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# Limb Idleness Index (LII): a novel measurement of pain in a rat model of osteoarthritis[1]

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SUMMARY

*Objectives:* Mechanical allodynia during ambulation in osteoarthritis (OA) animal models can be assessed as decreased extent of loading or decreased duration of loading. We propose to measure gait adaptation to pain by both mechanisms with the development of Limb Idleness Index (LII) in a rat model of knee OA.
*Methods:* Rats were assigned to anterior cruciate ligament transection (ACLT), Sham, or Normal group ($n = 6$). Gait data were collected at pre-injury, 1, 2, 3 and 6 months post-injury. Ratios of target print intensity, anchor print intensity, and swing duration were combined to obtain LII. The association of gait changes with pain was assessed by buprenorphine treatment at 3 and 6 months post-injury. At 6 months, OA-related structural changes in knee joints were examined by μCT and results from histological scoring were correlated with LII.
*Results:* As compared to pre-injury level (range 0.75–1.20), LII in ACLT group was increased at 6 months post-injury, which was significantly higher than that in Sham and Normal groups ($P = 0.024$). The increase in LII in ACLT group was effectively reversed by buprenorphine treatment ($P = 0.004$). ACLT group exhibited a significantly higher maximum Osteoarthritis Research Society International (OARSI) score as compared to Sham ($P = 0.005$) and Normal ($P = 0.006$) groups. Significant correlation was found between LII and side-to-side difference in OARSI score ($r = 0.893$, $P < 0.001$).
*Conclusions:* LII presents a good measurement for OA-related knee pain in rat model.


## Introduction

Osteoarthritis (OA) is a debilitating progressive disease manifested as pain in joints. The etiology and pathogenesis of OA is not completely understood. Animal models for investigations of OA are established to replicate the pathological changes observed in clinical cases by genetic modification, intra-articular injections of chemicals such as mono-iodoacetate or surgical destabilization of the joint[1,2]; yet there is no one gold standard model for OA that sufficiently represent human etiology. OA models induced by transection of anterior cruciate ligament (ACLT) or menisectomy may represent a good mimic of post-traumatic OA in humans, in spite of a faster disease progression[3]. In order to investigate new therapeutic strategies for OA, we need to assess both symptom-modifying and disease-modifying effects in these animal models.

Apart from histological and biochemical assessment, measurement of pain is regarded as one of the major outcome measures, which are available in several animal models,[2] in which half of them OA changes are chemically induced[4–6]. These methods successfully utilize paw elevation time[4] or weight-bearing[5,6] of injured limbs to measure OA pain based on avoidance of pain-triggering activities on the affected limbs (mechanical allodynia). Because the avoidance mechanisms of mechanical allodynia could be achieved by either decreasing duration of loading (increased paw elevation time) or re-distribution of loading (decreased weight-bearing), it is advisable to assess both mechanisms simultaneously. We propose to measure limb idleness by combining the information of swing time of target limb (increased paw elevation

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time to avoid loading), paw intensity on target limb (decreased weight-bearing to avoid loading) and paw intensity on anchor limb (increased sharing of weight from target limb), using a Catwalk animal gait analysis system. Because walking speed will affect all gait parameters and the rats are allowed to walk freely without constraint on speed, internal control for run-to-run variations in walking speed can be achieved by normalization to contralateral side. A Limb Idleness Index (LII) is calculated as a product of the ratios of target paw print intensity, anchor paw print intensity and swing duration. We hypothesize that LII is useful to detect limb idleness related to OA pain. In the present study, we measured LII in a rat model of knee OA induced by ACLT and determined its relationship to pain by analgesic reversal test. Moreover, whether the pain-related limb idleness was contributed by OA changes was evaluated by histological examination and micro-computed tomography (μCT).

## Methods

### *Rat OA model induced by ACL transection*

The animal experiments were approved by the Animal Ethics and Experimentation Committee of The Chinese University of Hong Kong (Reference No: 11/008/DRG) and all animals received humane care. Eighteen female Sprague–Dawley rats, 12 weeks old, with an average body weight of 220 g, were used in the present study. The rats were randomly assigned to Normal, Sham and ACLT groups ($n = 6$). In the ACLT group, a well-established protocol of ACL transection was adapted to induce knee OA[7,8]. In brief, the rats were anaesthetized by intra-peritoneal injection of ketamine and xylazine (75 and 10 mg/kg body weight respectively) A medial parapatellar arthrotomy was made, followed by a lateral displacement of patella and full flexion of the knee to expose the intra-articular joint space. The ACL was then transected with micro-scissors and successful ACL transection was assured by a positive anterior drawer test. After rinsing the joint space with saline, the joint capsule and the skin were closed in layers. In the Sham group, sham operation was performed with the same procedures except ACL transection. The rats were allowed free cage movement immediately after surgery. In the Normal group, no operation was performed.

### *Catwalk animal gait analysis*

Animal gait analysis was performed by Catwalk XT 9.0 (Noldus Information Technology, Wageningen, The Netherlands) at pre-injury, 1, 2, 3 and 6 months post-injury. The animals were put on the walkway to familiarize with the settings 1–2 days before the day of assessment. The camera was set at 60 cm from the walkway and the region of interest (ROI) (8 × 20 cm) for image capture was defined and calibrated. All rats were weighed before gait analysis, and the rat with a body weight closest to the average value was used to set thresholds to pick up the illuminated contact prints [Fig. 1(A)]. The rats were allowed to walk voluntarily back and forth inside the walkway in the dark; video recording of paw prints [Fig. 1(B)] was automatically triggered when the rats entered the ROI. Recorded runs [Fig. 1(C)] with a steady walking speed (variation <30%) were accepted as compliant runs for paw print auto-classification as left front (LF), right front (RF), left hind (LH) and right hind (RH) by the built-in software and the correctness of classification was further checked manually. Stance duration was detected as the time of paw contact [colored bar in Fig. 1(D)] and swing duration was detected as the time between consecutive paw contacts. Only runs with normal alternate footfall pattern (LF ⇒ RH ⇒ RF ⇒ LH) [Fig. 1(E)] were included for further analysis. Three to five runs were kept for calculation of gait parameters for every trial.

### *Calculation of LII*

Gait parameters were presented as ratios between target (injured) side and contralateral side to control for run-to-run and individual variations. The extent of limb loading during walking was evaluated by the paw print intensities. In the present study with injured RH in ACLT and Sham groups, if RH was idled during walking, the loading on target limb (RH) might be decreased with a dimmer or even smaller paw print; alternatively, the loading on the anchor limb (LF) might be increased to share the loading on RH. Thus the target print ratio (LH/RH) and the anchor print ratio (LF/RF) were increased in case of limb idleness on RH. Because a paw print is composed of a series of contact prints during stance phase [Fig. 1(F)], paw print intensity was calculated as the integration of

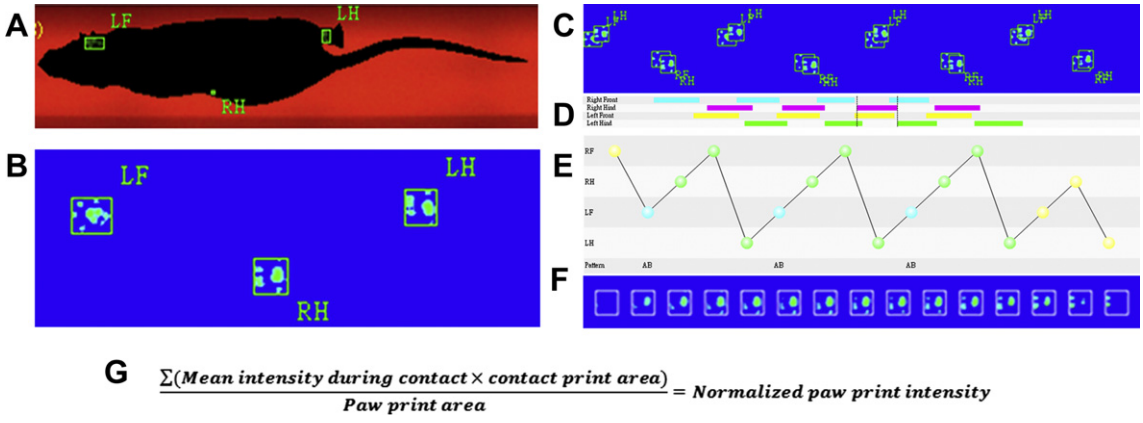


**Fig. 1.** Limb loading during walking is evaluated by capturing illuminated paw prints by Catwalk system (A). The paw prints are automatically classified after image capture (B). The resulting paw print patterns (C) are used to measure parameters such as print area, print intensity and swing duration for all four limbs. A run with steady speed has little variations in stance and swing durations as shown by the lengths of color bar in (D), which is qualified for further analysis. Normally rats walk in an alternate footfall pattern (E) and only the runs with this footfall pattern are included for calculation of LII. A paw print is composed of a series of contact prints (F) during stance phase. Paw print intensity is calculated as the integration of contact print intensities for all captured time frames during stance phase. Contact print intensity for every time frame is calculated by multiplying the mean intensity during contact with the contact print area (as shown in F). Normalized paw print intensity (G) is calculated by dividing the paw print intensity with paw print area (as shown in B), which is then used to calculate the target print ratio and anchor print ratio for consecutive pairs of front paws and hind paws respectively.

contact print intensities for all captured time frames during stance phase. Contact print intensity for every time frame was calculated by multiplying the mean intensity during contact with the contact print area (from the raw data output of the Catwalk software). The paw print intensity was normalized with paw print area to take account for the variations in the size differences in the left and right paws. The normalized paw print intensity was then used to calculate target print ratio and anchor print ratio for consecutive pairs of front paws and hind paws respectively [Fig. 1(G)]. Apart from decreased loading during stance phase, limb idleness during walking could be achieved by increasing the swing duration. Thus the swing duration ratio (RH/LH) also reflects limb idleness on target limb. Because target print ratio, anchor print ratio and swing duration ratio are three inter-dependent parameters representing different aspects of gait adaptation for an idled limb, a product of these three ratios will better reflect the extent of limb idleness regardless of the idling mechanisms, and we call this quantity LII. We have developed the calculation of LII with a pilot study on ACLT and sham-operated rats (data not shown). The result of the present study was a validation of the use of LII to measure pain-related gait changes in a rat OA model as compared to sham operation and untraumatized normal rats.

*Reversal test by buprenorphine injection*

After collection of gait data at 3 months and 6 months post-injury, reversal test by a single intra-peritoneal injection of buprenorphine (0.025 mg/kg body weight) was performed[9]. Gait analysis was performed again at 0.5 and 24 h after buprenorphrine administration.

*μCT analysis*

After the last session of gait analysis, the rats were euthanized by intra-peritoneal injection of overdose pentobarbital (20% w/v). The harvested knee joint segments were scanned with μCT (VivaCT 40, Scanco Medical AG, Brüttisellen, Switzerland) at a resolution of 35 μm. Analysis was performed by built-in software with optimized Gaussian filter (sigma: 0, support: 2) and threshold (>255). In brief, rectangular volume of interest in the dimensions of 2 mm (L) × 1 mm (W) × 0.5 mm (D) was put in the subchondral bone at medial and lateral tibial epiphysis[10]. Trabecular indices including bone volume/ tissue volume (BV/TV), connectivity density, trabecular thickness, trabecular number and trabecular separation were determined. The knee samples were then fixed in 10% buffered formalin solution overnight for subsequent histological processing.

*Histological examination*

The formalin-fixed knee samples were decalcified in 9% formic acid for 2 weeks. Frontal sectioning was performed to obtain six 5 μm sections at approximately 200 μm steps[11]. Hematoxylin and Eosin staining was performed for scoring of osteoarthritic changes according to the cartilage OA histopathology grading system from Osteoarthritis Research Society International (OARSI)[12]. In brief, the grade (0–6) and stage (0–4) of OA on the medial and lateral articular compartments (revealing both femoral and tibial surface in frontal sections) were scored by two-independent investigators in a single-blinded fashion. A semi-quantitative OARSI score was calculated by multiplying OA grade and OA stage, and the maximum OARSI score among four ROIs (medial/lateral side of femur/tibia) in the articular compartments in sampled frontal sections was considered as the most pathological regions for group comparisons. Toluidine blue staining was also performed to facilitate the examination of cartilage loss as one of the criteria of OARSI score.

*Statistical analysis*

Statistical analysis was done using Statistical Package for Social Science (SPSS) 16.0 (SPSS Inc, Chicago, USA). Because the ratio data follow a lognormal distribution, gait data were log-transformed for parametric tests. The coefficient of variation (CV) for LII measurement was calculated from the average of the variances of each set of repeated measurement (V), according to the equation $CV = (e^{V}-1)^{1/2}$ for log-transformed data. Repeated measure Analysis of Variance (ANOVA) was used to analyze the log-transformed gait data with respect to temporal changes/buprenorphine treatment (within-subject factor) and experimental groups (between-subject factor). Comparisons of gait data before and after buprenorphine treatment in each experimental group were performed by paired *t* test if the within-subject effect was statistically significant. For comparisons of end-point measurement at 6 months post-injury, μCT data were analyzed by one-way ANOVA with post-hoc Turkey's test after checking for data normal distribution; while gait data and OARSI scores were analyzed by non-parametric Kruskal–Wallis test, and post-hoc two-group comparison by Mann–Whitney test with Bonferroni correction if necessary. Correlation between LII and OARSI scores was performed by Spearman's rho test. Significant difference was determined at $P < 0.05$.

## Results

Among the 18 rats, 1 rat from Sham group died at 1 week post-injury due to post-surgical infection. Gait data for this rat were collected at pre-injury only.

*Gait changes in ACLT rat model*

The average of the variances of each set of repeated measurement for log-transformed LII was 0.018. The CV for LII was calculated as 13.3%, indicating acceptable reliability of the measurement. At pre-injury time point ($n = 18$), the mean LII was 0.97 with a range of 0.75–1.20. Although the between-subject effect (different experimental groups) on LII was not statistically significant (repeated measure ANOVA with between-subject effects, $P = 0.273$), the within-subject effects of time post-injury ($P = 0.042$) and time/group interaction ($P = 0.024$) was significant, indicating the temporal changes of LII were different among the experimental groups. Target print ratio, anchor print ratio and swing duration ratio did not show significant differences with respect to within-subject effects ($P = 0.100$, 0.816, 0.051, respectively) and between-subject effects ($P = 0.213$, 0.397, 0.201, respectively). Although there were obvious individual variations in the gait data, LII in ACLT group was significantly increased (Repeated measure ANOVA within-subject effect only, $P = 0.024$) until 6 months post-operation (Fig. 2), with all ACLT rats got higher LII as compared to pre-injury levels. One rat (rat54) in Sham group and two rats in Normal group (rat62, 63) also exhibited increased LII, but the temporal gait changes in Sham and Normal group were not significant ($P = 0.214$, 0.147, respectively). In ACLT group, the significant temporal gait change in LII was contributed mainly by an increased target print ratio ($P = 0.008$) and swing duration ratio ($P = 0.010$), but not by anchor print ratio ($P = 0.180$). When comparisons were made at 6 months post-operation by Kruskal–Wallis test, ACLT group exhibited a higher LII as compared to Sham and Normal groups ($P = 0.029$), while no significant difference was detected in target print ratio ($P = 0.081$), anchor print ratio ($P = 0.134$) and swing duration ratio ($P = 0.066$).

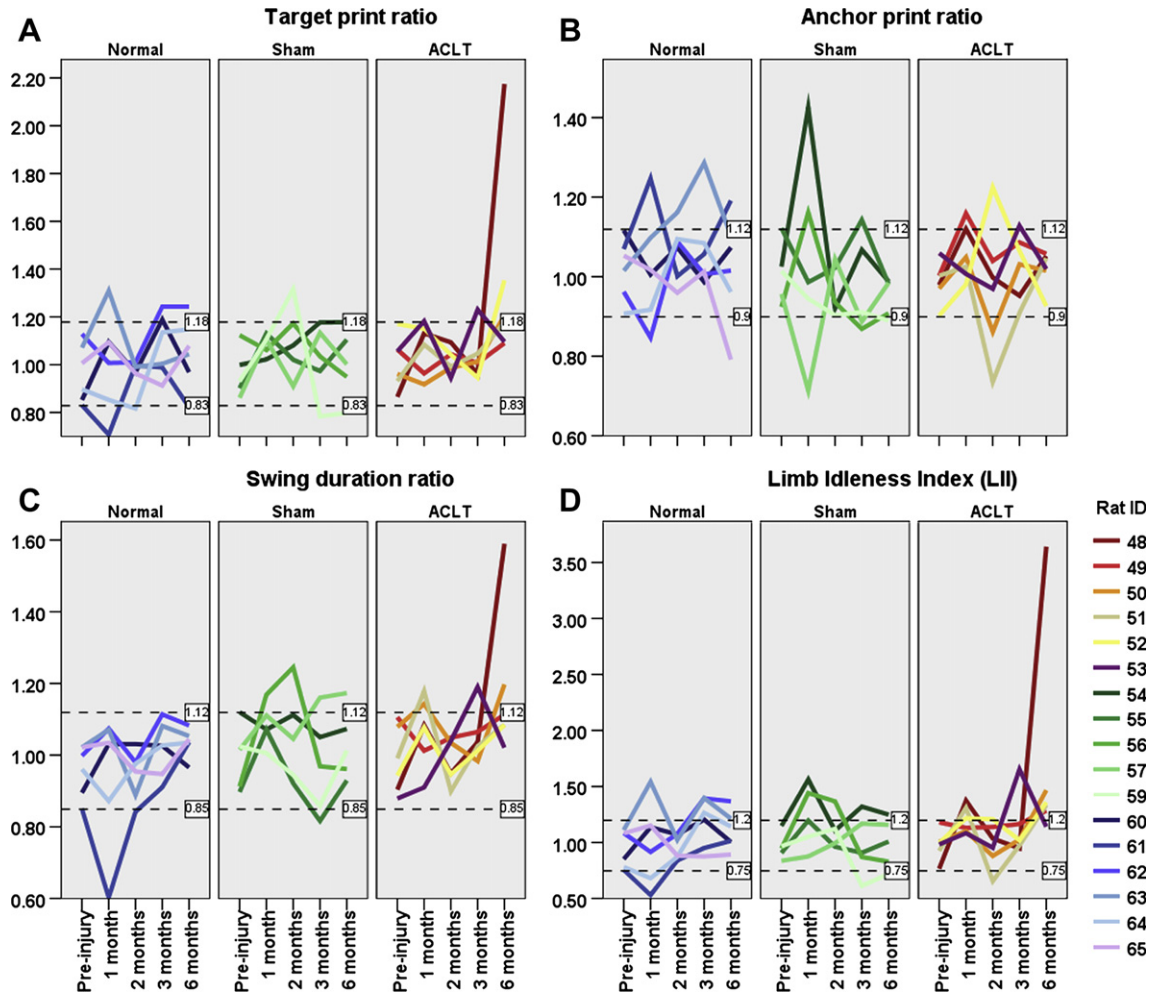


**Fig. 2.** The temporal changes of target print ratio (A), anchor print ratio (B), swing duration ratio (C) and LII (D) for individual rats are shown with reference lines indicating the normal range of the pre-injury levels.

*Detection of pain-related gait changes in response to analgesic treatment*

At 3 months post-injury, buprenorphine treatment did not significantly alter LII in all experimental groups (within-subject effect $P = 0.922$). At 6 months post-injury, buprenorphine treatment effectively decreased LII (within-subject effect $P = 0.004$) in ACLT group at 0.5 h after injection (paired $t$ test, $P = 0.041$), and LII was increased at 24 h after injection ($P = 0.014$). Sham and Normal groups did not respond to buprenorphine treatment (within-subject, $P = 0.750$, 0.555, respectively). The responses of individual rats to buprenorphine treatment with respect to different gait parameters were shown in Fig. 3. The LII of all ACLT rats responded to buprenorphine treatment, but target print ratio, anchor print ratio and swing duration ratio failed to reveal the analgesic response in some ACLT rats. The rats with high LII in Sham group (rat54) and Normal group (rat62, 63) also responded to buprenorphine treatment.

*μCT analysis of subchondral bone changes and correlation with LII*

In μCT measurements on tibial subchondral bone, BV/TV (Turkey's test, $P = 0.026$) and trabecular thickness ($P = 0.006$) in the lateral compartment was significantly lower in ACLT group as compared to Normal group, while the differences between Sham and ACLT was not statistically significant in these parameters ($P = 0.406$, 0.079, respectively). In the medial compartment, the trabecular thickness ($P = 0.018$) was also lower in ACLT group as compared to Normal group, but connectivity density was significantly higher ($P = 0.012$). The differences between Sham and ACLT were not statistically significant in these parameters ($P = 0.167$, 0.085, respectively). There was no significant difference between different groups with respect to trabecular number and trabecular separation (Table I).

*Histological scoring for OA changes and correlation with LII*

Histological scoring based on OARSI system showed that ACLT group exhibited a significantly higher maximum score among four ROIs (medial/lateral side of femur/tibia) on the target side, as compared to both Sham (Mann–Whitney test, $P = 0.005$) and Normal ($P = 0.006$) group (Table I); while the difference of OARSI scores on the contralateral side was not significant (ACLT vs Sham $P = 0.260$; ACLT vs Normal $P = 0.042$) (Bonferroni correction of Mann–Whitney $U$ test for three comparisons, statistical significance was accepted at $P < 0.0167$). The most pathological site in the target limb in ACLT group was the medial femur. It was characterized by complete erosion of hyaline cartilage with formation of sclerotic bone that involved 25–50% of articular surface in the observed sections [Fig. 4(A)]. The contralateral side in ACLT group

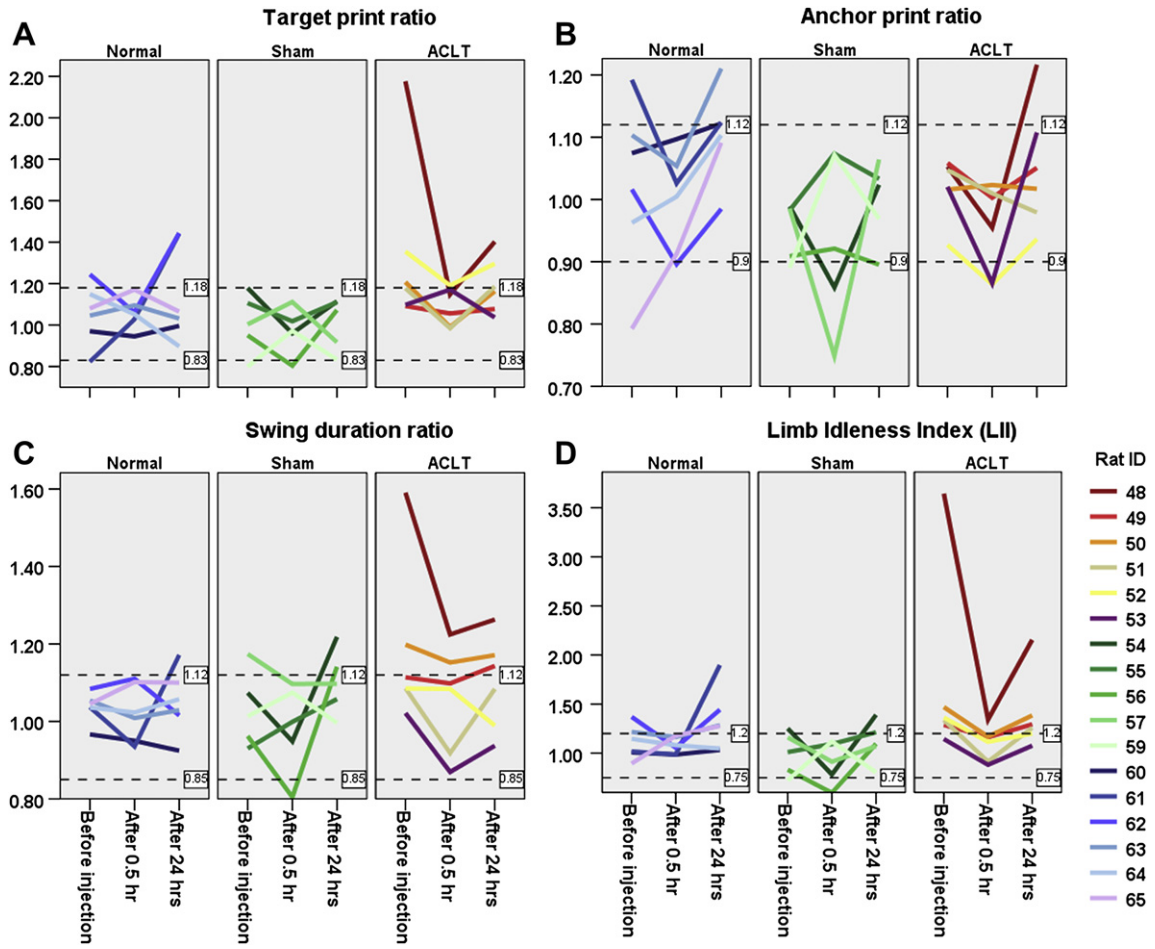


**Fig. 3.** The target print ratio (A), anchor print ratio (B), swing duration ratio (C) and LII (D) for individual rats at 6 months post-operation before buprenorphine injection, at 0.5 h and at 24 h after injection are shown with reference lines indicating the normal range of the pre-injury levels.

also exhibited significant osteoarthritic changes [Fig. 4(D)]. The OARSI scores in both target and contralateral sides ($P = 0.825$, 0.112, respectively) were not significantly different between Sham group and Normal group. Sham group showed pathological changes in the lateral tibia in both target and contralateral sides; which was characterized by mid zone excavation or abnormal cysts[13] in <10% of articular surface [Fig. 4(B and E)]. In Normal group, osteoarthritic change was detected in some rats. In rat64, mid zone excavation was found in medial tibia in the target side [Fig. 4(C)], while mild osteoarthritic change was noticed in the contralateral side [Fig. 4(F)]. The OARSI scores of target and contralateral sides of individual rats were shown in Fig. 5(A). The relationship of between LII and the side-to-side difference of OARSI scores (target to contralateral sides) was examined in a scatter plot [Fig. 5(B)]. Significant correlation was detected with ($r = 0.862$, $P < 0.001$) or without (Spearman's rho $r = 0.893$, $P < 0.001$) the outlier datum from rat48, in which tendon calcification was observed in the target side in addition to osteoarthritic changes [Fig. 5(C)]. The contralateral side of rat48 also got significant osteoarthritic changes without tendon calcification [Fig. 5(D)].

**Table I**
Results of μCT measurements and OARSI scores at 6 months post-injury

| Parameters | Side | Normal ($n = 6$) | Sham ($n = 5$) | ACLT ($n = 6$) | $P$ values |
|---|---|---|---|---|---|
| BV/TV | Medial | 0.454 (0.398–0.509) | 0.399 (0.345–0.454) | 0.386 (0.296–0.476) | 0.201 |
| | Lateral | 0.359 (0.302–0.416) | 0.303 (0.247–0.359) | 0.254 (0.173–0.335) | **0.033** |
| Connectivity density (1/mm$^3$) | Medial | 23.4 (13.4–33.5) | 30.2 (20.5–39.8) | 48.3 (29.4–67.2) | **0.014** |
| | Lateral | 40.4 (27.9–52.9) | 36.3 (23.7–48.9) | 52.7 (30.1–75.2) | 0.222 |
| Trabecular number (1/mm) | Medial | 8.44 (8.14–8.74) | 8.89 (7.83–9.95) | 8.48 (7.93–9.03) | 0.398 |
| | Lateral | 9.05 (8.47–9.62) | 8.55 (7.89–9.21) | 8.48 (7.90–9.06) | 0.185 |
| Trabecular thickness (mm) | Medial | 0.128 (0.115–0.141) | 0.120 (0.111–0.129) | 0.105 (0.090–0.121) | **0.022** |
| | Lateral | 0.104 (0.092–0.117) | 0.096 (0.082–0.110) | 0.079 (0.066–0.092) | **0.007** |
| Trabecular separation (mm) | Medial | 0.149 (0.136–0.163) | 0.155 (0.143–0.167) | 0.152 (0.139–0.1 | 0.709 |
| | Lateral | 0.161 (0.145–0.176) | 0.161 (0.148–0.173) | 0.163 (0.150–0.177) | 0.929 |
| Maximum OARSI score | Target | 3.5 (1–12) | 4 (0–4.5) | 18 (6–20) | **0.005** |
| | Contra-lateral | 1 (0–4) | 4 (1–4.5) | 4.5 (0–9) | 0.069 |

Data are presented as mean (lower and upper bounds of 95% confidence interval) for μCT data and median (minimum to maximum) for OARSI score. $P$ values for one-way ANOVA are shown for the μCT measurement; while $P$ values for Kruskal–Wallis test are shown for OARSI scores. Bold values signify $P < 0.05$.

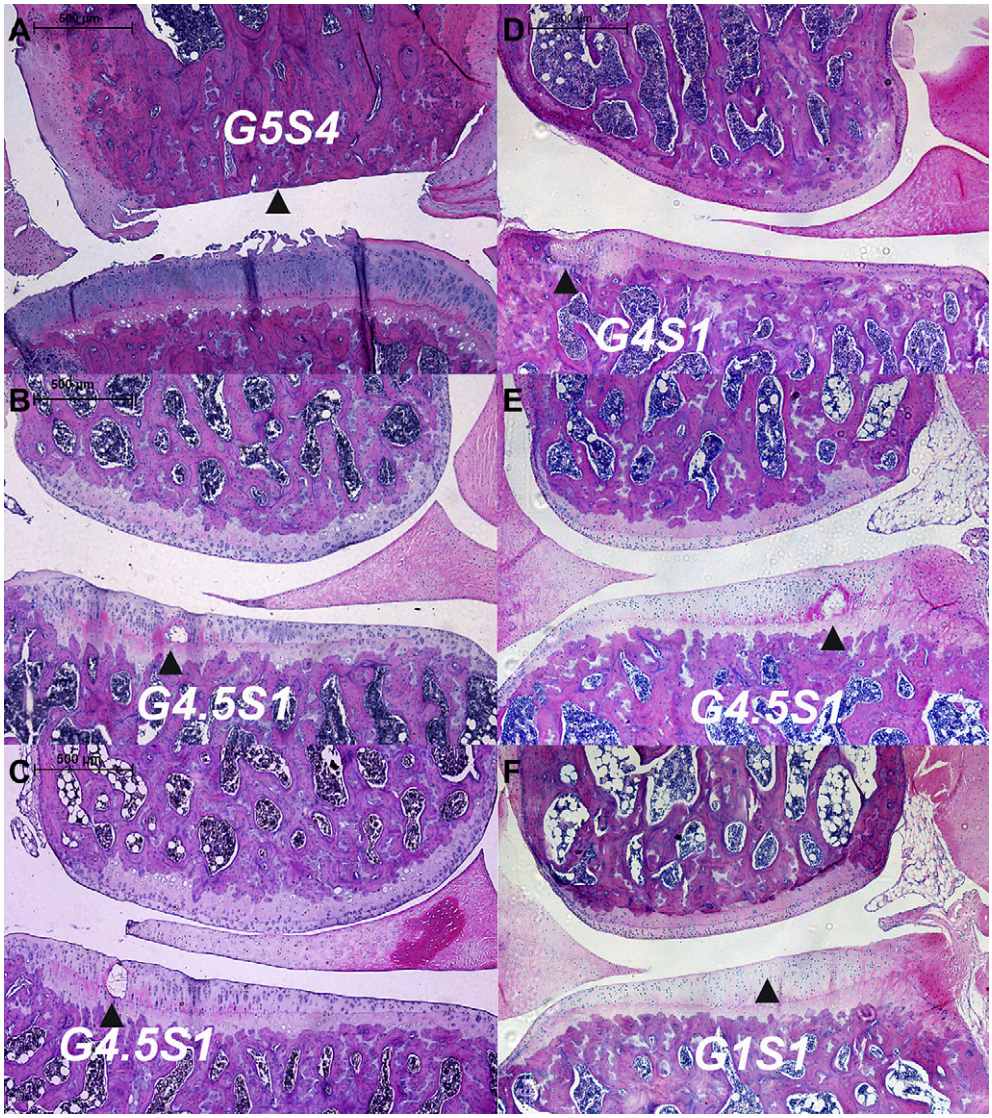


**Fig. 4.** H&E staining of frontal sections of the rat knees from ACLT (A, D), Sham (B, E) and Normal (C, F) groups (representative samples with median max OARSI scores on the target side) were shown. In ACLT rat (rat52), severe osteoarthritic changes with denudation was observed in the operate side (A); while mild superficial zone delamination was found in the contralateral knee (D). In Sham rat (rat55), mild cavity formation in a circumscribed cartilage volume (excavation) was noticed in the lateral tibia (B), and similar lesions were also observed in the contralateral side (E). In Normal rat (rat64), excavation was also observed in medial tibia (C), while mild osteoarthritic change was observed in the contralateral side (F). The grade and stage of OARSI scores are shown in the figures and the pathological sites are marked by ▲ (optical magnification: 50×).

## Discussion

Our results showed that LII is useful to reveal OA-related pain. The CV for LII is 13.3%, indicating acceptable reliability of the measurement. LII is in essence a measure of symmetry in walking gait. LII $>1$ means that the target limb was idled; in contrast, LII $<1$ reflects less activity in the contralateral limb. At pre-injury time point ($n = 18$), the mean LII was 0.97 with a range of 0.75–1.20. In the reversal test with buprenorphine injection, all rats with LII $\geq$1.20 were responsive to analgesic treatment, indicating limb idleness on the target sides was associated with pain. On the contrary, one rat from Sham group (rat59) with LII $\leq$0.75 indicated significant limb idling on the contralateral side. Rat59 also responded to buprenorphine in a reversed way as compared to other painful cases in target limbs (Fig. 3). Examination on histological samples from rat59 showed that the OARSI score in contralateral side (4.5) was higher than the target side (1). It indicates that rat59 might experience pain associated with osteoarthritic changes on the contralateral side. These observations suggest that LII is sensitive to detect the relative mechanical allodynia of both limbs. Thus it is necessary to consider both target and contralateral sides when we examine the relationship of LII and OA changes. The significant correlation between LII and the side-to-side difference of OARSI scores supports that LII is capable of detecting the relative severity of OA changes in both knees in the rat model of ACLT.

This is the first study measuring OA pain in a surgically induced OA animal model with a long follow-up (6 months), while all previous animal studies of OA pain were carried out in a relatively shorter duration (maximum follow-up ranging from 2 weeks[5] to 10 weeks[14]). In those studies with shorter follow-up time, pain due to acute joint inflammation rather than chronic arthritic changes was measured.[2] On the other hand, as compared to previous studies on rat ACLT model of knee OA,[15,16] our data showed a slower development of OA (symptomatic at 6 months post-operation), which might be due to the use of female rats (221.5 $\pm$ 11.9 g at pre-

A

| | Max OARSI target side | Max OARSI contralateral side |
|---|---|---|
| Rat48 | 16.5 | 8 |
| Rat49 | 6 | 0 |
| Rat50 | 18 | 4.5 |
| Rat51 | 20 | 4.5 |
| Rat52 | 20 | 4 |
| Rat53 | 18 | 9 |
| Rat54 | 4 | 0 |
| Rat55 | 4.5 | 4.5 |
| Rat56 | 4.5 | 4 |
| Rat57 | 4.5 | 2.5 |
| Rat59 | 1 | 4.5 |
| Rat60 | 2.5 | 0 |
| Rat61 | 1 | 0 |
| Rat62 | 12 | 1 |
| Rat63 | 5 | 1 |
| Rat64 | 4.5 | 1 |
| Rat65 | 1 | 4 |

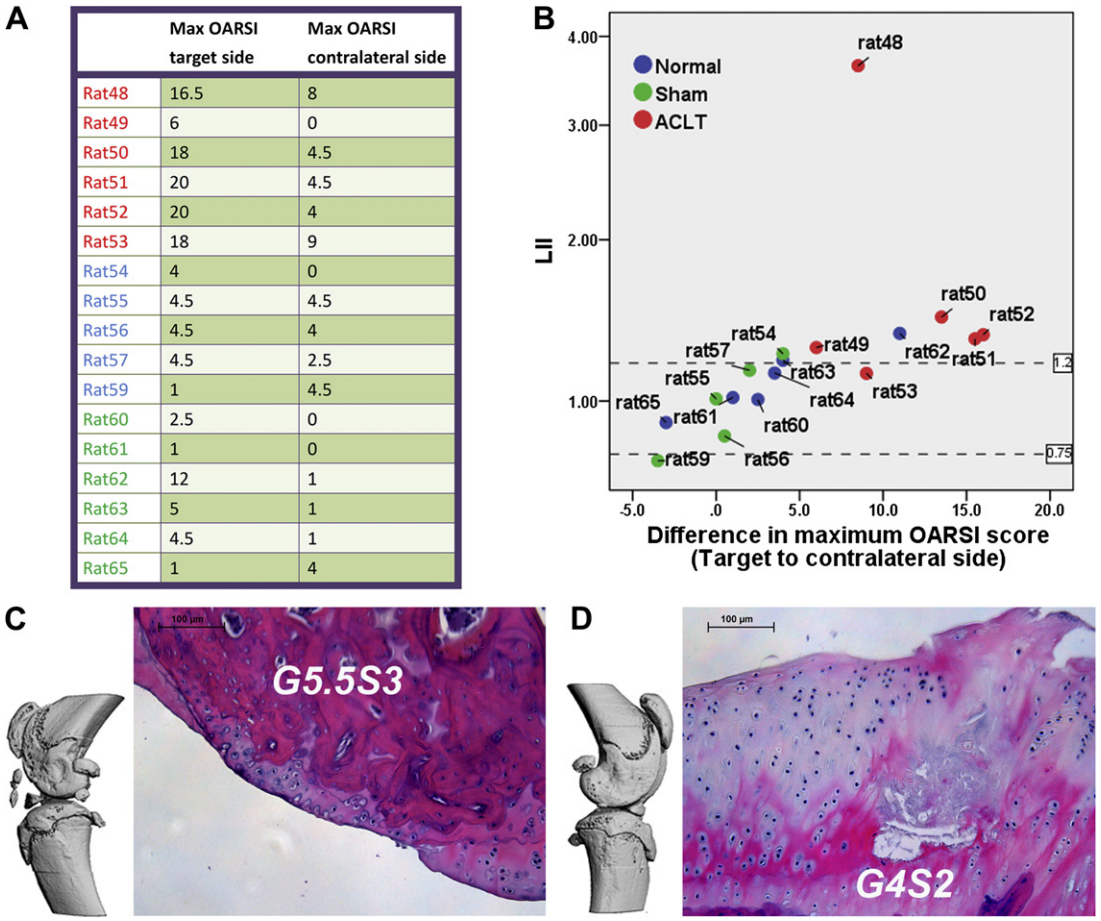


**Fig. 5.** Calcification of patellar tendon was detected in the operated side of rat48 (LII: 3.64) by µCT imaging in addition to osteoarthritic changes as shown in histological section (A), while no ectopic calcification was found in the contralateral side but significant osteoarthritic change was also observed (B). A scatter plot between LII and side-to-side difference in maximum OARSI score (C) suggested a positive correlation between pain-related gait adaptation and osteoarthritic changes. Reference lines are drawn to indicate the normal range of the pre-injury levels of LII. Maximum OARSI scores for individual rats were shown in (D).

injury and 286.4 ± 23.0 g at 6 months post-operation), as heavier and more active male rats (around 300 g at 8 weeks old) are used in previous studies[16]. Yet we also detected similar subchondral bone changes[10] and cartilage degeneration[15,16]. With such a long follow-up time, some spontaneous OA[17] were observed in the un-traumatized knees. The observed outcomes in ACLT group were probably contributed by both post-traumatic and primary OA. The significant intra-group variations in our data may affect the detection of between-subject group differences. However, these variations helped to explore the relationship of the painful responses as detected by gait adaptation and the osteoarthritic changes as revealed by histology. The correlation of LII to OARSI scores suggested that the gait adaptation to pain was associated with the OA-specific joint degeneration, with an outlier (rat48) which was also explainable by the co-existence of OA and tendon calcification. As abnormal LII was responsive to buprenorphine treatment, the use of LII in ACLT rat model may be helpful for the development of pain medication for OA patients, together with examination of OA changes by histology.

Gait analysis has been used to measure this movement-evoked pain in OA using Catwalk system[6]. All the built-in gait parameters did not show significant association with OA pain except for the percentage of ipsilateral paw intensity at standing, which is similar to the calculation of anchor print ratio and target print ratio in this study. We calculated mean integrated paw print intensity to estimate the extent of limb loading during ambulation, which might be more representative than maximum or mean print intensities at any time point during the stance phase (built-in gait parameters). As limb idleness can also result from decreasing time of loading (increasing paw elevation time) in addition to reducing loading during stance phase, LII presents a new parameter that integrates both mechanisms to avoid mechanical allodynia. The three component ratios of LII are chosen because they represent three different aspects to idle a target limb during walking, thus LII would be able to detect “limb idleness” when one of these ratios was increased. For example, rat53 would be regarded as “no change in limb idleness” if we accessed anchor print ratio and swing duration ratio; but with a higher target print ratio, we concluded that rat53 also experienced limb idleness on target limb at 6 months post-injury as revealed by LII (Fig. 2), which also responded to buprenorphine treatment (Fig. 3). Alternatively, false-positive cases of “limb idleness” detected by single ratio could be excluded when other ratios were changed in different directions. For example, rat55 got a high target print ratio but a low swing duration ratio, thus it would be difficult to judge whether rat55 was idling its target limb if we only based on single ratio. By combining these component ratios to yield LII, rat55 was still within the normal range and it did not respond to buprenorphine treatment (Fig. 3). This may explain why individual component ratios cannot detect the group difference at 6 months post-injury in contrast to LII, because the rats in one group may not use the same strategy to achieve limb idleness. Nevertheless, individual ratios should still be

presented along with LII in the gait pattern analysis to measure OA-related pain development in the ACLT model. If there is a switch of limb idling strategies or compensatory changes, evaluations on individual ratios and LII will provide more information.

There are several limitations for the use of LII to measure symptomatic OA in animal model. Firstly, it is only applicable for unilateral injury. Any unanticipated changes on the contralateral limb would have chance to confound the assessment of limb idleness, such as spontaneous development of primary OA. Buprenorphine-sensitive changes in LII may mean changes in pain levels in target side or contralateral side. Therefore, it is important to assess the status of contralateral side when LII is used to measure painful responses in animal model of unilateral injury. Secondly, although we detected significant correlations between LII and osteoarthritic structural changes, the mere co-existence of OA changes and gait changes at one single time point might not provide strong evidence for association. Longitudinal studies that include data from more time points would be more appropriate. Thirdly, LII only reveals activity-related pain, while pain in OA may have other elements which are not activity-related[18]. Thus LII may reveal only parts of the whole picture for the development of pain medication in animal model. Finally, other functional changes which are not related to pain may also affect limb idleness, for example, knee stiffness, extension deficit or altered limb coordination may lead to changes in gait parameters and affect symmetry in walking gait. Although LII cannot differentiate different painful conditions (OA vs tendinopathies) that trigger limb idleness, it is possible to extend the use of LII for evaluation of functional recovery as a restoration of gait symmetry after unilateral injury, such as tendon injuries, muscle weakness or some neural injuries. Further exploration on the use of LII to monitor limb functional recovery in animal models is possible.

## Conclusion

LII is a useful parameter to measure OA-related knee pain in a rat model.

## Author contributions

Experimental design and intellectual input: SCF, KMC, LKH.
Data acquisition, analysis, manuscript writing: SCF, YCC.
Final approval of manuscript: All authors.

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## Conflicts of interest

All authors declare no conflicts of interest.

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