Serum Amyloid A: Expression Throughout Human Ovarian Folliculogenesis and Levels in Follicular Fluid of Women Undergoing Controlled Ovarian Stimulation

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Background: Serum amyloid A (SAA) is an acute phase protein expressed primarily in the liver in response to various injuries and inflammatory stimuli and is recognized as a modulator of inflammation. Ovarian reproductive functions including folliculogenesis and ovulation use inflammatory processes; thus, studying SAA in this context is of interest.

Objectives: We investigated the expression and localization of SAA in ovarian developing follicles and its levels in follicular fluids.

Methods and Participants: Nonradioactive in situ hybridization and immunohistochemical staining were applied on ovarian paraffin tissue sections. ELISA and RT-PCR were applied on follicular aspirates and blood samples from women undergoing controlled ovarian stimulation for in vitro fertilization.

Results: Expression of SAA mRNA and protein was found in follicular cells at all stages of follicular development, from primordial and primary follicles through antral follicles and corpora lutea. Expression was observed in granulosa, theca and luteal cells, and oocytes. Expression of SAA was also found in granulosa cells recovered from follicular aspirates. The SAA protein was detected in follicular fluids. Its levels were somewhat lower than in peripheral blood with strong correlation between the two compartments and with significant correlation with patient’s body mass index. High follicular fluid SAA levels were associated with reduced pregnancy rate.

Conclusions: SAA is locally produced in ovarian developing follicles and is a constituent of follicular fluids, suggesting its role within the follicular environment. Elevated follicular SAA levels are associated with decreased pregnancy rate and may signify lower reproductive performance. (J Clin Endocrinol Metab 98: 4970–4978, 2013)

Serum amyloid A (SAA) is an acute phase protein whose level in the blood is elevated up to 1000-fold in response to various inflammatory stimuli, including tissue injury, infection, and neoplasia (1). The liver is the major site of SAA production; however, extrahepatic expression was described in the epithelium of normal tissues (2), in adipose tissues (3), in diseased tissues such as atherosclerotic lesions (4), and in various carcinomas including ovarian carcinoma (5, 6). The role of SAA in health and diseases is not well understood. In addition to its pathogenic role as the precursor protein in amyloid A-type amyloidosis and a role in cholesterol metabolism and transport,
being a high-density lipoprotein–associated apolipoprotein, SAA is viewed as a modulator of inflammatory processes (1). Its activities related to inflammation include inducing adhesion, migration, and tissue infiltration of leukocytes (7); enhancing production and activity of matrix-degrading enzymes metalloproteinases (8), and plasminogen activators (9); stimulating production of inflammatory cytokines: TNF-α, IL-1β, IL-8, IL-6 (10, 11); and stimulating angiogenesis, activation of inflammation-associated nuclear factor κB and MAPK signaling pathways (12).

Ovarian reproductive functions ie, folliculogenesis, ovulation, and corpus luteum formation are viewed as inflammatory processes (13). Folliculogenesis is the development of ovarian follicles from the primordial stage through a series of morphologically defined stages: primary, secondary, and antral follicles, culminating in pre-ovulatory follicle and formation of corpus luteum and corpus albicans. Follicular development involves cell recruitment and proliferation, angiogenesis, tissue remodeling, and steroid production, regulated by pituitary-derived gonadotropins and by locally produced growth factors and cytokines (14). Many of the regulatory proteins have been detected in follicular fluids surrounding developing oocytes and are thought to play a role in determining oocyte quality and subsequent fertilization and embryo development (15). Growth factors and cytokines found in follicular fluids of women undergoing in vitro fertilization (IVF) were suggested as biomarkers predicting IVF outcome (16, 17).

In view of the function of SAA as a modulator of inflammation, we sought to study its possible involvement in female reproduction. As a first step, we investigated the local expression of SAA in human ovarian follicles throughout folliculogenesis and its levels in follicular fluids of women undergoing controlled ovarian stimulation.

### Materials and Methods

**Ovarian tissues, follicular fluids, and blood samples**

Conventional serial sections of paraffin-embedded archival normal ovarian tissues from 12 women (age 26–52 years), operated for various diagnoses (uterus myomatosus, n = 8; prophylactic oophorectomy, n = 2; ovarian benign tumors, n = 2) between 1993 and 2003, were obtained from the Department of Pathology. Follicular aspirates were collected from 123 healthy normally cycling women (age 19–45 years) who underwent egg retrieval for IVF at the institutional IVF Unit, between 2008 and 2010. Patient and cycle characteristics are detailed in Table 1. Controlled ovarian stimulation and collection of follicular aspirates were performed as described (18, 19). Blood samples were drawn from 25 IVF patients at the time of follicular aspiration, and plasma was frozen until assayed. Informed consents were obtained for the use of follicular aspirate and blood samples.
Studies were approved by the Human Subjects Research Committee of the Hadassah-Hebrew University Medical Center.

Isolation of granulosa cells
Granulosa cells were isolated from follicular aspirates as described (18, 19). Briefly, after oocyte retrieval, follicular aspirates of each patient were pooled (aspirates with blood contamination were excluded) and centrifuged. The resulting supernatant was frozen in aliquots until assayed. The cell pellet was resuspended in PBS, and suspension was layered onto Ficoll-Paque Plus (GE Healthcare Bio-Sciences) and centrifuged at 500g for 30 minutes. Granulosa cells were recovered from the interface, washed with PBS, and kept frozen until analyzed.

In situ hybridization
Nonradioactive in situ hybridization for SAA mRNA expression was performed as described (5, 6). Briefly, SAA probe was prepared from pGEM transcription vector that contained a 110-bp sequence of mouse SAA1 cDNA (p125). This nucleotide sequence is 81% homologous with human SAA1 and SAA2 and 71% homologous with human SAA4 and therefore should hybridize with SAA1 and SAA2 but may not hybridize efficiently with SAA4 (4). The p125 was linearized with HindIII (antisense) or EcoRI (sense) and incubated in a transcription reaction containing digoxigenin-labeled UTP (Boehringer-Mannheim). A nonlabeled antisense probe was also generated using UTP instead of digoxigenin-labeled UTP. The antisense probe and the control probes (the sense probe and the antisense probe mixed with a 20-fold excess of nonlabeled antisense probe) were applied on parallel tissue sections and in situ hybridization was performed. Hybridization with the control probes resulted in substantially diminished signal.

Immunohistochemistry
Immunohistochemistry for SAA protein expression was performed using the Histostain-Plus SP kit (Zymed Laboratories Inc) as described (5, 6). Briefly, two anti-SAA monoclonal antibodies were used: clone mcl (DAKO Corporation) and clone mc29 (AmYmed). The antibody mc1 detects SAA1 and SAA2; mc29 detects SAA1, SAA2, and SAA4. The antibodies’ specificity was described (20, 21). Antibodies were diluted 1:20 (mc1) and 1:600 (mc29) in 0.1M Tris-HCl pH 7.6 and incubated 2 hours at room temperature. The two antibodies yielded similar staining pattern. For negative control, primary antibodies were replaced by normal mouse isotype–matched immunoglobulin G (IgG2a, κ; DAKO Corp), resulting in substantially diminished signal.

Quantification of the SAA protein
SAA concentrations in follicular fluids and in blood-plasma samples were determined using SAA-specific ELISA (BioSource International Inc). This ELISA detects SAA1 and SAA2. Isolated granulosa cells were subjected to protein extraction using RIPA lysis buffer containing protease and phosphatase inhibitors (UP-STATE). Total protein of granulosa cell extracts was determined using a modified Lowry protein assay (Sigma Aldrich), and SAA concentrations in these extracts were determined by the same ELISA.

RT-PCR
RNA was extracted from isolated granulosa cells using Tri Reagent (Sigma), and cDNA was amplified using primers specific for the human SAA genes: SAA1, SAA2, SAA4, and the control β-actin, as described (5, 6). Relative levels of SAA expression were measured by TaqMan real-time PCR. Commercially available primers (Applied Biosystems Life Technologies) were as follows: SAA1/2 (no. Hs00761940_s1, because of the high homology between SAA1 and SAA2, primers were designed to detect both genes), SAA4 (no. Hs01050250_m1), and internal control h-GUSB (glucuronidase β, no. Hs00939627_m1). Amplification was performed on 7900HT Real-Time PCR System (Applied Biosystems). Threshold cycle (Ct) values were normalized to h-GUSB (ΔCt), and relative gene expression was calculated by the comparative Ct method (2−ΔΔCt method) (22).

Statistical analysis
Comparison of patient and IVF parameters by levels of SAA was performed using Student’s t test, Wilcoxon rank sum test, and χ² test, as appropriate (Table 1). Correlations between concentrations of SAA in follicular fluids and those in blood and granulosa cells were calculated by linear regression analysis. One-way ANOVA was performed to find overall mean differences between three body mass index (BMI) levels and post-hoc multiple comparisons using Tukey’s method (Figure 3D). Analysis was conducted using SAS software version 9.1 (SAS Institute Inc). All tests were two-sided, and P values ≤.05 were considered significant. Statistical analysis was not applied on the in situ hybridization, immunohistochemistry, and RT-PCR results, as they were mostly descriptive not quantitative.

Results
Expression of SAA mRNA in ovarian follicles
SAA mRNA expression in ovarian follicles was studied by nonradioactive in situ hybridization applied on ovarian tissue sections. Follicles were classified as described (23). Staining was observed in granulosa cells of primordial, primary, and secondary follicles. In secondary follicles, the theca cell layer when apparent stained as well. Staining of the oocyte cytoplasm was also evident (Figure 1, A–D). In tertiary (antral) follicles, staining was observed in granulosa and theca ( interna and externa) cell layers. In mature antral follicles (Graafian), the cumulus granulosa cells, when preserved, showed weaker staining compared with mural granulosa cells. The follicular basal lamina (basement membrane) and the follicular fluid within the antrum did not stain for SAA mRNA, as they represent noncellular components. Staining was also observed in the oocyte (Figure 1, E and F). In corpora lutea, granulosa and thecalutein cells stained positively, whereas sprouting capillaries did not stain (Figure 1, G and H). In corpora albicantia, the densely packed collagen fibers (the fibrous scar) did not stain for SAA mRNA, as expected, whereas occasional admixed fibroblasts stained positively (Figure II). Staining of various intensities was observed in ovar-
within antral cavities (Figure 2E). In corpora lutea, SAA was immuno-detected in granulosa and theca-lutein cells, whereas sprouting capillaries did not stain (Figure 2F). In corpora albicantia, the densely packed collagen fibers stained less intensively and diffusely (Figure 2G). Staining of various intensities was observed in ovarian stroma throughout the ovary.

Levels of SAA protein in follicular fluids of IVF patients

We next examined follicular fluids of IVF patients (n = 123) by SAA-specific ELISA. SAA was detected in all follicular fluids studied. Its concentrations varied among patients, ranging between 1 and 102 µg/mL follicular fluid. Of the 123 patients, 66 patients (53%) had high SAA concentrations (≥15 µg/mL), namely higher than SAA concentration in the blood of healthy individuals. Of these 66 patients, 50 patients had slightly elevated SAA (range 15–49 µg/mL, mean 26.1 ± 1.3 µg/mL) and 16 patients had highly elevated SAA (range 50–102 µg/mL, mean 66.6 ± 4.0 µg/mL).

The following analyses were performed on follicular fluids chosen at random from the 123-patient group.

Levels of SAA concentrations in follicular fluids vs matched blood samples

Levels of SAA in follicular fluids of 25 patients were compared to levels in their peripheral blood (collected at the time of follicular aspiration). SAA levels in both compartments varied between patients, ranging between 1 and 102 µg/mL for follicular fluid and 4 and 130 µg/mL for blood (Figure 3A). Strong positive linear correlation (coefficient of determination $R^2 = 0.809$) was found between SAA levels in the two compartments (Figure 3B). The mean SAA concentration in follicular fluids was significantly lower than in blood, 27.4 ± 6 µg/mL and 43 ± 7.4 µg/mL, respectively ($P = .0001$) (Figure 3C). We divided the 25 patients into three groups according to BMI: <25 (normal weight), 25–29.9 (overweight), and ≥30 (obese) (Figure 3D). Overall P values for mean differences of SAA concentrations between the three BMI groups were 0.02.
Expression of SAA protein in granulosa cells

Granulosa cells recovered from follicular aspirates of an additional seven patients were subjected to RT-PCR using primers for the human SAA genes: SAA1, SAA2, SAA4, and β-actin, as control. Fragments of predicted sizes were amplified by the SAA1 and SAA4 primers. When the SAA2 primers were used, fragments were weak to barely detectable (Figure 5A). The identity of the SAA bands was confirmed by sequencing in previous studies (5, 6). RNA samples were also subjected to TaqMan quantitative real-time PCR. Primers for SAA1/SAA2 (detect SAA1 and 2) and SAA4 were used (Figure 5B). Together, RT-PCR analysis revealed expression of the SAA1 and SAA4 genes and minimal expression of SAA2. Expression intensity of the SAA genes varied between patients, consistent with variations seen in SAA protein expression.

High follicular fluid SAA levels are associated with reduced pregnancy rate

To look into a possible link between follicular fluid SAA levels and fertility potential, we initially divided the 123 patients into three groups according to SAA levels: <15 μg/mL (normal levels), 15–49 μg/mL (slightly elevated levels), and ≥50 μg/mL (highly elevated levels). Pregnancy rates in the normal and the slightly elevated SAA groups were comparable (43% and 42%), yet reduced by about 50% in the highly elevated SAA group (18.8%). Reproductive outcome parameters, other than pregnancy rate, were comparable in the three SAA groups (data not shown). Therefore, we continued our analysis by dividing the study cohort into two groups: SAA <50 μg/mL and SAA ≥50 μg/mL (Table 1). Pregnancy rate in the group with SAA ≥50 μg/mL was 56% lower than in the group with SAA <50 μg/mL (18.8% vs 43%, respectively). In the group with SAA ≥50 μg/mL, patient’s age and BMI were higher and estradiol level on
trigger day was lower. Other patient characteristics and reproductive parameters were comparable in the two SAA groups.

**Discussion**

We describe, for the first time, the expression of SAA in human ovarian follicles and its localization in cell subpopulations throughout the ovarian cycle. SAA expression was studied by nonradioactive in situ hybridization and immunohistochemistry, applied on ovarian tissue sections obtained from women with natural menstrual cycle. The SAA mRNA and protein were detected in follicular cells at all stages of folliculogenesis, from primordial to primary and secondary follicles through antral and mature (Graafian) follicles and corpora lutea. Expression was observed in granulosa, theca, and luteal cells as well as in oocytes. Local synthesis of SAA in ovarian follicular cells was further supported by studying preovulatory follicular aspirates from IVF patients. SAA protein was detected in granulosa cells isolated from follicular aspirates and RT-PCR analysis confirmed active transcription of the SAA genes in these cells. The data indicate local production of SAA in ovarian follicles both in natural cycle and during ovarian stimulation.

We also report, for the first time, the presence of SAA in the follicular fluid. SAA was detectable in follicular fluids of all 123 IVF patients studied. Sixty-six patients (53%) had higher than normal SAA concentrations (≥15 μg/mL). Of these 66 patients, 16 patients had SAA levels ≥50 μg/mL, levels which indicate persistent severe inflammatory activity (24). However, the increase in SAA concentrations in our patients is modest (up to 100 μg/mL), in contrast with levels in the blood during acute phase response or in pathological conditions, which can reach 1000 μg/mL and more (1). It is possible that lower SAA concentrations may be found in follicular fluids of patients with unstimulated spontaneous menstrual cycles, as reported for certain cytokines (25). Finally, high follicular SAA concentrations were associated with obesity, reinforcing the suggestion that obese patients have proinflammatory follicular environment (26).

Detecting SAA in the follicular fluid urged us to address whether follicular fluid SAA concentrations differ from

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Figure 3. Concentrations of SAA in follicular fluids vs matched blood samples. (A) SAA concentrations in follicular fluids (■) and matched blood samples (□) obtained from 25 IVF patients. (B) Strong positive linear correlation between SAA levels in the two compartments (coefficient of determination $R^2 = 0.809$). (C) The mean SAA concentration in follicular fluid is significantly lower than in blood (*, $P = .0001$). (D) SAA concentrations in follicular fluids (■) and matched blood samples (□) according to BMI ($n = 11, n = 8$, and $n = 6$ for BMI <25, 25–29.9, and ≥30, respectively). **, Overall $P$ values for mean differences (one-way ANOVA test) between the three BMI groups are 0.02 and 0.012 for follicular fluid and blood, respectively. $P$ values for multiple comparisons between BMI <25 and BMI ≥30 are 0.016 and 0.009 for follicular fluid and blood, respectively.
concentration in peripheral blood. We found that SAA concentrations in the two compartments are comparable (somewhat lower in follicular fluid) within the same range and strongly correlated. Such strong correlation suggests that SAA levels in the two compartments are closely and interdependently regulated. That is, factors inducing systemic SAA levels similarly affect follicular fluid SAA levels. As for blood SAA concentrations, of the 25 patients studied, 17 had SAA concentrations higher than normal (>) and the mean SAA concentration of the 25-patient group was about three times higher than normal (Figure 3C). The IVF patients studied were healthy with no known inflammatory diseases. Increased circulating SAA concentrations may be the consequence of hormonal treatment. In fact, controlled ovarian hyperstimulation is believed to induce inflammatory state as evidenced by a rise in C-reactive protein levels (27). Increased circulating SAA concentrations may be associated with obesity, as reported (28, 29). Indeed, we found that obese patients had significantly higher circulating SAA levels compared with normal weight patients.

Proteins found in follicular fluids are thought to originate from circulating plasma that enters extravascular spaces and follicular antrum and from ovarian local synthesis (15). In fact, the composition of follicular fluid is similar to serum with respect to low-molecular-weight plasma components, but proteins with sizes above 100 kDa are found at progressively lower concentrations than in plasma (30). As for SAA, although it is a small protein (~12 kDa) that can cross the blood-follicle barrier, in the plasma it circulates as a high-density lipoprotein–associated particle with high molecular weight (~200 kDa) (31). Therefore, as a large particle, a lower follicular concentration compared with plasma may be expected. In addition to circulating plasma as a source, local production of SAA in ovarian follicles, as we demonstrated, could also contribute to SAA concentrations in the follicular fluid. The relative contribution of each compartment is unknown at present.

The human SAA gene family comprises four genes, mainly expressed in the liver. SAA1 and SAA2 are viewed acute phase SAA (A-SAA) because they are induced (by inflammatory cytokines) during the acute phase response (32); SAA3 is a nonexpressed pseudogene (33), and SAA4 is viewed constitutive SAA (C-SAA) because low levels of its protein are constitutively expressed and it is induced...
minimally, if at all (34). These designations may differ in nonliver tissues. In ovarian carcinoma, SAA1 and SAA4 are both constitutively overexpressed (6). In adipose tissues, A-SAA (SAA1 and 2) is highly expressed during nonacute-phase conditions and in obesity contributes to the elevated plasma SAA levels (28). In mouse, unlike in human, SAA3 is an expressed gene, almost exclusively expressed in nonliver cells including granulosa cells (35).

Our staining procedures did not specify exactly which SAA gene is expressed in the different follicular cell types. However, expression of SAA1, SAA4, and low expression of SAA2 in granulosa cells (Figure 5) and in other nonliver cells ie, aortic smooth muscle cells (4), monocyte/macrophage cells (36), and ovarian cancer cells (6) suggest this expression pattern in all follicular cells. The ELISA we used detected the A-SAA proteins (SAA1 and SAA2). SAA1 is the predominant A-SAA in plasma. Because proteins in follicular fluid originate from plasma and from granulosa cells (15) and granulosa cells had minimal expression of SAA2, SAA1 is likely the predominant A-SAA also in follicular fluid. The levels of SAA4 protein have not been determined.

The role of SAA expressed in ovarian follicles is unknown at present. Considering the functions ascribed to SAA (mostly to SAA1) in other systems, it may affect in an autocrine and/or paracrine manner different aspects of follicular development. For example, SAA may enhance leukocyte recruitment, cytokine production, cell proliferation, and angiogenesis (7, 10–12), processes taking place during follicular growth and corpus luteum formation. It may enhance activity of matrix-degrading enzymes (8, 9), thereby enhancing the degradation and remodeling of follicular basal lamina and the extracellular matrix surrounding the follicle during follicular growth and rupture. It may have an effect on cell apoptosis (37), thus affecting follicular atresia and postovulatory regression. It may modulate cholesterol metabolism and transport (1), thereby influencing ovarian steroidogenesis and luteinization. Last, SAA expressed in oocytes may act as a paracrine factor regulating the development of surrounding somatic cells, as suggested for other oocyte-derived factors (38).

Is there a link between follicular fluid SAA concentration and fertility potential? We found that IVF patients with high SAA levels (≥50 μg/mL) have 56% reduction in pregnancy rate compared with patients with normal or slightly elevated SAA levels (<50 μg/mL). This difference was nearly significant (P = .06 two-sided χ² test; one-sided P = .03), probably due to the limited number of samples. SAA has been suggested as an inflammatory adipokine linking between increased adipose tissue mass, low-grade inflammation, and obesity-related metabolic complications (28). Hence, SAA may take part in the known association between obesity and infertility (39). However, obesity is not the only factor negatively affecting pregnancy rate in patients with high SAA as only 6 of the 16 patients (37.5%) were obese. Thus, SAA as an inflammatory mediator may decrease fertility potential independent of body weight. Older age may also contribute to lower pregnancy rate, yet in patients with high SAA only 6 of the 16 patients (37.5%) were over 40 years old, and the age by itself could not account for the reduced pregnancy rate. Because high follicular SAA levels correlated with high blood SAA levels (Figure 3B), others factors such as defective endometrial receptivity may contribute to lower pregnancy rate. Our findings that in patients with high SAA pregnancy rate was impaired but other reproductive parameters were not affected (Table 1) point to a possible alteration in the uterine environment in this cohort. Taken together, it is conceivable that patients with high SAA levels are exposed to inflammatory conditions that impede their pregnancy outcome. Concurring with this suggestion, another inflammatory indicator, C-reactive protein, was found at higher levels in IVF patients who failed to conceive (40).

In conclusion, our data indicate that SAA is locally produced in ovarian follicles and is a component of follicular fluids. Elevated follicular SAA levels were associated with obesity and with reduced pregnancy rate. Although our data need further validation in future studies, they support the hypothesis that SAA plays a role within the follicular environment and may serve as a marker whose elevated levels signify poorer reproductive potential.

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