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ORIGINAL ARTICLE

Genetic association study of systemic lupus erythematosus and disease subphenotypes in European populations

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Abstract Epidemiological studies suggest a strong contribution of genetic factors in the pathogenesis of systemic lupus erythematosus (SLE). In the last decades, many risk loci have been identified in several genetic association studies following both candidate gene and genome-wide approaches. The present work was conducted by GAPAID (Genes And Proteins for AutoImmunity Diagnostics) consortium with a dual aim: to replicate the association of several previously reported SLE susceptibility loci in an independent European sample and to explore their relation with some disease subphenotypes. A total of 48 single nucleotide polymorphisms (SNP) from 40 associated loci were typed in a cohort of 208 SLE patients and 152 controls from Rheumatology Units of the University Hospital of Pisa (Italy) and University of Pécs Medical Center (Hungary). Regression analyses were performed to detect disease susceptibility loci and to identify genes affecting specific disease manifestations (renal, neurological, or skin involvement; arthritis; secondary Sjögren syndrome; and secondary antiphospholipid syndrome). Association of previously described risk alleles from *HLA* locus has been replicated, while *IRF5*, *BLK*, *ITGAM*, and *IRF8* loci have been found to be consistent with previous published results. In addition, two new subphenotype-specific associations have been detected: SNP rs5754217 (*UBE2L3*) with skin involvement and rs3093030 (*ICAM1-ICAM4-ICAM5*) with hematological disorders. Overall, results from GAPAID project are consistent with previously established associations for *HLA*, *IRF5*, *BLK*, *ITGAM*, and *IRF8* SLE susceptibility loci and report for the first time two subphenotype-specific associations.

Keywords Clinical manifestations · Genetics · Single nucleotide polymorphism · Systemic lupus

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Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease affecting multiple organs, characterized by a wide spectrum of clinical manifestations and laboratory findings. Multiple lines of evidence support a strong genetic contribution to the development of the disease, and it is well accepted that SLE occurs in genetically predisposed individuals exposed to certain environmental stimuli. Evidence of familial clustering was the first indication of a genetic susceptibility to SLE. On the basis of twin studies, SLE heritability (that is the relative contribution of genetic variation to the liability of developing the disease) has been estimated to be about 66 % based on the higher monozygotic twins concordance rates (24–56 %) compared to dizygotic twins (2–5 %) [1]. Familial aggregation for SLE, measured by the sibling recurrent risk ratio, varies from 8 to 29 depending upon the disease prevalence in the population used as reference [2].

Several linkage studies and genome-wide association studies have highlighted the impact of genetic polymorphisms on the risk of developing the disease [3–9]. The strong contribution of HLA region is widely known; the risk conferred by *DRB1*1501* (*HLA-DR2*) and *DRB1*0301* (*HLA-DR3*) genes is confirmed in many European populations. Outside the MHC region, *IRF5* (interferon regulatory factor 5) is one of the most strongly and consistently SLE-associated loci; *STAT4* (signal transducer and activator of transcription 4) has been found to associate with SLE in multiple studies in European or Asian populations [10, 11].

Only a few studies have addressed the influence of disease-predisposing genes on SLE severity and outcome. Fc Rs I, II, and III have been consistently associated with both susceptibility and severity of SLE. In the case of FcgammaRIIa and Fc gammaRIIIa, the low affinity allele is predisposing not only to SLE but also to lupus nephritis [12, 13], while homozygosity for the valine allele of Fc gammaRIIIa is a risk factor for the progression of renal involvement to end-stage renal disease [14]. On the whole, only limited information is available on the association of risk genes for SLE with specific disease phenotypes.

The GAPAID (Genes And Proteins for AutoImmunity Diagnostics) consortium was created within the European Union's Seventh Framework Programme for Research and Technological Development (FP7), with the aim of developing a novel diagnostic/prognostic platform for patients affected by SLE, based on a genetic array, a serological protein array, and a software combining the clinical data with the genetic and serological information. In this context, the present study describes the genetic study performed on susceptibility to SLE, where 48 single nucleotide polymorphisms (SNP) from 40 different loci have been tested for SLE susceptibility and their association with disease subphenotypes.

Materials and methods

Ethic statement

This study was approved by the Ethics Committee of the University Hospital of Pisa (reference number: 45066/2012) and the Hungarian Scientific and Research Ethics Board (reference number: 24973-1/2012 EKU). The procedures followed were in accordance with the Helsinki Declaration of 1975. All the patients gave written informed consent.

SLE case-control population

A cohort of 208 SLE patients (cases) and 152 healthy blood donors (controls) was recruited between August 2012 and October 2013 from two centers, the Clinical Immunology Unit, Department of Clinical and Experimental Medicine of the University of Pisa (Italy) and the Department of Rheumatology and Immunology of the University of Pécs (Hungary) (Table 1). All SLE patients fulfilled the American College of Rheumatology (ACR) classification [15]. Gender and age data from all individuals were collected, as well as several clinical data retrospectively evaluated from SLE patients such as the age of patients at the onset of the disease, organ involvement, and secondary antiphospholipid syndrome (APS) (Table 1). The occurrence of arthritis (erosive or not) in clinical history was scored. Renal involvement was diagnosed on the basis of proteinuria, hematuria, and/or creatinine increase and/or hypertension and was in most cases (more than 80 % pts) confirmed by kidney biopsy. Hematological involvement was diagnosed in the presence of thrombocytopenia and/or hemolytic anemia and/or leucopenia. Neurological involvement was diagnosed in the presence of neuropsychiatric manifestations such as seizures,

Table 1 Clinical data of individuals included in the study

Variable	Italian cases ($N=62$)	Hungarian cases ($N=146$)	Total cases ($N=208$)	Italian controls ($N=100$)	Hungarian controls ($N=52$)	Total controls ($N=152$)
Age (at inclusion) (years)	43.6 (±14.5)	45.4 (±13.7)	44.9 (±13.9)	39.5 (±11.3)	39.7 (±9.9)	39.5 (±10.8)
Sex, female (%)	87.1	91.1	89.9	28	80.8	46.1
Age onset (years)	30.5 (±13.7)	32.3 (±13.6)	31.8 (±13.6)	n.d.	n.d.	n.d.
Nephritis (%)	70.7	39.0	48.0	n.d.	n.d.	n.d.
Central nervous system (CNS) involvement (%)	24.1	47.3	40.7	n.d.	n.d.	n.d.
Hematological disorders (%)	67.7	70.5	69.7	n.d.	n.d.	n.d.
Skin involvement (%)	71.4	63.7	65.8	n.d.	n.d.	n.d.
Arthritis (%)	52.6	61.0	58.6	n.d.	n.d.	n.d.
Secondary antiphospholipid syndrome (APS) (%)	30.5	39.0	36.6	n.d.	n.d.	n.d.

psychoses, cerebrovascular disease, and myelopathy. Skin involvement included generalized or malar rash, discoid lesions, and cutaneous vasculitis.

SNP selection and genotyping process

A total of 48 SNPs from 42 loci previously associated with SLE, in European ancestry populations, have been included in GAPAID project (Table 2). Genetic markers were principally selected from candidate gene, genome wide and replication association studies published at the time of the project. Some of the previously reported subphenotype-specific associations regarding those clinical features evaluated in the present study were also considered. The final list included several well known susceptibility loci such as 1q25.1, *PTPN22*, *TNFSF4*, *STAT4*, *PXK*, *BANK1*, *HLA*, *TNFAIP3*, *IRF5*,

Table 2 Selected SNPs

Gene	Location	SNP ID	Disease/subphenotype association
LOC100506023	1q25.1	rs10798269	Disease [4]
FCGR2A	1q23	rs1801274	Disease [4, 7]
FCGR2B		rs1050501	Disease [16]
FCGR3A		rs396991	Nephritis [17]
IL-10	1q31-q32	rs3024505	Disease [7]
LY9	1q23.3	rs509749	Disease [18]
NCF2	1q25	rs10911363	Disease [19]
PTPN22	1p13.2	rs2476601	Disease [4, 7]
TNFSF4	1q25	rs2205960	Disease, nephritis [7, 20]
CTLA4	2q33	rs231775	Disease [21]
NFE2L2	2q31	rs4894215	Immunologic disorders [22]
PDCD1	2q37.3	rs11568821	Disease [23]
STAT4	2q32.2-q32.3	rs7574865	Disease, age onset < 30, nephritis, immunologic disorders [4, 5, 7, 24]
PXK	3p14.3	rs6445975	Disease [4, 7]
TMEM39A	3q13.33	rs1132200	Disease [8]
BANK1	4q24	rs10516487 rs17266594	Disease [6, 7, 19]
IL21	4q26-q27	rs907715	Disease, hematological disorders [20, 25]
TNIP1	5q32-q33.1	rs6889239	Disease [19]
HLA (*CFB*)	6p21.3	rs1270942	Disease [4]
HLA (*HLA-DQA1*)		rs2187668	Disease [5]
HLA (*MSH5*)		rs3131379	Disease [4]
PERP	6q24	rs6922466	Disease [26]
PRMD1/*ATG5*	6q21	rs6568431	Disease [7]
TNFAIP3	6q23	rs2230926	Disease [3]
ICA1	7p22	rs10156091	Disease [4, 7, 19]
IKZF1	7p12.2	rs2366293	Disease [19]
IRF5	7q32	rs729302 rs10954213 rs2070197	Disease, skin lesions [4, 7, 27]
BLK	8p23-p22	rs13277113 rs2736340	Disease, nephritis [5, 7, 28]
LYN	8q13	rs2667978	Disease [4]
XKR6	8p23.1	rs4240671	Disease, nephritis [4, 28]
TRAF1-C5	9q33-q34	rs10818488	Disease [29]
TYRP1	9p23	rs1408806	Immunologic disorders [22]
PHRF1	11p15.5	rs4963128	Disease [4]
TRAF6	11p12	rs540386	Disease [30]
DLEU2	13q14.3	rs9568401	Immunologic disorders [22]
IRF8	16q24.1	rs450443 rs4843869 rs8046526	Disease [8]
ITGAM	16p11.2	rs1143679	Disease, nephritis, skin lesions [4, 20]
ZPBP2	17q12	rs1453560	Disease [8]
ICAM1-ICAM4-ICAM5	19p13.3-p13.2	rs3093030	Disease [31]
UBE2L3	22q11.21	rs5754217	Disease [4, 7]
MECP2	Xq28	rs17435	Disease, age onset < 30 [28, 32, 33]
TMEM187	Xq28	rs13397	Disease [33]

The gene or nearest gene from the analyzed SNPs is indicated

BLK, *IRF8*, or *ITGAM*, along with some less studied or controversial ones (e.g., *IL10*, *LY9*, *IL21*, *LYN*, *TRAF6*, *ICAM* region).

DNA from the buffy coat of all collected samples was purified by NucleoSpin 96 Blood Core Kit Macherey-Nagel). DNA quantity (ng/ul) and quality (260/280 and 260/230 absorbances) were checked with Qubit fluorometer and NanoDrop 8000 Spectrophotometer, respectively, before the genotyping process. Genotyping of selected SNP was performed by BioMark™ HD System (Fluidigm), based on the 5' exonuclease activity of the polymerase. For each array, 2 negative controls and 46 unknown samples were included. Fluidigm SNP Genotyping Analysis Software v.3 was used for allele assignation.

Before statistical analyses, three quality criteria were checked with *PLINK v.2.050* software [34]: SNP call rate (min. 95 %), sample call rate (min. 95 %), and conformity of genotype proportions to Hardy-Weinberg equilibrium (HWE) in the overall population.

Statistical analyses

Regression analyses were performed with the abovementioned software with the aim of detecting SLE susceptibility loci (case-control analysis) or specific polymorphisms for any of the measured subphenotypes (case-case analyses). All analyses were carried out under additive, dominant, and recessive genetic models. As differences in age and gender distribution between analyzed populations could limit the results of the study, both factors were included as covariates in all regression analyses. In the same manner, the origin of individuals was also considered as covariate in order to control the possible effect of genetic ancestry in the study. Finally, the results from the case-case analyses for renal, central nervous system (CNS), and skin involvement and presence of arthritis or secondary APS were also adjusted for the age of patients at the onset of the disease. The genetic model with the best *P* value has been chosen in each case. Corrections for multiple testing implemented in *PLINK v.2.050* software have been performed and significant associations were considered when adjusted *P* values < 0.05.

Results

After the genotyping process, SNPs rs396991 (*FCGR3A*), rs231775 (*CTLA4*), rs11568821 (*PDCD1*), rs6568431 (*PRMD1*/*ATG5*), and rs4963128 (*PHRF1*) were removed for subsequent statistical analyses due to their low call rate (<95 %). The same threshold was applied to remove 9 individuals (5 cases and 4 controls). In addition, SNP rs2187668 (*HLA-DQA1*) did not fit HWE in the overall population (*P* value < 0.001). Thus, a total of 42 SNPs and 351 individuals (203 cases and 148 controls) were included in the final case-control and case-case analyses.

Results from the regression analysis focused on the identification of SLE susceptibility loci (case-control analysis) are shown in Table 3. Two SNPs located in the *HLA* region (rs3131379 and rs1270942) appear significantly associated with the disease (adjusted *P* value < 0.05). Five additional SNPs in *IRF5* (rs729302 and rs2070197), *BLK* (rs2736340), *IRF8* (rs4843869), and *ITGAM* (rs1143679) loci show also a trend to be related with SLE susceptibility (nominal *P* value < 0.05).

Two significant subphenotype-specific associations (adjusted *P* value < 0.05) have been found between SNP rs5754217 (*UBE2L3*) and skin involvement, and SNP rs3093030 (*ICAM1-ICAM4-ICAM5*) and hematological manifestations (Table 4). Other 32 suggestive associations have also been observed among all the analyzed clinical features (nominal *P* value < 0.05).

Table 3 Results from regression analyses on the overall SLE case-control population

SNP ID	Locus	Minor allele (tested allele)	MAF Cases	MAF Controls	Genetic model	OR (95 % CI)	*P* value	Adjusted *P* value
rs3131379	*HLA* (*MSH5*)	A	0.165	0.064	DOM	2.99 (1.514–5.904)	0.0016	0.0345
rs1270942	*HLA* (*CFB*)	G	0.168	0.064	DOM	3.01 (1.526–5.947)	0.0015	0.0345
rs729302	*IRF5*	C	0.248	0.331	REC	0.24 (0.084–0.672)	0.0068	0.1728
rs2070197	*IRF5*	C	0.176	0.095	ADD	1.80 (1.053–3.073)	0.0316	0.2545
rs2736340	*BLK*	T	0.281	0.203	ADD	1.55 (1.019–2.349)	0.0405	0.2545
rs4843869	*IRF8*	A	0.148	0.160	DOM	0.53 (0.298–0.932)	0.0276	0.2743
rs1143679	*ITGAM*	A	0.212	0.160	ADD	2.01 (1.239–3.243)	0.0046	0.0660

P values have been adjusted for age, gender, and origin of individuals. Only those SNPs with a nominal *P* value <0.05 are shown

MAF minor allele frequency, *ADD* additive, *DOM* dominant, *REC* recessive

Table 4 Results from regression analyses on the different subphenotypes

Clinical feature	SNP ID	Gene	Minor allele (tested allele)	MAF Cases	MAF Controls	Genetic model	OR (95 % CI)	*P* value	Adjusted *P* value
Age onset (<30)	rs10911363	*NCF2*	T	0.395	0.389	REC	0.84 (0.378–1.857)	0.0373	1
	rs4894215	*NFE2L2*	G	0.500	0.454	DOM	3.31 (1.213–9.050)	0.0195	0.4829
	rs1143679	*ITGAM*	A	0.142	0.273	DOM	0.34 (0.133–0.858)	0.0225	0.4829
Nephritis	rs3131379	*HLA* (*MSH5*)	T	0.175	0.157	DOM	2.13 (1.055–4.298)	0.0349	0.5327
	rs1270942	*HLA* (*CFB*)	C	0.175	0.162	DOM	2.07 (1.022–4.168)	0.0433	0.5327
	rs2366293	*IKZF1*	G	0.130	0.221	DOM	0.40 (0.120–0.800)	0.0096	0.4108
	rs729302	*IRF5*	C	0.297	0.201	ADD	1.84 (1.050–3.225)	0.0331	0.7131
CNS involvement	rs1050501	*FCGR2B*	C	0.167	0.081	ADD	2.25 (1.167–4.348)	0.0155	0.3334
	rs3024505	*IL-10*	T	0.172	0.161	ADD	1.99 (1.175–3.383)	0.0105	0.3334
	rs6445975	*PXK*	G	0.340	0.225	DOM	2.08 (1.141–3.788)	0.0168	0.2265
	rs17266594	*BANK1*	C	0.191	0.259	DOM	0.52 (0.280–0.977)	0.0421	0.2265
	rs10516487	*BANK1*	T	0.191	0.259	DOM	0.52 (0.280–0.977)	0.0421	0.2265
	rs907715	*IL21*	A	0.241	0.326	ADD	0.58 (0.351–0.950)	0.0307	0.3591
	rs729302	*IRF5*	C	0.200	0.284	DOM	0.45 (0.245–0.841)	0.0120	0.2265
	rs4240671	*XKR6*	G	0.456	0.441	DOM	2.71 (1.290–5.710)	0.0085	0.2265
Skin involvement	rs509749	*LY9*	G	0.402	0.464	REC	0.45 (0.206–0.990)	0.0470	0.6955
	rs907715	*IL21*	A	0.273	0.319	REC	0.26 (0.081–0.817)	0.0213	0.6955
	rs729302	*IRF5*	C	0.217	0.312	ADD	0.53 (0.308–0.902)	0.0195	0.4133
	rs2070197	*IRF5*	C	0.209	0.123	ADD	1.96 (1.072–3.568)	0.0288	0.4133
	rs5754217	*UBE2L3*	T	0.301	0.130	ADD	2.91 (1.607–5.254)	0.0004	0.0177
Hematological disorder	rs4894215	*NFE2L2*	G	0.482	0.377	ADD	1.78 (1.127–2.806)	0.0133	0.1671
	rs907715	*IL21*	A	0.340	0.197	DOM	2.58 (1.376–4.828)	0.0031	0.1333
	rs6889239	*TNIP1*	C	0.326	0.443	ADD	0.63 (0.407–0.965)	0.0339	0.2914
	rs6922466	*PERP*	G	0.216	0.325	ADD	0.54 (0.332–0.890)	0.0156	0.1671
	rs8046526	*IRF8*	T	0.132	0.075	DOM	2.64 (1.070–6.516)	0.0351	0.3441
	rs4843869	*IRF8*	A	0.163	0.115	DOM	2.21 (1.019–4.799)	0.0446	0.3441
	rs450443	*IRF8*	G	0.261	0.312	REC	0.23 (0.059–0.878)	0.0317	0.4539
	rs3093030	*ICAM1-ICAM4-ICAM5*	C	0.461	0.361	ADD	2.22 (1.379–3.572)	0.0010	0.0442
Arthritis	rs10911363	*NCF2*	T	0.407	0.356	DOM	1.95 (1.080–3.527)	0.0270	0.4321
	rs6889239	*TNIP1*	C	0.326	0.406	DOM	0.53 (0.286–0.967)	0.0385	0.4321
	rs1270942	*HLA* (*CFB*)	C	0.195	0.131	DOM	1.98 (1.006–3.912)	0.0480	0.4321
	rs4240671	*XKR6*	G	0.431	0.444	DOM	0.39 (0.190–0.802)	0.0104	0.4321
APS	rs10911363	*NCF2*	T	0.431	0.367	DOM	2.04 (1.095–3.814)	0.0247	0.6789
	rs1408806	*TYRP1*	G	0.257	0.177	ADD	1.77 (1.055–2.968)	0.0307	0.8547

P values have been adjusted for age, gender, and origin of individuals. Only those SNP with a nominal *P* value <0.05 are shown

MAF minor allele frequency, *ADD* additive, *DOM* dominant, *REC* recessive

Discussion

SLE is an autoimmune disease affecting predominantly women characterized by a loss of tolerance to self-antigens, inflammation, and dysregulated immune responses leading to multi-organ damage [35]. Epidemiological studies suggest a strong contribution of genetic factors in the pathogenesis of the disease; many SLE risk loci have been identified in several genome-wide association studies (GWASs) and other association studies in the last decades [36]. In the present study, the strongest association has been detected for HLA region, concordantly with previous GWASs [4, 9]. Encoding more than 200 genes and subdivided into class I, II, and III regions, HLA complex was the first SLE susceptibility locus identified [37]. The two SNPs significantly associated with SLE in this study, rs3131379 and rs1270942, are located in *MSH5* and *CFB* immune genes, respectively, in the class III HLA region. The first one, previously reported as an SLE-associated gene in UK families and also in a study on SLE patients with African American background [38, 39], has recently also been related with cutaneous SLE in European populations [40].

The results found for *IRF5*, *BLK*, *IRF8*, and *ITGAM* could be also highlighted. Although the association level between these loci and SLE susceptibility does not reach the statistical significance in the present study, probably due to a limited sample size, all of them are well-known SLE susceptibility genes. The three SNPs from *IRF5* analyzed here (rs729302, rs2070197, and rs10954213) represent three haplotype blocks with an already described independent effect on SLE risk [41]. Regarding the related *IRF8*, Cunninghame Graham et al. [19] identified for the first time this gene associated with the disease in a European population, which was later robustly established with the independent effect of the three SNPs analyzed in the present study [8]. There are several studies reporting also the implication of *BLK* locus in SLE susceptibility, which encodes a tyrosine protein kinase involved in the proliferation, differentiation, and tolerance of B cells. The T allele from SNP rs2736340 has been associated with a major risk of the disease, as detected in the present study [42, 43]. Finally, the non-synonymous variant from *ITGAM* associated in the present study (rs1143679, R77H) has been proposed as one of the causal variants in this locus affecting the numerous ligand binding activities of ITGAM in monocytes, neutrophils, and dendritic cells and impairing C3-mediated phagocytosis [44, 45].

The analysis of SLE subphenotypes performed in the present study reveals two major findings. The association between *UBE2L3* gene and skin involvement is the strongest one. *UBE2L3*, encoding a ubiquitin conjugating enzyme, was suggested to be a SLE susceptibility locus for European populations by Harley et al. [4] and later confirmed in a large-scale replication study [7]. Both studies showed positive results for the intronic SNP rs5754217 which has been included in a SLE risk haplotype leading to a higher UBE2L3 mRNA expression [46]. Furthermore, T allele of rs5754217 has been associated with the presence of several autoantibodies in African-American and European SLE patients [47, 48], but this is the first study reporting its implication in organ involvement during the disease; this T allele appears to be associated with a higher risk of skin manifestations. The second novel association in the present study is that reported between *ICAM1-ICAM4-ICAM5* and hematological disorders. The ICAM locus, encoding intercellular adhesion molecule proteins that are expressed in vascular endothelium, macrophages, lymphocytes, red blood cells, and brain, confers susceptibility to SLE in multiple ancestry populations [31], but no association with disease subphenotypes has been so far reported.

Other several suggestive associations have been observed in this study near to the statistical significance. *XKR6* gene, encoding a transmembrane protein of the Kell blood group of antigens and related only to SLE nephritis up to now [28], appears to be linked with CNS involvement. On the contrary, the implication of *IL21* with hematological disorders has already been described in Europeans [20]. Other subphenotype-specific tendencies observed here match also previously reported results. Furthermore, the present paper is the first one suggesting some of these associations in European SLE patients, such as the relation between *IKZF1* and *IRF5* loci and lupus nephritis, *IRF8* and hematological disorders, or *NCF2* locus and arthritis, all of them already described in Chinese populations [49–52]. However, these new suggestive subphenotype-specific associations with moderate significance values should be taken with caution and replicated in independent populations.

Overall, and despite the possibility of some study design limitations, results from the present study are consistent with previously established associations for the SLE susceptibility loci *HLA*, *IRF5*, *BLK*, *ITGAM*, and *IRF8*. The analysis of disease subphenotypes shows new specific associations such as those between *UBE2L3* and skin involvement, and *ICAM1-ICAM4-ICAM5* and hematological manifestations, in addition to other several suggestive associations which need to be replicated in an independent and larger populations.

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Compliance with ethical standards

Ethic statement This study was approved by the Ethics Committee of the University Hospital of Pisa (reference number: 45066/2012) and the Hungarian Scientific and Research Ethics Board (reference number: 24973-1/2012 EKU). The procedures followed were in accordance with the Helsinki Declaration of 1975. All the patients gave written informed consent.

Disclosures None.

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