

Chromosomal Aberrations and HMGA2 Expression in Paroxysmal Nocturnal Hemoglobinuria

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Abstract

Somatic, inactivating *PIGA* mutations in haematopoietic stem cells, followed by an unknown autoimmune selection process in favor of the mutated clone, are thought to be important events in the pathogenesis of paroxysmal nocturnal hemoglobinuria (PNH). Recently, a second clonal event involving the *HMGA2* gene was reported in some PNH cases, and over expression of this gene was postulated to promote proliferation of the mutated clone. The present study investigated a total of 37 PNH-patients with two goals: First, single nucleotide polymorphism chip analysis was performed to detect novel recurrent copy number changes or allelic imbalances. A single deletion affecting the *PIGA* locus was found, but no other relevant or recurrent abnormalities were detected. Thus, although *PIGA* mutations are established as pathogenetic drivers of PNH, larger deletions of the locus are rare. Second, fluorescence *in situ* hybridization was conducted for the region containing the *HMGA2* gene in addition to expression analysis, to study the recurrence of changes at this locus. This revealed no aberration of the chromosomal region containing the *HMGA2* gene in the cohort, and *HMGA2* expression was surprisingly only detectable in a single case.

Keywords: PNH; SNP chips; Chromosomal aberrations; *HMGA2*

Abbreviations: BAC: Bacterial Artificial Chromosome; FISH: Fluorescence in-situ Hybridization; MNC: Mononuclear Cells; PNH: Paroxysmal Nocturnal Hemoglobinuria; SNP: Single Nucleotide Polymorphism; UPD: Uniparental Disomy

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematologic disorder with typical clinical manifestations, including intravascular hemolysis, venous thrombosis, and defective hematopoiesis [1]. Biochemically, PNH is a consequence of nonmalignant clonal expansion of hematopoietic stem cells with somatic mutation of *PIGA* [2,3], and mutant, inactive *PIGA* explains the deficiency of glycosyl phosphatidylinositol-anchored proteins [4]. Furthermore, preexisting bone marrow failure to various degrees seems to be required for the development of PNH [5,6]. PNH clones are present in a majority of patients with aplastic anemia, which is consistent with the strong immune component in the pathogenesis of this disease [7-9]. Furthermore, inhibition of the complement cascade is efficient in treating PNH-related symptoms [10]. Interestingly, the presence of a low number of PNH cells, even in healthy individuals, confirms the hypothesis that defective stem cells actually predate the stem cell failure and depletion [11]. Unlike dysplastic clones in myelodysplastic syndrome (MDS), which harbour intrinsic defects that predispose the cells to clonal evolution, external factors, such as pressure exerted by the immune system, have for a long time been speculated to be the main causes for clonal escape in PNH [5].

Inoue, *et al.* were the first to report a possible second clonal event. They described two patients with PNH with an acquired rearrangement of chromosome 12 affecting the *HMGA2* gene, which encodes a chromatin remodeling protein, in the *PIGA* mutant cells [12]. *HMGA2* is located in an area on chromosome 12q13 frequently involved in translocations and amplifications in benign, mesenchymal tumors. Emerging evidence also indicates an important role for *HMGA2* overexpression in tumorigenesis, including poorly differentiated solid tumors and some cases of leukemia [13-16]. The two PNH patients from the Inoue, *et al.* study had ectopic expression of *HMGA2* in the bone marrow. Based on this finding, *HMGA2* was considered to be a candidate driver gene, as ectopic expression induced cellular proliferation [12]. Another study identified additional PNH cases exhibiting elevated *HMGA2* expression [17]. However, the importance of *HMGA2* expression in PNH remains controversial [18]. Shen, *et al.* have recently sequenced the exome of affected cells in 12 PNH patients, and found a complex pattern of clonal evolution denoted by shared and distinct mutations in subclones, but not a single recurrent mutation besides *PIGA* [19].

Detection of further chromosomal aberrations in PNH clones has been hampered by the low mitotic index of cells, purity of samples, and coexistence of subclones [20]. Single nucleotide polymorphism (SNP) chips have the advantage of detecting aberrations in non-dividing cells and reveal genomic imbalances at a very high resolution. Furthermore, they are able to detect copy number-neutral loss of heterozygosity events, which are called (partial) uniparental disomies (UPD) [21]. Their resolution is also much greater than that of routine metaphase cytogenetics. SNP-based cytogenetic analysis of unbalanced genomic defects can be applied to study a variety of malignancies and germline defects [5,22,23].

Hence, the aim of the present study was to identify novel chromosomal aberrations detectable by SNP chips, to search for chromosomal aberrations involving the *HMGA2* gene by fluorescence in situ hybridization (FISH) and to investigate levels of *HMGA2* expression in PNH patients.

Patients and Methods

Patients

Thirty-seven patients with PNH from the Department of Hematology of the University of Duisburg-Essen who were diagnosed according to the World Health Organization (WHO) criteria were selected for this retrospective, single-centre analysis. Comprehensive clinical information, including treatment history, was available for all patients.

Samples

Peripheral venous blood samples in EDTA were obtained according to institutional guidelines. Peripheral blood mononuclear cells (MNC) were separated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Freiburg, Germany) and cryopreserved until further analysis. Granulocytes were isolated from fresh blood by using Polymorphprep (Axis-Shield, Oslo, Norway). Immunophenotypic analysis by flow cytometry was employed to document the percentage of abnormal GPI-deficient granulocytes as previously described [24]. RNA and DNA were extracted by using the respective QIAamp blood kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To retain RNA quality, RNA extraction was performed on ice.

FISH studies

FISH was performed on peripheral blood cells from 30 patients. For the detection of aberrations involving the *HMGA2* gene at chromosome 12q14.3, bacterial artificial chromosome (BAC) clones were selected by using bioinformatic resources available at the University of California at Santa Cruz website (<http://genome.ucsc.edu/cgi-bin/hgGateway>) as follows: RP11-125F18 (centromeric to the *HMGA2* gene locus, labelled in spectrum orange); RP11-462A13 (spanning the *HMGA2* gene locus, labelled in spectrum green); and RP11-945G8 (telomeric to *HMGA2*, labelled in spectrum green). BAC clones were prepared, BAC DNA was labelled, and the hybridization, washing, and evaluation steps were performed according to standard protocols, as previously described [25]. For the determination of the diagnostic thresholds of the new *HMGA2* probe set, 200 nuclei from blood samples of five healthy persons were evaluated to serve as negative controls. The cut-off level for gains or translocations involving the *HMGA2* gene locus was calculated as the mean of false-positive nuclei in controls plus three standard deviations and was set as 6%.

SNP chip analysis

For SNP array studies, genomic DNA was extracted from granulocytes isolated from 15 blood samples of PNH patients (cell number: $3.2 \pm 1.6 \times 10^7$), 7 buccal swabs from PNH patients (as imperfect germline DNA control; contamination by blood cell DNA cannot be excluded), and granulocytes from the peripheral blood samples of 6 normal donors (as normal reference) with the QIAamp blood kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Array experiments were performed according to the standard protocol for Affymetrix GeneChip SNP 6.0 arrays (Affymetrix, Santa Clara, CA, USA). Briefly, a 500-ng sample of DNA was digested with StyI and NspI, ligated to adaptors, amplified by PCR, fragmented with DNase I, and biotin-labelled. The labelled samples were hybridized to the Affymetrix GeneChip SNP 6.0 arrays, followed by washing, staining, and scanning.

Copy number and UPD analysis

The acquired signal data were loaded into the Affymetrix Genotyping Console Software, version 4 (Affymetrix). Genotyping and copy number analysis were performed with the manufacturer-supplied model and reference files for SNP 6.0 arrays, employing the human genome version 18. Parameters for the algorithms were left at their default values. The built-in Genome Browser was used for visualization of the detected aberrant regions.

Real-time RT-PCR for *HMGA2*

Total RNA was extracted from 25 samples from PNH patients, purified with the RNeasy Midi Kit (Qiagen), and quantified spectrophotometrically as previously described [26]. In eight patients where the purification of granulocytes was not successful, MNC were used instead (see Table 1). As a positive control, RNA was extracted from CD34⁺ cells from one patient without PNH. RNA from granulocytes of five healthy donors was used as a normal reference. The RNA isolation procedure included an on-column digestion step of residual genomic DNA using DNase I, as recommended by the manufacturer. One microgram of total

RNA was used for cDNA synthesis with oligo-dT primers (Roche, Mannheim, Germany) and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) [26]. Quantification of *HMGA2* was carried out by using a forward primer located in exon 2 (5'-GTCCCTCTAAAGCAGCTCAAAA-3') and a reverse primer located in exon 5 (5'-CTCCCTTCAAAAAGATCCAAGT-3'). All primers were manufactured by MWG Biotech (Ebersberg, Germany). PCR and primers for beta-actin were applied as previously described [26]. The PCR reaction mix was prepared with the Quantitect SYBR Green kit (Qiagen), according to the manufacturer's instructions. PCR was performed in duplicate on the ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, USA). An annealing temperature of 57 °C for 33 s and an extension step at 72 °C for 33 s were used. The relative expression of *HMGA2* in relation to beta-actin was quantified by using the formula $HMGA2/\beta\text{-actin}=2^{-\Delta\Delta Ct}$.

No.	Sex	Age at diagnosis (years)	Aplastic anemia	Thrombembolic history	Eculizumab therapy	Purity	FISH	HMGA2 delta CT	SNP-chip
1	M	25	no		yes	88	inconspicuous	undetermined	46XY
2	M	24	no		yes	80	inconspicuous	23.30 ^a	nd
3	M	38	no	DVT, MI	yes	MNC	inconspicuous	19.18	nd
4	F	17	no		yes	98	inconspicuous	undetermined	nd
5	M	19	yes		yes	87	inconspicuous	undetermined	46XY
6	M	42	no		yes	nd	inconspicuous	undetermined	nd
7	F	54	no	DVT	yes	97	inconspicuous	undetermined	nd
8	M	15	no		yes	99	nd	undetermined	46XY,-Xp22.2
9	F	15	no		yes	97	nd	undetermined	nd
10	M	19	yes		yes	85	inconspicuous	undetermined	46XY
11	M	27	no		yes	91	inconspicuous	undetermined	46XY
12	M	77	no	DVT	yes	MNC	inconspicuous	20.97 ^a	nd
13	F	42	yes		yes	88	inconspicuous	21.18 ^a	46XX
14	F	26	no	PE	yes	MNC	inconspicuous	undetermined	nd
15	M	11	yes	Thrombotic tumor ^b	yes	MNC	inconspicuous	nd	nd
16	F	18	no	DVT	yes	67	inconspicuous	undetermined	46XX
17	M	80	yes		yes	87	inconspicuous	undetermined	46XY
18	F	59	no		yes	MNC	inconspicuous	undetermined	nd
19	F	25	no	DVT, port thrombosis	yes	92	inconspicuous	undetermined	46XX
20	F	54	no		yes	MNC	inconspicuous	undetermined	nd
21	F	31	yes		no	MNC	inconspicuous	undetermined	nd
22	M	38	no		yes	MNC	inconspicuous	nd	nd
23	M	32	no		no	MNC	inconspicuous	nd	nd
24	M	21	no		no	74	inconspicuous	nd	nd
25	M	37	no		no	MNC	inconspicuous	nd	nd
26	F	18	no		no	MNC	nd	undetermined	nd
27	M	40	no		no	MNC	inconspicuous	27.40 ^a	nd
28	M	41	no	DVT	yes	79	inconspicuous	undetermined	46XY
29	F	23	no		yes	83	inconspicuous	undetermined	nd
30	F	20	no		yes	83	inconspicuous	undetermined	46XX
31	M	18	no		no	71	nd	nd	46XY
32	M	15	no	DVT	no	85	nd	nd	46XY
33	F	43	no	DVT	yes	92	nd	nd	46XX
34	M	34	yes	MI	yes	59	nd	nd	46XY
35	M	21	no		yes	MNC	inconspicuous	nd	nd
36	M	25	yes		no	MNC	inconspicuous	nd	nd
37	F	32	yes		yes	83	inconspicuous	nd	nd

Table 1: Clinical data and Results

Purity PNH clone size in extracted granulocytes, MNC mononuclear cells were investigated for HMGA2 PCR as not sufficient granulocytes were extractable, M male, F female, DVT deep vein thrombosis, MI myocardial ischemia, PE pulmonary embolism, nd not done, HMGA2 delta CT $HMGA2/\beta\text{-actin}=2^{-\Delta\Delta Ct}$

^a very low amounts of HMGA2 RNA and detected only in one of the duplicates

^bThrombotic tumor right heart

Results

The clinical data of 37 PNH patients (22 male; 59%) are summarized in Table 1. Patients had a median age of 26 years at the time of primary diagnosis. During the clinical course, 28 patients (76%) received eculizumab therapy and 16 patients (43%) were enrolled in clinical studies. Treatment was initiated, if indicated, at a median age of 37 years. Eleven patients (30%) suffered from thromboembolic complications and 9 patients (24%) had concomitant aplastic anemia.

To unravel novel and possibly recurrent chromosomal aberrations, SNP chip analysis was performed when enough DNA was obtained, resulting in 15 patients analyzed. The mean purity of granulocytes from which DNA was extracted after Ficoll Hypaque centrifugation was $88\% \pm 7\%$. The calculated PNH mean clone size was $83\% \pm 9\%$, which was determined by flow cytometry with fluorescent aerolysin. There is no accepted percentage of cell purity required to detect an abnormality by SNP chip analysis because the detection threshold depends on the size and magnitude of the aberrations. However, as described by Tiu, *et al.* and in accordance with our experience, at least 70% of cells should carry a monoallelic lesion to be reliably detected [5], which was a threshold in PNH clone purity surpassed in all PNH samples analyzed in this study.

In 7 of the 15 PNH patients, buccal swabs yielded sufficient DNA for simultaneous SNP chip analysis of the germline. As another reference for comparison, DNA from 6 healthy volunteers was extracted after granulocyte enrichment with the same method as was used for the PNH patients. One male PNH patient showed a deletion of 182 kb within the region Xp22.2 (Table 1; patient No. 8). Although the coding part of the *PIGA* gene itself was not deleted according to the copy number analysis, this aberration deleted material located upstream of the gene, which could remove portions of the promoter or other regulatory elements (Figure 1). However, no further relevant chromosomal aberrations, such as deletions, insertions, or UPDs, were detected in this case or in the other investigated PNH patients.

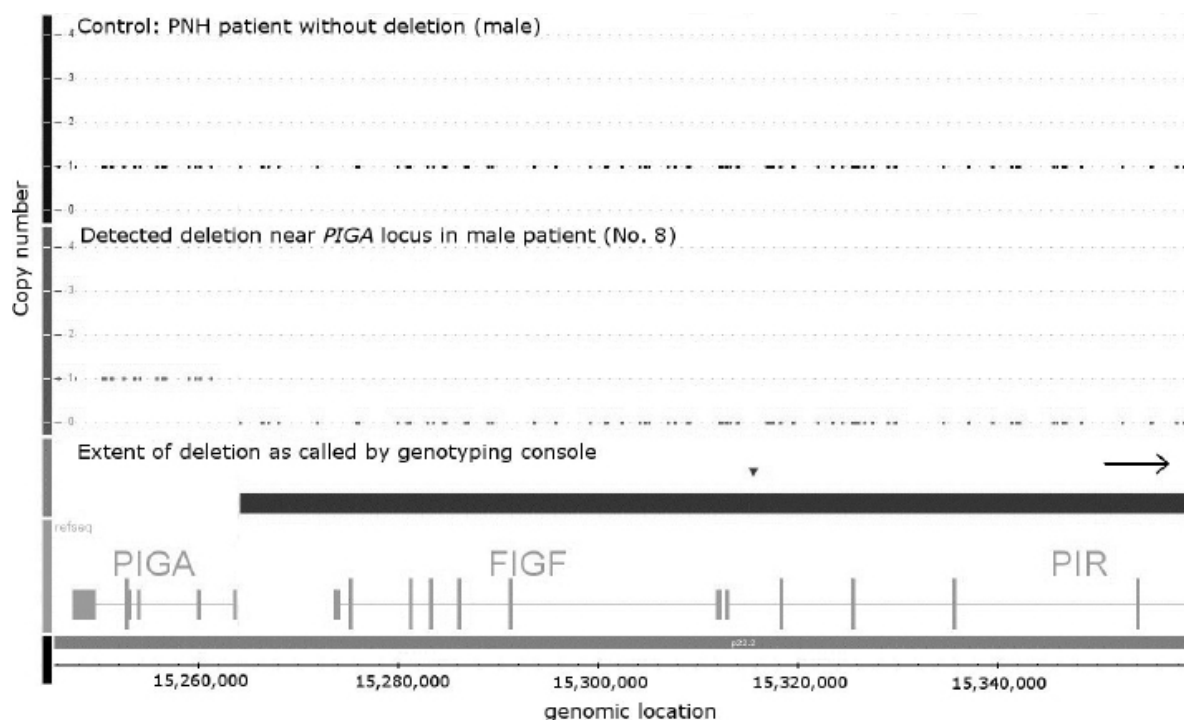


Figure 1: Deletion in Xp22 in a single PNH case

Fifteen PNH cases were evaluated by SNP chip analysis. PNH patient No. 8 exhibited a deletion of 182 kb in the region Xp22.2 (dotted line: copy number values for SNP probes in this region). One end of the deletion lies very close to the first exon of the *PIGA* gene, which could possibly remove regulatory or promoter sequences. For comparison, another SNP profile without gain or loss is shown above. The deletion is longer than the region displayed here

Cells from the peripheral blood of 30 patients were investigated by FISH for imbalances and structural aberrations involving the *HMGA2* gene locus in chromosomal region 12q14.3. All 30 samples showed inconspicuous FISH results for the *HMGA2* gene locus (Table 1). The *HMGA2* gene was originally postulated to be a candidate gene for PNH pathogenesis because it was found to be overexpressed in a PNH patient [17]. Interestingly, after quantitative RT-PCR of granulocytes or, if sufficient RNA could not be extracted, of MNC, significant expression of *HMGA2* in investigated cells was only detected in 1 out of 21 (5%) patients (Table 1; Patient No. 3). In 4 additional PNH patients (19%), very low amounts of *HMGA2* RNA were detected in one of the duplicates. The other 16 samples from PNH patients had no *HMGA2* expression at all, which was in concordance with control peripheral blood cells from five healthy donors. Moreover, the positive control (CD34-positive cells from a patient without PNH) showed strong expression of the gene, at a CT-value of 9.55.

Discussion

In addition to the known *PIGA* mutation that has been found in hematopoietic stem cells, followed by a speculative mechanism of immunological selection in favor of the PNH clone [6], a second clonal event was recently postulated in the pathogenesis of PNH. Two patients with PNH had a concurrent rearrangement of chromosome 12 affecting the *HMGA2* gene, which led to elevated expression of *HMGA2* compared to healthy volunteers [12]. However, the search for further chromosomal aberrations has been hampered by the low mitotic potency of cells harboring the PNH clone. Genome-wide SNP array-based methods could help to identify novel chromosomal aberrations that exclude reciprocal events. Indeed, we detected a deletion in Xp22.2 in one male patient out of 15 PNH patients in the present study. The affected region had a size of 182 kb, and begins upstream of the *PIGA* locus. It is tempting to speculate that this aberration is the cause for the loss of *PIGA* expression in this patient, although no firm conclusion can be reached with the data presented here. The other 14 patients analysed by SNP-Chip had no discernable chromosomal aberration, which is in contrast with another study of PNH patients that reported trisomy 6 and monosomy 7 in affected cells [27].

Total deletions of the *PIGA* gene have been rarely described, and most reported deletions were small [28-30], with only a single publication finding a large deletion of the entire locus [31]. Searching the Mitelman database for the occurrence of *PIGA* aberrations or alterations in Xp22 revealed genetic changes in one case of chronic myeloid leukemia (CML), one case of MDS, one case of chondroid hamartoma of the lung, one case each of adenocarcinoma of the prostate and ovary, three cases of acute lymphoblastic leukemia (ALL), and two cases of acute myeloblastic leukemia (AML), but no cases of PNH. All aberrations identified were translocations, with the exception of one ALL case, which was ins (X;12) (p22;q13q14). Interestingly, this case involved the *HMGA2* gene [32]. Therefore, somatic mutations of the *PIGA* gene remain the main mechanism of *PIGA* disruption. These mutations are located throughout the entire coding region, and most are frameshift mutations producing at most a nonfunctional *PIGA* protein [19, 29].

Inoue, *et al.* described two patients with rearrangements of chromosome 12 that affected the *HMGA2* gene. Searching the Mitelman database for *HMGA2* revealed 153 cases with clonal aberrations encompassing *HMGA2*. These cases included mostly benign illnesses, such as hamartoma, lipoma, and adenoma. However, myeloid neoplasms were also described [5,16,32]. *HMGA2* aberrations could also be clonal events in PNH. In this study, to estimate the frequency of *HMGA2* rearrangements, 30 PNH patients were evaluated with a specifically designed FISH probe for screening. However, no translocation was detected, demonstrating that this event is not common in PNH. In patients with concomitant PNH and MDS, other studies have found trisomy 8 in the fraction of GPI-deficient cells, suggesting that the *PIGA* mutation affected the stem cell first, and chromosomal aberrations evolved within a subclone thereafter [5,33,34]. Recently, it was shown that a stochastic model of clonal stem cell evolution could capture some of the features of PNH [35]. However, the exact mechanism of clonal dominance remains unclear in most PNH patients, and secondary clonal events seem to be rare, in line with recent findings by whole exome sequencing of PNH affected cells [19].

Overexpression of *HMGA2* was first reported in patients with myeloproliferative neoplasia [13]. All of these patients had translocations or inversions involving chromosomal bands 12q13-15 that resulted in the overexpression of *HMGA2*. Furthermore, truncated *HMGA2* transcripts containing only exons 1-3 have been reported to have oncogenic properties [36]. Murakami, *et al.* found significantly higher *HMGA2* expression in the peripheral blood of PNH patients than in healthy persons. However, there was no significant difference in the relative expression of *HMGA2* in the bone marrow [17]. Additionally, analyzing the genomic sequence of three patients, including one who had the highest *HMGA2* expression, revealed no mutation in the 3' untranslated region [17]. Two studies in transgenic mice overexpressing *HMGA2* lead the respective authors to speculate that overexpression of *HMGA2* leads to proliferative hematopoiesis with clonal expansion at the stem cell and progenitor levels and may account for the clonal expansion in PNH [17,37]. However, the clinical course of the mice was very benign, and the proportion of PNH cells was remarkably stable. Therefore, it was suggested that *HMGA2* overexpression itself may not be sufficient to induce clonal expansion of haematopoietic progenitor cells [18,38]. The present study found relevant *HMGA2* expression in 1 of 21 PNH patients (5%). Another 4 PNH patients showed very low *HMGA2* expression. These data suggest that *HMGA2* expression may at most be important in a small fraction of PNH patients, although the expression of shorter or truncated transcripts with unclear relevance cannot be excluded with the primers used in this study.

Clonal expansion has been proposed to be a result of genetic, epigenetic, immunologic, or stochastic events that, together with mutant *PIGA*, provide a proliferative advantage to the mutant PNH cells [39]. Recently, an autoreactive, CD1d-restricted, GPI-specific T-cell population was found to be expanded in PNH patients and was postulated as being responsible for bone marrow failure in PNH, indicating the importance of an autoimmune component in PNH [40].

Taken together, the present work shows that chromosomal aberrations could only be detected in single PNH cases by SNP chip analysis. We identified one PNH patient with the largest deletion adjacent to the *PIGA* gene region reported to date. Relevant novel or recurrent abnormalities were not detectable by SNP chip analysis. A second clonal event affecting the *HMGA2* gene was also not detectable in the present study and seems to be uncommon in PNH. It cannot be excluded that *HMGA2* expression plays a pathophysiological role in those PNH patients with overexpression of this gene, but this work suggests that this is not a regular feature in PNH.

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Authors' Contributions

A.R. and L.S. designed the research; L.S., S.G., L.K.H., H.N., R.S., and A.R. performed the experiments and collected the data; L.S., L.K.H., S.G., R.S., H.N., U.D. and A.R. analyzed and interpreted the data; L.S., R.S. and A.R. wrote the paper.

Compliance with Ethical Standards

The authors declare no conflict of interest. The study was approved by the Ethics Committee of the Faculty of Medicine at the University of Duisburg-Essen and is in accordance with the Helsinki Declaration of 1975 and its later amendments. All patients provided written informed consent.

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