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Use of Ultrasound-Guided Small Joint Biopsy to Evaluate the Histopathologic Response to Rheumatoid Arthritis Therapy

Recommendations for Application to Clinical Trials

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Objective. **To examine in a cohort of rheumatoid arthritis (RA) patients undergoing serial ultrasound (US)–guided biopsies of small joints in the context of clinical trials whether sufficient synovial tissue could be obtained at both baseline and second biopsy to: 1) accurately evaluate the synovial immune phenotype, 2) permit adequate RNA extraction to determine molecular signatures, and 3) sensitively detect change in the number of synovial sublining macrophages (CD68+) following effective therapy.**

Methods. **Synovial samples from RA patients undergoing US-guided biopsy of small joints as part of 2 clinical trials (Barts Early Arthritis Cohort [n = 18] and the Clinical and Pathological Response to Certolizumab Pegol (CLIP-Cert) study [n = 17]) were examined, and the quality and quantity of histologic samples and RNA extracted per joint were determined and compared to synovial thickness and power Doppler scores determined by US before biopsy. Modulation of the number of CD68+ sublining macrophages was correlated with clinical response to treatment.**

Results. **Good quality synovial tissue that accurately reflected the synovial immune phenotype of the total joint was obtained in 80% of US-guided procedures when synovial thickness (higher than grade 2) was documented before biopsy. In 100% of the procedures, sufficient RNA was extracted to permit molecular analysis. There was a significant correlation between change in CD68+ sublining macrophage number and clinical response to treatment.**

Conclusion. **This study provides minimum standards for sample retrieval for small joint biopsy. Furthermore, our findings confirm the clinical utility of the procedure in the largest reported cohort of US-guided small joint biopsies. The demonstration that small joint synovial tissue can be readily accessed by a technically simple, minimally invasive procedure is likely to facilitate critical advancements in the knowledge of RA pathobiology and personalized health care.**

The examination of synovial tissue has advanced the understanding of rheumatoid arthritis (RA) pathophysiology to an unprecedented level and has led to progress such as the development of targeted biologic therapies (1,2). It is recognized that small joint involvement is often predominant in early RA and, moreover, that patients with large joint involvement are likely to have more severe disease (3), making the acquisition of synovial tissue from small joints critical to expanding our understanding of disease pathobiology in relationship to disease progression and response to therapy (4).

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Table 1. Demographic characteristics of the patients with RA whose arthroscopic or arthroplastic samples were used for validation*

Patient	Joint	Procedure	Age, years	CCP status	RF status	Erosive	No. of blocks	Total no. of slides examined
1	Wrist	Arthroscopic	80	Positive	Positive	Yes	4	24
2	Wrist	Arthroscopic	80	Positive	Positive	Yes	3	18
3	Ankle	Arthroplastic	72	Negative	Negative	Yes	5	30
4	Wrist	Arthroscopic	39	Positive	Positive	Yes	4	24
5	MCP	Arthroplastic	47	NA	Positive	Yes	5	48
6	Wrist	Arthroplastic	44	NA	Positive	Yes	8	82

* Samples were used for validation of the quantity of synovial tissue required to obtain a representative image of immunohistochemical cell infiltration. RA = rheumatoid arthritis; CCP = cyclic citrullinated peptide; RF = rheumatoid factor; MCP = metacarpophalangeal; NA = not available.

However, in a clinical trial setting an arthroscopic approach to synovial biopsy is currently recommended as the gold standard technique for tissue acquisition (5), limiting recruitment of patients to those with only large joint involvement and to the relatively small number of centers where this technique is available. Though access to small joints can also be achieved by small-bore arthroscopy and blind needle biopsy (6), the former requires extensive training and is an invasive procedure, while the latter is less consistent in noninflamed joints and has primarily been used in diagnostic cohorts (7).

Thus, the development of a reliable, minimally invasive, and well-tolerated small joint biopsy technique remains an important goal. Synovial biopsy of small joints using ultrasound (US) guidance has been reported (8), and recently, a modification of this approach, using a Quick-Core biopsy needle (Cook Medical) under US guidance, was applied to small and large joint biopsy procedures in an early arthritis cohort (9). However, the performance of this technique in a clinical trial setting in RA patients undergoing serial biopsies of small joints following therapeutic intervention, when the degree of synovitis may be reduced dramatically, has not been established.

Therefore, the aim of this study was to examine the performance of serial US-guided needle synovial biopsies of small joints in 2 cohorts of patients with early or established RA who were treated with standard disease-modifying antirheumatic drugs (DMARDs) or a tumor necrosis factor inhibitor, respectively. The main objective was to determine whether sufficient synovial tissue (of sufficient quality) could be obtained both at baseline and at second biopsy in order to: 1) accurately examine the inflammatory cell infiltrate by standard immunohistochemical parameters, 2) enable adequate RNA extraction to determine molecular signatures, and 3) sensitively detect change in the number of synovial sublining macrophages (CD68+), a biomarker validated in arthroscopic biopsy samples for use in clinical trials, as an indication of response to therapy (10).

PATIENTS AND METHODS

Patients. Synovial samples from 6 small joints obtained either arthroscopically or following arthroplasty from patients with RA (according to the American College of Rheumatology 1987 criteria [11]) were used to standardize the minimum quantity of synovial tissue required to obtain a representative image of immunohistochemical cell infiltration (Table 1).

In order to determine whether sufficient synovial tissue could be obtained in patients recruited to clinical trials involving serial US-guided needle synovial biopsy of small joints, patients recruited to 2 observational clinical studies within Barts Health NHS Trust were included in the analysis. The studies are summarized in Table 2. Briefly, consecutive patients recruited to the Barts Early Arthritis Cohort (BEAC) (disease duration <12 months) all underwent a baseline synovial biopsy before beginning treatment and, following standard DMARD therapy, a second biopsy 6 months later. In addition, consecutive patients with DMARD-resistant severe active RA who were naive for biologic agents (Disease Activity Score in 28 joints [DAS28] >5.1) (12) were recruited to a second study, Clinical and Pathological Response to Certolizumab Pegol (CLIP-Cert). These patients underwent both a baseline biopsy and a second biopsy at 3 months of followup after treatment with certolizumab pegol and methotrexate. DAS28 scores were recorded at baseline and at 3 months (in the CLIP-Cert study) and at 6 months (in the BEAC) in order to determine response to treatment. Written informed consent was obtained from all patients, and the study was approved by the local ethics committee.

US-guided synovial biopsy. Prebiopsy standard US images of each biopsied joint were obtained as previously reported (9), using a General Electric Logiq 9 US machine with a 2-dimensional M12L transducer with gray-scale frequency of 12 MHz. Power Doppler settings were adjusted to the lowest permissible pulse repetition frequency to maximize sensitivity, and maximum color gain was used without creating noise artifact. Synovial thickness and power Doppler signal were scored sequentially by 2 observers (SK and NN), who were blinded with regard to patient identifiers, according to a previously validated semiquantitative score (0–3) (13).

US-guided synovial biopsy was performed as previously described (9). The joint selected for biopsy was determined by previously published criteria (9), with a second biopsy performed on the same joint unless contraindicated (e.g., joint tenderness, patient refusal). Three operators (FH, SK, and NN) performed all biopsies. A minimum of 12 synovial biopsies per joint (range 12–30) were performed to ensure

Table 2. Demographic characteristics of the patients with RA recruited to US-guided biopsy-driven studies*

	BEAC (n = 18)	CLIP-Cert (n = 17)
Disease stage	Early RA (<12 months)	Inadequate response to DMARDs
Therapeutic intervention	MTX + sulfasalazine	Certolizumab pegol + MTX
Time to second biopsy, months	6	3
Joint biopsied at baseline, no. of patients		
Wrist	14	12
MCP/PIP	4	4
Elbow	–	1
Joint biopsied at second biopsy, no. of patients		
Wrist	15	12
MCP/PIP	3	4
Elbow	–	1
Female, %	72	67
Age, mean ± SD years	53.8 ± 13.9	48.4 ± 12.2
Disease duration, mean ± SD years	0.6 ± 0.23	7.2 ± 6
Erosive disease, %	22	47
RF positive, %	66	41
Anti-CCP positive, %	72	59
DAS28, mean ± SD		
At baseline	6.3 ± 1.28	6.3 ± 0.9
At second biopsy	3.7 ± 2.4	4 ± 1.4
US synovial thickness score, mean ± SD		
At baseline	2.26 ± 0.72	–
At second biopsy	1.9 ± 0.7	–
US power Doppler score, mean ± SD		
At baseline	–	1.58 ± 1.1
At second biopsy	–	0.88 ± 0.95

* RA = rheumatoid arthritis; US = ultrasound; BEAC = Barts Early Arthritis Cohort; CLIP-Cert = Clinical and Pathological Response to Certolizumab Pegol; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate; MCP = metacarpophalangeal; PIP = proximal interphalangeal; RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; DAS28 = Disease Activity Score in 28 joints.

that at least 6 biopsy specimens were available for histopathologic analysis and at least 6 were available for RNA processing. In joints with sufficient synovial tissue, >12 biopsy specimens were obtained in order to increase overall tissue yield. The maximum number of biopsy specimens was determined by factors such as procedure time and patient tolerance.

Synovial sample processing. Following arthroplasty or arthroscopic synovectomy, all available synovial tissue was harvested and immediately fixed in 4% paraformaldehyde, with each piece embedded in separate paraffin blocks. Following US-guided synovial biopsy, tissue was immediately fixed in 4% paraformaldehyde for later paraffin embedding or immersed in 10:1 (volume/volume) RNAlater (Ambion) for storage at −80°C. A minimum of 2 samples were embedded per block, and 6 samples per vial were retained for RNA extraction.

After paraffin embedding, three 5-μm–thick sections from each arthroplastic/arthroscopic and US-guided biopsy specimen obtained 50 μm apart were mounted onto glass slides and examined at 40× magnification (using an Olympus BX60 microscope). Only sections with an intact visible lining layer were included in the analysis. If no intact lining layer was visible, a further three 5-μm–thick sections at least 50 μm apart were cut and examined. If no visible lining layer was seen, tissue was counted as ungraded.

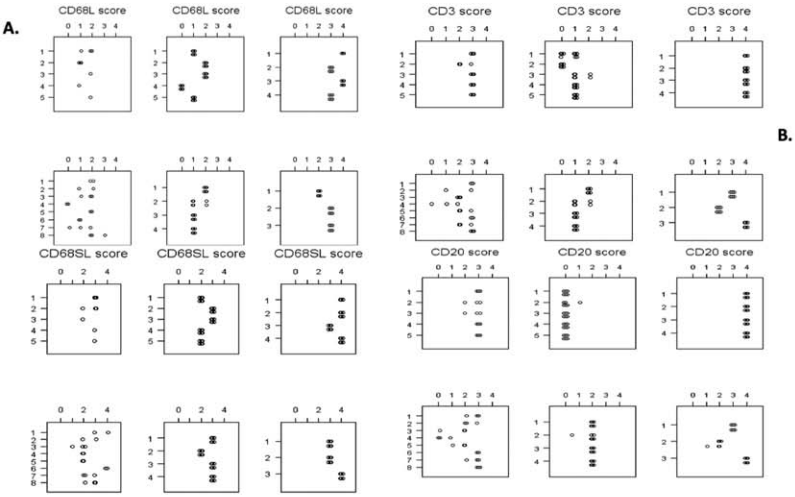
Immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections were deparaffinized and rehydrated and then stained for B cells (CD20), T cells (CD3), and macrophages (CD68), as previously described (14). Sequential sections from each cutting level from all arthroplastic/synovectomy specimens and all US-guided needle biopsy specimens were then scored semiquantitatively (on a scale of 0–4) for CD3, CD20, and CD68+ lining and CD68+ sublining macrophages, as previously described (15,16). Two trained readers (RH and VR) who were blinded with regard to clinical details scored all sections. When scores between the 2 readers did not match, the scores were resolved by mutual agreement.

RNA extraction. RNA was extracted from synovial tissue biopsy specimens using a TRIzol separation protocol as previously described (9).

Statistical analysis. Demographic characteristics of the patients are shown as the mean ± SD (17) or the relative frequency. Values for nonparametric variables are shown as the median (interquartile range) or the relative frequency. Chi-square test (with Yates' correction), Fisher's exact test, Mann-Whitney U test, or Kruskal-Wallis test was used when appropriate in order to determine whether the yield of synovial tissue was determined by baseline US-defined synovial thickness or power Doppler.

The variance of the score for each marker was deconstructed into 3 parts: variance between sections of individual biopsy specimens, variance between different biopsy sections from the same patient, and variance between patients. This variance was analyzed using analysis of variance. The F values for each analysis were provided, and P values less than 0.05 were considered significant. All statistical analyses were performed using SPSS (IBM version 19).

A.



B.

	Patients	Biopsies	Sections
CD3	60.9 (p<0.005)	1.6 (p=0.125)	0.528 (p=0.78)
CD20	85.9 (p<0.005)	2.423 (p<0.05)	0.476 (p=0.82)
CD68l	16.8 (p<0.005)	5.08 (p<0.005)	0.207 (p=0.974)
CD68sl	2.9 (P<0.05)	3.6 (p<0.005)	0.442 (p=0.850)

C.

Patient ID	CD3			CD20			CD68l			CD68sl		
	3 Bx	4 Bx	All Tx	3 Bx	4 Bx	All Tx	3 Bx	4 Bx	All Tx	3 Bx	4 Bx	All Tx
1	4	4	4 (+/0.4)	4	4	4 (+/-0.4)	3.6	3.5	3.42(+/-0.34)	3.7	3.75	3.7 (+/-0.37)
2	3	NA	3 (+/-0.3)	3	NA	2.94 (+/-0.29)	2.6	NA	2.6(+/-0.26)	3.3	NA	3.3 (+/-0.33)
3	0.67	0.75	0.76 (+/-0.08)	0*	0*	0.03 (+/-0.003)	1.6*	1.25	1.2(+/-0.12)	2.6	2.5	2.4 (+/-0.24)
4	1.33	1.25	1.33 (+/-0.13)	2	2	2 (+/-0.2)	1	1.25	1.3(+/-0.13)	3	2.75	2.75 (+/-0.25)
5	3	3	2.86 (+/-0.29)	3	3	2.86 (+/0.29)	1.6	1.5	1.44(+/-0.14)	2.3	2.5	2.77(+/-0.27)
6	2.66	2.5	2.28(+/0.23)	2.66	2.25	2.08(+/0.21)	1.66	1.25*	1.47(+/-0.15)	2.25*	2.33	2.53(+/-0.25)

Figure 1. Significant variation in cell infiltrate between patients and between biopsy specimens but not within biopsy specimens. **A,** Plot of semiquantitative scores for macrophages (CD68 lining [CD68L] and CD68 sublining [CD68SL]), T cells (CD3), and B cells (CD20) for individual biopsy specimens and sections of each specimen from 6 patients. **B,** Components of variance for each marker. F value and significance levels are shown for each marker in each component of variance. **C,** Mean values for semiquantitative scores for CD3, CD20, CD68+ lining macrophages, and CD68+ sublining macrophages when 3 biopsy specimens (Bx), 4 biopsy specimens, or all available tissue (Tx; ±10% of the mean) was examined for each patient. The mean fell within 10% of the mean in total tissue in 87.5% of the cases (21 of 24 cases) when 3 biopsy specimens were examined and in 91.6% of the cases (22 of 24 cases) when 4 biopsy specimens were examined. Asterisk indicates that the mean score lies outside ±10% of the mean in total tissue. NA = not available.

RESULTS

Patient demographics. Patient demographics are shown in Tables 1 and 2. In addition, a detailed breakdown of individual US-guided biopsy procedures is available from the corresponding author upon request, including the joint biopsied, the number of synovial biopsy samples obtained for histopathologic analysis, and the number of sections examined.

Significant variation in the CD3, CD20, and CD68 cell infiltrate between patients and between biopsy specimens but not within biopsy specimens. In order to determine the degree of variation of cellular infiltration of CD3, CD20, and CD68 among patients as well as between and within biopsy specimens, semiquantitative scores for individual markers were examined for all available sections from arthroscopic/arthroplastic procedures. The results for individual patients are shown in Figure 1A, and components of variance analysis are shown in Figure 1B. These results demonstrate that although there was significant variation in the degree of cellular infiltration between patients and between biopsy specimens from the same patient, there was no significant variation when multiple sections of the same biopsy specimen from the same patient were examined. This suggests that in order to obtain a representative image of the joint, examination of multiple sections of tissue from 1 biopsy specimen is not sufficient.

Examination of a minimum of 4 biopsy specimens gives a representative assessment of the CD3, CD20, and CD68 cellular infiltrate. The previous analysis suggested that examination of multiple biopsy specimens, rather than simply multiple sections from a single biopsy specimen, was required in order to obtain a representative image of the inflammatory cell infiltrate of the joint. Therefore, the mean total inflammatory score for each immune cell

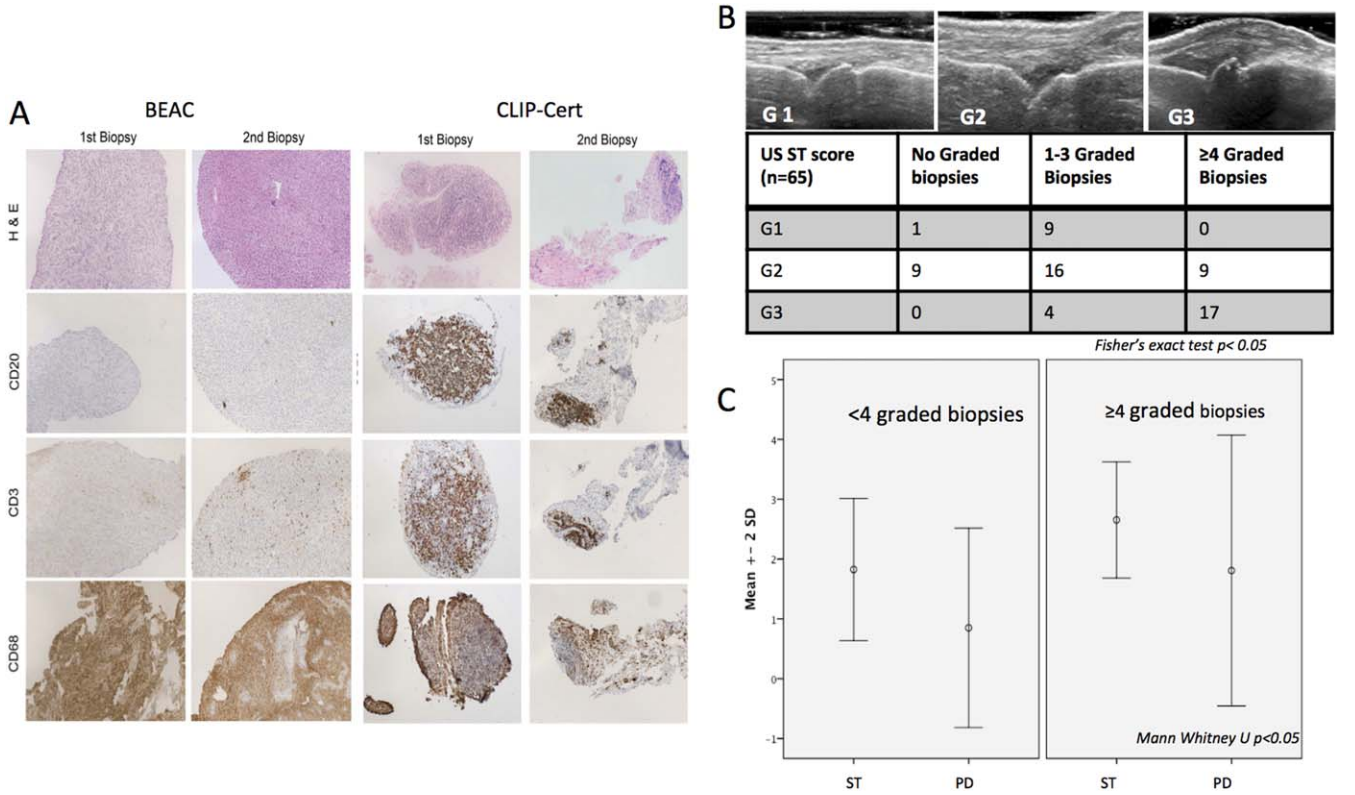
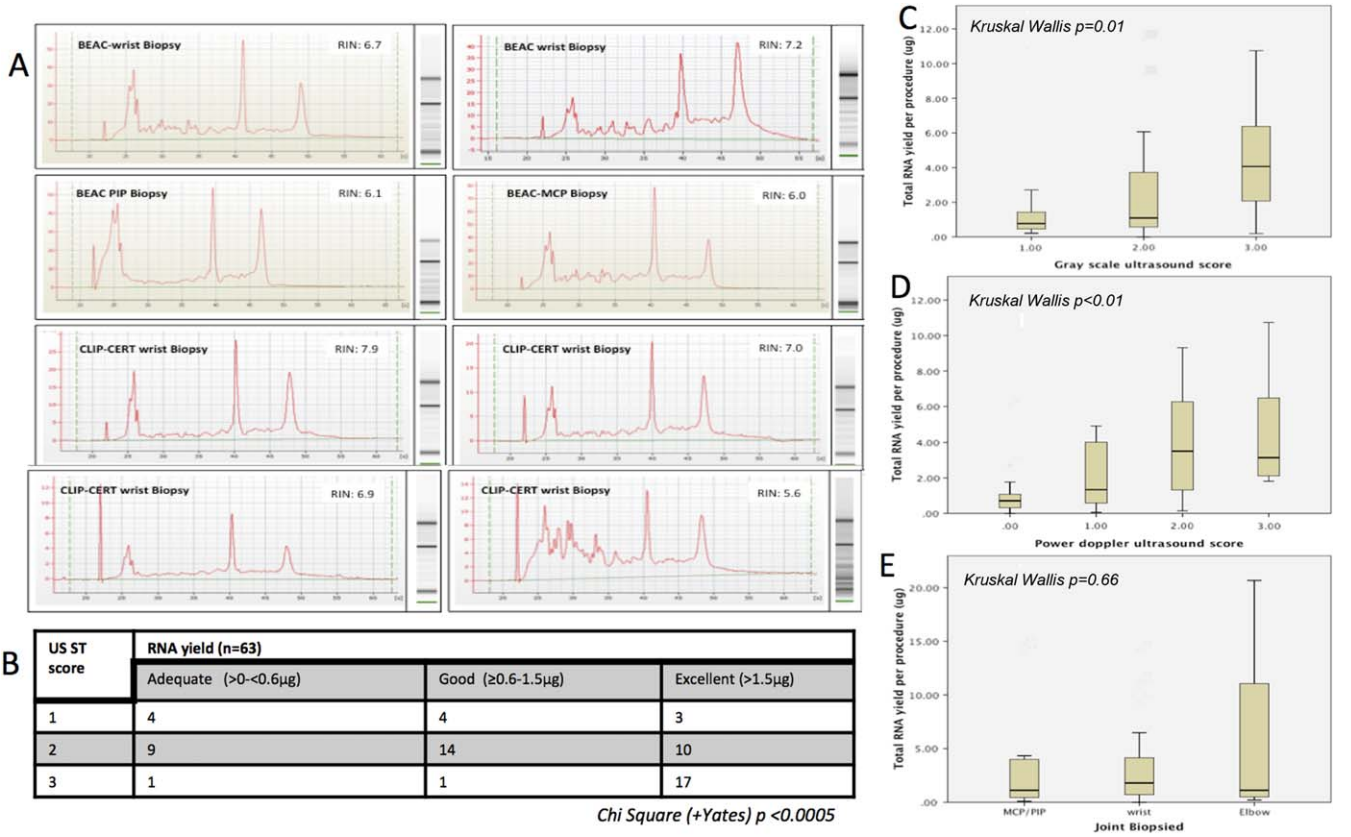


Figure 2. Prebiopsy synovial thickness determined by gray-scale ultrasound (US) is predictive of the quality of synovial tissue for histopathologic analysis. **A,** Representative images of synovial tissue obtained during first and second biopsies in each clinical trial (Barts Early Arthritis Cohort [BEAC] and Clinical and Pathological Response to Certolizumab Pegol [CLIP-Cert]). H&E = hematoxylin and eosin. **B,** Top, Representative gray-scale US images of grade 1 (G1), grade 2, and grade 3 synovial thickness. Bottom, Number of graded biopsy specimens obtained from patients with each synovial thickness grade. There was a significantly higher number of graded biopsy specimens from joints with a synovial thickness score of ≥2. **C,** Synovial thickness (ST) and power Doppler (PD) scores for biopsy procedures that yielded <4 or ≥4 graded synovial biopsy specimens. Synovial thickness scores and PD scores were significantly higher in procedures that yielded ≥4 graded synovial biopsy specimens ($P < 0.05$ for each comparison).

marker for each patient was calculated from all available tissue sections. Subsequently, the mean inflammatory score for each immune cell marker was determined per section from either 3 or 4 biopsy specimens from each patient. The results of this analysis are shown in Figure 1C. In 87.5% of the cases (21 of 24 cases), the sample mean fell within 10% of the total mean of the whole tissue if 3 biopsy specimens were analyzed. This proportion increased to 91.6% of the cases (22 of 24 cases) if 4 biopsy specimens were examined. It can thus be concluded that the examination of 4 biopsy specimens at 1 time point provides a reliable sample mean in the vast majority of samples.

Prediction of the quantity and quality of synovial tissue yields by prebiopsy synovial thickness determined by gray-scale US. In order to determine whether US-guided needle procedures yielded the minimum number of synovial biopsies (≥4) required to enable a representative estimate of the overall cellular infiltrate in small joints, the number of graded biopsy specimens (those with a detectable lining layer) (Figure 2A) at baseline and 6 months was determined as a proportion of the total number of biopsy specimens obtained (n = 70). This analysis demonstrated that while the mean ± SD number of synovial samples obtained per procedure was 11.1 ± 4.48, in 80% of all procedures (56 of 70), >1 graded biopsy specimen was retrieved, while >4 graded biopsy specimens were obtained in 39% of the procedures (27 of 70). In order to investigate whether increasing the number of biopsies per joint increased the number of gradable samples, procedures were stratified into those where <10 or ≥10 biopsy specimens were obtained and then categorized into those yielding <4 and ≥4 graded biopsy specimens. In those procedures in which <10 biopsy specimens were obtained, 21% (6 of 27) yielded ≥4 graded biopsy specimens, while when ≥10 biopsy specimens were obtained, 50% (21 of 43) yielded good tissue. As expected, this suggested that increasing the number of biopsy specimens obtained per joint improved tissue yield.



B

US ST score	RNA yield (n=63)		
	Adequate (>0-<0.6μg)	Good (≥0.6-1.5μg)	Excellent (>1.5μg)
1	4	4	3
2	9	14	10
3	1	1	17

Chi Square (+Yates) p <0.0005

Figure 3. Ultrasound (US)–guided small joint synovial biopsy procedures yield sufficient RNA for subsequent molecular analysis. **A,** Representative Agilent electropherogram profiles illustrating RNA quality from the first and second biopsy procedures in 4 patients (8 procedures) from each study (Barts Early Arthritis Cohort [BEAC] and Clinical and Pathological Response to Certolizumab Pegol [CLIP-Cert]). RIN = RNA integrity number. **B,** Number of biopsy procedures (of a total of 63) that yielded insufficient (0 μg), adequate (>0 μg to <0.6 μg), good (≥0.6–1.5 μg), or excellent (>1.5 μg) RNA, according to synovial thickness (ST) grade as determined by prebiopsy US. A significantly higher number of procedures in joints with a synovial thickness grade of ≥2 had a good or excellent RNA yield as compared to those with a synovial thickness grade of 1. **C,** RNA yields in biopsy procedures categorized according to synovial thickness grade as determined by prebiopsy US. There were significantly higher RNA yields in those with higher synovial thickness grades. **D,** RNA yields in biopsy procedures categorized according to prebiopsy power Doppler (PD) scores (range 0–3). There was a significantly higher RNA yield in procedures with higher prebiopsy PD scores. **E,** RNA yields in biopsy procedures categorized according to joint biopsied (metacarpophalangeal [MCP]/proximal interphalangeal [PIP] joint versus wrist versus elbow). No significant differences in RNA yield were found. In **C–E,** data are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.39235/abstract.

In order to examine whether the number of biopsy procedures performed was the result of synovial tissue volume or inflammatory activity (determined by prebiopsy US synovial thickness and/or power Doppler grade) and whether this predicted the tissue yield, semiquantitative scores for synovial thickness and power Doppler were analyzed when accurate records were available (65 of 70). As shown in Figure 2B, 77% of the procedures (34 of 44) performed on joints with grade 1 or 2 synovial thickness yielded at least 1 graded biopsy specimen, while, notably, the proportion rose to 100% of the joints with grade 3 synovial thickness. There was also a significantly higher number of patients with ≥4 graded biopsy specimens in the group of patients with a prebiopsy synovial thickness grade of 3 (17 of 21 [81%]) compared to those with a grade of 1 or 2 (9 of 44 [20%]) ($P < 0.05$) (Figure 2B). Finally, there was a significantly higher synovial thickness score (2.65 versus 1.82) ($P < 0.05$) and power Doppler score (1.8 versus 0.85) ($P < 0.05$) in those joints that yielded ≥4 graded biopsy specimens versus those with <4 graded biopsy specimens (Figure 2C).

When biopsy procedures were stratified according to the presence or absence of power Doppler signal and further stratified according to synovial thickness grade (range 1–3), there was a significantly higher proportion of patients with a synovial thickness grade of 3

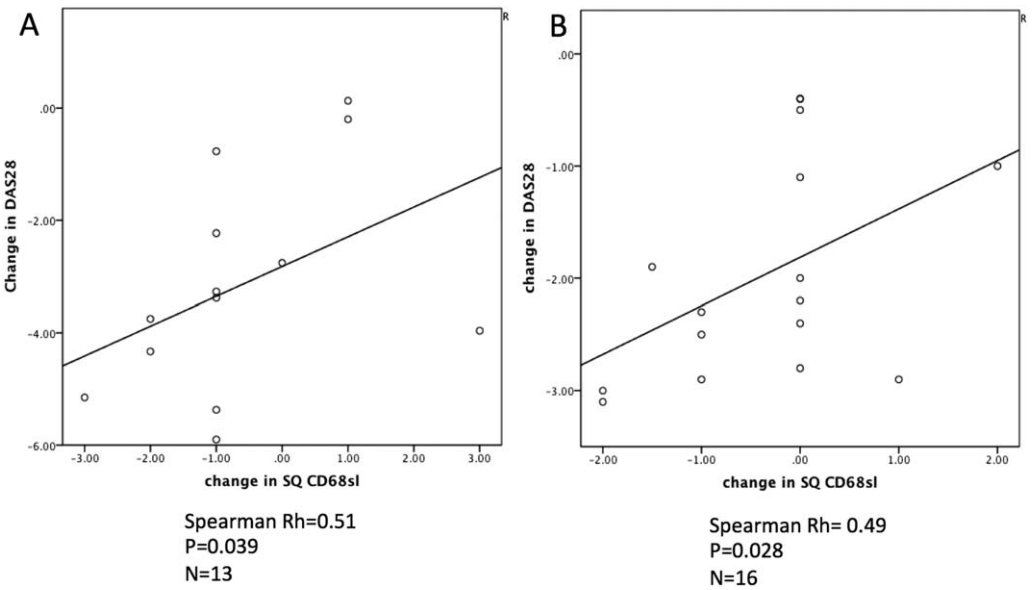


Figure 4. Significant correlation between clinical response and reduction in semiquantitative (SQ) synovial sublining macrophage (CD68+sl) number. The correlation between change in CD68+ sublining macrophage number and change in Disease Activity Score in 28 joints (DAS28) from baseline biopsy to second biopsy was examined for all patients from whom paired biopsy data were available in the Barts Early Arthritis Cohort (BEAC) cohort (n = 13) (**A**) and in the Clinical and Pathological Response to Certolizumab Pegol (CLIP-Cert) study (n = 16) (**B**). A significant correlation between a decrease in CD68+ sublining macrophage number and a decrease in DAS28 was seen for both the BEAC cohort and the CLIP-Cert cohort.

who demonstrated power Doppler positivity (19 of 21 [90%]) than patients with grade 2 synovial thickness (23 of 34 [68%]) or grade 1 synovial thickness (2 of 8 [25%]) ($P < 0.001$ by Fisher's exact test). Furthermore, all procedures in the grade 3 group and 90% (9 of 10) in the grade 1 group yielded ≥1 graded biopsy specimen, irrespective of power Doppler positivity. In the synovial thickness grade 2 group, 25 of 34 procedures yielded ≥1 graded biopsy specimens, and of these 25, 15 were power Doppler positive. In the group yielding no graded synovial tissue (9 of 34), 8 of the 9 were power Doppler positive, with no significant difference between the 2 cohorts ($P = 0.214$ by Fisher's exact test). This strongly suggests that the presence of power Doppler signal does not predict the success of biopsy procedure. Thus, prebiopsy US-defined synovial thickness rather than power Doppler signal or biopsy number is the main predictor of the quantity and quality of synovial tissue.

All US-guided synovial biopsy procedures in small joints yield sufficient RNA for subsequent molecular analysis. In order to determine whether synovial tissue acquired using US-guided synovial biopsy yielded sufficient RNA for subsequent molecular analysis, procedures in which synovial tissue had been retained for RNA extraction (n = 63) were categorized according to the total amount of RNA extracted into insufficient (0 μg), adequate (>0 to <0.6 μg), good (≥0.6–1.5 μg), or excellent (>1.5 μg) as determined by NanoDrop and Agilent electropherogram profiling for both first and second biopsies (Figure 3A). Notably, 100% of the procedures produced adequate, good, or excellent yields of RNA.

We next investigated whether prebiopsy synovial thickness was again predictive of RNA yield. To this end, the number of procedures producing insufficient, adequate, good, and excellent RNA was determined for each of the prebiopsy US synovial thickness grades (Figure 3B). There were a significantly higher number of patients with good or excellent RNA yield in those patients with grade 3 synovial thickness (18 of 19 [95%]), compared to those with grade 1 synovial thickness (7 of 11 [64%]) or grade 2 synovial thickness (24 of 33 [73%]) ($P < 0.0005$). In addition, when procedures were grouped according to prebiopsy synovial thickness or power Doppler score, there was a significantly higher RNA yield in those with higher grades of either synovial thickness ($P < 0.01$) (Figure 3C) or power Doppler ($P < 0.01$) (Figure 3D). However, there was no significant difference in RNA yield if procedures were grouped according to the type of joint biopsied (metacarpophalangeal/proximal interphalangeal joint versus wrist versus elbow) ($P = 0.66$) (Figure 3E). Thus, although a higher pre-

biopsy US synovial thickness and power Doppler score predicted a higher RNA yield, even in small joints with only grade 1 synovial thickness, 100% of US-guided procedures yielded sufficient RNA to allow identification of molecular signatures for future predictive models.

Significant correlation of reduction in synovial CD68+ sublining macrophage number with clinical response. Finally, in order to establish the performance of synovial biomarkers validated in the context of clinical trials using arthroscopy (18), we investigated changes in CD68+ sublining macrophages and clinical response (DAS28) in patients who had paired US-guided biopsy specimens available (n = 13 in the BEAC cohort and n = 16 in the CLIP-Cert study). The correlation between change in DAS28 and change in CD68+ sublining macrophage number between baseline and second biopsy was examined. A significant correlation was demonstrated between decrease in DAS28 and decrease in CD68+ sublining macrophage number for both patient cohorts (for BEAC, Spearman's $\rho = 0.51$, $P = 0.04$ [Figure 4A] and for CLIP-Cert, Spearman's $\rho = 0.49$, $P = 0.03$ [Figure 4B]). These data confirm that US-guided synovial biopsy yields synovial tissue of good quality to sensitively reflect changes in CD68+ sublining macrophages and, therefore, support its potential use in the context of clinical trials where synovial tissue is needed to investigate drug mechanisms of action.

DISCUSSION

The results presented herein demonstrate a number of important findings. First, a minimum of 4 synovial biopsy specimens of small joints are required to obtain accurate representation of synovial immune cell infiltration using semiquantitative analysis. Second, when increased synovial thickness (grade 3) is documented prebiopsy by US, sufficient synovial tissue can be obtained from small joints in 81% of US-guided procedures to accurately reflect the immunophenotype of the whole joint. Third, if 6 synovial biopsy specimens are processed per joint in 100% of all procedures, sufficient synovial tissue is obtained to permit RNA extraction and subsequent molecular analysis, and fourth, as in large joint arthroscopic biopsy samples, the number of CD68+ sublining macrophages, a validated biomarker of response to treatment in clinical trials (10), is consistent with clinical response to treatment.

This is the first study to illustrate the utility of US-guided biopsy of small joints to obtain high-quality synovial tissue from patients in clinical trials. The possibility of obtaining synovial tissue from any joint and from the majority of patients both before and after treatment through a simple, safe, and well-tolerated procedure offers a tremendous opportunity for mechanistic studies to advance mode of action understanding and drug development.

Though 6 synovial biopsies are recommended when sampling large synovial joints in order to overcome morphologic heterogeneity for both pathobiologic (19) and gene expression analysis (20), the extrapolation of these requirements to small joints in which synovial volume is much reduced was not previously validated. Notably, when pathologic heterogeneity of the immune cell infiltrate in large joints is systematically examined, the variability is highest between rather than within biopsy specimens (19), an observation that is consistent with the results of the present study of small joints. These observations strongly suggest that determining the number of synovial biopsies required, rather than simply the number of tissue sections per joint (as previously reported [5]), is critical to obtaining a representative estimate of the immune cell infiltration of the whole joint.

Thus, in order to define the minimum number of biopsy specimens required per joint, we undertook a further standardization exercise. The results presented herein demonstrate that examination of a minimum of 4 biopsy specimens per small joint is required for reliable histopathologic analysis. However, since this study used tissue obtained from arthroplastic/arthroscopic procedures in patients with considerable preoperative synovitis, it should be recognized that this may be too stringent a requirement in patients with minimal synovial thickness where, given the inherent lack of heterogeneity, it is conceivable that fewer biopsies would be required. Although further standardization would be ideal, including small joints with minimal synovitis in order to adjust for the degree of prebiopsy synovial thickness, it is extremely unlikely this can be achieved, since there would be no clinical indication for small joint synovectomy/arthroplasty in patients with minimal synovitis.

This is the largest reported cohort of patients with US-guided synovial biopsies of small joints, and importantly, our findings confirm the validity of the technique in the context of a clinical trial setting by demonstrating that graded synovial tissue is obtained in 80% of all procedures. It is important to acknowledge, however, that if a requirement for 4 graded biopsy specimens is set in order to permit reliable pathologic analysis, only 20% of patients with minimal synovial thickness reach this target versus 81% with grade 3 synovial thickness. We have previously described the importance of synovial thickness in predicting yield of synovial tissue in an early arthritis cohort (9) and, as has already been discussed, it may be that in patients with minimal syno-

vial thickness a standard of 4 biopsy specimens per joint is too stringent. Crucially, however, in 77% of the procedures performed in joints with minimal synovial thickness, graded synovial tissue was obtained. Furthermore, it should be stressed that in those patients with minimal synovial thickness, 100% of procedures yielded synovial tissue of sufficient volume for subsequent RNA extraction. This is particularly important in the context of clinical trials, in which simple histopathologic analysis is likely to be too crude a measure of synovial pathobiologic responses, and more comprehensive information is likely to be obtained by molecular analysis.

The validity of US-guided needle biopsy is further supported by the significant correlation, seen within both cohorts examined in this study, between change in CD68+ sublining macrophage number, a previously widely validated synovial biomarker (4), and clinical response to treatment. Finally, when considering the practical application of this technique to clinical trials or practice, the results from this study also suggest that in patients with minimal synovial thickness, increasing the number of biopsy specimens obtained per joint does not improve the yield of graded synovial tissue.

The results herein confirm that the technique of US-guided small joint biopsy is a reliable method of acquiring synovial tissue from small joints, including those with minimal synovitis, making serial US-guided small joint needle biopsy an attractive procedure for use in the context of clinical trials. We recognize, however, that before widespread adoption is recommended, it will be important to carry out additional head-to-head studies comparing US-guided needle biopsy with alternative approaches, including US-guided biopsy using a portal and forceps approach and small joint arthroscopy. Such studies are planned as part of the European League Against Rheumatism synovitis study group and Outcome Measures in Rheumatology research agenda. Directly comparing safety, tolerability, and feasibility as well as synovial yield, particularly in patients with minimal synovitis following effective treatment, would be an important goal to establish the clinical utility of a minimally invasive procedure that has the potential to change practice by bringing synovial tissue molecular pathology into future clinical decision, by stratifying patients according to prognostic and therapeutic response categories.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Pitzalis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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ROLE OF THE STUDY SPONSOR

UCB had no role in the study design or in the collection, analysis, or interpretation of the data, the writing of the manuscript, or the decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by UCB.

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