EXTENDED REPORT

Thermal signature analysis as a novel method for evaluating inflammatory arthritis activity

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Objective: To examine the potential usefulness of a novel thermal imaging technique to evaluate and monitor inflammatory arthritis activity in small joints using rat models, and to determine whether thermal changes can be used to detect preclinical stages of synovitis.

Methods: Three different rat strains were studied in a model of inflammatory arthritis of the ankle induced by an intra-articular (IA) injection of complete Freund's adjuvant (CFA), compared with the contralateral ankle injected with normal saline. Arthritis activity and severity scores, ankle diameters, pain related posture scores, and thermal images were obtained at 10 different times between 0 h (before induction) and day 7. The pristane induced arthritis (PIA) model was used to study preclinical synovitis. Thermal images were obtained at each time point using the TSA ImagIR system and were digitally analysed.

Results: Rats developed similar ankle arthritis detected six hours after the IA injection of CFA, which persisted for seven days. All ankle clinical indices, including arthritis activity and severity scores, correlated significantly with ankle thermal imaging changes in the monoarthritis model ($p < 0.003$). No thermal imaging changes were detected in preclinical stages of PIA. However, PIA onset coincided with increased ankle thermal signature.

Conclusions: Thermal measurements correlated significantly with arthritis activity and severity indices. The technique was highly sensitive and could measure directly two cardinal signs of inflammation (warmth and oedema, based on ankle diameter) in an area (ankle) that is less than half the size of a human interphalangeal joint, suggesting a potential use in drug trials or clinical practice.

Rheumatoid arthritis activity and severity in clinical practice and in drug trials are typically evaluated using composite scoring systems such as those developed by the American College of Rheumatology (ACR),¹ Paulus *et al*,² and Prevoo *et al* (the disease activity score, DAS).³ These arthritis scoring systems include laboratory indices such as levels of C reactive protein and the erythrocyte sedimentation rate, global physician and patient assessment of disease, and investigator dependent scoring of the number (and degree) of swollen and tender joints. While standardised, these clinical scoring systems have obvious inter-reader variability. An objective and consistent imaging technology would be very helpful for more consistent interinstitutional prospective evaluations of arthritis activity changes in drug trials, as well as in clinical practice.

There is histological evidence of synovitis before the onset of clinical arthritis, both in rheumatoid arthritis in humans⁴ and in collagen induced⁵ and pristane induced arthritis (PIA) in rats⁶ (Brenner *et al*, unpublished observations). Studying synovial tissue obtained at these preclinical stages during disease development could help identify novel pathways involved in disease pathogenesis. However, there are no objective indices for determining which joints have preclinical synovitis in order to guide closed needle or arthroscopic synovial biopsies for functional or gene expression studies.

Available imaging technologies such as magnetic resonance imaging (MRI)^{7–10} and scintigraphy^{8–11, 12} can detect inflammation and joint swelling but are expensive and not practical for repetitive use. Additionally, synovitis-like changes have been described in up to 9% of normal metacarpophalangeal joints studied with MRI,¹³ but the specificity of those findings remains to be determined. The use of articular ultrasound appears promising in the presence of joint swelling,^{8–10, 14} but has not been studied in preclinical synovitis. Additionally, articular ultrasonography is highly reader dependent and has not yet been standardised.

Warmth is a cardinal feature of inflammation and may be objectively measured by the use of infrared based thermography.¹⁵ Several studies have shown a good correlation between thermographic findings and disease activity indices in large but not small joints^{16–18} in patients with rheumatoid arthritis, thus limiting the potential value of these techniques in therapeutic trials or clinical practice. However, recent advancements both in infrared imaging and analytical technologies now allow for more precise temperature measurements in smaller surface areas.

We were interested in identifying novel and convenient imaging methods for evaluating and monitoring inflammation in rheumatoid joints as an objective index to measure disease activity, for use in both drug trials and clinical practice. Moreover, we were interested in identifying non-invasive methods for detecting the very early preclinical stages during the pathogenesis of arthritis. We therefore designed a pilot study in rats to test whether a novel thermal imaging system could be a useful tool for evaluating disease activity (and arthritis severity) in a surface area that is smaller than a typical human proximal interphalangeal joint (the rat ankle), and whether the thermal signature could serve as a biomarker for preclinical synovitis. Two rat models of arthritis were used in these studies.

METHODS

Rats

Eight to 12 week old female DA, ACI, and F344 rats were purchased from Harlan (Indianapolis, Indiana, USA) and used in all experiments. All the work done with rats was reviewed and approved by the North Shore–LIJ Research Institute Institutional Animal Care and Use Committee.

Abbreviations: CFA, complete Freund's adjuvant; HMF, high power magnification field; PIA, pristane induced arthritis; PRP, pain related posture

Figure 1 Thermal imaging work station and monoarthritis rats. (A) Work station with anaesthesia chamber (ac) and thermal imager acquisition station (as). The open compartment has a sliding platform where anaesthetised rats are positioned for image acquisition. (B) Rat with left ankle CFA induced monoarthritis (arrowhead) and normal saline injected right ankle. (C) Thermogenic image of a rat with left monoarthritis revealing increased temperature (bright yellow region labelled 2) while the saline injected right ankle has a temperature not differing from the tail.

Monoarthritis model

Induction of monoarthritis

This is a localised model of arthritis that begins within two to six hours after induction. This model was used to study the correlation between arthritis activity/severity and the thermal imaging data. Each rat received a single intra-articular left ankle injection of 40 µl of a mixture of Complete Freund's Adjuvant (CFA; Difco Inc, Dallas, Texas, USA) mixed with normal saline at 1:1,^{19 20} and compared with a saline injection in the right ankle.

Scoring indices used in the monoarthritis model

All variables outlined below were acquired at time 0 h, 6 h, 12 h, 24 h, and then once a day, until day 7.

- **Bilateral ankle diameters (latero-lateral and antero-posterior):** Diameters were measured with a digital caliper to the nearest 0.01 mm.
- **Clinical arthritis activity/severity scoring system:** Both ankles and mid-foot joints were scored in a range of 0–4 according to joint size and the ability to support weight, where 0 = no joint swelling, 1 = mild swelling, 2 = moderate swelling, 3 = severe (maximal) swelling, 4 = severe swelling plus non-weight bearing. This is an ankle and mid-foot focused version of a scoring system described in detail below, and commonly used to evaluate systemic autoimmune arthritis.^{21–23}
- **Pain related posture (PRP):** The posture of each animal's hind limb was scored according to a pain related behavioural scale (spontaneous pain rating score) (0–5), where 0 = normal, 1 = curling of the toes, 2 = eversion of the paw, 3 = partial weight bearing, 4 = non-weight

bearing and guarding, and 5 = avoidance of any contact with the hind limb.²⁴

Systemic autoimmune arthritis model (pristane induced arthritis)

Induction of pristane induced arthritis

Pristane induced arthritis (PIA) is a model of systemic autoimmune arthritis with disease onset typically 14 days after induction. DA rats are highly susceptible to PIA, with an incidence of arthritis of nearly 100%. Because of this predictable course, PIA was used to determine whether changes in the thermal imaging signature occurred before the onset of clinical disease, reflecting preclinical synovitis, and whether these changes could be useful markers to predict histological findings. DA rats were injected with 150 µl of pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich Chemical Co, Milwaukee, Wisconsin, USA).^{6 25} The dose was divided into two intradermal injections at the base of the tail.

Scoring of systemic polyarticular arthritis

Rats were scored daily between days 0 to 16 according to a well established scoring system.^{21–23} Specifically, the arthritis scoring system evaluates individual joints and weights the arthritis severity by joint size as follows: (1) wrist, mid-forepaw, ankle, and mid-foot joints were scored 0 to 4, where 0 = no swelling, 1 = mild swelling, 2 = moderate swelling, 3 = severe swelling, 4 = severe swelling and non-weight bearing; (2) the presence of arthritis was assessed in each of the three joints in the second to the fifth digits (two interphalangeal plus the metatarsophalangeal or metacarpophalangeal joints), where 0 = swelling absent, 1 = swelling present. The total score for each extremity was calculated by adding the scores of the individual joints. The maximum score for each extremity is therefore 20, and the maximum total joint score per rat is 80.

Figure 2 Arthritis activity and severity scores and thermogenic images in the monoarthritis model during a seven day observation period. (A) Arthritis scores (mean with SEM) of the left ankle of the three strains. Medians were compared with analysis of variance (ANOVA) on ranks. There were no significant differences between the three strains. (B) Temperature (°C) in DA, ACI, and F344 left (CFA injected) minus right (saline injected) ankles (mean with SEM) during the seven day observation period. Median temperature changes were compared among strains with ANOVA on ranks, and $p < 0.05$ was regarded as significant. *Significant difference between ACI and DA, and between ACI and F344; **significant difference between ACI and F344 only; †significant difference between ACI and DA only.

Thermal imaging acquisition and digital analyses

The Seahorse Bioscience TSA ImagIR Thermal Imaging system (Seahorse Bioscience, N Billerica, Massachusetts, USA) was used in accordance with the manufacturer's procedures to image rats at every time point (fig 1A). The TSA ImagIR employs a platinum silicide 256×256 pixel detector array filtered to be sensitive to infrared radiation in the $3\text{--}5\text{ }\mu\text{m}$ wavelength. This sensor returns signals to the processor that are proportional to the photons of infrared radiation detected. These data, combined with thermal reference devices, allow the instrument to detect the temperatures of all objects in the field of view simultaneously and in real time.^{26, 27}

Rats had anaesthesia induction in a sealed acrylic chamber by administration of 5% isoflurane in oxygen flowing at 2 l/min for approximately one minute. Following induction, rats were transferred from the acrylic induction chamber to the ImagIR imaging chamber's temperature controlled anaesthesia delivery module, where they received 2–3% maintenance isoflurane in oxygen. The rats' body temperatures were maintained at $36\text{--}37^\circ\text{C}$ using a temperature controlled imaging platform with a constant temperature of 35°C (accurate to $\pm 0.1^\circ\text{C}$). Legs were positioned in flexion and external rotation to expose the medial part of each ankle to

Table 1 Histological scores of left ankles collected seven days after CFA injection

	DA (n = 5)	F344 (n = 5)	ACI (n = 5)
Inflammatory infiltrate (0–3)	2.04 (0.35)	2.56 (0.17)	2.52 (0.16)
Synovial hyperplasia (0–3)	1.40 (0.68)	2.60 (0.24)	2.60 (0.40)
Pannus (0–3)	2.00 (0.32)	1.40 (0.24)	2.00 (0.32)
Fibrosis (0–3)	0.80 (0.37)	0.00 (0.00)	0.00 (0.00)
Vessels/HMF	7.28 (1.28)	7.60 (2.37)	8.80 (0.09)
Cartilage erosions (0–3)	0.60 (0.40)	1.20 (0.20)	1.20 (0.20)
Bone erosions (0–3)	1.40 (0.60)	1.80 (0.37)	2.20 (0.37)

Five rats per strain were randomly selected for histological analysis. Values are means (SEM). Differences were not statistically significant. CFA, complete Freund's adjuvant; HMF, high power magnification field.

the infrared sensor (fig 1B and 1C). Rats were imaged for a total of 10 minutes, with images captured every 15 seconds. Each captured image was generated by averaging 16 frames of video rate image data together to produce a clear still image. Both anaesthesia and the thermal imaging acquisition were done in a room with a constant temperature of $21\text{--}23^\circ\text{C}$.

The thermal imaging data were extracted from saved images using Animal Corral Software version 4.0.0 (Seahorse Bioscience). First, a region of interest (ROI) was drawn to delineate left and right ankles. The typical limits for the ROI were the ankle fur line (upper limit) and the midpoint of the mid-foot. The average temperature of all pixels within each ROI in each image was calculated and recorded automatically. Data quality at the beginning of each 10 minute imaging period was similar to that obtained at the middle and end of each imaging period. Additionally, it was determined that the initial images were the best reflection of pre-anaesthetic body and ankle temperatures (data not shown). Thus only data from the initial images were used, making the time to image each animal less than one minute and so minimising any anaesthetic induced effect on body temperature regulation.

Thermal images were obtained at 0 h (preinjection), 6 h, 12 h, 24 h, and daily on days 2 to 7 after intra-articular CFA (or saline) administration (monoarthritis model), and daily from day 0 to day 16 in PIA. In order to correct for each individual rat's own peripheral limb temperature, left ankle (CFA) thermal measurements were corrected for the right ankle (saline) measurements (left ankle minus right ankle) in the monoarthritis studies. In PIA, as both ankles are typically involved, thermal measurements were adjusted for the core temperature obtained with a rectal probe.

Histology and histological scoring

Hind paws were fixed in 10% formaldehyde at the end of the monoarthritis observation period (day 7). Rats studied for PIA were killed on days 7, 11, and 16 (four rats per time point), and their hind paws fixed as described above. Paws were then decalcified with a solution containing hydrochloric acid and 0.1 M EDTA (Cal-Ex, Fisher Scientific, Fairlawn, New Jersey, USA). Tissues were embedded in paraffin, sectioned, and stained with haematoxylin-eosin. We used a recently described comprehensive histological scoring system.^{28, 29} Briefly, tibio-talar, talus-calcaneal, and mid-foot joints were histologically scored for the following variables:

- **Synovial inflammation.** Five high power magnification fields (HMF) were scored for the percentage of infiltrating mononuclear cells as follows: 0 = absent, 1 = mild (1–10%), 2 = moderate (11–50%), 3 = severe (51–100%). The mean of the five HMF was used for analysis.

Table 2 Pearson's correlation coefficient matrix relating thermal signature and clinical indices in the intra-articular CFA monoarthritis model (left ankle)

		Arthritis activity and severity score			Ankle diameters	
		L ankle	L ankle + L mid-foot	L limb PRP	Lateral, L–R	AP, L–R
Ankle temperature (L–R)	r:	0.602	0.597	0.522	0.643	0.569
	p:	0.000427	0.000502	0.00309	0.000128	0.00104
L ankle arthritis activity and severity score	r:		0.985	0.834	0.908	0.902
	p:		4.16×10^{-23}	1×10^{-8}	4.47×10^{-12}	1×10^{-11}
L ankle+ L mid-foot arthritis activity and severity score	r:			0.875	0.857	0.898
	p:			2.63×10^{-10}	1.5×10^{-9}	1.7×10^{-11}
L limb pain related posture (PRP)	r:				0.687	0.819
	p:				0.0000272	3×10^{-8}
Ankle lateral diameter (L–R)	r:					0.892
	P:					3.9×10^{-11}

Thirty rats were studied (10 per strain). Correlation results include two values: correlation coefficient (*r*) and the *p* value. Data from 10 different time points (day 0, 6 h, 12 h, 24 h, and days 2–7) were included in the analyses.

CFA, complete Freund's adjuvant; L, left; L–R, left minus right (see text for definitions); PRP, pain related posture; R, right.

- *Synovial hyperplasia*. 0 = absent, 1 = mild (5–10 layers), 2 = moderate (11–20 layers), 3 = severe (>20 layers).
- *Extension of pannus formation*, based on the reader's impression. 0 = absent, 1 = mild, 2 = moderate, 3 = severe.
- *Synovial fibrosis*. 0 = absent, 1 = mild (1–10%), 2 = moderate (11–50%), 3 = severe (51–100%).
- *Synovial vascularity* (angiogenesis). The number of vessels was counted in five HMF of synovial tissue, and the mean used for analysis.
- *Cartilage erosions*. Percentage of the cartilage surface that was eroded: 0 = absent, 1 = mild (1–10%), 2 = moderate (11–50%), 3 = severe (51–100%).
- *Bone erosions*. 0 = none, 1 = minor (observed only at HMF), 2 = moderate (observed at low magnification), 3 = severe (transcortical).

Statistical analyses

Medians were compared with the Mann–Whitney rank sum test or with analysis of variance (ANOVA) on ranks with a pairwise multiple comparison procedure (Dunn's method). Variables were correlated with the Pearson's correlation coefficient. A probability (*p*) value of 0.05 or less was regarded as significant. All statistical analyses were done with SigmaStat 3.0 (SPSS, Chicago, Illinois, USA).

RESULTS

Thermal imaging signature v arthritis activity and severity

All DA, ACI, and F344 rats injected intra-articularly with CFA developed similar clinical monoarthritis which was detectable at six hours post-injection and reached its peak between days 1 and 3 (fig 2A). The arthritis scores of these three strains were not significantly different in any of the studied time points (fig 2A). None of the rats developed arthritis in the saline injected joints.

Left ankle thermal imaging readings (arthritic) were adjusted for the right ankle thermal readings (non-arthritic) for each individual rat (left minus right). Non-adjusted and adjusted temperatures consistently increased in arthritic ankles in all three strains (fig 2B). DA and F344 temperatures increased significantly more than ACI temperatures from 12 h (0.5 day) until day 3 (fig 2B). DA and ACI thermal readings remained significantly different from day 5 to day 7. These interstrain arthritic ankle temperature variations did

not translate into differences in arthritis activity and severity scores (fig 2A), or into differences in synovial inflammatory infiltration, synovial hyperplasia, synovial vascularisation, or cartilage and bone erosions (table 1).

The thermal imaging readings were significantly correlated with the arthritis activity and severity clinical scores, joint diameter, and PRP clinical scores ($p < 0.001$, Pearson's correlation coefficient matrix, table 2).

Thermal imaging signature in preclinical stages in PIA

We studied PIA in 12 female DA rats to determine whether daily thermal imaging readings changed in preclinical stages, and whether those changes could predict histological abnormalities. Groups of four rats were killed on days 7, 11, and 16 after the induction of PIA, and their paws prepared for histology. While histological abnormalities were detectable at day 11 post-induction, including inflammatory infiltration and synovial hyperplasia, the thermal signature did not change significantly before the onset of clinical arthritis (data not shown).

Thermal imaging signature correlated with arthritis severity scores in the PIA

Four rats used in the "preclinical stage" experiment described above with PIA developed clinical evidence of ankle arthritis between days 10 and 16. Despite the small number of rats there was a significant correlation between ankle arthritis scores and thermal signatures ($p < 0.001$).

DISCUSSION

We were interested in identifying novel, convenient, non-invasive, and inexpensive imaging methods to quantify joint inflammation in rheumatoid arthritis. New infrared based technologies that allow a more precise quantification of temperature variations in small surface areas, such as the interphalangeal joints, appeared promising. While older versions of infrared based thermographic analyses have been used to evaluate rheumatoid arthritis activity, none of those studies could reliably assess the hand joints, and typically the analyses were limited to knees, elbows, and wrists.^{16–18} In the present study we used a highly sensitive thermal imaging system that could reproducibly detect temperature variations in the rat ankle joint, an area that is less than half the size of a human proximal interphalangeal joint. Moreover, thermal variations correlated well with well established rodent

clinical arthritis activity and severity scores, as well as with changes in the articular diameter and pain posture in a monoarthritis model. Thermal imaging could also be used to measure joint diameter as an indicator of joint swelling/oedema (data not shown). These results suggest that the novel thermal imaging techniques could provide useful and objective measurements of joint inflammation based on two cardinal inflammatory signs—joint swelling/oedema (joint diameters) and warmth. The objective and prospective thermal imaging documentation could be helpful in drug trials as well as in clinical practice.

All three strains developed significant and similar histological abnormalities, showing that changes in the thermal signature predicted increased histological severity. There were interstrain variations in the magnitude of the thermal signature variation, with ACI rats having a less pronounced temperature increase than DA and F344 rats. However, there was a significant correlation between the thermal signature and clinical disease severity in all three strains. The interstrain difference in the magnitude of the thermal variations did not translate into significant clinical or histological differences in arthritis severity, and its relevance remains to be determined.

We were also interested in studying very early stages in the pathogenesis of synovitis and wanted to determine whether the thermal imaging analyses could identify preclinical stages of synovitis. The rapid and abrupt onset of arthritis in the monoarthritis model following the intra-articular injection of CFA does not allow any time for the development of preclinical synovitis. We therefore chose to study a systemic model of autoimmune arthritis, PIA, which is known to develop typically over a 14 day period. Ankle thermal signature changes were detected after the onset of clinical disease, but not before it. Ankle thermal signatures correlated with clinical arthritis severity scores.

Histological analyses of ankle joints obtained from rats with PIA have shown that synovial hyperplasia, synovial infiltration with mononuclear cells, and fibrin deposition can be detected in the synovial tissues around day 11, before the onset of clinical disease,⁶ and that increased numbers of neutrophils are present around the time of disease onset (Brenner M *et al*, unpublished observations). However, the early preclinical histological abnormalities did not correlate with any thermal imaging signature changes (data not shown). The association of disease onset with increased numbers of neutrophils in the synovial fluid and tissue in PIA suggests that these cells and their products are directly or indirectly central to the regulation of the articular thermal signature. As a result, thermal signature analysis did not appear to be helpful in detecting preclinical stages of synovitis.

Conclusions

The new generation of thermal imaging techniques has significantly improved resolution and temperature sensitivity, and generate reproducible measurements of surface areas smaller than an interphalangeal joint. We consider that this novel, less reader dependent, and non-invasive technology has the potential to become a useful and objective tool for measuring inflammatory arthritis activity, and for generating digital data that can be stored and analysed in both drug trials and clinical practice.

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