Ion channel remodeling is related to intra operative atrial refractory periods in patients

Chapter 5

Ion Channel Remodeling is Related to Intra-Operative Atrial Effective Refractory Periods in Patients with Paroxysmal and Persistent Atrial Fibrillation

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Abstract

Background  Sustained shortening of the atrial effective refractory period (AERP), probably due to reduction in L-type calcium current, is a major factor in the initiation and maintenance of AF. We investigated underlying molecular changes by studying the relation between gene expression of the L-type calcium channel and potassium channels and the AERP in patients with AF. Methods and Results mRNA and protein expression were determined in left and right atrial appendages of 13 patients with paroxysmal AF, 16 with persistent AF and 13 controls in sinus rhythm, by RT-PCR and slot-blot, respectively. The mRNA content of almost all investigated ion-channel genes was reduced in persistent but not in paroxysmal AF. Protein levels for L-type Ca\(^{2+}\) channel and five potassium channels (Kv4.3, Kv1.5, HERG, minK and Kir3.1) were significantly reduced in both persistent and paroxysmal AF. Furthermore, AERPs were determined intra-operatively with programmed electrical stimulation at 5 basic cycle lengths (BCLs) (between 250 and 600 ms). Patients with persistent and paroxysmal AF displayed significant shorter AERPs. Protein levels of all ion-channels investigated correlated positively with the AERP (at BCL: 600, 500, 400 and 300 ms) and with the rate adaptation of AERP. Patients with reduced ion-channel protein expression revealed shorter AERP duration and poorer rate adaptation.
Conclusions AF is predominantly accompanied by decreased protein contents of the L-type Ca\textsuperscript{2+} channel and several potassium channels. Reductions in L-type Ca\textsuperscript{2+} channel correlated with AERP and rate adaptation, and represent a probable explanation for the electrophysiological changes during AF.

Introduction

Atrial fibrillation (AF) is a common arrhythmia affecting millions of people worldwide.\textsuperscript{1} AF has the tendency to become more persistent and increasingly difficult to treat over time. During recent years, experimental and human studies showed that rapid shortening of the atrial effective refractory period (AERP) is an important factor contributing to the maintenance of AF.\textsuperscript{2-6} Rapid shortening of the AERP in AF involves functional changes in ion channels. Animal experimental data revealed that the L-type Ca\textsuperscript{2+} channel plays a main role in shortening of AERP and action potential duration (APD).\textsuperscript{7,8} These observations are supported by blocking of AERP shortening with the L-type Ca\textsuperscript{2+} antagonist, verapamil, in other experimental studies.\textsuperscript{9,10} In addition, human data on AF have demonstrated reductions in I\textsubscript{Cal}.\textsuperscript{11,12} and gene expression of L-type Ca\textsuperscript{2+} channel.\textsuperscript{13}

However, shortening of AERP could also be explained by an increase in (repolarizing) K\textsuperscript{+} channel activity. Indeed, one study found increased I\textsubscript{K,ACh} and I\textsubscript{K1} in isolated human atrial cells of patients with persistent AF.\textsuperscript{11} In contrast, other studies support a decrease in K\textsuperscript{+} channels in AF. In human atrial myocytes reductions in I\textsubscript{To} and I\textsubscript{Ksus} and a reduced gene expression of Kv1.5, Kv4.3, Kir3.1, Kir3.4 and Kir6.2\textsuperscript{13-15} were found.

Until now, the relationship between changes in AERP and ion channel gene expression has not been investigated in human tissue of patients with AF. The aim of the present study was to investigate the regulation of L-type Ca\textsuperscript{2+} channel and K\textsuperscript{+} channels and its relation to AERP in patients with persistent and paroxysmal AF. We included both patients with lone AF and with mitral valve disease (MVD), since the occurrence of MVD seems to prolong the AERP.\textsuperscript{16,17}

Materials and Methods

Patients and atrial tissue collecting

Prior to surgery, one investigator assessed the clinical characteristics of patients (Table 1). The patient’s arrhythmia history was classified according to Gallagher.\textsuperscript{18} The persistent and paroxysmal AF group contained patients with lone AF or AF with underlying MVD. All patients underwent MAZE surgery, were euthyroid and had normal left ventricular function. Coumarin therapy was interrupted 3 days before surgery and class I and III anti-arrhythmic drugs were discontinued for at least 5 half-times. During surgery the AERPs were determined with use of temporary epicardial pacing leads. AERPs were measured intra-operatively at 5 different basic cycle lengths (BCL; 600, 500, 400, 300 and
Ion channel remodeling is related to intra operative atrial refractory periods in patients 250 ms) at the right atrial appendage (RAA) and left atrial appendage (LAA) using pro-
grammed electrical stimulation.

LAAs and RAAs were obtained except for the control patients undergoing CABG from whom only the RAA was gathered. After excision, the RAAs and LAAs were imme-
diately snap-frozen in liquid nitrogen and stored at -85 °C. The study was approved by the Institutional Review Board and written informed consent was given by all patients.

<table>
<thead>
<tr>
<th>Table 1. Baseline characteristics of patients with lone paroxysmal AF, lone persistent AF and control patients in sinus rhythm</th>
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<td>SR (CABG)</td>
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<td>PAF</td>
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<td>CAF</td>
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<td>SR (MVD)</td>
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<td>PAF</td>
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<td>CAF</td>
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<tr>
<td>N</td>
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<tr>
<td>Age</td>
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<td>Previous duration of AF (median, range (months))</td>
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<td>Duration SR before surgery (median, range (days))</td>
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<tr>
<td>Underlying heart disease (n) and surgical procedure</td>
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<tr>
<td>Coronary artery disease/CABG</td>
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<td>Lone AF / MAZE</td>
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<tr>
<td>MVD/MV replacement/repair</td>
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<tr>
<td>New York Heart Association for exercise tolerance</td>
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<tr>
<td>Class I</td>
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<tr>
<td>Class II</td>
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<tr>
<td>Class III</td>
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<tr>
<td>Echocardiography</td>
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<tr>
<td>Left atrial diameter (parasternal) (mm)</td>
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<tr>
<td>Left ventricular end-diastolic diameter (mm)</td>
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<tr>
<td>Left ventricular end-systolic diameter (mm)</td>
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<tr>
<td>Medication (n)</td>
</tr>
<tr>
<td>Ace-inhibitors</td>
</tr>
<tr>
<td>Digitalis</td>
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<tr>
<td>Verapamil</td>
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<td>Beta-blocker</td>
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Values are presented as mean value ± SD or number of patients. ACE inhibitor indicates Angiotensin Converting Enzyme; AF, atrial fibrillation; CABG, Coronary Artery Bypass Grafting; CAF, chronic persistent atrial fibrillation; PAF, paroxysmal atrial fibrillation; SR, control patients in sinus rhythm.
RNA isolation and cDNA synthesis

Total RNA was isolated and processed as described previously. Briefly, first strand cDNA was synthesized by incubation of 1 µg of total RNA in reverse transcription 10x buffer, 200 ng of random hexamers with 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase, 1mM of each dNTP and 1 unit of RNase inhibitor (Promega, The Netherlands) in 20 µl. Synthesis reaction was performed for 10 minutes at 20 °C, 20 minutes at 42 °C, 5 minutes at 99 °C and 5 minutes at 4 °C. All the products were checked for contaminating DNA.

Semi quantitative PCR analyses

We described and validated these methods before. In short, the cDNA of interest and the cDNA of the ubiquitously expressed housekeeping gene glyceraldehyde-3-phos
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Phosphate dehydrogenase (GAPDH) were co-amplified in a single PCR. Primers (Eurogentec, Belgium) were designed for SCNA1, L-type calcium channel, Kv4.3, HERG, Kv1.5, Kir3.4 and Kir6.2 and the housekeeping gene GAPDH (Table 2).

The PCR products were separated on agarose gel by electrophoresis and stained with ethidium bromide. The density of the PCR products was quantified by densitometry. Linearity of the PCR was established by a good correlation between the number of cycles and the density of gene of interest and GAPDH (data not shown).

**Protein Preparation and Slot Blotting**

Frozen atrial appendages of patients in sinus rhythm, patients with paroxysmal AF and patients with persistent AF were homogenized in RadioImmunoPrecipitationAssay (RIPA) buffer as described before. The homogenate was centrifuged at 14.000 rpm for 20 minutes at 4°C. The supernatant was used for protein concentration measurement according to the Bradford method (Bio-Rad, The Netherlands) with bovine albumin used as a standard. Samples of 10 µg heat denatured protein were spotted on nitrocellulose membranes (Stratagene, The Netherlands) and checked by staining with Ponceau S solution (Sigma, The Netherlands). Blocking was performed for 20 minutes in blocking buffer (5% nonfat milk, TBS and 0.1% Tween 20). After washing three times in TBS with 0.1% Tween 20 the membranes were incubated with primary antibody against GAPDH (Affinity Reagents, USA), L-type calcium channel α1 subunit, Kv4.3, HERG, minK, Kir3.1 and Kv1.5 (all Alomone Labs, Israel). Immunodetection of the primary antibody was performed with peroxidase conjugated secondary antibody anti-mouse IgG (Santa Cruz Biotechnology, The Netherlands). The blot was incubated with the ECL-detection reagent (Amersham, The Netherlands) for 1 minute, and exposed to an X-OMAT x-ray film (Kodak, The Netherlands) for 15 to 90 seconds. The band densities were evaluated by densitometric scanning using a Snap Scan 600 (Agfa, The Netherlands). The amount of protein chosen was in the linear immunoreactive signal area and the specificity of the antibody was checked by SDS-PAGE and pre-incubation with the control peptide antigen.

**Rate adaptation coefficient**

To quantify the change in AERP at the different BCLs, we calculated the rate adaptation coefficient for individual patients as the slope of the linear regression after logarithmic transformation of BCL. Three patients were excluded, because AERP was obtained at less than 4 BCLs.

**Statistical Analysis**

All PCR and slot-blotting procedures were performed in duplo series and mean values were used for statistical analysis. Comparison between groups for normally distrib
uted variables was performed by one-way ANOVA and for skewed variables by Wilkoxon two-sample test. For determination of correlations the Spearman correlation test was used. The Mann-Whitney U-test was performed for group to group comparisons of small numbers. All p-values are two-sided, a p-value <0.05 was considered statistically significant. SPSS version 8.0 was used for all statistical evaluations.

**Results**

**mRNA Remodeling**

Changes in transcription of the gene of interest were determined by comparison of gene-of-interest/GAPDH ratios between patients with persistent AF, paroxysmal AF and their controls in sinus rhythm (Table 3). No differences in GAPDH amount between the groups were found for all the genes investigated (data not shown). Persistent, lone AF was associated with reductions in mRNA amount of Kv4.3, L-type Ca\(^{2+}\) channel and Kir3.4. The mRNA amounts of HERG and KvLQT1 showed an additional reduction in persistent AF with MVD. In general the reduction in mRNA expression was less pronounced in paroxysmal AF.

<table>
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<tr>
<th></th>
<th>mRNA</th>
<th>Protein</th>
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<tr>
<td></td>
<td>lone PAF +MVD PAF</td>
<td>lone CAF +MVD CAF</td>
</tr>
<tr>
<td>Na-channel</td>
<td>+35 ±6</td>
<td>-</td>
</tr>
<tr>
<td>Kv4.3</td>
<td>-20 ±4</td>
<td>-20 ±5</td>
</tr>
<tr>
<td>L-type calcium channel</td>
<td>-13 ±5</td>
<td>-27 ±4 -20 ±4</td>
</tr>
<tr>
<td>HERG</td>
<td></td>
<td>-22 ±4</td>
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<tr>
<td>Kv1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KvLQT1/minK</td>
<td>+82 ±8*</td>
<td>+56 ±6*</td>
</tr>
<tr>
<td>Kir3.4/Kir3.1</td>
<td>-27 ±5 -34 ±5</td>
<td>-41 ±7 -67 ±5*</td>
</tr>
<tr>
<td>Kir6.2</td>
<td>+28 ±4</td>
<td>-</td>
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Only significant changes (p<0.05) are given for the mRNA or protein content of interest/GAPDH. CAF means patients with chronic, persistent AF, PAF means paroxysmal AF, MVD means mitral valve disease; - means not significant; na means not available. *, means significant differences between lone PAF and PAF with MVD or lone CAF and CAF with MVD.
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Protein Remodeling

Proteins were isolated from RAA and LAA and used for immunological detection of L-type Ca$^{2+}$ channel, Kv4.3, HERG, Kv1.5, minK and Kir3.1. Changes in protein expression were studied in relation to protein levels of GAPDH and to total amount of protein spotted on the membrane. Because the GAPDH density and total protein amount density showed a highly significant positive correlation ($r=0.92$, $p<0.001$), we used the protein of interest/GAPDH ratio for further investigation. The protein expression of L-type Ca$^{2+}$ channel, Kv4.3, Kv1.5, HERG, Kir3.1 and minK was reduced in both patients with both persistent and paroxysmal AF (Figure 1 and 2, Table 3). Furthermore, ion-channel protein levels did not correlate with mRNA levels, duration of persistent AF or with the duration of sinus rhythm before surgery (data not shown).

Significant differences in protein remodeling between the lone AF group and the AF group with MVD were observed. Reductions in protein expression of Kv4.3, minK and Kir3.1 were more pronounced in patients with underlying MVD (Table 3).

**Figure 1.** Slot blot analysis of 10 µg of protein homogenates of 6 patients in sinus rhythm (SR), 6 patients with paroxysmal AF (PAF) and 6 patients with chronic, persistent AF (CAF). The immunoblots were done for (A) anti GAPDH, (B) anti L-type calcium channel α1 subunit, (C) anti Kv4.3, (D) anti-Kv1.5, (E) anti HERG, (F) anti minK and (G) anti-Kir3.1.
Chapter 5

Atrial Effective Refractory Period and Protein Remodeling

The AERP at 5 different basic cycle lengths (BCLs: 600, 500, 400, 300 and 250 ms) was determined in the RAA and LAA of patients during surgery. Patients with persistent and paroxysmal AF had significantly shorter AERPs than patients in sinus rhythm (Table 4). The relation between AERP and the amount of ion-channel protein was investigated, because protein amounts are anticipated to represent the amount of functional ion-channel closer than mRNA levels. A significant positive correlation was found at BCLs of 600, 500, 400 and 300 ms for all the proteins investigated in patients with AF (Figure 3, Table 5). Patients with reduced ion-channel protein expression exhibited the shortest AERP. Furthermore, no significant correlation was found between the GAPDH amount and AERP (data not shown).

Relation Rate Adaptation and Protein Remodeling

The rate adaptation coefficient was determined for every RAA and LAA. The rate adaptation coefficient was significantly reduced by 32% in persistent AF compared to sinus rhythm (mean persistent AF: 104 ± 53; paroxysmal AF: 133 ± 62 and sinus rhythm:

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**Figure 2.** Protein ratios for (A) L-type calcium channel, (B) Kv4.3, (C) Kv1.5, (D) HERG, (E) minK and (F) Kir3.1 for patients in sinus rhythm (SR), with paroxysmal AF (PAF) and with chronic, persistent AF (CAF). *, p<0.01, **, p<0.001
Ion channel remodeling is related to intra operative atrial refractory periods in patients with AF. Significant positive correlations were observed between ion-channel protein expression and the adaptation coefficient (Figure 4). AF patients with reduced ion-channel protein expression demonstrated poorer rate adaptation.

Furthermore, significant differences were observed between lone paroxysmal AF and patients with paroxysmal AF and MVD. Lone paroxysmal AF demonstrated a poorer rate adaptation compared to paroxysmal AF with MVD (109 ± 40 and 164 ± 76, p=0.04, respectively).

**Discussion**

Both experimental and human AF is accompanied by electrical remodeling and ion-channel remodeling. This is the first study which demonstrates in human paroxysmal and persistent AF (1) a positive correlation between the ion-channel protein remodeling and the AERPs, irrespective of the underlying heart disease, (2) a correlation between ion-channel protein remodeling and changes in rate adaptation and (3) dis-
crepancies between mRNA and protein remodeling. These data suggest ion-channel protein remodeling represent an important adaptation mechanism during AF, that may contribute to intractability of AF and inactivity of antiarrhythmic drugs instituted for the prevention of AF.

**Relation ion channel remodeling and AERP**

The observed ion-channel protein remodeling in this study is associated with the occurrence of AF. Patients with paroxysmal and persistent AF showed marked reductions in ion-channel protein expression of both L-type Ca\(^{2+}\) channel and several K\(^+\) channels. Furthermore, low ion-channel protein levels were associated with short AERP and poor
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Rate adaptation. This indicates that electrical remodeling\(^2\) and structural remodeling\(^{21}\) are paralleled by ion-channel protein remodeling as part of the adaptation mechanisms during AF. Furthermore, patients with paroxysmal AF showed a reduction in ion-channel protein expression comparable to persistent AF in the absence of mRNA reductions, suggesting that paroxysms of AF are able to induce changes in ion-channel protein expression via activation of a proteolytic system. Indeed, we have observed activation of the calpain system in human paroxysmal and persistent AF (Brundel et al., submitted).

As stated above, AF is accompanied by shortening of the AERP and action potential duration (APD). It has been suggested that the short-term decrease of APD and its reduced rate adaptation is mainly due to a ± 70% reduction of the L-type calcium current in animal experimental studies and human AF.\(^7,8,11,12\) This assumption is further supported by the observation that administration of the L-type Ca\(^{2+}\) channel agonist Bay K 8644 largely restored the plateau phase of the action potential in remodeled cells.\(^{22}\) If the main role for

![Figure 4. Correlation between the ion-channel protein expression and the rate adaptation coefficient for (A) L-type calcium channel, (B) Kv4.3, (C) Kv1.5, (D) HERG, (E) minK and (F) Kir3.1. (○) represents control patients in sinus rhythm undergoing CABG, (□) patients with lone paroxysmal AF, (●) patients with lone persistent AF, (■) patients in sinus rhythm with underlying MVD, (□) patients with paroxysmal AF and MVD, (■) patients with persistent AF and MVD.](image-url)


L-type Ca\(^{2+}\) channels in APD is correct, the observed reduction in protein expression of L-type Ca\(^{2+}\) channel in this study explains the present AERP shortening and decrease in its adaptation to rate.

The other possibility that may mediate AERP shortening is an increase in (repolarizing) K\(^+\) channel gene products and/or activity. However, we observed a reduction of K\(^+\) channel gene expression. Similar results were obtained in animal experimental studies showing reductions in I\(_{\text{to}}\) and Kv4.3 mRNA amount without reductions in delayed inward rectifier K\(^+\) current and Kir2.1 expression.\(^7\) The group of Van Wagoner et al. and our group examined the adaptation in gene expression of several potassium channels in patients with AF.\(^{13-15}\) The current of I\(_{\text{to}}\) and the protein expression of Kv1.5 were reduced rather than elevated during persistent AF.\(^{15}\) Our previous study, in a different patient group, showed reductions in gene expression of Kv4.3, Kv1.5, Kir3.1 and Kir6.2.\(^{14}\) Only one study in isolated RAA cells of patients with persistent AF showed that shortening of the human action potential by AF was related to a 70% reduction in I\(_{\text{cal}}\) and I\(_{\text{to}}\) and a 30% increase in I\(_{\text{ki}}\) and I\(_{\text{KACH}}\).\(^{11}\) The downregulation of potassium channel protein amounts observed in our study are in contrast with the few reports on the electrophysiological level. This possible inconsistency between decrease in protein level and increase in current density may be explained by a change in single channel properties in patients with persistent AF, such as an increase of mean open-time, an increase in channel conductance or a change in voltage dependency. Thus, a reduced expression of L-type Ca\(^{2+}\) channels probably plays a main role in AERP shortening. Secondary to this process, the myocardial cell may further adapt to high rate by reducing the expression of potassium channels to counteract the shortening of the AERP.

We did not find differences in ion-channel protein expression between AF patients with lone AF and AF with underlying MVD. Nevertheless, AERP was prolonged in MVD, as previously reported in experimental studies.\(^{6,16,17}\) Also an association between AF with MVD and severe cellular degeneration was observed.\(^{23}\) The results indicate that other factors beside AF are probably involved in the regulation of the duration of the effective refractory period. One of most likely candidates would be morphological changes, as AF is promoted by structural changes induced during experimental heart failure, which cause important local conduction abnormalities that could play an additional role in the vulnerability of AF.\(^{24,25}\)

**Post-transcriptional regulation?**

The observed discrepancy between alterations in mRNA and protein expression in patients with paroxysmal AF suggests the activation of proteolysis. Recently, we found that activation of the calpain system in human persistent and paroxysmal AF, in the absence of activation of the proteasome pathway (Brundel et al., submitted). As calpain are
Ion channel remodeling is related to intra operative atrial refractory periods in patients activated by calcium overload in the myocard cell\cite{26,27}, calpain activation would serve to protect the cells to additional damage by down-regulation of multiple ion-channels. However, this would be at the cost of proteolysis of several cytoskeletal, membrane-associated and regulatory proteins\cite{26,28-32}. Whether interference with the calpain system represents a valuable therapeutic strategy in AF remains to be investigated.

In conclusion, the observed correlation between ion-channel protein amounts and AERP strongly suggest that ion-channel protein remodeling, beside the electrical remodeling and structural remodeling\cite{33} may play an important role in the vulnerability of AF after restoration of sinus rhythm.

Limitations of the Study

The patients with lone AF included in this study represent patients who were difficult to treat and underwent finally MAZE surgery. Therefore, the present data cannot be extrapolated uncritically to all AF patients. Furthermore, it should be noted that in all groups the number of patients was small.

Acknowledgments

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References


