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Human host defence peptide LL37 and anti-cyclic citrullinated peptide antibody in early inflammatory arthritis

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ABSTRACT

Objective Antibodies to citrullinated peptides (anti-CCP) develop in individuals predisposed to rheumatoid arthritis (RA). Neutrophil extracellular traps are a major source of citrullinated antigens and the immunomodulatory host defence peptide LL-37. Vitamin D regulates LL-37 expression. This study assessed the associations of LL-37 and anti-CCP, vitamin D metabolites and vitamin D receptor (VDR) polymorphisms in early inflammatory arthritis (EIA).

Methods Serum LL-37, 25-hydroxy-vitamin D (25OHvitD) and anti-CCP were measured by ELISA in treatment naïve EIA (n = 181). VDR single nucleotide polymorphisms (Fok1, Bsm1, Apa1, Taq1, Cdx-2) and HLADRB1 shared epitope (SE) alleles were detected by DNA amplification. Associations were tested in multivariable models. Median (25%, 75%) or percentiles are reported.

Results Participants (70 % female, age 56 [45, 66] years, disease activity score [DAS28ESR3var] 3.7 [2.8, 4.8], 41 % anti-CCP positive, 68 % RA) had low serum 25OHvitD; 20.5 nmol/L (13.9, 29.0). In multivariable models, controlling for age, sex, SE, smoking and vitamin D deficiency, LL37 level (top quartile) associated with anti-CCP seropositivity (OR 22; 95% CI 4 to 104).

Conclusions Levels of circulating LL-37 are associated with anti-CCP seropositivity. LL37 activity may be one mechanism linking infection and toxin exposure to anti-CCP generation.

Key messages

What is already known about this subject?

- Human host defence peptide cathelicidin (LL-37) regulates inflammation and promotes autoimmune responses.

What does this study add?

- Circulating LL-37 associates with anti-CCP in early inflammatory arthritis.
- Circulating LL-37 does not associate with serum 25(OH)vitamin D or with common vitamin D receptor polymorphisms.

How might this impact on clinical practice?

- This suggests LL-37 may have a role in the development of inflammatory arthritis.

Dysregulated citrullination at articular and extra-articular sites leads to the generation of anti-citrullinated peptide autoantibodies in individuals with genetic susceptibility to rheumatoid arthritis (RA).[1] One source of citrullinated autoantigens in RA is activated neutrophils which release neutrophil extracellular traps (NETs) in response to infections and toxins.[2] NETs contain a high concentration of the human host defence peptide cathelicidin (LL-37)[3] a protein which regulates inflammation and promotes autoimmune responses.[4–8] However, it is not clear if the formation of autoantibodies to citrullinated proteins is enhanced by LL-37 in RA.

LL-37 expression can be regulated by vitamin D, which is also associated with autoimmune pathways relevant to RA.[9] Gene expression of hCAP18, the preproprotein of LL-37, is governed by the vitamin D/vitamin D receptor (VDR) complex.[10] Even though low serum vitamin D levels and VDR polymorphisms are associated with RA in some populations,[11] the association of vitamin D mediated expression of LL-37 with pathogenic events in RA remains unclear.

In this study we aimed to delineate the association between LL-37 with antibodies to citrullinated peptides, circulating vitamin D and VDR polymorphisms, in early inflammatory arthritis (EIA).

METHODS

Study participants

Participants with inflammatory arthritis for less than 1 year and naïve to disease modifying anti-rheumatic drug therapy were recruited from outpatient rheumatology clinics. Patients with RA,[12] undifferentiated arthritis (UA) (inflammatory arthritis not meeting criteria for defined arthropathy) or spondyloarthropathy (SpA) (predominantly psoriatic arthritis or reactive arthritis)

were included. Smoking status was assessed by self-report (never, past, current smoker). Arthritis activity was assessed using the disease activity composite score (DAS28ESR3var) and function using the modified health assessment questionnaire (mHAQ).

LL37 and 25-hydroxy vitamin D (25(OH)D)

Commercial ELISA kits were used to measure the concentrations of LL37 (HK321; Human LL-37 ELISA Kit, Hycult Biotechnology, Uden, the Netherlands) and 25(OH)D (AC-57F1; 25-hydroxy vitamin D, EIA Immunodiagnostic Systems, Scottsdale, Arizona, USA), in DMARD naïve serum samples. Levels are reported as median with (25%, 75% quartile).

Anti-cyclic citrullinated peptide (anti-CCP) antibody and rheumatoid factor

Anti-CCP2 was measured with a commercial ELISA kit (Inova Diagnostics, San Diego, California, USA) and positivity assessed according to the manufacture's guidelines.

Table 1 Demographics and clinical characteristics of study participants

	Rheumatoid arthritis	Undifferentiated arthritis	Spondyloarthropathy
N	123	33	25
Age years	61 (52, 68)	48 (40–53)	46 (38.5–56)
Sex (F)	71%	88%	52%
Anti-CCP ever positive	80/123 (65%)	2/33 (6%)	1/25 (4%)
RF and CCP positive	56%	0%	0%
HLADRB1 SE positive	73/110 (66%)	9/27 (33%)	8/22 (36%)
DAS28ESR3var	4.4 (3.2–5.2)	2.7 (1.9–3.8)	3.6 (2.9–4.5)
mHAQ	0.5 (0.0, 0.9)	0.2 (0–0.5)	0.5 (0.2–1)
Smoking status	n=102	n=29	n=20
Never smoker	34%	31%	40%
Ex-smoker	42%	38%	35%
Current smoker	24%	31%	25%
LL37 ng/mL	11.8 (5.9, 24.4)	12.1 (4.1–16.0)	11.9 (3.5–18.0)
Serum 25(OH)D mmol/L	21.5 (14.4–31.3)	17.3 (12.8–23.2)	19.6 (10.9–30.8)
25(OH)D deficient (%)	60%	82%	64%
Vit D receptor polymorphism			
FokI			
CC	45/118	15/33	8/24
CT	53/118	10/33	11/24
TT	20/118	8/33	5/24
Bsm1			
TT	28/112	8/33	4/23
CT	51/112	14/33	9/23
CC	33/112	11/33	10/23
Apa1			
TT	26/117	5/29	6/24
GT	64/117	16/29	14/24
GG	27/117	8/29	4/24
Taq1			
TT	42/117	12/29	12/24
TC	59/117	15/29	11/24
CC	16/117	2/29	1/24
Cdx1			
GG	0/52	0/14	0/14
GA	52/52	14/14	14/14
AA	0/52	0/14	0/14

Categorical variables are reported as n/N measured (%), continuous variables are median (25%, 75%)±range.
CCP, cyclical citrullinated peptide; RF, rheumatoid factor; SE, shared epitope.

Table 2 Association of LL37 level and anti-CCP antibody status*

Model	OR	95% CI OR
Model 1		
Age	1	1.0–1.1
Gender (female)	1	0.4–2.1
Vitamin D deficient†	1.4	0.7–2.8
LL37		
LL37 Q1	Ref	–
LL37 Q2	2.7	1.0–6.9
LL37 Q3	1.6	0.6–4.4
LL37 Q4	6.3	2.4–17.0
Model 2		
Age	1	1.0–1.0
Gender (female)	0.5	0.2–1.5
Vitamin D deficient†	2.2	0.8–5.9
Smoking status		
Never	Ref	
Quit	2.3	0.8–6.6
Current	3.4	1.0–11.8
Shared epitope positive	5.2	1.8–14.5
LL37		
LL37 Q1	Ref	
LL37 Q2	9.1	2.0–41.4
LL37 Q3	3.2	0.7–14.7
LL37 Q4	22.1	4.7–104.1

*Dependent variableanti-CCP seropositive status.
†Similar findings whenvitamin D status was assessed as vitamin D receptor haplotype (FokI, Bsm, Apa orTaq).
.Q, quartile; ref, reference category.

Rheumatoid factor (RF) was measured by nephelometry in the hospital laboratory and positivity defined as RF titre >40 IU.

HLA and VDR genotyping

HLA-DRB1 typing was performed by PCR using sequence-specific oligonucleotide primers and shared epitope (SE) bearing alleles defined as previously described.[13] VDR single nucleotide polymorphisms; Bsm1 (B/b (T/C)) (rs1544410), Apa1 (A/a (T/G)) (rs7975232), Taq1 (T/t (T/C)) (rs731236), Fok1 (F/f (C/T)) (rs10735810) and Cdx-2 (G/A) (rs11568820) were amplified from genomic DNA using published protocols and primers as previously described.[14]

Statistical analysis

Associations of serum LL37 with clinical disease and other parameters were tested using non-parametric methods. We tested the association of LL37 level (quartile) with anti-CCP positivity in logistic regression models. Model covariables were age, sex and vitamin D status measured as 25(OH)D level (deficient vs not deficient) or VDR. Subsequent models included self reported smoking status and SE status. Statistics were performed using SPSS V.24.

RESULTS

Study participants

Demographics, clinical characteristics and laboratory output of participants are shown in table 1. Study participants were predominantly female (70%) with a median (25th quartile; 75th quartile) age of 56 years (45,66) and 68% met criteria for RA. Nearly half (41%) of the participants were seropositive for anti-CCP of which 86% were also RF seropositive. Only 16% were seropositive for RF alone (table 1). Disease activity was moderate with median (25th, 75th percentile) DAS28ESR3var 3.7 (2.8, 4.8).

Serum LL-37, 25(OH)D, anti-CCP

Serum LL37 levels varied widely (median LL37 11.8 ng/mL [5.9, 22.3]) but were similar across RA, UA or SpA (median [25%, 75%; range]; RA 11.8 ng/mL [5.9,24.4; 0–2303], UA 12.1 ng/mL [4.1,16.0; 0–34.7]; SpA 11.9 ng/mL [3.5,18.0; 0–4050], p=0.4) (table 1). Therefore, subsequent analyses combined all three diagnostic categories. All participants had low serum 25(OH)D (median [25%, 75%; range]); 20.5 (20.4 nmol/L [13.6–29; 4.1–63.1]) and 64% of participants were vitamin D deficient with serum levels of 25(OH)D <25 nmol/L. LL37 and 25OH vitD levels did not correlate (rho −0.1, p=0.2). LL37 and anti-CCP2 titres correlated (rho 0.3, p<0.0001).

Multivariable models for independent association of LL-37 with anti-CCP

We tested the association of serum levels of LL-37 with anti-CCP using logistic regression models controlling for age, sex and vitamin D deficiency (table 2). Top quartile serum LL-37 was associated with positive anti-CCP status (OR 6.3 [CI 2.4 to 17.0]). The association of LL37 and anti-CCP remained significant when smoking and SE allele status were included in the model. Similar findings were seen when vitamin D status was assessed by VDR genotype, or when models included arthritis diagnosis (data not shown).

Association of LL-37 with clinical variables and VDR polymorphisms

LL-37 levels did not correlate with clinical disease activity (DAS28ESR3var Spearmans rho 0.07, p=0.4; ESR rho 0.04, p=0.6; swollen 28 joint count rho 0.1, p=0.1), mHAQ (rho 0.03, p=0.7) or with self-reported smoking (never [11.2 ng/mL; 4.9, 21.8; 0–4050], past [11.9 ng/mL; 7.2, 21.8; 0–1469.2], current [14.2 ng/mL; 8.6, 47.9; 0–2303], Kruskal-Wallis test p=0.4). The distribution of VDR polymorphisms (Fok1, BsM1, ApA1, Taq, Cdx1) was similar across the three diagnostic groups (RA, UA and SpA) (table 1) and 25(OH)D levels were similar across the different VDR polymorphisms (data not shown). HLA-DRB1 SE positivity was associated with anti-CCP

Table 3 Circulating levels of LL-37 based on antibody shared epitope and vitamin D status

Anti-CCP negative	10.9 (4.1, 16.7) 0–4050.2	P=0.001
Anti-CCP positive	17.1 (8.7, 43.3) 0–2303.1	
RF negative	10.4 (3.8, 14.3) 0–4050.2	P=0.001
RF positive	16.6 (8.2, 30.5) 0–2303.1	
Number of autoantibodies		
RF and anti-CCP negative	10.4 (3.8, 13.9) 0–4050.2	P<0.0001
RF or anti-CCP positive	10.9 (5.4, 23.4) 0–1073.4	
RF and anti-CCP positive	17.4 (9.24, 47.7) 0–2303.1	
Shared epitope positive	11.8 (6.1, 23.7) 0–2303	P=0.5
Shared epitope negative	10.9 (4.1, 21.5) 0–4050.2	
25(OH)D <25 nmol/L (deficient)	12.2 (7.2, 25.3) 0–4050.2	P=0.08
25(OH)D >25 nmol/L (adequate)	10.9 (4.1, 19.9) 0–1469.2	
Smoking status		
Never smoked	11.2 (4.9, 21.9)0.1–4050.2	P=0.4
Quit smoking	11.9 (7.2, 21.8) 0–1469.2	
Current smoking	14.2 (8.6, 47.9) 0–2303.1	
Vit D receptor polymorphism		
FokI	10.6 (5.3, 24.7) 0–2303	P=1.0
CC	11.9 (5.0, 21.6) 0.1–4050.1	
CT	12.3 (6.8, 19.4) 0–1469.2	
TT		
Bsm1	11.6 (8.5, 17.1) 0.2–550.8	P=0.3
TT	13.4 (6.5, 23.6) 0–4050.2	
CT	9.6 (4.1, 21.0) 0–1469.2	
CC		
Apa1	11.6 (7.4, 17.1) 0.23–550.8	P=1.0
TT	11.6 (5.0, 26.0) 0–4050.2	
GT	11.9 (4.9, 22.3) 0–1469	
GG		
Taq1	10.2 (4.7, 22.0) 0–1469.2	P=0.8
TT	11.9 (5.5, 23.9) 0–4050.2	
TC	12.5 (8.8, 17.3) 0.2–550.8	
CC		

Median (25th, 75th quartile) (range) reported. Statistical significance determined using Man-Whitney U non-parametric two group comparison or Kruskal-Wallis test for three group comparison.
CCP, cyclical citrullinated peptide; RF, rheumatoid factor.

(OR 5.2; CI 1.8 to 14.5) however, no associations were seen between LL-37 and HLA-DRB1 SE or VDR receptor polymorphisms (table 3). Similar findings were observed in analyses including only RA subjects (data not shown).

DISCUSSION

Cathelicidin LL37 expression is associated with anti-CCP seropositivity in patients with DMARD naïve EIA, however, serum LL37 levels do not associate with common VDR polymorphisms, circulating vitamin D or articular activity.

Several lines of evidence support a role for LL37 in autoimmune disease.[15] In lupus and psoriasis, LL-37 increases immunogenicity and stability of DNA facilitating plasmacytoid dendritic cell activation. Autoantibodies directed to LL37-DNA complexes are detected in multiple organs of lupus patients.[6 8] In psoriasis, LL37 is an autoantigen for pathogenic T helper cells and circulating levels are elevated over healthy controls but do not correlate with skin activity.[16] In psoriatic arthritis, synovial fluid has elevated LL37 protein levels and antibodies to carbamylated LL37, but not citrullinated LL37, correlate with articular disease activity.[7] In RA, LL37 and C1q enhance NET induced macrophage activation,[4] LL37 protein expression is elevated in serum and synovium[17 18]

but, as in this report, serum levels of LL37 protein are not correlated with disease activity and neither are antibodies to LL37.[18] LL37 induces induced osteoblast apoptosis,[19] suggesting potential to contribute to arthritis associated osteopenia. We report the association of elevated levels of LL37 with anti-CCP seropositivity in EIA.

The observed association of LL-37 with anti-CCP may reflect exposure to infectious or non-infectious factors implicated in RA pathogenesis and host responses. LL37 contributes to innate anti-microbial defences and although it is unclear if LL-37 citrullination occurs in vivo, citrullination of LL37 impairs its anti-infective activities in vitro[20] potentially increasing bacterial burden. In periodontal disease, a known risk factor for RA, LL37 gingival levels are elevated and RA-like hypercitrullination develops in neutrophils exposed to pore forming toxins produced by *A actinomycetemcomitans,* a periodontal pathogen associated with ACPA positive RA.[21–23] Smoking induced damage associated molecular pattern responses are enhanced by LL37 in patients predisposed to non-rheumatic lung disease.[24] Thus, LL37 may partly mediate immune responses relevant to RA that are induced from interactions between environmental factors and articular or non-articular host tissues.

Vitamin D has multiple effects on immune mechanisms and cellular metabolism potentially relevant for autoimmunity[9] and is important for LL37 expression.[10] However, we did not find robust associations of LL37 with serum 25OHvitD or VDR polymorphisms, even though both have been associated with RA in some populations. This may reflect uniform vitamin D insufficiency in our cohort, the small sample size or the VDR polymorphisms tested. Of the polymorphisms tested only Fok1 leads to functional changes in the receptor whereas the functional implications of the other polymorphisms are less clear; other VDR polymorphisms may be relevant to LL37 expression. However, metabolic stress independent of vitamin D can induce LL37 expression.[25] Alternatively, preformed LL37, or reduced LL37 degradation, may be sufficient to generate autoimmune responses. Data to support this are needed.

Our study has limitations. Samples were obtained in subjects with new onset arthritis prior to instituting immunomodulatory therapy. In this study, we cannot assess the trajectory of LL37 during the preclinical disease phase or after therapy. Our analysis of serum vitamin D is limited as all participants had low levels. We combined different arthropathies due to small sample sizes however, results were similar when we limited the analysis to only RA. Although the HK321 LL-37 ELISA does not discriminate between LL-37 and the precleaved hCAP18 protein, in general hCAP18 is restricted to intracellular granules thus in circulation the assay reflects LL37 protein.

In this cohort of EIA, cathelicidin (LL37) levels associate with seropositivity to anti-CCP, further supporting the role of LL37 in development of inflammatory arthritis.

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Contributors CAH, LL conceived and designed study. LL, XM conducted assays. CAH, HEG, LL interpreted data and drafted the manuscript. All authors approved final manuscript.

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Competing interests Carol Hitchon has received unrelated research funds from Pfizer, UCB Canada.

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