ORIGINAL RESEARCH

Systemic Inflammation Rapidly Induces Reversible Atrial Electrical Remodeling: The Role of Interleukin-6–Mediated Changes in Connexin Expression

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***Background*—**Systemic inflammation is a strong predictor of atrial fibrillation. A key role for electrical remodeling is increasingly recognized, and experimental data suggest that inflammatory cytokines can directly affect connexins resulting in gap-junction dysfunction. We hypothesized that systemic inflammation, regardless of its origin, promotes atrial electric remodeling in vivo, as a result of cytokine-mediated changes in connexin expression.

***Methods and Results*—**Fifty-four patients with different inflammatory diseases and elevated C-reactive protein were prospectively enrolled, and electrocardiographic P-wave dispersion indices, cytokine levels (interleukin-6, tumor necrosis factor-α, interleukin-1, interleukin-10), and connexin expression (connexin 40, connexin 43) were measured during active disease and after reducing C-reactive protein by >75%. Moreover, peripheral blood mononuclear cells and atrial tissue specimens from an additional sample of 12 patients undergoing cardiac surgery were evaluated for atrial and circulating mRNA levels of connexins. Finally, in vitro effects of interleukin-6 on connexin expression were studied in HL-1 mouse atrial myocytes. In patients with active inflammatory diseases, P-wave dispersion indices were increased but rapidly decreased within days when C-reactive protein normalizes and interleukin-6 levels decline. In inflammatory disease patients, both P-wave dispersion indices and interleukin-6 changes were inversely associated with circulating connexin levels, and a positive correlation between connexin expression in peripheral blood mononuclear cells and atrial tissue was demonstrated. Moreover, interleukin-6 significantly reduced connexin expression in HL-1 cells.

***Conclusions*—**Our data suggest that regardless of specific etiology and organ localization, systemic inflammation, via interleukin-6 elevation, rapidly induces atrial electrical remodeling by down-regulating cardiac connexins. Although transient, these changes may significantly increase the risk for atrial fibrillation and related complications during active inflammatory processes. **(*J Am Heart Assoc*. 2019;8:e011006. DOI: 10.1161/JAHA.118.011006.)**

Key Words: atrial electrical remodeling • atrial fibrillation • connexins • interleukin-6 • P-wave indices • systemic inflammation

Atrial fibrillation (AF) is the most common sustained arrhythmia in the general population, estimated to affect >30 million individuals worldwide.[1] Although several studies focused on the pathophysiology of this arrhythmia, detailed underlying mechanisms are still not completely understood, thereby explaining why current treatments are still suboptimal.[2]

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Accompanying Tables S1 through S6 and Figure S1 are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.118.011006

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Clinical Perspective

What Is New?

- In patients with active inflammatory diseases, P-wave dispersion indices are increased, but rapidly decrease within days when C-reactive protein normalizes and interleukin-6 levels decline.
- In these patients, both P-wave dispersion indices and interleukin-6 changes are inversely associated with circulating levels of connexin 40 and connexin 43, and a positive correlation between connexin expression in peripheral blood mononuclear cells and atrial tissue is demonstrated.
- Interleukin-6 significantly reduces connexin 40 and connexin 43 expression in HL-1 mouse atrial myocytes.

What Are the Clinical Implications?

- Regardless of specific etiology and organ localization, systemic inflammatory activation, via interleukin-6 elevation, rapidly induces atrial electrical remodeling by downregulating cardiac connexins; although transient, these changes may significantly increase the risk for atrial fibrillation and related complications during active inflammatory processes.
- A prompt treatment of concomitant inflammatory conditions, including intercurrent infections, in patients with specific risk factors for atrial fibrillation, such as preexisting cardiac disease or genetic predisposition, may be an important therapeutic measure, although to date largely overlooked.

Recently, a key role for both gap-junction changes[3] and inflammatory activation[4] in the pathogenesis of AF is increasingly recognized.

Gap-junctions are intercellular channels mediating electrical coupling between 2 adjacent cardiomyocytes, each one contributing to the junction with a hemichannel or connexon, formed by 6 transmembrane ion-channel proteins or connexins characterized by a rapid turnover (half-life, 1–5 hours).[5,6] Although a variety of connexins are expressed in the cardiac tissue, connexin 40 and connexin 43 are both expressed in the atrium and critically contribute to atrial conduction velocity and refractoriness heterogeneity, both representing major determinants of reentry mechanisms maintaining AF.[5,7] Accordingly, several genetic studies demonstrated association between connexin variants and the risk of AF, and abnormalities in connexin expression and distribution have been reported in animals and humans with AF.[3,8] Moreover, both connexin 40 and connexin 43 gene transfer preserves conduction velocity and prevents AF in porcine and canine models.[3]

A large body of evidence demonstrated that cardiac or systemic inflammation is a strong predictor of AF in the general population, as well as in patients who have undergone cardiac surgery, cardioversion, and catheter ablation.[9] An association between C-reactive protein (CRP) and inflammatory cytokine levels (tumor necrosis factor (TNF)-α, interleukin-1, interleukin-2, interleukin-6, interleukin-10) with the presence of AF has been reported.[4,9] The most recognized underlying mechanisms include acceleration of coronary atherosclerosis, myocardial injury, and fibrosis, leading to structural atrial remodeling and increased thrombogenicity.[4,9] An important role for electrical remodeling attributable to direct effects of cytokines on atrial ion channel expression and/or function is also increasingly recognized (inflammatory channelopathies).[4,10–12] Indeed, in vitro experiments and animal models provided evidence that TNF-α can induce gap-junction channel dysfunction via impaired atrial connexin 40 and connexin 43 expression and/or distribution.[13–15] Notably, these changes occur rapidly and are evident within hours.[16]

Atrial remodeling, both structural and electric, leads to atrial morphology and electrophysiological changes reflected early on the surface ECG by a number of P-wave indices, including P-wave duration, P-wave dispersion (PWD), and P-wave standard deviation (Psd or P-wave index).[17,18] Increase in these parameters, representative of slowed and inhomogeneous intra/interatrial conduction of sinus impulses has been associated in large population-based studies with a higher risk of AF.[17,19,20] Growing evidence indicates that P-wave indices correlate with markers of systemic inflammation[21–24] and are frequently increased in patients with inflammatory diseases of different pathogenesis and localization,[10,19] both chronic (ie, psoriasis,[24] chronic inflammatory arthritis,[23,25] inflammatory bowel disease[26]) and acute (sepsis).[27] Notably, large population-based studies demonstrated that in many of these conditions, AF incidence is higher than in the general population,[28–32] also correlating with flares and increased disease activity.[30,32]

As such, it seems conceivable that activation of systemic inflammation, regardless of its origin, may acutely induce atrial electrical remodeling, via direct cytokine-mediated effects on connexins. These functional changes could critically contribute to increased atrial arrhythmic risk during acute inflammation or flares of chronic inflammatory disease. To test this hypothesis, we studied the relationship between inflammatory markers, P-wave indices, and connexin expression during active disease and remission in patients with systemic inflammatory diseases of different origin.

Patients and Methods

The authors declare that all supporting data are available within the article and its online supplementary files. Local ethical committees approved the study, and patients from all groups gave their oral and written informed consent in accordance with the Principles of the Declaration of Helsinki.

Study Populations

To evaluate the impact of systemic inflammation on the electric properties of atrial myocardium, we prospectively enrolled 54 patients with elevated CRP levels resulting from different inflammatory conditions, including acute inflammatory processes, septic or aseptic, or chronic immune-mediated diseases during flares. In these patients, ECG recordings and blood sample withdrawals were performed during active disease (PRE), and after different therapeutic interventions, resulting in a CRP decrease >75% compared with the baseline (POST). Demographic and clinical characteristics of this population are detailed in Table 1.

In addition, to better interpret the clinical relevance of data obtained in patients with inflammatory diseases, 25 subjects comparable for age and sex, not having a history of systemic inflammation or cardiac disease, were enrolled as a control group (C) for ECG and laboratory parameters (Table S1).

Finally, a third additional sample of 12 patients undergoing cardiac surgery, different from the 2 cohorts described above, was specifically recruited to evaluate the correlation in terms of connexin expression between peripheral blood mononuclear cells (PBMCs) and atrial tissue. More information on this population is provided below and in Table S2.

ECG Recordings

A simultaneous 12-lead ECG (25 mm/s and 10 mV/cm) was recorded by means of a commercially available imaging system (Cardioline ECT WS 2000, Remco Italia, Vignate-Milano, Italy) in all subjects in a supine position (during spontaneous breathing) in the morning hours (between 8 AM and 12 PM). Paper-printed ECGs were scanned and digitized to achieve greater precision in detecting and measuring P-waves.[33] P-wave duration was measured from the beginning of the P-wave deflection from the isoelectric line to the end of the deflection, returning to the isoelectric line in all simultaneous 12 leads.[34] The following indices were derived from measurements of each ECG: the maximum P-wave duration (Pmax), the minimum P-wave duration (Pmin), P-wave dispersion (PWD, ie the difference between Pmax and Pmin in the 12 leads), and Psd (ie, the standard deviation of P-wave duration across all 12 leads, also representing an index of PWD). PWD values <40 milliseconds were considered normal,[19] while for Psd age- and sex-specific reference values in healthy subjects from the Framingham Heart Study were used to define normal ranges.[18]

Laboratory Analysis

Blood samples were centrifuged at 2840*g*, and serum samples were stored at −80°C. CRP was assayed by a particle-enhanced turbidimetric method (COBAS-6000 platform,

Table 1. Demographic and Clinical Characteristics, and Ongoing Therapies of Patients With Inflammatory Diseases

Patients, n	54
Age, y (range)	74.3±16.6 (24–98)
Females, n	30/54 (56%)
Definite inflammatory diseases, n	54/54 (100%)
Acute infections	39/54 (72%)
Pneumonia	17/39 (44%)
Sepsis	6/39 (15%)
Biliary tract infection	6/39 (15%)
Acute bronchitis	5/39 (13%)
Urinary tract infection	4/39 (10%)
Spondylodiscitis	1/39 (3%)
Skin infection*	1/39 (3%)
Immune-mediated diseases	13/54 (24%)
Rheumatoid arthritis	10/13 (77%)
Inflammatory bowel disease*	1/13 (8%)
Polymyalgia rheumatica	1/13 (8%)
Cryoglobulinemic vasculitis	1/13 (8%)
Other	2/54 (4%)
Acute microcrystalline arthritis	1/2 (50%)
Acute pancreatitis	1/2 (50%)
Therapeutic interventions for inflammatory disease, n	54/54 (100%)
Antibiotics	40/54 (74%)
Piperacillin/tazobactam	16/40 (40%)
Ceftriaxone	10/40 (25%)
Amoxicillin/clavulanate	4/40 (10%)
Levofloxacin	4/40 (10%)
Vancomycin	4/40 (10%)
Clarithromycin	3/40 (7%)
Metronidazole	3/40 (7%)
Imipenem	2/40 (5%)
Cefotaxime	1/40 (2%)
Ceftazidime	1/40 (2%)
Rifampicin	1/40 (2%)
Teicoplanin	1/40 (2%)
Meropenem	1/40 (2%)
Oxacillin	1/40 (2%)
Colistin	1/40 (2%)
Fluconazole	1/40 (2%)
Anti-inflammatory drugs	13/54 (24%)
Corticosteroids	10/13 (77%)
Tocilizumab	9/13 (69%)

Continued

Table 1. Continued

Methotrexate	2/40 (15%)
Cyclosporine	1/13 (8%)
Leflunomide	1/13 (8%)
Abatacept	1/13 (8%)
Colchicine	1/13 (8%)
Other	1/54 (2%)
Gabexate mesilate	1/1 (100%)

Age is expressed as mean±standard deviation (range).
*In one patient, both skin infection and inflammatory bowel disease were concomitantly detected. This patient has been counted in the "Immune-mediated disease" group.

Roche Diagnostics GmbH, Mannheim, Germany) and the values were expressed as milligrams per deciliter (normal values <0.5). Circulating levels of inflammatory cytokines interleukin-6, TNF-α, and interleukin-1 and the anti-inflammatory cytokine interleukin-10 were evaluated by multiplex assay for cytokine quantification (Bioplex, Bio-Rad, Hercules, CA). Cytokine concentrations were calculated using a standard curve established from serial dilutions of each cytokine standard as described in the manufacturer's protocol and expressed as picograms per milliliter. Because no established reference values for cytokine levels are currently available, an internal reference control group of 10 healthy subjects (mean age, 55.5±4.3 years) without clinical signs of ongoing acute infections was used. While CRP levels were measured in all patients of the study population, cytokine levels were evaluated in 41 of 54 patients. They were the last 41 subjects enrolled. After that, a preliminary evaluation on the first 13 patients recruited suggested potentially significant changes in P-wave indices in PRE versus POST conditions.

Correlation Study of Atrial Tissue and Circulating Levels of Connexins in Patients Undergoing Cardiac Surgery

Previous studies analyzed mRNA expression of sodium and potassium ion channels in PBMC as a reflection of the cardiomyocyte,[35,36] as the existence of a strong correlation between PBMC and myocardial expression has been demonstrated.[35] On the basis on this evidence, PBMC and atrial tissue specimens from the right or left appendage were obtained from 12 consecutive patients undergoing cardiac surgery, including valve surgery, coronary artery bypass surgery, and heart transplantation (Table S2). Blood samples were withdrawn 1 to 12 hours before surgical procedures, and atrial tissues were obtained before the establishment of extracorporeal circulation. Atrial tissue specimens were collected in Dulbecco's phosphate buffered saline 1% penicillin-streptomycin solution and immediately homogenized and stored at −80°C for further RNA extraction. Heparinized peripheral blood samples were diluted 1:1 with Dulbecco's phosphate buffered saline and PBMCs collected after a "lympholyte density gradient separation." PBMCs were washed twice in Dulbecco's phosphate buffered saline, and the pellet obtained was immediately lysed, frozen, and stored at −80°C for further RNA extraction.

For each of these patients, connexin mRNA levels were measured in both atrial tissue and PBMCs.

Circulating Connexin Levels in Patients With Inflammatory Diseases

In a subcohort of 16 consecutive, unselected patients belonging to the main cohort of 54 patients with inflammatory diseases, peripheral blood samples were withdrawn in PRE and POST conditions to isolate PBMCs and analyze connexin mRNA expression in these cells.

Analysis of Connexin 40 and Connexin 43 mRNA by Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated from heart tissue (30 μg) and PBMCs (10×10^6) using the RNeasy Lipid Tissue Mini Kit and miRNeasy Mini Kit, respectively, following the datasheet provided by the manufacturer. For cDNA synthesis, RT^2 First Strand Kit was used, according to the manufacturer's instructions. Quantitative reverse transcriptase polymerase chain reaction was performed using customized RT^2 polymerase chain reaction arrays with primers for connexin 40 (*GJA5*) and connexin 43 (*GJA1*). β-Actin and hypoxanthine phosphoribosyltransferase were selected and used as reference genes to normalize mRNA expression data obtained with quantitative reverse transcriptase polymerase chain reaction experiments.[35] Primers for the genes of interest were synthesized by the manufacturer and provided already dispensed in customized RT^2 polymerase chain reaction arrays. All kits and reagents were purchased at Qiagen S.p.A, Milano, Italy.

HL-1 Mouse Atrial Myocytes Culture

HL-1 mouse atrial myocytes were maintained in gelatin-fibronectin-coated flasks in Claycomb medium (Sigma-Aldrich, Boston, MA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Boston, MA), 0.1 mmol/L norepinephrine (Sigma-Aldrich, Boston, MA), 100 U/mL penicillin, 100 mg/mL streptomycin (Gibco, Invitrogen, Grand Island, NY), and 2 mmol/L L-glutamine (Gibco, Invitrogen, Grand Island, NY) and kept semiconfluent at all times.

Protein Extraction and Western Blotting on HL-1 Mouse Atrial Cardiomyocytes

HL-1 mouse atrial cardiomyocytes were untreated or treated with interleukin-6 (100 pg/mL, Sigma-Aldrich, Boston, MA)

for 24 and 48 hours and interleukin-6+monoclonal anti–interleukin-6 antibody (Thermo Fisher Scientific, Waltham, MA) complex was added. Cell pellets were collected after 48 or 72 hours, respectively. Likewise, interleukin-6 supplementation was stopped after 24 or 48 hours of interleukin-6 treatment, and pellets were collected at the 72-hour time point. All pellets were lysed in radioimmunoprecipitation assay buffer with protease inhibitors (25 mmol/L Tris-HCl [pH 7.6], 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo Fisher Scientific, Waltham, MA) for 30 minutes at 4°C and centrifuged at 19 722*g* for 15 minutes. The supernatant after centrifugation was resolved by SDS-PAGE on a 4% to 15% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA) and transferred on to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA). Blots were blocked with 5% milk for an hour and probed with anti–connexin 40 and anti–connexin 43 antibody (Sigma, St. Louis, MO; concentration: 12.5 μg and 20 μg/mL, respectively) and glyceraldehyde 3-phosphate dehydrogenase antibody (Sigma, St. Louis, MO; concentration: 5 μg/mL) overnight at 4°C. Further, it was probed with anti-rabbit immunoglobulin G horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX) at a 1:5000 dilution. The signal was detected with Clarity ECL substrate (Bio-Rad Laboratories, Hercules, CA), and blots were scanned in a C-Digit blot scanner (LI-COR, Lincoln, NE) at high sensitivity to obtain the image.

Statistical Analysis

Descriptive statistics is reported as frequency count and percentage for qualitative data, and mean±standard deviation or median and range of variation for quantitative data.

The following parametric or nonparametric statistical analyses were carried out: the 2-tailed Student paired *t* test, 2-tailed Wilcoxon matched-pairs test, 2-tailed Student unpaired *t* test, or 2-tailed Mann–Whitney test to evaluate differences in quantitative variables between 2 groups of data paired (changes in P-wave indices, CRP, cytokines, PBMC connexin expression in inflammatory patients, PRE versus POST; interleukin-6–dependent changes in connexin expression in mouse atrial cardiomyocytes versus baseline) or unpaired (comparisons of P-wave indices, CRP, cytokines, PBMC connexin expression in inflammatory patients versus controls), respectively; the Spearman rank correlation-test to verify possible statistical association between quantitative variables in patients with inflammatory diseases (P-wave indices versus CRP, neutrophil/lymphocyte ratio, cytokines, or PBMC connexin expression; CRP or cytokines versus PBMC connexin expression) and in patients undergoing cardiac surgery (atrial versus PBMC connexins expression); the 2-sided Fisher's exact test was performed to evaluate statistical association between categorical variables in patients with inflammatory diseases (P-wave indices, PRE versus POST) and in patients with inflammatory diseases versus controls (sex).

We also performed a sample size and power analysis to define the number of subjects to include in both groups of patients and controls. For the study, an effect size of 1 was considered clinically acceptable, corresponding to the same size of the average differences between sample groups and their group-pooled variations. This allows rejecting the null hypothesis of equivalence between samples with a statistical significance of 95% and a power of at least 90% with just 25 study cases and 25 controls, even using a nonparametric test. Nevertheless, all 54 study patients were used in the PRE-POST comparison, thus reducing the effect size. $P \leq 0.05$ was considered significant (GraphPad-InStat, version 3.06 for Windows 2000).

Results

Relationship Between Inflammatory Markers and P-Wave Indices in Patients With Inflammatory Diseases

During the active phase, most patients with inflammatory diseases showed increased values of P-wave dispersion indices, that is, PWD (41/54, 76%; median 48.5 milliseconds) and Psd (34/54, 63%; median 16.7 milliseconds). Therapeutic interventions, including antibiotics, anti-inflammatory drugs, or protease inhibitors (Table 1), depending on the specific inflammatory disease present, were associated with a rapid (mean follow-up time, 18.9±23.0 days; median, 10.0 [2–93] days) and significant reduction in CRP levels (from 13.8 [1.0–39.8] to 1.6 [0.01–6.3] mg/dL; median decrease 88.4%) as well as in Pmax (from 132.5 [88–168] to 122.8 [84–160] milliseconds), PW7D (from 48.5 [23–88] to 39.0 [18–61.5] milliseconds), and Psd (from 16.7 [5.8–34.6] to 13.4 [5.8–22.8] milliseconds) values. Of note, PWD and Psd reduced until reaching values observed in controls (Figure S1). Conversely, Pmin duration significantly prolonged (from 80.0 [58–122] to 86.0 [52–140] milliseconds) (Table 2; Figure 1). Accordingly, the number of patients showing increased values of PWD (22/54; 41%) and Psd (21/54; 39%) significantly decreased (Table 2). P-wave indices significantly correlated with CRP levels throughout the study duration, particularly Psd (ρ=0.35; P=0.0002), PWD (ρ=0.31; P=0.0010) and Pmax (ρ=0.25; P=0.0083) (Figure 2). On the contrary, no association was found between Pmin and CRP (Table S3). Moreover, echocardiography findings or other laboratory parameters, including electrolyte levels, did not show significant changes (Table 2). Notably, although patients with acute septic inflammatory processes treated with antibiotics reached, as expected, a CRP decrease >75% earlier than patients with

Table 2. Changes in Clinical, Electrocardiographic, Laboratory, and Echocardiographic Parameters in Patients With Inflammatory Diseases (n=54), During Active Disease (PRE), and After Therapeutic Interventions Resulting in a CRP Decrease >75% When Compared With the Baseline (POST)

	PRE	POST	*P* Value
CRP, mg/dL (r.v. <0.5)	13.8 (1.0–39.8)	1.6 (0.01–6.3)	<0.0001*
Neutrophil/lymphocyte ratio†	8.1 (2.1–15.7)	3.7 (1.4–9.5)	0.0017*
Interleukin-6‡, pg/mL (r.v. 0.49–1.25)	14.0 (0.2–156.5)	3.6 (0.05–66.1)	<0.0001*
Interleukin-1‡, pg/mL (r.v. 0.08–0.29)	0.37 (0.10–1.21)	0.24 (0.10–1.36)	0.025*
TNF-α‡, pg/mL (r.v. 0.60–3.24)	0.75 (0.15–13.75)	0.75 (0.15–12.98)	0.63
Interleukin-10‡, pg/mL (r.v. 0–3.60)	0.60 (0–2.35)	0.55 (0–2.23)	0.57
Pmax, ms§	132.5 (88–168)	122.8 (84–160)	0.006*
Pmin, ms§	80.0 (58–122)	86.0 (52–140)	0.012*
PWD, ms§	48.5 (23–88)	39.0 (18–61.5)	<0.0001*
Patients with high PWD (>40 ms), n	41 (76%)	22 (41%)	0.0004*
Psd, ms§	16.7 (5.8–34.6)	13.4 (5.8–22.8)	<0.0001*
Patients with high Psd, n‖	34 (63%)	13 (39%)	0.020*
Potassium, mEq/L (r.v. 3.5–5.5)	4.1±0.6	4.1±0.6	0.62
Calcium, mg/dL (r.v. 8–11)	8.5±0.7	8.7±0.6	0.96
Magnesium, mg/dL (r.v. 1.5–2.5)	2.0±0.3	1.8±0.3	0.19
Creatinine, mg/dL (r.v. 0.7–1.2)	1.1±0.5	0.9±0.3	0.10
pO_2, mm Hg (r.v. 70–100)	69.7±12.8	72.6±9.8	0.17
pH (r.v. 7.35–7.45)	7.43±0.05	7.44±0.04	0.95
Left atrial diameter, mm (r.v. <40)	39.9±3.1	39.8±3.1	0.99
Ejection fraction, % (r.v. >50)	56.4±4.5	57.5±4.3	0.23
Left ventricular internal dimension, mm (r.v. <56)	47.9±4.3	47.8±4.6	0.58
Estimated pulmonary artery pressure, mm Hg (r.v. <30)	33.3±6.9	30.1±5.9	0.10

Values are expressed as median (range), or mean±standard deviation, or frequency count and percentages. Differences were evaluated by the 2-tailed Student paired *t* test, or the 2-tailed Wilcoxon matched-pairs test. Difference in categorical variables were evaluated by the 2-sided Fisher's exact test. CRP indicates C-reactive protein; Pmax, maximum P-wave duration; Pmin, minimum P-wave duration; Psd, P-wave standard deviation; PWD, Pwave dispersion; r.v., reference values; TNF-α, tumor necrosis factor-α.

*Statistically significant *p* values.

†Data available for 19 of 54 patients.

‡Data available for 41 of 54 patients. Cytokine level range measured in an internal reference group of healthy controls.

§Reference values vary depending on age and sex (see Magnani et al[18]).

‖Based on age- and sex-specific reference values in healthy subjects from the Framingham Heart Study.[18]

chronic immune-mediated diseases (during flares) treated with immune-modulating/anti-inflammatory drugs (median time, 8.5 [2–28] versus 30.0 [4–93] days; *P*=0.0005; n=39 versus 13, 2-tail Mann–Whitney test), a significant and substantially similar decrease of P-wave dispersion indices was observed in both groups (PWD: from 49.0 [23–88] to 39.0 [20–61.5] milliseconds, *P*<0.0001 versus 47.0 [28–63] to 39.0 [18–52] milliseconds; *P*=0.0005, 2-tailed Student paired *t* test; Psd: from 17.0 [9.0–34.6] to 13.4 [5.8–22.8] milliseconds; *P*<0.0001 versus 15.0 [5.8–23.5] to 13.1 [6.7–18.7] milliseconds; *P*=0.029; 2-tailed Student paired *t* test).

Measurement of circulating inflammatory cytokines exhibited high interleukin-6 levels during active disease, over 20 times higher than healthy controls, but were markedly reduced after therapeutic interventions (Table 2; Figure 3A). A significant correlation was observed between interleukin-6 levels and P-wave indices throughout the study duration, particularly Psd (ρ=0.31; *P*=0.0047), Pmax (ρ=0.27; *P*=0.013) and PWD (ρ=0.22; *P*=0.048) (Figure 4A through 4C); conversely, no association was found between Pmin and interleukin-6 (Table S3). Although approaching normalization, median interleukin-6 levels in POST conditions did not fall below the reference value (ie, >1.25 pg/mL), also being significantly higher than in the control group (Table S1; Figure 3A). This was attributable to the fact that in a number of patients systemic inflammation was not completely suppressed after the treatment, as a result of the criterion used to perform POST measurements (CRP reduction >75%).

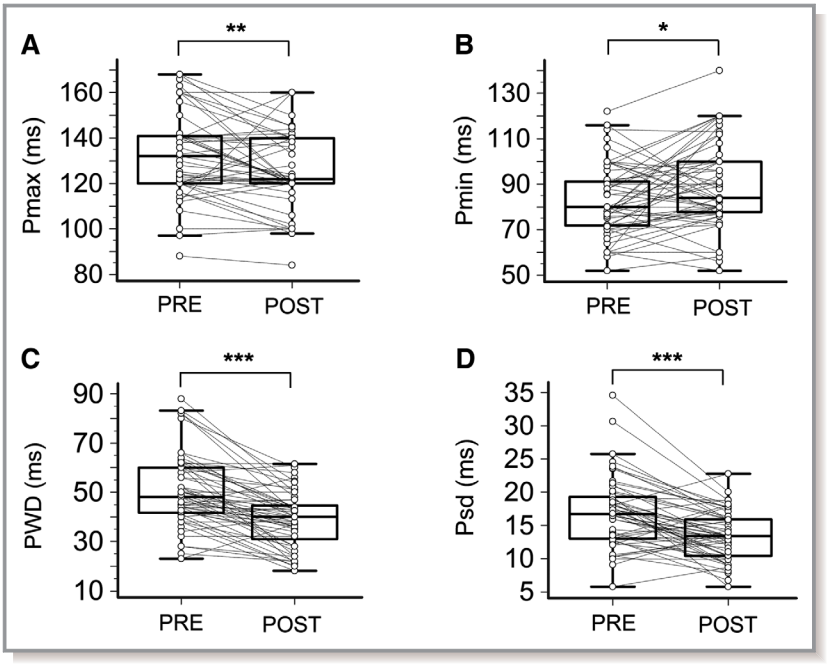

Figure 1. Changes in P-wave indices in patients with inflammatory diseases, during active disease (PRE) and after therapeutic interventions resulting in a CRP decrease >75% when compared with the baseline (POST). **A**, Maximum P-wave duration (Pmax); 2-tailed Wilcoxon matched-pairs test, $^{**}P<0.01$. **B**, Minimum P-wave duration (Pmin); 2-tailed Wilcoxon matched-pairs test, $^{*}P<0.05$. **C**, P-wave dispersion (PWD); 2-tail Wilcoxon matched-pairs test, $^{***}P<0.0001$. **D**, P-wave standard deviation (Psd); 2-tailed Student paired *t* test, $^{***}P<0.0001$. Patients, n=54.

Nevertheless, in 11 of 41 patients, interleukin-6 levels in POST conditions reached values <1.25 pg/mL (median value, 0.2 [0.05–1.23] pg/mL). With respect to the whole population, these subjects showed an additional slight decrease in POST conditions of both P-wave dispersion parameters, that is, PWD (from 48.5 [23–88] to 39.0 [18–61.5] milliseconds, −19.6% versus 48.0 [28–63] to 38.0 [22–61.5] milliseconds, −20.9%) and, particularly, Psd (from 16.7 [5.8–34.6] to 13.4 [5.8–22.8] ms, −19.9% versus 17.3 [9.9–23.5] to 12.4 [9.0–18.8] milliseconds, −28.4%), thus supporting a strict relationship between interleukin-6 levels and atrial electrical remodeling.

Median interleukin-1 levels were only slightly but significantly increased in patients during the active inflammatory phase when compared with controls, and only slightly reduced after therapeutic interventions resulting in a CRP decrease of >75%. Nevertheless, such a reduction reached the statistical significance (P=0.025) with respect to PRE conditions. Notably, like interleukin-6, interleukin-1 levels in POST conditions remained slightly but significantly higher than controls (Table 2; Figure 3B; Table S1). IL-1 levels did not correlate with P-wave indices, except Pmax (ρ=0.23; P=0.037) (Table S3). Conversely, mean TNF-α and IL-10 levels overlapped those detectable in controls without any appreciable change after treatment (Table 2; Figure 3C and 3D).

Correlation Between Atrial and Circulating Levels of Connexins in Patients Undergoing Cardiac Surgery

Connexins 40 and 43 are both expressed in PBMCs, where they play important roles in the development/recruitment of leukocytes and in the regulation of the immune response. In particular, they accumulate at the immunological synapse and contribute to T-cell activation.[37,38] Paired analysis of mRNA levels of connexins from atrial tissue and PBMCs demonstrated strong correlations for both connexin 40 (ρ=0.71; P=0.0091) and connexin 43 (ρ=0.70; P=0.0099) (Figure 5). These data provided evidence that PBMC-derived expression of connexins is correlative of the expression levels of these channels in the myocardial tissue of the atria.

Relationship Between Circulating Connexins Levels, P-Wave Indices and Inflammatory Markers in Patients With Inflammatory Diseases

Expression analyses were performed in PBMCs from 16 consecutive, unselected patients with inflammatory diseases in both PRE and POST conditions (mean follow-up time, 11.7±6.7 days; median, 12.0 [4–28] days). These subjects

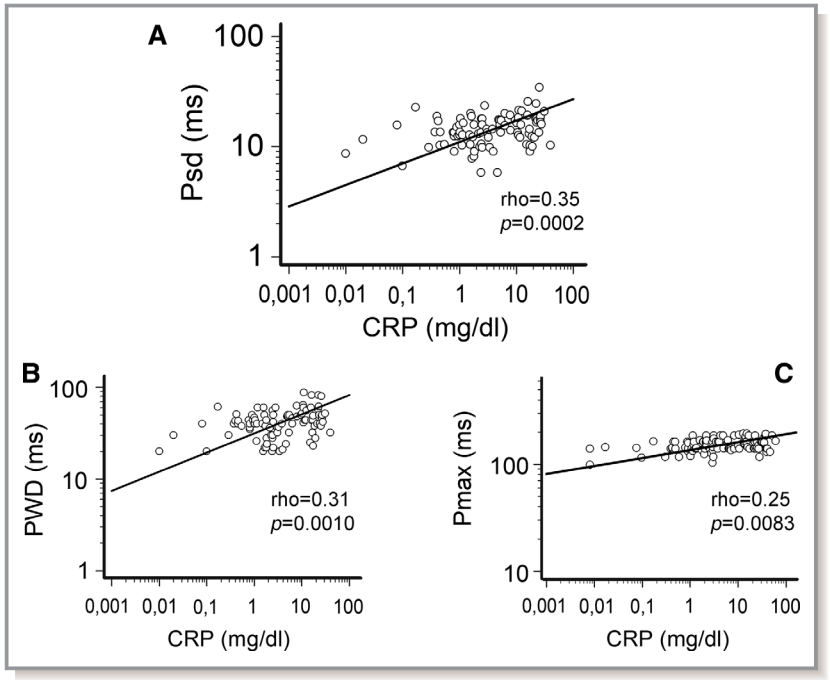

Figure 2. Correlation between P-wave indices and C-reactive protein (CRP) in patients with inflammatory diseases. **A**, Relationship between P-wave standard deviation (Psd) and CRP levels. **B**, Relationship between P-wave dispersion (PWD) and CRP levels. **C**, Relationship between maximum P-wave (Pmax) and CRP levels throughout the time. Spearman test. Patients, n=54.

were representative of the entire cohort, as demonstrated by the substantially overlapping values of P-wave dispersion indices (PWD, Psd) and inflammatory markers in PRE and POST conditions (Table S4). Although in these patients a slight increase in mRNA levels of connexins was observed after a CRP decrease >75% when compared with baseline, such differences were not statistically significant (total connexins, 1.67±0.75 [PRE] versus 1.88±1.39 [POST]; connexin 43, 0.98±0.50 [PRE] versus 1.14±0.79 [POST]; connexin 40, 0.79±0.38 [PRE] versus 0.85±0.65 [POST]; 2-tailed Wilcoxon matched-pairs test, all $P>0.05$). Nevertheless, in these patients, connexin levels (connexin 43, total connexins) were found to be significantly lower than controls in PRE, but not in POST conditions (Figure 6A through 6C). Moreover, connexin 43 expression significantly and inversely correlated with interleukin-6 concentrations throughout the study duration ($\rho=-0.35$; $P=0.044$; Figure 6D; Table S5, top panel).

On the basis of this evidence, we hypothesized that the relatively small sample size could have overestimated the weight of some patients showing, for different reasons, no (or less evident) inflammation-induced changes in the circulating connexin profile. In this regard, PRE/POST differences in connexin mRNA expression were emphasized when patients were grouped on the basis of the actual presence (or not) of evident P-wave indices modifications. Because the percent median reduction of PWD and Psd observed in all patients (n=54) were −19.6% and −19.9%, respectively, we defined that a patient experienced a *high P-wave dispersion indices decrease* when we observed a reduction of PWD and/or Psd values >25% in the POST condition compared with the PRE condition. Based on these criteria, patients were divided into the following 2 groups: the High-ΔP group (ie, high-PWD and/or Psd delta group; n=6) and the Low-ΔP group (ie, low-PWD and/or Psd delta group; n=10). As reported in Table S6 and showed in Figure 7, only in High-ΔP patients an increase in connexin expression (total connexins, connexin 43, and connexin 40) was demonstrated when inflammatory activation was reduced. As a result, mRNA levels in the 2 groups were substantially overlapping in PRE conditions, but in POST conditions they were significantly higher in the High-ΔP when compared with the Low-ΔP group (Figure 7A through 7C). In the High-ΔP group, a clear-cut inverse correlation (rho values up to >0.60) was demonstrated between connexin expression and P-wave indices, particularly Psd and total connexins/connexin 43 (Figure 8; Table S5, bottom panel). In addition, in these patients, connexins (particularly total connexins and connexin 43) and interleukin-6 levels were inversely associated, in a more robust manner than that observed in the whole population (rho from −0.35 in all patients, to −0.61 in the High-ΔP group) (Figure 8; Table S5, bottom panel). Conversely, no significant association between connexins and interleukin-1 was observed.

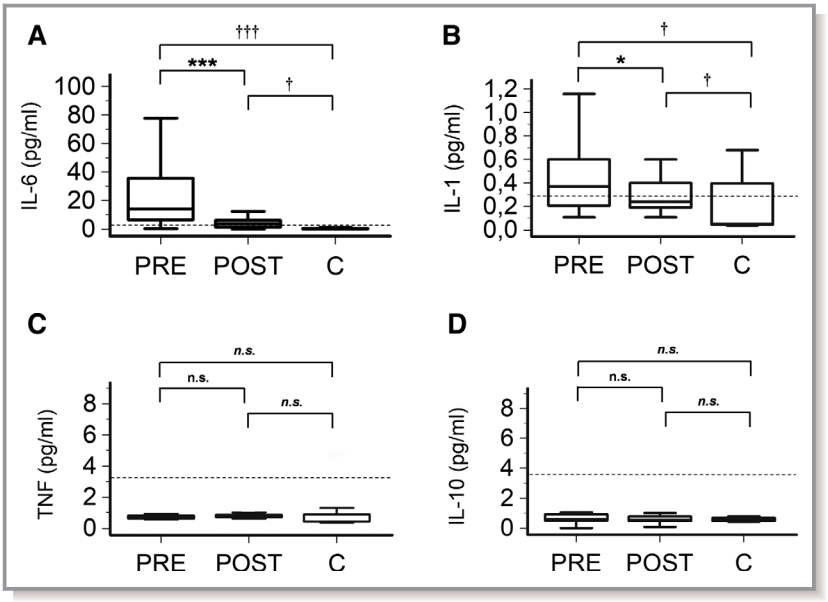

Figure 3. Changes in cytokines in patients with inflammatory diseases, during active disease (PRE) and after therapeutic interventions resulting in a CRP decrease >75% when compared with the baseline (POST), and controls (C). **A** through **D**, Interleukin-6, interleukin-1, tumor necrosis factor-α (TNF), and interleukin-10 levels during active disease (PRE), and after therapeutic interventions resulting in a CRP decrease >75% when compared with the baseline (POST), and controls (C). Two-tailed Wilcoxon matched-pairs test (ns, not significant, *P<0.05, ***P<0.0001.) or 2-tailed Mann–Whitney test (ns, not significant, $^{\dagger}P$<0.05, $^{\dagger\dagger\dagger}P$<0.001). Patients, n=41; controls (C) n=25. Horizontal dotted line indicates the upper limit values in a reference healthy population, that is, 1.25 pg/mL (interleukin-6), 0.29 pg/mL (interleukin-1), 3.24 pg/mL (TNF-α), and 3.6 pg/mL (interleukin-10).

Effect of Interleukin-6 on Connexin Protein Expression in HL-1 Atrial Myocytes

To further assess the effects of interleukin-6 on connexin protein expression, we evaluated connexin 43 and connexin 40 protein expression in HL-1 cells using western blots. HL-1 cells exhibit an ultrastructure and retain a pattern of gene expression characteristic to primary cultures of adult atrial cardiac myocytes.[39] Figure 9 shows that incubation of HL-1 cells for 24 and 48 hours with 100 pg/ml of interleukin-6 resulted in a significant decrease in both connexin 43 (61.3%, P<0.01 at 24 hours; and 57.9%, P<0.01 at 48 hours) and connexin 40 (39.1%, P<0.01 at 24 hours; and 39.0%, P=0.01 at 48 hours). IL-6 effects on connexin 43 were more prominent than those on connexin 40.

Effect of Inhibition of IL-6 With Monoclonal Antibody or Withdrawal of Interleukin-6 Supplementation

To further demonstrate the inhibiting effects of interleukin-6 on connexin 40 and connexin 43 protein expression in HL-1 cells, interleukin-6 complexed with a monoclonal anti–interleukin-6 antibody was added onto HL-1 cells previously treated with 100 pg/ml interleukin-6 for 24 or 48 hours. We observed that the interleukin-6–mediated decrease in connexin 43 and connexin 40 was reversed by the addition of the interleukin-6+anti–interleukin-6 antibody complex, and there was an increase in connexin 43 (Figure 10, interleukin-6+anti–interleukin-6 antibody added after 24 hours: +30.5%; P<0.05) and connexin 40 (Figure 10, interleukin-6+anti–interleukin-6 antibody added after 24 hours: +48%, P<0.01; after 48 hours: +61%, P<0.01) protein levels.

In addition, interleukin-6 supplementation was stopped after treatment with interleukin-6 for 24 or 48 hours and HL-1 cells were collected at 72 hours. We observed that there was an increase in connexin 43 (Figure 11, interleukin-6 supplementation stopped after 24 hours: +61%, P<0.01; after 48 hours: +12%, P<0.01) and connexin 40 (Figure 11, interleukin-6 supplementation stopped after 24 hours: +33%, P<0.05; after 48 hours: +49%, P<0.01) protein levels after interleukin-6 supplementation was stopped, suggesting that interleukin-6–mediated effects on connexin 43 and connexin 40 levels are specific.

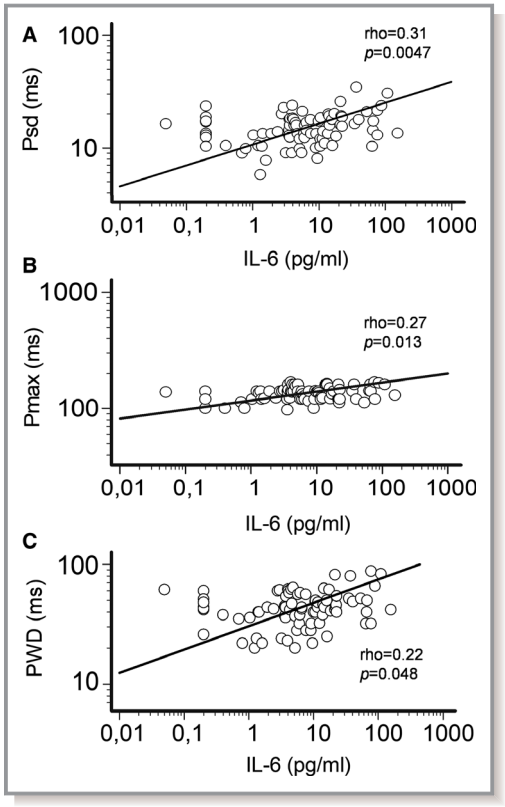

Figure 4. Relationship between interleukin-6 and P-wave indices in patients with inflammatory diseases. **A** through **C**, Relationship between P-wave standard deviation (Psd), maximum P-wave (Pmax), or P-wave dispersion (PWD) and interleukin-6 levels throughout the time. Spearman test. Patients, n=41.

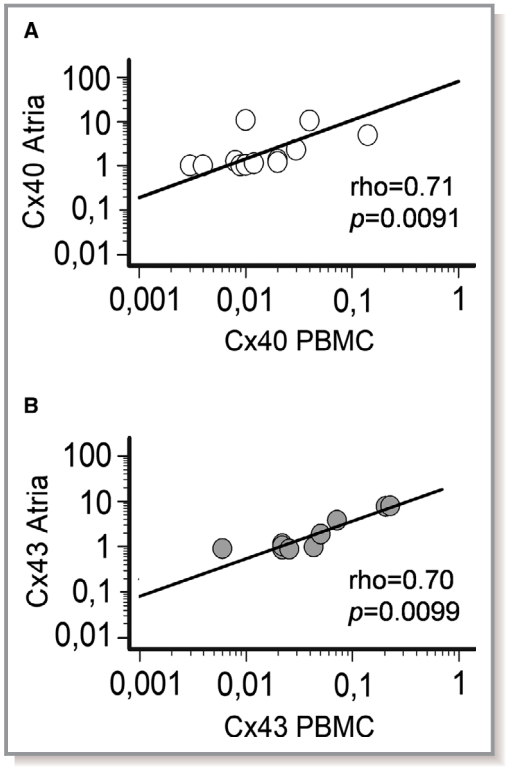

Figure 5. Correlation between atrial and circulating levels of connexins in patients undergoing cardiac surgery. **A**, Relationship between connexin 40 mRNA levels in atrial tissue and peripheral blood mononuclear cells (PBMCs). **B**, Relationship between connexin 43 mRNA levels in atrial tissue and PBMCs. Spearman test. Patients, n=12.

Discussion

In the present study, we provide evidence that in patients with elevated CRP levels from different inflammatory conditions, indices of P-wave dispersion are increased. CRP reduction was associated with a rapid (<20 days on average) and significant decrease of Pmax and P-wave dispersion indices, correlating with the decline of cytokine levels, particularly interleukin-6. Moreover, after demonstrating, for the first time, that connexin 40 and connexin 43 expression in PBMC and atrial tissue are highly correlated, we found that in patients with inflammatory diseases both P-wave indices and interleukin-6 concentration are inversely associated with circulating connexin levels throughout the duration of the study. Finally, in vitro experiments provided evidence that incubation of mouse atrial HL-1 cardiomyocytes with interleukin-6 is associated with a significant decrease in both connexin 40 and connexin 43 protein expression. Altogether, these data suggest that regardless of specific etiology and organ localization, systemic inflammation, via elevation of interleukin-6 levels, can cause atrial electrical remodeling, which is reversible and, at least in part, driven by changes in cardiac connexin expression.

Robust evidence exists that systemic inflammation is associated with an increased risk of AF. Indeed, large studies have demonstrated that inflammatory markers, particularly CRP and interleukin-6, are strong and independent predictors of AF both in patients with manifest cardiac diseases and apparently healthy subjects.[4,9] Furthermore, population-based studies provided evidence that in immune-mediated chronic inflammatory diseases and sepsis the incidence of AF is higher than in the general population,[10,28–32] also correlating with disease activity.[29,30,32] Accelerating effects on coronary artery disease and heart failure development are widely accepted mechanisms possibly linking long-lasting inflammation and AF risk.[4,9,10] Nevertheless, accumulating data indicate that systemic inflammation may also be per se arrhythmogenic by promoting cardiac remodeling, both structural (collagen deposition with myocardial fibrosis) and electric, as a result of direct effects of cytokines on the atrium.[10] These alterations, by modulating atrial impulse propagation, can significantly affect electrocardiographic P-

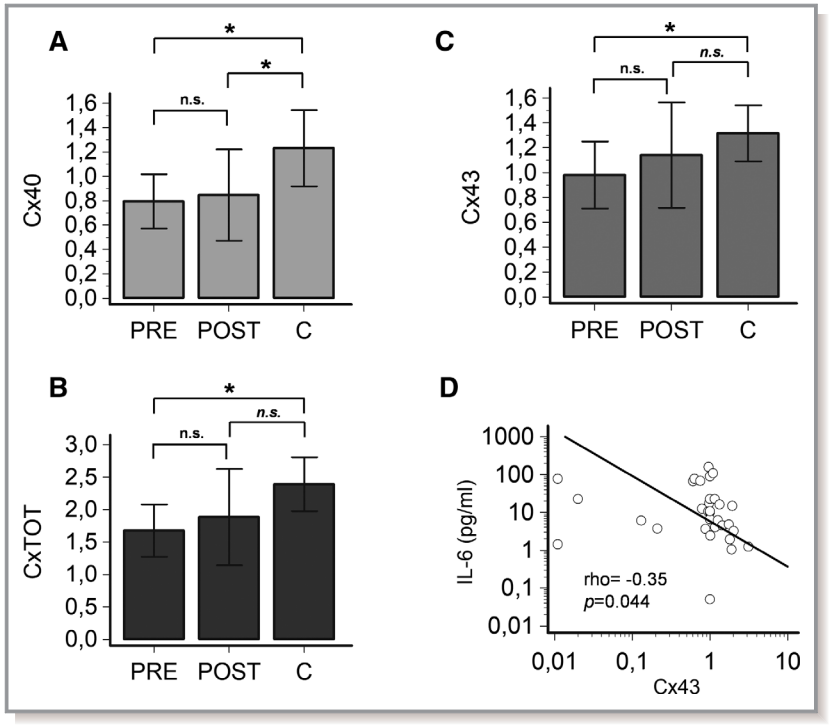

Figure 6. Comparison of circulating levels of connexins in patients with inflammatory diseases, during active disease (PRE) and after therapeutic interventions resulting in a C-reactive protein decrease >75% when compared with the baseline (POST), and controls (C). **A**, Connexin 40. **B**, Connexin 43. **C**, Total connexins. Two-tailed Wilcoxon matched-pairs test (n.s., not significant) or Mann-Whitney test (n.s., not significant, **P*<0.05). Error bars indicate 95% CI for mean. Patients, n=16; controls (C) n=25. **D**, Relationship between circulating interleukin-6 and connexin 43 mRNA levels in peripheral blood mononuclear cells of patients with inflammatory diseases. Spearman test. Patients, n=16.

wave indices, particularly PWD, which are increasingly recognized as surrogates for atrial remodeling in vivo.[17–19] Accordingly, an association between inflammatory activation reflected by CRP levels and PWD increase has been reported in several pathological conditions.[21–24] In our cohort of patients with different inflammatory diseases (infective, immune-mediated, or other) and elevated CRP levels, P-wave dispersion indices were found to be increased in most subjects during the active phase of the disease. In addition, we also demonstrated for the first time that the control of inflammatory activation following specific treatments (antibiotics, immune-modulating drugs, other) was associated with a rapid (days) and significant decrease of P-wave indices, particularly Pmax, PWD, and Psd. Notably, median values of PWD fell below 40 milliseconds, representing the currently accepted upper limit of normality.[19] These changes significantly correlated with the decrease of CRP levels, as well as circulating inflammatory cytokines, particularly interleukin-6. Indeed, while TNF-α/interleukin-1/interleukin-10 were substantially normal at baseline and did not change/slightly decreased when CRP was reduced, interleukin-6 levels were markedly elevated during the active phase (10 times higher than healthy controls) and almost normalized after treatment.

Moreover, interleukin-6 levels significantly correlated with Pmax, PWD, and Psd values throughout the duration of the study, whereas TNF-α/interleukin-1/interleukin-10 did not show any association with P-wave indices, except for interleukin-1 and Pmax. These data depict a predominant role for interleukin-6 among the mediators of inflammation-induced atrial remodeling in vivo. These findings are consistent with a recent study demonstrating that in patients with paroxysmal AF the presence of a single-nucleotide polymorphism in interleukin-6 promoter enhancing cytokine production is associated with increased PWD.[40] While the different behavior of interleukin-10 when compared with interleukin-6 may be in some way explained by the anti-inflammatory nature of this cytokine, it seems apparently less justified for the other proinflammatory cytokines interleukin-1 and TNF-α. Nevertheless, a proinflammatory cytokine profile similar to that observed in our patients was reported by several studies in different inflammatory diseases, including active rheumatoid arthritis[41,42] and septic processes,[43–46] where frequently TNF-α and interleukin-1

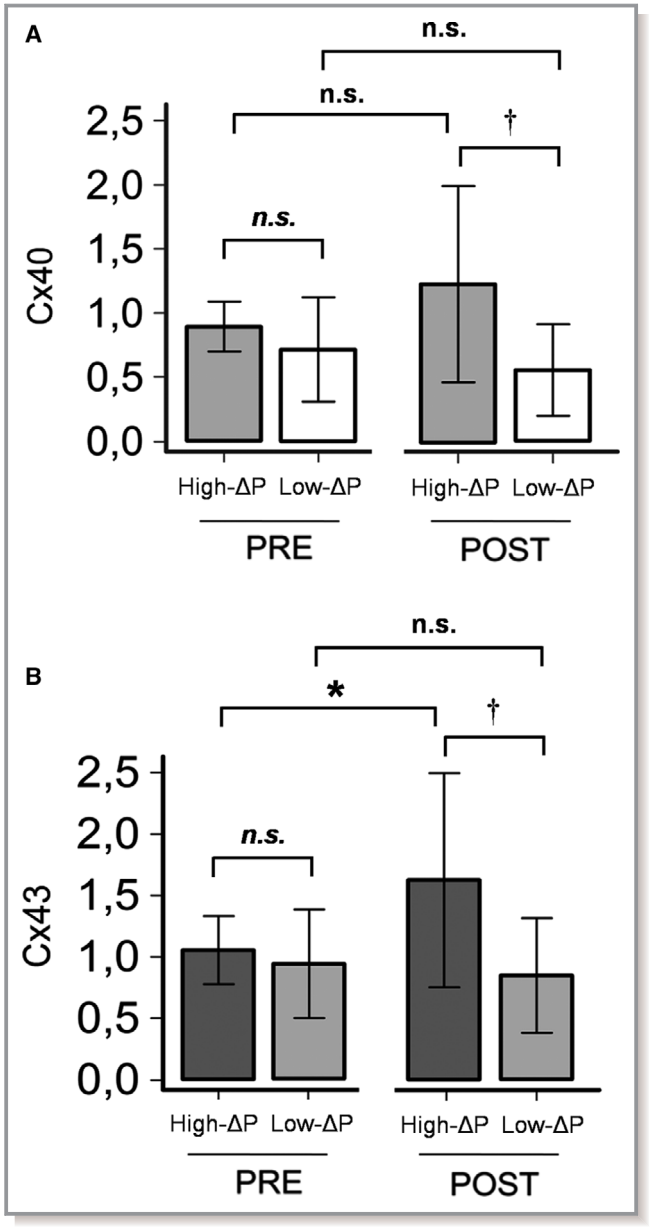

Figure 7. Comparison of PRE-POST changes in circulating connexins levels in inflammatory diseases patients with marked vs low P-wave dispersion indices decrease respect to baseline. **A**, Circulating connexin 40 mRNA levels in PRE and POST conditions in patients with marked vs low P-wave dispersion indices decrease (High-ΔP vs Low-ΔP). **B**, Circulating connexin 43 mRNA levels in PRE and POST conditions in High-ΔP vs Low-ΔP patients. **C**, Total circulating mRNA levels of connexins in PRE and POST conditions in High-ΔP vs Low-ΔP patients. Two-tailed unpaired *t* test (n.s., not significant, *$P \leq 0.05$) or Wilcoxon matched-pairs test (*n.s.*, not significant, $^{\dagger}P \leq 0.05$). High-ΔP patients, n=6; Low-ΔP patients, n=10.

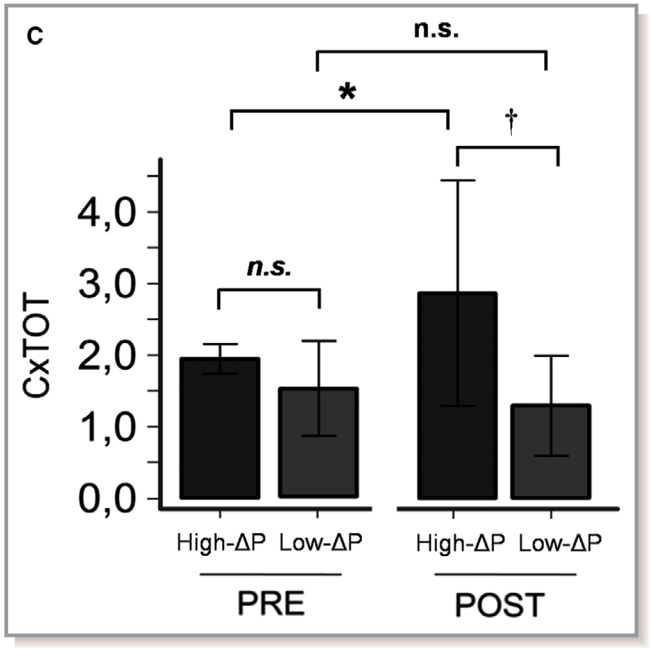

Figure 7. Continued

levels were either unchanged or slightly increased. Conversely, elevated interleukin-6 levels were consistently found throughout the studies in the literature, indicating that this cytokine remains stably elevated until the inflammatory process recovers.[43] This evidence, probably related to the role of TNF-α and interleukin-1 as early regulators of the immune response (peaking in the blood within a few hours, and then promoting downstream proinflammatory molecule release, mainly interleukin-6, in turn rapidly inhibiting TNF-α and interleukin-1 production)[47,48] supports the view that in the clinical setting, the actual impact of interleukin-6 and its electrophysiological effects on the atrium are probably higher than those exerted by TNF-α or interleukin-1.

Another important finding of our study is the time scale of the changes observed in P-wave indices. In fact, Pmax, PWD, and Psd reduced/normalized rapidly as soon as the inflammatory activation was controlled, in the course of some days/weeks only. In fact, although such changes occurred particularly early in patients with acute inflammatory processes (≈1 week to 10 days), a similar behavior was also observed, even if a bit more delayed, in subjects with reactivation of chronic immune-mediated diseases (≈4 weeks). This evidence, demonstrating that such alterations are largely reversible in the short term, in both acute and chronic inflammation during flares, strongly suggests the involvement of functional mechanisms, probably related to an electric remodeling of the atrium. In fact, this period of time is too short for structural remodeling associated with collagen deposition and tissue fibrosis. As such, we focused on connexin expression, also in consideration of preclinical data suggesting that the impact of inflammatory mediators on these proteins may be rapid. Indeed, in the hearts of lipopolysaccharide-treated rats, connexin 43 mRNA expression markedly decreases 6 hours after injection,[16] and in murine cardiomyocytes an overnight incubation with

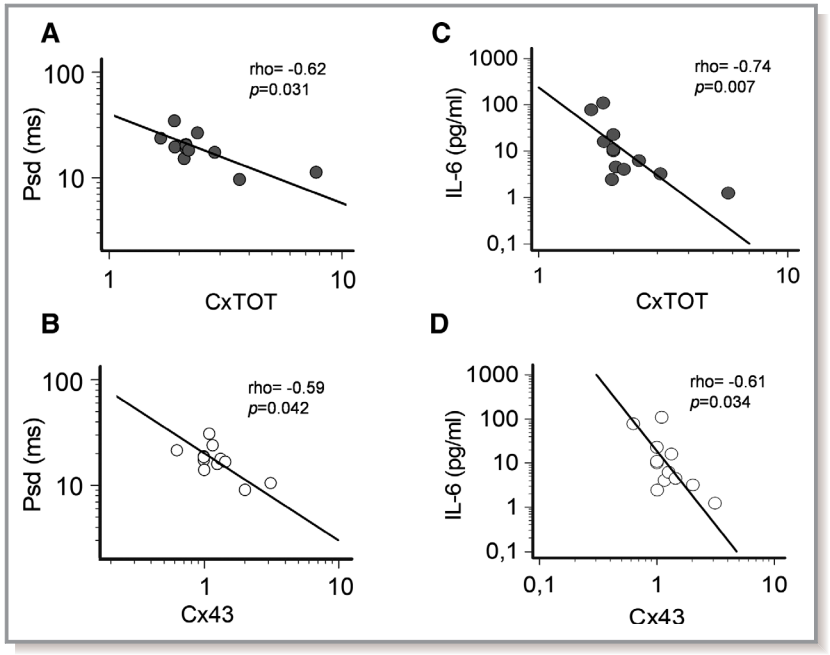

Figure 8. Correlation between P-wave indices, circulating connexins and interleukin-6 levels in patients with inflammatory disease showing marked P-wave dispersion indices decrease with respect to baseline. **A**, Relationship between P-wave standard deviation (Psd) and circulating mRNA levels of total connexins in peripheral blood mononuclear cells (PBMCs) throughout the time. **B**, Relationship between P-wave standard deviation (Psd) and connexin 43 mRNA levels in PBMCs throughout the time. **C** and **D**, Relationship between interleukin-6 levels and circulating mRNA levels of connexins (total connexins, connexin 43) throughout the time. Spearman test. Patients, n=6.

interleukin-1 is enough to significantly reduce connexin 40 membrane expression.[49] As we provided evidence that mRNA connexin 40/ connexin 43 levels in PBMCs are a reflection of connexin expression in the atria, mRNA connexin 40/connexin 43 changes were analyzed also in peripheral cells from a subgroup of consecutive patients during inflammatory activation and remission. Compared with the active phase, PBMC connexin expression tended to rapidly increase as soon as inflammation was controlled (mean follow-up, ≈12 days). These changes became particularly evident in those patients who actually developed marked reduction of P-wave dispersion indices in POST versus PRE conditions (High-ΔP group). Accordingly, we found throughout the study duration a significant inverse correlation between circulating levels of interleukin-6 and connexins, specifically connexin 43 ($\rho=-0.35$). The strength of such an association markedly increased when it was selectively analyzed in the High-ΔP group ($\rho=-0.61$). At the same time, in this latter group connexin levels (connexin 43, total connexins) also significantly and inversely correlated with P-wave dispersion indices, particularly Psd. Of note, among the P-wave indices, Psd showed the strongest correlation also with CRP and IL-6 levels, thus suggesting a particular sensitivity and accuracy of this parameter in reflecting inflammation-induced atrial electric changes in vivo. Notably, a large retrospective analysis on over 40 000 patients identified Psd as one of the strongest independent ECG predictors of AF in the general population.[20] Here, we also demonstrated for the first time that incubation of cultured HL-1 atrial cells with interleukin-6 for 24 to 48 hours was sufficient to significantly reduce both connexin 43 and connexin 40 protein expression, both of which may directly affect atrial conduction and propagation. These effects were reversible after withdrawal of interleukin-6 supplementation or upon preincubation of interleukin-6 with a monoclonal anti–interleukin-6 antibody, thus suggesting specificity of interleukin-6 effects on connexins 40 and 43. Altogether, our data strongly suggest that during active inflammatory processes systemically released cytokines, particularly interleukin-6, act on the atria, inhibiting connexin expression and inducing electric remodeling. The kinetics of these changes, rapidly reversing after interleukin-6 level normalization, seems to be rapid (hours/days), thus putatively accounting for a transient but significant increased risk of AF during active phases of inflammation.

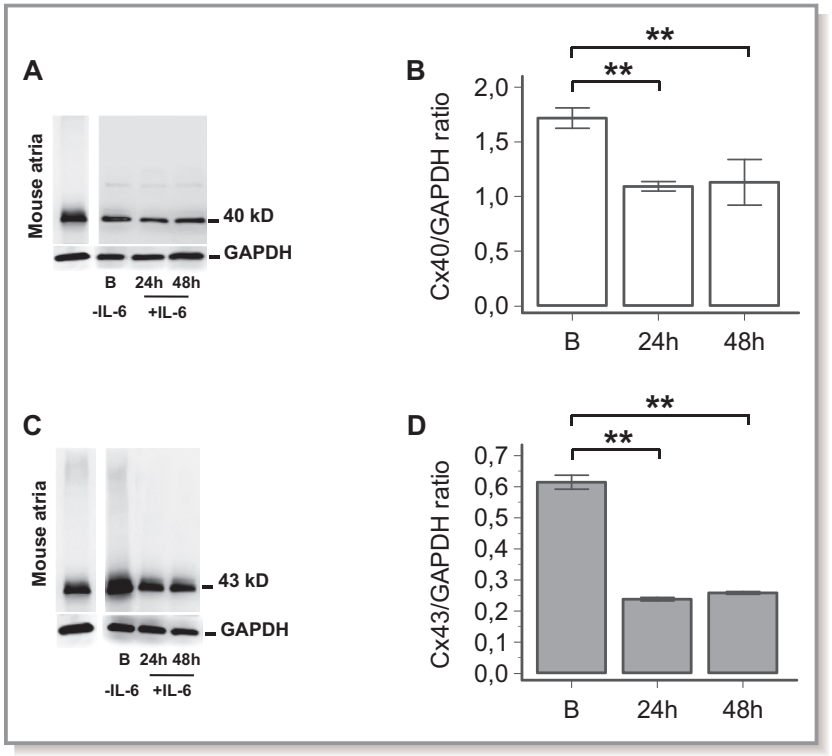

Figure 9. Effects of interleukin-6 on connexin 40 and connexin 43 protein expression in HL-1 cells. **A**, Western blot for connexin 40 at baseline (**B**, ie, without interleukin-6), and after 24 and 48 hours of treatment with 100 pg/ml interleukin-6 and (**B**) the corresponding histograms showing band intensities; (**C**) Western blot for connexin 43 at baseline (**B**, ie, without interleukin-6), and after 24 and 48 hours of treatment with 100 pg interleukin-6 and (**D**) the corresponding histograms showing band intensities. Western blots were performed on technical positive control (mouse atria, **A** and **C**). Two-tailed Student paired *t* test, $^{**}P \leq 0.01$. Histograms represents mean±standard deviation of 3 different experiments. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.

Mounting evidence from epidemiological studies in patients with different inflammatory diseases supports this point of view. In particular, in a nationwide study on 24 499 patients with inflammatory bowel diseases, it was found that the increased risk of AF observed when compared with the general population was mainly driven by a higher incidence during flares or persistently high disease activity.[30] Moreover, in a prospective study on 687 patients with septic shock, Meierhenrich et al[50] demonstrated that new-onset AF was preceded by a steady and significant increase in CRP levels. Finally, also in patients undergoing major abdominal or thoracic surgery, new-onset AF occurrence was significantly associated with maximum CRP and interleukin-6 levels in the early postoperative period.[51,52]

In conclusion, our data for the first time provide evidence that systemic inflammatory activation is associated in the short term with significant electric atrial remodeling, and that interleukin-6–induced down-regulation of atrial connexins is mechanistically involved in these changes. Although rapidly reversible, such functional modifications may lead to an increased risk of AF shortly after an acute inflammatory process activates (or a chronic inflammatory disease reactivates). Besides providing a new insight further supporting a link between inflammation and AF, our findings may contribute to explaining why the increased AF risk observed in different inflammatory diseases seems to be mainly attributable to a higher incidence during flares or persistently elevated disease activity. Our data also support the recommendation to translate into clinical practice that the potential impact of a systemic inflammatory state, regardless of its origin, on atrial arrhythmic risk and related complications must always be carefully considered. In this regard, a prompt treatment of concomitant inflammatory conditions, including intercurrent infections, in patients with specific risk factors for AF, such as preexisting cardiac disease or genetic predisposition, may be an important therapeutic measure, although to date largely overlooked. Targeting inflammatory mediators, particularly interleukin-6,

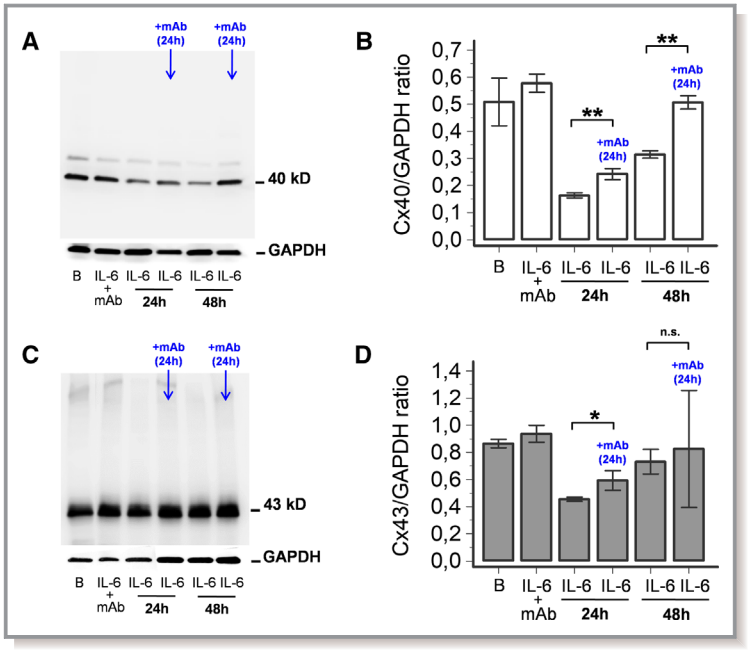

Figure 10. Effects of interleukin-6 inhibition on connexin 40 and connexin 43 protein expression in HL-1 cells. **A**, Western blot for connexin 40 at baseline (**B**, ie, cells cultured for 24 hours without intervention); after the addition of interleukin-6 plus anti–interleukin-6 monoclonal antibody (interleukin-6+mAb, added at 0 hours and collected at 24 hours); after the addition of interleukin-6 for 24 hours (interleukin-6 added at 0 hours and collected at 24 hours); after addition of interleukin-6 for 24 hours and then anti–interleukin-6 monoclonal antibody for 24 hours (interleukin-6 added at 0 hours; at 24 hours interleukin-6 was removed and then added the interleukin-6+mAb complex; collected at 48 hours); after the addition of interleukin-6 for 48 hours (interleukin-6 added at 0 hours and collected at 48 hours); after the addition of interleukin-6 for 48 hours and then anti–interleukin-6 monoclonal antibody for 24 hours (interleukin-6 added at 0 hours; at 48 hours interleukin-6 removed and then added the interleukin-6+mAb complex; collected at 72 hours), and (**B**) the corresponding histograms showing band intensities; (**C**) Western blot for connexin 43 at baseline (B, ie, cells cultured for 24 hours without intervention); after addition of interleukin-6 plus anti–interleukin-6 monoclonal antibody (interleukin-6+mAb, added at 0 hours and collected at 24 hours); after the addition of interleukin-6 for 24 hours (interleukin-6 added at 0 hours and collected at 24 hours); after the addition of interleukin-6 for 24 hours and then anti–IL-6 monoclonal antibody for 24 hours (interleukin-6 added at 0 hours; at 24 hours interleukin-6 removed and then added the interleukin-6+mAb complex; collected at 48 hours); after the addition of interleukin-6 for 48 hours (interleukin-6 added at 0 hours and collected at 48 hours); after addition of interleukin-6 for 48 hours and then anti–interleukin-6 monoclonal antibody for 24 hours (interleukin-6 added at 0 hours; at 48 hours interleukin-6 removed and then added the interleukin-6+mAb complex; collected at 72 hours), and (**D**) the corresponding histograms showing band intensities. Two-tailed Student paired *t* test, **P*<0.05, ***P*<0.01; n.s., not significant. Histograms represents mean±standard deviation of 3 different experiments. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.

may represent in perspective an innovative approach in antiarrhythmic therapy.[53]

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Disclosures

None.

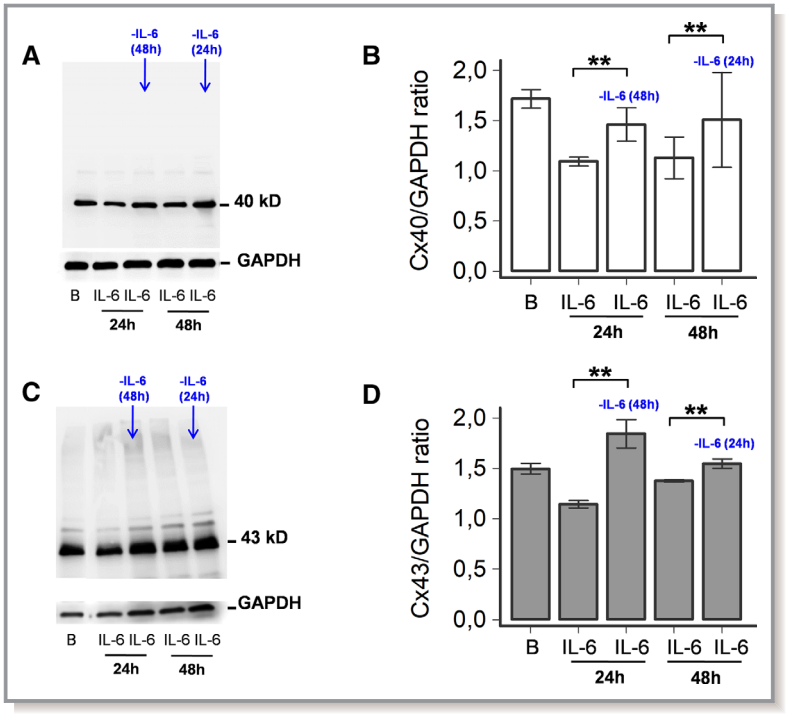

Figure 11. Effects of withdrawal of interleukin-6 supplementation on connexin 40 and connexin 43 protein expression in HL-1 cells. **A**, Western blot for connexin 40 at baseline (**B**, ie, cells cultured for 24 hours without intervention); after the addition of interleukin-6 for 24 hours (interleukin-6 added at 0 hours and collected at 24 hours); after the addition of interleukin-6 for 24 hours and then interleukin-6 withdrawal for 48 hours (interleukin-6 added at 0 hours; at 24 hours interleukin-6 removed; collected at 72 hours); after the addition of interleukin-6 for 48 hours (interleukin-6 added at 0 hours and collected at 48 hours); after the addition of interleukin-6 for 48 hours and then interleukin-6 withdrawal for 24 hours (interleukin-6 added at 0 hours; at 48 hours interleukin-6 removed; collected at 72 hours), and (**B**) the corresponding histograms showing band intensities; (**C**) Western blot for connexin 43 at baseline (B, ie, cells cultured for 24 hours without intervention); after addition of interleukin-6 for 24 hours (interleukin-6 added at 0 hours and collected at 24 hours); after addition of interleukin-6 for 24 hours and then interleukin-6 withdrawal for 48 hours (interleukin-6 added at 0 hours; at 24 hours interleukin-6 removed; collected at 72 hours); after the addition of interleukin-6 for 48 hours (interleukin-6 added at 0 hours and collected at 48 hours); after the addition of interleukin-6 for 48 hours and then interleukin-6 withdrawal for 24 hours (interleukin-6 added at 0 hours; at 48 hours interleukin-6 removed; collected at 72 hours), and (**D**) the corresponding histograms showing band intensities. Two-tailed Student paired *t* test, $^{**}P<0.01$. Histograms represents mean±standard deviation of 3 different experiments. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.

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Supplemental Material

Table S1. Demographic, electrocardiographic and laboratory characteristics of subjects in the control group.

Subjects, n	25
Age, years (range)	72.8±5.2 (64-83)
Females, n	14
Pmax, ms	120.1 (82-152)
Pmin, ms	80.0 (50-109)
PWD, ms	39.9 (31-49)
Psd, ms	13.4 (7.4-17.1)
CRP, mg/dl	0.11 (0.03-0.64)
Neutrophil/lymphocyte ratio	1.64 (1.0-6.1)
IL-6, pg/ml	0.09 (0.05-12.2)
IL-1, pg/ml	0.05 (0.04-1.67)
TNFα, pg/ml	0.42 (0.36-4.76)
IL-10, pg/ml	0.55 (0.44-9.30)
Cx40	1.23±0.52
Cx43	1.31±0.67
CxTOT	2.39±0.96

Pmax: maximun P-wave duration; Pmin: minimum Pwave duration; PWD: Pwave dispersion; Psd: P wave standard deviation; CRP: C-reactive protein (reference values <0.5 mg/dl); IL-6: interleukin-6 (reference values 0.49-1.25 pg/ml)*; IL-1: interleukin-1 (reference values 0.08-0.29 pg/ml)*; TNFα: tumor necrosis factor alpha (reference values 0.6-3.24 pg/ml)*; IL-10: interleukin-10 (reference values 0-3.6 pg/ml)*; Cx 43: connexin 43 mRNA levels in peripheral blood monuclear cells; Cx 40: connexin 40 in peripheral blood monuclear cells; CxTOT: connexin 43 + connexin 40 mRNA levels in peripheral blood monuclear cells.

Values are expressed as median (range), or mean ±standard deviation. Age is expressed as mean ±standard deviation (range).

*Cytokine level range measured in an internal reference group of healthy controls.

Table S2. Demographic, clinical, laboratory and echocardiography characteristics of patients studied for correlation of connexins expression between atrial tissue and blood.

Patient	Atrium	Age, years	Sex	Diagnosis	EF, %	proBNP, pg/ml	CRP, mg/dl
1	Left	63	Male	Ischemic cardiomyopathy	<20	842	0.15
2	Right	59	Male	Aortic valve prosthetic endocarditis	60	3149	5.68
3	Left	79	Female	Aortic valulopathy	60	131	0.08
4	Right	79	Male	Mitral and aortic valulopathy	55	58	0.57
5	Right	81	Male	Mitral and aortic valulopathy	55	821	1.15
6	Right	60	Male	Mitral and aortic valulopathy	60	132	0.03
7	Left	60	Female	Mitral valulopathy	70	77	0.46
8	Right	73	Female	Aortic valulopathy	50	346	0.01
9	Left	55	Male	Ischemic cardiomyopathy	<20	813	4.06
10	Right	56	Male	Interatrial communication with partial anomalous pulmonary venous drainage	55	1697	0.35
11	Right	75	Female	Mitral and aortic valulopathy	55	155	0.53
12	Left	57	Male	Mitral valulopathy	60	131	0.03

EF: ejection fraction; proBNP: pro-brain natruiretic peptide; CRP: C-reactive protein.

Table S3. Correlations between P-wave indices and inflammatory markers showing significant changes in PRE/POST conditions, in the whole population of inflammatory patients (n=54).

	Pmax	Pmin	PWD	Psd
CRP	**rho=0.25** ***p*=0.0083****	rho=0.0004 *p*=0.96	**rho=0.31** ***p*=0.0010****	**rho=0.35** ***p*=0.0002*****
N/L ratio	rho=0.01 *p*=0.99	rho= - 0.14 *p*=0.37	rho=0.07 *p*=0.65	rho= - 0.01 *p*=0.92
IL-6	**rho=0.27** ***p*=0.013***	rho=0.06 *p*=0.53	**rho=0.22** ***p*=0.048***	**rho=0.31** ***p*=0.0047****
IL-1	**rho=0.23** ***p*=0.037***	rho=0.12 *p*=0.28	rho=0.11 *p*=0.30	**rho=0.18** ***p*=0.094**

PRE condition: during active disease; POST condition: after therapeutic interventions resulting in a CRP decrease >75% when compared to the baseline; Pmax: maximun P-wave duration; Pmin: minimum Pwave duration; PWD: Pwave dispersion; Psd: P wave standard deviation; CRP: C-reactive protein; N/L ratio: neutrophil/lymphocyte ratio†à; IL-6: interleukin-6††; IL-1: interleukin-1††.

Correlations were evaluated by the Spearman test. In bold p<0.10; *p<0.05, **p<0.01, ***p<0.001.

† Data available in 19 out of 54 patients.
††Data available in 41 out of 54 patients

Table S4. Changes in laboratory and electrocardiographic parameters in a sub-cohort of patients with inflammatory diseases (n=16) underwent expression analysis of circulating connexins, during active disease (PRE) and after therapeutic interventions resulting in a CRP decrease >75% when compared to the baseline (POST).

	PRE	POST	*p*
CRP,mg/dl	14.5 (9.4-34.5)	1.8 (0.4-6.3)	**<0.0001**
IL-6, pg/ml	15.9 (4.0-156.5)	3.7 (0.05-66.1)	**0.002**
IL-1, pg/ml	0.38 (0.10-1.21)	0.29 (0.10-1.26)	0.20
TNFα, pg/ml	0.80 (0.15-13.75)	0.70 (0.15-12.98)	0.62
IL-10, pg/ml	0.60 (0.40-1.64)	0.54 (0.19-1.70)	0.46
PWD, ms	50.0 (32-88)	41.3 (20-61.5)	**0.0016**
Patients with high PWD (>40 ms), n	14 (87%)	8 (50%)	0.053
Psd, ms	17.3 (12.1-30.6)	14.2 (9.0-18.7)	**0.0028**
Patients with high Psd,* n	11 (69%)	6 (37%)	0.155

Pmax: maximun P-wave duration; Pmin: minimum Pwave duration; PWD: Pwave dispersion; Psd: P wave standard deviation; CRP: C-reactive protein (reference values <0.5 mg/dl); IL-6: interleukin-6 (reference values 0.49-1.25 pg/ml); TNFα: tumor necrosis factor alpha (reference values 0.6-3.24 pg/ml); IL-1: interleukin-1 (reference values 0.08-0.29 pg/ml). Cytokine level range measured in an internal reference group of healthy controls.

Values are expressed as median (range), or frequency count and percentages.

Differences were evaluated by the two-tail Student's paired "t" test, or the two-tail Wilcoxon matched pairs test. Difference in categorical variables were evaluated by the two-sided Fisher's exact test.

*Based on age- and sex-specific reference values in healthy subjects from the Framingham Heart Study.[18]

Table S5. Correlations between circulating connexins expression, P-wave indices and inflammatory markers showing significant changes in PRE/POST conditions, in a sub-cohort of inflammatory patients.

Top: analysis on the whole population (n=16); **Bottom:** analysis in patients showing marked P-wave dispersion indices decrease respect to baseline (n=6).

	Pmax	**Pmin**	**PWD**	**Psd**	**CRP**	**IL-6**	**IL-1**
Cx40	rho=0.17 *p*=0.37	rho=0.07 *p*=0.69	rho=0.14 *p*=0.46	rho=0.11 *p*=0.56	rho= - 0.04 *p*=0.81	rho=0.02 *p*=0.88	rho=0.01 *p*=0.94
Cx43	rho=0.23 *p*=0.19	rho= - 0.21 *p*=0.23	rho=0.07 *p*=0.69	rho= - 0.15 *p*=0.38	rho=0.08 *p*=0.65	**rho= - 0.35** ***p*=0.044***	rho= 0.22 *p*=0.21
CxTOT	rho=0.09 *p*=0.59	rho=0.12 *p*=0.49	rho=0.04 *p*=0.81	rho= - 0.07 *p*=0.69	rho=0.08 *p*=0.65	**rho= - 0.30** ***p*=0.088**	rho=0.23 *p*=0.20

	Pmax	**Pmin**	**PWD**	**Psd**	**CRP**	**IL-6**	**IL-1**
Cx40	rho=0.13 *p*=0.67	**rho=0.57** ***p*=0.051**	rho= - 0.35 *p*=0.26	rho= - 0.35 *p*=0.25	rho= - 0.42 *p*=0.17	rho= - 0.47 *p*=0.12	rho=0.34 *p*=0.26
Cx43	rho= - 0.07 *p*=0.82	rho= - 0.41 *p*=0.18	rho= - 0.37 *p*=0.22	**rho= - 0.59** ***p*=0.042***	rho= - 0.44 *p*=0.15	**rho= - 0.61** ***p*=0.034***	rho= 0.14 *p*=0.65
CxTOT	rho= - 0.31 *p*=0.32	**rho=0.61** ***p*=0.036***	**rho= - 0.50** ***p*=0.091**	**rho= - 0.62** ***p*=0.031***	rho= - 0.46 *p*=0.13	**rho= - 0.75** ***p*=0.007****	rho=0.46 *p*=0.13

PRE condition: during active disease; POST condition: after therapeutic interventions resulting in a CRP decrease >75% when compared to the baseline; Pmax: maximun P-wave duration; Pmin: minimum Pwave duration; PWD: Pwave dispersion; Psd: P wave standard deviation; CRP: C-reactive protein; N/L ratio: neutrophil/lymphocyte ratio†à; IL-6: interleukin-6††; IL-1: interleukin-1††.

Correlations were evaluated by the Spearman test. In bold p<0.10; *p<0.05, **p<0.01.

Table S6. Changes in circulating mRNA levels of connexins in patients with inflammatory diseases during active disease (PRE) and after therapeutic interventions resulting in a CRP decrease >75% versus baseline (POST): comparison of subjects showing high P wave dispersion indices decrease (High-ΔP) vs subjects showing low P wave dispersion indices decrease (Low-ΔP).

	High-ΔP (n=6)	**Low-ΔP** (n=10)	*p*
PWD, ms			
PRE	64.2±18.5	48.8±11.4	
POST	41.5±11.4	42.4±9.3	
Δ	22.7±14.8 (-35%)	6.5±7.1 (-11%)	**0.0099**
Psd, ms			
PRE	21.4±5.3	16.4±3.7	
POST	14.3±11.4	14.7±2.3	
Δ	7.1±2.9 (-33%)	1.8±3.6 (-8%)	**0.0095**
CxTOT			
PRE	1.94±0.19	1.51±0.92	0.28
POST	2.86±1.49	1.29±0.97	**0.022**
Cx43			
PRE	1.06±0.26	0.94±0.61	0.66
POST	1.62±0.83	0.85±0.65	**0.05**
Cx40			
PRE	0.89±0.18	0.72±0.49	0.51

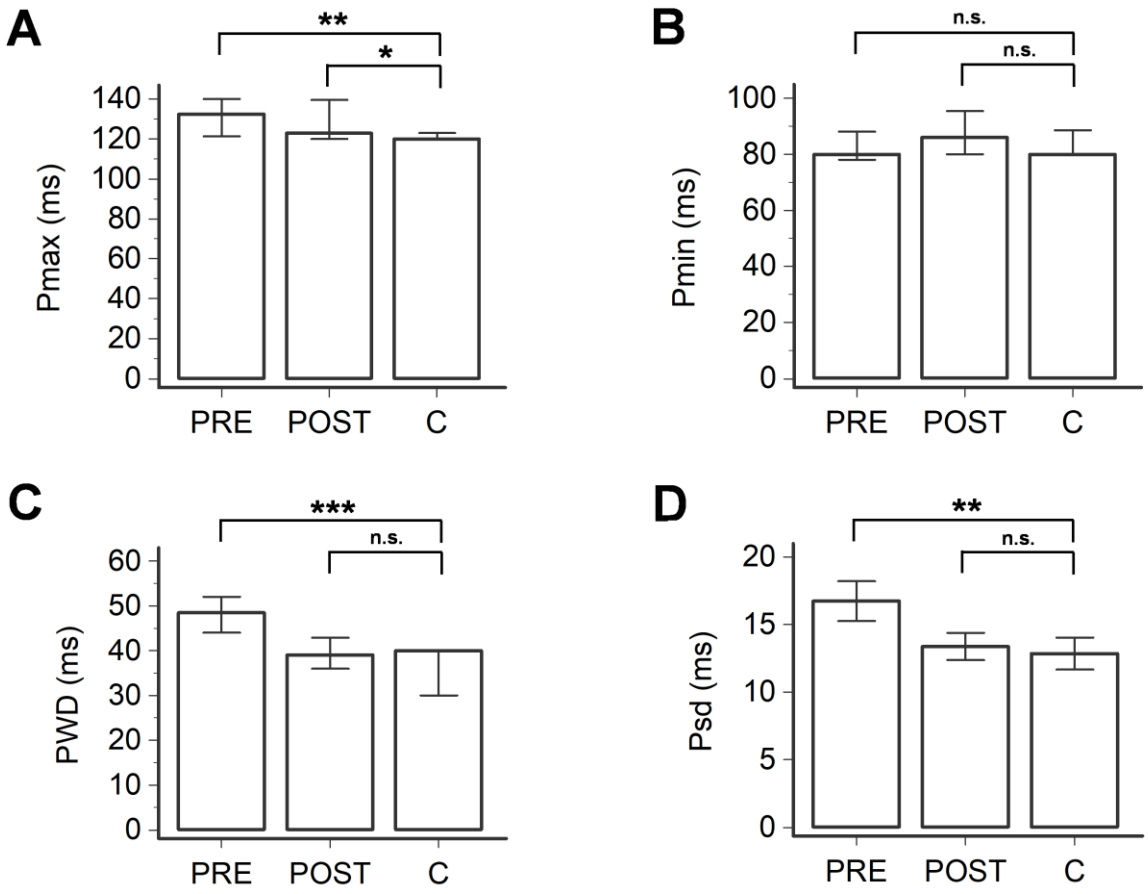
POST	1.24±0.73	0.55±0.43	**0.047**

PWD: Pwave dispersion; Psd: P wave standard deviation; Cx 43: connexin 43 mRNA levels in peripheral blood monuclear cells; Cx 40: connexin 40 in peripheral blood monuclear cells; CxTOT: connexin 43 + connexin 40 mRNA levels in peripheral blood monuclear cells.

Values are expressed as mean ±standard deviation.

Differences were evaluated by the two-tail unpaired “t” test.

Figure S1. Comparison of P-wave indices in patients with inflammatory diseases, during active disease (PRE) and after therapeutic interventions resulting in a CRP decrease >75% when compared to the baseline (POST), and controls (C).



(**A**) Maximum P-wave duration (Pmax); two-tail Mann-Whitney test, *p<0.05, **p<0.01,. (**B**) Minimum P-wave duration (Pmin); two-tail Mann-Whitney test, n.s. not significant. (**C**) P-wave dispersion (PWD); two-tail Mann-Whitney test, n.s. not significant, ***p<0.0001. (**D**) P-wave standard deviation (Psd); two-tail Student's unpaired "t" test, n.s. not significant **p<0.01, Error bars indicate 95% Confidence Interval for median (Pmax, Pmin, PWD) or for mean (Psd). Patients, n=54; controls (C) n=25.