CHAPTER V

PLACENTAL COMPLEMENT ACTIVATION IS RELATED TO PLACENTAL DYSFUNCTION IN THE SETTING OF PREECLAMPSIA

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Abstract

INTRODUCTION Increasing evidence suggests that the complement system is involved in the pathogenesis of preeclampsia. We have recently demonstrated a strong association between preeclampsia and placental complement activation. However, it remains unknown whether placental complement activation in the setting of preeclampsia is related to disturbed placental function and the different related clinical manifestations.

METHODS We collected 111 placentas from women whose pregnancy was complicated by preeclampsia. Placentas were stained immunohistochemically for C4d, a stable tissue marker for complement activation. C4d staining patterns were related to pregnancy outcomes and the clinical and histopathological manifestations of preeclampsia.

RESULTS C4d was absent in 43.3%, present in a focal staining pattern in 36.0% and in a diffuse staining pattern in 20.7%. The presence of diffuse C4d was associated with a significantly lower mean gestational age at delivery ($p<0.05$). Importantly, the presence of placental C4d was also associated with intra-uterine fetal death ($p<0.01$), significantly lower placenta weight ($p<0.05$), and various histopathological manifestations of preeclampsia, including placental infarction ($p<0.01$), increased syncytial knots ($p<0.01$) and deciduitis ($p<0.01$).

DISCUSSION The current study confirms that preeclampsia is frequently associated with placental C4d deposits. Importantly, a relation between preeclampsia-associated intra-uterine fetal death and placental C4d was established. The presence of placental C4d was also associated with significantly smaller placentas and with hallmarks of reduced placental perfusion, suggesting that complement activation is related to placental dysfunction in the setting of preeclampsia.

Introduction

Preeclampsia is a devastating pregnancy complication that is characterized—in its simplest form—by new-onset hypertension and proteinuria after 20 weeks of gestation. Maternal health can be further threatened by seizures (eclampsia) and the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets)[1], whereas fetal outcomes are frequently compromised by intra-uterine growth restriction, placental abruption and occasionally even intra-uterine fetal death.[2] Importantly, preeclampsia is associated with a risk for recurrence in subsequent pregnancies, particularly in women with severe, early-onset preeclampsia in their initial pregnancy.[3] Preeclampsia remains a leading cause of maternal and perinatal mortality and morbidity, as no therapy is available other than (iatrogenic) delivery.[4]

Although the etiology of preeclampsia is probably as heterogeneous as its presentation, several lines of evidence suggest that the complement system is involved in the pathogenesis of preeclampsia. The complement system is an ancient part of innate immunity that helps to eradicate invading pathogens. However, the system can also damage host tissues if excessively activated or inadequately regulated. In animal models of preeclampsia, complement activation results in the development of preeclampsia symptoms, while complement inhibition prevents the manifestations of preeclampsia.[5,6] Women with preeclampsia have higher levels of circulating complement components.[7,8] In addition, mutations in complement regulatory proteins predispose to preeclampsia and of the preeclamptic women with these mutations, many have severe, early-onset preeclampsia and/or preeclampsia complicated by intra-uterine growth restriction or HELLP syndrome.[8] Furthermore, increased quantities of several complement activation products are observed in placentas of women with preeclampsia.[9-11] Illustratively, our group has recently demonstrated that C4d—a stable tissue marker for complement activation—is frequently observed in

placentas from preeclamptic women, whereas it is rarely present in placentas from women with uneventful pregnancies.[11]

In settings other than preeclampsia, such as antiphospholipid syndrome, placental complement activation is associated with profound placental injury, both in experimental models and human placentas.[12-14] Experimental models of preeclampsia have demonstrated a similar relation between complement activation and placental injury and dysfunction.[5] Therefore, we hypothesized that women with preeclampsia and placental complement activation have increased placental injury and dysfunction compared to preeclamptic women without placental complement activation. To test this hypothesis, we stained 111 placentas obtained from preeclamptic women for C4d and related the presence of placental C4d to the clinical manifestations and the histopathological lesions that characterize preeclampsia.

Methods

PATIENTS AND PLACENTAS We studied 111 placentas obtained from women who were diagnosed with preeclampsia and who delivered at the Obstetrical Department of the Leiden University Medical Center (LUMC) between 2001 and 2011. Preeclampsia was defined as the presence of hypertension and proteinuria after 20 weeks of gestation.[15] All clinical data was derived from the case-records of the LUMC. We obtained clinical information on maternal age, gravidity, parity, height of blood pressure, amount of urinary protein, gestational age at delivery, birth- and placenta weight, mode of delivery and maternal and fetal morbidity (Table 1).

ETHICS All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (*Code for Proper Secondary Use of Human Tissue*, Dutch Federation of Medical Scientific Societies).

PLACENTA PATHOLOGY AND IMMUNOHISTOCHEMISTRY
All placentas were formalin fixed and paraffin embedded. Paraffin sections were routinely stained with haematoxylin and eosin (H&E). To relate placental complement activation to the clinical and histopathological manifestations of preeclampsia, immunohistochemical staining was performed for C4d. Sections were deparaffinized and rehydrated, after which heat induced antigen retrieval was performed in Tris-EDTA buffer (pH 9.0). After blocking for endogenous peroxidase, sections were incubated for 1 hour with polyclonal rabbit anti-human C4d (1:50, Biomedica Gruppe, Austria). Binding of the primary antibody was visualized with appropriate secondary antibodies and diaminobenzidine as a chromogen. Sections were counterstained with Haematoxylin. Renal tissue of a patient with humoral rejection served as a positive control. For the negative control, the primary antibody was omitted and replaced by Rabbit Immunoglobulin Fraction (DakoCytomation).

QUANTIFICATION OF PLACENTAL MORPHOLOGY AND IMMUNOHISTOPATHOLOGY Sections were evaluated by an experienced pathologist, who scored the slides blinded to the patients' clinical data. Each placenta was scored separately for the presence or absence of histopathological changes associated with decreased uteroplacental perfusion: acute and/or chronic deciduitis; decidual necrosis; increased syncytial knots; accelerated villous maturity; accellerated maturity; avascular villi; villous infarcts; retroplacental haematomas; intervillous thrombi; and decidual vasculopathy.

Positivity for immunohistochemical staining was scored semi-quantitatively by two independent observers. Staining intensity around syncytiotrophoblast was scored as 0, 1 or 2, with 0 representing absence of staining (<10%), 1 representing focal staining (10-50%) and 2 representing diffuse staining (>50%).[16]

STATISTICAL ANALYSES Categorical variables were compared using the Chi-square test. When comparing numerical data from >2 independent groups, a one-way ANOVA was performed. All analyses were performed using SPSS statistical software package (version 20; Chicago, IL). A p-value < 0.05 was considered statistically significant.

Results

C4d is frequently observed in placentas of women with preeclampsia C4d was observed in 63 out of 111 (56.7%) placentas. When present, C4d was observed at the syncytiotrophoblast in either a focal or a diffuse staining pattern. Focal C4d deposits were observed in 40 (36.0%) placentas, whereas 23 (20.7%) placentas showed a diffuse C4d staining pattern. Table 2 shows the incidence of the different C4d staining patterns among women with preeclampsia and Figure 1 shows representative examples of the different C4d staining patterns.

PLACENTAL C4D IS ASSOCIATED WITH PLACENTAL DYSFUNCTION When subdividing women according to their C4d staining pattern (Table 3), women with placental C4d had a significantly lower mean gestational age at delivery, as compared to women without placental C4d. Furthermore, intra-uterine fetal death was significantly more common among women with placental C4d as compared to women without placental C4d ($p<0.01$). In addition, the mean birth weight at delivery and the average placenta weight were significantly lower in women with placental C4d. To exclude the confounding effect of gestational age on birth weight, we looked up birth weight percentiles and placenta weight percentiles. Placenta weight percentiles were significantly lower among women with placental C4d ($p<0.05$) No statistically significant difference was observed between the groups with respect to birth weight percentiles. To investigate whether placental complement activation is primarily related to placental dysfunction, or also to the maternal

manifestations of preeclampsia, we compared the height of blood pressure and the amount of urinary protein between preeclamptic women with and without placental C4d. A trend was observed between the amount of placental C4d and the amount of urinary protein (Table 3). No significant differences were observed with respect to the height of blood pressure, eclampsia and the prevalence of HELLP syndrome.

PLACENTAL C4D IS RELATED TO PLACENTAL INJURY
The histopathological changes associated with decreased uteroplacental perfusion were frequently observed in the placentas included in the study. When subdividing placentas according to their C4d staining pattern, a strong relation ($p<0.01$) was observed between the presence of C4d and the presence of placental infarction (Figure 2). Placental C4d was frequently observed around areas of infarction, of which a representative image is provided as supplementary data (Figure S1). The presence of placental C4d was also associated with a higher incidence of acute and/or chronic deciduitis ($p<0.01$). Furthermore, a higher incidence of increased syncytial knots was observed in placentas in which C4d was present ($p<0.01$). Additionally, the presence of C4d was related to the presence of decidual vasculopathy ($p<0.05$). Avascular villi were observed in 37% of preeclamptic women without IUFD and in 61% of women with both preeclampsia and IUFD. Its presence was significantly associated with C4d deposits ($p<0.05$).

PATIENTS OF WHICH PLACENTAS OF SUBSEQUENT PREGNANCIES WERE AVAILABLE Of two patients included in the study, paraffin-embedded placenta material from a subsequent pregnancy was available. These two placentas were also stained for C4d to investigate whether the presence or absence of C4d is consistent among subsequent pregnancies. The first patient initially had a preeclamptic pregnancy for which a Caesarean Section was performed at 34+6 weeks. Macroscopically, the placenta consisted

of two lobes with a combined weight of 351 g. On macroscopic examination, no obvious signs of infarction were observed. Microscopically, micro-infarcts were observed, as well as pronounced syncytial knotting, which is frequently observed in the setting of placental hypoxia. No C4d was observed in this placenta. In the placenta of the subsequent pregnancy, there was no C4d either. The patient delivered at 41 weeks gestational age of a healthy son (3500 g). The placenta (460 g) was sent in for examination because of a suspected placenta accreta, which necessitated manual removal. Some placental infarction (<5%) but no signs of placenta accreta were observed. The second patient initially had a pregnancy complicated by fetal loss in the setting of severe preeclampsia, severe intra-uterine growth restriction (IUGR) and HELLP syndrome at 23 weeks of gestation. The placenta was removed manually and it was severely damaged. Microscopically, fibrosis of the villious stroma was observed, as well as fibrin deposition and some infarction. The C4d staining showed intense and diffuse C4d deposits along the syncytiotrophoblast of all villi. The subsequent pregnancy was also complicated by IUGR and hypertension for which the patient delivered by Caesarean section at 30+6 weeks gestational age. The placenta (184 g) showed increased syncytial knotting, as well as accelerated villous maturity; findings that are consistent with increased placental maturity and ischemia. Strikingly, this placenta also showed C4d deposits, although less prominent than in the placenta from the previous pregnancy.

Discussion

In this study, which includes 111 placentas obtained from preeclamptic women, we have shown that the presence of complement activation (C4d) is associated with a significantly lower gestational age at delivery and intra-uterine fetal death. Moreover, the presence of C4d is also associated with significantly

lower placenta weight percentiles and a significantly higher prevalence of several histopathological changes associated with reduced uteroplacental perfusion. These data suggest that placental complement activation is related to placental injury and dysfunction in the setting of preeclampsia.

We show a strong relation between placental classical complement activation and preeclampsia-associated intra-uterine fetal death (IUFD). Placental pathology plays a notable role in IUFD, both in developing nations and in high-income countries.[17,18] However, the exact mechanisms underlying IUFD in women with preeclampsia remain largely unknown. Placental classical pathway activation has long been recognized as an important cause of pregnancy complications in women with the autoimmune disorders systemic lupus erythematodes and antiphospholipid syndrome. Adverse pregnancy complications in these women include frequent early and late fetal loss.[19,20] In human placentas of women with antiphospholipid syndrome, particularly the classical pathway marker C4d is strongly related to IUFD. IUFD in the setting of antiphospholipid syndrome is associated with classical pathway activation at the fetal-maternal interface, in a similar pattern as observed in the current study.[21] In a murine model of immune-mediated fetal loss, complement activation leads to increased fetal loss and complement inhibition prevents this.[12] Altogether, both murine and human studies have demonstrated that complement activation is involved in anti-phospholipid antibody induced fetal loss. The strong association between the presence of C4d and IUFD in this study suggests that IUFD in the setting of preeclampsia may also be complement mediated. It has indeed been observed that IUFD in the setting of preeclampsia is accompanied by activation of the complement system, as indicated by elevated maternal plasma concentrations of complement split products.[22] However, this is the first study that reports an association between IUFD and complement activation at the placental level in women with preeclampsia. We also show a relation between placental complement

activation and placenta size. Importantly, preeclampsia is associated with significantly smaller placentas.[23] Our results suggests that among preeclamptic women, the smallest placentas are among the women with placental complement activation. This is evidenced by the fact that in the presence of placental C4d, the average placenta weight percentile is significantly lower compared to placentas from preeclamptic women without placental C4d. Complement activation could affect placental development and function, as previously shown in an animal model of preeclampsia[6], thereby resulting in smaller placentas.

The current study also demonstrates a strong association between placental complement activation and various histopathological findings associated with reduced uteroplacental perfusion. The presence of C4d was strongly related to placental infarction. Placental infarction is a frequent finding in preeclampsia and the extent of placental infarction correlates with the severity of preeclampsia.[24] Complement activation could contribute to the development and extent of infarction, like previously demonstrated in the setting of experimental myocardial infarction.[25] The presence of C4d was also strongly correlated to the incidence of increased syncytial knots. Syncytial knots are the main placental production site of sFlt-1, suggesting that these knots play an important role in the pathogenesis of preeclampsia.[26] Because inflammatory cytokines stimulate the release of trophoblasts from the placenta[27], it is not unlikely that complement activation may also be involved in the formation and release of syncytial knots. Furthermore, C4d deposits were associated with decidual vasculopathy, which is in line with a previous report.[28] Like in the setting of atherosclerosis[29], excessive complement activation could contribute to the development of decidual vasculopathy. Placental complement activation is not only associated with injury to the maternal vasculature, as the presence of C4d was also related to avascular villi, which results from fetal vascular damage. Finally, placental C4d was associated with deciduitis; a placental inflammatory reaction. This is in line

with observations in a murine model of preeclampsia, in which complement activation is associated with placental inflammation.[6] Of two patients included in the current study, placentas from subsequent pregnancies were available. The first patient showed no C4d in both placentas. The other patient, on the contrary, showed placental C4d deposits in both placentas, which were derived from pregnancies that were complicated by severe, early-onset hypertensive disorders of pregnancy as well as severe IUGR, in one case complicated by fetal loss. Placental C4d appears to be related to the extent of placental dysfunction in preeclampsia and these two cases suggest that the presence or absence of C4d may be consistent among subsequent pregnancies. Severe, early-onset preeclampsia is most likely to recur in subsequent pregnancies.[3] Particularly those women with severe preeclampsia as well as placental C4d may have the highest chance of developing complications during later pregnancies. It would be interesting to investigate whether placental C4d can serve as a biomarker to predict adverse pregnancy outcomes in future pregnancies among women with preeclampsia in an initial pregnancy.

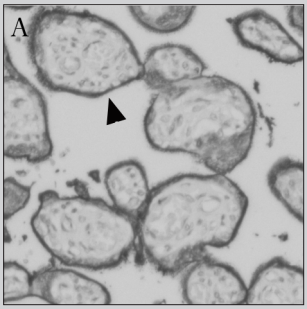
The findings in the current study confirm the association between preeclampsia and placental classical pathway activation, as previously reported by our group.[11] It must be acknowledged that it is difficult to determine whether complement activation is a cause or a consequence of preeclampsia. In the setting of IUFD, C4d deposits could be a late event, as severely damaged or apoptotic tissue can induce classical pathway activation.[30] However, placental C4d is frequently absent in cases of IUFD due to chromosomal abnormalities, indicating that IUFD does not necessarily result in complement activation.[21] Furthermore, C4d is also observed in placentas of live-born children, indicating that C4d may have been present before the fetus died. Moreover, complement inhibition prevents fetal loss in a mouse model for preeclampsia, suggesting a causal relationship between complement activation and fetal loss.[5] Therefore, complement activation could also precede IUFD.

Importantly, a rise in complement factor Bb precedes the onset of preeclampsia, suggesting that complement activation is an early event in the development of preeclampsia.[7] Furthermore, complement activation in pregnant mice results in a variety of pregnancy complications and local blockade of complement activation at the fetal-maternal interface rescues both fetal loss and preeclampsia in mice.[5] Although it remains uncertain whether placental C4d is a cause or a consequence of the pathophysiological processes taking place in preeclampsia, the current study does underline the importance of further investigating the role of the complement system in the pathogenesis of preeclampsia.

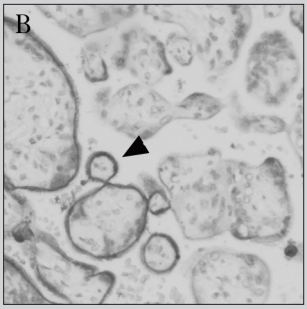
In conclusion, our results have demonstrated a strong relationship between placental complement activation and the prevalence of several placenta-related manifestations and complications of preeclampsia such as IUFD and small for gestational age placentas. In addition, we have shown that placental complement activation in preeclamptic women is associated with a higher prevalence of histopathological hallmarks of reduced placental perfusion. Altogether, placental classical pathway activation appears to be related to the extent of placental dysfunction and placental injury in women with preeclampsia. If complement activation contributes to the pathophysiology and/ or the severity of preeclampsia, it may be worth while exploring its possibilities as a disease marker or even a target for therapy.

FIG 1 DIFFERENT C4D STAINING PATTERNS

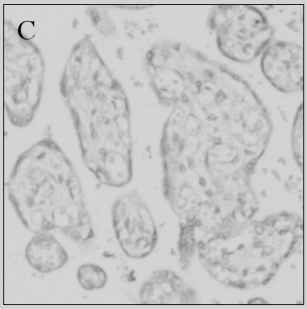
(full colour version inside cover)



diffuse C4D staining



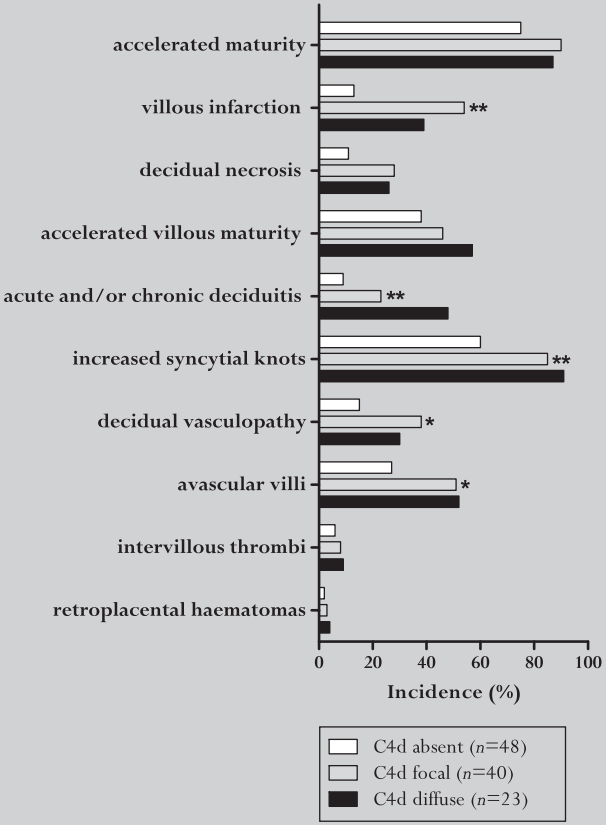
focal C4D staining



negative C4D staining

Overview showing representative images of the different C4d staining patterns. Staining was scored as 'diffuse placental C4d staining' (A), 'focal placental C4d staining' (B) or 'no or minimal placental C4d staining' (C). Images A and B clearly illustrate that C4d deposits are present at the surface of the syncytiotrophoblast (arrows), and not within villi.

BAR GRAPH OF SPECIFIC PLACENTAL HISTOLOGICAL SCORES AS DESCRIBED IN RESULTS **FIG 2**



Bar graph of specific placental histological scores as described in Results, giving an indication of the abundance of various lesions in the different C4d groups. The differences between groups are represented by *$p<0.05$ and **$p<0.01$.

TABLE 1 PATIENT CHARACTERISTICS

CHARACTERISTICS	
Mean maternal age in years (SD)	32.0 (6.5)
Mean gravidity (SD)	2.2 (1.6)
% gravida 1	44.1
Mean parity (SD)	0.6 (1.1)
Nulliparous (%)	68 (61.3)
Mean highest diastole (mmHg) (SD)	111.1 (11.4)
Mean proteinuria (g/24h) (SD)	6.5 (6.3)
Mean gestational age at delivery (weeks + days, SD in days)	30 + 2 (25.5)
Mean birth weight (g) (SD)	1166.7 (573.1)
Birth weight percentile	
<5 (%)	14 (13.5)
5-10 (%)	11 (10.6)
10-20 (%)	31 (29.9)
20-50 (%)	32 (30.8)
>50 (%)	16 (15.4)
Mean trimmed placenta weight (g) (SD)	226.2 (95.7)
Mode of delivery	
Caesarean section (%)	83 (74.8)
Spontaneous delivery (%)	4 (3.6)
Induction (%)	24 (21.6)
Complications	
Intra-uterine fetal death	18 (16.2)
HELLP syndrome (%)	31 (27.9)
Eclampsia (%)	12 (10.9)

TABLE 2 DISTRIBUTION OF DIFFERENT C4D STAINING PATTERNS AMONG WOMEN WITH PREECLAMPSIA

C4D STAINING PATTERN	n = 111
No C4d (%)	48 (43.3)
Focal C4d (%)	40 (36.0)
Diffuse C4d (%)	23 (20.7)

CLINICAL CHARACTERISTICS OF WOMEN WITH PREECLAMPSIA AND NO, FOCAL OR DIFFUSE PLACENTAL C4D **TABLE 3**

	No C4d (n = 48)	Focal C4d (n = 40)	Diffuse (n = 23)
Mean maternal age in years (SD)	31.5 (6.1)	32.2 (7.3)	32.5 (6.1)
Mean gravidity (SD)	2.3 (1.5)	2.1 (1.4)	2.1 (2.1)
G1 (%)	19 (39.6)	16 (40.0)	14 (60.9)
Mean parity (SD)	0.5 (0.8)	0.7 (1.0)	0.8 (1.7)
Nulliparous (%)	30 (62.5)	22 (55.0)	16 (69.6)
Mean highest diastole (mmHg) (SD)	110.3 (12.0)	111.6 (10.2)	111.7 (12.7)
Mean proteinuria (g/24h) (SD)	5.3 (5.0)	6.5 (5.7)	8.8 (8.8)
Mean gestational age at delivery (weeks + days, SD in days)	31 + 1 (25.2)	29 + 5 (26.5)	29 + 1 (21.7)*
Birth weight (g) (SD)	1359.3 (595.9)	1067.3 (551.8)*	933.5 (433.1)*
Birth weight percentile			
<5 (%)	6 (12.8)	5 (14.3)	3 (13.6)
5-10 (%)	2 (4.3)	5 (14.3)	4 (18.2)
10-20 (%)	15 (31.9)	10 (28.6)	6 (27.2)
20-50 (%)	19 (40.4)	9 (25.7)	4 (18.2)
>50 (%)	5 (10.6)	6 (17.1)	5 (22.7)
Placenta weight (g) (SD)	261.7 (98.3)	207.1 (91.4)*	181.1 (68.0)*
Placenta weight percentile †			
<3 (%)	14 (32.6)	24 (63.2)	10 (43.5)
3-10 (%)	12 (27.9)	10 (26.3)	7 (30.4)
>10 (%)	17 (39.5)	4 (10.5)	6 (26.1)
unknown (%)	5	2	0
Mode of delivery			
Caesarean section (%)	42 (87.5)	24 (60.0)	17 (73.9)
Spontaneous delivery (%)	2 (4.2)	2 (5.0)	0 (0.0)
Induction (%)	4 (8.3)	14 (35.0)	6 (26.1)
Maternal complications			
HELLP (%)	10 (20.8)	12 (30)	9 (39.1)
Eclampsia (%)	5 (10.4)	6 (15.4)	1 (4.3)
Fetal complications			
Intra-uterine fetal death (%) †	0 (0)	12 (30)	6 (26.1)

* denotes a statistically significant difference when compared with no C4d

† denotes a statistically significant difference in overall comparison

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