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Editor

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Screening for Calcifying Nanoparticles in Dermatological Diseases

Rauno J. Harvima[1*], Ilkka T. Harvima[1], K. Maria Kajander[2,3], Anita Naukkarinen[4], and E. Olavi Kajander[5,6]

[1]Department of Dermatology, University of Eastern Finland and Kuopio University Hospital, Kuopio, Finland
[2]Kuopio University Hospital, Hospital Pharmacy, Wellbeing Services County of North Savo, Kuopio, Finland
[3]University of Eastern Finland, School of Pharmacy, Kuopio
[4]Department of Pathology, Kuopio University Hospital, Kuopio, Finland
[5]Sulkava Health Care Center, Sulkava, Finland
[6]Faculty of Medicine, Department of Biomedicine, University of Eastern Finland, Kuopio, Finland

[*]**Corresponding author:** Rauno J. Harvima, MD, PhD, DSc, Department of Dermatology, University of Eastern Finland and Kuopio University Hospital, FIN-70210 Kuopio, Finland. Fax: +358-17-174420. Email: rauno.harvima@kuh.fi

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Dear Editor

Pathological deposits are found in various diseases. Calcification causing agent, described as calcifying nanoparticles (CNPs), has been our interest [1,2]. Recent study by Colboc et al. has revealed that abnormal skin deposits, at submicrometer level, can be studied in more detail using physico-chemical methods, such as Field Emission Scanning Electron Microscopy coupled with Energy Dispersive X-ray Spectroscopy, X-ray fluorescence and vibrational spectroscopy. It is of great interest that skin calcifications of nanometer scale, remaining undetected through classic staining procedures, can be revealed with these novel approaches [3]. Authors press the importance of accurate characterization of skin deposits in improving the diagnosis and pathogenesis mechanisms of many skin diseases. Our work on screening of CNPs, sub-micrometer sized mineralized particles, in various skin diseases might bring new insights and methods for detecting and characterizing submicrometer-sized calcified skin deposits.

Study inclusion criteria was a referral to Kuopio University Hospital Dermatology Clinic due to skin disease. The study was approved by the Ethical Committee of the Kuopio University Hospital (Permit 9/1995 on Jan 17, 1995). The study was a preliminary screening study, and thus, patients from wide variety of skin diseases were recruited during 1995-1998 using consecutive sampling. 70 patients were screened for the study and 46 patients (28 females and 18 males, age range 16-85 years, median age 48 years) who gave consent for analyzing sampling both skin biopsy and serum sample were included in the study. Controls (5 females and 7 males, age range 31-46 years, median age 39 years) were healthy volunteers from Hospital staff without diagnosed skin disease. Biopsy samples were collected under local anesthesia from skin lesion and healthy skin from patients, and from healthy skin from two controls. Skin samples were stored frozen at -20°C until fixed and stained using 8D10 anti-CNP antibody [1,4]. Serum samples were collected by venipuncture and stored at -20°C prior to analysis. Serum samples were analyzed using CNP culture[1] and using in house ELISA test for CNP antigen and IgG[5] and IgM antibodies against CNPs. CNP culture positivity was confirmed using double staining method for CNPs [1].

Diseases studied are shown in Table 1. Altogether 20 patients (43 %) were positive in IFA. Positive finding in IFA was present in dermatitis (75%), lichen ruber planus (67%), urticaria and pruritus (50%), psoriasis (31%, see an example in Figure 1) and necrobiosis lipoidica patients (17%), none of the positives were found in prurigo nodularis whereas CNP culture positivity was detected in all these diseases. Diseases with only 1-2 samples studied are shown only for reference, no conclusions can be withdrawn from them. Only 2 skin samples from controls were obtained and they both were negative in IFA. CNP culture positivity was found in 32/46 patients (70 %), positive finding in ELISAs was detected in 15/46 (33 %) for CNP antigen, 18/46 (40 %) and 6/46 (13 %) patients for anti-CNP IgG and IgM antibodies, respectively. Serum markers for CNP culture and IgM antibodies in controls are at similar levels as in patients. Serum CNP antigen in ELISA has higher positivity rate in patients (33% vs 14%), whereas IgG antibodies are more common in controls (57%) than in patients (40%).

Table 1: Samples studied for CNP markers in the screening study. Values above cutoff values are reported as positive in ELISA tests, calculated values are not shown.

	Sex	Age	IFA	Culture	Antigen ELISA	IgG ELISA	IgM ELISA
Psoriasis (n=12)	F	33	Neg	Pos	Pos	Neg	Pos
	F	41	Pos	Pos	Pos	Neg	Neg
	F	42	Pos	Pos	Neg	Neg	Neg
	F	45	Neg	Pos	Neg	Neg	Neg
	F	85	Neg	Neg	Neg	Neg	Neg
	M	22	Neg	Pos	Neg	Pos	Neg
	M	28	Neg	Pos	Pos	Neg	Neg
	M	28	Pos	Neg	Neg	Neg	Neg
	M	45	Neg	Pos	Neg	Neg	Neg
	M	47	Neg	Pos	Pos	Neg	Neg
	M	58	Neg	Neg	Neg	Neg	Neg
	M	60	Pos	Pos	Neg	Neg	Pos
Lichen ruber planus (n=6)	F	43	Pos	Neg	Neg	Pos	Neg
	F	64	Neg	Pos	Neg	Pos	Neg
	F	73	Pos	Pos	Neg	Neg	Neg
	M	42	Pos	Pos	Pos	Neg	Neg
	M	50	Pos	Neg	Neg	Pos	Neg
	M	70	Neg	Pos	Neg	Neg	Neg
Necrobiosis lipoidica (n=6)	F	16	Pos	Neg	Neg	Neg	Pos
	F	31	Neg	Pos	Neg	Pos	Neg
	F	37	Neg	Neg	Neg	Pos	Neg
	F	50	Neg	Pos	Neg	Neg	Pos
	F	75	Neg	Neg	Neg	Neg	Neg
	M	72	Neg	Neg	Neg	Pos	Neg
Urticaria and puritus (n=5)	F	25	Pos	Pos	Pos	Pos	Neg
	F	60	Neg	Pos	Neg	Neg	Neg
	F	72	Neg	Pos	Neg	Pos	Neg
	F	73	Pos	Pos	Pos	Pos	Neg
	M	69	Pos	Neg	Pos	Pos	Neg
Dermatitis NUD and nummulare (n=4)	F	81	Pos	Pos	Neg	Neg	Neg
	M	33	Pos	Neg	Neg	Neg	Neg
	M	41	Neg	Pos	Pos	Pos	Neg
	M	75	Pos	Neg	Neg	Neg	Neg
Purigo Nodularis (n=3)	F	47	Neg	Neg	Neg	Pos	Neg
	F	48	Neg	Pos	Neg	Neg	Neg
	F	65	Neg	Pos	Neg	Pos	Neg
Miscellaneous							
Amyloidosis	F	48	Pos	Pos	Neg	Pos	Neg
Amyloidosis	F	79	Neg	Pos	Pos	Neg	Neg
Autonomic circulation dysfunction	F	34	Pos	Pos	Neg	Neg	Pos
Erythema induratum Bazin	F	64	Neg	Pos	Neg	Neg	Neg
Erythema induratum Bazin	F	75	Neg	Neg	Pos	Neg	Neg
Granuloma annulare	M	46	Pos	Pos	Neg	Pos	Neg
Lichen sclerosus	M	32	Neg	Pos	Pos	Neg	Pos
Pyoderma gangrenosum	F	40	Pos	Pos	Pos	Pos	Neg
Scleroderma	F	70	Neg	Pos	Pos	Pos	Neg
Scleroderma	M	59	Pos	Pos	Pos	Neg	Neg
Controls (n=7)	F	31	Neg	Neg	Neg	Neg	Pos
	F	33	NA	Neg	Neg	Neg	Neg
	F	39	NA	Neg	Neg	Pos	Neg
	F	45	Neg	Neg	Neg	Pos	Neg
	F	46	NA	Neg	Neg	Neg	Neg
	M	35	NA	Pos	Neg	Pos	Neg
	M	39	NA	Neg	Pos	Pos	Neg

NA= Sample not available for analysis

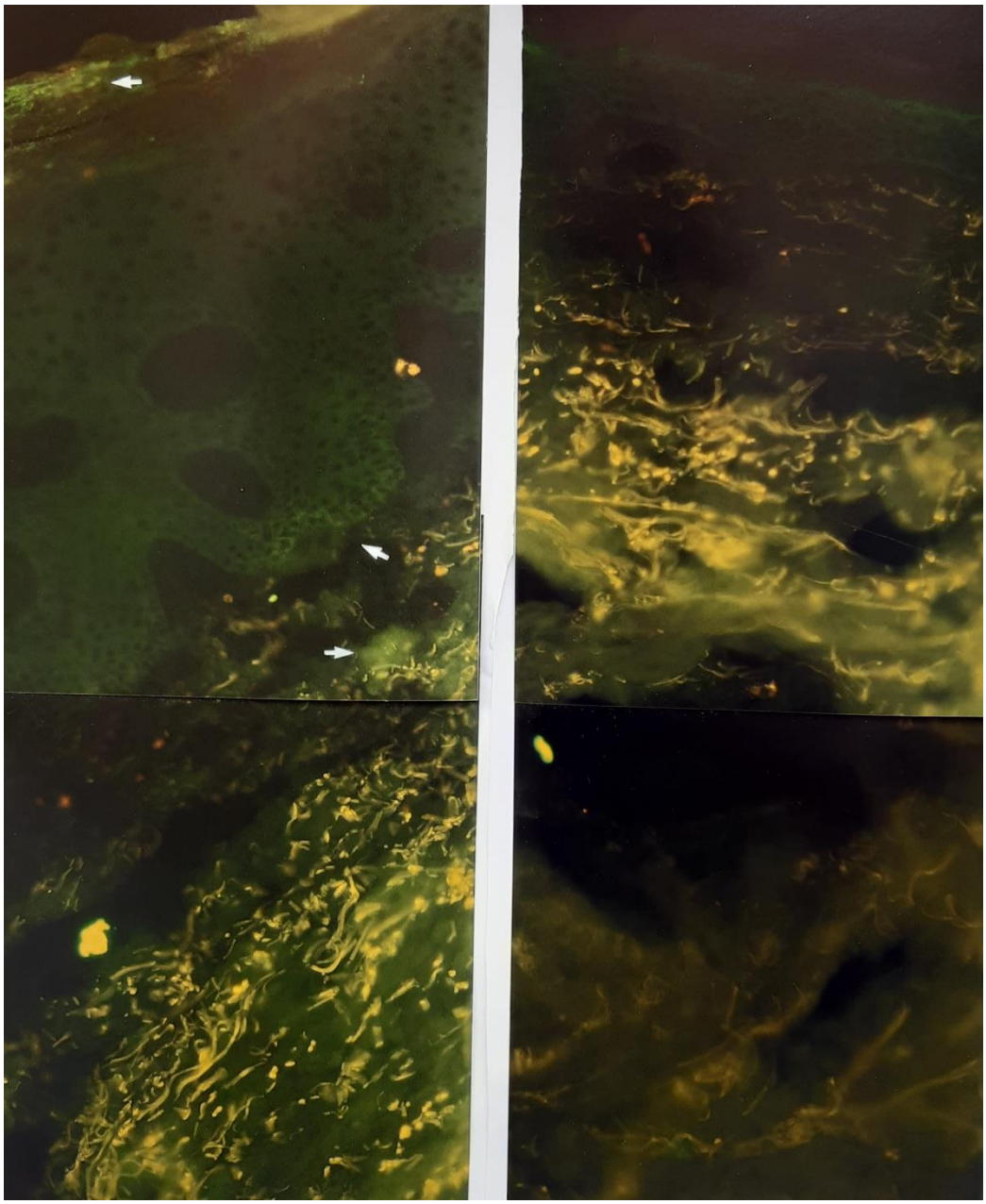

Figure 1: Green-colored fluorescence-positive CNPs shown with white arrows in a 53-year-old male patient of psoriasis vulgaris lesional skin (left), and non-lesional skin (right), taken at edge about 1 cm apart.

CNPs have been found in several pathological calcifications [2]. CNPs have been criticized to be only physiological mineral precipitates, supported by many studies made using calcium and phosphate concentrations well exceeding normal serum levels [6,7]. Ability to replicate is a key characteristic of CNPs in cell culture conditions [1], simple CaP precipitates dissolve in subculture whereas CNPs replicate new particles in subculture [7]. The important role of serum proteins in biological characteristics of calciprotein particles has been recently shown. Calcium phosphate containing calciprotein particles formed in the presence of serum are immunogenic and causing cellular response different from calcium phosphate precipitates [6]. This finding is supported by immunogenicity observed after laboratory exposure to CNPs via eye [8] and cellular response of cultured fibroblasts exposed to CNPs [9]. As discussed by Colboc et al, the presence of nano-sized mineral deposits in skin diseases raises questions whether these apatite deposits are involved in the disease process, or they are products of the actual disease process [3]. Serum of patients and controls contains culturable CNPs. Thus, tissues can be exposed to CNPs via vasculature and/or CNPs can form in tissues and be released to the blood. If CNPs are capable to replicate new particles in tissues under physiological conditions or nanoparticles present in tissues serve as promoters of calcification process, new insights could be achieved on how the nano-scale calcifications form and how they participate in the pathogenesis of various calcification diseases.

Study weakness is the preliminary screening approach. Classical skin diseases with calcifications are rare, thus our sample population is limited. Calciphylaxis and sarcoidosis reported by Colboc et al[3], were not studied, thus it is not exactly known if the CNPs detected with IFA in the skin samples are the same as Colboc et al[3] have found with their analytical methods. Their electron microscopic results are in concordance at anatomical locations with our findings by IFA. Comparative study could

give new information of nanoscale calcium phosphate particles observed in the skin. Our preliminary screening approach is limited in solving mechanism, development and progression of disease with respect to role of CNPs. Medication is a significant confounder as the disease activity is often diminishing during the treatment period, and therapy affects the disease process. Our small sample size is limiting statistical evaluation with respect to comorbidities of study population and physiological markers, e.g., for kidney function.

In summary, this study shows that the CNP antigen can be detected in skin samples from variety of skin diseases using IFA method. Positive serum markers for CNP are present in both skin diseases and controls. This preliminary study provides new insights into research of skin diseases of unknown suspectedly infectious origin and/or with calcifications. How the presence of antigen and antibodies is related to disease activity and prognosis, cannot be answered by this screening study, and further studies are encouraged.

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Conflicts of Interest
The authors have nothing to disclose.

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Data Sharing Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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