The autoimmunePTPN22 variant rs2476601 is associated with rheumatoid arthritis risk but not with systemic bone loss.

Abstract:

Introduction: PTPN22 is a powerful inhibitor of signaling cascades initiated in various immune cells. The association between the *PTPN22* variant rs2476601 and rheumatoid arthritis (RA) varies by ethnicity. *PTPN22 rs2476601* exhibits the strongest non-HLA association with RA in Caucasians. However, data in the Tunisian population are inconsistent.Among patients with RA, *PTPN22* rs2476601 has been shown to be associated with bone erosion and autoantibodies. However, no association with systemic bone loss (SBL) has been investigated so far. The aim of this study was to assessthe association of PTPN*22 rs2476601* with RA risk and SBL.

Methods:In this case-control cross-sectional study, PTPN*22 rs2476601* was genotyped by PCR-Restriction Fragment Length Polymorphism in 363 Tunisian patients with RA and 217 controls. Bone mineral density was assessed by dual-energy X-ray absorptiometry in 216 RA patients.

Results:*PTPN22 rs2476601* was associated with RA, with the minor T allele increasing the risk of RA (OR = 14.27 (95% CI = 3.44-59.13), p=3.45 x 10^{-6}). *PTPN22 rs2476601* was not associated neither with autoantibodies nor with bone erosion. In the univariate analysis, only a trend towards association was observed between *PTPN22 rs2476601* and total femur osteopenia (OR=7.56 (0.91-62.53), p=0.034). No association was revealed by the multivariate analysis.

Conclusion:Our study confirms the association between *PTPN22* rs2476601 and RA but did not reveal an association between this variant and SBL. The exact impact of PTPN22 rs2476601 on SBL needs to be assessed in large scale studies.

Key words: Rheumatoid arthritis, PTPN22, rs2476601, systemic bone loss.

Introduction

RA is a progressivedisabling diseaseprimarilyaffecting the joints; it is however, a systemic disease with significant extra-articular manifestations, including systemic bone loss (SBL). SBL culminates with osteoporosis which affects 30-50% of patients with RA(1, 2). RA is a health problem worldwide and poses a substantial economic burden (3). Patients with RA are at a significantly higher risk of osteoporotic fractures; notably, a recent Mendelian randomization study highlighted the causal association between RA and fracture (4).Studies have shown that reducing RA risk factors and treating incipient cases is significantly less costly than hospitalization, surgery, and work disability (5).

The etiology of RA is not fully understood. However, significant progress has been made in identifying the main contributing genetic factors. The *PTPN22* gene located on chromosome 1 is the main non-human leukocyte antigen (HLA) gene involved in RA in Caucasians. This gene encodes for PTPN22 or LYP, a protein tyrosine expressed in hemopoietic cells. PTPN22 is considered as a powerful inhibitor of signaling cascades initiated in T cells, B cells, and innate immune cells (6-8). Its inhibitory role has been most extensively studied in T cells, where it regulates T-cell activation by dephosphorylating kinases involved in proximal antigen receptor (TCR) signaling (9-11).*PTPN22*-deficient mice exhibit increased T-cell proliferation with significant expansion of the effector and memory T-cell compartments. More recently, PTPN22 has been shown to regulate platelets activation and control formation of thrombus(12). By

contrast, PTPN22 activates innate immunity by triggering type I interferon release by dendritic cells and macrophages(13). In addition, PTPN22 seems to regulate Th17 differentiation by controlling IFN-induced signaling (14).

The association between *PTPN22* rs2476601 and RA varies by ethnicity and reveals inconsistencies. *PTPN22* rs2476601 is associated with the risk of RA in Caucasians (15), but in Tunisians and Asians, data are inconsistent (15-18).*PTPN22* rs2476601 has been shown to be associated with bone erosion and the presence of autoantibodies, however no association with systemic bone loss (SBL) has been investigated so far. This study investigated the association between *PTPN22* rs2476601 and RA risk in the Tunisian population. Moreover, it assessed the relationship between *PTPN22* rs2476601 and SBL in RA patients.

Patients and metods

Study population

Our case control cross-sectional study enrolled 363 patients with RA and 217age and gender matched healthy controls without any personal or family history of autoimmune diseases. Patients with RA were recruitedbetween 2018 and 2021. RA diagnosis was based on the ACR EULAR 2010 criteria for RA (19).Patients taking anti-osteoporosis regimes (bisphosphonates) were excluded. Data regarding menopause, comorbidities, smoking, and alcohol intake were collected. RA duration was calculated as the time period between the initial self-reported joint symptoms and diagnosis. Body mass index (BMI) was calculated bydividing the weight (in kilograms) by the height (in meters squared).Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Disease Activity Score 28 with ESR (DAS 28), Sharp–van der Heijde score and patient management details (cumulative dose of glucocorticoids, conventional synthetic disease-

modifying antirheumatic drugs (csDMARDs), biological DMARDs (bDMARDs), and osteoporosis regimens were also collected.

The patients' written informed consents were acquired. The study was approved by the Ethical committee of Farhat Hached Hospital (IRB protocol number CEMR-16-157, approval date 15/07/2016).

Methods

Bone mineral density measurement

Bone mineral density (BMD) was measured at three sites: femoral neck (FN), total femur (TF), and lumbar spine (LS) by dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance Scans, GE Healthcare, USA). BMD was calculated by dividing bone mineral content (g) by bone area (cm^2). Osteoporosis andosteopenia were diagnosedbased upon T-scores in accordance with WHO criteria:osteoporosis was diagnosed in patients with T-score ≤ -2.5 standard deviation (SD) and osteopenia in patients with -1 SD <T score >- 2.5 SD (20).

The association of *PTPN22* rs2476601 with SBL was also investigated using the Z-score at various cut-offs.

PTPN22 rs2476601 genotyping

Genomic DNA was extracted from peripheral whole blood by salting out method. Samples were stored at −80ºC until analysis. Genotyping was conducted using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP). The following primers were used to amplify the extracted genomic DNA: 5'TCA CCA gCT TCC TCA ACC ACA3 (forward primer) and 5′gAT AAT gTTgCT TCA ACggAA TTT A3′ (reverse primer). The PCR reaction was performed in a total volume of 20 µL using 0.5 µL of each primer (10 µM), 0.2 µL dNTP (20mM), 1.5 µL MgCl2 (25 mM), 1 unit of Taq polymerase, and 100 ng of genomic DNA.

Amplification was done with a denaturation step at 95 ºC for 5 min, then 34 cycles of denaturation at 95 ºC for 30s, annealing at 56 ºC for 30 s, extension at 72 ºC for 30s, and a last cycle of extension at 72 ºC for 7 min. Digestion of the obtained amplification product by the restriction enzyme *Xcm*I was performed in a volume of 20 µL, containing 4 µL of PCR product, 0.4 µL of *Xcm*I (5,000 U/mL, New England Biolabs Inc.), 2 µL of 10× enzyme buffer, and 13.6 µL of ddH_2O. The amplified PCR fragment was 215 bp in size. Only amplified fragments with the *PTPN22 rs2476601* T allele were cut with *Xcm*I, generating two fragments of 46 bp and 169 bp. After incubation overnight at 37ºC, the restriction digestion products were separated on 3% agarose gel stained by ethidium bromide next to a DNA size marker and visualized under UV light.

Autoantibodies measurements

Serum anti-citrullinated protein antibodies (ACPAs) were assessed using a second-generation ELISA (Euroimmun, Lubeck, Germany). Serum IgM rheumatoid factor (IgM RF) was measured by ELISA (Orgentec, Hamburg, Germany). Tests were performed according to the manufacturers' instructions. Optimal antibody cut-off points were determined as described before (21).

Statistical analysis

Statistical analysis was conducted by SPSS 26.0 for Windows statistical software (SPSS, Chicago, IL). Odds ratios (ORs) were calculated using epi info^{TM}version 7.2.5. Atlanta, GA:CDC.The Hardy–Weinberg equilibrium was checked in patient and control groups using chi-square test (χ^2). Continuous variables were expressed as mean or median as appropriate. Student's *t* test was used to compare the means of variables with a normal distribution and Mann-Whitney test was applied for variables that did not follow normal distribution. Chi square test

and Fisher test were used for qualitative data. To identify risk factors associated with SBL,a multivariate analysis was conducted. Independent variables included in the multivariate analysis were variable with $p < 0.2$ among the following variables: gender, *PTPN22 rs2476601*, age,BMI, smoking, alcohol intake, diabetes, dysthyroidism, disease duration, CRP, ESR, DAS28, ACPAs, and cumulative dose of glucocorticoids. Statistical power was estimated using OpenEpi. A *p* value < 0.05 was considered as significant.

Results

RA Patients Characteristics

Among the 363 patients, 306 (84.3%) were females and 57 (15.7%) were males. The mean age of the patients was 53.4 (±11.5) years. Clinical data were available in 230 patients (**Table 1**). Among these, 198 (86.1%) were females. The mean age was 54.9 (±11.5) years.Most Females patients were post-menopausal (80.3%). Majority ofpatients had active disease with a median CRP of 20 (8-50) mg/L and a mean DAS28 of 5.5 (±1.5).

PTPN22 rs2476601 and RA

The C allele was present in 93.8 % of patients and 99.5% of controls, whereas the T allele was present in 6.2 % of patients and in 0.5% of controls. The total T allele frequency in patients and controls was 4%. The genotype frequencies in both patients and controls were in Hardy–Weinberg equilibrium ($\chi^2 = 2.1$ and $\chi^2 = 0.05$, respectively). The statistical power was estimated to be 97.5%. An association between *PTPN22 rs2476601* and RA was found with the T allele conferring a 14.27-fold increased risk of RA (OR= 14.27 (95% CI = 3.44-59.13), $p=3.45 \times 10^{-6}$ (**Table2**). The OR for heterozygote CT carriers *vs.* CC carriers was 13.06 (95% CI = 3.12-54.65, $p = 15 \times 10^{-6}$).

ACPAs were tested in 229 patients among the 230 and were positive in 180 (78.6%) patients (**Table 1**). ACPAs were positive in 171 (77.7) patients among the 220 carrying the CC genotype and in all the 9 (100%) patients carrying the CT genotype. RF IgM was performed in 204 patients and was positive in 137 (67.1%) patients. It was positive in 131 (67.1%) patients among the 195 harboring the CC genotype and in 6 (66.6%) patients among the 9 harboring the CT genotype. No associationbetween *PTPN22 rs2476601*and ACPAs or RF was observed. Titers of ACPAs and RF were comparable between patients with CC and CT genotypes: median ACPA levels were 95 (14–200) RU/mL vs. 85 (15–145) RU/mL (p = 0.1), and median IgM RF levels were 325 (109–500) IU/mLvs. 138(118–297) IU/mL (p = 0.2), respectively.

Erosion was detected in 203 (91.8%) of the 221 patients investigated. Among these patients, 195 (96.1%) had the CC genotype and 8 (3.9%) had the CT genotype. All the 18 (100%) patients with no erosion had the CC genotype. *PTPN22 rs2476601* was not significantly associated with erosive arthritis (p = 1). The median Sharp–van der Heijde score was lower in patients with the CC genotype compared to patients with the CT genotypes: 87 (52–121) vs.102 (82–206), with no statistical significance (p=0.1). Analysis of the relationship between PTPN*22 rs2476601* and erosion after stratification by autoantibodies was not possible statistically because of the low number of patients harboring the T allele.

PTPN22 rs2476601 and SBL

FN absolute BMD values were available for 207 patients whereas TF and LS BMD values were available for all the 216 patients. **Table 3** shows the distribution of absolute BMD values, osteopenia, and osteoporosis at the three measured sites. Osteopenia at FN, TF, and LS was observed in 101 patients (48.7%), 107 patients (49.5%), and 75 patients (34.7%), respectively. Osteoporosis was observed at the FN in 48 patients (23.1%), at the TF in 36 patients (16.6%),

and at the LS in 87 patients (40.2%). Out of 107 patients with TFosteopenia, 100 (93.5%) carried the CC genotype and 7 (6.5%) the CT genotype whereas out of the 109 patients without osteopenia, 108 (99.1%) carried the CC genotype and 1 (0.9%) the CT genotype.

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Discussion

This study revealed that *PTPN22 rs2476601*was associated with risk of development of RA but not with SBL in the Tunisian population. *PTPN22 rs2476601* exhibits a strong association with autoimmune diseases (22).However, a meta-analysis indicated that the strength of this association varies across different diseases. *PTPN22 rs2476601* is strongly associated with RA, type 1 diabetes, systemic lupus erythematosus, systemic sclerosis, psoriatic arthritis, juvenile idiopathic arthritis, and some forms of vasculitis. By contrast, its association with other autoimmune diseases, such as multiple sclerosis, ankylosing spondylitis, psoriasis, pemphigus vulgaris, celiac disease, and ulcerative colitis, is weak (23).Our study confirmed the association between *PTPN22 rs2476601* and RA in the Tunisian population. Two previous Tunisian studies

on *PTPN22* rs2476601 and the risk of RA have been limited by small sample sizes which may have affected the statistical power and explain the discordant results of these studies (16, 17).

The *PTPN22 rs2476601* variant is most common in Northern Europeans, particularly Scandinavians (23, 24)among whom it is present in approximately 10% of the population (25)whereas it is rare in Asians and virtually absent in individuals of African origin (26, 27).In our study, this variant was present in 4% of patients and controls.The association of*PTPN22 rs2476601* and RA was initially reported in 2004, with an OR of 2.63 for the homozygous TT genotype versus the CC genotype (28). In 2019, Mustelin et al.(26) estimated the OR for heterozygote CT carriers to be 1.7 for RA. According to previous reports, the OR is higher in patients who are RF-positive (29), in our study, the OR for Tunisian heterozygote CT carriers versus CC carriers was 13.06. Several studies assessed the relationship between PTPN22 rs2476601 and autoantibodies with discrepant results (30-33). The present study revealed no association between *PTPN22 rs2476601* and ACPAs or RF.

Substantial evidence shows that autoimmunity and inflammatory processes in RA play a critical role in bone homeostasis. Considering the influence of *PTPN22 rs2476601* on innate and adaptive immunity, we hypothesized that *PTPN22 rs2476601* may impact SBL. Indeed, in mouse models, *PTPN22* is essential in maintaining tolerance (34).Along with its role in T- and B-cell regulation, PTPN22 triggers type 1 IFN production through TRAF3 ubiquitination. Moreover, *PTPN22* regulates gut homeostasis and suppresses inflammatory arthritis (13). Loss of tolerance towards citrullinated proteins and ACPA formation are critical in RA pathogenesis. Peptidyl-arginine deiminases (PADs) are enzymes controlling the transformation of arginine into citrulline and neutrophil extracellular traps (NETs) formation. ACPAs and excessive NETformation promote proinflammatory cytokine secretion, enhance RANKL induced

osteoclastogenesis (35)and stimulate local and SBL (36-40).PADI4-deficient mice exhibit less severe inflammatory arthritis and reduced autoantibody levels (41). PTPN22 interacts with PAD4 and inhibits its activity (42).Interestingly, *PTPN22 rs2476601* was associated with hypercitrullination and increased propensity for forming NETs (25). Unlike most variants associated with RA risk, which are non-coding, *PTPN22 rs2476601*is a missense coding variant located in exon 14 of the *PTPN22* gene. This variant convert arginine (R620) to a tryptophan (W620) and alters the structure of the P1 proline-rich motif of PTPN22. This motif is responsible for high-affinity binding to the Src homology 3 (SH3) domain of Csk (6).*PTPN22 rs2476601* not only impairs the ability of PTPN22 to interact with Csk but also with TRAF3 and PAD4 (13, 42).These data indicate that *PTPN22 rs2476601* might be associated with SBL in RA patients.No association between*PTPN22 rs2476601 and SBL* was observedin this study. Similarly, no association was detected between *PTPN22 rs2476601* and bone erosion. The low number of patients with non-erosive RA carrying the T allele in our cohort prevented us from refining the analysis by evaluating the relationship of *PTPN22 rs2476601* and bone erosion in ACPA-negative and ACPA-positive patients. Data describing relationship between *PTPN22 rs2476601* and bone erosion are conflicting. Whereas some reports confirm an association between this variant and bone erosion (43,44)others failed to detect such a relationship (45-47).In a study comprising 593 European ACPA-positive patients with RA and a replication cohort of 397 North American ACPA-positive patients with RA followed up to 6 years, no association between *PTPN22 rs2476601* and erosion at baseline or higher rate of radiological progression was reported (48).

Although our study included more Tunisian patients to explore the association of *PTPN22 rs2476601* and RA, it had limitations. The number of patients carrying the *PTPN22 rs2476601* T allele was relatively small, and BMD could not be measured in all patients. In order to identify independent variables influencing SBL, we conducted a multivariate analysis including the main

confounding factors known to be associated with SBL. However, we could not account for all confounding factors.

This study confirms the association between *PTPN22 rs2476601* and RA in the Tunisian population. We did not find an association between this variant and SBL. The exact impact of PTPN22 rs2476601 on SBL needs to be assessed in large scale studies.

List of abbreviations

RA: rheumatoid arthritis

SBL: systemic bone loss

BMI: Body mass index

ESR: Erythrocyte sedimentation rate

CRP: C-reactive protein

DAS 28: Disease Activity Score 28 with ESR

csDMARDs: conventional synthetic disease-modifying antirheumatic drugs

bDMARDs: biologicaldisease-modifying antirheumatic drugs

BMD: bone mineral density

PCR-RFLP: Polymerase Chain Reaction–Restriction Fragment Length Polymorphism

ACPAs: anti-citrullinated protein antibodies

IgM RF: IgM rheumatoid factor

NETs: neutrophil extracellular traps

Declarations

Ethics approval and consent to participate: The patients' written informed consents were acquired. The study was approved by the Ethical committee of Farhat Hached Hospital (IRB protocol number CEMR-16-157, approval date 15/07/2016).

Consent for publication: not applicable

Availability of data and material: hedatasetsused and/or analysedduring the currentstudy are availablefrom the correspondingauthor on reasonablerequest.

Competing interests: not applicable

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Authors' contributions: RS designed the project.EB recruited the patients. AA, AS, and RG performed the assays. RS and ZS drafted the manuscript, RS, TD, IS, FS, RZ performed and reviwed the analysis. All authorsreviewed and approved the final version of the manuscript.

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Table 1. Characteristics of patients with rheumatoid arthritis

Variable	All patients (n=230)	Patients with the CC genotype (n=221)	Patients with the CT genotype (n=9)	*p*	p^+
Femalegender, n (%)	198 (86.1)	191 (86.4)	7 (77.7)	0.3	-
Age, mean (SD), years	54.9 (11.5)	54.7 (11.6)	58.7 (8.1)	-	0.3
Menopause	159 (80.3)	153 (80.1)	6 (85.7)	1	-
Body mass index, median (SD), kg/m^2	27.1 (22.7-31.8)	27.2 (22.6-31.9)	26.1 (22.4-29.5)	-	0.3
Disease duration, median (IQR), months	60 (24-135)	60 (24-144)	84 (24-129)	-	0.6
Corticosteroids*, number (%)	224 (97.3)	216 (97.7)	8 (88.8)	0.1	-
Corticosteroids cumulative dose, median (IQR), mg	12387 (3375-27543)	12387 (3300-27375)	18337 (8025-43587)	-	0.7
csDMARDs*,number (%)	198 (93.0)	189 (92.7)	9 (100)	1	-
bDMARDs, number (%)	30 (13,0)	28 (12,6)	2 (22.2)	0.3	
Erythrocyte sedimentation rate, mean (SD), mm/1 h	48.0 (34.6)	54.5 (34.7)	52.0 (34.7)	-	0.8
C reactive protein, median (IQR), mg/L	20 (8-50)	20 (8.7-50)	21.5 (4.5-74.7)	-	0.7
Disease activity score in 28 joints, mean (SD)	5.5 (1.5)	5.5 (1.5)	4.8 (1.8)	-	0.1
ACPA*, median (IQR), RU/mL, n (% of positive)	93 (14-200), 180 (78.6)	95 (14-200), 171 (77.7)	85.3 (15.5-145), 9 (100)	0.2	1
IgM RF*, median (IQR), IU/mL, n (% of positive)	315 (110-500), 137 (67.1)	325.0 (109.0-500.0), 131 (67.1)	138 (118-297), 6 (66.6)	1	0.2

p: significance of Chi squared test, p^+: significance of mean or median comparison tests, SD: standard deviation, IQR: interquartile, DMARDS: disease modifying anti-rheumatic drugs, RF: rheumatoid factor, ACPA: anti-citrullinated peptides/proteins antibodies, *: upon availability

Table 2. PTPN22 rs2476601 alleles and genotypes distribution in the Tunisian patients and controls

	Allele		Genotype			OR (CI 95%)	*p*
	C (%)	**T (%)**	**CC (%)**	**CT (%)**	**TT (%)**		
Patients (n=363)	681 (93.8)	45 (6.2)	321 (88.4)	39 (10.8)	3 (0.8)	14.27 (3.44-59.13)	3.45×10^{-6}
Controls (n=217)	432 (99.5)	2 (0.5)	215 (99.1)	2 (0.9)	0 (0.0)		

OR: odds ratio, CI: confidence interval

Table 3. Bone mineral density, osteopenia and osteoporosis distribution in RA patients

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Variable	All patients (n=216)	Patients carrying the CC genotype (n=208)	Patients carrying the CT genotype (n=8)	*p*	p^+
FN BMD*, median (IQR), g/cm^2	0.805 (0.723–0.900)	0.806 (0.726–0.902)	0.786 (0.656-0.835)	-	0.7
FN Z-score*, mean (±SD)	-0.47 (1.02)	-0.46 (1.03)	-0.75 (0,55)	-	0.5
FN T-score*, median (IQR)	-1.60 (-2.40–-0.90)	-1.60 (-2.30– -0.90)	-1.95 (-2.80- -1.55)	-	0.3
FNosteopenia*, n (%)	101/207(48.7)	97/199(48.7)	4/8 (50.0)	0.9	-
FNosteoporosis*, n (%)	48/207 (23.1)	45/199 (22.6)	3/8 (37.5)	0.3	-
TF BMD, median (IQR), g/cm^2	0.840 (0.760–0.940)	0.840 (0.760–0.945)	0.840 (0.673-0.882)	-	0.7
TF Z-score, median (IQR)	-0.70 (-1.30–0.00)	-0.70 (-1.37–0.00)	-1.10 (-1.27--0.17)	-	0.3
TF T-score, median (IQR)	-1.50 (-2.20– -0.72)	-1.50 (-2.20– -0.70)	-1.55 (-2.12- -1.42)	-	0.3
TFOsteopenia, n (%)	107 (49.5)	100(48.0)	7 (87.5)	*0.034*	-
TFOsteoporosis, n (%)	36 (16.6)	35(16.8)	1 (12.5)	1	-
LS BMD, mean(±SD), g/cm^2	0.928 (0.180)	0.928 (0.181)	0.924 (0.147)	-	0.9
LS Z-score, mean (±SD)	-1.16 (1.38)	-1.16 (1.39)	-1.08 (1.17)	-	0.8
LS T-score, mean (±SD)	-1.96 (1.49)	-1.96 (1.50)	-2.01 (116)	-	0.9
LSosteopenia, n (%)	75 (34.7)	70(33.6)	5 (62.5)	0.1	-
LSosteoporosis, n (%)	87(40.2)	85(40.8)	2 (25.0)	0.4	-

p: *p* value of proportions comparison, p^+:*p* value of medians or means comparison,FN: femoral neck, TF: total femur, LS: lumbar spine, *: n=depending on availability, IQR: interquartile, SD: standard deviation