



Polyclonal Anti-D Antibodies Significantly Reduce the Rate of Miscarriages in Rh(D) positive Women with Recurrent Pregnancy loss

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Abstract

Background: Macrophages play a key role in all environmental conditions surrounding pregnancy. Coating of autologous red blood cells (RBCs) with polyclonal antibodies to Rh(D) antigen may result in an immunomodulation and improved outcome in Rh(D) positive women with recurrent pregnancy loss (RPL).

Methods: A total of 60 Rh(D) positive women (age 23 to 45 years) with a history of RPL and ineffective treatment with low molecular weight heparin (LMWH) and/or aspirin were included in this retrospective study. In addition to this treatment, Anti-D (300 µg) was given subcutaneously to each woman either prior to pregnancy and/or two times within 12 weeks of gestation.

Results: Treatment with Anti-D in non-responders to heparin/aspirin resulted in successful pregnancies in 67% of all cases. The remaining women had only abortions (23%) or did not become pregnant (10%). None of the treated women has developed anemia due to this treatment or any other significant adverse reaction. The rate of successful pregnancies does not appear to be influenced by the administration of: Anti-D prior to pregnancy, age, thrombophilia or previous alive births.

Conclusion: The improved outcome following the administration of Anti-D in women with RPL might be explained by immune modulations induced by different immune reactions including polarization of decidual macrophages. The results obtained in this study clearly indicate that Anti-D is safe and highly effective in treatment of Rh(D) positive women with RPL. However, further studies are required to support our results and to find out the optimal dose and timing of Anti-D administration

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Introduction

Recurrent pregnancy loss (RPL) is defined as two or more consecutive pregnancy losses prior to 20–24 weeks gestation. It affects about 1–2 % of pregnancies and is often associated with serious psychological complications. During the last few decades, many factors including age, inheritable and acquired genetic and/or anatomical abnormalities, infections, and endocrine dysfunctions have been identified to play a crucial role in this field. However, the true cause remains unclear in most cases. Until now, there is no specific or even generally accepted treatment in such cases.[1],[2] In general, pharmacological treatment is only recommended for anti-phospholipid

syndrome, and factor V mutation.[3-5] Dependent on clinicians and patients, this treatment may also be employed in the presence of other congenital thrombophilias, such as prothrombin mutation, deficiencies in anti-thrombin, protein C, and protein S.[1],[6],[7]

Based on the fact that pregnancy reflects an immunological miracle combining immune defense/response on the one hand and immune tolerance on the other hand, it is not surprising that immune imbalance during pregnancy may result in complications. In fact, several studies have shown that RPL could be related to immune abnormalities in roughly 50% of affected women.[8-12] Current studies have focused on the role of decidual macrophages. These cells mature during early stages of pregnancy and persist during the entire gestation period.[13] In the first trimester, about 40 % of all cells in the decidua are leukocytes and 20–30 % of these cells are macrophages, which have the plasticity to alter their function in response to various environmental signals. They are also sensitive to small changes in the microenvironment[13-15], they represent the most crucial immune cells for pregnancies due to their involvement in regulation of implantation, placentation, fetal development, and most importantly vascular remodeling at the maternal-fetal interface. These properties make those cells attractive as a therapeutic target in immune related diseases.

Polyclonal Anti-D is successfully used to prevent alloimmunization in Rh(D) negative pregnant women as well as in treatment of patients with autoimmune thrombocytopenia (ITP). Until now, the mechanisms by which these antibodies are operating in vivo are poorly understood. In addition to Fc mediated phagocytosis of IgG coated RBCs, Anti-D has exciting immunomodulatory effects, which could not completely be explained yet.[16],[17]

In this retrospective study, Anti-D has been shown to increase the rates of successful pregnancies in Rh(D) positive women who remained abortive despite treatment with LMWH and/or aspirin.

Patients and Methods

Serological testing including blood group, antibody screening, and direct antiglobulin test were performed by standard techniques using the gel cards (Biorad, Cressier sur Morat, Switzerland and Grifols SA, Barcelona, Spain). Only Rh(D) positive women who had at least two pregnancy losses have been included in this retrospective study. 59 women (age 23 - 45 years) had inheritable genetic abnormalities (thrombophilias), one woman did not give consent to genetic testing but had thrombophilia history in her anamnesis (table 1). ^a number of thrombophilia associated mutations; ^b Anti-D injections prior (pre) or after (after) conception; *patient did not give consent to genetic testing, therefore no proof of inherited thrombophilia, but increased risk of thrombosis by anamnesis); unk: unknown; nd: not done

All women had ineffective pretreatment with LMWH and/or aspirin. They were treated on an outpatient basis between 2016 and 2021 at our practice, the Haemostaseologikum Mitte in Berlin. All medical records and investigations were retrospectively reviewed.

Depending on presentation, unpregnant women were introduced to admit themselves Anti-D (300 µg Rhophylac, CSL Behring) five to ten days prior to the expected ovulation, and two times within the first twelve weeks of pregnancy. All other women were presented within short time when they became aware of their pregnancy, and received, similar to the other group, two times Anti-D.

All women were clinically examined and investigated for underlying diseases which might be responsible for the RPL. In addition, they were tested for thrombophilia.

Informed written consent was obtained for participation, and the local ethics review board was briefed about this study.

Statistical analyses were performed using the IBM SPSS statistics 28.0 software.

Results

As expected, administration of Anti-D resulted in a weakly positive direct antiglobulin test in all cases, but none of the treated women developed significant hemolysis.

Most importantly, the majority of these women had not only unremarkable pregnancy, but also normal delivery. Only six women did not get pregnant (10%), and 40 of the remaining 54 women gave a live birth (60% of all treated women and 74% of all pregnant women, respectively) (figure 1).

Impact of previous abortions/births on outcome

Despite treatment with LMWH and/or aspirin, 36 of the 60 women had two or three abortions prior to Anti-D treatment, and the remaining 24 had four or more abortions (figure 1). Following treatment with Anti-D, 27 women of the first group had successful pregnancies (75%), nine had abortions (25%), and three did not get pregnant (8%). Of the second group of 24 women, 13 gave birth (54%), nine had abortions (38%), and three did not get pregnant (13%).

Prior to treatment with Anti-D, 30 women had no birth, and 30 women had at least one birth (table 2). Of the first group, 22 women had successful pregnancies (73%), eight had abortions (27%), and one woman did not get pregnant (3%). Of the other 30 women, 18 women gave birth (60%), ten had abortions (33%), and five did not get pregnant (8%) (table 2).

A chi-square test was used to compare number of abortions prior to treatment with Anti-D and successful pregnancy under treatment with Anti-D. No expected cell frequencies were below 5, and the results showed no significant correlation

Table 1: Relevant data of women treated with Anti-D

woman no.	age [years]	blood group	mutation ^a [numbers]	only LMWH/aspirin		plus Anti-D			
				abortion	birth	injections ^b	gravity	abortion	birth
1	23	B	2	4	0	after	1	0	1
2	27	unk	3	3	1	pre and after	0	0	0
3	29	A	3	2	1	after	1	0	1
4	29	A	3	3	0	pre and after	1	1	0
5	30	A	2	4	1	pre and after	1	0	1
6	31	B	4	2	1	after	1	0	0
7	31	A	3	4	0	pre	0	0	0
8	32	A	4	2	0	pre and after	2	0	2
9	33	A	1	2	0	unk	1	0	1
10	33	B	2	4	0	pre and after	1	0	0
11	34	A	1	3	0	after	1	0	1
12	34	A	3	3	0	after	3	1	2
13	34	0	4	3	0	after	1	0	1
14	34	B	2	2	0	after	1	0	0
15	35	0	3	2	0	pre and after	2	0	2
16	35	0	3	2	1	pre and after	2	1	1
17	35	B	2	2	1	unk	1	0	1
18	35	A	6	2	1	unk	1	0	1
19	35	A	4	4	0	after	3	2	1
20	35	A	2	3	0	after	1	0	1
21	36	A	2	2	1	pre and after	4	1	2
22	36	0	3	2	0	pre and after	2	0	2
23	36	B	2	4	1	after	1	0	1
24	36	A	2	2	3	pre	0	0	0
25	36	A	4	4	0	pre and after	2	2	0
26	36	AB	1	2	1	after	1	0	1
27	37	0	2	3	0	pre and after	1	0	1
28	37	unk	1	5	1	after	1	0	1
29	37	A	2	2	2	pre and after	1	0	1
30	38	A	1	2	0	after	1	0	1
31	38	0	4	3	0	after	1	0	0
32	39	A	1	4	0	after	1	0	1
33	39	A	5	4	2	pre and after	1	0	1
34	39	AB	1	2	1	pre	0	0	0
35	39	0	3	2	1	after	1	0	1
36	40	A	3	6	2	after	4	3	0
37	40	B	2	7	2	pre and after	2	1	0
38	40	0	1	4	0	pre and after	1	0	1
39	40	A	2	2	3	unk	1	0	1
40	41	0	1	7	1	after	2	0	2
41	41	A	1	3	0	pre and after	1	0	1
42	41	A	4	5	2	pre	0	0	0
43	41	0	1	2	0	after	1	0	1
44	41	unk	3	4	1	pre	0	0	0

45	41	A	1	5	1	after	1	1	0
46	42	O	4	2	0	after	1	0	1
47	42	A	2	7	0	after	1	0	1
48	42	A	3	3	5	pre and after	1	1	0
49	42	A	2	3	1	after	1	0	1
50	42	O	1	3	0	unk	1	0	1
51	42	A	3	4	1	pre and after	1	1	0
52	43	A	1	3	1	pre and after	1	0	1
53	43	B	1	3	0	unk	1	0	1
54	43	A	nd*	4	3	unk	1	0	1
55	43	A	2	18	0	pre and after	1	0	1
56	44	O	3	4	4	after	1	0	0
57	44	O	3	5	2	pre and after	3	1	1
58	44	AB	4	3	0	after	1	0	1
59	44	A	1	4	0	unk	1	1	0
60	45	B	4	2	0	pre and after	1	1	0

^a number of thrombophilia associated mutations; ^b Anti-D injections prior (pre) or after (after) conception; *patient did not give consent to genetic testing, therefore no proof of inherited thrombophilia, but increased risk of thrombosis by anamnesis; unk: unknown; nd: not done

with $\chi^2(1) = 0.303$, $p(\chi^2) = 0.582$, $p(\text{Fisher's exact test}) = 0.784$, $V = 0.071$.

Impact of age

There have been 23 women at ages between 23 and 35 years, and 37 at ages between 36 to 45 (table 1). Of the first group, 17 women had successful births (74%), six had abortions (26%), and two women did not get pregnant (9%). Of the second group, 27 women gave births (73%), 11 had abortions (30%), and four did not get pregnant (11%) (table 2).

A chi-square test was used to compare age groups and successful pregnancies under treatment with Anti-D. No expected cell frequencies were below 5, and the results showed no significant correlation with $\chi^2(1) = 0.881$, $p(\chi^2) = 0.348$, $p(\text{Fisher's exact test}) = 0.408$, $V = 0.121$.

Impact of Anti-D dose

Of the 25 women who received Anti-D only after conception, 19 gave birth (76%), and 8 had abortions (32%) (table 2). Of the 21 women who received Anti-D prior to pregnancy, 14 gave birth (67%), ten had abortions (48%). Six women did not get pregnant. For eight women full data of Anti-D administration were not available (table 2).

In summary, the prophylactic administration of Anti-D also prior to pregnancy does not appear to be more effective than the administration only post gestation.

A chi-square test was used to compare pre and after conception injections of Anti-D with successful pregnancies. No expected cell frequencies were below 5. Results showed no significant correlation with $\chi^2(1) = 0.801$, $p(\chi^2) = 0.371$, $p(\text{Fisher's exact test}) = 0.522$, $V = 0.132$.

Impact of blood group

There have been 31 women with blood group A, from whom 28 got pregnant, 21 gave birth (68%), ten had abortions (32%). Of the nine women with blood group B, four gave birth (44%), and 5 had abortions (56%). Of the 14 women with blood group O, 12 gave birth (86%), and four had abortions (29%). Only three women had blood group AB, from whom two gave birth (67%), no woman had an abort (0 %), and one did not get pregnant (table 2).

A chi-square test was used to compare blood group and successful pregnancies. Four cell frequencies were below 5, and the results showed no significant correlation with $\chi^2(1) = 2.401$, $p(\chi^2) = 0.493$, $p(\text{Fisher's exact test}) = 0.518$, $V = 0.205$.

Impact of thrombophilia associated mutations

All women showed thrombophilia associated mutations, except one who did not give consent for this kind of genetic testing. 41 women had a PAI1 mutation, 39 a prothrombin 19911 mutation, 27 a fibrinogen alpha mutation, 19 a fibrinogen gamma mutation, seven a factor V Leiden mutation, six a HR2 mutation, two a prothrombin 20210 mutation, two a FSAP mutation, one a MTHFR mutation, and none had a tPA mutation.

From 16 women with only one mutation 13 gave births (81%), two had abortions (13%), and one did not get pregnant (6%). From 16 women with two mutations 12 gave births (75%), three had abortions (19%), and one did not get pregnant (6%). Three mutations were found with 15 women, of whom seven gave births (47%), seven had abortions (47%), and three did not get pregnant (20%). Four to six mutations were found

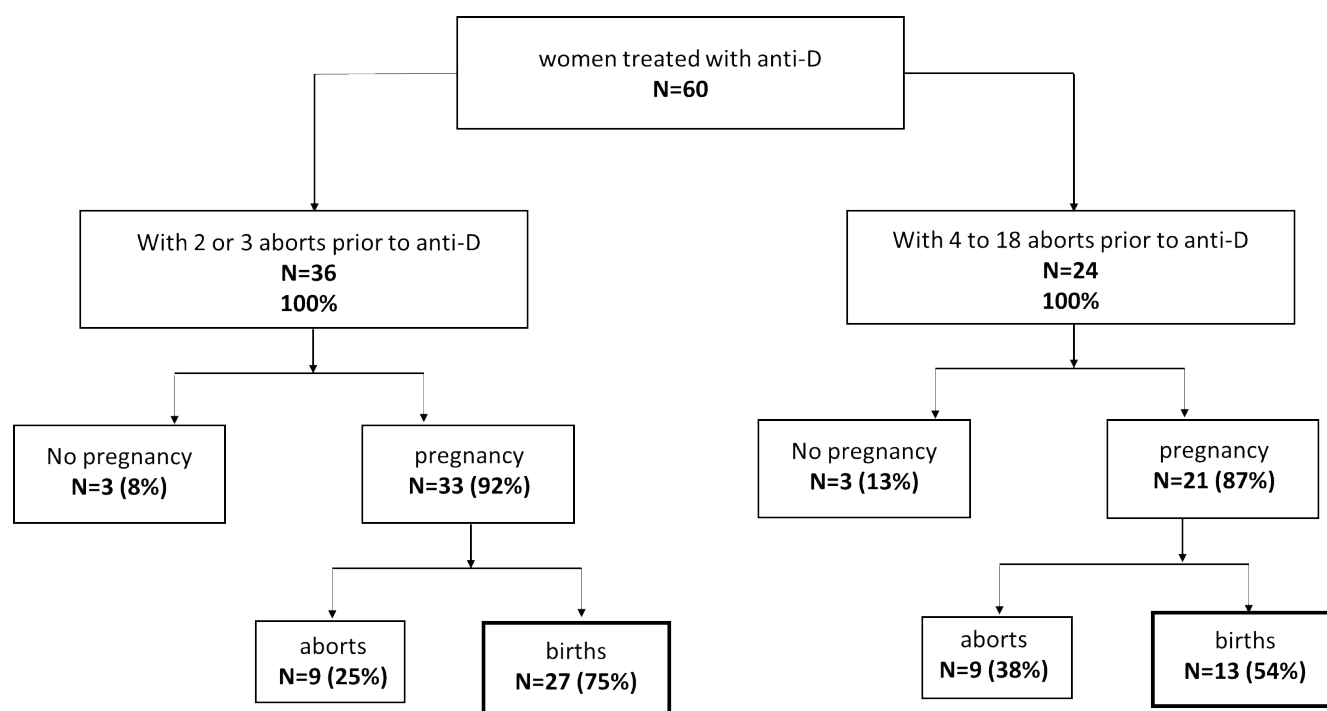


Figure 1: Outcome of women treated with Anti-D in addition to LMWH/ and or aspirin.

As some women had both, birth(s) and abort(s), sum of values for births and abortions can be higher than 100%.

Table 2: Efficacy of treatment with Anti-D

	age [years]		Injections of Anti-D ^a			Birth prior to Anti-D		Thrombophilia associated mutations [numbers]			
	≤35	>35	pre and after	after	unknown	no	yes	1	2	3	≥4
total	23 (100%)	37 (100%)	21 (100%)	25 (100%)	8 (100%)	30 (100%)	30 (100%)	16 (100%)	16 (100%)	15 (100%)	11 (100%)
pregnancy	21 (91%)	33 (89%)	21 (100%)	25 (100%)	8 (100%)	29 (97%)	25 (83%)	15 (94%)	15 (94%)	12 (80%)	10 (91%)
no pregnancy	2 (9%)	4 (11%)	0 (0%)	0 (0%)	0 (0%)	1 (3%)	5 (17%)	1 (6%)	1 (6%)	3 (20%)	1 (9%)
birth	17 (74%)	23 (62%)	14 (67%)	19 (76%)	7 (88%)	22 (73%)	18 (60%)	13 (81%)	12 (75%)	7 (47%)	7 (64%)
abort	6 (26%)	11 (30%)	10 (48%)	8 (32%)	1 (12%)	8 (27%)	10 (33%)	2 (13%)	3 (19%)	7 (47%)	5 (45%)

Because some women had birth(s) and abort(s), sum of values for births and abortions can be higher than 100%.

^a in relation to time of conception. Six women did not get pregnant, and did only receive Anti-D prior to conception.

in 12 women. Of these women, seven gave births (64 %), five had abortions (45%), and one woman did not get pregnant (9%) (table 2).

A chi-square test was used to compare number of thrombophilia associated mutations (one or two versus three or more) and successful pregnancies. No expected cell frequencies were below 5. Results showed no significant correlation with $\chi^2(1) = 1.701$, $p(\chi^2) = 0.192$, $p(\text{Fisher's exact test}) = 0.276$, $V = 0.170$.

Discussion

The vast majority of women treated with Anti-D had unremarkable pregnancy and delivery. More than 70% of all

pregnant women delivered healthy children. Interestingly, the efficacy of this treatment does not appear to be dependent on age, thrombophilia, and prior treatment. Nevertheless, by these data there could be a relation between those parameters and outcome of a pregnancy, but case numbers are too low for significant statistical analysis. Treatment with Anti-D was safe with no signs of significant hemolysis.

Worldwide, RPL represents a serious and unresolved problem [1],[2],[6]. Immune pathways play a key role in the pathophysiology of RPL. Already at early stage, gestation induces a major immune response which presumably involves all immune cells, including lymphocytes, macrophages and decidual dendritic cells. Thus, the question

whether immunotherapy may help in management of RPL is increasingly focused in new studies[18].

Macrophages are presumably the main player in regulation of pregnancy, including implantation, placentation, fetal development, parturition, and most importantly vascular remodeling at the maternal-fetal interface. Thus, some modulation of macrophages may prevent miscarriages related to immune imbalance. The question then arose to whether subtle attraction of these cells by coating of autologous red blood cells in such affected women may result in successful pregnancies. In fact, the results obtained during observation were encouraging. The vast majority of treated women had unremarkable pregnancy and delivery. Interestingly, the efficacy of this treatment does not appear to be dependent on age, thrombophilia, and prior treatment. Nevertheless, by these data there could be a relation between those parameters and outcome of a pregnancy, but case numbers are too low for significant statistical analysis. Since all treated women received simultaneously LMWH and/or aspirin, the question remains open whether the beneficial effect was solely related to Anti-D or to a combined effect of both drugs. This is supported by the finding that LMWH beyond its anti-coagulating effect may induce immunomodulation.[19] Until now, a monotherapy with Anti-D has not been undertaken. However, it has been shown that treatment with intravenous immunoglobulins (IVIg) that somewhat resembles Anti-D, may improve the outcome in pregnant women with miscarriages.

Similarly, there is evidence that polyclonal Anti-D has an immunomodulatory effect, which could not fully be explained yet.[20],[21] In our experience, this treatment is safe when it is given subcutaneously or at least slowly by intravenous route.[22] In previous studies we have demonstrated that the administration of LMWH and/or aspirin results in live births in most women with RPL.[7] In this retrospective study, we used low Anti-D doses (2 to 3 times 300 µg) for treatment of RPL in Rh(D) positive women who remained abortive despite treatment with LMWH and/or aspirin.

At first glance, the question may arise whether this treatment could be justified in Rhesus positive pregnant women. Based on the following facts, this treatment is, we think, rational as long as no alternative therapy is available for such affected patients. Anti-D is used in treatment of adults and children for four decades with little or no side effects, if the drug was carefully injected.[22] The injection of 300 µg is harmless compared to that dose given in ITP (50-75 µg/kg body weight), and to that amount reaching Rh(D) positive infants in Rh(D) negative pregnant women who receive at least two times 100 - 300 µg prophylaxis.

The positive influence of Anti-D on RPL is evident as reflected by the significantly increased rates of successful pregnancies in the majority of treated women in our practice. This effect does not appear to be dependent on age, inherited

hemophilias or blood group. The question whether Anti-D per se or only in combination with LMWH is effective is a matter of speculation. Since the mechanisms by which LMWH influences the outcome in RPL is largely unknown, a synergic effect cannot be excluded. Ultimately, LMWH has been shown to induce in vitro and in vivo a proinflammatory profile on RPL. It has been suggested that this effect could be valuable at the implantation stage.[7] Thus, it remains unclear whether Anti-D alone could also be effective as well as the combination with LMWH.

The question why this treatment was ineffective in 26% of the treated pregnant women remains to be answered. A possible explanation could be related to unknown predisposition factors that cannot be influenced by immunological modulations. Similarly, the phenomenon that the treatment may again result in miscarriage in some women who had been shown to be responsive remains also obscure. Whether this ineffectiveness could be related to the giving dose or timing of Anti D administration remains to be determined.

Though, the most possible explanation for the successful pregnancy following treatment with Anti-D may be attributed to an immunomodulatory effect, some other questions remain open. For example, when and by which mechanisms Anti-D may induce the tolerance of the semiallogeneic fetus. There is no doubt that pregnancy is highly controlled by immune reactions which simultaneously maintain immune response (defense) and tolerance (semi-allogeneicity), respectively. [10],[11],[23].

Changes during pregnancy include an increase in phagocytic cells and their functional capacity to ingest IgG coated RBCs,[24] an increase of T and B regulatory cells, reduction of natural killer cells, and polarization of decidual macrophages.[23],[25-28] It has been demonstrated that RPL is associated with an imbalance of pregnancy related immune haemostasis,[10],[11],[29] which could, at least in part, be normalized by immunomodulation.[18],[30]

Anti-D administration into Rh(D) positive pregnant women results in coating of autologous RBCs with IgG antibodies. This happening represents a dangerous signal, and attracts macrophages to recognize IgG opsonized RBCs. Although the attracted macrophages do not appear to ingest a significant amount of the IgG coated RBCs, they could be influenced in the presence of IgG-coated RBCs.

In addition, polyclonal Anti-D may contain antibodies other than Anti-D which may have influence on macrophages, independent of phagocytosis, i.e. HLA and anti-idiotypic antibodies. Thus, it would not be surprising that the reactions to Anti-D may lead to polarization of decidual macrophages. These cells exhibit wide plasticity and are sensitive to small microenvironmental changes, including nutrition, smoking, toxins, inflammation, and physiological stress.[13-15]

It seems that the M1 subtype predominates over the M2 subtype in RPL. Coating of autologous RBCs with antibodies represents unphysiological condition in vivo, which may lead to polarization of M1 to M2 phenotype. The latter cells seem to be responsible for immunotolerance, which is a prerequisite for successful implantation a fetal development.

This hypothesis can only be proved by phenotyping of decidual macrophages prior to and following treatment. Alternatively, an extended investigation of cytokines and changes of peripheral immune cells may give an explanation of this phenomenon.[8-11],[26],[28]

Conclusions

We are aware of the limitation of the presented study. It is mainly based on retrospective data. In addition, the number of treated women is relatively small and too low for significant statistical analysis, and an optimal dose and timing of Anti-D remained speculative.

Anti-D could be a new therapeutic option in women with recurrent pregnancy loss who are not responding well to low molecular heparin and/or aspirin alone.

Thus, new well-designed studies may gain deeper insights in this field, and into the maternal-fetal immune interface.

Statement of Ethics

This is a retrospective analysis of patient data. The Ethics Committee of the Berlin Medical Association has confirmed that no ethical approval is required.

Author Contribution statement

FR: literature search, data collection, data analysis, data interpretation, writing, critical revision, accessed and verified all data; FL: data collection; HK: treatment of patient, critical revision; JS: writing, critical revision; BH: treatment of patients, critical revision; AK: data collection; RH: treatment of patient, critical revision; CFS: treatment of patient, critical revision, SM: treatment of patient, critical revision; GH: treatment of patient, critical revision; AS: treatment of patient, literature search, data interpretation, writing, critical revision, accessed and verified all data

All authors confirm that they had full access to all the data in the study and accept responsibility to submit for publication of the manuscript.

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