Standardized *Aronia melanocarpa* extract regulates redox status in patients receiving hemodialysis with anemia

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Abstract
The aim of our study was to investigate the effects of one-month consumption of polyphenol-rich standardized *Aronia melanocarpa* extract (SAE) on redox status in anemic hemodialysis patients. The study included 30 patients (Hb < 110 g/l, hemodialysis or hemodiafiltration > 3 months; > 3 times week). Patients were treated with commercially available SAE in a dose of 30 ml/day, for 30 days. After finishing the treatment blood samples were taken to evaluate the effects of SAE on redox status. Several parameters of anemia and inflammation were also followed. After the completion of the treatment, the levels of superoxide anion radical and nitrites significantly dropped, while the antioxidant capacity improved via elevation of catalase and reduced glutathione. Proven antioxidant effect was followed by beneficial effects on anemia parameters (increased hemoglobin and haptoglobin concentration, decreased ferritin and lactate dehydrogenase concentration), but SAE consumption didn’t improve inflammatory status, except for minor decrease in C-reactive protein. The consumption of SAE regulates redox status (reduce the productions of pro-oxidative molecules and increase antioxidant defense) and has beneficial effects on anemia parameters. SAE could be considered as supportive therapy in patients receiving hemodialysis which are prone to oxidative stress caused by both chronic kidney disease and hemodialysis procedure. Additionally, it could potentially be a good choice for supplementation of anemic hemodialysis patients. TRN: NCT04208451 December 23, 2019 “retrospectively registered”

Keywords *Aronia melanocarpa* (Rosaceae) · Oxidative stress · Inflammation · Anemia · Hemodialysis

Introduction
The population of patients receiving hemodialysis is at high risk for developing complications, the most common include anemia, bone disease, heart disease and electrolyte imbalance [1, 2]. The cause of anemia is complex and refers to both erythropoietin deficiency and reduction of red blood cell survival and what is more, anemia is associated with high morbidity and mortality [3]. Oxidative stress and inflammation are involved in pathogenesis of many complications of chronic kidney disease (CKD) particularly anemia [4]. Additionally, oxidative stress and inflammation interaction, along with the several factors such as efficacy and biocompatibility of dialysis membrane, patient’s age and concomitant diseases play a critical role in CKD progression. Hemodialysis contributes to the occurrence of oxidative stress in patients, since during this procedure molecules with antioxidant potential are being removed. Furthermore, the reduction of serum levels of antioxidant vitamin E and
C, and antioxidant enzymes activity are typically observed in these patients [5, 6]. Deficiency in antioxidant molecules and the disproportionate formation of oxidative compounds leads to inflammation through the activation of nuclear factor-κB (NFκB) and increased production of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP) in hemodialysis patients [7, 8]. On the other hand, activated leukocytes and macrophages, present in inflammation, induce the release of reactive oxygen species (ROS) and subsequent oxidative stress [9].

The prevalence of all stages of chronic kidney disease is between 7% and 12% in the world population [10]. Oxidative stress along with inflammation plays a crucial role in the pathogenesis of almost all complications related to hemodialysis. The pathogenesis of anemia, as one of the most common following complication in CKD, is multifactorial and includes reduced lifetime of erythrocytes, impaired erythropoietin production, damaged intestinal iron absorption and chronic blood losses. Erythropoietin (EPO) is recommended therapeutic strategy of anemia in CKD patients with hemoglobin levels above 110 g/mol. However, regardless of EPO administration, erythropoietin resistance develops in 5 to 10 percent of these patients [11]. Additionally, these patients are usually treated with iron intravenously. The long-term of parenteral iron usage may lead to oxidative stress, risk of inflammation and iron overload [12].

Different studies investigated the effects of treatment with various vitamins with the antioxidant potential on oxidative stress and inflammation in the patients on hemodialysis. The results of these studies are inconsistent; very often even contradictory [13, 14]. On the other hand, the studies which investigated the effects of polyphenol-rich interventions on oxidative stress and inflammation in the hemodialysis patients demonstrated very interesting results. Namely, the usage of pomegranate juice and extract, soy protein, grape powder, turmeric and cocoa flavanols could reduce oxidative stress parameters in patients on hemodialysis [15–17]. On top of that, the usage of pomegranate juice and turmeric induced the reduction of inflammation in these patients [18–20].

*Aronia melanocarpa*, commonly known as chokeberry, is a fruit which belongs to the *Rosaceae* family [21]. In recent years, black chokeberries have gained popularity due to their high content of polyphenols, particularly anthocyanins and flavonoids, with antioxidant activity, which are mostly responsible for its therapeutic potential [22–24]. Previous studies showed that chokeberry fruit and extracts, rich in polyphenols such as queretin, and epicatechin [25, 26] exert wide beneficial effects in chronic diseases, especially in diseases connected with oxidative stress [27–29]. Our recent studies showed that standardized *Aronia melanocarpa* extracts (SAE) could reduce oxidative stress and increase the levels of iron in rats with metabolic syndrome [30]. Although the antioxidant and anti-inflammatory potential of a certain number of polyphenol-rich plants has been investigated in hemodialysis patients, to our best knowledge the effectiveness of SAE has not been elucidated so far in this specific population.

Taking into account all aforementioned facts, the aim of our study was to investigate the effects of one-month consumption of Standardized *Aronia melanocarpa* extract on redox status in anemic hemodialysis patients.

**Methods**

**Patients and plant extract**

All the research procedures were conducted in accordance with the Helsinki Declaration on Medical Research and approved by the Ethics Committee of the Clinical Center Kragujevac No 01-14-3039. The study enrolled 30 patients with chronic kidney disease on dialysis treatment (written informed consent was obtained before study enrollment) at the Center for Nephrology and Dialysis of the Clinical Center Kragujevac. Inclusion criteria were: regular dialysis treatment for more than 3 months, 3 times a week and hemoglobin values between 80 g/L and 110 g/L. Exclusion criteria were: the usage of antioxidants and immunosuppressants, presence of malignancies, proven active bleeding and presence of systemic inflammation or active infection. All patients were treated with Epoetin alfa 2000 IU, 3 times per week.

The extraction of SAE was performed by EU-Chem Company (Belgrade, Serbia). This product contains 400 mg/30 ml of polyphenols. Patients included in the study consumed SAE 30 ml/day (the recommended daily dosage) for 30 days [30]. Exact phenolic content of SAE was previously published [31], and antocyanins and flavonoids were shown to be most abundant in SAE.

Blood samples were taken from all the patients at two points of interest: day 0, before SAE consumption and day 30, after the completion of chronic SAE treatment. Collected blood samples were used for determination of hematological, inflammatory and redox status.

**Evaluation of systemic redox state**

**Determination of pro-oxidant molecules (NO\(_2^−\), O\(_2^−\), H\(_2\)O\(_2\))**

The NO\(_2^−\) level was measured using Griess’s reagent. 0.1 ml 3 N perchloride acid, 0.4 ml 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.2 ml plasma were put on ice for 15 min, then centrifuged for 15 min at 6000 rpm. After pouring off the supernatant, 220 μl K\(_2\)CO\(_3\) was added. Nitrites were measured at 550 nm [30]. Superoxide anion radical
concentrations in plasma samples were measured using the NTB (Nitro Blue Tetrazolium) reagent in TRIS buffer (assay mixture). The measurement was performed at a wavelength of 530 nm [25]. The measurement of H2O2 was based on the oxidation of phenol red by H2O2 in a reaction catalyzed by horseradish peroxidase. The level of H2O2 was measured at 610 nm [30].

**Determination of antioxidant molecules (GSH, SOD, CAT)**

The level of reduced glutathione (GSH) was determined based on GSH oxidation via 5,5-dithiobis-6,2-nitrobenzoic acid. 0.1 ml 0.1% EDTA and 750 μl precipitation solution (containing 1.67 g metaphosphoric acid, 0.2 g EDTA, 30 g NaCl, and filled with distilled water until 100 ml) was added in 400 μl RBC lysate. The level of GSH was measured at 420 nm [30]. CAT buffer, prepared RBC lysate sample, and 10 mM H2O2 were used for CAT determination. Detection was performed at 360 nm. SOD activity was determined by the epinephrine method. RBC lysate was mixed with carbonate buffer, and then epinephrine was added. Detection was performed at 470 nm [30].

**Evaluation of the inflammatory status, the hemathological and serum parameters**

In order to evaluate the inflammatory status of patients, following parameters were measured at both points of interest (0 and 30th day): serum C-reactive protein concentration—CRP and tumor necrosis factor concentration—TNF-α. Turbidimetric method (Olympus AU680) was used to determine the serum CRP concentration. For the determination of plasma TNF-α concentration we used commercially available high sensitivity indirect sandwich enzyme-linked immunosorbent assay (ELISA, Human Tumor Necrosis Factor α ELISA Kit, Sigma Aldrich) and after finishing the protocol absorbance was read at 450 nm of wavelength. Several hemathological parameters were also determined: erythrocytes (Er), hemoglobin (Hb), hematocrit (Hct), erythrocyte index- mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), serum lactate dehydrogenase concentration (LDH), serum haptoglobin concentration as well as iron status parameters: serum iron concentration, ferritin; total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), transferrin saturation (TSAT). Erythrocytes and MCV were counted using a Beckman Coulter LH780 hematology counter. Namely, the passage of cells suspended in solution through a crack of a given diameter changes the electrical resistance that the counter registers. The cyanometemoglobin method was used to determine hemoglobin, which is based on the creation of a cyanometemoglobin complex between iron in hemoglobin and added cyanide ions. The complex thus obtained gives an absorption maximum at a wavelength of 540 nm, which is measured spectrophotometrically. The hematocrit was calculated mathematically as the product of the average volume and number of erythrocytes. MCHC is calculated based on the ratio of hemoglobin concentrations in erythrocytes and hematocrit values. MCH was calculated as the ratio of the average hemoglobin concentration in 1 l of erythrocytes and the number of erythrocytes. LDH was measured as the change in NADH absorbance. If there is lactate in the reagent, an increase in absorbance is recorded, and if there is pyruvate in the reagent, a decrease in absorbance to 340 nm is recorded. Ferritine was measured using turbidimetric method on biochemical analyzer Beckman Coulter AU680. A variant of the Shade method was used for determination of Fe, while UIBC was determined via colorimetric method. TIBC was calculated as the sum of Fe and UIBC, while TSAT was calculated as the Fe and TIBC ratio in percents. All of these parameters (Fe, UIBC, TIBC and TSAT) were measured on Beckman Coulter AU680 biochemical analyzer.

**Statistical analysis**

IBM SPSS Statistics 20.0 Desktop for Windows was used for statistical analysis. Shapiro–Wilk test was used to check the distribution of data. Statistical comparisons between two moments of measurement were performed using the Wilcoxon signed-rank test. Values of $p < 0.05$ were considered to be statistically significant.

The total sample calculation was based on the results of previously published studies [31]. A t-test for related samples was used for the calculation, double, with the assumption of an alpha error of 0.05 and a study power of 0.8 (beta error 0.2) and using an appropriate computer program G*Power. Taking into account the results of these studies, the total number of patients was calculated at 25. Since some patients might be excluded from the study, the total number of patients was recalculated at 30.

**Results**

**Patients**

Patient’s characteristics, comorbidities and chronic therapy are provided in Table 1. Namely, most common comorbidities were diabetes and hypertension, while most frequent chronic therapy of these patients were ACE inhibitors and ARBs.
Effects of one-month administration of SAE on anemia in hemodialysis patients

One-month treatment with SAE led to a significant increase in hemoglobin and ($p = 0.018$), haptoglobin levels ($p = 0.038$) after the SAE consumption, as well as the significant decrease in LDH level (0.042). Iron status has also changed after the treatment, mostly via significant decrease in and ferritin ($p = 0.009$) levels. (Table 2).

Effects of one-month administration of SAE on redox status of hemodialysis patients

The levels of superoxide anion radical were significantly decreased ($p = 0.034$) after the one-month treatment with SAE, as well as the concentration of nitrites ($p = 0.000$) (Fig. 1a, b). On the other hand, SAE didn’t induce statistically significant changes in hydrogen peroxide measured levels (Fig. 1c). Supplementation with SAE has also recovered enzymatic antioxidant defense system. Significant increase in the activity of catalase (CAT; $p = 0.017$) and level of reduced glutathione (GSH; $p = 0.022$) were noticed after the treatment period (Fig. 2b, c). (Fig. 2a).

### Table 1

| Variable               | Mean values (X ± SD)/ Frequency |
|------------------------|---------------------------------|
| Patients characteristics|                                 |
| Gender                 |                                 |
| Males n = 19           |                                 |
| Females n = 11         |                                 |
| Age (years) 62.93 ± 11.28 |                                 |
| Body weight (kg) 74.45 ± 16.13 |                                 |
| Body height (m) 1.7 ± 0.09 |                                 |
| BMI (kg/m2) 25.82 ± 5.02 |                                 |
| HD HD length (years) 6.33 ± 2.42 |                                 |
| Comorbidities          |                                 |
| Diabetes mellitus n = 2 |                                 |
| Hypertension n = 21    |                                 |
| Diabetes mellitus + hypertension n = 7 |             |
| Chronic therapy        |                                 |
| ACE inhibitors/ARBs n = 22 |                 |
| β blockers n = 13      |                                 |
| Ca antagonists n = 11  |                                 |
| Diuretics n = 12       |                                 |
| Nitrates n = 5         |                                 |
| Antiaggregation drugs  n = 15 |                   |

Data are presented as mean values ± SD or frequency i.e. Number of patients with certain characteristic

HD hemodialysis; ACE inhibitors angiotensin converting enzyme inhibitors; ARBs angiotensin receptor blockers; β blockers beta receptor blockers; Ca antagonists calcium channel antagonists

### Table 2

| Measured parameter | Before SAE (X ± SD) | After SAE (X ± SD) | Statistical significance (p-value) |
|--------------------|---------------------|--------------------|-----------------------------------|
| Er                 | 3.28 ± 0.07         | 3.45 ± 0.07        | 0.195                             |
| Hct (%)            | 31.27 ± 0.68        | 32.87 ± 0.59       | 0.162                             |
| MCV (fL)           | 95.64 ± 0.98        | 93.46 ± 2.66       | 0.819                             |
| MCH (pg)           | 31.20 ± 0.34        | 31.27 ± 0.38       | 0.468                             |
| MCHC (g/l)         | 325.50 ± 1.05       | 327.54 ± 0.74      | 0.165                             |
| Hb (g/l)           | 101.17 ± 2.09       | 108.21 ± 1.93 *    | 0.018 *                          |
| HP (ng/ml)         | 1.14 ± 0.09         | 1.38 ± 0.10 *      | 0.038 *                          |
| LDH (U/l)          | 347.80 ± 11.92      | 322.36 ± 13.09 *   | 0.042 *                          |
| Fe (µmol/l)        | 8.99 ± 0.52         | 9.81 ± 0.59        | 0.117                             |
| Ferritin (ng/ml)   | 785.90 ± 38.22      | 656.54 ± 42.13 *   | 0.009 *                          |
| Transferrin (g/l)  | 1.54 ± 0.05         | 1.45 ± 0.06        | 0.762                             |
| TSAT (%)           | 26.97 ± 1.23        | 27.64 ± 1.43       | 0.510                             |
| TIBC (µmol/l)      | 33.40 ± 1.03        | 35.36 ± 0.95       | 0.069                             |
| UIBC (µmol/l)      | 24.33 ± 0.82        | 25.57 ± 0.80       | 0.167                             |

Statistical comparisons between two moments of measurement were performed using the Wilcoxon signed-rank test. *Statistical significance at the level of $p < 0.05$ between groups. Data are presented as mean values ± SD.

Hematocrit (%), MCV mean corpuscular volume (fL), MCH mean corpuscular hemoglobin (pg), MCHC mean corpuscular hemoglobin concentration (g/l), Hb hemoglobin (g/l), HP haptoglobin serum concentration (ng/ml), LDH lactate dehydrogenase serum concentration, Fe Iron serum concentration (µmol/l), FER Ferritin serum concentration (ng/ml), TSAT transferrin saturation (%), TIBC total iron binding capacity, UIBC unsaturated iron binding capacity.
Fig. 1 The effects of one-month administration of SAE on the plasma levels of the prooxidants in hemodialysis patients: (A) superoxide anion radical, (B) nitrites, (C) hydrogen peroxide. Statistical comparisons between two moments of measurement were performed using the Wilcoxon signed-rank test. *Statistical significance at the level of \( p < 0.05 \) between groups. Data are presented as mean values ± SD.

Fig. 2 The effects of one-month administration of A. melanocarpa extract on the levels of the antioxidants in hemodialysis patients: (A) superoxide dismutase, (B) catalase, (C) reduced glutathione. Statistical comparisons between two moments of measurement were performed using the Wilcoxon signed-rank test. *Statistical significance at the level of \( p < 0.05 \) between groups. Data are presented as mean values ± SD.
Effects of one-month administration of SAE on inflammatory status of hemodialysis patients

Regarding inflammation, no significant changes after SAE treatment were noticed in the levels of C-reactive protein, leukocytes and TNF-α. (Fig. 3a-c).

Discussion

Our previous research confirmed the fact that patients on hemodialysis are in higher risk for oxidative stress and anemia [32]. Antioxidant potential of SAE, as a specific formulation, was recently demonstrated in an animal model of metabolic syndrome. Taking into account that oxidative stress is one of the underlying mechanisms in anemia associated with hemodialysis, we wanted to investigate the effects of SAE in this specific group of patients. So, the aim of our study was to explore the effects of one-month consumption of Standardized Aronia melanocarpa extract on oxidative stress in anemic hemodialysis patients.

Considering the facts that polyphenol-rich formulations could reduce oxidative stress and that SAE is one of them, we analyzed and compared the redox status of these patients before and after the SAE consumption. Namely, SAE consumption decreased superoxide anion radical and nitric oxide production and didn’t influence the hydrogen peroxide production. The possible explanation for this might lay in the fact that CAT activity, which catalyses degradation of hydrogen peroxide into water and oxygen, was increased. Additionally, the activity of superoxide dismutase (SOD) was slightly increased and reduced glutathione (GSH) were significantly increased after SAE consumption. Several mechanisms might support the antioxidant effect of SAE. Firstly, direct radical scavenging activity of polyphenols present in SAE, such as rutine, izoquercetin, hyperozide [33, 34]. Polyphenols modulate oxidative stress in different ways: they prevent the formation of ROS, enable the removal of already formed radical particles and increase the activity or concentration of molecules that participate in antioxidant protection. Except for beneficial effects of polyphenols, antioxidant properties of anthocyanins (the main constituents of chokeberry juice) may be associated with their influence on purine base metabolism [25]. Anthocyanins can limit the activity of xanthine oxidase, and thus, reduce the production of superoxide anion radical and hydrogen peroxide, which in turn can lead to reduced production of hydroxyl radicals and consequent cell damage. Additionally, antioxidant properties of anthocyanins, as well as polyphenols, might originate from the ability to remove free radicals and chelate transition metal ions, mainly iron [26].

The exact mechanism of anthocyanins’ antioxidant action has not been fully elucidated, but it has been shown that they affect the signaling pathways, especially the signaling pathways of nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a major regulator and transcription factor,
involved in the regulation of gene expression for antioxidant proteins. Keap1 is a cysteine-rich protein that suppresses Nrf2 signaling, serving as a bridge between Nrf2 and ubiquitin ligase kulin-3, which is required for protein degradation in proteasomes [35]. Pro-oxidants induce covalent modification of Keap1 cysteine residues and therefore inhibit ubiquitin-dependent degradation and increase Nrf2 nuclear accumulation, resulting in increased synthesis of endogenous antioxidants [35]. Dietary polyphenols are not present in sufficient quantities to act as free radical scavengers in vivo, instead they are converted to electrophilic quinones and hydroquinones after contact with free radicals, which can interact with Keap1 and activate Nrf2 [36]. SAE consumption led to a decline in the levels of nitrites. The possible explanation for this effect might lie in the fact that anthocyanins inhibit NO biosynthesis. Pergola and coworkers suggested that anthocyanins could inhibit inducible nitric oxide synthase (iNOS) protein expression and concomitantly decrease iNOS activity [36]. It has also been shown that anthocyanins, particularly cyanidin-3-glucoside upregulates eNOS [37]. While increased iNOS expression leads to the pro-inflammatory effects of NO and induction of inflammatory cytokines, generation of NO by endothelial nitric oxide synthase (eNOS) has important role in reducing endothelial dysfunction.

Beneficial effects of this plant are documented in a study on athletes where consumption of chokeberry juice reduced inflammatory markers, and improved total antioxidant and iron status [38]. When speaking about the effects of polyphenol on inflammation in patients on hemodialysis, the opinions of authors are opposite. These results could be explained by the large heterogeneity of polyphenol therapy types (soy isoflavones, pomegranate juices and extract, cocoa flavanols, turmeric powder, and grape powder) and different time of exposition (from 4-weeks to 1 year) [39]. To our best knowledge the effectiveness of Aronia melanocarpa has not been elucidated so far in this specific group of patients, and our results present the first data about the effects of this polyphenol-rich extract in anemic hemodialysis patients. After the four-week consumption of SAE the levels of C-reactive protein were slightly decreased, CRP < 5 mg/l (Fig. 3a), but observed drop was not statistically significant. On the other hand, the levels of TNF-α and white blood cells were slightly decreased, CRP < 5 mg/l (Fig. 3a), but observed increment also wasn’t statistically significant. Anti-inflammatory potential of Aronia melanocarpa was confirmed in different pathologies, such as colitis, pneumonitis diabetes. Several studies found that Aronia extract decreases the production of pro-inflammatory cytokines (IL-6 and TNF-α) [40]. These findings are not in accordance with our results, and it may be due to insufficient one-month length of SAE consumption, or eventual comorbidities of the patients involved in the study, which may count as our study’s limitations. Modulation of cell growth, cellular defense, and signal transduction mechanisms may contribute to the anti-inflammatory effects of polyphenolic compounds in SAE. Some investigations highlighted the significant decrease of malondialdehyde and NF-κB DNA binding activity, as a possible mechanism of mixed antioxidant anti-inflammatory effects of polyphenols, whereas, increase in GSH may be a consequence of enhanced expression of γ-GCL by flavonoids/polyphenols [41].

Since our study included specific subgroup of patients on hemodialysis additional goal was to assess the influence of SAE consumption on the regulation of anemia. Increased concentration of ferritin in serum and functional iron deficiency are important factors for the development of erythropoietin resistance [42]. The results of our study showed that after one-month of SAE consumption the level of hemoglobin was significantly increased and the levels of ferritin were decreased (Table 2). Thus, our results are in favor of that SAE consumption reduced erythropoietin resistance in these patients. Haptoglobin is generally decreased during hemolysis, where little intravascular lysis of structurally altered erythrocytes escaped from reticuloendothelial clearance may be present [43]. On the other hand the values of the lactate dehydrogenase (LDH) are increased during the hemolysis [44]. The results of our study, increment of haptoglobin and decrement of LDH indicate that rate of hemolysis is reduced after one-month consumption of SAE (Table 2). The reduction of hemolysis occurs as a consequence of decrement in the permeability of the capillary wall and erythrocytes membranes stabilization. Knowing the cellular and molecular mechanisms through which each compound of the Aronia melanocarpa extract acts in anemia in hemodialysis patients requires further research.

According to results of our study we could make certain conclusions: (1) The consumption of SAE regulates redox status (reduce the productions of pro-oxidative molecules and increase antioxidant defense). (2) Four-week consumption of SAE has beneficial effects on anemia parameters in patients on hemodialysis (increased hemoglobin and haptoglobin concentration, decreased ferritin and lactate dehydrogenase concentration). (3) The results of our study did not generate sufficient evidence to support efficacy of SAE as anti-inflammatory agent for anemic hemodialysis patients. (4) The extension of SAE consumption should be considered to improve its beneficial effects. Taken in consideration all afore mentioned facts and results, chronic supplementation with antioxidant polyphenol-rich SAE in anemic hemodialytic patients may be useful due to its beneficial effects on anemic and redox status.

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Data availability Data are available from the correspondence author upon reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval Study is approved by the Ethics Committee of the Clinical Center Kragujevac, Serbia No 01-14-3039.

Consent to participate All patients gave written informed consent to participate in the study.

Consent for publication All patients gave written informed consent for publication of data.

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