with Dunnett's multiple comparison test was performed for each time point, comparisons made against the CFA-injected vehicle treated group. *=p<0.05; **=p<0.01; ***=p<0.001
BL – baseline measurement; 1pre – 1st day test before dosing; 1post – 1st day test after dosing; p.o. – per oral; i.p. – intra peritoneal; qd – once a day

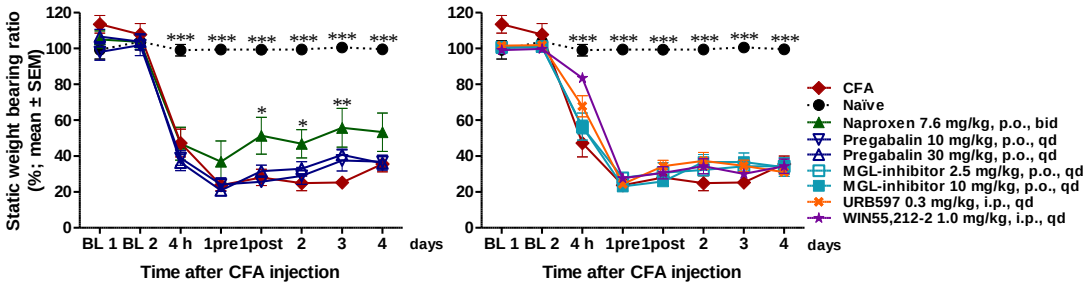

Figure 19: Effects of reference and test compounds on weight distribution between hind paws while standing in two separate studies. Data are shown as mean ± SEM (n = 8/group). 1-way ANOVA with Dunnett's multiple comparison test was performed for each time point, comparisons made against the CFA-injected vehicle treated group. *=p<0.05; **=p<0.01; ***=p<0.001
BL – baseline measurement; 1pre – 1st day test before dosing; 1post – 1st day test after dosing; p.o. – per oral; i.p. – intra peritoneal; qd – once a day; bid – twice a day

8.6 Long term study

The four-week study demonstrated that monoarthritis induced with i.a. CFA injection lasted only a short time period suggesting pharmacological testing to be implemented right after the monoarthritis induction. At least when using this rat model for analyzing locomotor abilities, detecting the CatWalk gait parameters revealed that action of CFA injection starts to decrease after the first week, disappearing entirely by the time the drug administration was started (Figure 20). The monoarthritis influence stayed longer when measured with static weight bearing but nevertheless no statistically relevant pharmacological treatment effects were observed (Figure 21). Even though naproxen treatment has been shown to be effective in five-day studies, it had no effect when started 21 days after CFA injection.

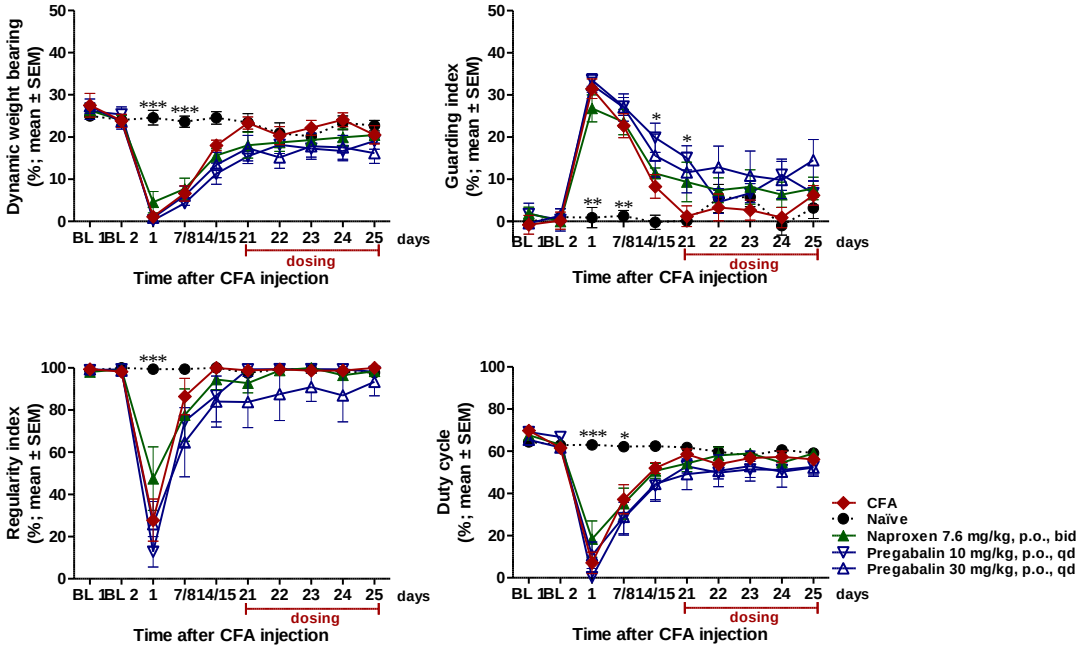

Figure 20. Permanency of CFA impact and effectiveness of naproxen and pregabalin on gait parameters when treatments started 21 days after monoarthritis induction. Dynamic weight bearing and duty cycle graphs are calculated from affected hind paws (data of other paws not shown). Data are shown as mean ± SEM (n = 8/group). 1-way ANOVA with Dunnett's multiple comparison test was performed for each time point, comparisons made against the CFA-injected vehicle-treated group. *=p<0.05; **=p<0.01; ***=p<0.001
BL – baseline measurement; p.o. – per oral; qd – once a day; bid – twice a day

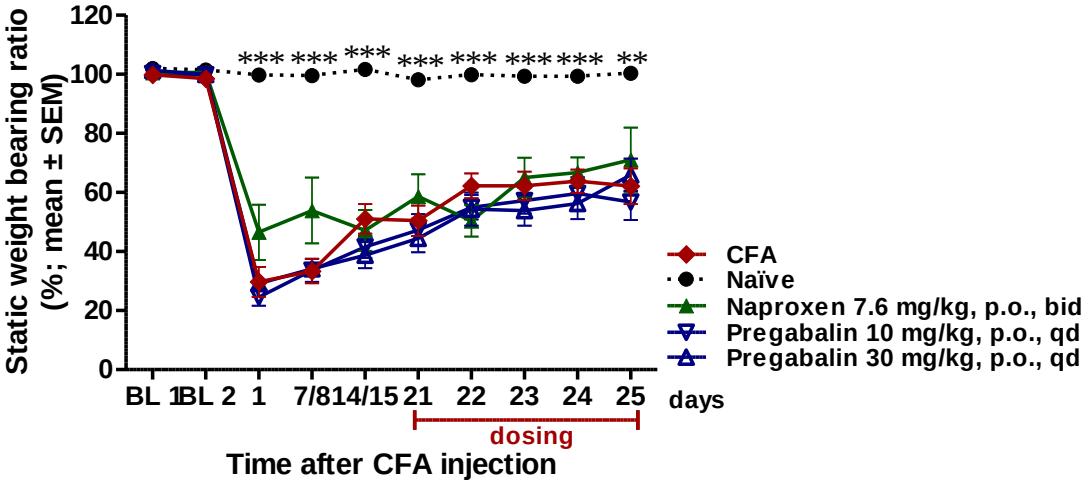

Figure 21. Permanency of CFA impact and effectiveness of naproxen and pregabalin on weight bearing between the hind paws while standing when treatments started 21 days after monoarthritis induction. Data are shown as mean ± SEM (n = 8/group). 1-way ANOVA with Dunnett's multiple comparison test was performed for each time point, comparisons made against the CFA-injected vehicle treated group. *=p<0.05; **=p<0.01; ***=p<0.001
BL – baseline measurement; p.o. – per oral; qd – once a day; bid – twice a day

9 Discussion of experimental study design, implementation and results

One of the main objectives of current studies was to evaluate the i.a. CFA rat model, a commonly used rodent inflammatory pain model, for studying OA and arthritis pain. Another, equally important aim, was to evaluate usefulness of the CatWalk XT apparatus in pain research. Gait analysis had previously been used for models of spinal cord injury and neuropathic disorder and more recently tested with rodent pain models (Ängeby Möller, Berge and Hamers, 2008; Ängeby Möller *et al.*, 2012, 2015) with promising results. Due to prior positive results from the same research field, our validation of model and method was justified and the results of our studies shared the feasibility of the CatWalk XT apparatus. After the i.a. CFA-induced monoarthritis rat model and gait analysis with the CatWalk XT apparatus had been proven to be proper, it was possible to compare guarding behavior in walking and in standing. Already for a long time it had been feasible to measure static weight bearing and guarding behavior in standing with the well-known Incapacitance tester apparatus. However, results from the Incapacitance tester alone have not been strong enough to forecast effectiveness of investigational treatment in clinical environment. Instead, together with weight bearing measurements resulted from the CatWalk it became possible to assess pain-like behavior in both a static (while standing) and dynamic (while walking) situation in rats which raises the predictive power of the results of pre-clinical studies. However, when comparing the two methods it is good to notice that sensation of pain and avoidance of painful hind paw loading were more obvious and also lasted longer when measured with static than dynamic weight bearing, or other gait parameters. This possibly reflects the ability of rats to compensate one tender limb with other limbs and tail when moving, but they lack similar compensation possibility when standing still with two limbs. Or when standing on two limbs, the weight targeted to the sore limb is higher and the need to keep it lifted is also higher. This could explain why pharmacological effects were more clearly seen with dynamic than static setting.

The huge amount of parameters gained from the CatWalk have been challenging to sort out and understand which ones can be translated to human pain conditions, since the use of it in the field of OA research has been limited. The dynamic weight bearing and guarding index derived from it have been considered as the most prescribing and translational parameters for OA and arthritis pain purpose. These parameters have been shown to be robust, straight-forward and objective and promote reproducibility and produce more relevant behavioral outcomes. Especially the method of calculating dynamic weight bearing during voluntary locomotion of animals (Ängeby Möller *et al.*, 2012) has been shown undisputed validity and correlation to the assessment of walking pain in OA patients. Calculating the dynamic weight bearing in the presented way, many of the factors causing possible variability to that parameter were prevented. In addition to these two parameters, there are many interesting parameters without straight counterparts in patients but still with relevance in pre-clinical studies. For example, the number of normal step sequences, duration of step placement on the ground and stride length and swing speed of individual paws could serve additional information of pain when comparing results between separate animal studies.

In the field of *in vivo* studies, the quality of experimental design, statistical analysis and reporting of research using animals have raised increasing concern. It has been stated that only appropriately and precisely planned, conducted and analyzed pre-clinical animal experimentations will advance understanding of disease pathophysiology and contribute to development of successful therapies for patients (Smith, Clarke and Little, 2017). Different recommendations and advise have introduced guidance for improving accuracy and transparency of reporting and publications of pre-clinical animal studies but they are generally followed with poor success. The most well-known and coherent is the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines which was developed as part of an NC3Rs (UK National Centre for the Replacement, Refinement and Reduction of animals in research) desiring to maximise information published and minimise unnecessary studies. The guidelines were published in 2010 (Kilkenny *et al.*, 2010) and are thereafter endorsed by scientific journals, major funding bodies and learned societies. In

addition, the DEPART (Design and Execution of Protocols for Animal Research and Treatment) was developed and planned to be used with the ARRIVE guidelines hoping to improve the rigor, utility and translation of animal studies of OA as DEPART usage would facilitate ARRIVE compliance (Smith, Clarke and Little, 2017). Even though the checklist format of the DEPART and ARRIVE guidelines or the gold standard publication checklist (Hooijmans, Leenaars and Ritskes-Hoitinga, 2010) were not used in our experiments, designing, implementing, analyzing and reasoning of future experiments were always thoroughly thought and openly discussed with the whole research team and followed well the principles of both guidelines. However, some lessons were left to be learned afterwards to further improve our research manners and so raise the quality of studies. E.g. the randomization of long term experiment could have been more homogenous, if it would have been done by taking into account the results of guarding index gained by the time of randomization, instead of just ensuring that cage mates are randomized into different treatment groups. However, the reporting of studies fully met the requirements of the ARRIVE guidelines. In addition, besides private in-house reporting and public reporting of studies in this master's thesis, an article has been published from the studies implemented for advancing the knowledge and improving the research in the field of OA (Ängeby Möller *et al.*, 2017).

9.1 Study substances

In the validation experiments two known drugs were used to detect their effects on rats' performance and on stability of CFA-induced inflammation. These two drugs, naproxen and pregabalin, were chosen to be positive and negative control because their pain-relieving actions are known and they belong in different drug classes. After validation, the intention was to study the possible analgesic feature and the impacts on inflammation of investigational drugs of Orion Pharma's interest.

According to the existing literature naproxen is a NSAID analgesic that affects especially inflammation pain. It acts by inhibiting prostaglandin synthase

enzymes, cyclooxygenases (COX1 and COX2), and has strong responses to inflammation factors such as many cytokines, for example IL-1 and TNF-alpha, and also IL-2, IL-6 and IL-8 which contribute to the inflammation (Burke, Smyth and FitzGerald, 2006). Concentrations of these factors are often increased in the synovia of inflammatory arthritis. Curative effects of naproxen are clearly visible in results collected from behavioral tests regarding locomotion and standing still (Figures 17 and 19). Furthermore, beneficial outcomes of naproxen treatment were shown to be reproducible since the results of all of our experiments were comparable (Figures 15 and 16). Besides inter-study in-house reproducibility, we were able to show the effects of naproxen being similar compared to studies in the literature (e.g. Ängeby Möller *et al.*, 2015). However, to further enhance the translatability of our studies and to strengthen the study outcomes, it might be worthwhile to measure pathological changes, such as inflammatory markers from synovial fluid samples.

Pregabalin is a drug licensed for the treatment of peripheral and central neuropathic pain (Moore *et al.*, 2009). Its exact mechanism of action is still unknown but it is proposed to act via binding to the $\alpha_2\delta$ protein subunit of voltage-gated calcium channels. By modulating calcium influx, it may reduce the excitatory neurotransmitter release and has anticonvulsant, analgesic and anxiolytic properties. Structurally pregabalin is a γ-aminobutyric acid (GABA) analogue and shares structural and functional similarity with gabapentin (Blommel and Blommel, 2007). Presumably pregabalin has no effects on inflammation pain but is more effective to chronic and especially neuronal pain. Meta-analysis of a systematic review indicated that there is no reliable evidence to support the use of pregabalin for acute pain (Moore *et al.*, 2009). Our experiment with pregabalin was in line with the previous knowledge.

The first drug under investigation was MGL inhibitor which was chosen as a study substance based on the results from previous experiments conducted at the Orion Pharma Research and Development (R&D). The information gathered from those experiments indicated possible effects on pain and inflammation. Similarly, the next two substances, URB597 and WIN55,212-2, had shown previous possible analgesic effectiveness and were therefore interesting and valid to test further with this particular model and methods.

9.2 Development of weight

During the experiments, it was visually observed that rats with CFA-induced monoarthritis receiving only vehicle treatment did not gain weight in similar manner than naïve ones. The reason was considered to be reduced appetite due to pain caused by monoarthritis. For confirmation, the weights of naïve and CFA-injected vehicle-treated animals were compared. However, based on the weight data collected from the long-term experiment over the five-week period showed no statistically significant differences. The graph of weights shows a slight slowdown and greater variance among individuals in CFA-injected rats and also slightly greater weight increase in naïve rats but the weights of both groups increased evenly (Figure 10) and differences did not reveal statistical significance (p-values were > 0.05) when carried out unpaired Student's *t*-test. The pain caused by CFA-injection could affect the appetite a little but based on weight comparison results it can be said that it did not influence the experiments and parameters measured.

9.3 Comparison of animals from two breeders of Harlan

During the experimental phase the breeding location had to be changed from Harlan Netherlands to Harlan United Kingdom. Except for the breeding location, the animals were graded to be equal, but to ensure their similarity, the same naproxen (7.6 mg/kg) dosing was repeated with Harlan UK RccHan:WIST rats that was previously done with Harlan Netherlands RccHan:WIST rats. Data of naïve rats and CFA-induced monoarthritic rats with vehicle and naproxen treatments of these two studies were then used to evaluate the behavioral similarity of the animals. When comparing the results gained from two separate studies implemented in the same manner (data of guarding index shown, Figure 11) only little statistically significant differences could be observed by using unpaired Student's *t*-test. Mild difference was observed between groups with CFA-induced monoarthritis and vehicle treatment. However, the naïve animals from both breeding locations behaved similarly and naproxen treatment restored gait-related behavior in a similar manner in animals from both breeding

locations. Variability was observed between the monoarthritic but only vehicle-treated groups from the two different studies, but it could be explained also by other factors than breeding locations. The variations can be related to success of monoarthritis induction and its stable remaining rather than origin of used animals. Since naïve animals and monoarthritic naproxen-treated animals showed no significant differences between breeding locations, and since monoathritic vehicle-treated animals from both breeding locations followed a similar pattern despite mild variation, it can be concluded that the breeding location of the animals has no significance on this research and its results.

9.4 Gait analysis

Effects of pain to the movement can be observed in many ways and with many different parameters but one of the clearest and simplest is to detect how pain affects the will to put weight on the sore limb. This is described in Figure 12 showing the results of i.a. injection of CFA: rats with induced inflammation and vehicle treatment do not want to put weight on to the left, injured hind paw but instead compensate it by putting more weight to the other paws, especially to the right, healthy hind paw. Figure 12 also shows how the relative weight bearing of the injected hind paw decreased dramatically from roughly 27 % before CFA injection to mean value of only 7 % during the first post-injection measurement and after that to mean values between 0,2 % and 2,5 % during next three post-injection days. At the same time, relative weight bearing of non-injected hind paw increased up to 40 % representing the compensation effect. Additionally, a slight increase in front paw values occurred.

Comparison of weight bearings between hind paws, the guarding index, was considered to be one of the best prescribing parameters of the CatWalk when detecting the possible differences caused by monoarthritis. The shift of weight from injected hind paw to the non-injected hind paw increased with monoarthritic rats after the induction (Figure 12B), revealing the pain and unwillingness to put weight on the arthritic paw.

Effects of naproxen to the guarding index are clearly visible and repeated in every experiment where it was used (Figure 15). The dose of naproxen, selected based on the first validation experiment carried out with the CatWalk, was 7.6 mg/kg p.o. twice a day and this dose showed effect already after the first dose. Naproxen partially and markedly restored the weight bearing between hind paws even though it did not completely reach up to the naïve animals' level. This is clearly visible, for example, on the results of the experiment where both naproxen and pregabalin were used (Figure 17). The same figure also shows the inefficacy of two doses of pregabalin: neither of these doses had a beneficial effect to the gait at least when measured with weight bearing, guarding index, regularity index or duty cycle. When comparing the curves of pregabalin against the curves of vehicle-treated group it can be seen that they do not differ from each other at all. Therefore, it can be said that pregabalin had no favorable effects on inflammation pain when measured with gait parameters.

When the CatWalk XT and i.a. CFA monoarthritis rat model were being validated, some pre-clinically relevant reference molecules under investigation were studied. None of the compounds studied, MGL inhibitor, URB597 and WIN55,212-2, showed effectiveness on joint inflammation pain. Consequently, the studies carried out and the results gained (guarding and regularity index shown, Figure 18) do not support the role of endocannabinoid system in monoarthritic pain. According to good scientific manners, these negative results were reported and will be taken into account when considering future experiments with these agents.

The parameter describing cycle of the steps of an animal and how regular the cycle is and how evenly each paw is used is called regularity index in the CatWalk XT. It is easily seen from the regularity index figure whether the animals used all their paws evenly or whether they avoided using some paw which in turn reflects the feeling of pain. Figure 13A shows how regularity index of naïve animals' group stayed about 100 % for the whole experiment reflecting the normal gait but instead, the rats with CFA-induced monoarthritis avoid the use of the sore limb. Therefore, the left hind paw was not used as frequently as the three other paws which were used irregularly in a way that both front paws were placed on the ground more often and for a shorter duration than the non-

injected hind paw. Due to that the regularity index dropped dramatically after CFA-injection being about 11 % at the lowest and staying clearly under the normal 100 % regular step cycle for the entire study. Effects of naproxen to the regularity of gait and even usage of each paw were significant and it normalized the whole step cycle (Figure 17). In contrast, neither dose of pregabalin or MGL inhibitor nor URB597 or WIN55,212-2 improved the normality and regularity of step cycle (Figures 17 and 18).

Duty cycle is a parameter that also shows the will to use the paws and distribute the usage of all paws evenly. It describes the relative duration that each paw is in contact to the surface. Due to the pain i.a. injection of CFA is causing to the left hind paw, the value of the duty cycle parameter of vehicle-treated rats changes (Figure 13B). Duration of paw placement for the injected paw decreased in contrast to the non-injected hind paw as well as the front paws increased the time they were placed on the floor during locomotion. Naproxen showed its efficacy to inflammation pain also when measured with duty cycle parameter (Figure 17). It clearly increased the duration of the left hind paw kept on the ground. It also decreased the paw-floor contact of other paws describing reduced pain of the left hind paw and reduced the need to compensate the walk by more intensive use of the other paws (data of other paws not shown). Pregabalin did not show any clear evidence on analgesic effect in monoarthritic rats when compared with the graphs of the results from naïve, vehicle and naproxen groups (Figure 17). There was no clear improvement on the duty cycle parameter with either dose, so the paw-floor contact of left hind paw was not improved.

Swing speed describes how willing a rat is to put a paw on the ground and how fast it circulates steps. If swing speed is high and regular, the rat does not guard any paw but uses them evenly and regularly and distributes even force to each paw. With high swing speed walking is usually quite fast too, whereas if swing speed for some paw is low the rat is probably guarding it by holding it in the air and placing it to floor contact much fewer and shorter times than normally. Swing speed of the left, injected, hind paw remarkably dropped after the CFA injection compared to baseline levels (Table 5). At the same time swing speed of the non-injected, right hind paw increased. Swing speeds of the front paws

changed too to a much lesser degree and the change was in the opposite direction than for the hind paws; left front paw, the paw on the injected side, moved slightly faster and right front paw, the paw of the non-injected side, moved slightly slower. This may refer to slight compensation: when the left hind paw moves slower its ipsilateral counterpart, the left front paw has to move a little faster and the other way round on the right side.

Acute, short-term nature of CFA as a substance for inducing arthritis and pain was pointed out with the studies carried out, since both the rat model used and the behavioral testing implemented with the CatWalk worked well with the five-day studies but not anymore with the long-term four-week study. Inflammatory pain components cleared off already after one and especially after two and three weeks of monoarthritis induction leading to testing of the pharmacological treatments to be invalid: even though naproxen was very efficient to recover locomotion in the five-day studies, similar results could not be shown in the long-term study since the CFA-injected rats from the vehicle treatment group had recovered their gait back to pre-injection level already before the treatment period started (Figure 20).

Altogether, the validation of the rat model was successful and it can be said that CFA-induced monoarthritis was a suitable model for detecting joint pain during walking. Also the CatWalk XT device offered a proper, semi-automatic and objective testing method and locomotor assay with multiple parameters to be used in studies demanding information related to gait. The CatWalk XT apparatus showed its usefulness for the arthritis study field and it was shown to be valid at least when used with the CFA-induced monoarthritis rat model. Even though every animal is always an individual and some rats were more sensitive than others and some spend more time in the starting end exploring the entrance than others, the testing method of the CatWalk XT was still equal for all because when a rat was placed on the entrance it decided itself when and how it walked without the person testing being able to affect the run. Furthermore, the CatWalk revealed strictly and reliably with many different parameters the capability of different pharmacological treatments to recover the weight bearing among the four paws and loading of the damaged paw.

9.5 Static weight bearing

Since human arthritic pain is measured both dynamic and static manner, these experimental studies implemented similarly, and therefore behavioral tests of gait and static position were both carried out. However, testing with the Incapacitance tester was more subjective than with the CatWalk because the the rat's position had to be affected to make it stand only on its hind paws to ensure maximal accuracy of the static weight bearing results. Albeit being the more subjective method, the Incapacitance tester is a well-known and long and extensively used apparatus and the static weight bearing parameter received from the measurements provided comparable and reproducible data for comparing naïve and CFA-injected rats which had received either vehicle or naproxen treatment (Figure 16). The static weight bearing dropped from 100 % level down to 24 % mean values with rats which had received CFA-injection into the ankle joint of the left hind paw and only vehicle treatment, thus revealing the unwillingness to set weight on that leg (Figure 14). During the maximum decrease only a quarter of the total weight was placed on the sore limb describing the pain sensed when arthritis was induced. Also the lack of analgesic properties of MGL inhibitor, URB597 and WIN55,212-2 shown with the CatWalk parameters were proven with static weight bearing as well (Figure 19).

The objective of the long-term four-week study was to detect if the CFA-induced monoarthritis rat model could also serve studies of chronic and neuropathic pain, but similarly to gait analysis, the results of static weight bearing did not support that. Therefore, studies of chronic pain or alteration of inflammatory pain into neuropathic were neither real nor feasible with this model. It must be noted that the test method used affected persistence of effects and arthritis state, since the recovery was much quicker when measured with gait parameters compared to static measurement. Even though the CFA-injected rats from the vehicle-treatment group recovered their gait back to pre-injection level before the treatment period started but when measured at standing position the static weight bearing did not return to 100 % but instead, it remained around 63 % until the end of the study period. Nevertheless, the

inflammatory component must have diminished during the three weeks since analgesic naproxen did not show any improvement on static weight bearing and presumably no neuropathic pain component had been developed because pregabalin did not work either (Figure 21).

10 Conclusion of the experimental phase

The relevance of the used i.a. CFA monoarthritis rat model and behavioral test methods were proved with naproxen and pregabalin treatments. Well-known and clinically used NSAID naproxen showed partial improvement in the behavioral effects in monoarthritic rats as expected, whereas pregabalin that is mainly neuropathic pain medication in clinical use showed no effect on CFA-induced pain, so both findings were in line with our set assumptions. The consequent testing of the three investigational drugs, MGL inhibitor, URB597 and WIN55,212-2, showed no detectable effect for any parameter measured with behavioral tests indicating that their mechanisms of action were not activated in the arthritis condition. Even though the outcomes were undesired there is no room for doubt because the studies were known to be reliable due to the well conducted validation of the model and testing methods.

The results achieved from the implemented studies support the use of the i.a. CFA model to study inflammatory joint pain in rodents, and since the testing methods with the CatWalk and the Incapacitance tester were shown to measure different modalities of pain, i.e. pain at walking and pain at standing, which translated well into complaints from pain patients, the evaluation and validation aims can be said to be successfully met. The reliability of both, the model and behavioral testing methods, were established when corresponding results of rats from naïve, vehicle-treated and naproxen-treated groups were able to be reproduced study after study. In addition, the gait-related results from CatWalk XT were shown to be in line with the precursor, private PawPrint system which supports and enables the use of gait analyzing method and apparatus available for everyone. Also, the reliable and translational nature of the CatWalk setup, and the particularly dynamic weight bearing parameter were proven since the efficacies of the tested treatments corresponded with their real-life efficacies tested and reported in clinical environment. In conclusion, our data support the use of this animal model and introduced pain-like behavioral testing methods in translational joint pain research and in novel analgesics research and development.

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APPENDIX

WOMAC Osteoarthritis Index.

Dimensions and questions included in Standard WOMAC hip/knee osteoarthritis questionnaire. WOMAC (LK) survey form available online, last visited 11.3.2017: https://www.hss.edu/files/New_patient_Hip_WOMAC.PDF

WOMAC Survey Form Name: _____

Instructions: In Sections A, B, and C, questions will be asked about your hip or knee pain. Please mark each response with an X. If you are unsure about how to answer a question, please give the best answer you can.

A. Think about the pain you felt in your hip/knee during the last 48 hours.

Question: How much pain do you have?	None	Mild	Moderate	Severe	Extreme
1. Walking on a flat surface	☐	☐	☐	☐	☐
2. Going up and down stairs	☐	☐	☐	☐	☐
3. At night while in bed, pain disturbs your sleep	☐	☐	☐	☐	☐
4. Sitting or lying	☐	☐	☐	☐	☐
5. Standing upright	☐	☐	☐	☐	☐

B. Think about the stiffness (not pain) you have in your hip/knee during the last 48 hours. Stiffness is a sensation of decreased ease in moving your joint.

	None	Mild	Moderate	Severe	Extreme
6. How severe is your stiffness after first awakening in the morning?	☐	☐	☐	☐	☐
7. How severe is your stiffness after sitting, lying, or resting in the day?	☐	☐	☐	☐	☐

C. Think about the difficulty you had in doing the following daily physical activities due to your hip/knee during the last 48 hours. By this we mean your ability to move around and look after yourself.

Question: What degree of difficulty do you have?	None	Mild	Moderate	Severe	Extreme
8. Descending stairs	☐	☐	☐	☐	☐
9. Ascending stairs	☐	☐	☐	☐	☐
10. Rising from sitting	☐	☐	☐	☐	☐
11. Standing	☐	☐	☐	☐	☐
12. Bending to the floor	☐	☐	☐	☐	☐
13. Walking on flat surfaces	☐	☐	☐	☐	☐
14. Getting in and out of a car, or on or off a bus	☐	☐	☐	☐	☐
15. Going shopping	☐	☐	☐	☐	☐
16. Putting on your socks or stockings	☐	☐	☐	☐	☐
17. Rising from the bed	☐	☐	☐	☐	☐
18. Taking off your socks or stockings	☐	☐	☐	☐	☐
19. Lying in bed	☐	☐	☐	☐	☐
20. Getting in or out of the bath	☐	☐	☐	☐	☐
21. Sitting	☐	☐	☐	☐	☐
22. Getting on or off the toilet	☐	☐	☐	☐	☐
23. Performance heavy domestic duties	☐	☐	☐	☐	☐
24. Performing light domestic duties	☐	☐	☐	☐	☐

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