Study on Diagnostic Value of Serum Amyloid A Protein During Late- Onset Sepsis in Preterm and Full Term Neonates

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Abstract: Objective: 1. To assess the diagnostic value of serum amyloid A protein (SAA) during late-onset sepsis (LOS) in preterm and full term neonates. 2. To compare SAA efficiency with C- reactive protein(CRP) and hematological parameters as total leucocytic count (TLC) and immature to total neutrophil ratio (I:T) in detection of LOS. Design: Case control study. Setting: Neonatal Intensive Care Unit (NICU), Abu El-Reish Hospital, Cairo University. Patients: 30 neonates (36.7±2.6 weeks) presenting with sepsis manifestations after first week of life, and 30 healthy neonates (36.9±1.69 weeks) were included in the study as a control group. Methods: 1 ml of venous blood was withdrawn from each neonate , centrifuged and serum was separated for complete blood count, SAA, C- reactive protein (CRP), total leucocytic count (TLC), and immature to total neutrophil ratio (I:T) on the first day of admission and repeated (for patients) 72 hours after treatment. Blood cultures were done to all patients. Results: SAA levels were significantly higher in patients than controls (p<0.01), these levels continued to be elevated 72 hours after treatment. On comparing SAA level to other markers of sepsis such as TLC, I/T ratio and CRP, SAA was the most sensitive marker (86%), and was more specific (93.3%) than CRP, TLC, but less specific than the I: T ratio (100%). However, SAA level showed the highest negative predictive value (87.5%) compared to all other inflammatory markers. High significant elevation of SAA protein level was obtained in the non- survivors compared to survivors (p<0.01). Conclusion: SAA was the most sensitive marker with the highest negative predictive value in comparison with other markers of LOS, while .I:T ratio was the most specific marker with the highest positive predictive value. Therefore, the combining use of SAA and I:T ratio will enhance the diagnosis accuracy of both markers in neonatal LOS. This study also suggests that SAA may be useful marker for follow up of these cases.

Key words: Neonatal sepsis, SAA, CRP, NICU.

INTRODUCTION

Late- onset sepsis (LOS) in newborn infants is defined as sepsis occurring after the first 72 hours of life and is a major cause of infant mortality (Polin & Randis, 2010).

Despite advances in medicine, diagnosis of neonatal sepsis remains a major challenge (Arnon et al, 2007). Early clinical signs are non-specific and the laboratory criteria are also not fully reliable. Warning signs and symptoms are often subtle and can easily be confused with non- infective causes such as apnea, hypothermia, and acute exacerbation of chronic lung disease (Aggarwal et al, 2001).

Hematological and biochemical markers such as total leucocytic count(TLC), immature/ total neutrophil ratio(I:T), platelet count (PLT), C- reactive protein (CRP), various cytokines, procalcitonin and tumor necrosis factor-α (TNF- α) have been proposed as useful indicators for early identification of the septic infants (Lam & Ng, 2008). However, these tests have a wide range of sensitivities and specificities(Gerdes,2004). Additionally, elevations of CRP may not occur until 8 to 48 hours after the first clinical suspicion of infection, and blood culture results mostly are available after 48–72 hours(Suiclathangam et al,2012).

Recently, serum amyloid A protein (SAA) that refers to a group of polymorphic apolipoproteins 12-14 KDa, mainly produced by the liver, has been proposed as a new discriminative marker of bacterial infection (Goldman et al,2005). Expression of SAA is principally regulated at transcriptional level by cytokines (TNF- α and interleukins 1 and 6) or glucocorticoids (Malle et al,2009).

The aim of this study was; 1) to determine the role of serum amyloid A (SAA) in the diagnosis of LOS in neonates. 2) To compare the efficiency of SAA with total leucocytic count, I:T ratio, C- reactive protein in the diagnosis of LOS.

Patients and Methods:

This case control study was conducted on 60 neonates admitted to the Neonatal Intensive Care Unit (NICU) at Abu El-Reish Hospital, Cairo University in the period from (Mars 2007-Mars 2008).

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The study protocol was approved by the ethical committee of the National Research Center (NRC). All neonates were included after a written consent from their parents.

Newborns Included in the Study were Grouped as Follows:
Study Group:
This group comprised 30 neonates with gestational age of 36.7±2.6weeks, who presented with clinical manifestations of sepsis after the first week that was proved by blood culture.

This group was further subdivided into Gram negative and Gram positive subgroups according to the cultured organism.

Control Group:
This group comprised 30 normal healthy neonates. They were matched for age, sex and weight.

Exclusion Criteria:
- Patients less than 32 weeks of gestation.
- Patients presenting during first week of life
- Patients with intra-ventricular hemorrhage (IVH)
- Patients with hypoxic ischemic encephalopathy (HIE).
- Patients presenting with life threatening congenital anomalies.

Criteria for Diagnosis of Late –Onset Neonatal Sepsis:
Clinical diagnosis of neonatal sepsis was based on the presence of clinical manifestations that included; lethargy, poor feeding, fever or hypothermia and signs of cardio-respiratory dysfunction such as increased incidence of apnea, bradycardia, hypotension, poor skin perfusion as well as pallor presenting during the second to fourth weeks of life (Levy et al, 2003).

Sample Collection:
From each newborn included in the study, 1 ml of venous blood was obtained by a sterile venipuncture on admission and serum was separated after centrifugation of blood for 10 minutes at room temperature and stored in 200ul aliquots at -20ºC until assayed. Blood culture samples were used immediately. Another sample was obtained from each patient 72 hours from the first sample collection for performing complete and differential blood cell counts, C-reactive protein and SAA protein.
Only one blood sample was obtained from each healthy control subject.

All the Studied Cases were Subjected to:
Full history taking and complete clinical examination with special emphasis on the clinical signs of sepsis.

The specimens of blood were obtained from each neonate prior to the commencement of the antibiotics for sepsis work up, included hematological parameters like total leukocyte count TLC, the absolute neutrophil count, immature to total neutrophil count ratio I:T, platelets count, blood culture, C-reactive protein (CRP) and serum amyloid A protein (SAA). Blood culture was done to identify the etiological agents as well as to differentiate Gram negative and Gram positive patients.

SAA Protein Levels:
SAA Protein was estimated by using Enzyme Linked-Immuno-Sorbent Assay (ELISA) Kit . (Bio Source International: Human SAA ELISA kit protocol; 2001: 1-2).

The cut off level of SAA for controls was set at (< 10 μg/mL) according to   the manufacturer's instructions.

Statistical Analysis:
- Data were statistically described in terms of mean ± standard deviation (mean± SD), frequencies (number of cases) and relative frequencies (percentages) when appropriate. Comparison of quantitative variables between the study groups was done using Student *t* test for independent samples. Chi-square test was used for comparison of qualitative variables (not normally distributed).
- Mann-Whitney test was used for inter-group analysis for continuous non- parametric data.
- Correlation between continuous variables was performed using spearman correlation coefficient(r) and the probabilities (p).

A probability value (*p* value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2003 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows. ROC curves were done to determine the cut off values for all studied markers (Ng, 2004).

Results:

Table 1: Descriptive data of patients and controls.

	Cases n=30	Control n=30	P- value	Significance
Sex Male Female	17(56.7%) 13(43.3%)	20(66.7%) 10(33.3%)	0.42	NS
Gestational age(weeks)	36.7±2.6	36.99±1.7	0.68	NS
Birth weight (grams)	2812.3±960.1	2982.7±529.7	0.39	NS
Duration of admission (days)	24.3±10.7	5.5±2.0	0.00	HS
NS: non-significant, p value>0.05. S: significant, p value <0.05. HS: highly significant, p value<0.01				

The descriptive findings in patients and controls are illustrated in table (1). No significant difference was found between cases and controls regarding sex, gestational age and weight (p>0.05). A highly significant difference was found between cases and controls in the duration of hospital stay (p<0.01).

Table 2: Descriptive data of the studied groups of patients.

	Gram negative group n=13	Gram positive group n=17	P -value	Significance
Sex Male Female	8(61.5%) 5(38.5%)	9(52.9%) 8(47.1%)	0.64	NS
Gestational age(weeks)	35.2±2.4	37.8±2.1	0.003	HS
Birth weight (grams)	2295.4±1001.5	3207.7±731.7	0.013	S
Duration of admission (days)	30.3±10.7	19.7±8.4	0.004	HS
NS: non-significant, p value>0.05. S: significant, p value <0.05. HS: highly significant, p value<0.01				

Among patients' subgroups, the gram negative (-ve) group had a significant lower birth weight compared to gram positive group (+ve) (p<0.05), in addition they showed a high significant lower gestational age and a longer duration of hospital stay when compared to gram +ve group (p<0.01) (table 2).

The most common risk factors for   late onset sepsis among the studied babies included; the presence of intravenous lines in all patients (100%), sixteen patients were on mechanical ventilation (53.3%), and eleven patients gave low Apgar score (36.7%). Eleven patients were preterm (<37 wks)   (36.7%).Five cases were very low birth weight (VLBW) (16.7%) and 4 cases (13.3%) were low birth weight (LBW).

Lethargy, decreased Moro reflex and intolerance to feeding were the most important presentations for all cases. Hypothermia and tachypnea were significantly more common in the gram negative group (p<0.01).

All cases showed a significant low mean value of platelet count (132.6±30.6x 10^3 /mm^3) when compared to the control group (148.5±27.6 x 10^3 /mm^3) (p<0.05). However, the TLC and I:T ratio were significantly higher in patients than in controls (17.5±6.2 x 10^3 /mm^3 and (0.35±0.12) versus 11.2±5.4 x 10^3 /mm^3 and (0.1± 0.06) respectively) (p<0.01).

All cases with LOS had positive blood cultures. Thirteen neonates had Gram negative organisms (43.3%). Klebsiella was the most common Gram negative organism (25%), followed by E coli (10%) and Pseudomonas (10%). Seventeen neonates had Gram positive organisms (56.7%). Coagulase-negative staphylococci (CONS) (13%), were the most common isolated organisms followed by Staph epidermis(12%), Streptococci (12%) and Staph haemolyticus (10%).

Table 3: SAA protein levels in cases before and after treatment compared to controls:

	Cases		Controls (3)	P value (1) &(2)	P value (1) & (3)	P value (2) & (3)
	1st sample (1) (before treatment)	2nd sample (2) (72 hours after treatment)				
SAA µg/mL Mean ± SD Minimum-Maximum	40.16±35.17 15-120	39.43±33.46 13-120	6.45±2.42 2-10	p>0.05*	P < 0.01**	P < 0.01**
NS: non-significant, p value>0.05*. S: significant, p value <0.05. HS: highly significant, p value<0.01**						

A high significant elevation in mean SAA was present in patients at onset of sepsis compared to controls (p<0.01). After 72 hours of treatment initiation, the mean SAA levels in patients continued to be higher than that of controls (p<0.01).

Table 4: Comparison of SAA protein levels between the patients' subgroups:

	Gram negative group (n=13)	Gram positive group (n=17)	P value	significance
SAA µg/mL Mean ± SD Minimum-Maximum	60.00±35.124 13-120	24.99±27.430 11-120	<0.01	HS
HS= Highly significant = P < 0.01				

Regarding the patients' subgroups, the gram negative group showed a high significant difference in the mean level SAA compared to gram positive group (p<0.01).

Table 5: Comparison between sensitivity and specificity of SAA and other markers of sepsis according to ROC curve:

		SAA µg/mL	CRP(mg/dl)	TLC	I:T
Mean ± SD	cases	40.16±35.17	13.20±10.84	17.47±6.17	0.359±0.12
	controls	6.45±2.42	7.60±2.69	11.24±5.44	0.104±0.06
Cut off value		10	6	10	0.21
Sensitivity %		86.67	60.00	80.00	80.00
Specificity %		93.33	73.33	53.33	100.00
PPV%		92.86	69.23	63.16	100.00
NPV%		87.50	64.71	72.73	83.33
accuracy		90.00	66.67	66.50	90.00
PPV%=positive predictive value%, NPV%= negative predictive value, SAA= serum amyloid A protein, CRP= C-reactive protein, TLC= total leukocytic count, I:T= immature to total neutrophil ratio.					

The receiver-operating characteristic (ROC) curve was done to determine the cut off level of other sepsis markers.

The normal cut off level for CRP was set at < 6 µg/ml, the normal cut off level for TLC was set at ($<10\times10^3$ /mm^3) cells, that of I: T ratio was set at <0.20, and the normal cut off level for PLT was set at (148×10^3 /mm^3).

Table 6: SAA level in non-survivors versus survivors:

SAA µg/mL	Non-survivors	Survivors	P- value	Significance
Mean ± SD Minimum –Maximum	67.25±29.49 22-120	22.10±26.15 11-120	0.000	HS
HS=Highly significant = P < 0.01				

In this study eighteen neonates (60%) with LOS improved, while twelve neonates (40%) died. Mortality was significantly higher in the Gram negative group 10 /13 (77%), compared to the Gram positive group where only 2/17 cases died accounting for 12% of the patients in this group. (P value < 0.01).

By using the receiver-operating characteristic (ROC) curve (figure 1), comparison of the SAA protein data to the tested other markers of sepsis including CRP, TLC,and I: T ratio, showed that the SAA protein was the most sensitive marker (86.67 %) compared to the sensitivity of TLC (80%), of I: T ratio (80%), and CRP (60%).

As regarding the specificity of the markers I: T ratio was the most specific (100%) marker followed by SAA protein s (93.33%). The specificity of CRP was (73.33 %), TLC was (53.33%).

The positive predictive value (PPV) of SAA in this study was (92.86%) which was higher than the PPV of CRP (69.23%),and the PPV of TLC (63.16%), but lower than the PPV of I: T ratio (100%). The SAA protein had the highest negative predictive value (NPV) (87.50%), compared to all other markers.

Correlation between SAA protein and risk factors; showed that SAA protein level was significantly elevated in septic neonates with high risk factors including; low gestational age (r = -0.608, p = 0.000) LBW (r = -0.641, p = 0.000), low Apgar score   (r = -0.796, p = 0.000), and those who were ventilated (r = 0.665, p = 0.000).

Correlation between SAA protein level and other markers of sepsis: high significant positive correlation between SAA protein levels was noticed with I: T ratio (r = 0.755) (p value < 0.01); (Figure 2), and CRP level (r = 0.483, p= <0.01) (Figure 3); whereas, a non-significant positive correlation was observed between the SAA protein level and the TLC in the studied cases (r = 0.203, P = > 0.05).
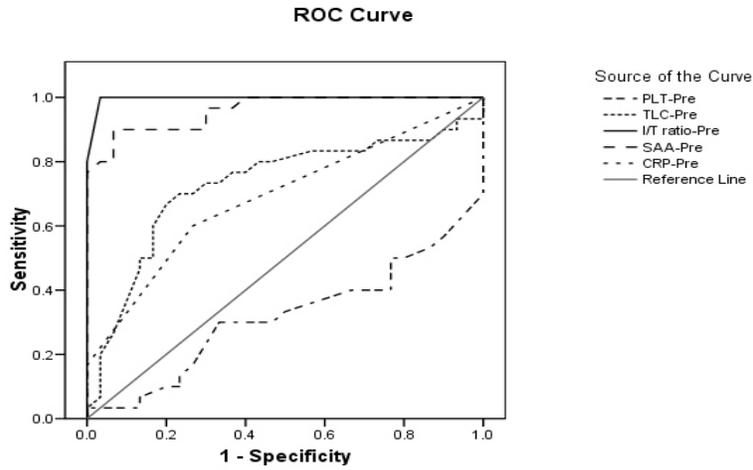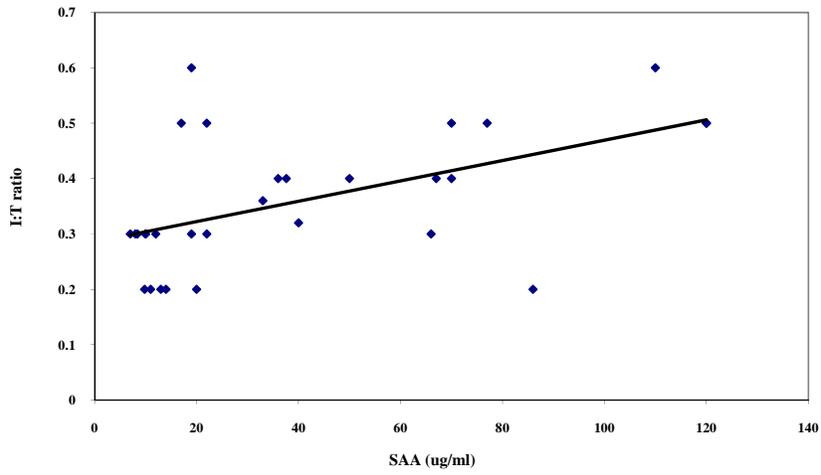
Fig. 1: ROC curve for markers of sepsis.

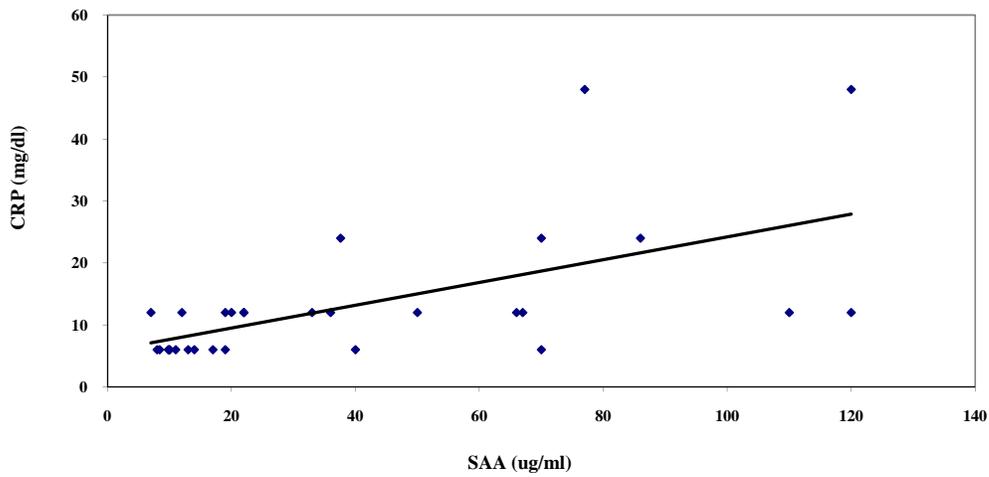**Fig. 2:** Correlation between SAA (ug/ml) and I: T ratio among cases.

Fig. 3: Correlation between SAA (ug/ml) and CRP (mg/dl) among cases.

Discussion:

Neonatal sepsis with its high mortality rate, still remains a diagnostic and treatment challenge for the neonatal health providers. Therefore, the need persists for improved diagnostic indicators of neonatal sepsis *(Sucilathangam et al, 2012)*.

C- reactive protein has been the most analyzed parameter for the detection of bacterial infection for years. It increases 12 to 24 hours after the onset of infection, it is usually used in combination with other markers as procalcitonin and SAA. There are conflicting results about their superiority to each other in diagnosis of neonatal sepsis *(Cetinkaya et al, 2009)*.

This study showed that the mean level of the SAA protein in neonates with late-onset sepsis was significantly higher at time of presentation compared to the control group (P value < 0.01), which indicates that SAA was elevated in septic cases. This finding was concordant with literature. *Malle and De Beer, (1996)* found that SAA levels show an early increase (even as much as 2 days before the onset of clinical signs), reach a peak and then, in absence of inflammatory stimuli, revert to normal in a space of few days. *Arnon et al (2005)* reported that SAA could be used as a reliable marker for early detection of LOS in preterm infants. They established that SAA had higher levels, and rose earlier and sharper than CRP in those neonates. *Cetinkaya et al, (2009),* found that inspite SAA protein increased in septic neonates at onset of sepsis, in comparison with CRP, yet that increase was insignificant. These data were in contrast to the results of Ucar *et al, (14)*, who found that the production of SAA may not be adequately stimulated in newborns due to defects in IL-1β production.

SAA has inhibitory effects on inflammation by reducing the production of prostaglandins E2 and the oxidative respiration of neutrophils, counteracting the pyrogenic effect of a number of cytokines and inhibiting platelet activation *(Pizzini et al, 2000)*.

In the present work, SAA levels were still high 72 hours after treatment initiation. This result was in contrast to that of *Arnon et al (2005) and Cetinkaya et al,(2009),* who showed that SAA level, returned faster to normal (at 24 hours, 48 hours respectively) in response to treatment.

By comparing the SAA level in the subgroups it was highly elevated in the Gram negative group compared to the Gram positive group (P value < 0.01). This finding is in agreement with *Ray and Ray (1997) and Arnon et al, (2002),* who found that Gram negative sepsis produced a more pronounced elevation of SAA levels than what occurred with Gram positive sepsis. Ray and Ray *(1997),* explained this elevation by the direct lipopolysaccharide (LPS) induction of the transcription factors by the Gram negative organisms that lead to SAA synthesis. *Shah et al (2006),* demonstrated that SAA binds to many Gram negative bacteria through an outer membrane protein A (Omp A); which is found across almost all Gram negative bacteria. They established the concept that SAA act as an innate immune protein that has the ability to opsonize Gram negative bacteria and enhance neutrophil respiratory burst and macrophage tumor necrosis α (TNFα) and interleukin 10 (IL-10) productions. This finding can be very helpful in clinical practice since elevated SAA protein levels can be used to initiate wide spectrum antibiotic therapy for suspected cases of LOS.

The present work showed that there was a positive correlation between the elevated SAA level in septic neonates and the risk factors for LOS; low gestational age, LBW, low Apgar score, and in those who were ventilated (P value < 0.01).

To compare the diagnostic validity of SAA and routine markers for LOS (TLC, I: T ratio, and CRP). ROC curves were performed for markers of sepsis. They showed that SAA was the most sensitive marker (86 %) for sepsis compared to TLC (80%), I: T ratio (80%),CRP (60%).This result came in agreement with that of *Arnon et al, (2005),* who showed that SAA had significantly largest AUC compared to CRP at zero hour of sepsis evaluation. Contrary to our results, *Cetinkaya et al, (2011)* study on newborns with necrotizing enterocolitis , found no significant difference with respect to the areas under the curve for SAA and CRP at all times of measurements.

As regards specificity, I:T ratio was the most specific septic marker compared to SAA, CRP, and TLC . These findings disagree with those of <u>*Walliullah et al, (2009)*</u>, who showed low specificity of I/T ratio (56%) for the diagnosis of sepsis. This great difference could be explained by individual variation in counting band cells and variation in number of patients. The positive predictive value (PPV) of SAA in this study was higher than that of CRP, and TLC, but lower than that of the I: T ratio. These results came in agreement with *Arnon et al, (12),* who showed a high PPV of SAA compared to that of CRP (86%) and IL-6 (64%).

However, SAA gave the highest negative predictive value (NPV) in comparison to NPV of I: T ratio, TLC, and CRP. In concordance to the study of *Arnon et al, (12)*, the NPV of SAA was 97%, which was greater than that of CRP (74%), and IL-6 (88%). On contrary, *Cetinkaya et al (2009),*found no significant differences between CRP and SAA in terms of AUC, PPV, NPV and specificity. Negative predictive value is helpful in rulling out the possibility of LOS in neonates.

By studying the correlations of SAA with other markers it was observed that a highly significant positive correlation was found between SAA and CRP and I: T ratio. This was in agreement with *Cetinkaya et al (2009).*Conversely, *Arnon et al, (2005)* showed no correlation between SAA and CRP.

The mortality rate was significantly higher in the Gram negative group (P value < 0.01) compared to neonates with gram positive sepsis, which is a fact previously confirmed in the literature *(Graham et al, 2006)*.

In the present work, the level of SAA among non-survivors was significantly higher compared to that of the survivors (p<0.01). The stimulation of SAA production promotes macrophage TNFα (pro-inflammatory) and IL-10 (Anti-inflammatory) production, however, overproduction of these mediators can result in tissue destruction and thus leads to poor prognosis and increased mortality *(Shah et al,2006)*.

In conclusion: SAA is a reliable marker for early diagnosis and follow up of late onset sepsis in neonates. It is more sensitive than CRP, TLC and I:T ratio in detecting LOS cases.I:T ratio can still be employed as a useful test to distinguish the infected from non infected neonates as it has a high specificity and a high positive predictive value compared to other markers

Therefore, the combining use of SAA and I:T ratio will enhance the diagnosis accuracy of both markers in neonatal LOS.

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