Association between gene polymorphism in programmed cell death 1 (PD1.1) and susceptibility for rheumatoid arthritis in an Iraqi patients? Case control study.

العلاقة بين تعدد الاشكال الجيني في جين موت الخلية المبرمج (PD-1.1) و القابلية لتطوير التهاب المفاصل الرثوي في المرضى العراقيين. دراسة الحالات والشواهد.

Anfal Abdulhussain Jawad
Dept of Microbiology /College of Medicine/ Kufa University
a.a.j.alkhafaff91@gmail.com
Prof. Dr. Salman Azeez Addoos
Dept of Microbiology/ College of Medicine/ Kufa University
Dr.salmanazeez@gimail.com
Prof. Dr. Hashim Raheem Tarish
Dept of Microbiology/ College of Medicine/ Kufa University
Hashimr.tarish@uokufa.edu.iq

Summary:

Programmed cell death-1 (PDCD1) gene is a negative regulator of T-cell to maintain peripheral tolerance and is a key molecule in the development of autoimmune diseases. Although gene polymorphism in PDCD1 was reported to be associated with rheumatoid arthritis (RA), replication studies later on showed conflicting results. This study aimed to determine whether SNP PD1.1 in PDCD1 gene is associated with susceptibility for RA. **Methods:** Clinical diagnosis of the RA patients was confirmed by the Rheumatology Center of Al Sadder Medical Teaching Hospital in Al-Najaf Al-Ashraf city. Genomic DNA was extracted from the whole blood samples using commercial kit (FavorPrep™ Blood Genomic DNA Extraction Mini Kit). Programmed cell death-1 (PD1.1 G/A SNP) genotyping was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to 60 RA patients and 70 non-autoimmune control group. The genotype and allele frequencies of these SNP were analyzed by statistical tests. **Results:** There were no significant differences in alleles and genotypes of PD1.1 SNP in PDCD-1 gene between RA cases and control group. **Conclusions:** PD-1.1 polymorphism in the PDCD1 gene was not associated neither with susceptibility for RA nor with clinical course in rheumatoid arthritis patients (at least in an Iraqi population particularly in AL-Najaf city).

Key words: programmed cell death 1 gene, rheumatoid arthritis, PD-1.1 single-nucleotide polymorphism, RFLP.

الخلاصة

جين موت الخلية المبرمج-1 (PDCD1) هو منظم سلبي للخلية التائية للحفاظ على التحمل المحيطي وهو جزيء رئيسي لتطوير امراض المناعة الذاتية. على الرغم من وجود دراسات تدل على ان تعدد الاشكال الجيني مرتبط مع التهاب المفاصل الرثوي، لاحقا ظهرت دراسات متكررة نتائج متضاربة. وهدفت هذه الدراسة إلى تحديد ما إذا كانPD-1.1 SNP في جين PDCD-1 ترتبط مع قابلية لتطور االتهاب المفاصل الرثوي. **المنهجية:** التشخيص السريري لمرضى التهاب المفاصل الرثوي اكده مركز امراض الروماتيزم في مستشفى الصدر التعليمي في محافظة النجف الاشرف. تم استخراج الحمض النووي من عينات الدم باستخدام كت استخلاص (FavorPrep™ Blood Genomic DNA Extraction Mini Kit). وقد تم معرفة التنميط الجيني لجين موت الخلية المبرمج 1 A / G 1 (PD1.1 SNP) باستخدام تفاعل سلسلة البلمرة المعتمد على طريقة RFLP 60 مريض بالتهاب المفاصل الرثوي و 70 مجموعة السيطرة لم يثبت لديهم امراض المناعة الذاتية .وقد تم تحليل التركيب الوراثي وتكرار الاليلات من هذه SNP بواسطة استخدام اختبارات احصائية. **النتائج:** لم تكن هناك فروق ذات دلالة إحصائية في الأليلات والأنماط الجينية من PD1.1 SNP في جين PDCD-1 بين حالات التهاب المفاصل الرثوي ومجموعة السيطرة. **الاستنتاجات :**تعدد الأشكال من PD1.1 SNP في الجين PDCD1 لم يكن مرتبطا مع القابلية لالتهاب المفاصل الرثوي ولا مع المسار السريري في مرضى التهاب المفاصل الروماتويدي (على الأقل في المرضى العراقيين وخاصة في مدينة النجف) .

Introduction

Rheumatoid arthritis is an inflammatory systemic disease that affecting about 1-2% of populations depending on the geographical distribution in the world **[1]**. It characterized by non-organ-specific self-reactive antibodies production and chronic synovitis leading to the destruction of the cartilage and bone. So, RA is consider a huge socioeconomic and psychological problem subsequent in disability, loss in quality of existence, and early mortality **[2]**. More than double mortality rates higher in RA patients than normal individuals [3]. And since RA is a chronic T cell mediated autoimmune disease **[2,4]**. Therefore, the genes involved in the regulation of T lymphocyte response may be one determinants of susceptibility for RA [5].

Programmed cell death-1 (PDCD-1) is a negative regulator for T- lymphocyte to maintain peripheral tolerance, belong to the CD28 family and is inducibly expressed on activated T- and B-lymphocytes, when PDCD1 interaction by its ligands (PDL-1 and PDL-2) leads to inhibit T-cell proliferation and cytokine production of formerly stimulated lymphocytes **[6,7]**.

Elevated levels of autoantibodies (ACPA and RF) in serum are linked to chronic disease, joint destruction, and functional disability **[8]**. Recently, the study of anti-ccp autoantibodies lightening autoimmune pathways in RA pathogenesis. In the clinical field, they have proven to be biomarkers for the diagnosis and assessment of prognosis of RA **[9]**. The aim of this research to determine whether SNP PD1.1 in PDCD1 gene is associated with susceptibility of rheumatoid arthritis.

Study protocol

-Subjects and methods

Out of sixty patients with RA (51 females and 9 males) with an age average 44.6± 12.5 that fulfilled the 2010 American College of Rheumatology / European League Against Rheumatism classification criteria for RA **[10]**. All of the subjects were patients of the rheumatology center in Al Sadder Medical Teaching Hospital at Al-Najaf Al-Ashraf city and 70 apparently healthy individuals with no history of rheumatic disease or any autoimmune disease have been enrolled in this study, RA patients and control group were age and sex matched. Blood sample were collected in EDTA tubes from patients and control group. DNA was extracted from the blood using commercial kit (FavorPrep™ Blood Genomic DNA Extraction Mini Kit) and the PDCD-1 (PD-1.1 SNP) polymorphism were determined in RA patients and control group by applying polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Table (1): Genotyping method and primers

SNP	Amplicon	Method	RCR primers sequence		Restriction enzyme	Restriction site	Company / Country
PD-1.1	552bp	PCR-RFLP	F	5'-TCTAGCCTCGCTTCGGTTA-3'	MspI	G/A	Biolab/ UK
			R	3'-CTCAACCCCACTCCCATTCT-5'			

SNP single nucleotide polymorphism; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; F forward; R reverse.

-Genotyping

One SNP, namely PD-1.1 from PDCD-1 investigated in this study were defined by restriction fragment length polymorphism (RFLP) technique using their corresponding restriction enzyme (Fermentase) [11]. Primers were designed based on the sequence of the human PDCD1 gene, as shown in Table 1. PCR was set up for amplification of fragments involving desired polymorphism. PCR products were then digested with specific restriction enzyme for this SNP, Table 1. PCR was achieved by following conditions: initiation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and an extension at 72°C for 1 min

and then a final extension step at 72°C for 5 minute. PCR master mix (AccuPower PCR PreMix Kit, Bioneer / Korea) contained 5µl of genomic DNA as template, 10 pmoles (1.5µl) of each primer and 12µl of PCR water. Then components of PCR master mix transferred to PCR tube that contain all other components required to PCR reaction depending on standard AccuPower PCR PreMix Kit such as (dNTPs, Taq DNA polymerase, Tris-HCl pH: 9.0, KCl, MgCl2, loading dye, and stabilizer). The PCR product sizes were 552 bp which weas digested with MspI, restriction enzyme (Fermentase). This restriction enzyme digestion was performed at 37°C overnight.

The genotypes of individuals were identified by length of digested fragments subsequent to 2.5% agarose gel electrophoresis stained by ethidium bromide. The size of specifically digested fragments were achieved as follows: 227 bp for G allele and 282 bp for A allele as showed in figure 1. Anti-cyclic citrullinated peptide (ACCP) antibodies was measured using Aeskulisa kit (Germany), and rheumatoid factor (RF) antibodies was detected using Rheumatoid factor latex agglutination (Spinreact / S.A.). The chi square test was used for categorical variables. P value < 0.05 were considered statistically significant.

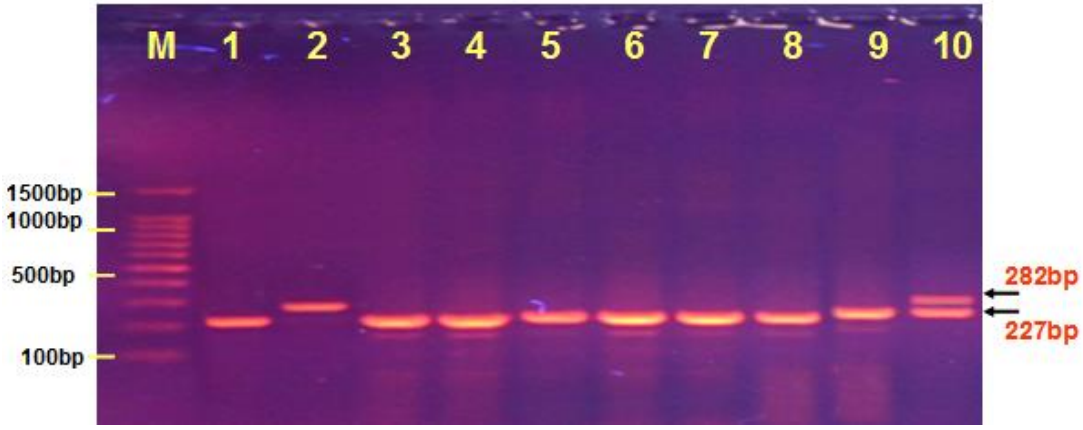

Figure (1): Agarose gel electrophoresis image that show the RFLP-PCR product analysis of PD1.1 gene by using MspI restriction enzyme from some blood RT patient samples. Where M: marker (1500-100bp), lane (1, 3-9) as (GG) homozygote at 227bp, lane (2) as (AA) homozygote at 282bp and lane (10) as (G/A) heterozygote at 227bp and 282bp.

Results:

-Genotype frequency

The genotype distributions according to Agarose gel electrophoresis images was compared between study groups as summarized in Table (2), the genotype frequencies of PD1.1 SNP was not significantly difference between the RA patients and control group.

-Allele frequencies

The allelic frequencies of PD-1.1 were compared between study groups which as brief in Table (2). In this study, the allele frequencies of PD1.1 SNP was not significantly difference between the RA patients and controls group (P > 0.5).

Table (2): Distribution of PD1.1 polymorphism in RA patients and control group.

Genotype	Allele (2N)	RA cases N=60		Control N=70		P	OR (95% CI)
		No.	%	No.	%		
GG		38	63.3	42	60		1
AA		12	20	14	20	0.9	0.9 (0.4-2.3)
GA		10	16.7	14	20	0.6	0.4 (0.2-1.2)
	A	42	30	34	28.3	0.8	0.9 (0.5-1.6)
	G	98	70	86	71.7		

RA rheumatoid arthritis; OR odds ratio; 95% CI=95% confidence interval; P-values for each allele and genotype frequencies are calculated by chi-square test.

To assess the possible association of PD-1.1 allele frequencies with clinical course of RA, this study also classify RA patients depending on the presence of the ACPA and RF. There are no association were observed between PD-1.1 polymorphism and RA Table 3.

Table (3): Association analysis in RA patients and control group with regard to PD1.1 genotype and allele frequency.

PD1.1 SNP	Genotypes			Total	MAF	Allele G versus allele A	
	GG	GA	AA			OR (95% CI)	P
Patients	38	10	12	60	0.283	0.922(0.539-1.578)	0.768
RF+	32	6	10	48	0.270	0.867 (0.487-1.544)	0.627
RF-	6	4	2	12	0.333	1.167 (0.464-2.935)	0.743
ACPA+	32	8	11	51	0.294	0.972 (0.556-1.700)	0.921
ACPA-	6	2	1	9	0.222	0.667 (0.207-2.145)	0.494
Controls	42	14	14	70	0.3		

SNP single-nucleotide polymorphism; MAF minor allele frequency was A allele; OR odds ratio; CI: confidence interval; RF rheumatoid factor; ACPA Anti- citrullinated peptide antibody.

Discussion

This present study is the first to investigate the role of programmed cell death 1 (PDCD-1) polymorphism in genetic susceptibility for RA in an Iraqi population. One SNP of PDCD1, namely PD1.1, was investigated in this study.

In spite of its association with RA disease in Iranian , Chinese and Caucasian populations, the findings of this current study suggest that this SNP is not associated with the susceptibility for RA in the Iraqi population. Association analysis in regard to PD1.1 allele frequencies also could not determine the association of PD1.1 SNP with RA compared to the previous studies **[5,11,12]**. While this current study was supported by other researchers who not found any significant association between allele/genotype distributions of PD-1.1 and susceptibility for RA **[13,15]**.

Investigation of the genetic determinants for common diseases that previously determined association is not necessary significant **[13]**. There are more than one cause for this failure to confirm an association: First, different genetic background and/or it is likely that this SNP included

in susceptibility for RA affects with insignificant degree on susceptibility in our population, which might underpowered the study [**14**]. Second, some of the major difficulties in the analyzed of genes involved in susceptibility to RA linked with differences in ethnicities as well as attributable to the heterogeneity of the disease **[16]**.

Third, It is well known that the HLA locus which has been estimated with 37 % of the heritability, has a significant effect on RA susceptibility, so the disease-associated genes that located out the HLA locus may have a small effect with limited relative risk on disease **[17,18]**. The most significant genetic risk factors for RA are changes in HLA genes, specifically the HLA-DRB1 and modifications in other genes appeared to have a smaller effect on a person's overall risk of developing the condition **[19]**.

Regarding to the genotypes distribution of PD-1.1 in PDCD1 gene, this current study found that PD-1.1GG were 63.3% vs. 60% in RA patients and control group respectively (OR= 1), PD1.1AA were the similar percentage for RA patients and control group 20% (P= 0.9, OR= 0.9, 95% CI 0.4-2.3) and, PD1.1G/A were 16.7% vs. 20% in RA patients and controls group respectively (*P*= 0.6, OR= 0.4 for 95% CI 0.2-1.2). These findings show that there is no significant differences between genotypes distribution of PD1.1 and susceptibility for RA.

This current study result is compatible with Iwamoto et al. , Tahoori *et al*. and Siwiec *et al*. which show genotypes distribution of PD1.1 was not associated with susceptibility for RA **[12,13,15]**. On the other hand, Liu *et al*, reported that homozygote genotype of rs36084323 (PD1.1GG) was associated with high risk for development of RA (*P*= 0.049, OR = 1.70, 95% CI 1.11–2.61), while Kong *et al*, observed that homozygote of the SNP PD-1.1AA genotype are associated with a decreased risk for RA development in Hong Kong Chinese RA patients (*P*= 0.034, OR= 0.38, 95% CI 0.15-0.99) **[5,11]**.

However, this current study revealed that frequency of the PD1.1G allele was (71.7%) and is considered a major allele in Iraqi population, this observation is agreement with Tahoori *et al*, which observed that the PD-1.1G allele is a common allele in Iranian population, while the PD1.1A allele is a common in Chinese and Mexican-Indian populations [5,11,12,20]. Whereas, our results found that frequency of the PD1.1A allele was 30% *vs*. 28.3% in patients and controls respectively (*P*= 0.8, OR= 0.9, CI= 0.5-1.6) and is considered minor allele.

Indeed, this current study result demonstrated that the allele frequencies of SNP PD1.1 was not associated with susceptibility for RA. This result was incompatible with Tahoori *et al*. who reported that the frequency of PD-1.1A allele located in the promoter region of the PDCD1 gene at position -538 is a risk allele in Iranians patients (2.9% vs. 0.7%, OR= 3.735, 95% CI= 0.956–14.588, P=0.046) and Liu *et al* which reported that PD1.1A (rs36084323) allele is associated with risk of RA [5,12].

However, the studies by Lin et al. (PD-1.5T), Kong, et al. (PD-1.1 AA), Tahoori et al. (PD-1.1A) and Liu et al.(PD-1.1GG & PD-1.1A) in Asian populations, identified the association between PDCD1 and RA. While this study (PD-1.1) which supported by study carried by Iwamoto et al. (PD-1.1 & PD-1.5) did not prove relationship between PDCD1 gene and RA disease. This inconsistency among different populations even among the same population it might be possible due to population-specific differences in Asians.

A chinese study carried by Hua *et al* reported that PD-1.1 was associated with risk of sporadic breast cancer [20].

A PDCD-1 gene SNP (PD-1.3A) in intron 4 within European inhabitants is associated with the developing RA in Sweden population (P= 0.053, OR= 1.18), but not in Poles population [14,15]. While PD-1.3 within Asian inhabitants was either non-polymorphic among patients with RA and control group in both Chinese and Japanese or not associated with RA development in Iranian population [5,11-13]. It may be possible that the polymorphism studied in the Chinese and Japanese population have no direct impact on susceptibility because the etiological mutation present in the Swedish is not present in their population.

No association for SNP PD-1.5 in exon 5 of PDCD1 gene with RA was found among Hong Kong Chinese, Han Chinese and Japanese, the opposite is proven by Lin, *et al*. in Taiwan Chinese

who found that PD1.5T allele associated with RA susceptibility, but not SLE [5,11,13,22]. Additionally, Tahoori et al. and Liu et al. tested genotypes and alleles distribution of SNP PD-1.9 in exon 5 but they did not prove their relationship with rheumatoid arthritis [5,12]. While Siwiec *et al* observed that the homozygous genotype of PD-1.5CC and PD-1.9CC SNPs are associated with RA susceptibility in Poles population [15].

This discrepancy among different populations even among the same population due to heterogeneity of the disease as well as to different ethnicities [16].

It should be pointed out that a recent study found a highly significant levels of sPD-1 in plasma and SF among RA patients compared with healthy control. Remarkably, An Egyptian study demonstrated that the plasma and synovial fluid levels of soluble PD-1 significantly correlated with ACPA titers in RA patients hinting at the possibility of its involvement in the pathogenesis RA [23].

In conclusion, we could not confirm associations of PD-1.1 polymorphism in PDCD-1 with RA patients at least in the Iraqi population. However, further studies are required to test the possibility that other polymorphisms within the PDCD-1 gene may be involved in the susceptibility for RA.

References

1. **Arthritis Foundation Malaysia** (2012). website at http://www.afm. org.my. Last assessed on 23 July 2012.
2. **McInnes, I.B. & Schett, G.** (2011) "The pathogenesis of rheumatoid arthritis". N Engl J Med; 365: 2205–19.
3. **Aletaha, D., Neogi, T., Silman, A.J., et al.** (2010) "Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis & Rheumatism". 62 (9): 2569–2581.
4. **Gol-Ara, Maryam., Jadidi-Niaragh, Farhad., Sadria, Reza., Azizi, Gholamreza., Mirshafiey, Abbas.** (2012). "The role of different subsets of regulatory T cells in immunopathogenesis of rheumatoid arthritis" Arthritis, vol. 2012, Article ID 805875, 16 pages.
5. **Liu, CuiPing., Jiang, JueAn., Gao, Li., et al.** (2014). "A Promoter Region Polymorphism in PDCD-1 Gene Is Associated with Risk of Rheumatoid Arthritis in the Han Chinese Population of Southeastern China". Hindawi Publishing Corporation International Journal of Genomics. Article ID 247637, 8 pages.
6. **Bennett, F., Luxenberg, D., Ling, V., et al.** (2003) "Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokinedriven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses". J Immunol 170:711–718.
7. **Keir, Mary, E., Butte, Manish, J., Freeman, Gordon, J., & Sharpe, Arlene, H.** (2008) "PD-1 and its ligands in tolerance and immunity" Annual Review of Immunology. 26: 677–704.
8. **Malaviya, A.N., Collings, J.B., Makol, A., Kaushik, P.** (2009) "Effect of baseline rheumatoid factor (RF) levels on DAS28- response to standard DMARD-combination treatment in patients with rheumatoid arthritis (RA)". Indian Journal of Rheumatology. 4(2):51-5.
9. **Sarantopoulos, Alexandros & Boura, Panagiota** (July 2013). "Anti- Cyclic Citrullinated Autoimmunity In Pathophysiology And Diagnosis Of Rheumatoid Arthritis. International Trends In Immunity. 1 (3).
10. **American College of Rheumatology** (Feb. 2016). "Rheumatoid arthritis".
11. **Kong, Eric Kai-Pang., Prokunina-Olsson, Ludmila., Wong, Wilfred Hing-Sang., et al.** (2005). "A new haplotype of PDCD1 is associated with rheumatoid arthritis in Hong Kong Chinese". Arthritis Rheum. 52: 1058–62.
12. **Tahoori,M.T.,Pourfathollah,A.A.,Akhlaghi,M.,Daneshmandi,S.,Nicknam,M.H.,Soleimani far,N.**(2011)."Association of programmed cell death-1(PDCD-1)gene polymorphisms with rheumatoid arthritis in Iranian patients".Clin. Exp. Rheumatol. 29: 763-767.

13. **Iwamoto, Takuji., Ikari, Katsunori., Inoue, Eisuke., et al.** (2007). "Failure to confirm association between PDCD1 polymorphisms and rheumatoid arthritis in a Japanese population". The Japan Society of Human Genetics and Springer. J Hum Genet. 52: 557–560.
14. **Sfar, Imen., Dhaouadi, Tarak., Krichen, Henda., et al. (2010)** "Genotyping CTLA-4 exon 1 (+49 A/G) and HLA-DRB1 polymorphisms and susceptibility to systemic lupus and rheumatoid arthritis in Tunisian population". Journal of Medical Genetics and Genomics Vol. 2(1) pp. 008-017
15. **Siwiec, Anna and Majdan, Maria** (2015). "The role of the PD-1 protein in pathogenesis of autoimmune diseases, with particular consideration of rheumatoid arthritis and systemic lupus erythematosus". Postepy Hig Med Dosw (online), tom 69: 534-542.
16. **Prokunina, Ludmila., Padyukov, Leonid., Bennet, Anna., et al.** (2004). "Association of the PD-1.3A allele of the PDCD1 gene in patients with rheumatoid arthritis negative for rheumatoid factor and the shared epitope". Arthritis Rheum. 50: 1770.
17. **Van der Woude D., Houwing-Duistermaat, J.J., Toes, R.E., et al.** (2009). "Quantitative heritability of anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis". Arthritis Rheum. 60(4): 916–923.
18. **Gene home reference, 2015**
19. **Jutley, G., Raza, K., & Buckley, C.D.** (2015) "New pathogenic insights into rheumatoid arthritis". Curr Opin Rheumatol. 27: 249–55.
20. **Prokunina, L., Castillejo-Lopez, C., Oberg, F., et al.** (2002) "A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans". *Nat Genet* ; 32: 666-9.
21. **Hua, Zhang., Li, Dalin., Xiang, Gao., et al.** (2011) "PD-1 polymorphisms are associated with sporadic breast cancer in Chinese Han population of Northeast China". Breast Cancer Research and Treatment. 129 (1): 195–201.
22. **Lin, Shih-Chang., Yen, Jeng-Hsien., Tsai, Jaw-Ji., et al.** (2004) "Association of a programmed death 1 gene polymorphism with the development of rheumatoid arthritis, but not systemic lupus erythematosus". Arthritis Rheum. 50: 770.
23. **Hassan, Waleed A., Baraka, Eman A., & Fouad Nehad A.** (2015). "Clinical significance of soluble programmed death-1(sPD-1) in rheumatoid arthritis patients: Relation to disease activity and functional status". The Egyptian Rheumatologist. 37: 165–169.