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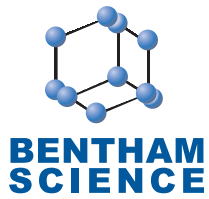

Structure and Expression of Different Serum Amyloid A (SAA) Variants and their Concentration-Dependent Functions During Host Insults

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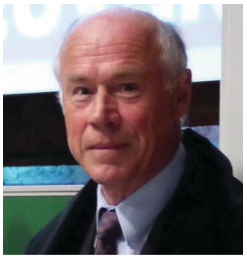

Jo Van Damme

Abstract: Serum amyloid A (SAA) is, like C-reactive protein (CRP), an acute phase protein and can be used as a diagnostic, prognostic or therapy follow-up marker for many diseases. Increases in serum levels of SAA are triggered by physical insults to the host, including infection, trauma, inflammatory reactions and cancer. The order of magnitude of increase in SAA levels varies considerably, from a 10- to 100-fold during limited inflammatory events to a 1000-fold increase during severe bacterial infections and acute exacerbations of chronic inflammatory diseases. This broad response range is reflected by SAA gene duplications resulting in a cluster encoding several SAA variants and by multiple biological functions of SAA. SAA variants are single-domain proteins with simple structures and few post-translational modifications. SAA1 and SAA2 are inducible by inflammatory cytokines, whereas SAA4 is constitutively produced. We review here the regulated expression of SAA in normal and transformed cells and compare its serum levels in various disease states. At low concentrations (10-100 ng/ml), early in an inflammatory response, SAA induces chemokines or matrix degrading enzymes *via* Toll-like receptors and functions as an activator and chemoattractant through a G protein-coupled receptor. When an infectious or inflammatory stimulus persists, the liver continues to produce more SAA ($\geq$ 1000 ng/ml) to become an antimicrobial agent by functioning as a direct opsonin of bacteria or by interference with virus infection of host cells. Thus, SAA regulates innate and adaptive immunity and this information may help to design better drugs to treat specific diseases.

Keywords: SAA variants, FPR2, TLR2, leukocytes, chemotaxis, cytokines, inflammatory diseases, amyloidosis.

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1. INTRODUCTION

The acute phase response is a highly orchestrated defense mechanism of vertebrates against infectious and inflammatory insults. The initiation of this response is predominantly driven by the endogenous cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, induced in macrophages and other leukocytes by exogenous (viral, bacterial) agents binding to toll-like receptors (TLR). Besides systemic and metabolic changes, such as fever and anorexia, these cytokines also provoke the induction of acute phase proteins in the liver. In humans, one of the major acute phase proteins is serum amyloid A (SAA). The concentration of this highly conserved acute phase protein increases 100- to 1000-fold, reaching maximal plasma concentrations superior to 1 mg/ml [1, 2]. Despite the fact that SAA has been studied extensively in the past, its exact role is still not fully understood. Recently, however, more evidence of the mechanisms by which SAA exerts its biological activities has been provided.

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In this review, knowledge about SAA structure/ function relationships is complemented with insights into the distinct regulation of extrahepatic *SAA* gene expression and the association between serum levels and physiological and pathological conditions is discussed. Furthermore, the various biological functions ascribed to SAA variants and the roles played by these molecules in chronic diseases are reviewed.

2. STRUCTURE OF HUMAN SERUM AMYLOID A (SAA)

2.1. Structure of the *SAA* Genes

In humans, four different genes encoding SAA1, SAA2, SAA3 and SAA4 are clustered in a segment spanning 150 Kb within the p15.1 region of chromosome 11 [3, 4]. The genes contain four exons and three introns [5-7], except for the *SAA3* gene, which has a three exon – two intron structure [8].

The *SAA1* and *SAA2* genes are located 15 to 20 Kb from each other [4]. These genes contain the allelic variants *SAA1α* (*SAA 1.1*), *SAA1β* (*SAA 1.2*), *SAA1γ* (*SAA 1.3*), *SAA2α* (*SAA 2.1*) and *SAA2β* (*SAA 2.2*) [9]. The nucleotide sequences of the human coding regions are shown in Fig. (**1**). *SAA2α* and *SAA2β* differ from *SAA1α* in 12 and 13 nucleotides, respectively. In contrast, the nucleotide sequences of *SAA2α* and *SAA2β* are essentially identical, except for a single-base substitution in codon 71 (adenine versus guanine, respectively, Fig. **1**). Since *SAA1* and *SAA2* are almost identical, they probably originate from gene duplication during evolution [10, 11]. Their promotor regions contain nuclear factor (NF)-κB (GGGACTTTCC) and NF-IL6 (AGGTTACACAACTG) transcription factor recognition sequences, allowing the induction by IL-1, IL-6 and TNF [12-14], as well as an SAA-activating sequence (SAS), which is a binding site for SAS-activating factor (SAF) [15].

The third gene, *SAA3*, is situated 110 Kb downstream of the *SAA4* gene. It was initially considered to be a pseudogene by Kluve-Beckerman *et al.* [16], who discovered that it contains a defective promotor and a single base insertion in codon 31, provoking a translation stop signal (TGA) at codon 43 (Fig. **1**). However, later on, Larson *et al.* [8] reported local transcription of the *SAA3* gene in mammary gland epithelial cells (*vide infra*).

The *SAA4* gene was identified a few years after the discovery of the *SAA1* and *SAA2* genes. It is located only 9 Kb downstream of the *SAA2* gene [4] and differs from the other *SAA* genes by not containing in its promotor region the sequence CTGGGA, typical for some acute phase proteins, nor an NF-IL6 binding site. Furthermore, the gene encloses only a truncated NF-κB recognition sequence (GACTTT), which is present at full length (GGGACTTTCC, *vide supra*) in other *SAA* genes [7]. The *SAA4* gene has eight extra codons between codon 69 and 70 in comparison with the nucleotide sequence of the *SAA1* and *SAA2* genes (Fig. **1**) [17].

2.2. Structure of SAA Proteins

Each of the *SAA* genes encodes a different protein: SAA1, SAA2, SAA3 and SAA4. SAA1α, SAA1β, SAA1γ, SAA2α and SAA2β originate from their corresponding allelic variants of the *SAA1* and *SAA2* genes. The amino acid sequences of the mature SAA isoforms are shown in Table **1**. All the precursor SAA forms contain a signal peptide of 18 amino acids in length, which is cleaved by the signal peptidase (docking enzyme) during secretion. The different (mature) SAA proteins were isolated from plasma of patients, undergoing an acute phase response, or identified from cDNA clones derived from human liver libraries. The mature SAA1 and SAA2 proteins are 104 amino acids in length and their sequences are for more than 90% identical to each other. Within SAA1, the variants differ in only a few amino acids: SAA1α contains valine (V) and alanine (A) at positions 52 and 57, respectively, whereas SAA1β is characterized by alanine (A) at position 52 and valine (V) at position 57 [20]. Both positions 52 and 57 are occupied by alanine (A) in SAA1γ [21]. Beach *et al.* [22] described the same SAA1β variant as Parmelee *et al.* [20], but reported aspartic acid (D) at position 72 instead of glycine (G), as was already observed by Kluve-Beckerman *et al.* in a family of Turkish origin [23]. Since the other SAA1 and SAA2 variants all contain glycine (G) at position 72, the SAA1β protein described by Parmelee and coworkers was adopted. The two allelic variants of SAA2 are identical, except for the substitution of histidine (H) for arginine (R) at position 71 in SAA2β, provoked by the single base mutation in the corresponding codon in the gene structure. SAA2α and SAA2β have seven and eight amino acid substitutions, compared with SAA1α, resulting from the 12 and 13 nucleotide differences in the *α*- and *β*-allelic variants of the *SAA2* gene. Besides the same two amino acid substitutions at positions 52 and 57 as in SAA1β, SAA2α and -β both contain the following amino acid substitutions: aspartic acid (D) for asparagine (N) at position 60, phenylalanine (F) for leucine (L) and threonine (T) at positions 68 and 69, respectively, glutamic acid (E) for lysine (K) at posi-

```
         -18                                -10
SAA1α    ATG AAG CTT CTC ACG GCC CTG GTT TTC TGC TCC TTG GTC CTG GGT GTC AGC
SAA1ß
SAA1γ
SAA2α                                                          A
SAA2ß                                                          A
SAA3     ATG AAG CTC TCC ACT GGC ATC ATT TTC TGC TCC CTG GTC CTG GGT GTC AGC
SAA4     ATG AGG CTT TTC ACA GGC ATT GTT TTC TGC TCC TTG GTC ATG GGA GTC ACC

         -1   +1                                10
SAA1α    AGC CGA AGC TTC TTT TCG TTC CTT GGC GAG GCT TTT GAT GGG GCT CGG GAC
SAA1ß
SAA1γ
SAA2α
SAA2ß
SAA3     AGC CAA GGA TGG TTA ACA TTC CTC AAG GCA GCT GGC CAA GGG ACT AAA GAC
SAA4     AGT GAA AGC TGG CGT TCG TTT TTC AAG GAG GCT CTC CAA GGG GTT GGG GAC

                  20                                      30
SAA1α    ATG TGG AGA GCC TAC TCT GAC ATG AGA GAA GCC AAT TAC ATC GGC TCA GAC
SAA1ß
SAA1γ
SAA2α
SAA2ß
SAA3     ATG TGG AAA GCC TAC TCT GAC ATG AAA GAA GCC AAT TAC AAA AAA TTC AGA
SAA4     ATG GGC AGA GCC TAT TGG GAC ATA ATG ATA TCC AAT CAC CAA AAT TCA AAC

                               40                                        50
SAA1α    AAA TAC TTC CAT GCT CGG GGG AAC TAT GAT GCT GCC AAA AGG GGA CCT GGG
SAA1ß
SAA1γ
SAA2α
SAA2ß
SAA3     CAA ATA CTT CCA TGC TTG GGG GAA CTA TGA
SAA4     AGA TAT CTC TAT GCT CGG GGA AAC TAT GAT GCT GCC CAA AGA GGA CCT GGG

             52      54          57          60
SAA1α    GGT GTC TGG GCT GCA GAA GCG ATC AGC GAT GCC AGA GAG AAT ATC CAG AGA
SAA1ß         C                   T
SAA1γ         C
SAA2α         C         C         T          A
SAA2ß         C         C         T          A
SAA3
SAA4     GGT GTC TGG GCT GCT AAA CTC ATC AGC CGT TCC AGG GTC TAT CTT CAG GGA

         68  69  70  71                        77  78      80              84
SAA1α    TTC TTT GGC CAT GGT GCG GAG GAC TCG CTG GCT GAT CAG GCT GCC AAT GAA
SAA1ß
SAA1γ
SAA2α    C   ACA                                   C                     A
SAA2ß    C   ACA      G                            C                     A
SAA3
SAA4     TTA ATA GAC TAC TAT TTA TTT GGA AAC AGC AGC ACT GTA TTG GAG GAC TCG


                            90                                      100
SAA1α    TGG GGC AGG AGT GGC AAA GAC CCC AAT CAC TTC CGA CCT GCT GGC CTG CCT
SAA1ß
SAA1γ
SAA2α                        G
SAA2ß                        G
SAA3
SAA4     AAG TCC AAC GAG AAA GCT GAG GAA TGG GGC CGG AGT GGC AAA GAC CCC GAC

                 104                     110
SAA1α    GAG AAA TAC TGA
SAA1ß
SAA1γ
SAA2α
SAA2ß
SAA3
SAA4     CGC TTC AGA CCT GAC GGC CTG CCT AAG AAA TAC TGA
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Fig. (1). Nucleotide sequences of the coding regions from the human *SAA* genes.

(Legend Fig. 1) contd….

Nucleotide sequences of the coding regions from *SAA1α* [18], *-β* [19] and *-γ* [19], *SAA2α* [18] and *-β* [18], *SAA3* [16] and *SAA4* [7], arranged in codons, are shown. Identical nucleotides with *SAA1α* are not shown in the sequences of the other *SAA1* and *SAA2* genes. The sequence coding for the signal peptide (18 amino acids) is included. The stop codon (TGA) is marked in bold. Because of the single base insertion in the *SAA3* gene, the stop signal already occurs in codon 43. Nucleotides in *SAA3* and *SAA4* differing from *SAA1α* are underlined. Compared to the *SAA1α* gene sequence, a single base insertion in codon 31 and an insertion of eight codons (codons 70-77) are present in the *SAA3* and *SAA4* genes, respectively. Exon boundaries are situated in between the shaded nucleotides. The exon boundaries within the *SAA1β* and *-γ* and *SAA2α* and *-β* genes are identical to those of the *SAA1α* gene.

Table 1. Protein sequences of the mature human SAA variants.

Form	AA	AA sequence[a]	References
SAA1α	**19-122**	**RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGGV WAAEAISDAR ENIQRFFGHG AEDSLADQAA NEWGRSGKDP NHFRPAGLPE KY**	[9, 18, 20, 24, 39]
SAA1β	**19-122**	**RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGGA WAAEVISDAR ENIQRFFGHG AEDSLADQAA NEWGRSGKDP NHFRPAGLPE KY**	[20]
SAA1γ	**19-122**	**RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGGA WAAEAISDAR ENIQRFFGHG AEDSLADQAA NEWGRSGKDP NHFRPAGLPE KY**	[21]
SAA2α	**19-122**	**RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGGA WAAEVISNAR ENIQRLTGHG AEDSLADQAA NKWGRSGRDP NHFRPAGLPE KY**	[9, 18, 24]
SAA2β	**19-122**	**RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGGA WAAEVISNAR ENIQRLTGRG AEDSLADQAA NKWGRSGRDP NHFRPAGLPE KY**	[5, 9, 18, 24]
SAA3	**19-60**	**QG WLTFLKAAGQ GTKDMWKAYS DMKEANYKKF RQILPCLGEL**	[8]
SAA4	**19-130**	**ES WRSFFKEALQ GVGDMGRAYW DIMISNHQNS NRYLYARGNY DAAQRGPGGV WAAKLISRSR VYLQGLIDYY LFGNSSTVLE DSKSNEKAEE WGRSGKDPDR FRPDGLPKKY**	[17]

[a]Amino acids (AA) different from those of SAA1α are shaded. The inserted octapeptide in SAA4 is underlined.

tion 84 and lysine (K) for arginine (R) at position 90. SAA2β also contains arginine (R) instead of histidine (H) at position 71. SAA1 and SAA2 isoforms lacking their amino terminal arginine (R) and/or serine (S), co-existing with the full length mature proteins, are described [9, 18, 21, 22, 24]. These processed forms originate from proteolytic cleavage of the proteins, which occurs mainly extracellularly [9, 18].

SAA3 was initially thought to be not expressed at all, since its encoding gene was believed to be a pseudogene (*vide supra*). Nonetheless, Larson *et al.* [8] detected SAA3 mRNA in two human mammary gland epithelial cell lines, coding for a predicted 42 amino acid polypeptide. This predicted peptide is only 40% identical to the related part of SAA1 and SAA2. In fact, the single base insertion in codon 31 of the *SAA3* gene (Fig. **1**) leads to a frame shift in the translation of the protein, modifying thereby practically all of the last ten amino acids of SAA3. A decade later, Tomita *et al.* [25] reported for the first time the detection of the protein *via* a specific sandwich ELISA in conditioned media from one of the human mammary gland epithelial cell lines, previously used by Larson *et al.* [8]. Indeed, SAA3 is the main SAA form being expressed extrahepatically in non-human mammals [26]. Considerable levels of full length SAA3 were already detected in bovine, equine and ovine colostrum [27].

The mature SAA4 protein consists of 112 amino acids, since the insertion of eight codons between codons 69 and 70, when compared to the nucleotide sequences of *SAA1* and *SAA2*, gives rise to an additional eight amino acids between the 69th and 70th position [17]. Only 52% of the amino acid sequence of SAA4 is identical to that of SAA1α. This protein is constitutively present in the blood and differs thereby from SAA1 and SAA2, which are mainly induced when inflammation occurs [17]. Therefore, SAA4 is also denominated "constitutive SAA" or "C-SAA", whereas SAA1 and

SAA2 are named "acute phase SAA" or "A-SAA". The last two amino acids from the inserted octapeptide in SAA4 are part of an N-linked glycosylation site, consisting of the sequon asparagine (N) – serine (S) – serine (S) [17]. This tripeptide is responsible for the coexistence of unglycosylated (14 kDa) and glycosylated (19 kDa) forms of SAA4 in serum.

When secreted into the blood circulation, SAA associates with high density lipoprotein (HDL), predominantly with the HDL_3 fraction, displacing thereby apolipoprotein (Apo) A-I, which is the major component of HDL in the blood circulation [28-30]. During the acute phase response, acute phase SAA (*i.e.* SAA1 and SAA2) thus becomes the major apolipoprotein associated with HDL. Although a minority of the SAA1 and SAA2 proteins also binds to low density lipoprotein (LDL) and very low density lipoprotein (VLDL) [28], conflicting data exist about the association of SAA4 with lipoproteins. For instance, SAA4 binds to HDL and VLDL, but whether it associates with LDL is not clear [30-32].

Initially, Benditt *et al.* [33] and Betts *et al.* [34] described the formation of two α-helices within the SAA protein. However, recent findings by Lu *et al.* [35] indicate that SAA1α forms four antiparallel α-helices and that six SAA1α molecules together form a hexamer that is stable in solution. In SAA1α, these α-helices span residues 1-27, 32-47, 50-69 and 73-88 of the mature protein, respectively. The C-terminal loop of SAA1α (spanning residues 89-104) forms strong salt bridges and hydrogen bonds with the α-helices and is important for the stability of the four-helix bundle. The authors also defined two positively charged binding sites for glycosaminoglycans (GAGs) in A-SAA, specifically for heparin and heparan sulphate. The first of these two GAG-binding regions is formed by three arginines (R) at positions 15, 19 and 47 of the mature SAA protein. The second GAG-binding site consists of arginine (R) at positions 1 and 62 and of histidine (H) at residue 71. One of these two regions is a shared binding site for GAGs and HDL, although the exact HDL-binding site(s) in SAA is/are not yet determined. This suggests that, at sites of inflammation, SAA dissociates from HDL by competition with GAGs [35]. More binding sites are defined by other authors. Turnell *et al.* [36] delineated the first 11 amino terminal residues of A-SAA as a lipid binding site and the glycine (G) – proline (P) – glycine (G) – glycine (G) region at residues 48-51 as a calcium binding sequence. This calcium binding site is conserved in all the SAA variants. Between amino acid residues 39 and 41, all SAA molecules also contain an arginine (R) – glycine (G) – asparagine (N) sequence, which is similar to a cell binding domain [arginine (R) – glycine (G) – aspartic acid (D)] of the extracellular matrix protein fibronectin [37]. Likewise, SAA1 and SAA2 have an amino acid sequence [tyrosine (Y) – isoleucine (I) – glycine (G) – serine (S) – aspartic acid (D)] between residues 29 and 33, comparable to that of the cell binding site of laminin [tyrosine (Y) – isoleucine (I) – glycine (G) – serine (S) – arginine (R)], which is also an extracellular matrix protein. Recently, amino acids 86-104 and particularly the proline (P) residues were found to be important for binding of cystatin C to A-SAA [38]. No data are yet available about receptor-binding sites in the SAA proteins.

3. PRODUCTION OF HUMAN SAA VARIANTS

3.1. Expression of SAA Variants in Various Tissues

Like for the other acute phase proteins, the major site of synthesis of SAA is the liver [37, 40-43]. Indeed, cultured hepatocytes were shown to effectively express SAA mRNA or protein [42-50]. The synthesis of human A-SAA in the liver is reviewed by De Buck *et al.* [51] and is principally induced by the cytokines IL-1β, IL-6 and TNF-α. These cytokines can synergize with each other or with glucocorticoids to greatly potentiate A-SAA production. However, not all authors agree with the liver being the foremost site of SAA synthesis. Sjöholm *et al.* [52] and Yang *et al.* [53] pointed out that adipocytes from obese individuals, not suffering from any other disease, expressed more A-SAA mRNA than hepatocytes.

Extrahepatic expression of SAA was reported in different species, including mice, rabbits and minks [26]. In humans, extrahepatic expression of SAA was also shown, but mostly at the mRNA level (Tables **2-5**). The expression of mRNA and/or protein of SAA (not specified which SAA types expressed) and specific expression of SAA1, SAA2 (A-SAA) and/or SAA4 (C-SAA) was demonstrated in various histologically normal human tissues, predominantly in epithelium (Table **2**) [42, 43, 46, 54-56]. Moreover, SAA was expressed in lymphocytes and plasma cells of many normal tissues and in lymphoid follicles [43]. Likewise, both A-SAA and/or C-SAA were at least transcribed in certain non-stimulated cells or cell lines (Table **3**) [45, 48, 52, 55-60]. Conflicting data are nevertheless published, reporting that A-SAA was not detected neither at the mRNA, nor at the protein level in normal brain, stomach, small intestine, colon, heart, kidney, lung, prostate, skeletal muscle, spinal cord, spleen, testis, uterus and

Table 2. Extrahepatic production of SAA variants in histologically normal tissues.

Tissue	SAA[a]		A-SAA[b]		SAA1		SAA2		SAA3		SAA4 (C-SAA)		References
	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	
Adipocytes			+	+									[53]
Brain	+		-	+									[43, 52]
Breast	+	+			+	+	+	+	-	-	+	+	[42, 43]
Colon			-	+	+		-		-		+		[52, 54]
Esophagus					+		+		-		+		[43]
Heart			-										[52]
Kidney	+		-	+	+	-	+		-		+		[42, 43, 52, 62]
Large intestine	+			+	+		+		-		+		[43]
Lung	+	+/-	-										[42, 43, 52, 70, 71]
Ovary					-						+		[56]
Pancreas	+			+									[43]
Pituitary gland	+	+			+		+						[43, 46]
Placenta	+			+									[43]
Prostate	+		-	+									[43, 52]
Sinonasal mucosa	+												[72]
Skeletal muscle			-										[52]
Skin	+			+									[43, 73]
Small intestine	+		-	+									[43, 52]
Spleen	+		-		+		+		-		+		[43, 52]
Stomach	+	+	-										[42, 43, 52]
Synovial membrane			+	+									[55]
Testis			-										[52]
Tonsil	+												[43]
Uterus			-	-									[52, 61]

[a]Not specified which SAA type produced
[b]A-SAA production without discrimination between SAA1 and SAA2
The presence or absence of SAA, A-SAA, SAA1, SAA2, SAA3 and/or SAA4 (C-SAA) mRNA and/or protein in non-hepatic tissues is indicated by + or -, respectively. Note that sometimes conflicting data, indicated by +/-, exist

macrophages (Table **2**) [52, 61, 62]. There are no reports showing detectable SAA3 protein expression in histologically normal and diseased tissues (Tables **2** and **4**) and in unstimulated cell types (Table **3**). It should be noted that SAA production is often observed in cancer (Tables **3** and **4**) and may be related to paraneoplastic dysregulated expression.

Local SAA1 and/or SAA2, but not SAA4 expression was found to be upregulated in diseased tissues or in cells stimulated by inflammatory mediators (Tables **4** and **5**) [42, 45, 54-57]. This is in accordance with the fact that SAA1 and SAA2 originate predominantly from induction by inflammatory stimuli and that SAA4 is not inducible [17]. Only two research groups quantitatively compared local SAA production between normal and diseased state. Firstly, O'Hara *et al.* [63] found a two- to four-fold increase in A-SAA protein expression in synovial cells from patients with rheumatoid

Table 3. Extrahepatic expression of SAA variants in non-stimulated cell types.

Cell type / cell line	SAA[a]		A-SAA[b]		SAA1		SAA2		SAA4 (C-SAA)		References
	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	
Aortic smooth muscle cells			+						+		[57]
Macrophages			-								[52]
U937 (monocytic cell line)			+						+		[48]
MG-63, Saos-2 (osteosarcoma)			+	+	-		-		+	+	[45]
OVCAR-3 (ovarian carcinoma)		-			+		+		+		[56]
RT4/31 (bladder papilloma)			+						+		[48]
SW13 (adrenal cortex carcinoma)			+						+		[48]
SW480, SW620 (colon adenocarcinoma)	+	+									[58, 59]
Jeg-3 (placental choriocarcinoma)			+	+							[46]
KB (oral epidermal carcinoma)			+		+		+		+		[48, 55, 60]

[a]Not specified which SAA type expressed
[b]A-SAA expression without discrimination between SAA1 and SAA2
The presence or absence of SAA, A-SAA, SAA1, SAA2 and/or SAA4 (C-SAA) mRNA and/or protein in non-hepatic cell types is indicated by + or -, respectively

arthritis (RA), compared to synoviocytes from healthy individuals. Secondly, Cocco *et al.* [61] stated that uterine serous papillary carcinoma cells produced low (11.2 ng/ml/10^5 cells after 72 h), but detectable amounts of SAA, whereas production by normal fibroblasts was ten-fold lower. A gradual increase in local SAA mRNA and protein expression was also noted in tumors upon progression to a more malign phenotype [54, 56, 62, 64]. Nonetheless, in a study of two tumor cell lines derived from the same patient with colon cancer, the highest SAA mRNA expression was detected in the primary tumor [59].

SAA4 mRNA or protein was expressed in all the tissues or cells, in which its presence has been studied [31, 43, 45, 48, 52, 54, 56, 57, 65-67] (Tables **2-5**), except in the brain from patients with Alzheimer's disease (Table **4**) [68]. In addition, Murphy *et al.* [69] reported a human case of SAA4 amyloidosis in the kidney, caused by a point mutation in the *SAA4* gene sequence, leading to substitution of glycine (G) for tryptophan (W) at position 22. Moreover, in the few studies conducted, SAA3 was not observed at the mRNA or the protein level in healthy or diseased tissues (Tables **2** and **4**) [43, 52, 54, 67]. Larson *et al.* [8] identified SAA3 mRNA in two mammary gland epithelial cell lines, stimulated with either prolactin or lipopolysaccharide (LPS). Later on, Tomita *et al.* [25] reported the detection of low quantities of SAA3 (231 pg/l) in conditioned media from the mammary gland epithelial cell line T47D stimulated with IL-1β, IL-6 and dexamethasone for three days (Table **5**). In contrast to humans, SAA3 is the major extrahepatically expressed SAA variant in other mammals [26] and locally produced SAA3 was even reported to be the etiologic agent of uterine amyloid A (AA) amyloidosis in end-term pregnant goats [87]. In pigs, SAA3 is the only SAA variant that has been isolated from extrahepatic and hepatic tissues thus far [88].

3.2. SAA Levels in Physiological and Pathological Conditions

It is frequently mentioned that the concentrations of acute phase reactants, including C-reactive protein (CRP) and A-SAA, increase up to 1000-fold during an inflammatory event, such as florid infections or severe burns. However, significant but smaller increases may also be relevant as diagnostic or prognostic markers for specific disease states. For this reason, a short overview of the quantitative studies with maximal information on SAA protein concentrations follows hereafter.

Table 4. Extrahepatic production of SAA variants in diseased tissues.

Tissue	Disease / inducer	SAA[a]		A-SAA[b]		SAA1		SAA2		SAA3		SAA4 (C-SAA)		References
		mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	
Adipocytes	obesity		+	+	+		+			-		+		[52, 53, 74]
	diabetes	+												[75]
Artery wall	atherosclerotic lesions	+					+		+				+	[31, 66]
Bone and bone marrow	OA					+		-				+		[45]
Brain	Alzheimer's disease		+	+								-		[68, 76-78]
	multiple sclerosis		+											[76]
	sarcoidosis		+											[71]
Cartilage	OA				+									[79]
Colon	neoplasia		+			+						+		[54]
Kidney	carcinoma	+				+	+							[62, 80]
	amyloidosis												+	[69]
Lung	COPD	+	+											[70, 81]
	sarcoidosis		+											[71]
	carcinoma		+											[42]
Ovary	neoplasia					+		-		-		+		[56]
Sinonasal mucosa	chronic rhinosinusitis	+												[72]
Skin	psoriasis	+	+											[73]
	sarcoidosis		+											[71]
Synovial fluid	RA / OA / PsA				+									[82, 83]
Synovial membrane	RA / OA / PsA / arthritis			+	+							+		[55, 63, 65, 79, 82]
Uterus	carcinoma	+	+											[61]

Abbreviations: COPD: chronic obstructive pulmonary disease, OA: osteoarthritis, PsA: psoriatic arthritis, RA: rheumatoid arthritis

[a] Not specified which SAA type produced

[b] A-SAA mRNA or protein production without discrimination between SAA1 and SAA2

The presence or absence of SAA, A-SAA, SAA1, SAA2, SAA3 and/or SAA4 (C-SAA) mRNA and/or protein in extrahepatic tissues is indicated by + or -, respectively.

Table 5. Extrahepatic production of SAA variants by various cell types, stimulated by (anti-)inflammatory mediators.

Cell type / cell line	Disease	Inducer	SAA[a]		A-SAA[b]		SAA1		SAA2		SAA3		SAA4 (C-SAA)		Ref.
			mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	
Aortic smooth muscle cells	n/a	IL-1β / IL-6 / TNF-α / IL-1β + IL-6 + TNF-α			-										[57]
	n/a	dex / cortico / aldo / IL-1β + dex			+	+									[57]
Cultured smooth muscle cells	n/a	dex / IL-1 + dex / IL-6 + dex					+		+				+		[66]

(Table 5) contd….

Cell type / cell line	Disease	Inducer	SAA[a]		A-SAA[b]		SAA1		SAA2		SAA3		SAA4 (C-SAA)		Ref.
			mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	
Multipotent adipose-derived stem cells	obesity	IL-1β + dex / TNF-α + dex / IL-1β + TNF-α + dex		+											[74]
Mesenchymal stem cells	arthritis	IL-1 / IL-6 / TNF-α					+		-				+		[45]
Osteoblast-like differentiated mesenchymal stem cells	arthritis	IL-1 / IL-6 / TNF-α					+		+				+		[45]
Primary chondrocytes	n/a	IL-1β			+										[79]
	OA	pred				+									[83]
	OA	IL-1β / IL-6 / TNF-α				-									[83]
Cultured synoviocytes	RA	IL-1β + dex			+								+		[65]
Synovial fibroblasts	arthritis	IL-1β / TNF-α			+										[55]
	OA	pred				+									[83]
	OA	IL-1β / IL-6 / TNF-α				-									[83]
MRC5 (fetal lung fibroblast)	n/a	dex / MoCM ± dex			+								+		[48]
ECV304 (umbilical cord endothelial cells)	n/a	MoCM ± dex / IL-1α / IL-1β / TNF-α / IL-1α ± IL-6 + dex / IL-1β ± IL-6 + dex / TNF-α ± IL-6 + dex / IL-1β + TNF-α + dex			+								+		[48]
Primary keratinocytes	n/a	IL-17A	+	+											[73]
Int-407 (small intestinal epithelium)	n/a	IL-1β + IL-6 + TNF-α + dex		+											[50]
RT4/31 (bladder papilloma)	n/a	MoCM ± dex			+								+		[48]

(Table 5) contd….

Cell type / cell line	Disease	Inducer	SAA[a]		A-SAA[b]		SAA1		SAA2		SAA3		SAA4 (C-SAA)		Ref.
			mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	
Caco-2 (colorectal adenocarcinoma)	n/a	IL-1β / dex / IL-1β + IL-6 ± dex / IL-1β + IL-6 + TNF-α ± dex / MoCM ± dex		+	+								+		[48, 50]
	n/a	IL-6 / TNF-α		-											[50]
HCT-8 (ileocecal carcinoma)	n/a	MoCM			+								+		[48]
Hela Ohio (cervical carcinoma)	n/a	MoCM ± dex			+								+		[48]
JAR (placental choriocarcinoma)	n/a	IL-1α ± dex / IL-1β ± dex / TNF-α			+	+									[46]
	n/a	IL-6 / dex			-	-									[46]
Jeg-3 (placental choriocarcinoma)	n/a	IL-1α / IL-1β / IL-6 / TNF-α / dex			+	+									[46]
OVCAR-3 (ovarian carcinoma)	n/a	IL-1β + IL-6		+											[56]
A549 (lung carcinoma)	n/a	IL-1β + IL-6					+		+						[42]
KB (oral epidermal carcinoma)	n/a	IL-1β ± dex / IL-6 ± dex / TNF-α ± dex / dex / IL-1β + IL-6 ± dex / IL-6 + TNF-α / dex / MoCM ± dex			+		+		+				+		[48, 60, 63, 84]
MCF-7 (mammary gland adenocarcinoma)	n/a	prolactin / LPS									+				[8]
T47-D (mammary gland ductal carcinoma)	n/a	prolactin / LPS / IL-1β + IL-6 + dex									+	+			[8, 25]
SW13 (adrenal cortex carcinoma)	n/a	MoCM			+								+		[48]

(Table 5) contd….

Cell type / cell line	Disease	Inducer	SAA[a]		A-SAA[b]		SAA1		SAA2		SAA3		SAA4 (C-SAA)		Ref.
			mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	mRNA	prot	
MG-63, Saos-2 (osteosarcoma)	n/a	IL-1α / IL-1β / TNF-α			+	+	+		-				+	+	[45]
	n/a	IL-6					-		-				+	+	[45]
THP-1 (monocytic cell line)	n/a	dex ± vit. D3 / IL-1β + dex ± vit. D3 / vit. D3 + IL-6 + dex ± IL-1 / LPS ± dex / vit. D3 + LPS + dex	+	+			+	+	+		-		+		[67, 70, 85, 86]
U937 (monocytic cell line)	n/a	MoCM ± dex / dex / PMA + dex			+								+		[48, 67]

Abbreviations: aldo: aldosterone, cortico: corticosterone, dex: dexamethasone, LPS: lipopolysaccharide, MoCM: monocyte conditioned medium, n/a: not applicable, OA: osteoarthritis, PMA: phorbol 12-myristate 13-acetate, pred: prednisolone, RA: rheumatoid arthritis

[a]Not specified which SAA type produced

[b]A-SAA mRNA or protein production without discrimination between SAA1 and SAA2

The presence or absence of SAA, A-SAA, SAA1, SAA2, SAA3 and/or SAA4 (C-SAA) mRNA and/or protein in extrahepatic cell types is indicated by + or -, respectively

3.2.1. A-SAA (SAA1 and SAA2) Serum Levels

In most studies conducted to measure serum SAA levels, it was not clear against which form of SAA the antibodies were raised. Shortly after the discovery of SAA in serum, antibodies were produced against protein AA, which was found later on to be a derivative of A-SAA, but not of C-SAA [37, 89]. Since there are no reports on SAA3 expression *in vivo* in humans and since particularly A-SAA is induced upon inflammatory stimulation, most probably in the studies not specifying which SAA form was measured, SAA1 and/or SAA2 (which differ in only a few amino acids) are the SAA forms detected.

Reported physiological SAA serum levels vary substantially. When summarizing all the data, we can conclude that the overall serum concentration of SAA in healthy individuals is about 1-2 μg/ml [2, 90-96]. Serum SAA concentrations in the ng/ml range (100-200 ng/ml) are also reported [1]. In contrast, some authors reported relatively high physiological levels of SAA (15-40 μg/ml) [42, 97-100]. This variation may be the result of subclinical infection or inflammation or of a lack of specificity of the antibodies used to detect SAA in serum. We speculate that the association of SAA with HDL may also mask the epitopes, recognized by the antibodies, and could therefore cause an underestimation of the SAA concentrations.

Several authors, comparing SAA levels in serum between healthy and diseased individuals, showed an increase in SAA concentration during various diseases (*e.g.* acute bacterial or viral infections, inflammatory and autoimmune diseases, neoplasia), trauma and surgery [1, 2, 32, 42, 56, 61, 83, 90-92, 94, 96-98, 100-105]. Individuals with benign tumors did not have significantly higher serum SAA levels than healthy persons [61], but SAA levels increased significantly as cancer progressed [56, 61, 90, 101, 104, 106]. The same effect was seen in patients with RA or osteoarthritis, whose serum SAA concentrations increased with the severity of the disease [83, 102, 107, 108]. Marhaug and Dowton [93] concluded that the serum SAA concentration is a very sensitive, but non-specific marker both in diagnosis, prognosis and monitoring of inflammatory and infectious diseases, just as in cancer [56, 61, 90, 105, 106].

Like in healthy individuals, serum SAA concentrations in patients vary between studies, ranging from about 10 μg/ml to about 500 μg/ml, up to even 1 mg/ml [2, 56, 61, 82, 83, 90, 92, 94, 97, 98, 100-102, 107-110]. In general, we can state that under pathological conditions (*i.e.* inflammatory or infectious disease or neoplasia) A-SAA concentrations of more than 10 μg/ml and up to 1 mg/ml or more are found in serum. Nonetheless, there are also reports of serum SAA levels of 'just' a few hundreds of ng/ml in diseased per-

sons [1, 101, 106]. These low levels can be due to inadequate sensitivity of the antibodies against SAA and/or to insufficient recognition of serum SAA bound to HDL. Alternatively, low levels of SAA in serum from diseased individuals may be significant in view of the short (1-1.5 day) half-life of SAA [100].

Other factors potentially influencing serum SAA concentrations are gender, age, pregnancy and the body mass index (BMI). Rosenthal and Franklin [1] mentioned an increase in SAA levels during pregnancy. In contrast, this observation was not made by Ostensen *et al.* [111]. The former authors also found a significant correlation between serum SAA concentrations and age, increases starting at 30 years of age and reaching the highest levels at the age of more than 70 years. However, other authors did not support this correlation [90, 99, 100, 112]. With regard to gender, d'Eril *et al.* [95] and Wang *et al.* [90] did not find any significant differences in serum SAA levels between women and men. Moreover, serum SAA concentrations were not influenced by genetic factors in patients with atherosclerosis [98]. Finally, a significant positive correlation was found between serum SAA concentrations and change in BMI. Moreover, when obese individuals experienced diet-induced weight loss, serum SAA levels decreased significantly [52, 53, 113].

3.2.2. C-SAA (SAA4) Serum Levels

There are only a few articles discussing SAA4 levels in human serum. Average SAA4 concentrations in healthy individuals ranged between 42 and 86 μg/ml [30-32, 114], which is relatively high in comparison with basal serum A-SAA levels (1-2 μg/ml, *vide supra*). SAA4 concentrations in serum were also investigated in individuals suffering from specific diseases. A direct comparison between healthy and diseased persons was made by Yamada *et al.* [31], who found that patients with coronary artery diseases (angina pectoris and/or myocardial infarction) had serum SAA4 concentrations similar to those of healthy controls. On the other hand, these authors reported significantly higher SAA4 concentrations (114 μg/ml) in serum from patients with hypertriglyceridemia. SAA4 concentrations in serum were also compared between pre- and post-surgical states in persons who received (successful) renal transplants [32]. SAA4 levels rose up to about 100 to 150 μg/ml after surgery, which is a three- to four-fold increase above the pre-surgical basal SAA4 concentrations in these patients. Finally, Kumon *et al.* [109] measured average concentrations of about 100 μg/ml of SAA4 in serum of patients with RA, but did not compare these levels with healthy controls. In summary, basal serum SAA4 concentrations vary around 50 μg/ml under physiological conditions and can rise two- to four-fold under some inflammatory conditions.

4. BIOLOGICAL FUNCTIONS OF SAA VARIANTS

4.1. Pro-and Anti-Inflammatory Properties of SAA

Since the SAA proteins are highly conserved through evolution [10], important biological roles may be expected. Throughout all the studies on SAA function done in the past, different sources of SAA were used. In most studies carried out during the past decades, recombinant SAA was used. A non-natural hybrid between SAA1α and SAA2β (rSAA) was mostly chosen to conduct the studies. Besides containing an additional amino-terminal methionine (M), this hybrid molecule contains two amino acid substitutions in the sequence of SAA1α, which correspond to the amino acids in SAA2β: at position 60, aspartic acid (D) is replaced by asparagine (N) and histidine (H) is substituted by arginine (R) at position 71. More recently, a recombinant SAA corresponding to SAA1α (except for an additional amino-terminal M, rSAA1α) was commercialized. We found that rSAA1α and rSAA have similar potencies at exerting chemotactic activity (*vide infra*). Since the primary structure of the various SAA subtypes (SAA1α, -β and -γ and SAA2α and -β) is slightly different, this might affect their tertiary structure [35] and hence, these molecules may have distinct functional characteristics. It has been shown that natural SAA from serum or plasma lacks most pro-inflammatory effect shown for recombinant SAA (*vide infra*).

Several functions have been described for A-SAA (Table **6**), but an all-round function of SAA is still unclear. Putative functions of C-SAA have not yet been studied, whereas both pro- and anti-inflammatory activities are reported for A-SAA. The most prominent pro-inflammatory activities of A-SAA, requiring the lowest minimal effective SAA concentration, are related to leukocyte migration *via* its chemokine inducing capacity and its direct chemotactic effect, which both occur at SAA concentrations as low as 10 ng/ml. A-SAA is a chemoattractant for monocytes, immature dendritic cells (DCs), neutrophils and T cells *via* binding to the G protein-coupled receptor (GPCR) formyl peptide receptor (FPR) 2 (Fpr2 in mice) [115-119]. Although this direct chemotactic activity is generally weak, SAA1α is able to greatly enhance the migration of monocytes in an autocrine way and that of neutro-

Table 6. Various biological functions of A-SAA.

Function	SAA (ng/ml)[a]	References
Chemotaxis	12.5	[115-117, 120]
Induction of chemokines	10	[53, 117, 121-123]
Induction of cytokines	500	[121, 124-127]
Induction of matrix degrading enzymes	100	[83, 110, 128, 129]
Inhibition of the oxidative burst in neutrophils	100	[130, 131]
Opsonin for gram-negative bacteria	1000	[132]
Formation of ion-channels in membranes	1000	[133, 134]
Inhibition of hepatitis C virus entry into hepatocytes	2000	[135, 136]
Retinol binding protein	3200	[137]
Induction of M2 macrophages	6000	[138]
Role in cholesterol transport	10000-20000	[139, 140]
Stimulation of angiogenesis	10000	[82, 125, 141-143]
Suppression of antibody production	20000	[144]
Inhibition of platelet activation and aggregation	50000	[145]

[a] Minimal effective SAA concentration required to exert biological activity

phils in a paracrine fashion by inducing CC and CXC chemokines (*e.g.* CCL3, CXCL8) by binding to TLR2 (Fig. **2**) [51, 116, 117].

Human blood monocytes and mouse bone marrow-derived macrophages exposed to SAA express macrophage M2 markers, including IL-10, Ym1, 'found in inflammatory zone-1' (Fizz-1), mannose receptor C type 1 (MRC1), IL-1RA and CCL17. SAA also increases arginase 1 (Arg1) activity and enhances macrophage efferocytosis of apoptotic neutrophils. This induction of M2 macrophages by SAA depends on MyD88 signaling and also requires IFN regulatory factor (IRF) 4, which is quickly induced by SAA. The *in vivo* relevance of these observations was illustrated by the observation that a single peritoneal injection of 25 μg human SAA in mice upregulates IL-10, IL-1RA, Fizz-1, CCL17, MRC1 and Arg1 mRNA in peritoneal macrophages [138]. The effect of SAA may not be limited to M2 differentiation. Indeed, SAA is expressed locally in chronic obstructive pulmonary disease (COPD) and this is accompanied by accumulation of macrophages distinct from classical M1/M2 cells. *In vitro*, human monocytes differentiated in the presence of SAA, produce IL-6 and IL-1β along with the M2 markers IL-10 and CD163. In mice, *in vivo* SAA administration induced the amplification of $CD11b^{high}$ $CD11c^{high}$ cells producing increased IL-6, IL-1β and GM-CSF levels after LPS stimulation [146].

In addition, A-SAA has been described as an angiogenic protein. In contrast to the low concentrations (10-100 ng/ml of SAA) required for chemotactic activity, only 1000-fold higher concentrations (10-60 μg/ml of SAA, Table **6**) can stimulate endothelial cell proliferation, adhesion, invasion and formation of capillary-like structures [82, 125, 141, 143]. In fact, A-SAA was also found to stimulate *in vivo* neovascularization [82, 125]. Furthermore, A-SAA at physiological levels (1 μg/ml) functions as an opsonin for gram-negative bacteria *via* binding to Outer membrane protein A (OmpA), thereby enhancing phagocytosis of bacteria [132]. Moreover, A-SAA is able to form ion-channels in synthetic and reconstituted membranes, potentially disturbing the cellular ionic homeostasis and causing bacterial lysis [133, 134]. A-SAA also reduces the bacterial burden in tissues, since the spleen and liver of $A\text{-}SAA^{-/-}$ mice injected with *Salmonella enterica* contained more bacteria than wild type mice treated with these bacteria [137]. The authors ascribed this antibacterial effect to the binding of A-SAA (3 μg/ml) to retinol, thereby taking over the role of retinol binding protein (RBP). Indeed, RBP is important to transport retinol to inflammatory sites at epithelial barriers to participate in repair processes. During acute infections, serum RBP levels decrease, whereas its requirement increases. Hence, A-SAA putatively helps RBP in its retinol transport function. Besides an antibacterial role, A-SAA seems to

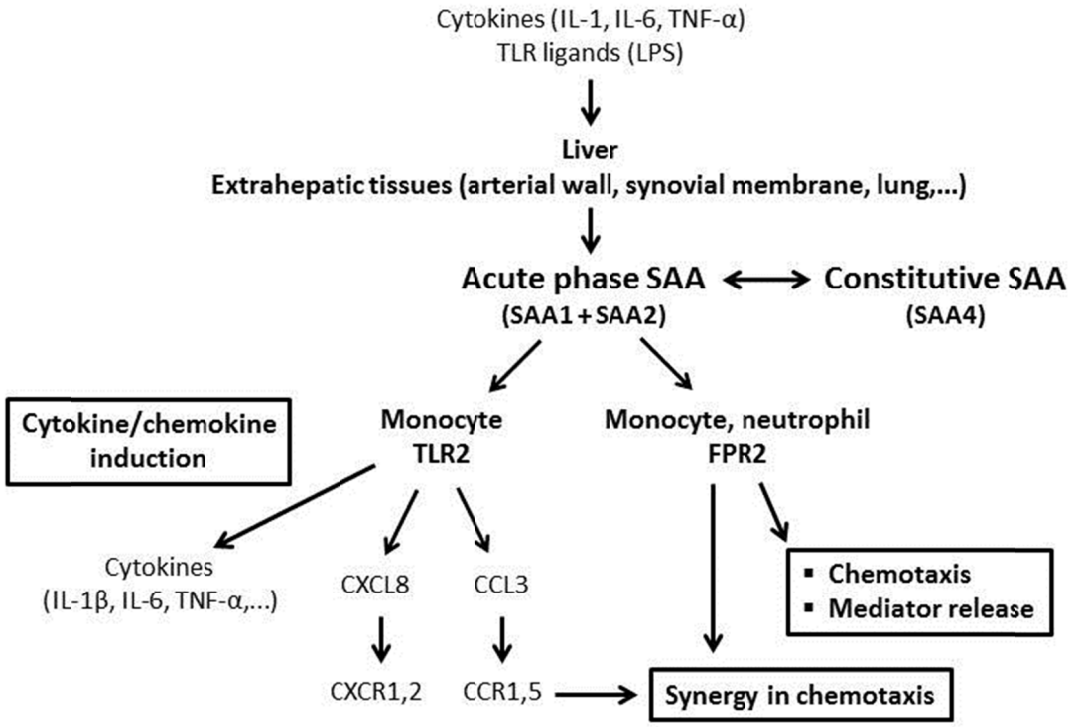

Fig. (2). Production of A-SAA and its chemotactic and cytokine inducing activities.

During the acute phase response, the production of A-SAA (SAA1 + SAA2), but not of C-SAA (SAA4), in the liver and in extrahepatic tissues is stimulated by exogenous TLR ligands (*e.g.* LPS) and endogenous cytokines (IL-1, IL-6 and TNF-α). SAA1α and probably also SAA2 are chemotactic for neutrophils and stimulate the release of mediators from these cells *via* activation of the GPCR FPR2. At the same time, these acute phase proteins activate TLR2 on monocytes, leading to induction of chemokines (*e.g.* CXCL8, CCL3) and other cytokines in monocytes. These SAA-induced chemokines synergize with each other and with SAA to attract leukocytes by binding to their proper chemokine receptors (CXCR, CCR).

have antiviral activities as well. A-SAA (≥ 2-10 μg/ml) dose-dependently inhibited hepatitis C virus entry into hepatoma cells by interacting with envelope glycoproteins of this virus, to reach a complete inhibition of infection at 100 μg/ml [135, 136]. However, this antiviral activity of SAA is probably restricted to hepatitis C virus, since A-SAA did not interfere with the infectivity of other RNA viruses.

Along with its antibacterial and antiviral actions, other activities have been reported for A-SAA. In particular, the capacity of A-SAA to induce matrix degrading enzymes is highlighted by several researchers. SAA (0.1-10 μg/ml) induced significant amounts of matrix metalloproteinase (MMP)-1, -2, -3, -9 and -13 in synovial fibroblasts from patients with RA or psoriatic arthritis [55, 110, 129, 147]. In chondrocytes, similar levels of SAA induced MMP-1, -2, -3, -10 and -13 [83, 110]. At physiological concentrations (0.1-10 μg/ml), SAA induced 3 ng/ml to 80 μg/ml of MMP in synovial fibroblasts and chondrocytes [55, 83, 110]. Induction of MMP-9 and of MMP-2 and -9 has also been observed in monocytes [128] and in renal cell carcinoma cell lines [62], respectively. Further, SAA (50 μg/ml) has been reported to inhibit thrombin-induced platelet activation and aggregation, probably *via* competition with thrombin for binding to its GPCR, *i.e.* protease-activated receptor (PAR-1), on platelets. Platelet aggregation was not inhibited when induced by collagen or ADP, nor did SAA inhibit the enzymatic activities of thrombin, such as in the coagulation cascade [145]. The inhibition of the oxidative burst in neutrophils is another anti-inflammatory activity of A-SAA (100 ng/ml) [130, 131]. The release of reactive oxygen species from fMLF-stimulated neutrophils was inhibited, but not that of phorbol 12-myristate 13-acetate (PMA)-stimulated or unstimulated neutrophils. Moreover, only low SAA concentrations exhibited this effect and SAA levels of ≥ 50 μg/ml paradoxally stimulated the oxidative burst, indicating that, at levels found in pathological conditions, A-SAA modulates the mode of action of neutrophils from an anti-inflammatory to a pro-inflammatory state [130]. In addition, murine A-SAA (≥ 20 μg/ml) is reported to suppress antibody production in mice *via* inhibition of

macrophage-T cell interactions or *via* induction of a suppressive factor in both macrophages and T-cells [144].

Another function of A-SAA that is often discussed in the literature is its role in cholesterol transport, although the reports are contradictory. On one hand, A-SAA (10 μg/ml) is reported to inhibit cholesterol efflux from cells [37, 139] and to reduce cholesterol delivery to damaged cells, which, however, require more cholesterol to promote tissue repair [37]. In addition, A-SAA was found to bind to the scavenger receptor SR-BI (or to its human equivalent CLA-1), one of the cholesterol efflux receptors, which could impair HDL binding to this receptor and which could thus inhibit cholesterol efflux [139, 148]. Some studies affirm that there is a reduction in cholesterol efflux during acute inflammation, but it was found that this inhibition was independent of SAA [149, 150]. On the other hand, some researchers reported that A-SAA (10 μg/ml) promotes cholesterol efflux [151, 152]. In fact, the group of Kisilevsky [140, 153] stated that HDL-bound A-SAA directs the HDL towards macrophages and that A-SAA subsequently modulates the enzymatic activity in macrophages, resulting in a shift towards unesterified cholesterol, which is able to leave the cell to be accepted by the HDL particle, brought to the macrophage by SAA. Moreover, when compared with normal Apo-I-bound HDL, SAA-bound HDL (HDL/SAA) had a reduced affinity for hepatocytes, whereas that for macrophages increased [154]. In line with these results, the number of binding sites for HDL/SAA increased on macrophages whereas it decreased on hepatocytes [154]. Hence, there is a shift in cholesterol efflux capacity and in HDL cholesterol carrying capacity from the liver towards macrophages, supporting the hypothesis that SAA helps recycling cholesterol from damaged cells.

Whether the binding of A-SAA to HDL in the blood circulation alters the above proposed functions of SAA is poorly studied. The association between SAA and HDL in the blood circulation could hamper the chemotactic and the cytokine inducing capacities of SAA. Although its chemotactic effect seems to be affected even at high SAA/HDL molar ratios, as observed during an acute phase response, SAA still induces cytokines and chemokines at the same high molar ratios, but this activity decreases as SAA/HDL molar ratios diminish [115, 116, 119, 155]. However, this subject requires further investigation, since contradictory reports exist [118, 126]. Hari-Dass and co-workers [156] reported that HDL does not influence binding of SAA to bacteria, but whether its opsonic activity is also conserved is not known. Nonetheless, its antiviral activity is indeed affected by association with HDL, but since both SAA and hepatitis C virus particles are synthesized in the liver, interaction between both molecules before contact of SAA with HDL is possible [136]. Similarly, inhibition of platelet aggregation, observed when gel-filtered platelets were used, was not seen when platelet-rich plasma was used, indicating that serum contains some SAA-inhibiting factors [145]. Furthermore, normal serum had a weaker inhibitory effect on the oxidative burst in fMLF-stimulated neutrophils than acute phase serum [131]. Because of the substantially higher SAA concentration in acute phase serum, more lipid-free (*i.e.* not associated with HDL) SAA is present than in normal serum, suggesting that the association with HDL could be responsible for the diminished neutrophil activating activity of SAA in normal serum. We conclude that the observed effects of SAA are dose-dependent and, because the range of serum concentrations spans almost four orders of magnitude, the discrepancies in the literature may be easily explained by taking into account that specific *in vivo* biological effects of SAA really are dependent on the actual concentrations, whether in serum or in specific local microenvironments.

Similar to the chemotactic potency of SAA (*vide supra*), other functions may be mediated by induction of cytokines and chemokines by SAA itself and may therefore also imply indirect activities of SAA. For example, Connolly *et al.* [82] discovered that the SAA-stimulated adhesion of monocytes to endothelial cells was in fact mediated by induction of the inflammatory chemokine CCL2. The SAA-mediated induction of matrix degrading enzymes could certainly also be an indirect effect *via* induction of cytokines [157]. Nonetheless, MMP-3 induction in SAA-stimulated synovial fibroblasts was not inhibited by antibodies against IL-1β and TNF-α [129]. In fact, SAA and amyloid proteins are reported to activate the inflammatory response by stimulating the NLRP3 inflammasome pathway, leading to the caspase-1 dependent activation of cytokines, such as IL-1β [158-161]. Further research should be conducted to elucidate whether SAA executes its functions directly or in an indirect way, *via* induction of cytokines and/or chemokines.

4.2. SAA Receptors and Drug Design

In view of the evidenced pro-inflammatory properties of SAA, which are predominantly mediated by TLR2 (for cytokine/chemokine induction) and by FPR2

(for direct chemotactic activity), it makes sense to block either SAA production or its activity to treat excessive inflammation. First, this could be achieved by reducing the production of SAA inducers, *i.e.* the cytokines IL-1, TNF-α and IL-6, for which antagonists/inhibitors (*e.g.* monoclonal antibodies) are already clinically used. Second, the chemokine inducing capacity of SAA could be prevented by TLR2 antagonists. Third, its direct chemotactic effect could be blocked by FPR2 antagonists. Indeed, SAA binding to Fpr2 is shown to be involved in several inflammatory processes, such as allergic airway inflammation (see section 6 "Remarks and conclusions") [162, 163]. Small molecules, which are potent receptor blockers, are already used in animal models (*vide infra*), but have not yet entered clinical trials. However, both TLR2 and FPR2 do recognize a number of other endogenous as well as exogenous ligands, which are depicted in Table **7**. The CC chemokine CCL23β is an alternatively spliced variant of CCL23, containing 116 instead of 99 amino acids. This chemokine is chemotactic for monocytes, neutrophils, DCs and T lymphocytes *via* activation of CCR1 [164, 165]. A synthetic N-terminal truncated form of CCL23β, consisting of 92 amino acids, was reported to stimulate the migration of neutrophils *via* binding to FPR2 [166]. This truncated form of CCL23β was the only FPR2 agonist, tested amongst others [*e.g.* SAA, amyloid β (Aβ)(1-42)], that was fully able to disrupt the binding of the synthetic peptide WKYMVm (a potent FPR2 agonist [167]) to FPR2-transfected cells [166]. In contrast to Aβ(1-42), which is involved in the pathogenesis of Alzheimer's disease, humanin has neuroprotective properties by competing with Aβ(1-42) for binding to FPR2 [168]. Both endogenous polypeptides are also chemotactic for specific leukocyte subsets [168-171]. Human mitochondrial formylated peptides (in particular the hexapeptide fMMYALF), which are cleavage products of mitochondrial proteins, are also reported to chemoattract neutrophils through activation of FPR2 [172]. In addition, cleavage products of the antibacterial cathelicidin, *i.e.* LL-37 and its murine homolog cathelin-related antimicrobial peptide (CRAMP), are also FPR2 agonists involved in the recruitment of leukocytes to sites of inflammation [173-176]. Similarly, potent FPR2-agonistic synthetic peptides (*e.g.* WKYMVm, MMK-1) with chemotactic activity on monocytes and neutrophils have been identified [167, 177-179]. FPR2 agonists are predominantly peptides or proteins, whereas the structures of ligands binding to TLR2 are very diverse. Lipoteichoic acid and peptidoglycan are components of the cell membrane of gram-positive bacteria and induce, in analogy with SAA, the activation of neutrophils and/or the production of cytokines in leukocytes [180-184]. The polysaccharide zymosan originating from *Saccharomyces cerevisiae* and bacterial lipoproteins also stimulate the induction of cytokines in several leukocyte subsets *via* activation of TLR2 [185-188].

Taken together, most of the FPR2 agonists, like SAA, do exert chemotactic activity and, likewise, most of the TLR2 ligands are pro-inflammatory, indicating that drug design to block the receptors as anti-inflammatory treatment makes sense. Efforts for drug development to inhibit these receptors are currently still limited and should deserve more attention (Table **8**). For example, WRW_4 is a potent antagonist able to selectively block the inflammatory effects of most FPR2 ligands [190]. Actually, Meng *et al.* [192] were able to prevent the development of lethal shock-like syndrome in mice infected with *Bacillus subtilis* by administration of anti-TLR2 antibody before or even several hours after infection. In general, studies using specific monoclonal antibodies blocking TLR2 or FPR2, as well as receptor knockout mice revealed the pro-inflammatory properties of these receptors. Indeed, owing to the availability of genetically engineered mice and clinically relevant disease models, the presumed function of Fpr2 as a chemoattractant receptor has been increasingly recognized in infection, immune responses and inflammation. It is noteworthy that Fpr2 possesses a unique biochemical property of recognizing structurally diverse chemoattractants, including pathogen-associated chemotactic molecular patterns (PAMPs) and damage-associated chemotactic molecular patterns (DAMPs).

5. ROLE OF SAA IN DISEASES

Serum concentrations of A-SAA are upregulated more than three orders of magnitude in several inflammatory, infectious and neoplastic diseases (*vide supra*). In some chronic inflammatory diseases, SAA is thought to take part in the pathogenesis (Table **9**), but the exact role of this acute phase protein in disease development and whether elevated SAA levels are causative or more a consequence of these diseases is mostly not well understood.

5.1. Rheumatoid Arthritis

Probably one of the most discussed diseases in which A-SAA plays a role, is RA. Several observations support the association between SAA and the pathogenesis of this disease. Significantly higher amounts of A-SAA mRNA and protein were detected in synovial tissues from patients with RA than in tissues of healthy

Table 7. Selective agonists of the SAA receptors FPR2 and TLR2.

Receptor	Inflammatory effect	Target cells	Ligand/agonist	Structure	Reference
FPR2	Chemotaxis, mediator release	Leukocyte subsets	SAA	Acute phase protein (104 aa)	[110, 116, 117, 120]
	Chemotaxis	Monocytes, neutrophils, T lymphocytes	CCL23β	CC chemokine (92 aa)	[166]
	Chemotaxis	Phagocytes	Aβ (1-42)	Polypeptide of β amyloid (42 aa)	[170, 171]
	Chemotaxis	Monocytes	Humanin	Polypeptide (24 aa)	[168, 169]
	Chemotaxis	Leukocytes	Mitochondrial formyl peptides	Oligopeptide (6 aa)	[172]
	Chemotaxis, dendritic cell maturation	Monocytes, neutrophils, T lymphocytes, dendritic cells	LL-37/CRAMP	Polypeptide (37/34 aa)	[173-176]
	Activation	Monocytes, neutrophils, B lymphocytes	WKYMVm, WKYMVM	Oligopeptide (6 aa)	[167, 177, 189]
	Chemotaxis	Monocytes, neutrophils	MMK-1	Polypeptide (13 aa)	[178, 179]
TLR2	Cytokine induction	Monocytes	SAA	Acute phase protein (104 aa)	[51]
	Cytokine induction	Macrophages, monocytes	Peptidoglycan	Polymer of glycan strands cross-linked by peptide bridges	[182, 184]
	Induction/release of reactive oxygen species, cytokine induction	Phagocytes, monocytes	Lipoteichoic acid	Chains of up to 30 glycerol or ribitol residues with phophodiester links	[180, 181, 183]
	Cytokine induction	Macrophages, monocytes	Bacterial lipoprotein	Membrane-anchored protein (several hundreds aa)	[185, 187]
	Cytokine induction	Macrophages, dendritic cells	Zymosan	β-glucan (*Saccharomyces cerevisiae*)	[186, 188]

Abbreviations: aa: amino acids, Aβ: amyloid β, CRAMP: cathelin-related antimicrobial peptide, CCL23: CC chemokine ligand 23

Table 8. Selective antagonists of the SAA receptors FPR2 and TLR2.

Receptor	Antagonist	Structure	Reference
FPR2	WRW_4	Oligopeptide (6 aa)	[190]
	PBP10	Phosphoinositol-binding peptide	[191]
TLR2	Anti-TLR2 antibody	Antibody	[192]
	TIR domain inhibitors	Peptides	[193]
	Curcumin	Diarylheptanoid	[194]
	CU-CPT22	Benzotropolone	[195]
	SSL3	Protein	[196]
	Hsp60	Protein	[197]
	Lipolanthionine	Two thioether-linked alanine residues, bound to lipid	[198]

Abbreviations: Hsp: heat shock protein, SSL3: staphylococcal superantigen-like protein 3

Table 9. Chronic inflammatory diseases, in which SAA is thought to play a role.

Disease	Effect	References
Rheumatoid arthritis	Pro-inflammatory (marker), angiogenic	[55, 82, 107, 199]
Obesity	Pro-inflammatory, insulin resistance	[52, 53, 74, 113]
Diabetes type II	Causative?, pro-inflammatory	[148, 200-202]
Atherosclerosis	Causative?, pro-inflammatory	[66, 203-206]
Pulmonary diseases	Pro-inflammatory	[81, 158, 207, 208]
Cancer	Anti-/pro-tumoral?, acute phase marker	[104, 209-211]
Crohn's disease	Acute phase marker	[212, 213]
Alzheimer's disease	Not obvious	[68, 76-78]
Amyloid A amyloidosis	Causative	[37, 214, 215]

individuals [55, 63, 65, 82]. Plasma or serum from these patients also contains higher A-SAA levels than observed in healthy individuals [83, 216]. In fact, SAA is a more sensitive acute phase marker for inflammatory joint diseases than CRP [107, 216]. In addition, A-SAA levels in plasma and synovial fluid were found to correlate [83]. This last notion suggests that SAA enters the joint *via* diffusion from the blood circulation. However, local expression of SAA has been demonstrated in synoviocytes, macrophages and endothelial cells from synovial tissues of patients with RA [55, 63]. Moreover, Connolly *et al.*, [82] found that expression of SAA was even higher in synovial fluid than in plasma of diseased individuals. Indeed, about 5-100 μg/ml of SAA was detected in synovia from patients with RA [82, 109, 217]. Unstimulated synovial fibroblasts from patients with RA produced also more A-SAA (about 150-350 ng/ml) than control synoviocytes (about 85 ng/ml) [55]. In addition, A-SAA stimulates angiogenesis, which is an important process in pannus formation [82]. Another observation, suggesting that SAA plays a pro-inflammatory role in RA, is its capacity to potently induce cytokines (*e.g.* IL-1β, TNF-α) and chemokines (*e.g.* CXCL8, CCL2), contributing to the pathogenesis of RA [51, 199]. Moreover, SAA-induced cytokines and SAA itself potently induce matrix degrading enzymes in synovial fibroblasts from patients with RA [55, 110, 129]. These matrix degrading enzymes support tissue repair on short term, but if chronically upregulated, these proteases contribute to joint destruction.

5.2. Obesity

Another pathological condition, in which A-SAA seems to play a role, is obesity. Higher A-SAA mRNA expression was observed in adipocytes from obese individuals than in liver, suggesting the importance of local production of SAA in adipose-rich tissues [52, 53]. Adipocytes from obese persons also produce chemokines and cytokines (*e.g.* IL-6, TNF-α), chemoattracting leukocytes and inducing additional local synthesis of SAA [53, 74, 113, 218, 219]. SAA in turn further stimulates the local production of more chemokines and cytokines [51]. This positive feedback loop contributes to a maintained inflammatory state in adipose tissue from obese persons. Moreover, SAA expression in adipose tissue was correlated with macrophage infiltration [74]. Macrophages can produce SAA directly [67] or can stimulate other cells to synthesize SAA in a paracrine way *via* production of SAA-inducing cytokines. Furthermore, a significant positive correlation was found between, on one hand, A-SAA expression in adipocytes or serum and, on the other hand, the BMI and insulin resistance [52, 53, 113]. Weight loss resulted in a decreased A-SAA protein expression in adipocytes and serum. At the same time, decreased serum SAA concentrations correlated with an increase in insulin sensitivity, since SAA stimulated lipolysis, resulting in increased levels of free fatty acids in the blood circulation, accompanied by raised insulin resistance [53].

5.3. Diabetes Type II

SAA also putatively influences the pathogenesis of non-insulin dependent diabetes or diabetes type II. SAA levels in plasma from patients with diabetes type II were significantly higher than those of healthy individuals [220]. Moreover, a significant correlation was observed between plasma levels of this acute phase protein and the development of diabetes type II. This correlation was independent from risk factors for development of the disease, such as BMI, smoking and

education [202]. Significantly higher serum SAA concentrations were also observed in patients suffering from diabetes type II with nephropathy compared to patients without nephropathy [148, 220]. Monocytes and neutrophils from patients with diabetes type II produced higher amounts of IL-1β and CXCL8 and were more responsive to SAA in terms of chemotactic response than those of healthy subjects [200]. As a consequence, a general inflammatory state is sustained, with a higher risk for cardiovascular disease, such as atherosclerosis. The risk of atherosclerosis may also be influenced by a redistribution of SAA from HDL to LDL and VLDL, detected only in obese individuals with diabetes type II, but not in obese individuals not having any other disease [201]. However, the biological consequences of this redistribution are not known.

5.4. Atherosclerosis

Chronic inflammatory disorders, such as obesity, diabetes and RA, as discussed above, are associated with the development of cardiovascular disease [203]. Logically, SAA also seems to be implicated in the pathogenesis of atherosclerosis. Patients suffering from this disease show a significant increase in serum A-SAA levels compared to healthy controls [98]. Both brief and chronic elevations in serum A-SAA concentration have been reported to predispose for the development of atherosclerosis. Indeed, mice injected with a viral vector encoding human SAA1 developed increased atherosclerosis compared to control mice, independently of single or multiple injections of the viral vector [205]. Just as in RA and in obese individuals, SAA mRNA has been locally detected in atherosclerotic lesions, especially in macrophages, foam cells, endothelial cells and smooth muscle cells [66]. A-SAA (10-100 μg/ml) stimulates the proliferation and migration of smooth muscle cells [221] and is directly and indirectly (*via* induction of chemokines such as CXCL8 and CCL2) chemotactic for neutrophils and monocytes [115-118], important cell types in the pathogenesis of atherosclerosis. Once the acute phase protein has entered the vessel wall, A-SAA stimulates foam cell formation *via* induction of the LDL scavenger receptor LOX-1 [204]. Moreover, SAA increases the *in vitro* and *in vivo* synthesis of proteoglycans (which bind LDL) *via* induction of transforming growth factor (TGF)-β and increases the GAG chain lengths of the proteoglycans and the binding affinity of LDL to proteoglycans [205, 206]. These effects of SAA lead to an increased LDL retention in atherosclerotic lesions. In addition, inhibition of reverse cholesterol transport may also contribute to the pro-atherogenic role of SAA, but this issue is still controversial (*vide supra*).

5.5. Pulmonary Diseases

Serum SAA levels are increased in COPD [207] and may contribute to the local inflammation by promoting IL-6 production, which in turn stimulates T helper 17 cell accumulation and production of neutrophil attracting chemokines by these cells. A causal link was established by the observation that anti-IL-17A antibody impaired neutrophil lung infiltration induced by SAA. This effect of SAA is thought to be mediated through FPR2 as the induction of IL-6 was inhibited by lipoxin A4, an FPR2 ligand [81]. In these experiments, IL-17A was induced by SAA in both T cells, predominantly γδ innate T cells, and $Epcam^{+}$ $CD45^{-}$ epithelial cells. A link between IL-17A and SAA was also described in patients with chronic rhinosinusitis in whom a significant correlation between the two factors was noted [222]. Interestingly, in polyp epithelial cells, IL-17A induced SAA mRNA expression. These results suggest that the dual SAA-IL-17A interaction represents an important axis in the innate immune network involved in COPD regulation.

SAA has also been reported to play a role in lung alterations induced by cigarette smoking. Lung inflammation actually persists for many years after smoking cessation. Interestingly, the mediators involved in the inflammatory process may vary between the acute smoking period and the time after smoking cessation. In fact, SAA, along with IL-17A, was predominantly detected in the lungs of cigarette smokers, who have stopped smoking, contrary to CXCL1, CXCL2 and G-CSF, which were predominantly expressed during the active smoking period. The SAA/IL-17A program may therefore be the main cause for the long-lasting lung inflammation of former cigarette smokers [81, 208].

Another mechanism connecting SAA to airway alterations was reported in asthma. SAA was found to activate the NLRP3 inflammasome which enhances IL-1β secretion by DCs and macrophages, thus increasing the local inflammatory reaction [158]. Finally, SAA interacts with extracellular matrix (ECM) components and was found to enhance mast cell adhesion to the ECM, thereby further contributing to enhanced allergic reactions [223].

5.6. Cancer

As previously reviewed, SAA is involved in neoplastic processes [104]. Although it is not clear whether increased serum levels of SAA are the effect of cancer-

associated inflammation or the cause of oncological mechanisms or are simply a paraneoplastic consequence of cancer, these levels are a very sensitive serum marker that is upregulated in many cancers, including gastric cancer, multiple myeloma and osteosarcoma [45, 224, 225]. Systemic SAA expression increases significantly as tumors progress from stage 1 to stage 4. Moreover, serum SAA concentrations predict the prognosis of neoplasia. Patients having a serum SAA concentration below 10 μg/ml have a significantly greater chance of survival than those with higher levels [209]. Furthermore, SAA is locally expressed in tumors and in cell lines of neoplastic origin [42, 104]. The biological significance of this local expression has not yet been elucidated. Nonetheless, evidence exists that SAA can inhibit tumor invasion by binding to ECM components, blocking thereby the binding of cells to those components. On the other hand, SAA-mediated induction of matrix degrading enzymes (*vide supra*) might also have a repercussion on tumor cell invasion [42, 104]. Other pathogenic mechanisms connecting SAA and cancer include stimulation of the production of ECM adhesion proteins like laminin and heparin/heparan sulfate proteoglycans [226], enhanced ECM transition, angiogenesis and production of TGF-β and IL-10 [227]. SAA could also favor tumor development by limiting immune anti-tumor activity as it promotes expansion of myeloid-derived suppressor cells (MDSCs) as shown for SAA3 in mice [210] and stimulates growth of regulatory T cells in a process involving IL-1β and IL-6 induction in monocytes [211]. Considering these data, it is not surprising that SAA has evolved as a very sensitive marker of tumor development.

5.7. Other Chronic Diseases

Other diseases conceivably influenced by SAA are Crohn's disease, Alzheimer's disease and sleep apnea. Patients with Crohn's disease have higher plasma SAA levels than patients with other inflammatory bowel diseases. Just as in RA, SAA is a more sensitive acute phase marker for both diagnosis and monitoring of Crohn's disease than CRP [212, 213]. The brain of patients with Alzheimer's disease also expresses more SAA protein at affected sites than controls [68, 76-78]. SAA protein was specifically detected in myelin sheaths (white matter) and in neuritic plaques (grey matter) and co-localized with expression of β amyloid protein, which is associated with the pathogenesis of Alzheimer's disease [76, 77]. Furthermore, serum SAA levels of patients with moderate to severe obstructive sleep apnea were significantly higher than those of controls (healthy or suffering from mild obstructive sleep apnea), suggesting that SAA may contribute to the higher risk for cardiovascular dysfunction in these patients [228].

5.8. Amyloid A Amyloidosis

SAA plays unmistakably a role in the pathogenesis of secondary or AA amyloidosis. In fact, AA amyloidosis was known long before the serum component causing this disease, *i.e.* SAA, was discovered *via* antibodies against AA protein, cross reacting with SAA [229]. AA proteins are typically 76 amino acids long, but variable lengths are reported. They originate from proteolytic cleavage of SAA, the N-terminal part being AA protein. The mechanism of AA protein generation out of SAA is until now not well understood, but recurrent or persistently high serum SAA concentrations, as observed in chronic diseases such as RA, are necessary to promote AA amyloidosis [214]. Several hypotheses of AA amyloid formation have been proposed: incomplete digestion of SAA in macrophages [230], proteolytic cleavage of SAA by enzymes other than in macrophages [214], at-risk allelic SAA variants (*vide infra*) [37, 214, 231], induction of a seeding-nucleation process [232, 233] and mutant amyloid-prone SAA sequences, as observed in a case of SAA4 amyloidosis in the kidney [69]. The GAG heparan sulphate, which is consistently found in amyloid deposits, seems to be essential in amyloidogenesis [234, 235].

AA amyloidosis is characterized by folding of AA protein derived from SAA into extremely hydrophobic β-sheets which aggregate to oligomers. These oligomers form insoluble, proteolysis resistant fibrils in multiple organs (*e.g.* kidney, spleen and liver). Accumulation of these fibrils in organs impairs their normal function and leads eventually to death [214, 236]. However, not all patients with chronic or recurrent disease develop AA amyloidosis. The reason for this fact is not yet known. Nonetheless, SAA1 is the predominant SAA form found in amyloid deposits and Caucasian and Japanese patients who are homozygote for SAA1α or SAA1γ, respectively, have a higher risk for developing amyloidosis [37, 214]. Moreover, Armenian patients with Familial Mediterranean fever of the male gender (independent of SAA1-allelic variation) as well as homozygous patients for SAA1α had a four- and seven-fold higher risk for developing renal AA amyloidosis, respectively [231].

6. REMARKS AND CONCLUSIONS

As a major human acute phase protein, highly induced during inflammatory events, SAA is believed to

play an important role in host defense. Although several functions have been attributed to A-SAA, the predominant role of this acute phase protein remained thus far unclear. In this review, we have provided a critical overview of the various functions ascribed to SAA and of its potential role in the pathogenesis of different chronic inflammatory diseases. We contend that one needs to see the function(s) of SAA as dependent on its *in vivo* concentrations. To simplify many unanswered questions about the biological functions of SAA, we took a new approach by defining SAA functions on the basis of specific activities in biological assays (Table **6**). This comparative analysis revealed that, at very low tissue concentrations (10-100 ng/ml), early in an inflammatory response, SAA acts as a chemotactic and inducing cytokine. When a strong infectious or inflammatory stimulus persists, the liver continues to produce more SAA and concentrations of 1000 ng/ml or more are reached. In such conditions, SAA exerts antibacterial and antiviral activities by functioning as an opsonin for bacteria and by interfering with virus infection of host cells. Such direct antimicrobial activities are aimed at reducing infectious triggers, whereas high SAA concentrations (above 10 µg/ml) may also be regarded as mainly anti-inflammatory negative feedback mechanisms to protect the host from collateral damage. These latter feedback controls include effects on platelets, cholesterol transport and adaptive immune responses. SAA is thus an important concentration-dependent effector and assists in the regulation of innate and adaptive immune responses.

One could wonder how SAA leaves the blood circulation to exert its functions locally in tissues. In the blood circulation, the association of SAA with HDL could hamper the extravasation of the acute phase protein. However, GAGs and HDL have shared binding sites on the SAA protein. Both molecules compete with each other for binding to SAA and GAGs are actually able to disrupt the association of SAA with HDL [35]. Moreover, two GAG binding sites on SAA were discovered and not all SAA molecules are bound to HDL in acute phase serum, increasing the chance of SAA binding to GAG-rich endothelium in inflamed tissues [35, 237]. Besides the proven capability of SAA to bind to GAGs, the enhanced permeability of inflamed blood vessels causes leakage of plasma proteins into the inflamed tissues. Even albumin with a molecular weight of 66 kDa, which is much higher than that of SAA (12 kDa), exudates easily to local tissues [238]. Finally, several researchers reported local non-hepatic production of SAA. Immunohistochemical studies revealed the presence of SAA in various histologically normal tissues, as well as in macrophages, endothelial cells and other cell types in diseased tissues (Tables **2** and **4**) [43, 55, 66]. Sjöholm *et al.* [52] found a positive correlation between serum A-SAA levels and local A-SAA mRNA expression levels in subcutaneous adipocytes and even suggested that increased serum A-SAA concentrations are the result of increased local synthesis of A-SAA, but no firm proof was provided for this hypothesis.

The A-SAA proteins are expressed in various histologically normal tissues, most importantly in the epithelium [43], as well as in non-stimulated epithelial cell lines [48, 55, 60, 84] (Tables **2** and **3**). Nonetheless, the significance of its presence in epithelia is not clear. Does A-SAA play a role in the preservation of epithelia? Does A-SAA function as a local antibacterial component? Antibacterial and antiviral properties are indeed ascribed to high concentrations of SAA in that it functions as an opsonin for gram-negative bacteria, is able to form ion-channels in membranes, inhibits hepatitis C virus entry into hepatocytes and limits the bacterial burden in tissues by contributing to retinol transport [132, 133, 135-137]. Besides its role in the development of an immune response, retinol also supports the epithelial integrity [137, 239]. Another theory concerning the inflammatory role of SAA is that it functions (at extremely low concentrations) as a signal relay by inducing chemokines. SAA induces several CC and CXC chemokines, such as CCL2, CCL3 and CXCL8 [116-118], which transmit the chemotactic message to surrounding tissues, extending its action to recruit leukocytes to sites of inflammation [240].

When discussing the function of SAA, one should also be conscious about the fact that the different A-SAA variants (*i.e.* SAA1 and SAA2) may have distinct functions. This idea is supported by the reports about different expression patterns of both A-SAA variants in some cell lines or tissues. SAA2 was predominantly expressed in cytokine-stimulated HepG2 cells [60, 241]. In contrast, in ovarian carcinoma, mesenchymal stem cells and the osteosarcoma cell line MG-63, particularly SAA1 was expressed [45, 56]. Moreover, C-SAA (*i.e.* SAA4), which is constitutively present in serum at a concentration of about 50 µg/ml, may play a distinct role from A-SAA, since serum concentrations of A-SAA vary between 1-2 µg/ml under non-inflammatory conditions and 1 mg/ml under pathological circumstances. However, the role of SAA4 has scarcely been studied. In the future, more (comparative) studies should thus be conducted to investigate the role of the distinct SAA forms.

The influence of the source of SAA on the results obtained in functional studies is of increasing interest. In most studies, a recombinant SAA was used. Christenson *et al.* [242] and van den Brand *et al.* [243] directly compared the recombinant SAA1α (rSAA1α) and the recombinant hybrid between SAA1α and SAA2β (rSAA) for their chemokine inducing capacity. More CXCL8 was induced when stimulating synovial fibroblasts or neutrophils with rSAA than with rSAA1α. We also compared both recombinant SAA variants in chemotaxis experiments, but no significant difference in their chemotactic potency for monocytes and neutrophils was found [116, 117]. Furthermore, various A-SAA subtypes were expressed in *E. coli* and their chemotactic and cytokine inducing capacities were compared by Chen *et al.* [174]. Recombinant SAA1α and SAA2β were significantly more potent chemoattractants for the rat basophilic leukemia cell line RBL-2H3 than recombinant SAA1β and SAA1γ. In contrast, recombinant SAA1β and SAA1γ induced significantly more cytokines in murine macrophages than recombinant SAA1α and SAA2β. In addition, recombinant SAA variants and natural SAA are also reported to have different cytokine inducing and neutrophil activating abilities. In contrast to rSAA, SAA purified from patients with high serum SAA titers or SAA-rich plasma were neither able to induce CXCL8, nor to activate neutrophils [242, 244]. Binding of endogenous SAA to HDL may be responsible for the loss of activity of the acute phase protein [116]. In contrast, HDL-associated recombinant SAA was capable of inducing IL-1β and IL-1RA in THP-1 cells, similar to synthetic SAA1α, not associated with HDL [126]. Data obtained from experiments using recombinant SAA variants, in particular non-natural recombinant variants, should thus be interpreted with care since these different SAA variants may have distinct activities.

Studies of Fpr2 in disease models have revealed different patterns of leukocyte recruitment by sequentially expressed chemoattractant receptors on the cell surface and local production of chemoattractants on the route of cell trafficking, termed chemotaxis signal relay in which Fpr2 is positioned in different stages. For instance, in *Listeria*-infected mice, Fpr2 as well as its prototype receptor Fpr1 are vital for host resistance to infection and mediate the rapid first wave of neutrophil infiltration into the bacteria-laden liver in response to bacteria-derived chemotactic peptides. This function of Fpr1/2 antecedes the neutrophil specific chemokine receptor CXCR2, in that the production of CXCR2-binding chemokines in the liver, as a response triggered by TLR2 agonists, such as bacterial lipoproteins, was detectable long after the peak of neutrophil accumulation [163]. Fpr2 also interacts with endogenous chemotactic agonists released by injured tissue in concert with the chemokine receptor CXCR2, to mediate neutrophil swarming subsequently to the initial cell recruitment controlled by the leukotriene B4 receptor [245]. Similarly, Fpr2 contributes to the rapid healing of skin wounds by mediating neutrophil infiltration in response to skin-derived chemotactic agonists [246]. In an allergic airway inflammation model, which requires a complex host response to antigens, Fpr2 was found to cooperate with chemokine receptors CCR2 and CCR7 to guide DC trafficking. After antigen immunization and challenge, CCR2 mobilizes DC precursors from the circulation to the perivascular regions in the lung. In these perivascular regions, CCR2 expression decreases, whereas that of Fpr2 increases. By responding to the endogenous chemotactic ligand CRAMP, immature DCs migrate into the peribronchiolar regions in the inflamed lung where the cells become mature upon contact with pro-inflammatory stimulants, such as TLR ligands and cytokines. Matured DCs then express a high level of CCR7 to guide these cells toward the draining lymph nodes. The contribution of Fpr2 and CRAMP to DC trafficking in allergic airway inflammation was demonstrated by the greatly reduced severity of asthmatic syndrome and by the lack of DC infiltration in the peribronchiolar region and the mediastinal draining lymph nodes in Fpr2-deficient mice [162, 173, 174]. Interestingly, Fpr2 and CRAMP are not only involved in DC trafficking in inflamed lung, they also promote the maturation of DCs in response to conventional stimulants, further illustrating the multi-facet functions of Fpr2 and its ligands in host defense. It is interesting to note that despite its primary function as a leukocyte sensor of chemotactic ligand gradient generated by invading bacteria or by injured tissues, Fpr2 has recently been found to be expressed by mouse and human colon epithelial cells and in a mouse inflammatory bowel disease model. Fpr2 was shown to contribute to the homeostasis, inflammation, repair, and antitumor defense of the colon mucosa, presumably by interacting with commensal bacteria components [163, 173]. This paradigm-changing discovery has greatly expanded the functional scope of the SAA receptor Fpr2 as a mere chemoattractant receptor and may have important implications in better understanding disease processes and identifying novel therapeutic targets.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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