Persistence of Crystals in Stored Synovial Fluid Samples.

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ABSTRACT

Background and aims: The lack of immediate access to a polarized light microscope is often used as an argument to justify the clinical diagnosis of crystal-related arthritis. The aim of this study was to assess the influence of time since sampling and preservation methods on crystal identification in synovial fluid samples under polarized light microscopy.

Methods: Prospective, longitudinal, observational factorial study, analyzing 30 synovial fluids samples: 12 with monosodium urate crystals (MSU) and 18 with calcium pyrophosphate (CPP) crystals. On extraction, each fluid sample was divided into four subsamples (120 subsamples in total). Two were stored in each type of tube—heparin or ethylenediaminetetra-acetic acid (EDTA) as preserving agents -, at varying temperatures - room temperature or refrigerated at 4°C (39.2°F). Samples were analyzed the following day (T1), at three days (T2), and at seven days (T3) by simple polarized light microscopy, and the presence of crystals was recorded.

Results: The identification of crystals in the MSU group was similar between groups, with crystals observed in 11/12 (91.7%) of room temperature samples and in 12/12 (100%) of refrigerated samples at T3. However, the identification of CPP crystals tended to decrease in all conditions, especially when preserved with EDTA and kept at room temperature (12/18 [66.7%] at T3), while less reduction was seen in refrigerated heparin-containing tubes.

Discussion: Preserving samples with heparin in refrigerated conditions allows a delayed microscopic examination for crystals. Avoiding crystal-proven diagnosis due to the immediate unavailability of a microscope no longer appears justified.

INTRODUCTION

Synovial fluid (SF) analysis under polarized microscope is an immediate, reliable, reproducible bedside procedure, no requiring staining or fixation, that enables a definitive diagnosis of crystal-related arthritis (1,2). The technique is included within the American College of Rheumatology's Core Curriculum for specialty training (3). Microcrystals that usually trigger arthritis—monosodium urate (MSU) and calcium pyrophosphate (CPP)—are identified based on their shape and birefringence. Formally, a compensated polarized light microscope is required, although access can be limited. MSU crystals are recognizable by their needle shape and strong birefringence, while CPP crystals appear as parallelepipeds (rhomboidal, rods) with varying intensity of birefringence, often lower than MSU (4–6).

The time elapsed from joint aspiration to microscopic evaluation is a relevant consideration in SF analysis for crystals. Early visualization is usually recommended to prevent leukocyte degradation and potential crystal alteration, and reach a rapid diagnosis (4). However, only a few studies have set out to establish the correct timing and sample preservation methods for SF analysis.

Table 1 describes the studies retrieved on SF analysis during the literature review that preceded performance of the present study (7–13). Results differed significantly between studies, reflecting methodological differences in the type of crystal assessed, methods, storage temperature, and timing. This heterogeneity makes direct comparison difficult and precludes a clear conclusion.

Thus, the influence of the time between SF extraction and examination as well as the sample preservation method on the persistence of crystal visualization under an optical microscope has yet to be determined. The primary objective of the present study was to assess the persistence of crystals (MSU or CPP) over time in SF following extraction, according to the sample method and storage temperature.

METHODS

We designed a prospective, longitudinal, observational factorial study. SF samples were obtained from clinical practice at the Rheumatology section of Hospital General Universitario de Alicante (Alicante, Spain). No clinical data were collected, making ethics evaluation unnecessary according to current regulations in Spain.

Sample collection.

The recruitment period was from October 2017 to April 2018. Inclusion criteria were SF samples showing MSU or CPP crystals under polarized light microscope and identified by a rheumatologist with expertise in the field (MA). Samples containing both types of crystals were excluded.

Following sample collection in our clinic, the SF obtained was divided into four subsamples. Two were stored in each type of tube— containing heparin or ethylenediaminetetra-acetic acid (EDTA) as preserving agents— at either room temperature (20°C) [68°F] or refrigerated at 4°C [39.2°F]. In all cases, the tubes were protected from light and enumerated in a non-consecutive manner. The list of numbers was kept separately, and the study started when two synovial fluid samples were available on the same day. To prevent bias, three control fluids without crystals were also included.

Samples were analyzed using a simple polarized light microscope (Olympus CX41, Japan) at 400× magnification under bright and simple (non-compensated) polarized light. Crystals were classified based on their shape and intensity of birefringence (4). Neither other magnifications nor red compensator were used. Samples were labelled as lacking crystals after examining a minimum of 30 separated x400 fields on the slide. Observations were performed the following day (T1), at 3 days (T2), and at 7 days (T3). The observer was a medical student in the final year of her studies; she received specific training prior to the study and reviewed the technique after every 10 fluids analyzed (14). Observations were recorded and submitted separately for each time point, and they could not be consulted again until the end of the study.

The primary outcome variable was the presence of crystals. Secondary outcome variables were the type of crystal (MSU or CPP) and their predominant location (intracellular, extracellular, inside conglomerates, or various, depending on where the principal location could be established). Explanatory variables were the time elapsed between sample collection and analysis (T1, T2, and T3), the preserving agent (EDTA versus heparin) and the storage temperature (20°C versus 4°C).

Statistical analysis.

Descriptive data were expressed as means (± standard deviation [SD]) for quantitative variables, and as absolute and relative frequencies for qualitative variables.

To assess the influence of the study variables in the identification of crystals, a generalized linear model for repeated measures was built, with fixed effects (type of crystal; temperature; preserving agent) and random effects (sample observations with the time). Presence of crystals was considered the dependent variable. In case of significant results, interactions between explanatory variables were assessed accordingly.

Statistical analyses were performed using Microsoft Excel and Google Colab with Jupyter notebooks, libraries Pyreadstat v0.2.0, Pandas v0.23.3, and Statsmodels v0.10.1. The level of significance was established at $p < 0.050$.

Quality of data assessment.

The Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) tool (15,16) was used to evaluate the quality of the studies identified from the literature search and the present study. This tool assesses risk of bias according to the following domains: patient selection; index test (storage temperature, conservation and time); reference standard (crystals at baseline); and flow and timing. Except for the last domain, applicability was assessed as well. Two authors (SP and JAB) independently applied the QUADAS-2 tool, resolving disagreements by consensus.

RESULTS

Thirty SF samples with crystals (MSU in 12, CPP in 18) were included and divided into 120 subsamples at baseline, so 360 observations were carried out at T1, T2, and T3. The observer correctly identified the three fluids without crystals, and these were excluded from the analysis.

Microscopic examinations were performed at the different time points as follows: T1, mean 31.0 h (SD 10.3); T2, mean 90.5 h (SD 29.3); and T3, mean 179.2 h (SD 16.4). By type of crystal, the mean time between sampling and analysis was, for MSU: T1, 31.2 h (SD 11.8); T2, 99.2 h (SD 34.9); and T3, 182.9 h (SD 14.8). For CPP, mean time elapsed was: T1, 30.8 h (SD 9.2); T2, 84.7 h (SD 23.3); and T3, 176.8 h (SD 17.1).

Table 2 and **Figure 1** show the results of the microscopy observations for each type of crystal (presence and location) at each time point, in relation to the preservative used and storage temperature, as well as the results of the statistical comparisons. MSU crystals seem to persist in almost all samples, regardless of temperature and preserving agent (**Figure 1**). However, the persistence of CPP crystals tends to progressively decline in all samples (**Figure 1**), especially when kept within EDTA and stored at room temperature, with only 60% showing crystals by microscopy.

The results of the generalized linear model confirmed the descriptive results (**Table 3**). Time to visualization showed a significant, inverse association with crystal identification in the stored samples. Besides, in comparison to CPP, MSU crystals were positively associated with the identification. No association with temperature or preserving agents was found. Afterwards, the analysis was stratified for the type of crystal (**Table 3**). For MSU crystal, no explanatory variable was found associated with the visualization, in keeping with our descriptive results of crystal persistence regardless of time, temperature or preserving agent. However, for CPP crystals, time showed a significant, inverse association, while refrigerated samples showed a direct association. Interestingly, a significant interaction between both variables was noted. No association was found for the type of preserving agent employed.

The **table S1** (supplementary material) shows the results of the association analysis incorporating only the T1 to T3 observations.

DISCUSSION

Synovial fluid analysis under polarized light microscope remains the reference standard for diagnosing crystal-related arthritis (1,2), and is an essential procedure when confronting arthritis of unknown origin (17). However, clinicians sometimes avoid performing it in practice (18,19), citing the lack of immediate access to a microscope as justification. Our results support the identification of crystals in SF, especially in the case of MSU, up to one week after sampling, regardless of the storage temperature or the preserving agent. Regarding CPP crystals, their identification decreased over time, especially when kept at room temperature and preserved using EDTA. However, samples that were refrigerated and preserved in heparin showed excellent persistence of CPP crystals at three days (100%) and good persistence at seven days (83.3%). Thus, microscope analysis can be safely used for up to several days after the sample is taken due to crystal persistence and the apparent absence of de novo formation. Regarding the findings on CPP crystals, samples can be refrigerated in a heparinized medium (common tubes for biochemical tests) to ensure the persistence of crystals when they are present.

Compared to other reports in the literature (**Table 1**), our findings are in keeping with Gálvez *et al*. and Tausche *et al.*, except for the gradual reduction in CPP crystals identification, linked to room temperature and EDTA preservation. These differences may be attributable to several factors. First, previous studies used a timeframe of three days, while our samples were stored for up to seven days, allowing more time for the degradation of crystals (in fact, comparisons from baseline to T2 showed no significant differences). Second, unlike other studies, ours maintained strict blinding during the examination of samples. Besides numerically labelling the study samples, the results of each observation were delivered and sealed—impeding comparisons with previous registers—and fluids with no crystals were introduced as controls. These measures help to reduce risk of bias, strengthening the certainty of the evidence. **Table 4** shows the quality assessment of the published studies along with a self-assessment of this one. Most were deemed to provide low- or moderate-quality evidence, except for Galvez *et al.*, Tausche *et al*., and this study, which were at low risk of bias.

MSU crystals remained identifiable throughout the seven-day study period, independently of the storage conditions. Artificial MSU crystals can be synthetized in the lab using oversaturated concentrations of urate and sodium. However, this solution may remain metastable for long periods until crystallization occurs. Factors such as albumin enhance MSU nucleation, while alkaline pH delays it (20). After formation, solubility of MSU crystals mostly and directly depend on temperature (20–24). Here tubes were kept at lower temperatures (4° and 20°C), likely reducing crystal dissolution and contributing to their persistence in stored samples. Storage of

tubes at normal body temperature (36°C) may be of further research interest to assess MSU crystal degradation. Theoretically, crystals could form continuously in stored samples, as SF urate levels tend to be higher than serum levels in untreated gout patients, widely exceeding the saturation point for urate (25). However, while *de novo* crystallization of MSU in vitro may occur in sealed glass slides, it is rare in stored samples (24). Besides, this is not in keeping with the current understanding of how MSU crystals form and deposit. Urate probably requires a complementary structure (most likely proteins) to crystallize as MSU (26). In vitro studies have suggested that gammaglobulins or collagen serve as this kind of template (27), with the latter a firm candidate considering the usual deposition of MSU crystals on the cartilage surface, as seen by ultrasound (28), arthroscopy or in SF fragments (29).

Regarding CPP crystals, we observed a significant decrease in the crystal persistence in study samples, especially when stored at room temperature and likely when preserved with EDTA. Despite being widely mentioned in published reviews (30,31), just one study supports the effect of EDTA as a solvent for CPP crystals. Bennet *et al*. (32) analyzed the influence of several factors (pH, crystal size, citrate, albumin, and others) on synthetic CPP crystals solubility under 37°C. Regarding pH, higher solubility was seen at pH 8.0-9.0. Smaller CPP crystals appear to dissolve quicker. Increasing ionized calcium concentrations decreased CPP solubility, while for ionized inorganic pyrophosphate (iPP), solubility rates followed a J-shaped curve, being lower at normal SF values (2-25μM). Higher concentrations likely induce dissolution by Ca^{++} chelation. PPi hydrolysis by pyrophosphatases also increased CPP solubility. EDTA played a key role here considering its known effect as a calcium chelator (33). Our results are in keeping with this observation, indicating that the best storage method for SF samples with CPP crystals is refrigeration and preservation with heparin. The potential applicability of this finding to clinical practice, where CPP crystals cannot be dissolved and the management of CPP crystal arthritis is based only on controlling the inflammatory manifestations (34), needs to be further addressed. In the study by Bennet *et al*. (32), when EDTA was applied to CPP patients through joint lavages, it triggered severe, acute CPP flares.

The quality of the evidence presented here is strengthened by rigorous efforts to reduce observer bias through masking of the samples (numbered labelling, control samples with no crystals), including preventing comparisons between observations until study end. Moreover, the observer underwent short training sessions in SF analysis at the beginning of the study and periodic reviews throughout (every 10 samples visualized). No formal reliability assessment was carried out for intra or interrater agreement, and this might be taken as limitation, especially as the observer was a medical student. However, polarized light microscopy for crystals is a reliable

technique, as reported by rheumatologists (35,36), and with laboratory registrars after brief training (14). In the present study, similar training was followed before starting the study and repeated during it; proper crystal identification was verified at these times. Moreover, the observer properly identified the control samples containing no crystals. These strategies likely ensure the value of the study data, which this was later confirmed in the multivariable analyses, which were identical regardless of incorporating the T0 visualizations. As observations were performed on consecutive days, the observer might expect progressive crystal degradation; however, tubes were masked using nonconsecutive enumeration, and while noting significant differences with either refrigerated or MSU crystals, EDTA-containing tubes indicate minimal impact. The sample size of 30 may be considered small and could have had an impact on the non-significant results; however, given the separation of the samples into four different tubes, results were based on 120 subsamples and 360 observations. In addition, the use of paired samples reduces the sample size needed to detect differences. Despite no formal evaluation, the observer's impression was that the cells present in the samples progressively lysed and died, mainly at room temperature, which can hamper crystal identification (specially to assess CPP shape). Here, such identification was performed according to standards, though future studies may aim to replicate our results using techniques with higher sensitivity—i.e. centrifugation (37)—or methods that eliminate the observer-related variability—i.e. Raman spectroscopy (38). This research would be of special interest for CPP crystals because of the observed reduction in its detection.

In summary, although an early analysis of SF samples is advisable for establishing the diagnosis and initiating proper management, our data indicate that visualization may be delayed up to one week after sampling. The MSU crystals persisted during the study period regardless of the storage method. For CPP crystals, storage refrigerated instead of at room temperature favored persistence; using EDTA and not heparin as preserving agent appeared to hamper visualization, but this was not confirmed in the statistical analysis. Avoiding crystal-proven diagnosis due to the immediate unavailability of a microscope no longer appears justified.

FIGURE LEGENDS

Figure 1. Persistence of MSU (top) and CPP crystals (bottom) at each time point, according to preservative and storage temperature.
EDTA=ethylendiaminetetraacetic acid, HEP= heparin.

REFERENCES

1. Zhang W, Doherty M, Pascual E, Bardin T, Barskova V, Conaghan P, et al. EULAR evidence based recommendations for gout. Part I: Diagnosis. Report of a task force of the Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT). Ann Rheum Dis 2006;65:1301-11.

2. Zhang W, Doherty M, Bardin T, Barskova V, Guerne P-A, Jansen TL, et al. European League Against Rheumatism recommendations for calcium pyrophosphate deposition. Part I: terminology and diagnosis. Ann Rheum Dis 2011;70:563-70.

3. ACR. Core curriculum outline for rheumatology fellowship programs [Internet]. 2015. Available from: https://www.rheumatology.org/Portals/0/Files/Core%20Curriculum%20Outline_2015.pdf

4. Pascual E, Sivera F, Andrés M. Synovial fluid analysis for crystals. Curr Opin Rheumatol 2011;23:161-9.

5. Dieppe P, Swan A. Identification of crystals in synovial fluid. Ann Rheum Dis 1999;58:261-3.

6. Andrés M, Vela P, Jovaní V, Pascual E. Most needle-shaped calcium pyrophosphate crystals lack birefringence. Rheumatology (Oxford) 2019;58:1095-8.

7. Bible MW, Pinals RS. Late precipitation of monosodium urate crystals. J Rheumatol 1982;9:480.

8. Kerolus G, Clayburne G, Schumacher HR. Is it mandatory to examine synovial fluids promptly after arthrocentesis? Arthritis Rheum 1989;32:271-8.

9. McKnight KM, Agudelo C. Comment on the article by Kerolus et al. Arthritis Rheum 1991;34:118-20.

10. McGill NW, Swan A, Dieppe PA. Survival of calcium pyrophosphate crystals in stored synovial fluids. Ann Rheum Dis 1991;50:939-41.

11. Gálvez J, Sáiz E, Linares LF, Climent A, Marras C, Pina MF, et al. Delayed examination of synovial fluid by ordinary and polarised light microscopy to detect and identify crystals. Ann Rheum Dis 2002;61:444-7.

12. Tausche A-K, Gehrisch S, Panzner I, Winzer M, Range U, Bornstein SR, et al. A 3-day delay in synovial fluid crystal identification did not hinder the reliable detection of monosodium urate and calcium pyrophosphate crystals. J Clin Rheumatol 2013;19:241-5.

13. Kienhorst LBE, Janssens HJEM, Eijgelaar RS, Radstake TRDJ, van Riel PLCM, Janssen M. The detection of monosodium urate crystals in synovial fluid after long-term and varying storage conditions. Joint Bone Spine 2015;82:470-1.

14. Lumbreras B, Pascual E, Frasquet J, González-Salinas J, Rodríguez E, Hernández-Aguado I. Analysis for crystals in synovial fluid: training of the analysts results in high consistency. Ann Rheum Dis 2005;64:612-5.

15. Whiting PF, Rutjes AWS, Westwood ME, Mallett S, Deeks JJ, Reitsma JB, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med 2011;155:529-36.

16. Graf SW, Buchbinder R, Zochling J, Whittle SL. The accuracy of methods for urate crystal detection in synovial fluid and the effect of sample handling: a systematic review. Clin Rheumatol 2013;32:225-32.

17. Landewé RBM, Günther KP, Lukas C, Braun J, Combe B, Conaghan PG, et al. EULAR/EFORT recommendations for the diagnosis and initial management of patients with acute or recent onset swelling of the knee. Ann Rheum Dis 2010;69:12-9.

18. Perez Ruiz F, Sanchez-Piedra CA, Sanchez-Costa JT, Andrés M, Diaz-Torne C, Jimenez-Palop M, et al. Improvement in Diagnosis and Treat-to-Target Management of Hyperuricemia in Gout: Results from the GEMA-2 Transversal Study on Practice. Rheumatol Ther 2018;5:243-53.

19. Roddy E, Packham J, Obrenovic K, Rivett A, Ledingham JM. Management of gout by UK rheumatologists: a British Society for Rheumatology national audit. Rheumatology (Oxford) 2018;57:826-30.

20. Perl-Treves D, Addadi L. A structural approach to pathological crystallizations. Gout: the possible role of albumin in sodium urate crystallization. Proc R Soc Lond, B, Biol Sci 1988;235:145-59.

21. Loeb JN. The influence of temperature on the solubility of monosodium urate. Arthritis Rheum 1972;15:189-92.

22. Allen DJ, Milosovich G, Mattocks AM. Inhibition of monosodium urate needle crystal growth. Arthritis Rheum 1965;8:1123-33.

23. Fiddis RW, Vlachos N, Calvert PD. Studies of urate crystallisation in relation to gout. Ann Rheum Dis 1983;42 Suppl 1:12-5.

24. de Médicis R, Dansereau JY, Ménard HA, Lussier A. [Diagnosis of gout: problems caused by crystallization “in vitro” of sodium urate]. Union Med Can 1979;108:810, 812, 814 passim.

25. Beutler AM, Keenan GF, Soloway S, Norden D, Luchi M, Schumacher HR. Soluble urate in sera and synovial fluids from patients with different joint disorders. Clin Exp Rheumatol 1996;14:249-54.

26. Pascual E, Addadi L, Andrés M, Sivera F. Mechanisms of crystal formation in gout-a structural approach. Nat Rev Rheumatol 2015;11:725-30.

27. McGill NW, Dieppe PA. The role of serum and synovial fluid components in the promotion of urate crystal formation. J Rheumatol 1991;18:1042-5.

28. Grassi W, Meenagh G, Pascual E, Filippucci E. “Crystal clear”-sonographic assessment of gout and calcium pyrophosphate deposition disease. Semin Arthritis Rheum 2006;36:197-202.

29. Pascual E, Ordóñez S. Orderly arrayed deposit of urate crystals in gout suggest epitaxial formation. Ann Rheum Dis 1998;57:255.

30. Announ N, Guerne P-A. Treating difficult crystal pyrophosphate dihydrate deposition disease. Curr Rheumatol Rep 2008;10:228-34.

31. Sivera F, Andrés M, Pascual E. Current advances in therapies for calcium pyrophosphate crystal arthritis. Curr Opin Rheumatol 2016;28:140-4.

32. Bennett RM, Lehr JR, McCarty DJ. Factors affecting the solubility of calcium pyrophosphate dihydrate crystals. J Clin Invest 1975;56:1571-9.

33. Schubert J. Chelation in medicine. Sci Am 1966;214:40-50.

34. Zhang W, Doherty M, Pascual E, Barskova V, Guerne P-A, Jansen TL, et al. EULAR recommendations for calcium pyrophosphate deposition. Part II: management. Ann Rheum Dis 2011;70:571-5.

35. Pascual E, Tovar J, Ruiz MT. The ordinary light microscope: an appropriate tool for provisional detection and identification of crystals in synovial fluid. Ann Rheum Dis 1989;48:983-5.

36. Bernal JA, Andrés M, López-Salguero S, Jovaní V, Vela P, Pascual E. Compensated polarized microscopy for crystal identification shows high reliability among multiple observers [abstract]. 2017. p. 1132.

37. Boumans D, Hettema ME, Vonkeman HE, Maatman RG, van de Laar MA. The added value of synovial fluid centrifugation for monosodium urate and calcium pyrophosphate crystal detection. Clin Rheumatol 2017;36:1599-605.

38. Li B, Singer NG, Yeni YN, Haggins DG, Barnboym E, Oravec D, et al. A Point-of-Care Raman Spectroscopy-Based Device for the Diagnosis of Gout and Pseudogout: Comparison With the Clinical Standard Microscopy. Arthritis & Rheumatology (Hoboken, NJ) 2016;68:1751-7.

Table 1. Published studies evaluating the storage of synovial fluid samples for crystal analysis.

Study	No samples	Analyzed crystals	Preservation method	Storage temperature	Time period	Conclusions
Bible *et al*, 1982 (7)	50	MSU	NA	Refrigerated (temperature NA) vs room temperature	24 h	· Crystals were visible at 24 h · No differences at different storage temperatures
Kerolus *et al*, 1989 (8)	50 (5 CPP, 7 MSU, 8 lipids, 6 apatite)*	CPP, MSU, others (lipids, apatite, hematoidin)	Heparin	4°C and 22°C	2 months	· CPP shows dissolution over time · MSU crystals persist but in smaller number · Emergence of artefacts
McKnight and Agudelo, 1991 (9)	6	CPP	Unclear (EDTA, heparin, saline and no anticoagulant for samples from the same patient)	Room temperature	4 weeks	· CPP persistence at room temperature
McGill *et al*, 1991 (10)	11	CPP	No anticoagulant, some with heparin	Room temperature, 4°C, and −70°C	8 weeks	· Persistence with a slight decrease in crystals counts at room temperature and 4°C · No new crystals
Galvez *et al*, 2002 (11)	91 (31 MSU, 30 CPP, 30 no crystals)	MSU, CPP, no crystals	Heparin, EDTA, no anticoagulant	4°C (plus −80°C with no anticoagulant)	24 h vs 72 h 2 months later, at −80°C	· High probability of detecting crystals if those were present first ** · EDTA associated with less agglomeration and better cellular delineation
Tausche *et al*, 2013 (12)	75 (16 MSU, 6 CPP, 5 MSU + CPP, 48 no crystals)	MSU, CPP	EDTA or no anticoagulant	20°C and 4°C	3 days	· No changes in crystal counts for both types, regardless of storage conditions. · No new crystals
Kienhorst *et al*, 2015 (13)	10	MSU	No anticoagulant	−20°C, 4°C, and 20°C	24 weeks	· Persistence in the identification of crystals with no differences between storage temperature
Present work	30 (12 MSU, 18 CPP)	MSU, CPP	EDTA or heparin	20°C and 4°C	7 days	· Persistence of MSU regardless of storage conditions · Decrease of CPP visualization at room temperature and with EDTA preservation

EDTA = ethylenediaminetetraacetic acid, NA = not available, CPP = calcium pyrophosphate, MSU = monosodium urate.

** Each type of crystal is analyzed independently, and there may be samples without crystals or with several types of them. Therefore, the total number of samples is not consistent.*
*** In samples at −80°C visualized after 2 months, the observed decrease in intracellular CPP could be explained by the cell degradation over time.*

Table 2. Results of sample analysis for crystals at each time point, by type of crystal, preservative and storage temperature.

			T0	T1		T2		T3	
			Presence	*Presence*	*Location*	*Presence*	*Location*	*Presence*	*Location*
Crystal	**Temperature**	**Preservative**							
MSU	*20°C*	*EDTA*	100% (12)	91.7% (11)	No: 8.3% (1) IC: 16.7% (2) EC: 33.3% (4) CO: 0% (0) V: 41.7% (5)	91.7% (11)	No: 8.3% (1) IC: 0% (0) EC: 33.3% (4) CO: 0% (0) V: 58.3% (7)	91.7% (11)	No: 8.3% (1) IC: 0% (0) EC: 33.3% (4) CO: 0% (0) V: 58.3% (7)
		Heparin	100% (12)	91.7% (11)	No: 8.3% (1) IC: 16.7% (2) EC: 33.3% (4) CO: 0% (0) V: 41.7% (5)	91.7% (11)	No: 8.3% (1) IC: 0% (0) EC: 33.3% (4) CO: 0% (0) V: 58.3% (7)	91.7% (11)	No: 8.3% (1) IC: 0% (0) EC: 16.7% (2) CO: 8.3% (1) V: 66.7% (8)
	4°C	*EDTA*	100% (12)	100% (12)	No: 0% (0) IC: 8.3% (1) EC: 41.7% (5) CO: 0% (0) V: 50.0% (6)	100% (12)	No: 0% (0) IC: 0% (0) EC: 25.0% (3) CO: 8.3% (1) V: 66.7% (8)	100% (12)	No: 0% (0) IC: 0% (0) EC: 25% (3) CO: 8.3% (1) V: 66.7% (8)
		Heparin	100% (12)	91.7% (11)	No: 8.3% (1) IC: 8.3% (1) EC: 33.3% (4) CO: 0% (0) V: 50.0% (6)	100% (12)	No: 0% (0) IC: 0% (0) EC: 41.7% (5) CO: 0% (0) V: 58.3% (7)	91.7% (11)	No: 8.3% (1) IC: 0% (0) EC: 25.0% (3) CO: 0% (0) V: 66.7% (8)
CPP	*20°C*	*EDTA*	100% (18)	88.9% (16)	No: 11.1% (2) IC: 66.7% (12) EC: 5.6% (1) CO: 0% (0) V: 16.7% (3)	77.8% (14)	No: 22.2% (4) IC: 55.6% (10) EC: 0% (0) CO: 0% (0) V: 22.2% (4)	66.7% (12)	No: 33.3% (6) IC: 55.6% (10) EC: 0% (0) CO: 0% (0) V: 11.1% (2)
		Heparin	100% (18)	100% (18)	No: 0% (0) IC: 55.6% (10) EC: 5.6% (1) CO:11,1% (2) V: 27.8% (5)	83.3% (15)	No: 16.7% (3) IC: 44.4% (8) EC: 0% (0) CO: 5.6% (1) V: 33.3% (6)	77.8% (14)	No: 22.2% (4) IC: 16.7% (3) EC: 0% (0) CO: 16.7% (3) V: 44.4% (8)
	4°C	*EDTA*	100% (18)	83.3% (15)	No: 16.7% (3) IC: 77.8% (14) EC: 0% (0) CO:0% (0) V: 5.6% (1)	94.4% (17)	No: 5.6% (1) IC: 61.1% (11) EC: 0% (0) CO:0% (0) V: 33.3% (6)	77.8% (14)	No: 22.2% (4) IC: 38.9% (7) EC: 0% (0) CO: 11.1% (2) V: 27.8% (5)
		Heparin	100% (18)	88.9% (16)	No: 11.1% (2) IC: 27.8% (5) EC:0% (0) CO: 5.6% (1) V: 55.6% (10)	100% (18)	No: 0% (0) IC: 22.2% (4) EC: 0% (0) CO: 27.8% (5) V: 50.0% (9)	83.3% (15)	No: 16.7% (3) IC: 16.7% (3) EC: 0% (0) CO: 16.7% (3) V: 50.0% (9)

Data shown as % (n). CO= conglomerates, CPP= calcium pyrophosphate, EC= extracellular, IC= intracellular, MSU= monosodium urate, V= various

Table 3. Results of the association analysis by generalized linear models, with presence of crystals as the dependent variable.

Sample	Variable	Coefficient (95%CI)	*P-value*
Whole	***Time***	-0.0013 (-0.001 to -0.002)	<0.001
	Type of crystals		
	CPP	Ref.	-
	MSU	0.0603 (0.011 to 0.110)	0.018
	Temperature		
	Room	Ref.	-
	Refrigerated	0.0391 (-0.009 to 0.088)	0.114
	Preserving agent		
	EDTA	Ref.	-
	Heparin	0.0237 (-0.025 to 0.072)	0.338
MSU crystals	***Time***	0.0007 (-0.000 to 0.002)	0.170
	Temperature		
	Room	Ref.	-
	Refrigerated	0.0402 (-0.032 to 0.113)	0.277
	Preserving agent		
	EDTA	Ref.	-
	Heparin	0.0548 (-0.018 to 0.127)	0.138
CPP crystals	***Time***	-0.0026 (-0.001 to -0.004)	<0.001
	Temperature		
	Room	Ref.	-
	Refrigerated	0.1715 (0.062 to 0.281)	0.002
	Preserving agent		
	EDTA	Ref.	-
	Heparin	-0.0851 (-0.195 to 0.024)	0.128
	Time*temperature	-0.0021 (-0.004 to -0.001)	0.006
	Time*preserving	0.0010 (-0.001 to 0.002)	0.206

95%CI: 95% confidence interval, EDTA = ethylenediaminetetraacetic acid, CPP = calcium pyrophosphate, MSU = monosodium urate.

Table 4. QUADAS-2 quality assessment of diagnostic accuracy test studies evaluating the storage of synovial fluid samples.

Study	Domain	Patient selection	Index test	Reference Standard (crystals at baseline)	Flow and timing
Bible 1982	Risk of bias	☺	Storage temperature: ☹ Conservation: ☹ Time: ☺	?	☺
	Applicability concerns	☹	Storage temperature: ☹ Conservation: ☹ Time: ☹	☹	-
Kerolus 1989	Risk of bias	☺	Storage temperature: ☺ Conservation: ☺ Time: ☺	?	☺
	Applicability concerns	☹	Storage temperature: ☺ Conservation: ☹ Time: ☹	☹	-
Mc Knight y Agudelo 1991	Risk of bias	?	Storage temperature: ☹ Conservation: ? Time: ☺	?	?
	Applicability concerns	☹	Storage temperature: ☺ Conservation: ? Time: ☹	☹	-
Mc Gill 1991	Risk of bias	?	Storage temperature: ? Conservation: ☹ Time: ☹	?	?
	Applicability concerns	☹	Storage temperature: ☺ Conservation: ☹ Time: ☹	☹	-
Gálvez 2002	Risk of bias	☺	Storage temperature: ☺ Conservation: ☺ Time: ☺	?	☺
	Applicability concerns	☹	Storage temperature: ☺ Conservation: ☺ Time: ☺	?	-
Tausche 2013	Risk of bias	☺	Storage temperature: ☺ Conservation: ☺ Time: ☺	?	☺
	Applicability concerns	☹	Storage temperature: ☺ Conservation: ☹ Time: ☹	☺	-
Kienhorst 2015	Risk of bias	☺	Storage temperature: ☺ Conservation: ☺ Time: ☹	?	☹
	Applicability concerns	☹	Storage temperature: ☹ Conservation: ☹ Time: ☹	?	-
Present study	Risk of bias	☺	Storage temperature: ☺ Conservation: ☺ Time: ☺	☺	-

Low risk is represented as emoji ☺ , High risk as ☹ , and Unclear as "?" (14)

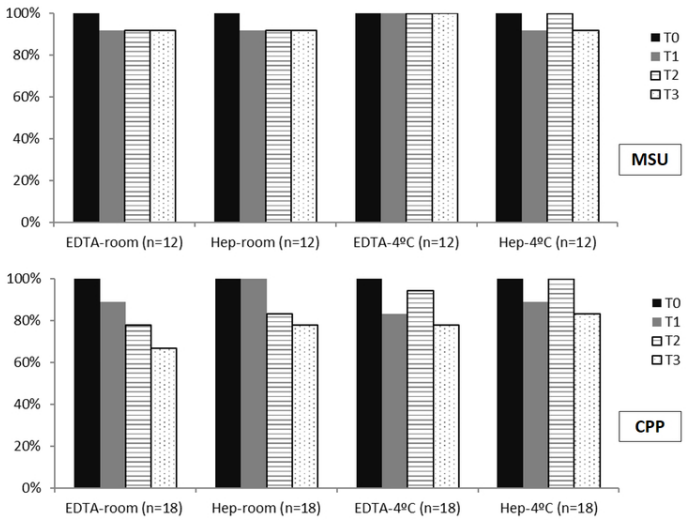

Figure 1. Persistence of MSU (top) and CPP crystals (bottom) at each time point, according to preservative and storage temperature.
EDTA=ethylendiaminetetraacetic acid, HEP= heparin.

90x50mm (300 x 300 DPI)