



Human serum albumin: From bench to bedside

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This paper is dedicated to Prof. T. Peters Jr. who pioneered albumin structure–function relationships.

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ABSTRACT

Human serum albumin (HSA), the most abundant protein in plasma, is a monomeric multi-domain macromolecule, representing the main determinant of plasma oncotic pressure and the main modulator of fluid distribution between body compartments. HSA displays an extraordinary ligand binding capacity, providing a depot and carrier for many endogenous and exogenous compounds. Indeed, HSA represents the main carrier for fatty acids, affects pharmacokinetics of many drugs, provides the metabolic modification of some ligands, renders potential toxins harmless, accounts for most of the anti-oxidant capacity of human plasma, and displays (pseudo-)enzymatic properties. HSA is a valuable biomarker of many diseases, including cancer, rheumatoid arthritis, ischemia, post-menopausal obesity, severe acute graft-versus-host disease, and diseases that need monitoring of the glycemic control. Moreover, HSA is widely used clinically to treat several diseases, including hypovolemia, shock, burns, surgical blood loss, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, hemodialysis, acute liver failure, chronic liver disease, nutrition support, resuscitation, and hypoalbuminemia. Recently, biotechnological applications of HSA, including implantable biomaterials, surgical adhesives and sealants, biochromatography, ligand trapping, and fusion proteins, have been reported. Here, genetic, biochemical, biomedical, and biotechnological aspects of HSA are reviewed.

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Abbreviations: AFM, afamin; AFP, α -fetoprotein; A/G, albumin/globulin ratio; Alb A, albumin A; Alb B, albumin B; B, basic form; bp, base pair; BSA, bovine serum albumin; CD, circular dichroism; CEA, carcinoembryonic antigen; CVVHD, continuous venous hemodialysis; DBP, vitamin D binding protein; E, extended form; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure spectroscopy; F, fast migrating form; FA, fatty acid; FDH, familial dysalbuminemic hyperthyroxinemia; FeP(II), ferrous tetraphenylporphyrinatoiron; FT₄, free T₄; Hb, hemoglobin; HDL, high density lipoproteins; heme–Fe(III), ferric heme; HPX, hemopexin; HPX–heme, heme–hemopexin; HSA, human serum albumin; HSA–heme–Fe(II)–NO, ferrous nitrosylated HSA–heme; HSA–heme–Fe(III), ferric HSA–heme; HSA–heme–Fe(III)–NO, ferric nitrosylated HSA–heme; HSA–heme, human serum heme–albumin; HSA–heme–Fe(II), ferrous HSA–heme; kbp, kilobase pairs; LDL, low density lipoproteins; MARS, molecular absorbent recirculating system; Mb, myoglobin; MBS-A, primary multimetal binding site or Cadmium site A; MBS-B, secondary multimetal binding site or Cadmium site B; mRNA, messenger RNA; N, neutral form; NMR, nuclear magnetic resonance; NTS, N-terminal binding site; OsrHSA, *Oryza sativa* recombinant HSA; OTU, operational taxonomic units; pdHSA, plasma-derived HSA; Pc, phthalocyanine; PEG–HSA, hexa-polyethyleneglycolylated–HSA; Poly(A), polyadenylation; PyPU, pyrimidine–purine residues; PU, purine rich; rHSA, recombinant HSA; RNS, reactive nitrogen species; ROS, reactive oxygen species; SA, serum albumin; SA–heme, serum heme–albumin; scFvs, single-chain variable fragments; SNPs, single nucleotide polymorphisms; SPAD, single pass albumin dialysis; SR-A, class A scavenger receptor; T₃, triiodothyronine; T₄, thyroxine; tHSA, truncated HSA; Trx, thioredoxin; TrT₃, total reverse T₃; TT₃, total T₃; TT₄, total T₄.

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1. Introduction

The name albumin derives from the early German term *albumen*, generally indicating proteins. *Albumen*, on the other hand, derives from the Latin word *albus* (white) indicating the white part of the cooked egg surrounding the yolk. The protein component of the egg white makes part of a heterogeneous group of proteins, broadly indicated as albumins, including serum albumin (SA), milk albumin, urinary proteins, and the secretion of the snail. More specifically, SA is a member of a family of homologous proteins characterized by distinctive structural features and peculiar ligand binding properties. Members of this family are α -fetoprotein (AFP), afamin (AFM; also named α -albumin), and vitamin D binding protein (DBP) (Peters, 1996; Fasano et al., 2007).

Human serum albumin (HSA), the most abundant protein in plasma, is a monomeric multi-domain macromolecule, representing the main determinant of plasma oncotic pressure and the main modulator of fluid distribution between body compartments (Peters, 1996; Evans, 2002; Mendez et al., 2005a). HSA displays an extraordinary ligand-binding capacity, providing a depot and carrier for many endogenous and exogenous compounds. Indeed, HSA represents the main carrier for fatty acids (FA), affects pharmacokinetics of many drugs, provides the metabolic modification of some ligands, renders potential toxins harmless, accounts for most of the anti-oxidant capacity of human plasma, and displays (pseudo)-enzymatic properties (Sudlow et al., 1975; Carter and Ho, 1994; Peters, 1996; Bertucci and Domenici, 2002; Curry, 2002, 2009; Kragh-Hansen et al., 2002; Sakurai et al., 2004; Sułkowska et al., 2006; Ascenzi et al., 2005a, 2006a; Bocedi et al., 2005; Fasano et al., 2005; Ghuman et al., 2005; Fanali et al., 2007; Yang et al., 2007; Ascenzi and Fasano, 2009, 2010). HSA is a valuable biomarker of many diseases, including cancer, rheumatoid arthritis, ischemia, post-menopausal obesity, severe acute graft-versus-host disease, and diseases that need monitoring of the glycemic control (Gupta and Lis, 2010; Koga and Kasayama, 2010; Sbarouni et al., 2011). Moreover, HSA is widely used clinically to treat several diseases, including hypovolemia, shock, burns, surgical blood loss, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, hemodialysis, acute liver failure, chronic liver disease, nutrition support, resuscitation, and hypoalbuminemia (Tullis, 1977a,b; Alexander et al., 1982; Erstad et al., 1991; Hastings and Wolf, 1992; Haynes et al., 2003; Alderson et al., 2004; Liberati et al., 2006). Recently, biotechnological applications of HSA, including implantable biomaterials, surgical adhesives and sealants, biochromatography, ligand trapping, and fusion proteins, have been reported (Bertucci et al., 2010; Furukawa et al., 2011; Komatsu et al., 2011a).

Some pathophysiological properties of HSA were recognized in 400 BC by Hippocrates of Cos, who reported in *Aphorisms* that a foamy urine, in all likelihood caused by the presence of HSA, indicates kidney disease (Coar, 1822). SA was named a century and half ago (Denis, 1859) and deeply investigated over the last century (Peters, 1996). In 1932, HSA was separated from plasma proteins (Race, 1932); in 1934, HSA was crystallized (Hewitt, 1936); in 1939, the neutral-to-acid (*i.e.*, N-to-F) conformational transition was detected (Luetscher, 1947); in 1940, HSA was purified for intravenous use as a blood substitute (Cohn, 1941); in 1954, the first two cases of analbuminemia were reported (Bennhold et al., 1954); in 1960, the HSA “domain” structure was proposed (Foster, 1960); in 1975, the primary structure of HSA was deduced (Meloun et al., 1975), and the characterization of specific drug binding sites started (Sudlow et al., 1975); in 1979, the HSA gene was isolated (Sargent et al., 1979); in 1981, the nucleotide sequence of HSA cDNA was reported (Lawn et al., 1981); in 1986, the complete gene sequence of HSA was determined (Minghetti et al., 1986), and the expression, the cleavage, and the secretion of HSA from cultured yeast *Saccharomyces cerevisiae* was reported (Hinchcliffe and Kenney, 1986); starting from 1989, many HSA mutations were localized (Peters, 1996; Minchiotti et al., 2008; Otagiri and Chuang, 2009); in 1992, the “heart-shaped” three-dimensional structure of HSA was determined (He and Carter, 1992); in 1995, the ferrous tetraphenylporphyrinatoiron (FeP(II)) was incorporated in HSA to obtain a red blood cell substitute (Komatsu et al., 1995); in 1999, the role of HSA in heme-Fe scavenging was highlighted (Miller and Shalkai, 1999), and human serum heme-albumin (HSA-heme) was engineered to become a O₂ carrier (Carter et al., 1999; Komatsu et al., 2004a); and starting from 2001, the detoxifying role of HSA-heme-Fe towards reactive nitrogen species (RNS) and reactive oxygen species (ROS) was underlined (Monzani et al., 2001; Ascenzi and Fasano, 2007; Ascenzi et al., 2009a).

Here, structure-function relationships of HSA are reported and discussed with an emphasis to issues related to human health.²

2. From gene to protein

2.1. Human serum albumin gene

HSA synthesis is governed by a single copy gene (*ALB*; GenBank reference sequence NC_000004.11) lying on the long arm of chromosome 4, near the centromere at position q11–22, where the genes of the other members of the SA family are located (*i.e.*, *AFP*, *AFM*, and *DBP*) (Mikkelsen et al., 1977; Harper and Dugaiczky, 1983; Minghetti et al., 1986). The genes of the SA family are recognized to occur in tandem also on rat chromosome 14 (Cooke et al., 1987), on mouse chromosome 5 (Yang et al., 1990), on pig chromosome 8 (Johansson et al., 1992), and on chicken chromosome 6 (Palmer and Jones, 1986). In humans, the *AFP* gene lies 14.5 kilobase pairs (kbp) downstream (in the 3′-phosphodeoxyribose direction) from *ALB* (Urano

² Most of data concerning HSA obtained prior than 1996 have been reported in the invaluable book entitled “All about Albumin: Biochemistry, Genetics, and Medical Aspects” by Peters (1996).

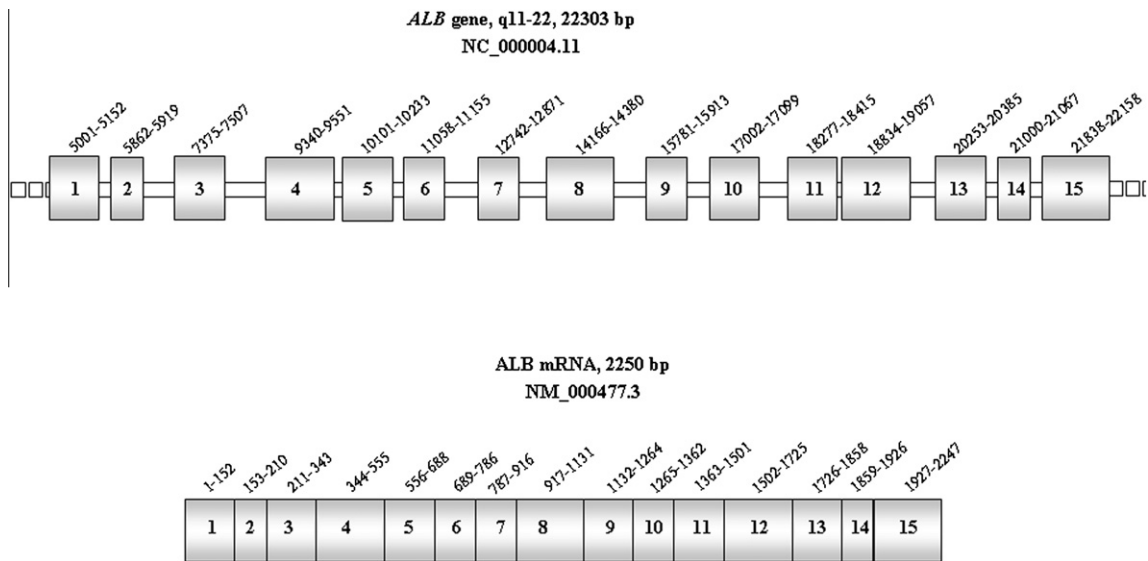


Fig. 1. *ALB* gene and mRNA organization. The *ALB* gene lies on the long arm of chromosome 4, near the centromere at position q11-22, and is composed of 22306 base pair (bp) subdivided in 15 exons and 14 introns. The exons and introns numbering is based on the GenBank reference sequence NC_000004.11. The *ALB* transcript is composed of 2250 bp; the exons bp numbering is based on the GenBank reference sequence NM_000477.3. For details, see Sections 2.1. and 2.2.

et al., 1984), and the *AFM* gene lies 10 kbp downstream from *AFP* (Bélanger et al., 1994); whether the *DBP* gene lies upstream or downstream of the other three is still nor clear (Peters, 1996). *Alb* spans 22303 nucleotides from the putative “cap” site to the first poly(A) addition site, and contains 15 exons separated by 14 introns (Fig. 1). The symmetrical location of intervening sequences within the structure of the HSA protein is an impressive feature of this gene. Indeed, the introns split the polypeptide chain between codon positions 3 and 1 and between codon positions 2 and 3 in an alternating pattern, which is repeated in all three domains of HSA, thus strengthening the argument that *ALB* arose via triplication of a single primordial domain (Sargent et al., 1981).

In humans, the *Alu* elements (180–300 base pairs (bp) in length) occur twice in intron 2 and once in introns 7, 8, and 11. These appear to be recent invaders, occurring since the mammalian radiation during evolution, and may be the cause of the human *ALB* gene being longer than the corresponding rat gene (Sargent et al., 1981; Minghetti et al., 1986; Peters, 1996).

The *ALB* gene shows several DNA mutations that may result in the presence of a HSA genetic variant (bisalbuminemia or alloalbuminemia) or in the virtual absence of HSA (analbuminemia) (Peters, 1996) (see Table 1). In addition to these HSA genetic variants, 91 single nucleotide polymorphisms (SNPs) without any known clinical or biological effect are currently reported in the SNP data bank of NCBI (Minchiotti et al., 2008).

2.2. Human serum albumin transcript

Although HSA as isolated from the blood contains 585 amino acids, when first translated from its messenger RNA (mRNA) (GenBank reference sequence NM_000477.3) it carries at the *N*-terminus the pro-peptide, composed of six amino acids, which is preceded by the signal peptide, composed of 18 residues; both peptides are removed during processing in the hepatocyte cytoplasm, before the mature HSA is secreted (see Section 2.7). Thus, the translated portion of the mRNA contains 1827 nucleotides, corresponding to 609 amino acids (Fig. 1). Actually, the mRNA includes about 2250 nucleotides, because about 400 nucleotides at its ends are untranslated (Peters, 1996). Each hepatocyte contains about 3000 molecules of HSA mRNA processed from pre-mRNA from a single gene; the splicing process to remove the 14 introns appears to be highly efficient (Peters, 1996).

The 5' region of the *ALB* gene contains the conserved features found in many eukaryotic promoters up to position –250 from the transcription start site, implying a functional role in gene expression. Upstream from the –250 position, the homology drops off abruptly. Located at position –88 lies the CAT box (Benoit et al., 1980), and at position –32 lies the TATA box (Goldberg, 1979). An identical TATA box sequence is located also at position –793, but an obvious CAT box does not appear to accompany this upstream sequence. Therefore, the –793 TATA box may be functional *in vivo* or possibly represents a relic promoter element. Furthermore, a purine-rich (Pu)₃₉ stretch has been found at position –904, and a stretch of alternating pyrimidine-purine residues (PyPu)₂₄ lies at position –511 in the *ALB* gene. In addition to the presence of these alternating PyPu nucleotides in the 5' region of the gene, both a (CA)₁₂ sequence within intron 4 and a (PyPu)₃₂ region within intron 11 have been described (Minghetti et al., 1986). The latter sequence is located on the immediate 5' side of an *Alu* repetitive element. Overall, these sequences possess the potential to form Z-DNA structures (Wang et al., 1979). Note that left-handed helical conformation of Z-DNA has been implicated in genetic recombination (Miesfeld et al., 1981), higher order structure of chromosomal DNA (Arndt-Jovin et al., 1983), and regulation of genetic expression (Nordheim et al., 1981; Nordheim and Rich, 1983).

Table 1

Human serum albumin natural occurring genetic variants.

Mutation name	Base change ^a	Exon (E) ^a Intron (I)	Protein change	Domain	Functional effects	References
<i>Pro-albumin (proAlb) variants</i>						
Malmö I (also named Ildut, Kaikoura, Tradate, Redhill)	67C>T	E1	Arg23Cys	IA	Low Ni (II) affinity	Rousseaux et al. (1982) Brand et al. (1984) Hutchinson and Matejtschuk (1985) Brennan et al. (1990a) Brennan et al. (1990b) Galliano et al. (1990) Carlson et al. (1992)
Lille also named Pollibauer, Somalia, Tokushima, Taipei, Fukuoka-2, Varese, Wu Yang, Mayo, Komagone-3)	68G>A	E1	Arg23His	IA	Low Ni (II) affinity	Abdo et al. (1981) Matsuda et al. (1986) Takahashi et al. (1987c) Arai et al. (1989b) Galliano et al. (1990) Madison et al. (1991) Zan et al. (1993) Kragh-Hansen et al. (1994)
Christchurch (also named Gainesville, Honolulu-2, Fukuoka-3, Mayo, Shizuoka, Zagreb)	71G>A	E1	Arg24Gln	IA	Low Cu (II) and Ni (II) affinity	Brennan and Carrell (1978) Brennan and Carrell (1980) Abdo et al. (1981) Fine et al. (1983) Takahashi et al. (1987c) Arai et al. 1989b Arai et al. (1989c) Madison et al. (1991) Kragh-Hansen et al. (1994) Kragh-Hansen et al. (2004)
Takefu (also named Honolulu-1)	71G>C	E1	Arg24Pro	IA	Low Ni (II) affinity	Takahashi et al. (1987c)
Jaffna	71G>T	E1	Arg24Leu	IA	No effect	Galliano et al. (1989)
<i>Albumin (Alb) variants</i>						
Blenheim (also named Bremen, Malmö-II, Iowa City-2)	74A>T	E1	Asp25Val	IA	Low Ni (II) affinity	Brennan et al. (1989) Arai et al. (1990) Madison et al. (1991) Kragh-Hansen et al. (1994)
Larino	79C>T	I1	His27Tyr	IA	Low HSA stability (10–12% of total HSA)	Madison et al. (1994)
Analb Baghdad	79+1G>A	E1	Undetected splicing defect	IA	Analbunemia	Campagnoli et al. (2002)
Nagasaki-3	81C>A/G	E1	His27Gln		Low Ni (II) affinity	Takahashi et al. (1987c)
Analb Codogno	166C>T	E2	Gln56X	IA	Analbunemia	Watkins et al. (1994b)
Analb Kayseri	228_229delAT	E3	Val78CysfsX2	IA	Analbunemia	Galliano et al. (2002)
Torino	250G>A	E3	Glu84Lys	IA	No effect	Galliano et al. (1990)
Dalakarlia-1	259G>A	E3	Asp87Asn	IA	N-glycosylated at Asn87 High thermal stability	Arai et al. (1990) Carlson et al. (1992) Sakamoto et al. (1995) Kragh-Hansen et al. (2005)
FDH-1	269T>C	E3	Leu90Pro	IA	High T3 affinity	Sunthornthepvarakul et al. (1998)
Vibo Valentia	316G>A	E3	Glu106Lys	IA	No effect	Arai et al. (1989c) Galliano et al. (1990)

Yanomama-2	412C>G	E4	Arg138Gly	IB	Low bilirubin affinity	Lorey et al. (1984)
Analb Bethesda	412C>T	E4	Arg138X	IB	Analbuminemia	Takahashi et al. (1987b)
Nagoya	427G>A	E4	Glu143Lys	IB	No effect	Watkins et al. (1994b)
Tregasio	437T>A	E4	Val146Glu	IB	No effect	Campagnoli et al. (2005a)
Komagome-2	455A>G	E4	His152Arg	IB	No effect	Arai et al. (1990)
Asola	491A>G	E4	Try164Cys	IB	No effect	Minchiotti et al. (1997)
Corea	593A>T	E5	Lys198Ile	IIA	Bisalbuminemia	Madison et al. (1991)
Zonguldak	597T>A	E5	Undetected splicing defect	IIA	Analbuminemia	Minchiotti et al. (1995)
Hawkes Bay	602G>T	E5	Cys201Phe	IIA	Low stability (5% of total HSA)	Kim et al. (2010a,b)
Analb Vancouver	714-2A>G	I6	Undetected splicing defect	IIA	Analbuminemia	Caridi et al. (2008)
Analb Seattle	713+1G>A	I6	Trp238X	IIA	Analbuminemia	Brennan and Fellowes (1993)
FDH-2	725G>A	E6	Arg242His	IIA	High T4 affinity	Ruffner and Dugaiczuk (1988)
FDH-3	725G>C	E6	Arg242Pro	IIA	Low warfarin affinity	Watkins et al. (1994b)
Tradate-2	745A>C	E6	Lys249Gln	IIA	High T4 affinity	Petersen et al. (1994)
Herborn	790A>G	E7	Lys264Glu	IIA	Low warfarin affinity	Petersen et al. (1995)
Analb El Jadida	802G>T	E7	Glu268X	IIA	No effect	Sunthornthepvarakul et al. (1994)
Analb Roma	872insA	E7	Asn291LysfsX8	IIA	No effect	Madison et al. (1994)
Skaane SA	875A>G	E7	Glu292Arg	IIA	No effect	Minchiotti et al. (1993)
Niigata (also named Nagasaki- 1)	878A>G	E7	Asp293Gly	IIA	High prostaglandin affinity	Campagnoli et al. (2005b)
Caserta	900G>C	E7	Lys300Asn	IIB	High stability (60-65% of total HSA)	Watkins et al. (1994a)
Bologna	920delT	E8	Leu331ArgfsX22	IIB	Analbuminemia	Carlson et al. (1992)
Canterbury (also named Tagliacozzo, Cooperstown, New Guineal, IRE-1)	1011G>T	E8	Lys337Asn	IIB	Low drug affinity	Sugita et al. (1987)
					Low thermal stability	Arai et al. (1989c)
						Kragh-Hansen et al. (1990)
						Madison et al. (1994)
						Dagnino et al. (2010a)
						Tárnoky and Lestas (1964)
						Galliano et al. (1986b)
						Brennan and Herbert (1987)
						Huss et al. (1988b)
						Arai et al. (1990)
						Kragh-Hansen et al. (1990)
						Sakamoto et al. (1991)
						Carlson et al. (1992)
						Madison et al. (1994)
						Savva et al. (1998)
						Kragh-Hansen et al. (2005)
Bergamo	1013A>G	E8	Asp338Gly	IIB	No effect	Minchiotti et al. (1997)
Brest	1013A>T	E8	Asp338Val	IIB	High FA affinity	Minchiotti et al. (1999)
Orebro SW (also named Malmö-4)	1026C>G	E8	Asn342Lys	IIB	No effect	Carlson et al. (1992)
Redhill	1030G>A	E1;E8	Arg23Cys; Ala344Thr	IIB	High FA affinity N-glycosylated at Asn342	Abdo et al. (1981)
						Brand et al. (1984)
						Hutchinson and Matejtschuk (1985)
						Brennan et al. (1990a)
						Nielsen et al. (1997)
Roma	1033G>A	E8	Glu345Lys	IIB	No effect	Galliano et al. (1998)

(continued on next page)

Table 1 (continued)

Mutation name	Base change ^a	Exon (E) ^a Intron (I)	Protein change	Domain	Functional effects	References
Sondrio	1069G>A	E8	Glu357Lys	IIB	No effect	Porta et al. (1980)
Hiroshima-1	1132A>G	E9	Glu378Lys	IIB	No effect	Minchiotti et al. (1992)
Coari I (also named Porto Alegre-1)	1144G>A	E9	Glu382Lys	IIB	No effect	Arai et al. (1989c)
Trieste	1149G>T/C	E9	Lys383Asn	IIB	Low thermal stability	Arai et al. (1989a)
Parklands	1165G>C	E9	Asp389His	IIIA	Low drug affinity	Galliano et al. (1998)
Iowa City-1	1166A>T	E9	Asp389Val	IIIA	No effect	Kragh-Hansen et al. (2005)
Naskapi (also named Mersin, Komagone-1)	1186A>G	E9	Lys396Glu	IIIA	No effect	Brennan (1985)
Nagasaki-2	1195G>A	E9	Asp399Asn	IIIA	No effect	Kragh-Hansen et al. (1990)
Milano Slow	1195G>C	E9	Asp399His	IIIA	No effect	Madison et al. (1991)
Tochigi	1198G>A	E9	Glu400Lys	IIIA	No effect	Franklin et al. (1980)
Malmö-3	1198G>C	E9	Glu400Gln	IIIA	No effect	Takahashi et al. (1987a)
Hiroshima-2	1216G>A	E9	Glu406Lys	IIIA	No effect	Madison et al. (1991)
Analb Roma-2	1225C>T	E9	Glu409X	IIIA	Analbunemia	Takahashi et al. (1987b)
Monastir	1275C>A	E10	Tyr425X	IIIA	Analbunemia	Galliano et al. (1998)
Liprizzi	1300C>T	E10	Arg434Cys	IIIA	High S-nitrosylation	Caridi et al. (2009)
Analb Fondi	1427A>G	E11;E12	Tyr476SerfsX13	IIIA	Analbunemia	Galliano et al. (1995)
Analb Bartin	1428+2T>C	I11	Leu431TyrrfsX5	IIIA	Analbunemia	Galliano et al. (1998)
Dublin	1507G>A	E12	Glu503Lys	IIIB	No effect	Ishima et al. (2007)
Casebrook	1552G>A	E12	Asp518Asn	IIIB	N-glycosylated at Asn518 High FA affinity	Campagna et al. (2005)
Vancouver (also named Brimingham, Adana, Porto Alegre-II, Manaus-I, Lambadi, Kashmir)	1573G>A	E12	Glu525Lys	IIIB	No effect	Dolcini et al. (2007)
Ortonovo	1585G>A	E12	Glu529Lys	IIIB	No effect	Sakamoto et al. (1991)
Tubingen	1595C>T	E12	Arg533X	IIIB	Analbunemia	Peach and Brennan (1991)
Analb Locust Valley	1610delT	E12	Ile537AsnfsX21	IIIB	Analbunemia	Haynes et al. (1992)
Safranbolu	1614_1615delCA	E12	Leu540PhefsX2	IIIB	Analbunemia	Minchiotti et al. (1997)
Maddaloni	1669G>A	E12	Val557Met	IIIB	No effect	Nielsen et al. (1997)
Castel di Sangro	1678A>G	E12	Lys560Glu	IIIB	No effect	Galliano et al. (1993)
Maku (also named Wapishana, Oriximina-1)	1693A>G	E12	Lys565Glu	IIIB	High FA affinity	Ruhoff et al. (2010)
Mexico	1721A>G	E12	Asp574Glu	IIIB	No effect	Davis et al. (2008)
Dalakarlia-2 (also named Malmö-62)	1721A>C	E12	Asp574Ala	IIIB	No effect	Dagnino et al. (2010b)
Church Bay	1750A>G	E13	Lys584Glu	IIIB	No effect	Minchiotti et al. (1997)
						Minchiotti et al. (1990)
						Takahashi et al. (1987b)
						Arai et al. (1989a)
						Nielsen et al. (1997)
						Kragh-Hansen et al. (2005)
						Takahashi et al. (1987a)
						Carlson et al. (1992)
						Chua et al. (1998)

Fukuoka-1 (also named Ube-1, Varese-2, Paris-2)	1759G>A	E13	Asp587Asn	IIIB	High FA affinity	Arai et al. (1990) Minchiotti et al. (1992) Madison et al. (1994) Nielsen et al. (1997)
Osaka-1	1765G>A	E13	Glu589Lys	IIIB	No effect	Arai et al. (1990)
Bazzano	1771delT	E13	Cys591AlafsX17	IIIB	High FA affinity	Madison et al. (1994) Nielsen et al. (1997)
Verona (also named B, Oliphant, Osaka-24, Phnom Penh, Nagano, Iowa City-3, Mayo, Victoria (East India), Saitama-1)	1780G>A	E13	Glu594Lys	IIIB	Low thermal stability	Winter et al. (1972) Minchiotti et al. (1987) Arai et al. (1989b) Arai et al. (1989c) Arai et al. (1990) Madison et al. (1991) Madison et al. (1994) Kragh-Hansen et al. (2005)
Rugby Park	1785+1G>C	I13	Gly596_Leu 609 delins7	IIIB	High FA affinity	Peach et al. (1992) Nielsen et al. (1997)
Banks Peninsula	1786-15T>A	I13	Gly596_Leu609 delinsSerLeu CysSerGly	IIIB	No effect	Brennan et al. (1999)
Venezia	1786_1814+ 1delinsAAAAT	E13;I14	Gly596ProfsX10	IIIB	Low thermal stability	Minchiotti et al (1989) Watkins et al. (1991) Kragh-Hansen et al. (2005)
Krapina (also named Milano Fast)	1789A>G	E13	Lys597Glu	IIIB	No effect	Iadarola et al. (1985) Madison et al. (1994) Kragh-Hansen et al. (2004)
Vanves	1794A>T/C	E13	Lys598Asn	IIIB	No effect	Minchiotti et al. (1987)
Kenitra	1794dupA	E13	Leu599ThrfsX30	IIIB	O-glycosylated at Thr62 Low thermal stability	Minchiotti et al. (2001) Kragh-Hansen et al. (2005)
Catania	1810delC	E13	Gln604LysfsX4	IIIB		Galliano et al. (1986b) Watkins et al. (1991)

^a Base change and exon numbering are based on the mRNA sequence NM_000477.3 (updated 04/10/2011). Subtract 24 from amino acid numbers to convert to starting at the Asp1 of mature HSA.

Exon 1 begins with the “cap” site (*i.e.*, codon AGC) where transcription starts. Exon 1 is only partially translated, beginning with the initial Met at bp 40. In the untranslated portion comprised between the bp –14 and –29, lies the hypothetical site for the 18S ribosomal rRNA binding to the mRNA (Minghetti et al., 1986; Urano et al., 1986). Binding sites for the H1 histone lie in the antisense DNA strand of intron 1, overlapping into the preceding exon 1 (Sevall, 1988).

Exon 14 lies at the other end of the molecule and is only partially translated. In particular, its first 42 nucleotides codify for 14 codons that come before the terminator codon (*i.e.*, codon TAA). Exon 15, corresponding to the 3' untranslated region of the *ALB* gene, is completely untranslated and contains three AATAAA polyadenylation (poly(A)) signals necessary for mRNA termination, and modulating mRNA stability in the cytoplasm (Birnstiel et al., 1985; Conway and Wickens, 1985; Minghetti et al., 1986; Peters, 1996). The first poly(A) signal, producing the shortest HSA mRNA (Dugaiczky et al., 1982), is located 165 bp downstream from the translation termination codon. About 150 bp downstream is the second functional poly(A) signal (Lawn et al., 1981), while the third poly(A) signal is located at further 70 bp downstream, but its role is still obscure (Minghetti et al., 1986; Peters, 1996). In addition to these three signals, other 18 poly(A) signals are scattered throughout the introns of the *ALB* gene. Since these 18 sequences are not functional, there must be other elements, bordering the functional AATAAAs signals, recognized by the processing machinery. It has been speculated that transcription of the *ALB* gene continues well beyond the poly(A) addition site, as shown for other eukaryotic genes (Hagenbüchle et al., 1984; Falck-Pedersen et al., 1985; Rohrbaugh et al., 1985; Minghetti et al., 1986) therefore it would require processing at the 3' terminus. Several other motifs able to form potential secondary structures (*i.e.*, the consensus sequence TTTTCACTGC, the GT clusters, and the short inverted repeats) have been described at the 3' untranslated region of the *ALB* gene (Benoist et al., 1980; Birnstiel et al., 1985; Minghetti et al., 1986).

Splicing of pre-mRNA transcripts is a critical step in the cascade of gene expression. In humans, the single-copy *ALB* gene must be “turned on” constitutively in order to produce constant circulating levels of HSA. Thus, it has been speculated that the *ALB* gene must have not only efficient promoter and 3' processing signals, but also splicing signals that are effectively recognized by the cell splicing machinery. The “non-coding” exon 15 seems to be conserved within species and to have a yet unclear role during the polyadenylation reaction (Minghetti et al., 1986).

2.3. Human serum albumin genetic variants

In 1955, a double HSA band was recognized in the electrophoretic pattern of a 25-year-old woman with diabetic ketosis (Scheurlen, 1955); the abnormal (slower) band disappeared in a few months, after her condition improved. In 1959, it was found that several close relatives of the patient also showed a second HSA, and in one of these individuals the second band was not transient (Wuhrmann, 1959). Meanwhile, slower HSA electrophoretic bands were reported, each band amounting to half of normal HSA (also termed Albumin A; Alb A) concentration (Knedel, 1957; Nennstiel and Becht, 1957). The transient HSA band seems to result from the alteration of the protein mobility due to either a bound ligand(s) or a proteolytic cleavage(s) or from leakage of pro-albumin (proAlb) from the liver cell (Peters, 1996). Alb Naskapi (Lys372Glu) was next discovered during a survey of gene frequencies in the North American Indians and Eskimos (Melartin and Blumberg, 1966). This study established hereditary bisalbuminemia as the result of expression of both alleles of the single *ALB* gene. Penetrance is complete because each allele codifies for half of the total HSA, and there is no suppression by the presence of a mutation. Remarkably, while in the transient bisalbuminemia the abnormal band is generally significantly weaker than Alb A, in the hereditary form both bands are of equal intensity (Peters, 1996).

Since only about one-third of HSA amino acids are charged, approximately half of single residue substitutions do not alter the net HSA charge, being thus undetectable by electrophoresis. To date, only the HSA variant with increased affinity for L-thyroxine (T₄, 3,3',5,5'-tetraiodo-L-thyronine), leading to familial dysalbuminemic hyperthyroxinemia (FDH), displays an altered function (Peters, 1996; Petitpas et al., 2003) (see Section 8.3.2).

In 1958, a slow HSA band, termed Albumin B (Alb B), was found in the plasma of 25 of 56 members of an American family of Norwegian heritage (Earle et al., 1958). Afterwards, the practice of naming HSA variants on the basis of the geographical area of their discovery was established (Tárnoky and Lestas, 1964).

Bisalbuminemia (also named alloalbuminemia) is an autosomal dominant rare disorder or acquired abnormality, characterized by either the occurrence of two distinct HSA bands (representing the wild type and the variant protein) or by a single widened HSA band (Tárnoky, 1980). Despite the fact that bisalbuminemia is observed with a frequency of 0.0003–0.0010 in the average population, its role in various pathological states has not yet been clearly defined. Remarkably, HSA variants could display abnormal binding affinities for different ligands with possible metabolic effects (see Sections 2.3 and 8.3).

To date, 83 genetic variants of HSA (alloalbumins) have been described. Among these, 66 mutations result in bisalbuminemia and 17 are responsible of the virtual absence of the protein from the blood (analbuminemia) (see Table 1) (Minchiotti et al., 2008; Caridi et al., 2009; Dagnino et al., 2010a,b; Kim et al., 2010a; Ruhoff et al., 2010). Of these, 21 genetic variants have been found in domain I (15 in subdomain IA, and 6 in subdomain IB), 25 in domain II (13 in subdomain IIA, and 12 in subdomain IIB), and 37 in domain III (13 in subdomain IIIA, and 24 in subdomain IIIB) (see Table 1). Note that mutations affecting the HSA binding sites are less likely to be detected because they would occur in intramolecular regions poorly accessible to solvent (Minchiotti et al., 2008).

All the HSA variants are localized within either the coding regions or the intron-exon junctions of *ALB* (Dolcini et al., 2007; Minchiotti et al., 2008; <http://www.albumin.org>). Overall, these mutations can be classified as follows: 60 missense mutations leading to the synthesis of a HSA variant having one amino acid changed (Peters, 1996; Caridi et al., 2008; Minchiotti et al., 2008; Kim et al., 2010a); 7 nonsense mutations creating a premature stop codon (Papi et al., 1983; Watkins et al.,

1994a,b; Campagna et al., 2005; Campagnoli et al., 2005a,b; Caridi et al., 2009; Ruhoff et al., 2010); 11 frameshift mutations leading to premature stop codons (Galliano et al., 1986a, 2002; Minchiotti et al., 1989, 2001; Watkins et al., 1991, 1994a; Madison et al., 1994; Campagna et al., 2005; Dolcini et al., 2007; Davis et al., 2008; Dagnino et al., 2010a,b); one insertion leading to a defective intron splicing and to the creation of a premature stop codon (Alb Rugby Park; Gly596_Leu609delins7) (Peach et al., 1992); one insertion leading to the activation of a cryptic acceptor splice site, the deletion of 15 bp and the consequent synthesis of a shorter HSA variant (Alb Banks Peninsula; Gly596_Leu609delinsSerLeuCysSerGly) (Brennan et al., 1999); and three undetected splicing variants (Alb Baghdad, Alb Zonguldak, Alb Vancouver) (Ruffner and Dugaiczky, 1988; Campagnoli et al., 2002; Caridi et al., 2008) (Table 1).

The majority of the HSA variants results from single residue substitutions and almost all have net charges which differ from normal HSA at physiological pH: Alb Vibo Valentia (Glu82Lys), Alb Roma (Glu321Lys), Alb Sondrio (Glu333Lys), Alb Vancouver (Glu501Lys), Alb Ortonovo (Glu505Lys), and Alb Verona (Glu570Lys) are all +2 variants (*i.e.*, they have two positive charges more than wild type HSA); Alb Blenheim (Asp1Val), Alb Niigata (Asp269Gly), Alb Brest (Asp314Val), Alb Parklands (Asp365His), Alb Milano Slow (Asp375His), and Alb Fukuoka-1 (Asp563Asn) are +1 variants (*i.e.*, they have one positive charge more than wild type HSA); Alb Tregasio (Val122Glu), Alb Caserta (Lys276Asn), Alb Canterbury (Lys313Asn), and Alb Trieste (Lys359Asn) are –1 variants (*i.e.*, they have one negative charge more than wild type HSA); and, Alb Tradate-2 (Lys225Gln), Alb Herborn (Lys240Glu), Alb Maku (Lys541Glu), Alb Church Bay (Lys560Glu), and Alb Krapina (Lys573Glu) are –2 variants (*i.e.*, they have two negative charges more than wild type HSA) (Kragh-Hansen et al., 2005) (Table 1).

Since the HSA synthesis is governed by a single copy gene codominantly expressed, heterozygous subjects carrying point mutations usually show the presence of the wild type and the variant proteins in the 1:1 ratio. Remarkably, Alb Larino (His3-Tyr), representing only 10–12% of the total HSA, is an exception to this rule. Its low expression is probably due to the fact that the mutated base is the last nucleotide of exon 1 and consequently its mutation could alter the splicing (Minchiotti et al., 2008). Furthermore, Alb Hawkes Bay (Cys177Phe) represents only 5% of total circulating HSA. Its low plasma level is probably due to molecular instability caused by the loss of the normal Cys168–Cys177 sulphydryl bridge and the rearrangement of one of the pre-existing Cys–Cys bridges (Brennan and Fellowes, 1993). Even more unusual is the finding for Alb Caserta (Lys276Asn), the amount of which is higher than that of normal HSA, thus suggesting an increased stability of the variant. Indeed, the Lys276 residue is exposed and may play an important role in the chemical modification of HSA during degradation (Madison et al., 1994).

Until 1980s, electrophoresis on cellulose acetate at pH 8.6 was the primary method used for the detection of HSA variants, since sharper separations could be obtained than using a filter paper, even if the identification by the position of the band was still difficult. Performing migration at three pH values (*i.e.*, 5.0, 6.9, and 8.6) allowed to classify about 50 HSA variants (Weitkamp et al., 1973; Porta et al., 1979; Fine et al., 1982). In 1985, the “CISEL” group (Comitato Italiano di Standardizzazione in Medicina di Laboratorio) proposed to operate HSA variants classification on electrophoretic migration relative to that of human transferrin as a reference (Burlina et al., 1985). Actually, HSA genetic variants are usually identified by electrophoresis under non-denaturing conditions, since almost all the identified amino acid substitutions are located on the surface of the protein molecule and are exposed to the solvent. Isoelectric focusing has been applied as a second stage of classification of HSA variants on the basis of its isoelectric point. This procedure allows a distinction of HSA variants and could be a useful technique when employed as a second step in the identification of HSA variants prior to determine the structural change(s) characterizing the variant (Rochu et al., 1991).

2.3.1. Pro-albumin variants

Among HSA variants, proAlb Malmö I (Arg-2Cys), proAlb Lille (Arg-2His), proAlb Christchurch (Arg-1Gln), proAlb Takefu (Arg-1Pro), and proAlb Jaffna (Arg-1Leu) are characterized by the retention of the pro-peptide. In fact, the missense mutations prevent the proteolytic cleavage of the HSA pro-peptide, but not protein secretion. Therefore, proAlb variants, in contrast to wild-type proAlb, can be isolated from plasma. The sequence of the pro-peptide shows that even though Arg-2 has been substituted by either His or Cys, and Arg-1 has been changed to either Gln or Pro or Leu, the net charge of proAlb is less negative than that of Alb A at physiological pH (Nielsen et al., 1997).

2.3.2. C-terminal human serum albumin variants

More extensive alterations of the polypeptide chain were observed in six C-terminal HSA mutants: Alb Bazzano (Cys567AlafsX17), Alb Rugby Park (Gly572_Leu585delins7), Alb Banks Peninsula (Gly572_Leu585delinsSerLeuCysSerGly), Alb Venezia (Gly572ProfsX10), Alb Kenitra (Leu575ThrfsX30), and Alb Catania (Gln580LysfsX4) (Table 1). These mutants are caused by either single nucleotide deletions and subsequent frameshifts (Alb Bazzano (Cys567AlafsX17) and Alb Catania (Gln580LysfsX4)) (Galliano et al., 1986a; Watkins et al., 1991; Madison et al., 1994; Nielsen et al., 1997) or by a single A duplication and the subsequent frameshift (Alb Kenitra; Leu575ThrfsX30) (Minchiotti et al., 2001; Kragh-Hansen et al., 2005) or by a 30-bp deletion and a 5-bp insertion resulting in exon skipping (Alb Venezia; Gly572ProfsX10) (Minchiotti et al., 1989; Watkins et al., 1991; Kragh-Hansen et al., 2005) or by two splicing errors (GT to CT splice mutation in intron 13 (Alb Rugby Park; Gly572_Leu585delins7) (Peach et al., 1992; Nielsen et al., 1997), and the insertion of an alternative splice site in intron 13 (Alb Banks Peninsula; Gly572_Leu585delinsSerLeuCysSerGly) (Brennan et al., 1999)).

Alb Kenitra (Leu575ThrfsX30) is peculiar because represents the only elongated variant of HSA so far identified (601 residues instead of 585 for the mature polypeptide chain), with a completely different amino acid sequence from residue 575 to

the C-terminal end. This new sequence contains two additional disulfide bridges and is partially O-glycosylated at Thr596 (Minchiotti et al., 2001; Kragh-Hansen et al., 2005) (see Section 2.3.3).

Although it is unclear whether the integrity of the C-terminal end of the protein is crucial for HSA secretion or for preventing its degradation in plasma, all the C-terminal truncated or elongated variants of HSA identified so far (ranging in size from 576 to 601 amino acid residues) are present in the plasma of heterozygous individuals in levels ranging from 2% to 30% of the total HSA amount. This led to the conclusion that any major structural alteration of the C-terminal region of the molecule is probably detrimental to the stability of the protein (Minchiotti et al., 2001). It has been proposed that the reduced recycling of HSA variants modified at the C-terminal region could be the reason for their diminished plasma concentration (Andersen and Sandlie, 2007). Normally, after cellular uptake, part of the HSA is bound to the intracellular neonatal Fc receptor FcRn, this protects HSA from intracellular proteases thus enabling its return to the circulation. Because the native structure of domain III of HSA is important for the protein–receptor interaction, mutations in domain III could reduce binding and thereby shorten the half-life of these HSA variants (Minchiotti et al., 2008).

2.3.3. Glycosylated human serum albumin variants

HSA is normally unglycosylated since its primary structure lacks the canonical Asn-X-Thr/Ser tripeptide acceptor sequence required for N-glycosylation. To date, three HSA N-glycosylated variants have been identified, namely Alb Dalakarla-1 (glycosylated at Asp63) (Arai et al., 1990; Carlson et al., 1992; Sakamoto et al., 1995), Alb Redhill (glycosylated at Asn318) (Brennan et al., 1990a), and Alb Casebrook (glycosylated at Asp494) (Peach and Brennan, 1991; Haynes et al., 1992; Minchiotti et al., 1997; Nielsen et al., 1997) (Table 1).

The structures of the oligosaccharides bound to Alb Dalakarla-1 (Asp63Asn) (Sakamoto et al., 1995) and Alb Casebrooks (Asp494Asn) (Haynes et al., 1992) have been found to be identical (i.e., $\alpha(2-6)$ -desialylated complex biantennary glycan). On the contrary, the glycan bound to Alb Redhill is largely unknown, but all carbohydrates are negatively charged due to the presence of sialic acid (Brennan et al., 1990a; Haynes et al., 1992; Sakamoto et al., 1995). Moreover, Alb Redhill is unique since it is characterized by the presence of two mutations: the Arg-2Cys missense mutation results in the normal hydrolysis of the pre-pro-albumin within the liver cell and in the formation of HSA still possessing an Arg at position -2, and the Ala320Thr mutation that leads to the glycosylation of the Asn318 residue (Brennan et al., 1990a).

Among the glycosylated HSA variants, Alb Kenitra (Leu575ThrfsX30) has not only an elongated polypeptide chain (see Section 2.3.2), but the four additional Cys residues form two new Cys–Cys bridges that determine a partial O-glycosylation at Thr596 by a monosialylated Hexose-N-Acetyl-hexosamine (HexHexNAc) structure (Kragh-Hansen et al., 1996, 2005; Minchiotti et al., 2001).

2.3.4. Human serum albumin variants with altered fatty acid binding properties

Under physiological conditions, more than 99% of the nonesterified FAs in plasma are bound to HSA (Spector, 1986). HSA is the transport and depot protein for nonesterified FAs in the blood, acting not only as a passive carrier in the circulation, but also facilitating FA removal from donor cells (Trigatti and Gerber, 1995; Peters, 1996). The association equilibrium constants for FA binding to HSA increase, but not linearly, with the chain length (Nielsen et al., 1997). To get information about the position of the FA high-affinity sites, 13 recombinant HSA isoforms mutated in four different subdomains were synthesized (Kragh-Hansen et al., 2006). Results obtained indicate that octanoate and decanoate bind preferentially to a single site in subdomain IIIA, laurate binds to sites in subdomains IIIA and IIIB, whereas myristate binds in subdomains IB and IIIB. Moreover, results obtained from the analysis of the recombinant mutants and of the natural genetic variants showed that FA binding is affected by amino acid substitutions (Kragh-Hansen et al., 2006). Amino acid substitutions of alloalbumins are almost all situated at the surface of HSA and usually have no effect on FA binding (Nielsen et al., 1997). Binding of FAs to different high affinity sites, and the sensitivity of the sites to amino acid substitutions elsewhere in the protein (and perhaps also to other types of modifications) are important factors in cases of simultaneous binding of ligands to HSA (see Section 4). These circumstances have to be taken into account, e.g., when patients are treated with drugs binding to HSA (Kragh-Hansen et al., 2006).

The most pronounced changes in FA binding to HSA are caused by mutations in domain III. Indeed, Alb Krapina (Lys573-Glu) and Alb Maku (Lys541Glu) are characterized by decreased FA affinity. However, laurate binding to Alb Catania (Gln580LysfsX4) and Alb Venezia (Gly572ProfsX109) is normal, thus indicating that the HSA C-terminus is not important for FA binding (Kragh-Hansen et al., 2004).

The FA binding properties of HSA can be affected by glycosylation, indeed different effects on FA binding are caused not only by different glycan structures but also by different locations of attachment (Kragh-Hansen et al., 2001). Glycosylation in subdomain IA (i.e., Alb Dalakarla-1; Asp63Asn) does not alter FA binding. By contrast, glycosylation in subdomain IIB (i.e., Alb Redhill; Arg-2Cys or Arg-2Cys,Ala320Thr) or subdomain IIIB (i.e., Alb Casebrook; Asp494Asn) results in increased FA binding. In particular, Alb Redhill binds FA 1.7 times the normal load, whereas Alb Casebrook has 8.6-fold the amount carried by its normal counterpart. Among the C-terminal variants, Alb Rugby Park (Gly572_Leu585delins7) and Alb Bazzano (Cys567AlafsX17) bind FA 3.9 and 1.5 times, respectively, the amount bound to their corresponding Alb A (Nielsen et al., 1997).

Overall, five structurally different alloalbumins bind more FAs (i.e., proAlb Jaffna (Arg-1Leu), Alb Maku (Lys541Glu), Alb Fukuoka-1 (Asp563Asn), Alb Bazzano (Cys567AlafsX17), and Alb Rugby Park (Gly572_Leu585delinsSerLeuCysSerGly)), 8 alloalbumins bind less FAs (i.e., proAlb Malmö-I (Arg-2Cys), proAlb Lille (Arg-2His), Arg-Alb (a variant of Alb Malmö I, possessing an extra Arg at the N-terminus (Brennan et al., 1990a)), Alb Torino (Glu60Lys), Alb Tradate-2 (Lys225Gln), Alb Caserta (Lys276Asn), Alb Parklands (Asp365Val), and Alb Vancouver (Glu501Lys)), and 10 alloalbumins bind FAs amounts comparable to that of

their normal counterpart (*i.e.*, Alb Dalakarlia-1 (Asp63Asn), Alb Hernborn (Lys240Glu), Alb Niigata (Asp269Gly), Alb Roma (Asn267LysfsX8), Alb Sondrio (Glu333Lys), Alb Ortonovo (Glu505Lys), Alb Verona (Glu570Lys), Alb Malmö-95 (the glycosylated form of Alb Dalakarlia-1), Alb Catania (Gln580LysfsX4), and Alb Venezia (Gly572ProfsX10)). Except for two cases (*i.e.*, proAlb Jaffna (Arg-1Leu) and Alb Redhill (Arg-2Cys or Arg-2Cys,Ala320Thr)), all the variants with increased FA binding capacity are modified in domain III. On the other hand, HSA variants with reduced FA binding capacity are characterized by a structural change in either domain I or II (Nielsen et al., 1997).

Overall, genetic modifications of HSA can affect, quantitatively and/or qualitatively, the *in vivo* FA binding properties; a severe hyperlipidemia and a marked lipodystrophy can occur in patients affected by analbuminemia (Peters, 1996).

2.3.5. Human serum albumin variants with altered metal binding properties

Changes of Ni(II), Ca(II), and Zn(II) affinity for six genetic HSA variants (*i.e.*, proAlb Lille (Arg-2His), proAlb Christchurch (Arg-1His), proAlb Blenheim (*i.e.*, Alb Blenheim (Asp1Val) to which the normal pro-peptide is still bound) (Brennan et al., 1989), Alb Blenheim (Arg1Val), Arg-Alb, and Alb Redhill (Arg-2Cys or Arg-2Cys,Ala320Thr)), for Alb A isolated from the same heterozygote carriers has been reported (Kragh-Hansen et al., 1994). However, different molecular bases for Ni(II), Ca(II), and Zn(II) binding and biological consequences for metal transport and delivery occur (Peters, 1996).

When Ni(II) binds to the N-terminal region of HSA, partially blocked by the presence of either a pro-peptide (as in proAlb Lille (Arg-2His) and in proAlb Christchurch (Arg-1Gln)) or of only an extra Arg residue (as in Arg-Alb and in Alb Redhill (Arg23Cys or Arg-2Cys,Ala320Thr)), values of the association equilibrium constant decrease by more than one order of magnitude (Brennan et al., 1989). Also Alb Blenheim (Arg1Val) is characterized by a value of the association equilibrium constant for the Ni(II) binding reduced by 50% when compared to Alb A (Kragh-Hansen et al., 1994).

Similarly, the value of the association equilibrium constant for Ca(II) binding decreases by about 60–80% by the presence of the pro-peptide, as observed in the Alb Blenheim variant (Asp1Val) (Kragh-Hansen et al., 1994). On the contrary, Alb Redhill (Arg23Cys or Arg-2Cys,Ala320Thr) binds Ca(II) more strongly than the normal protein, the value of the association equilibrium constant increasing by 125%. Probably the Ala320Thr mutation neutralizes the effect of the Arg-2Cys mutation, thus determining an increased affinity for Ca(II). Overall, since the N-terminal region of HSA, actively involved in metal ion binding, is partially blocked by the presence of the pro-peptide, the reduced Ni(II) and Ca(II) affinity could be explained by the persistence of this sequence in the proAlb variants (Kragh-Hansen et al., 1994; Otagiri and Chuang, 2009).

In contrast with Ni(II) and Ca(II) binding, HSA shows heterogeneity with regard to Zn(II) binding. In fact, the association equilibrium constant for Zn(II) binding increases to 125% in the case of proAlb Lille (Arg-2His), decreases to 50–60% in the case of proAlb Christchurch (Arg-1Gln) and Alb Redhill (Arg23Cys or Arg-2Cys,Ala320Thr), and is normal in the case of pro-Alb Blenheim, Alb Blenheim (Asp1Val), and Arg-Alb (Kragh-Hansen et al., 1994).

2.3.6. Human serum albumin variants with altered hormone binding properties

Alb Niigata (Asp269Gly), Alb Roma (Glu321Lys), Alb Parklands (Asp365His), and Alb Verona (Glu570Lys) are all characterized by normal progesterone-binding properties, whereas Alb Canterbury (Lys313Asn) displays a 1.5 times higher binding association constant for progesterone with respect to normal HSA. Alb Roma (Glu321Lys) displays a lower affinity for testosterone with respect to normal HSA, whereas Alb Niigata (Asp269Gly) binds prostaglandin F_{2α} with a 2.4 times higher affinity with respect to Alb A. Lastly, the T₄ affinity for all five variants of HSA and their corresponding Alb A are comparable (Kragh-Hansen et al., 1990a).

The effects of mutations on ligand-binding to HSA are generally small, and probably not clinically significant. Exceptions are three HSA variants (*i.e.*, FDH-1 (Leu66Pro), FDH-2 (Arg218His), and FDH-3 (Arg218Pro)), which form strong binding sites for either triiodothyronine (T₃) and/or T₄ (see Table 1). The FDH-1 variant gives rise to the familial dysalbuminemic hypertriiodothyroninemia syndrome, and the FDH-2 and FDH-3 variants result in the familial dysalbuminemic hyperthyroxinemia syndrome (see Section 8.3.2). This syndrome is the most common cause of inherited euthyroid hyperthyroxinemia in Caucasian populations. A very similar third syndrome has been found and named familial hyperzincemia (see Section 8.3.3); however, the HSA mutation causing this condition is unknown (Failla et al., 1982).

2.4. Albumin variants and human populations

The prevalence of HSA alleles detected by electrophoresis in the average human population is low (*i.e.*, 0.0003–0.001) (Neel et al., 1988; Carlson et al., 1992; Peters, 1996). The variant with the widest geographical distribution is Alb Coari I (Glu358Lys), found broadly in Brazil, India, United Kingdom, and Canada (Franco et al., 1999). Alb Vancouver (Glu501Lys) is the form most often reported among Caucasians living in India, Japan, Cambodia, and several European countries (Carlson et al., 1992).

In Europe, the predominant HSA variant alleles are proAlb Lille (Arg-2His) and proAlb Christchurch (Arg-1His) (Arai et al., 1990), having a total heterozygotes frequency of 0.003. A survey of nearly 200,000 plasma samples in Sweden found proAlb Lille (Arg-2His) plus Alb Canterbury (Lys313Asn) with a frequency of 0.006 (Carlson et al., 1992). A high number of different variants have been reported in Italy with respect to any other country; however, this seems to reflect the magnitude and duration of the survey rather than the greater frequency of bisalbuminemia in Italy (Minchiotti et al., 2008). In particular, records of the distribution of nine allotypes in Italy indicates the dominance of a specific HSA variant in a local area: Alb Milano slow (Asp375His) in Lombardia; Alb Milano slow (Asp375His), Alb Verona (Glu570Lys), and Alb Venezia (Gly572ProfsX10) in Veneto; and Alb Catania (Gln580LysfsX4) in Sicily (Burlina et al., 1985; Porta et al., 1992). Three homozygotic

subjects have been found in Italy and Sweden as the result of intermarriage of parents both carrying proAlb Lille (Arg-2His) (Peters, 1996).

Many genetic mutants of HSA have been described also in the Japanese, Brazilian, New Zealand, and Swedish populations, because of the intensive survey and the extensive genetic study performed in these countries. In particular, Asian surveys assessing radiation-induced changes, including 16,000 specimens from Hiroshima and Nagasaki, found two variants from Hiroshima (Glu354Lys and Glu382Lys) and three from Nagasaki (His3Gln, Asp269Gly, and Asp375Asn) (too few to place the blame on excessive radiation), with a total of 13 alleles in Japan and a frequency of 0.0016; of these 13 alleles, 10 are unique to that country (Neel et al., 1988). Other Asian forms are proAlb Lille (Arg-2His), Alb Canterbury (Lys313Asn), Alb Naskapi (Lys372Glu), and Alb Vancouver (Glu501Lys) (Arai et al., 1990).

Only one HSA variant from Africa has been reported, i.e., proAlb Lille (Arg-2His) in a Somalian living in Italy (Peters, 1996).

The polymorphic occurrence of Alb Naskapi (Lys372Glu) has helped to document the migration of Indian tribes in North America. In fact, it has been hypothesized that ancestral Americans migrated across the Bering Strait 15,000–20,000 BC, and then branched eastward to Canada and southward toward Mexico (Schell et al., 1978; Kaur et al., 1982). Although the frequency of alloalbumins is low, a polymorphic frequency has been found in several interrelated groups. Alb Naskapi has a prevalence of 0.138 in Indian tribes of Labrador and of 0.022 in Alberta (Canada), and has been found also in Turkey and in the Punjab region of India (Takahashi et al., 1987a). Alb Naskapi mixes with Alb Mexico (Asp550Gly) in Apaches of the United States desert southwest, and is found in some tribes in Brazil. Alb Yanomama-2 (Arg114Gly) is found at frequency 0.08 in this stone-age Brazilian tribe. Alb Maku (Lys541Glu) and Alb Makiritare-1 (mutant site not yet known) are other forms which are polymorphic among Brazilian tribes (Peters, 1996; Franco et al., 1999).

Remarkably, the apparently low prevalence of mutations in some populations probably reflects the indifference of investigators to benign mutations rather than their true incidence (Peters, 1996).

2.5. Recombinant albumin

To date, HSA has been produced by fractionation of human plasma, which is generally in limited supply. As the source of the blood can vary, there is the potential risk of HSA contamination by blood-derived pathogens. The development of an alternative, industrial method of preparation greatly improved the general movement toward self-sufficiency in blood and blood products. Although expected to play an important role, recombinant DNA technology faces a number of problems in the large-scale production of pharmaceutical grade recombinant HSA (rHSA) (Kobayashi, 2006; He et al., 2011).

The non-glycosylated feature of HSA has made it possible to screen a wide range of host organisms for correct structure and high productivity potential. With the aim of establishing an rHSA-producing strain for industrial use, studies of secretion systems using *Escherichia coli* (Lawn et al., 1981), *Bacillus subtilis* (Saunders et al., 1987), *Hansenula polymorpha* (Hodgkins et al., 1990), *Kluyveromyces lactis* (Fleer et al., 1991), *Pichia pastoris* (Clare et al., 1991; Kobayashi, 2006), *S. cerevisiae* (Sleep et al., 1991), transgenic plants (Sijmons et al., 1990; Farran et al., 2002; Fernández-San Millán et al., 2003; Huang et al., 2005; He et al., 2011), and transgenic animals (Barash et al., 1993) have been performed. Interestingly, yeast- and rice-based expression systems represent the main options for rHSA production (Kobayashi, 2006; He et al., 2011).

The methylotrophic yeast *P. pastoris* seems to be the most attractive candidate for HSA production as its system usually possesses a high production level (Clare et al., 1991; Kobayashi, 2006). Moreover, *P. pastoris* is capable of several post-translational modifications performed by higher eukaryotic cells, such as proteolytic processing, folding, disulfide bond formation, and glycosylation (Cregg et al., 2009). *P. pastoris* is a highly successful system for the production of a wide variety of recombinant proteins, since they result correctly folded and efficiently secreted into the medium from the *P. pastoris* cells. Because *P. pastoris* secretes only low levels of endogenous proteins and because its culture medium contains no added proteins, a secreted heterologous protein comprises the vast majority of the total protein in the medium (Tschopp et al., 1987; Barr et al., 1992). Thus, secretion serves as a major first step in purification, separating the foreign protein from the bulk of cellular proteins. Secretion requires the presence of a signal sequence on the foreign protein to target it to the secretory pathway. Although several different secretion signal sequences have been used successfully, including the native secretion signal present on some heterologous proteins, success has been variable. The secretion signal sequence from the *S. cerevisiae* α -factor pre-pro-peptide has been used with the most success (Cregg et al., 2009).

Secreted rHSA is purified from the culture broth of *P. pastoris* by a combination of several chromatographic and membrane filtration techniques (Sumi et al., 1999). It should be noted that the clinical dosage of HSA often exceeds 125 g. Since an extremely high degree of HSA purity is required, immunologically active contaminants, such as *P. pastoris* antigens, must be completely removed. Indeed, even at a rHSA purity of 99.999%, 1 mg of impurities injected by the administration of 100 g of rHSA represents enough contaminating material to elicit an adverse immune response (Ohtani et al., 1998; Kobayashi, 2006). rHSA purified from *P. pastoris* is structurally identical to plasma-derived HSA (pdHSA) (Ikegaya et al., 1997; Kobayashi, 2006), and has shown comparable safety, tolerability, pharmacokinetics, and pharmacodynamics (Bosse et al., 2005; Kobayashi, 2006). In 2007, rHSA expressed in *P. pastoris* has been approved as a medicine, and in 2008 this rHSA has been launched in Japan for therapeutic purpose (Murasugi, 2010).

Plant seeds, especially cereal crop seeds, are promising vehicles for producing recombinant proteins, because they can achieve high levels of stable recombinant protein and are well controlled on a production scale (Lau and Sun, 2009; Boothe et al., 2010).

Very recently, *Oryza sativa* recombinant HSA (OsrHSA) from transgenic rice seeds has been produced (He et al., 2011). OsrHSA is highly and stably expressed in the rice seed endosperm. OsrHSA is equivalent to pdHSA from both structural and functional viewpoints. The efficiency of OsrHSA in promoting cell growth and treating liver cirrhosis in rats is similar to that of pdHSA. Furthermore, OsrHSA displays similar *in vitro* and *in vivo* immunogenicity as pdHSA. The level of OsrHSA reached 10.58% of the total soluble protein of the rice grain. Large-scale production of OsrHSA generated protein with a purity >99% and a productivity rate of 2.75 g/kg brown rice. Transgenic rice shows a very low frequency (0.04–0.80%) of pollen-mediated gene flow between genetically modified rice and adjacent non-genetically modified plants. As a whole, a rice seed bioreactor produces cost-effective rHSA that is safe and can help to satisfy the increasing worldwide demand for HSA (He et al., 2011).

For time being, the main use of rHSA will be: (i) a substitute for the pdHSA in clinical applications; (ii) an excipient or stabilizer in other recombinant and biotechnology based products; (iii) a component in serum-free cell culture media; (iv) a component in special products such as imaging agents; and (v) an agent in therapeutic apheresis where plasma exchange might be desirable. rHSA will thus open new doors in its applications and contribute to self-sufficiency in blood and blood products (Kobayashi, 2006).

2.6. Artificial human serum albumin mutants

Gene manipulation techniques open up the possibility of producing rHSA mutants to investigate the HSA physico-chemical properties as well as to explore desirable therapeutic and biotechnological applications. Table 2 reports the main artificial mutants of HSA.

2.6.1. Artificial human serum albumin mutants for physico-chemical studies

The *in vitro* production of rHSA maintaining the pdHSA binding properties represents a relevant tool to investigate the role and importance of residues involved in the binding process, and to understand the functional links between different sites of HSA. This aspect is essential to avoid critical and unexpected changes in the pharmacokinetic properties of therapeutic drugs (Varshney et al., 2010)

2.6.1.1. Glycated artificial human serum albumin mutants. Functional studies have been performed with HSA glycated with D-glucose, but disagreement seems to exist on whether or not glycation affects the unique ligand binding properties of the molecule. Thus, reports of increased, decreased, and unmodified HSA ligand binding capacity have appeared (He and Carter, 1992; Vorum et al., 1995; Peters, 1996; Bourdon et al., 1999; Sugio et al., 1999). In cases of effects on binding, the issue of whether the effect is caused by the presence of the covalently bound glucose, blockage of charged residue(s) of the protein or a combination of both mechanisms has not been evaluated. The principal site of glycation of HSA is Lys525, but Lys residues at positions 199, 281, and 439 are also susceptible to glycation. In addition, six other residues of less importance have been identified (Shaklai et al., 1984; Iberg and Fluckiger, 1986; Robb et al., 1989). In an attempt to shed some light on the above aspects, non-glycated and glycated wild-type rHSA have been produced, and the single-residue mutants (i.e., Lys199Ala, Lys439Ala, and Lys525Ala) as well as the triple-residue mutant have been generated (Nakajou et al., 2003). Results obtained showed that the substitution of three important Lys residues for Ala affects HSA in principally the same way as glycation. This finding suggests that the effect of glycation on the conformation, function, and biological fate of HSA can, to a great extent, be explained by removal of the positive charges of Lys at positions 199, 439, and 525. However, the triple-residue mutant is not a perfect mimic for glycated HSA. The quantitative differences in the effects may be due to the fact that glycation also affects Arg residues and additional Lys residues. Perhaps a perfect mimic for glycation can be made by mutating a proper combination of Lys and Arg residues. Therefore, the effect of glycation on structure, function, and metabolism of HSA seems to be primarily caused by the blockage of positively charged amino acid residues (Nakajou et al., 2003).

2.6.1.2. Truncated human serum albumin. The truncated form of HSA (tHSA), produced in *P. pastoris* and including domains I and II (Asp1–Glu382), represents a valuable model to investigate the allosteric properties of HSA. Indeed, by removal of the C-terminal domain III, a number of contacts that involve domain I (containing the heme site) and domain II (containing the warfarin site) are lost; nevertheless, the allosteric linkage between the heme and warfarin sites is maintained. Remarkably, this simplified model of HSA maintains the allosteric properties of the wild type protein with a reduced number of binding sites (Fanali et al., 2009) (see Sections 4 and 7).

2.6.2. Artificial human serum albumin mutants with potential pharmaceutical properties

HSA plays a relevant role as drug carrier in clinical settings. Indeed, the possibility to synthesize HSA *in vitro*, as well as its known structure, has made HSA an attractive candidate for use in recombinant fusion protein in order to extend half-life of small proteins that would otherwise be rapidly cleared (see Section 9.5). However, the design of HSA mutants analogues having pharmaceutical properties can be a difficult task since the protein may undergo multiple post-translational modifications (see Otagiri and Chuang, 2009).

2.6.2.1. Human serum albumin mutants with prolonged half-life. Since oxidation induces the elimination of HSA (Iwao et al., 2006a), a oxidation-resistant rHSA dimer has been produced. This rHSA, which was cross-linked by reaction of the thiol group of Cys34 with 1,6-bis(maleimido)hexane, is characterized by: (i) an half-life approximately 1.5 times greater than that

Table 2

Main artificial mutants of HSA.

Type of modification	Mutation	Effect	References
Glycosylation	Triple mutants	Partially mimics naturally glycosylated HSA	Nakajou et al. (2003)
Truncation	Removal of the 383–585 sequence	Maintains the allosteric properties of full-length HSA	Fanali et al. (2009)
Dimerization	Cross-linking of the thiol group of Cys34 with 1,6-bis(maleimido)hexane	Oxidation resistant Extended half-life Reduced body clearance Increased retention rate in blood Reduced vascular permeability	Matsushita et al. (2006)
S-nitrosylation	S-nitrosylation of the Arg410Cys mutant	Anti-bacterial activity against <i>S. typhimurium</i> Cytoprotective effects <i>in vitro</i> and <i>in vivo</i> Extended half-life of the Arg410Cys mutant	Ishima et al. (2007)
Aminoacid substitution	Triple mutant (Ile142His-Tyr161Leu-Leu185Asn)	O ₂ binding	Komatsu et al. (2009a)

of monomeric HSA (15–20 days); (ii) a significant decrease in the total body clearance and distribution volume at steady state than monomeric HSA; (iii) an increased retention rate in blood than monomeric HSA; and (iv) a lowered vascular permeability than monomeric HSA. Therefore, dimeric HSA may be used in critically ill patients with hypovolemia, burns, and hypoalbuminemia (Matsushita et al., 2006).

2.6.2.2. Anti-bacterial human serum albumin mutants. Alb Liprizzi displays the Arg410Cys mutation in subdomain IIIA (Galliano et al., 1988). S-nitrosylation of Cys410 confers to Alb Liprizzi a potent anti-bacterial activity against *Salmonella typhimurium* and cytoprotective properties *in vitro* and *in vivo* (Ishima et al., 2007); the cytoprotective effect appears enhanced by FA binding (Ishima et al., 2008). Moreover, S-nitrosylation of Cys410 prolongs the half-life of Alb Liprizzi in circulation, probably through reduced liver uptake (Otagiri and Chuang, 2009). In contrast, the Arg410Ala mutation shortens the half-life of rHSA (Iwao et al., 2006a).

2.6.2.3. Oxidation-resistant human serum albumin mutants. Since HSA oxidation compromises its ligand-binding properties and shortens its half-life, it should be relevant to use an oxidation-resistant HSA analogue for the purpose of HSA-based dialysis (Otagiri and Chuang, 2009). Oxidation resistance could be conferred to HSA through mutation of Cys and/or Met residues, which are susceptible to free radical attack. Moreover, it should be desirable to design oxidation-resistant HSA mutant(s) facilitating the removal of radical species in HSA-based dialysis (Wratten et al., 2000; Otagiri and Chuang, 2009; Anraku et al., 2011). In this respect, it should be mentioned that HSA–heme–Fe derivatives scavenge RNS (Ascenzi and Fasano, 2007; Ascenzi et al., 2009a, 2010a, 2011a; di Masi et al., 2011) (see Section 5.3.2).

2.6.2.4. O₂-carrying human serum albumin mutants. In order to generate an artificial heme-based oxygen carrier, the FA1 site (i.e., the heme pocket; see Section 4.1.1) of HSA has been engineered to bind heme–Fe in the globin-like fashion. Thus, the heme–Fe–atom of HSA–heme–Fe(II) mutants (showing His142 or His185 as the proximal heme–Fe–atom ligand) does not undergo to oxidation and bind reversibly O₂ (Komatsu et al., 2007). Although the pharmacokinetic profile of wild-type and mutated HSA–heme–Fe should be investigated, the oxidation-resistant HSA–heme–Fe(II) derivative mutants may represent an invaluable heme-based oxygen carrier (Otagiri and Chuang, 2009; Tsuchida et al., 2009).

Transport of O₂ by rHSA–heme–Fe(II) may be of great medical importance not only as a blood alternative, but also as an O₂-therapeutic fluid. By focusing on modification on the distal side of the heme pocket, rHSA triple mutant–heme–Fe(II) complexes with a broad range of O₂ binding affinity have been produced. The rHSA–heme–Fe(II) triple mutant showing the highest O₂ affinity is Ile142His/Tyr161Leu/Leu185Asn (Komatsu et al., 2009a).

2.7. Human serum albumin structure

The biosynthesis of HSA starts in the hepatocyte with the production of pre-pro-albumin that is modified into pro-albumin in the lumen of the endoplasmic reticulum. Then, furin cleaves an N-terminal 6-amino acid long oligopeptide in the trans-Golgi network to obtain the mature plasma protein, constituted by a single chain of 585 residues, with a molecular weight of 66,438 Da (Peters, 1996). During its long life-time (28–36 days), HSA may undergo several modifications that could affect, among others, ligand binding and anti-oxidant properties (Roche et al., 2008) (see Sections 3 and 4).

HSA contains a single Trp residue at position 214; Met, Gly, and Ile residues are low, whereas Cys, Leu, Glu, and Lys are abundant. The large number of ionized residues gives to HSA a high total charge (i.e., 215 ions per molecule at pH 7.0), which facilitates its solubility. Moreover, the acidic amino acid residues outnumber the basic ones in HSA, resulting in a negative net charge per molecules of about –15, at pH 7.0, HSA contains 35 Cys residues forming 17 disulfide bridges, with the only free Cys residue located at position 34. The disulfide bridges significantly contribute to the stability of HSA and make clear its long biological life-time (Peters, 1996).

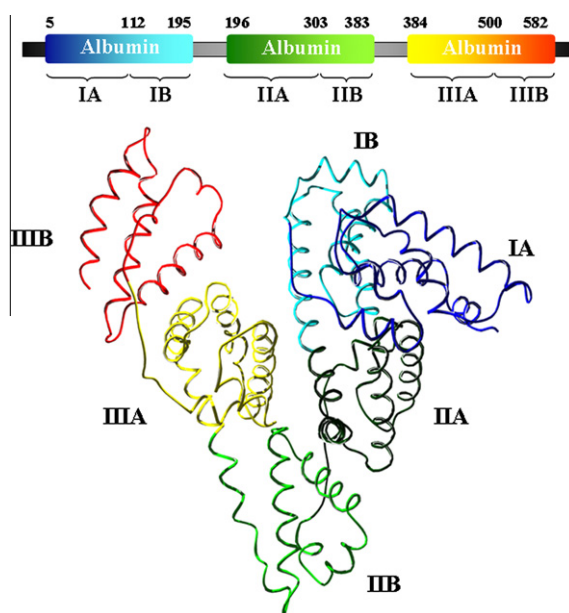


Fig. 2. Modular domain organization of HSA. The upper panel shows the architecture of the SA sequence, with three repeated conserved domains, as assigned by the NCBI CDD entry cd00015. The lower panel shows the three-dimensional structure of HSA with the subdomains rendered with different colors (domain IA, in blue; domain IB, in cyan; domain IIA, in dark green; domain IIB, in light green; domain IIIA, in yellow; domain IIIB, in red). Atomic coordinates were taken from the PDB entry 1AO6 (Sugio et al., 1999). Ribbon representation of HSA was drawn with the Swiss-PdbViewer (Guex and Peitsch, 1997). For details, see Section 2.7.

The secondary structure of HSA is dominated by α -helices (68%), without any β -sheet element. HSA is arranged in a globular heart-shaped conformation (Fig. 2) containing three homologous domains usually indicated as I (1–195), II (196–383), and III (384–585). The three domains are comparable in the amino acid sequence as well as in the secondary and tertiary structure (Curry, 2002, 2009; Fasano et al., 2005; Ascenzi and Fasano, 2010; Varshney et al., 2010). The three-dimensional structures of HSA in the absence and presence of endogenous and exogenous ligands are summarized in Table 3. Remarkably, the HSA conformation is grossly maintained even in the presence of a wide variety of ligands (see Section 4) and is common to the structure of all vertebrate SA (see Section 4.6).

The three domains of HSA assemble asymmetrically and resemble a heart shape, with approximate dimensions of $80 \times 80 \times 30$ Å (Sugio et al., 1999). Each domain includes ten helices that are packed in two separate subdomains (named A and B) constituted of six (h1–h6) and four (h7–h10) α -helices, respectively, connected by a long extended loop (Fig. 2). The polypeptide chain folding and disulfide bond topology are similar between the subdomains. Subdomain IIA is connected to the interface region between subdomain IA and IB by hydrophobic interactions and hydrogen bonds. This feature leads to the T-shaped arrangement of domains I and II that are almost perpendicular to each other. Domain III, that protrudes from subdomain IIB to form a Y-shaped assembly for domains II and III, interacts only with subdomain IIB. Domains I and III are separated by a big channel created by subdomains IB, IIIA, and IIIB and are connected by few contacts. Terminal regions of sequential domains contribute to the formation of inter-domains, 9-turn-long helices linking domain IB to IIA (residues 173–205) and IIB to IIIA (residues 336–398). Despite their structural similarity, each domain interacts with the neighbor domain(s) in different ways. Therefore, the orientation of domains I–II with respect to domains II–III constitutes a highly asymmetric environment where a variety of ligand binding sites is located (Fig. 3) (Curry et al., 1998; Sugio et al. 1999; Ascenzi and Fasano, 2010).

As a consequence of the high number of acidic and basic residues, the HSA structure undergoes reversible conformational transitions at different pH values. At pH lower than 2.7, HSA occurs in the extended (E) conformation. Between pH 2.7 and 4.3, HSA assumes the fast-migrating (F) form, characterized by an increase in viscosity, much lower solubility, and the loss of α -helix with respect to physiological conditions (i.e., the neutral (N) form). Between pH 4.3 and 8.0, HSA displays the N form that is characterized by the heart-shaped structure. At pH greater than 8.0, HSA changes its conformation to the basic form (B) characterized by the loss of α -helix and an increased affinity for some ligands with respect to the N form (Peters, 1996; van der Vusse, 2009; Ascenzi and Fasano, 2010).

By comparing the unliganded HSA structures with those obtained in the presence of FAs, a remarkable conformational change(s) becomes evident. Indeed, FA binding produces a relative domain rotation within the molecule. In particular, both domains I and III appear to pivot around a point close to their interface with domain II, so that the most extreme subdomains (IA and IIIB) display the greatest deviations in position as a consequence of FA binding. The long interdomain helices connecting domain I with II and domain II with III are tilted by 16° after FA binding, thus determining the rotation of domain I with respect to domain III (Ascenzi and Fasano, 2010).

Table 3

Three-dimensional structures of HSA.

PDB code	Resolution (Å)	Ligands	Ligand binding sites	References
<i>Crystal structures</i>				
1AO6	2.50	Ligand free	n.a.	Sugio et al. (1999)
1BJ5	2.50	Myristic acid	FA1, FA2, FA3, FA4, FA5	Curry et al. (1998)
1BKE	3.15	Myristic acid	FA1, FA2, FA3, FA4, FA5	Curry et al. (1998)
		Tri-iodobenzoic acid	FA1 ^a , FA7	
1BM0	2.50	Ligand free	n.a.	Sugio et al. (1999)
1E78	2.60	Ligand free	n.a.	Bhattacharya et al. (2000b)
1E7A	2.20	Propofol	FA3–FA4, FA5	Bhattacharya et al. (2000b)
1E7B	2.38	Halothane	FA3, FA6	Bhattacharya et al. (2000b)
1E7C	2.40	Myristic acid	FA1, FA2, FA3, FA4, FA5	Bhattacharya et al. (2000b)
		Halothane	FA2, FA6, FA7, IA/IB	
1E7E	2.50	Capric acid	FA1, FA2, FA3, FA4, FA5, FA6 ^b , FA7, FA8, FA9	Bhattacharya et al. (2000a)
1E7F	2.43	Lauric acid	FA1, FA2 ^b , FA3, FA4, FA5, FA6, FA7	Bhattacharya et al. (2000a)
1E7G	2.50	Myristic acid	FA1, FA2, FA3, FA4, FA5, FA6, FA7	Bhattacharya et al. (2000a)
1E7H	2.43	Palmitic acid	FA1, FA2, FA3, FA4, FA5, FA6, FA7	Bhattacharya et al. (2000a)
1E7I	2.70	Stearic acid	FA1, FA2, FA3, FA4, FA5, FA6, FA7	Bhattacharya et al. (2000a)
1GNI	2.40	Oleic acid	FA1, FA2, FA3, FA4, FA5, FA6, FA7	Petitpas et al. (2001)
1GNJ	2.60	Arachidonic acid	FA1, FA2, FA3, FA4, FA5, FA6, FA7	Petitpas et al. (2001)
1H9Z	2.50	Myristic acid	FA1, FA2, FA3, FA4, FA5, FA6	Petitpas et al. (2001)
		R-(+) warfarin enantiomer	FA7	
1HA2	2.50	Myristic acid	FA1, FA2, FA3, FA4, FA5, FA6	Petitpas et al. (2001)
		S-(−) warfarin enantiomer	FA7	
1HK1	2.65	Thyroxine	Tr-1 ^c , Tr-2 ^d , Tr-3 ^e , Tr-4 ^f	Petitpas et al. (2003)
1HK2 ^g	2.80	Thyroxine	Tr-1 ^c , Tr-2 ^d , Tr-3 ^e , Tr-4 ^f	Petitpas et al. (2003)
1HK3 ^h	2.80	Thyroxine	Tr-1 ^c , Tr-2 ^d , Tr-3 ^e , Tr-4 ^f	Petitpas et al. (2003)
1HK4	2.40	Myristic acid, Thyroxine	FA1, FA2, FA3, FA4, FA5, FA6, FA7 Tr-5 ⁱ	Petitpas et al. (2003)
1HK5 ^g	2.70	Myristic acid Thyroxine	FA1, FA2, FA3, FA4, FA5, FA6, FA7 Tr-5 ⁱ	Petitpas et al. (2003)
1N5U	1.90	Myristic acid	FA2, FA3, FA4, FA5, FA6	Wardell et al. (2002)
		Heme	FA1	
1O9X	3.20	Myristic acid Heme	FA2, FA3, FA4, FA5, FA6, FA7 FA1	Zunszain et al. (2003)
1TF0	2.70	Citric acid	FA7 ^a	Lejon et al. (2004)
		Decanoic acid	FA6 ^b , FA7	
		GA module of <i>P. magnus</i> PAB	IIA/IIIB	
1UOR	2.80	Ligand free	n.a.	Carter and Ho (1994)
2BX8	2.70	Azapropazone	FA1 ^a , FA7	Ghuman et al. (2005)
2BXA	2.35	3-Carboxy-4-methyl-5-propyl- 2-furanpropanoic acid	FA3–FA4, FA7	Ghuman et al. (2005)
2BXB	3.20	Oxyphenbutazone	FA7	Ghuman et al. (2005)
2BXC	3.10	Phenylbutazone	FA7	Ghuman et al. (2005)
2BXD	3.05	Warfarin	FA7	Ghuman et al. (2005)
2BXE	2.95	Diflunisal	FA3–FA4, FA6, FA7	Ghuman et al. (2005)
2BXF	2.95	Diazepam	FA3–FA4	Ghuman et al. (2005)
2BXC	2.70	Ibuprofen	FA3–FA4, FA6	Ghuman et al. (2005)
2BXH	2.25	Indoxyl sulfate	FA3–FA4, FA7 ^a	Ghuman et al. (2005)
2BXI	2.50	Myristic acid	FA2, FA3, FA4, FA5, FA6	Ghuman et al. (2005)
		Azapropazone	FA1, FA7	
2BXK	2.40	Myristic acid	FA1, FA2, FA3, FA4, FA5, FA6	Ghuman et al. (2005)
		Azapropazone	FA7	
		Indomethacin	FA7 ^a	
2BXL	2.60	Myristic acid	FA1, FA2, FA3, FA4, FA5, FA6	Ghuman et al. (2005)
		3,5-diiodosalicylic acid	FA7	
2BXM	2.50	Myristic acid	FA1, FA2, FA3, FA4, FA5, FA6, FA7	Ghuman et al. (2005)
		Indomethacin	FA1, FA7 ^a	
2BXN	2.65	Myristic acid	FA1, FA2, FA3, FA4, FA5, FA6	Ghuman et al. (2005)
		Iodipamide	FA7 ^b , FA9	
2BXO	2.60	Myristic acid	FA1, FA2, FA3, FA4, FA5, FA6	Ghuman et al. (2005)
		Oxyphenbutazone	FA5 ^a , FA7	
2BXP	2.30	Myristic acid	FA1, FA2, FA3–FA4, FA5, FA6, FA8	Ghuman et al. (2005)
		Phenylbutazone	FA7	
2BXQ	2.60	Myristic acid	FA2, FA3, FA4, FA5, FA6,	Ghuman et al. (2005)
		Phenylbutazone	FA7	
		Indomethacin	FA1, FA8	
2I2Z	2.70	Myristic acid	FA1, FA2, FA3, FA4, FA5	Yang et al. (2007)
		Aspirin	FA7	
2I30	2.90	Myristic acid	FA1 ⁱ , FA2, FA3, FA4, FA5	Yang et al. (2007)
		Salicylic acid	FA1 ⁱ , FA7	

Table 3 (continued)

PDB code	Resolution (Å)	Ligands	Ligand binding sites	References
2VDB	2.52	Capric acid S-Naproxen GA module of <i>P. magnus</i> PAB	FA1, FA2, FA3, FA4, FA6, FA7 FA1 IIA, IIB	Lejon et al. (2008)
2VUE	2.42	4Z,15E-bilirubin-IX- α FA1		Zunszain et al. (2008)
2VUF	3.05	Fusidic acid	FA1, Tr-4 ^f	Zunszain et al. (2008)
2XSI	2.70	Myristic acid Dansyl-L-glutamate	FA1, FA2, FA3, FA4, FA5, FA6 FA1, FA7	Ryan et al. (2011a)
2XVQ	2.90	dansyl-L-sarcosine	FA3–FA4	Ryan et al. (2011a)
2XVU	2.60	Dansyl-L-asparagine	FA3–FA4, FA7	Ryan et al. (2011a)
2XVV	2.40	Myristic acid Dansyl-L-asparagine	FA1, FA2, FA3, FA4, FA5, FA6 FA1, FA7	Ryan et al. (2011a)
2XVW	2.65	Myristic acid Dansyl-L-arginine	FA1, FA2, FA3, FA4, FA5, FA6 FA1, FA7	Ryan et al. (2011a)
2XW0	2.40	Dansyl-L-phenylalanine	FA3–FA4, FA7	Ryan et al. (2011a)
2XW1	2.50	Dansyl-L-norvaline	FA3–FA4	Ryan et al. (2011a)
2YDF	2.75	Iophenoxic acid	FA1 ^a , FA3–FA4, FA7, IIIA–IIIB	Ryan et al. (2011b)
3A73	2.19	Myristic acid D12-prostaglandin J2	FA2, FA3, FA4, FA5, FA6, FA7 FA1 ^b	Yamaguchi et al. (2010)
3B9L	2.60	Myristic acid AZT	FA1, FA2, FA3, FA4, FA5, FA6, FA7 FA1, FA7	Zhu et al. (2008)
3B9M	2.70	Myristic acid AZT	FA1, FA2, FA3, FA4, FA5, FA6 FA7	Zhu et al. (2008)
3CX9	2.80	Salicylic acid Myristic acid Lisophosphatidylethanolamine	FA1 ^l , FA7 FA2, FA3, FA4, FA5, FA6 FA7	Guo et al. (2009)
3JQZ	3.30	Lidocaine	cleft	Hein et al. (2010)
3JRY	2.30	Sulfate	IIIA	Hein et al. (2010)
3LU6	2.70	Compound 1 ^m	FA6, FA7	Buttar et al. (2010)
3LU7	2.80	Compound 2 ⁿ	FA7	Buttar et al. (2010)
3LU8	2.60	Compound 3 ^o	FA1	Buttar et al. (2010)
3SQJ	2.05	Myristic acid	FA1, FA2, FA3, FA4, FA5, FA6, FA7	He et al. (2011)
<i>Solution structures</i>				
1YSX	n.a.	Thioethylamino-2,4-dimethylphenyl	FA3–FA4	Oltsersdorf et al. (2005)
2ESG	n.a.	Immunoglobulin IgA1	Cys34	Almogren et al. (2006)

^a Partial occupancy.^b two ligand molecules in the site.^c Tr-1 largely corresponds to FA7. The partial occupancy of this site does not allow to say that it matches with fatty acid binding site.^d Tr-2 largely corresponds to FA3–FA4. The partial occupancy of these sites do not allow to say that they matches with fatty acid binding sites.^e Tr-3 largely corresponds to FA5. The partial occupancy of this site does not allow to say that this site matches with fatty acid binding site.^f Tr-4 is located in the proximity of FA5.^g Arg218His.^h Arg218Phe.ⁱ Tr-5 corresponds to FA9.^l myristic acid and salicylic acid occupy two contiguous regions forming the heme site^m [(1R,2R)-2-[(5-fluoro-1H-indol-2-yl)carbonyl]amino]-2,3-dihydro-1H-inden-1-yl]acetic acid.ⁿ 4-[(1R,2R)-2-[(5-fluoro-1H-indol-2-yl)carbonyl]amino]-2,3-dihydro-1H-inden-1-yl]butanoic acid.^o N-[5-(5-[(2,4-dimethyl-1,3-thiazol-5-yl)sulfonyl]amino)-6-fluoropyridin-3-yl)-4-methyl-1,3-thiazol-2-yl]acetamide.

Although the HSA structure in the B form was never characterized by X-ray crystallography, there are converging evidences that the conformational transition induced by FA binding reproduces at least in part the conformational transition induced by the pH increase over 8.0 (Curry, 2002, 2009; Ascenzi and Fasano, 2010). In this view, FAs shift the N to B conformational equilibrium towards the B state (van der Vusse, 2009; Ascenzi and Fasano, 2010) (see Section 7).

3. Human serum albumin chemical modifications

HSA undergoes several chemical modifications not only altering its binding properties (see Sections 4 and 7) but also conferring antigenicity properties (Honma et al., 1991; Peters, 1996).

3.1. Human serum albumin acetylation

HSA is unusual in the family of plasma proteins because it has no carbohydrates. This makes the amino acid residues of HSA more accessible to acetylation. The reaction between aspirin (at a 3×10^{-4} M concentration, comparable with therapeutic blood levels) and HSA results in covalent binding of the acetyl group to the ϵ -N-amino groups of Lys199, Lys402, Lys519, and Lys545. Treatment with 2×10^{-2} M aspirin results in acetylation of 26 Lys residues. Acetylated Lys residues were stable for at least 21 days (Liyasova et al., 2010).

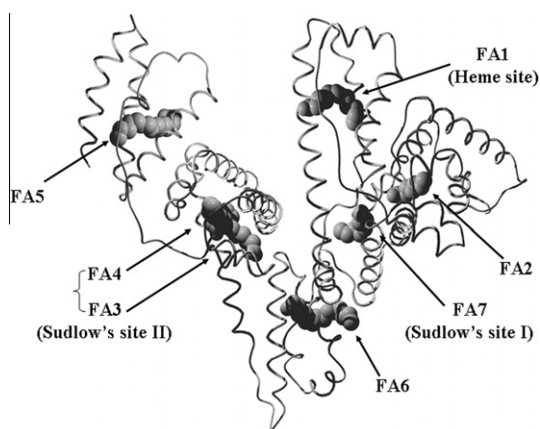


Fig. 3. FA binding sites. Ribbon representation of the heart-shaped structure of HSA complexed with myristate in FA binding sites. Myristate is rendered as space-fill (gray). Atomic coordinates were taken from PDB entry 1E7G (Bhattacharya et al., 2000b), 1O9X (Zunsain et al., 2003). Ribbon representation of HSA was drawn with the Swiss-PdbViewer (Guex and Peitsch, 1997). For details, see Section 4.1.

Ligand binding properties of HSA could be modulated by acetylation. Indeed, the HSA affinity for phenylbutazone increases, and bilirubin binding is inhibited. Moreover, binding of aspirin to HSA reduces prostaglandin affinity, accelerating the clearance of prostaglandins and serving as an additional mechanism of the aspirin anti-inflammatory effect (Liyasova et al., 2010).

3.2. Human serum albumin cysteinylation, homocysteinylation, and glutathionylation

3.2.1. Cys34 cysteinylation, homocysteinylation, and glutathionylation

Cys34 represents the largest fraction of free thiol in human plasma, being HSA the most abundant protein in plasma; remarkably, Cys34 is completely conserved within mammalian SAs. Cys34 is located at the surface of HSA, close to Asp38, His39, and Tyr84. These three residues affect the ionization state of Cys34, thus modulating its reactivity (Ascenzi et al., 2000).

In healthy adults, about 70–80% of the HSA molecules have the Cys34 residue with the free sulfhydryl group, whereas about 25–30% of the HSA molecules have Cys34 forming a mixed disulfide with either cysteine or homocysteine or glutathione, thus affecting the Cys34 redox potential. While the concentration of free glutathione is in the lower micromolar range, HSA-bound glutathione may represent a significant pool. Whether the glutathione adduct merely serves either as a kind of storage and transport of glutathione or has a functional role in modulating the redox potential of Cys34 still remains unclear (Oetl and Stauber, 2007).

Cys34 acts as a physiological anti-oxidant, participating in radical scavenging (e.g., NO; see Section 5.3.1), and as a binding site for a wide variety of endogenous ligands (e.g., NO; see Section 3.4.1), metal ions (i.e., Au(I), Pt(II), Ag(I), and Hg(II); see Section 4.4.2), and drugs (i.e., ethacrynic acid, organomercurials, and many drugs which bind as mixed disulfides, including the anti-hypertensive captopril and the anti-alcohol-abuse drug disulfiram) (Gutteridge, 1986; Christodoulou et al., 1995; Kragh-Hansen et al., 2002; Oetl and Stauber, 2007).

Modifications of Cys34 modulate allosterically and inhibit competitively HSA reactivity. Indeed, the oxidation of Cys34 with cysteine induces allosterically the fast *N*-homocysteinylation of Lys525 (Bertucci et al., 1999; Stewart et al., 2005). Moreover, the formation of a mixed disulfide may inhibit HSA *S*-nitrosylation at Cys34 (see Section 3.4.1).

Oxidation of Cys34 leads to the formation of sulfenic acid, which is further oxidized to sulfinic or sulfonic acid. Remarkably, sulfenic acid displays a pivotal role in both the reversible and irreversible redox modulation of protein actions by reactive species (Turell et al., 2008, 2009; Alvarez et al., 2010). Sulfenic acid in HSA was recently involved in mixed disulfide formation, supporting a role of HSA as an important redox regulator in extracellular compartments (Carballal et al., 2007).

The Cys34 residue represents an important anti-oxidant against peroxynitrite, as the thiol group was shown to be oxidized to sulfenic acid (Carballal et al., 2003). Subsequently, the Cys34–SOH group can be converted to a disulfide and then back to Cys34–SH (Quinlan et al., 2005). Moreover, HSA is able to scavenge strongly oxidant compounds such as hypochlorous acid and hypothiocyanous acid. Cys34 is oxidized preferentially by hypochlorous acid and hypothiocyanous acid with the formation of the corresponding sulfenyl derivative (Arnhold et al., 1990; Hawkins et al., 2008). Also Tyr and Lys HSA residues undergo oxidation by hypochlorous acid (Kettle, 1996; Temple et al., 2006) (see Section 3.8). Remarkably, West Nile virus is neutralized by hypochlorous acid-modified HSA that binds to domain III of the viral envelope protein E (Vossmann et al., 2008). Moreover, HSA may neutralize hypochlorous acid-mediated oxidation of α_2 -macroglobulin (Khan and Khan, 2004).

3.2.2. Lys homocysteinylation

HSA undergoes to non-enzymatic *N*-homocysteinylation, the main targets being Lys4, Lys12, and Lys525 residues. *N*-homocysteinylation of HSA accounts for 0.26–0.36% of HSA in circulation in healthy subjects and increases in pathologic conditions (Jakubowski, 2008). Major pathophysiological consequences of HSA *N*-homocysteinylation include the induction of anti-*N*-homocysteinylation HSA auto-antibodies and thrombogenesis, which contribute to atherosclerosis and cardiovascular diseases in humans (Perla-Kajan et al., 2007; Gerasimova et al., 2008a). Lastly, the RNA-hydrolyzing activity of HSA is decreased by *N*-homocysteinylation (Gerasimova et al., 2008a).

3.3. Human serum albumin glycosylation and glycation

Even though ancestors of the SA superfamily are glycosylated (see Section 4.6), HSA is not a glycoprotein, being one of the few plasma proteins devoid of carbohydrate groups. Indeed, HSA lacks the Asn-X-Ser/Thr sequence needed for *N*-glycosylation. In three HSA variants (see Section 2.3.3), mutation has created the site Asn-X-Ser/Thr and the variant allele is normally glycosylated, without apparent effect on its function (Nakajou et al., 2003).

The spontaneous, non-enzymatic reaction of a monosaccharide with a protein is referred to as glycation (Brownlee, 1995). The glycation of HSA occurs via Schiff base reaction of the sugar carbonyl group with amino groups of HSA in a reversible manner. Arg410 and Lys525 are the principal sites of HSA glycation. Other sites of minor modifications are Arg114, Lys186, Lys199, Arg218, Lys281, Arg428, and Lys439 (Ahmed et al., 2005; Otagiri and Chuang, 2009). The Schiff base undergoes Amadori rearrangement with the irreversible formation of a secondary amine (*i.e.*, fructosamine) and the introduction of a carbonyl group on the surface of the protein (contributing to the formation of carbonylated HSA) (Baynes et al., 1989).

HSA glycation is associated with oxidation of His and Trp residues, main chain fragmentation, and loss of both secondary and tertiary structure (Coussons et al., 1997; Iwao et al., 2006b). Glycation decreases the intrinsic fluorescence of HSA, inducing a conformational change that alters the physicochemical properties of the Trp214 environment (Ahmed and Thornalley, 2002; Mendez et al., 2005b). The levels of advanced glycation of HSA are lowered by diclofenac, a non-steroidal anti-inflammatory drug, that interacts non-covalently with the protein. The same behavior is observed in the presence of aspirin (van Boekel et al., 1992).

HSA glycation alters the binding of endogenous and exogenous ligands to Sudlow's sites I and II, and the interaction with FAs. In particular, glycation of Lys525 can affect the protein conformation, thus modifying ligand binding to Sudlow's sites I and II. Moreover, glycation of Lys199 enhances warfarin binding, but decreases bilirubin affinity (Oetli and Stauber, 2007). The structural changes induced by FA binding prevent the ability of Lys ϵ -N-groups to undergo the Amadori rearrangement for the formation of the stable fructosamine adduct. This does not imply that increased levels of FAs are clinically advantageous, although the increased plasma FA concentration is advisable for a decrease in glyated HSA level (Lautenslager et al., 2011). Lastly, glycation decreases RNase-like activity of HSA (Gerasimova et al., 2008a).

The level of glyated HSA in normal humans is about 10%; however, this proportion typically increases to 20–30% in hyperglycemic patients (Rondeau and Bourdon, 2011) (see Section 8.2.6). HSA glycation in patients affected by diabetes mellitus impairs both ligand binding and anti-oxidant properties (Bourdon et al., 1999; Sakata et al., 2002; Cohen, 2003; Van Campenhout et al., 2006; Barzegar et al., 2007; Faure et al., 2008a). Notably, the Cu(II) binding capacity of glyated HSA is lower than that of the non-glyated protein (Sakata et al., 2002). Thus, glyated HSA exacerbates Cu(II)-induced LDL oxidation, probably by the generation of superoxide (Bourdon et al., 1999; Sakata et al., 2002). Moreover, the Fe(III)-binding anti-oxidant capacity of HSA is markedly reduced in diabetes mellitus patients (Van Campenhout et al., 2006). Lastly, binding and transport of Trp by glyated HSA is reduced (Barzegar et al., 2007).

Scavenging of glyated HSA by a class A scavenger receptor (SR-A) may contribute to the pathogenesis of diabetic nephropathy induced by advanced glycation end products (Nakajou et al., 2005a,b; Iwao et al., 2008). In fact, several receptors for advanced glycation end products, including glyated HSA, initiate intracellular signaling and enhance the formation of ROS (Goldin et al., 2006; Mera et al., 2007; Roche et al., 2008). Remarkably, HOCl-mediated carbonylation of Lys residues (*i.e.*, formation of α -amino adipic semialdehyde residues) of glyated HSA represents a major antigenic advanced glycation end product in hyperglycemia and in inflammation (Mera et al., 2007). Then, advanced glycation end products, including glyated bovine SA, impair vascular endothelial NO synthase activity *in vivo* and *in vitro* (Xu et al., 2003).

Structural modification of HSA induced by glucose or free radicals impairs its anti-oxidant properties (Sakata et al., 2002). HSA of type 1 diabetes mellitus patients is conformationally altered, with more exposure of its hydrophobic regions. In addition, hydroxyl radical modified HSA is highly immunogenic in rabbits as compared with native HSA. Sera from elderly type 1 diabetes mellitus patients having smoking history and advanced disease show substantially stronger binding to hydroxyl radical-modified HSA over normal HSA. These findings suggest that oxidation of plasma proteins, especially HSA, might enhance oxidative stress in type 1 diabetes mellitus patients (Rasheed and Ali, 2006). HSA of type 2 diabetes mellitus patients has decreased anti-oxidant properties that may aggravate oxidative stress (Faure et al., 2008a).

Several diabetes patients (30–40%) develop nephropathies that require hemodialysis treatment (see Section 8.2.6). Both di-Tyr and carbonyl contents were found increased in HSA isolated from patients on hemodialysis. Such damaged HSA has impaired ligand-binding capacity and anti-oxidant properties (Lim et al., 2007).

Very recently, alteration of the HSA anti-oxidant properties has been observed in patients affected by the obstructive sleep apnea syndrome. This reflects the impaired anti-oxidant HSA activity, which is associated with the enhanced glycation level (Faure et al., 2008b).

Lastly, glycated HSA displays a toxic effect on microglial cells associated with impairments of cellular proteolytic systems, this possibly could reflect the role of advanced glycation end products in neurodegeneration (Vitek et al., 1994; Cohen, 2003; de Arriba et al., 2006; Stolzing et al., 2006).

3.4. Human serum albumin nitrosylation and nitration

HSA acts as a NO depot and a NO transducer (Ascenzi et al., 2001; Sampath et al., 2001; Harohalli et al., 2002; Stamler, 2004; Ishima et al., 2009). Remarkably, 82% of NO in blood ($\sim 7 \mu\text{M}$) is transported as a S-nitrosothiol bound at the HSA residue Cys34 (Peters, 1996). Additionally, HSA could bind NO at Trp and Tyr residue(s) (Khan et al., 1997; Yamakura and Ikeda, 2006; Léger et al., 2008).

3.4.1. Cys34 nitrosylation

Since the various biological activities of NO can be preserved in stable form as S-nitrosothiols (Rafikova et al., 2002), S-nitrosylated HSA may represent a circulating endogenous reservoir of NO and may act as a NO donor in therapeutic applications (Stamler et al., 1992; Keaney et al., 1993). Remarkably, Cys34 S-nitrosylation is modulated by hydrogen bond formation and dissociation with Asp38, His39, and Tyr84 residues (Ascenzi et al., 2000).

HSA may be involved in various biological NO-based reactions such as *trans*-nitrosylation and S-thiolation processes (Akaike et al., 1997; Hogg, 2000). S-Nitrosylated HSA acts primarily as a venodilator *in vivo* and represents a stable reservoir of NO that can be released when the concentrations of low-molecular-weight thiols are elevated (Orie et al., 2005). S-Nitrosylated HSA has been shown to reduce either ischemia or reperfusion injury in the pig and rabbit heart after unprotected warm ischemia through long-lasting release of NO (Semsroth et al., 2005; Hallström et al., 2008). Recently, S-nitrosylated HSA has been reported to display anti-tumor activity through apoptosis induction without causing hepatic or renal damage (Katayama et al., 2008, 2010). In a rat ischemia/reperfusion model, the protective effect on liver cells has been observed to be enhanced by oleate binding to S-nitrosylated HSA. Indeed, oleate binding increases the accessibility of the Cys34 residue of HSA improving denitrosylation of HSA–Cys34–NO by HepG2 cells. Remarkably, oleate enhances the anti-apoptotic effect of HSA–Cys34–NO on HepG2 cells exposed to anti-Fas antibody. This may lead to preparation of an S-nitrosylated HSA dosage form that could produce better treatment outcome (Ishima et al., 2008).

Lastly, S-nitrosylation of Cys34 decreases allosterically the affinity of Cu(II) ions, phenolsulphophthalein, and palmitic acid for HSA (Kashiba-Iwatsuki et al., 1997). Moreover, mutation of Trp214 leads to an increase in Cys34 nitrosation, indicating possible competition between these two residues for the reaction of HSA with N_2O_3 , the reactive nitrosating species formed in aqueous acidified NO_2^- solutions (Harohalli et al., 2002).

3.4.2. Tyr nitration

Formation of 3-nitrotyrosine (Tyr-3- NO_2) by the reaction between RNS and Tyr residues in proteins has been analyzed extensively and it is used widely as a biomarker of physiological and pathological conditions mediated by RNS (Ducrocq et al., 1999; Alvarez and Radi, 2003; Ischiropoulos, 2003; Ascenzi et al., 2010b).

In the systemic circulation, low density lipoprotein (LDL) occurs in the form of a weakly nitrated LDL–HSA complex. A loss of Tyr-3- NO_2 , higher with low-LDL–HSA than with rich-LDL–HSA or high density lipoprotein (HDL), occurs only in the presence of Cu(II). Note that γ -tocopherol loading of the lipoprotein fraction inhibits Tyr-3- NO_2 loss. The Tyr-3- NO_2 loss is stoichiometrically equivalent to the NO_3^- production, reflecting the presence of an LDL- and HDL-associated nitratase activity that denitrates Tyr-3- NO_2 residues of HSA. Remarkably, both the LDL- and HDL-associated nitratase activity and HSA contribute to beneficially convert peroxynitrite (ONOO^-) into non-bioactive NO_3^- . However, under inflammatory conditions, xanthine oxidoreductase is expressed leading to detrimentally reduce O_2 and NO_3^- into $\text{O}_2^{\cdot-}$ and $\cdot\text{NO}$ that in turn may interact, reconstituting the ONOO^- pool. As a whole, LDL and HDL are able to denitrate the HSA Tyr-3- NO_2 residues (Khan et al., 1997; Léger et al., 2008).

Tyr residues in bovine SA are nitrated and chlorinated (see Sections 3.4.2 and 3.8) by $\text{NO}_2^-/\text{HOCl}$ and synthetic Cl-NO_2 . The formation of Cl-NO_2 and/or Cl-ONO *in vivo* may represent mediators of inflammation-mediated protein modification and tissue injury, and offers an additional mechanism of Tyr nitration independent of ONOO^- (Eiserich et al., 1996).

3.4.3. Trp nitrosylation and nitration

Modifications of the Trp residue by RNS are relevant in physiological and pathological conditions (Ducrocq et al., 1999; Alvarez and Radi, 2003; Yamakura and Ikeda, 2006; Ascenzi et al., 2010b; Nuriel et al., 2011). Free Trp can be modified to several nitrated products (1-, 4-, 5-, 6-, and 7-nitrotryptophan), to 1-N-nitrosotryptophan, and to several oxidized products by reaction with various RNS, depending on the conditions used. Among them, 1-N-nitrosotryptophan and 6-nitrotryptophan are the most abundant products in the reaction with peroxynitrite, 6-nitrotryptophan being the most abundant product in the reaction with the peroxidase/hydrogen peroxide/nitrite systems (Yamakura and Ikeda, 2006; Bregere et al., 2008; Nuriel et al., 2011).

6-Nitrotryptophan has been observed as the most abundant nitrated product of the reactions between peroxynitrite or myeloperoxidase/hydrogen peroxide/nitrite and Trp residues of several proteins, including bovine SA (Yamakura and Ikeda, 2006). Although several oxidized products have been identified in modified proteins, no 1-N-nitrosotryptophan and 1-N-nitrotryptophan have been observed in proteins that have been reacted either with peroxynitrite or with the

myeloperoxidase/H₂O₂/nitrite system. Trp modifications in proteins may occur at a more limited number of sites *in vivo* than that of Tyr residues, since Trp residues are more buried inside proteins and exist less frequently in proteins, generally. However, since surface-exposed Trp residues tend to participate in protein–protein interactions, their modifications may result in modulation of (macro)molecular recognition (Yamakura and Ikeda, 2006; Bregere et al., 2008; Nuriel et al., 2011).

3.5. Human serum albumin oxidation

Oxidation of HSA by ROS is age related, HSA becoming more oxidized in the elderly than in young subjects (Era et al., 1995). Mild oxidation of HSA has no detectable effect on the binding of drugs to the FA7 site in subdomain IIA (see Section 4.1.6). In contrast, the ligand-binding properties of the FA3–FA4 site in subdomain IIIA (see Section 4.1.3) and the esterase-like activity of oxidized HSA (see Section 6.1) are decreased, most probably due to conformational changes in this subdomain (Anraku et al., 2001). Remarkably, liver and spleen act as scavengers of oxidized HSA (Iwao et al., 2006b); the oxidation of key amino acid residues such as Arg410 may promote the elimination of HSA (Iwao et al., 2006a).

3.5.1. Cys and Met oxidation

The Cys34 residue in domain I of HSA provides anti-oxidant activity and constitutes the largest portion of free thiol in blood (see Section 3.2.1) (Stewart et al., 2005). Also Met87, Met123, Met298, Met329, Met446, and Met548 residues of HSA have been reported to provide anti-oxidant activity (Bourdon et al., 2005; Otagiri and Chuang 2009). In particular, Met and Cys residues account for 40–80% of total anti-oxidant HSA activity (Bourdon et al., 2005). Remarkably, in patients with acute lung injury or sepsis syndrome, HSA administration favorably influences plasma thiol-dependent anti-oxidant status, as well as levels of protein oxidative damage (Quinlan et al., 1998, 2004). In fact, while Cys34 chiefly works as a free radical scavenger, Met residues mainly act as a metal chelator (Bourdon et al., 2005). Lastly, the major structural change in oxidized HSA in healthy human plasma is a disulfide-bonded Cys at the thiol of Cys34 (Kawakami et al., 2006).

Oxidation of Met side-chains leads to the sulfoxide derivative. Oxidation of Met-sulfoxide to Met-sulfone can be obtained under conditions not occurring usually in biological systems. Met-sulfoxide can be reversed back to Met with mild reductants or by Met-sulfoxide reductases, whereas Met-sulfone formation is biologically irreversible (Berlett and Stadtman, 1997; Bourdon and Blache, 2001; Bourdon et al., 2005). Remarkably, the oxidation and reduction cycle of Met residues in biological systems has been hypothesized to serve as a ROS scavenging system to protect proteins from extensive modifications (Levine et al., 1996, 1999). Lastly, HSA is able to scavenge HOCl acid preventing alteration of its preferential biological target α_1 -antiprotease (Halliwell, 1988).

3.5.2. Lys oxidation

Pulmonary hypertension in sickle cell anemia is characterized by decreased NO bioavailability that might, in part, be related to oxidative stress. The Lys159 residue of HSA has been reported to be a site of malonyldialdehyde modification that is differentially present in patients with idiopathic pulmonary arterial hypertension and pulmonary hypertension in sickle cell anemia (Odhiambo et al., 2007).

3.5.3. Protein carbonylation

HSA is subjected to oxidative stress resulting in the modification of protein carbonyls (Temple et al., 2006; Matsuyama et al., 2009; Colombo et al., 2010).

Metal- and HOCl-catalyzed oxidation of HSA lead to the conversion of Lys residues to 2-amino adipic semi-aldehyde residues. Under metal-catalyzed oxidation conditions, only Lys97 and Lys186 residues appear to be modified. With HOCl, the Lys130, Lys257, Lys438, Lys499, and Lys598 modification occurs (Temple et al., 2006).

Recently, increased levels of carbonylated plasma proteins have been observed in smokers, HSA being the major carbonylated protein in the bronchoalveolar lavage fluid of older smokers. In fact, α,β -unsaturated aldehydes present in the whole-phase cigarette smoke extract induce HSA carbonylation. These chemicals induce the depletion of HSA Cys34 free thiol and the marked decrease of free Lys residues by formation of covalent carbonyl adducts. Indeed, acrolein and crotonaldehyde Michael adducts at Cys34, His39, Lys351, and Lys525 occur; moreover, Lys541 and Lys545 form a Schiff base with acrolein. The carbonyl scavenger drugs (*i.e.*, hydralazine and pyridoxamine) partially prevent HSA carbonylation by cigarette smoke extract. Carbonylation of HSA associated with cigarette smoking might result in modifications of its anti-oxidant properties and transport functions of both endogenous and exogenous compounds (Colombo et al., 2010). The preferred site of HSA modification is dependent on the nature of the oxidant and on the structural motifs in the protein to direct the oxidation. The high selectivity provides insights into the factors that drive the selective carbonylation of HSA in *in vivo* systems under oxidative stress (Temple et al., 2006; Aldini et al., 2008; Colombo et al., 2010).

3.6. Human serum albumin phosphorylation

Although HSA phosphorylation does not occur *in vivo*, the 4-*N,N*-dimethylaminopyridine derivative of p(U)₆ reacts with HSA resulting in phosphorylation of Lys and Tyr residues, the amidophosphate bond between Lys and p(U)₆ being the most stable. Remarkably, HSA phosphorylation by the 4-*N,N*-dimethylaminopyridine derivative of p(U)₆ inhibits oligonucleotide cleavage (Gerasimova et al., 2008a).

3.7. Human serum albumin biotinylation

Although HSA biotinylation does not occur *in vivo*, biotinylated bovine SA shows heterogeneous binding to distinct vascular subsets in different lymphoid tissue and organs. In lymph nodes, the strongest labeling has been observed in the medullary sinuses. In the thymus, the most prominent labeling of capillaries was restricted to the corticomedullary area where it has been found to be less intense compared with the splenic reaction. In the spleen, the reaction is restricted to the red pulp sinuses, the white pulp vessels and the marginal sinus being negative for biotinylated bovine SA. The use of biotinylated SA may offer a reliable probe for the histological identification of selected microvascular endothelia in lymphoid tissues (Balogh and Petz, 2005).

3.8. Human serum albumin chlorination

HOCl chlorinates Tyr residues in peptides and proteins to produce chloro-Tyr. When HSA is exposed either to HOCl *in vitro* or to HOCl produced by myeloperoxidase or to HOCl produced by stimulated neutrophils, Tyr residues are converted to chloro-Tyr. About 2% of the HOCl generated by neutrophils accounts for the formation of chloro-Tyr. Approximately 20% decrease in the chloro-Tyr content of HSA occurs over 1 h in SA-knockout rats. This indicates that the dechlorination of chloro-Tyr is the major metabolic pathway to eliminate this modified amino acid *in vivo*. Lastly, chloro-Tyr appears to be a useful marker for establishing a role of HOCl in host defence and inflammation (Kettle, 1996; Mani et al., 2007).

4. Albumin binding properties

The extraordinary ligand binding properties of HSA reflect its multidomain organization. The complex mechanism modulating ligand binding to HSA represents one of the most important structure–function correlations ever reported for monomeric proteins. HSA is known to carry almost every small molecule, thus it is a potential contender for being a molecular cargo/or nanovehicle for clinical, biophysical, and industrial purposes (Fasano et al., 2005; Ahmed-Ouameur et al., 2006; Ascenzi et al., 2006a; Varshney et al., 2010; Ascenzi and Fasano, 2010).

Under physiological conditions, HSA binds not only endogenous and exogenous low molecular weight compounds but also peptides and proteins (Fasano et al., 2005). Thirty-five proteins were found to be associated to HSA including both known high and low abundant proteins (e.g., angiotensinogen, apolipoproteins, ceruloplasmin, clusterin, hemoglobin (Hb), plasminogen, prothrombin, and transferrin). Any binding of proteins and peptides to HSA impacts proteomics and biomarker discovery studies, since the presence of both unbound and bound states of proteins in serum can affect both the clearance and the detection of the free-state proteins and peptides. The fraction of peptides and proteins bound HSA is defined as “albuminome” (Gundry et al., 2007).

4.1. Fatty acid binding sites

The first evidence of FA-binding properties of HSA dates at 1941, when it has been reported that: “Albumin which was crystallized four times was still yellow by the presence of plasma pigments. It was suspected that this pigment might be lipoidal in nature” (Kendall, 1941). Several studies carried out in the following years allowed to better characterize the ability of HSA to bind FA (Curry, 2009; van der Vusse, 2009).

HSA is able to bind up to nine equivalents of long chain FAs, which represent the primary physiological ligands at multiple binding sites (i.e., FA1–FA9). These sites are distributed throughout the protein in an asymmetric way (Fig. 3) and show different affinity (Bhattacharya et al., 2000a; Simard et al., 2006). FA4 and FA5 are high-affinity sites for FAs. FA2, a medium affinity site which lies at the interface between subdomains IA and IIA, is entirely contained within the N-terminal half of the protein, while sites FA4 and FA5 are entirely contained within domain III. These sites provide the most enclosed binding environments on HSA that allow the methylene tail of the FA to bind in a nearly linear conformation while the FA carboxyl forms specific salt-bridge interaction(s) with at least one basic amino acid side-chain (Simard et al., 2006). FA8 and FA9 are usually considered as supplementary binding sites, as they show ligand occupancy only in the presence of short-chain FAs (i.e., FA8) or in the presence of saturating FA concentration (i.e., FA9) (Bhattacharya et al., 2000a).

FA binding sites also provide accommodation of several endogenous and exogenous ligands, including a wide variety of drugs, displaying appreciable affinity for one or more binding sites of HSA. This issue is of great relevance as binding to HSA improves plasma solubility and half-life of drugs, but at the same time reduces their free active concentration (Ascenzi et al., 2006a). Crystallographic structures of HSA are summarized in Table 3.

4.1.1. FA1 (heme pocket)

FA1 is located in a D-shaped cavity in the centre of the four-helix bundle of subdomain IB (Fig. 4A). In the absence of any ligand, the binding pocket is closed by Tyr138 that stacks with Tyr161.

4.1.1.1. Heme binding. FA1 partially corresponds to the heme binding site (Fig. 4B). Heme–Fe(III) binding induces a dramatic conformational change(s) with reorientation of Tyr138 and Tyr161 residues that provide π – π stacking interaction with the

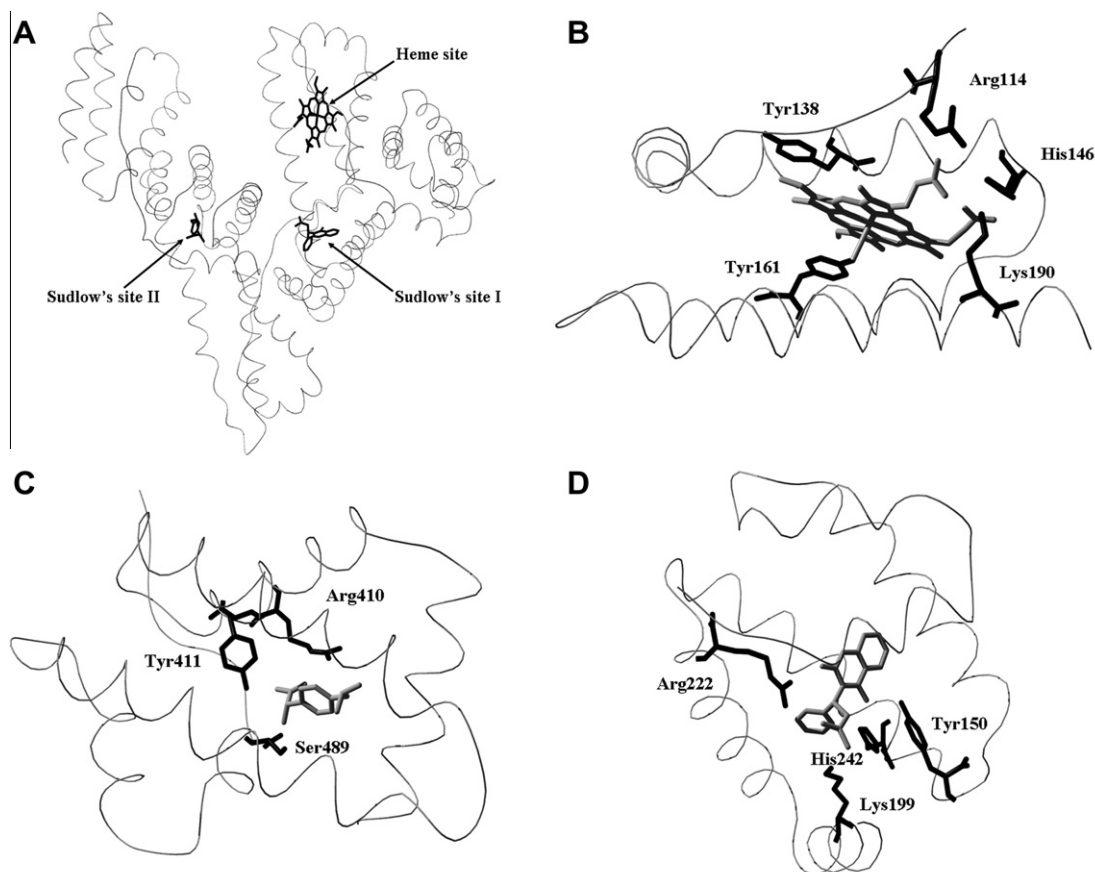
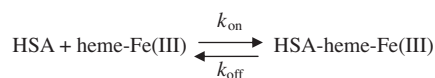


Fig. 4. Three-dimensional structure of HSA complexed with prototypical ligands. Panel A. Ribbon representation of the heart-shaped structure of HSA complexed with heme-Fe(III) in FA1 (*i.e.*, heme site), warfarin in FA7 (*i.e.*, Sudlow's site I), and ibuprofen in FA3–FA4 (*i.e.*, Sudlow's site II). Heme, ibuprofen, and warfarin are rendered as sticks (black). Atomic coordinates were taken from PDB entry 1O9X (Zunzain et al., 2003), 2BXD and 2BXG (Ghuman et al., 2005). For details, see Section 4.1. Panel B. Heme-Fe(III) binding to the FA1 site. Heme-Fe(III) (gray) is bound in the D-shaped cavity in the centre of the four-helix bundle of subdomain IB. Residues Arg114, Tyr138, His146, Tyr161, and Lys190 are shown. Atomic coordinates were taken from the PDB entry 1O9X (Zunzain et al., 2003). For details, see Section 4.1.1. Panel C. Ibuprofen primary binding to the FA3–FA4 cleft. Ibuprofen (gray) is bound in the center of FA3–FA4 pocket (*i.e.*, Sudlow's site II). Residues Tyr411, Arg410, and Ser489 are shown. Atomic coordinates were taken from the PDB entry 2BXG (Ghuman et al., 2005). For details, see Section 4.1.3. Panel D. Warfarin binding to the FA7 site. Warfarin (gray) is bound in the hydrophobic cavity of subdomain IIA (*i.e.*, FA7, Sudlow's site I). Residues Tyr150, Lys199, Arg222 are shown. Atomic coordinates were taken from the PDB entry 2BXD (Ghuman et al., 2005). For details, see Section 4.1.6. Ribbon representation of HSA was drawn with the Swiss-PDB Viewer (Guex and Peitsch, 1997).

porphyrin and supply a donor oxygen (from Tyr161) for the penta-coordinate heme-Fe-atom. Heme-Fe(III) is secured by the long IA–IB connecting loop that fits into the cleft opening. Heme-Fe(III) propionates point toward the interface between domains I and III and are stabilized by salt bridges with Arg114, His146, and Lys190 residues (Wardell et al., 2002; Zunzain et al., 2003).

Heme-Fe(III) binds to its primary cleft, encompassing FA1, according to the minimum reaction mechanism depicted by Scheme 1 (Bocedi et al., 2005):



Scheme 1. Minimum reaction mechanism for heme-Fe(III) binding to HSA.

where k_{on} is the second order rate constant for the formation of the HSA-heme-Fe(III) complex, k_{off} is the first order rate constant for the dissociation of the HSA-heme-Fe(III) complex, and $K_d (=k_{\text{off}}/k_{\text{on}})$ is the dissociation equilibrium constant.

Values of k_{on} , k_{off} , and K_d ($=k_{\text{off}}/k_{\text{on}}$) are $7.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $9.6 \times 10^{-3} \text{ s}^{-1}$, and $1.2 \times 10^{-8} \text{ M}$, respectively, under para-physiological conditions (Adams and Berman, 1980; Peters, 1996; Bocedi et al., 2005). Remarkably, heme-Fe(III) binding to HSA is modulated allosterically and inhibited competitively (see Section 7.3) (Bocedi et al., 2005; Ascenzi and Fasano, 2010).

4.1.1.2. Synthetic porphyrin and phthalocyanine binding. HSA binds not only the heme-Fe but also synthetic Fe(II) porphyrins (Fe(II)P) and Al(III) phthalocyanines (Al(III)Pc) (Gantchev et al., 1999; Tsuchida et al., 2009).

HSA incorporates Fe(II)P yielding to red HSA-Fe(II)P hybrids which can reversibly bind and release O_2 under physiological conditions in approximately the same way as Hb (see Section 5.2.1). HSA hosts a maximal eight Fe(II)P molecules, their step-wise dissociation equilibrium constants ranging between 1×10^{-6} and $1 \times 10^{-4} \text{ M}$. The HSA-Fe(II)P solution properties are almost identical to those of HSA itself. Moreover, HSA-Fe(II)P complexes have no effect on the morphology of blood cells and do not engender immunological reaction and platelet activation (Tsuchida et al., 2009).

Phthalocyanines, which are extensively studied as tumor localizing photosensitizers for photodynamic therapy, are transported by the blood circulatory system to target tissues. Al(III)Pc occupy one strong binding site and eight weak clefts of HSA. Al(III)Pc binding to the high affinity site depends on the sulfonation and isomeric composition. Al(III)Pc without SO_3^- groups on adjacent iso-indole rings exhibit a high affinity ($K_d = 3 \times 10^{-8} \text{ M}$) and a non-cooperative binding behavior. On the other hand, Al(III)Pc with two or three adjacent SO_3^- groups display a lower affinity ($K_d \sim 3 \times 10^{-7} \text{ M}$) and a cooperative binding behavior. Al(III)Pc binding to HSA induces protein conformational changes (as reported for heme-Fe(III) binding to HSA; see Section 4.1.1.1) depending on the degree of sulfonation and isomeric composition (Gantchev et al., 1999). Interestingly, since the ferrous Pc complex (Fe(II)Pc) binds reversibly CO and NO (Ercolani et al., 1981; Ascenzi et al., 1987), O_2 carriers based on HSA-Fe(II)Pc might be developed.

4.1.1.3. Bilirubin binding. HSA binds bilirubin, an insoluble yellow-orange pigment derived from heme-Fe catabolism, in sub-domain IB, in close proximity to the heme site (see Section 4.1.1.1). However, due to steric hindrance of the open tetrapyrrole, bilirubin accommodates in the preformed L-shaped pocket indicatively located at the opening of the FA1 cavity (Zunzain et al., 2008).

The pigment is evidently “strapped” into place by the extended polypeptide that connects subdomains IA and IB (residues Glu110–Glu119) and by the formation of salt-bridges with residues Arg117 and Arg186, which lie at the entrance of the binding cleft. The endo-vinyl dipyrinone group of the 4Z,15E-bilirubin-IX α isomer, containing the 4Z double bond, maintains a *syn*-planar conformation and is accommodated in the deepest part of the binding cleft, largely shielded from solvent. It makes close contacts with the apolar side-chains of residues from helices h8–h10 (Ile142, Phe149, Leu154, Phe157, Gly189, and the aliphatic portion of Lys190). The carbonyl oxygen of the lactam ring is hydrogen bonded to the O atom of the Tyr138 side-chain. The propionate carboxylate group of the exo-vinyl dipyrinone group makes a salt-bridge interaction with the side-chain of Arg117 (Zunzain et al., 2008).

The carboxylate group of the endo-vinyl dipyrinone group of the 4Z,15E-bilirubin-IX α isomer is partially shielded from solvent and is oriented towards the entrance to the pocket, making a salt-bridge with the Arg186 side-chain. A planar conformation for the exo-vinyl dipyrinone group that contains the 15E double bond is unfavorable because of steric interactions of the methyl substituents. It occurs in a twisted *syn*-periplanar conformation in a more exposed part of the cleft formed by the polypeptide “strap” and helices h7 and h8. The inner pyrrole is bound directly underneath the polypeptide strap, pinned by apolar contacts with the Leu115 and Arg117 side-chains, while the outer lactam ring is more exposed to solvent. The E configuration allows the methyl and vinyl substituents on the lactam ring to pack against the hydrophobic flank of this part of the pocket (composed largely of the side-chains of Pro118, Met123, Phe134 and Phe165), but its amide and carbonyl groups are solvent exposed and appear to make no contact with the protein. The NH atom of the inner pyrrole may form an unfavorable hydrogen bond with the carbonyl O atom of Leu115. Thus, the exo-vinyl dipyrinone moiety of 4Z,15E-bilirubin makes no specific hydrogen bond interactions with the protein. Modeling studies suggest that also binding of the 4Z,15Z-bilirubin isomer, the most abundant in human plasma, could be achieved in the same pocket (Zunzain et al., 2008).

4.1.1.4. Fatty acid binding. All FAs bind to FA1 in the same orientation, with the carboxylate group hydrogen-bonded to Arg117 and to a water molecule that is also coordinated by both the side-chain hydroxyl group of Tyr161 and the carbonyl oxygen atom of Leu182. For long-chain saturated FAs, the tail curls around the inside surface of the cavity so that the tip of the hydrophobic tail gradually approaches His146 at the lower end of the cavity opening (Bhattacharya et al., 2000a).

4.1.1.5. Comparative binding geometry of FA1 ligands. The binding mode of heme-Fe(III), of the 4Z,15E-bilirubin isomer, and of FAs to the HSA subdomain IA is markedly different. In fact, FAs and heme-Fe(III) binding induces a significant conformational rearrangement of subdomain IB (Zunzain et al., 2008). In particular, binding of FAs and heme-Fe(III) causes the rotation of the Tyr138 and Tyr161 side-chains of about 90° to opposite sides of the binding cavity, thus re-shaping the binding cleft. Tyr138 moves to form the outside flank of the pocket and Tyr161 lies against the other wall of the cavity. Thus, Tyr138 and Tyr161 are placed flat in contact with the bound FAs and heme-Fe(III), holding it in place. The FAs- and heme-Fe(III)-induced cavity is D-shaped, and can fully accommodate the planar porphyrin ring of heme-Fe(III) (see Section 4.1.1.1). The methylene tails of FAs bound to this pocket curve around the back wall; this leaves sufficient room for co-binding of aromatic drugs or drug-like molecules such as indomethacin and triiodobenzoic acid (see Table 3) (Ghuman et al., 2005;

Bhattacharya et al., 2000b). Nevertheless, bilirubin binding does not induce the large rotations of Tyr138 and Tyr161 which open up the D-shaped cavity described above for heme–Fe(III) binding (see Section 4.1.1.1), whereas the pigment is significantly distorted from the ridge-tile conformation (though it broadly maintains an L-shaped conformation) (Zunzain et al., 2008).

4.1.2. FA2

FA2 is located between subdomains IA and IIA and it is one of the most enclosed FA binding sites of HSA. Several evidences indicate that FA binding to this modulatory site stabilizes the HSA B-conformation and it is responsible for the ligand-induced conformational transition. Indeed, the formation of a contiguous pocket to accommodate FA requires the rotation of domain I relative to domain II (see Section 2.7). As a consequence, this rearrangement is stabilized by ligand binding to FA2 (Fanali et al. 2005, 2007; Ascenzi and Fasano, 2010).

The carboxylate head-groups of FAs are anchored in subdomain IIA by hydrogen bond interactions to the side-chains of Tyr150, Arg257, and Ser287. The methylene tail extends linearly within the narrow hydrophobic cavity formed by realignment of IA and IIA subdomains (Bhattacharya et al., 2000a).

Eventually, FA2 has been suggested to be a third binding site of ibuprofen on the basis of solution studies addressing the effect of this drug on heme–Fe(III) affinity and HSA–heme–Fe spectroscopic and reactivity properties (Nicoletti et al., 2008; Ascenzi et al., 2009a,b).

4.1.3. FA3–FA4 (Sudlow's site II)

FA3 and FA4 are composed of six helices and are located in a large cavity in subdomain IIIA that as a whole constitutes Sudlow's site II (Fig. 4C). This cleft is preferred by aromatic carboxylates with an extended conformation, the non-steroidal anti-inflammatory drug ibuprofen representing the prototypical ligand (Sudlow et al., 1975, 1976; Carter and Ho, 1994; Peters, 1996; Curry et al., 1998; Yamasaki et al., 1999; Petitpas et al., 2001; Curry, 2002, 2009; Hamilton, 2004; Ghuman et al., 2005).

In FA3, the head-groups of FAs are hydrogen bonded to Ser342 and Arg348 from the IIB subdomain, and Arg485 from the IIIA subdomain. In FA4, the carboxylate head-groups of FAs are hydrogen bonded by Arg410, Tyr411, and Ser489, which lie on the exterior surface on one side of subdomain IIIA, whereas the methylene tail protrudes further into the hydrophobic tunnel that runs through subdomain IIIA (Bhattacharya et al., 2000a; Ghuman et al., 2005; Curry, 2009).

Actually, Sudlow's site II is composed of the apolar region occupied by the methylene tail of FA bound to FA3 and the polar patch that interacts with the carboxylate moiety of FA bound to FA4. No drug is observed to access the long, narrow hydrophobic tunnel of FA4 that accommodates the methylene tail of FAs. Moreover, FAs bound to FA3 do not interact with the polar patch centered on Tyr411 (Bhattacharya et al., 2000a; Ghuman et al., 2005; Curry, 2009).

Drugs (e.g., ibuprofen) cluster in the center of Sudlow's site II, interacting with the hydroxyl group of Tyr411; Arg410 and Ser489 residues also contribute with salt-bridge and hydrogen-bond interactions to drug binding. Thus, the presence of a single basic polar patch located at one end of the apolar binding pocket is at the root of the specificity for drugs with a peripherally located electronegative group (Ghuman et al., 2005; Curry, 2009).

The dissociation equilibrium constant for ibuprofen binding to FA3–FA4 (K_d) is 1×10^{-6} M, under para-physiological conditions (Kragh-Hansen, 1981; Cheruvallath et al., 1997). Remarkably, ibuprofen binding to HSA is inhibited competitively (Ghuman et al., 2005).

Lastly, fluorescent dansyl-L-norvaline, dansyl-L-phenylalanine, and dansyl-L-sarcosine have been reported to bind selectively to the FA3–FA4 cleft, representing invaluable specific tools for ligand binding. The dansyl group provides the most important anchoring interactions for binding of L-amino acids which are pinned between the side-chains of Asn391 and Phe403 on one side and Leu453 on the other (Ryan et al., 2011a).

4.1.4. FA5

FA5 is formed by a hydrophobic channel located in subdomain IIIB. A single FA molecule binds in an extended conformation; the carboxylate head-group of the bound FA interacts with the side-chains of Tyr401 and Lys525 while the methylene tail extends into the tunnel (Bhattacharya et al., 2000a). HSA complexed with arachidonic acid in FA5 provides a comprehensive look on the peculiar structure of this site, where the long linear cavity is evident (Petitpas et al., 2001). On the other hand, complexes with small ligands (e.g., propofol) highlight the occurrence of small sub-cavities within this cleft (Bhattacharya et al., 2000b).

4.1.5. FA6

FA6 is located at the interface between subdomains IIA and IIB and it is occupied by both medium and long-chain FAs. This cleft is significantly different from FA1–FA5 sites; in fact, there is not a cluster of amino acid side-chains that stabilize electrostatically the FA carboxylate. The side-chains of Arg209, Lys351, and Ser480 transiently recognize the FA carboxylate. The middle portion of the methylene tail is well anchored by salt-bridges from Arg209 to both Asp324 and Glu354. Only capric acid binds to FA6 in a different mode, since two molecules are observed in a linear tail-to-tail configuration. In general, the absence of clear ligands for the carboxylate groups of FAs at this rather open site suggests that the binding affinity is relatively low (Bhattacharya et al., 2000a; Simard et al., 2006). Noteworthy, FA6 has been reported to be the ibuprofen secondary site (Ghuman et al., 2005).

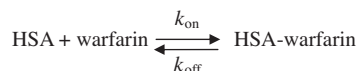
4.1.6. FA7 (Sudlow's site I)

The hydrophobic cavity of subdomain IIA hosts the seventh FA binding site (i.e., FA7 or Sudlow's site I). This site binds preferentially bulky heterocyclic anions, the prototypical ligand being warfarin (Fig. 4D) (Sudlow et al., 1975, 1976; Carter and Ho, 1994; Peters, 1996; Curry et al., 1998; Petitpas et al., 2001; Curry, 2002, 2009; Hamilton, 2004; Ghuman et al., 2005; Zhu et al., 2008). This site is smaller than the analogous binding cavity in subdomain IIIA (i.e., FA3–FA4 or Sudlow's site II) (Ghuman et al., 2005; Curry, 2009).

The FA carboxylate is stabilized by polar interaction(s) with the Arg257 residue, thus providing a bridge between FA2 and FA7 sites. Basic residues Lys199, Arg218, Arg222, and His242 do not appear to be directly involved in FA binding, but could actively participate to the stabilization of other ligands in the cavity. FAs bind to FA7 in a curved configuration, their tail being co-planar with aromatic drugs bound to this site, thus suggesting that FA7 is a low affinity cleft (Bhattacharya et al., 2000a; Simard et al., 2006).

Drugs (e.g., warfarin) cluster in the center of Sudlow's site I, having a planar group pinned snugly between the apolar side-chains of Leu238 and Ala291. In contrast, there is much greater variation in the drug position within the plane perpendicular to the line between these two residues. Different compounds occupy the apolar compartments of Sudlow's site I to different extents, e.g., warfarin occupies the right-hand and the front sub-chambers. All the compounds are positioned to make a hydrogen bond interaction with the hydroxyl group of Tyr150, this residue assuming a central role in drug interactions. The R-(+) and S-(−) enantiomers of warfarin bind in essentially the same position as one another and appear capable of making a total of three hydrogen bonds with Tyr150, His242, and either Lys199 or Arg222. The similarity of the binding environments for the enantiomers helps to explain the poor stereo-selectivity of HSA for warfarin (Petitpas et al., 2001; Ghuman et al., 2005; Curry, 2009).

At low drug concentration, warfarin binds to FA7 according to the minimum reaction mechanism depicted by Scheme 2 (Fitos et al., 2002):



Scheme 2. Minimum reaction mechanism for warfarin binding to HSA.

where k_{on} is the second order rate constant for the formation of the HSA-warfarin complex, k_{off} is the first order rate constant for the dissociation of the HSA-warfarin complex, and $K_d (=k_{\text{off}}/k_{\text{on}})$ is the dissociation equilibrium constant. Values of k_{on} , k_{off} , and K_d are $3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, 10 s^{-1} , and $3 \times 10^{-7} \text{ M}$, respectively, under para-physiological conditions (Fitos et al., 2002). On the other hand, at high drug concentration, a rate limiting step, probably reflecting protein rearrangements, controls kinetics of warfarin association. Although both R- and S-warfarin enantiomers display similar thermodynamic and kinetic parameters, the rate limiting step for binding of R- and S-warfarin enantiomers is different ($k_{\text{lim}} = 32 \text{ s}^{-1}$ and 400 s^{-1} , respectively) (Petitpas et al., 2001; Fitos et al., 2002; Ghuman et al., 2005; Curry, 2009). Remarkably, warfarin binding to HSA is modulated allosterically and inhibited competitively (Baroni et al., 2001; Fanali et al., 2010) (see Section 7.3).

Recently, dansyl-L-asparagine, dansyl-L-arginine, and dansyl-L-glutamate have been reported to accommodate selectively in the FA7 site; due to their intrinsic fluorescence, these dansyl-L amino acids can be used as specific markers for ligand binding. The dansyl group provides the most important anchoring interactions for binding of L-amino acids in the center of FA7 between the side-chains of Ala291 and Leu238 (Ryan et al., 2011a).

Lastly, HSA can covalently bind different FA7 ligands thanks to the presence of Lys199, which can act as a nucleophile. Some molecular dynamic simulations of the FA7 binding site show that the ionization state of the Lys199 and Lys195 residues influence the structure and dynamics of the FA7 site (Díaz et al., 2001).

4.1.7. FA8

FA8 is located at the base of the gap between subdomains IA–IB–IIA on one side and subdomains IIB–IIIA–IIIB on the other side. Due to volume restrictions, FA8 can only accommodate short-chain FA molecules, such as capric acid. The methylene tail of capric acid bound to FA6 contributes to the formation of the hydrophobic end of FA8. At the other end of the cavity some polar residues (i.e., Lys195, Lys199, Arg218, Asp451, and Ser454) form an open ring that stabilizes the polar head of the ligand (Bhattacharya et al., 2000a).

4.1.8. FA9

FA9 lies in an upper region of the gap between subdomains IA–IB–IIA on one side and subdomains IIB–IIIA–IIIB on the other side, thus providing a rather open binding environment. A salt-bridge between Glu187 of domain I and Lys432 of domain III contributes to keep the ligand in place (Bhattacharya et al., 2000b). Noteworthy, FA9 is formed upon the FA-induced conformational transition, thus providing an additional binding site in FA-saturated HSA (Bhattacharya et al., 2000a; Petitpas et al., 2003).

4.2. Thyroxine binding sites

T₄ binds to FA-free HSA at four sites, labeled Tr-1 to Tr-4; Tr-1 is located in subdomain IIA, Tr-2 in subdomain IIIA, and Tr-3 and Tr-4 in subdomain IIIB (Fig. 5A). The Tr binding clefts partially overlap the FA7, FA3–FA4, and FA5 binding sites, the latter showing double occupancy (Petitpas et al., 2003).

T₄ binding to Tr-1 does not significantly affect the side-chains of residues Trp214, Arg218, and Arg222. A specific hydrogen bond interaction occurs between the phenolic hydroxyl of T₄ and the side-chains of Tyr150 and Arg257. The iodine atoms of the phenyl rings of T₄ form hydrophilic contacts with side-chains and main-chain carbonyl oxygen atoms, although they are accommodated within a predominantly hydrophobic pocket. However, the ether oxygen in the center of the hormone does not form hydrogen bond(s) with HSA. The T₄ carboxylate moiety makes a salt-bridge interaction with Lys199; Lys195 may also contribute an electrostatic interaction, while the amino group is located at the mouth of FA7. On the basis of structural considerations, Tr-1 has been hypothesized to be the high affinity T₄ binding site, thus being responsible for the observed dissociation equilibrium constant $K_d = 2.3 \times 10^{-6}$ M (Petitpas et al., 2003).

T₄ binding to Tr-2 is sterically hindered and the hormone binds at the entrance of FA4, pointing out toward the solvent. The outer ring of the hormone is located in the deepest part of the binding site, where the phenolic hydroxyl makes hydrogen

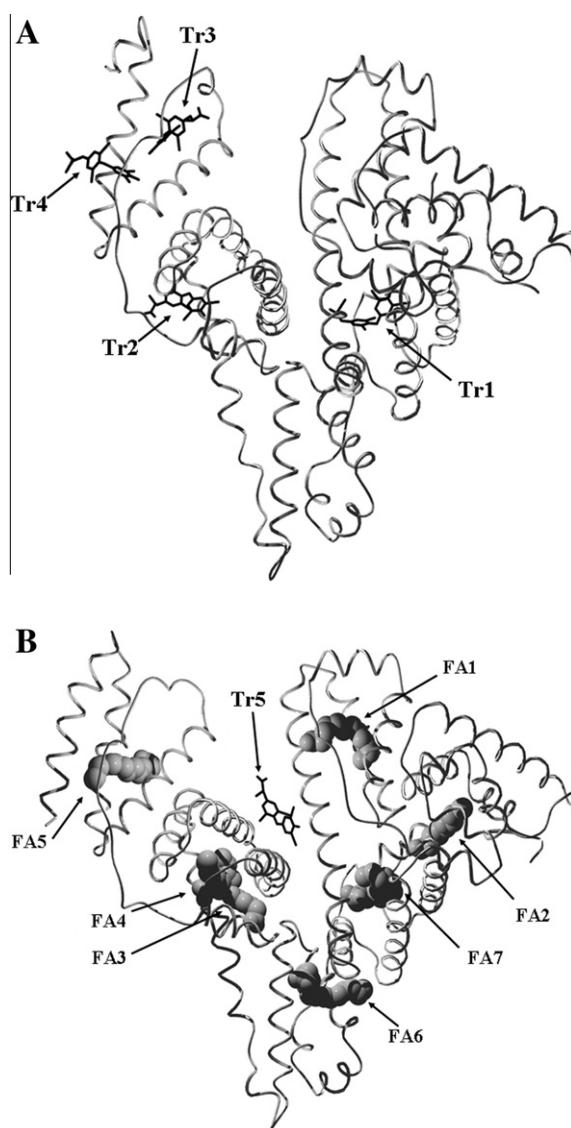


Fig. 5. Three-dimensional structure of HSA complexed with T₄ in the absence (panel A; PDB entry 1HK1) and in the presence (panel B; PDB entry 1HK4) of myristate (Petitpas et al., 2003). Myristate (gray) is rendered as space-fill. T₄ is rendered as sticks. For details, see Section 4.2. Ribbon representation of HSA was drawn with the Swiss-PdbViewer (Guex and Peitsch, 1997).

bonds to the side-chains of Tyr411 and Ser489, whereas the inner ring establishes van der Waals contacts with the side-chains of Glu390, Asn391, Leu394, Ala406, and Arg410. The aminopropionic acid moiety of T₄ does not make hydrogen bonds with the protein. Probably, the partial occupancy of Tr-2 is consistent with the hypothesis that this site is a low-affinity cleft for T₄ (Petitpas et al., 2003).

T₄ molecules bind to Tr-3 and Tr-4 with a configuration that partially overlaps FA binding to FA5 (Curry et al., 1998). The occupation of this site induces a significant structural modification of the flank of the subdomain IIIB (residues 502–514). T₄ forms some hydrophobic contacts in the Tr-3 site that is open at both ends, whereas the phenolic hydroxyl is exposed to solvent and does not form specific interactions with HSA. The carboxylate group makes a hydrogen bond to a water molecule stabilized by Asp301. In Tr-4, only the outer ring of T₄ contacts the protein, and the C-terminal helix that is a very flexible part of the structure serves to enclose T₄ at this site. The Tr-4 site is considered a low affinity site for T₄ because the phenolic hydroxyl faces an intra-helical main-chain hydrogen bond (between Ala528 and Leu532) and does not have a hydrogen bonding partner (Petitpas et al., 2003).

FAs compete with T₄ for binding to all four Tr sites, and high mole ratios of FA prevent T₄ binding. At the same time FAs are responsible for the conformational changes that produce the rearrangement of the three-dimensional structure of HSA (Fig. 5B) (Curry et al., 1998; Curry, 2002, 2009) with the formation of the fifth T₄ binding site located in the cleft between domains I and III (i.e., FA9). This open site, called Tr-5, is composed by the long helix connecting domains I and II and by a pair of helices from subdomain IIIA. T₄ binds to Tr-5 with a dissociation equilibrium constant ($K_d = 4.9 \times 10^{-6}$ M) similar to that observed for the primary binding site (i.e., Tr-1) in defatted HSA (Petitpas et al., 2003).

Two HSA mutants (i.e., Arg218His and Arg218Pro in subdomain IIA; see Section 2.3.6) show a higher affinity for T₄ than wild type HSA (Petersen et al., 1994; Sunthornthepvarakul et al., 1994; Wada et al., 1997). This causes a reduction of the T₄ availability in plasma with the consequent increase of T₄ synthesis. This leads to the increase of the total plasma T₄ level which is associated with familial dysalbuminemic hyperthyroxinemia (Petitpas et al., 2003) (see Section 8.3.2).

4.3. The bacterial protein binding site

HSA has been reported to bind bacterial surface: in some isolates of *Finnegoldia magna*, the protein responsible for HSA binding is called PAB (de Château and Björck, 1994). PAB contains a domain (i.e., GA module) showing high sequence similarity (60%) to the albumin binding domains (ABDs) of protein G, a bacterial protein that binds the Fc domain of IgGs (Björck and Kronvall, 1984).

The interaction of the GA module with HSA occurs between residues from the second α -helix of the GA module and the two loops surrounding it and the HSA domain II region delimited by h2–h3 and h7–h8 together with the loop region preceding h7. Only one-fourth of the total GA surface area is in contact with HSA in a flat surface of $\sim 700 \text{ \AA}^2$. The binding surface consists of a hydrophobic core flanked by two hydrogen bond networks. The hydrophobic core of the interface is lined with residues Phe228, Ala229, Ala322, Val325, Phe326, and Met329 from HSA and residues Phe27, Ala31, Leu44, and Ile48 from the GA module. A hydrogen bond network is present between h7 in the HSA domain IIB and the loop preceding the second helix in GA. The HSA residue Glu321 forms two hydrogen bonds, one each with the main chain nitrogens of Thr24 and Ser25 in the GA module. Furthermore, the side-chain hydroxyl group of Ser25 in the GA module forms another hydrogen bond with the side-chain oxygen of Asn318 from HSA, that is also involved in a hydrogen bond with the side-chain hydroxyl group of Tyr28 in the GA module. At the opposite end of the binding interface, a second hydrogen bond network is formed between residues of the loop connecting the second and third helix in the GA module and residues of h2–h3 present in the HSA domain IIA. Here, HSA residues Glu230 and Asn267 form hydrogen bonds to the N α atom and the side-chain hydroxyl group of Thr37, respectively. Glu230 also interacts with the main chain oxygen of Ala35 (Lejon et al., 2004).

Although the biological function(s) of the GA module is not known in detail, the acquisition of the GA module seems to add selective advantages to the bacterium in terms of growth and to increase its virulence, probably by providing the bacteria with FAs and, possibly, other nutrients transported by HSA (Lejon et al., 2004).

4.4. Metal binding sites

HSA displays a wide variety of binding sites for several metal ions, including Mg(II), Al(III), Ca(II), Mn(II), Co(II/III), Ni(II), Cu(I/II), Zn(II), Cd(II), Pt(II), Au(I/II), Hg(II), and Tb(III) (Peters, 1996; Sokołowska et al., 2009, 2010; Deng et al., 2010; Duff and Kumar, 2009). So far, three major binding sites endowed with appropriate residues matching for the different metal geometries have been observed. The first site (usually labeled as N-terminal binding site, NTS) is located at the N-terminus, where Cu(II), Co(II), and Ni(II) are coordinated by nitrogen donor atoms from Asp1, Ala2, and His3 (Sadler et al., 2005). The second site is represented by the free Cys34 thiol that binds Au(I), Hg(II), and Pt(II) ions (Shaw, 1989; Peters, 1996). The third metal binding site is the so-called primary multimetal binding site or Cadmium site A (MBS-A), which involves His67, Asn99, His247, and Asp249 residues. Due to its pseudo-octahedral geometry, this cleft is able to stably allocate different metal ions. Indeed, MBS-A has been suggested to be the primary site of Zn(II) and Cd(II) and the secondary cleft for Cu(II) and Ni(II) (Blindauer et al., 2009). Despite the current lack of experimental evidence, a secondary site for Cd(II) binding, called secondary multimetal binding site or Cadmium site B (MBS-B), has been postulated (Mothes and Faller, 2007). Eventually, other metal ions bind either unspecifically or to undefined regions (Peters, 1996).

4.4.1. The N-terminal binding site

NTS was the earliest metal binding site to be identified. The site is primarily specific for Cu(II) and Ni(II), which are strongly bound in a square-planar ligand arrangement formed by the three N-terminal amino acid residues Asp1-Ala2-His3. Cu(II) and Ni(II) are held tightly in a chelate ring embracing the $N\alpha$ atom of Asp1, the deprotonated $N\alpha$ atoms of Ala2 and His3, and the $N\delta$ atom of His3 (Sadler et al., 2005; Sokołowska et al., 2009). The Cu(II) affinity for HSA is very high, the value of the dissociation equilibrium constant for the HSA-Cu(II) complex formation being 6.7×10^{-17} M. The Ni(II) affinity for HSA is lower than that of Cu(II), the value of the dissociation equilibrium constant for the HSA-Ni(II) complex formation being 2.5×10^{-10} M. Addition of HSA to a Cu(II) solution is accompanied by a clearly visible spectral shift, the blue of free Cu(II) being replaced by a purple color, with $\lambda_{\max} = 525$ nm and $\epsilon_{\max} = 1.01 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$. The color of the HSA-Ni(II) complex solution is yellow with $\lambda_{\max} = 420$ nm and $\epsilon_{\max} = 1.37 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$ (Glennon and Sarkar, 1982; Masuoka et al., 1993; Peters, 1996). In plasma, only about 10% of the total Cu(II) is bound to HSA, the majority being incorporated into ceruloplasmin. Since the Cu(II) concentration is about 2×10^{-6} M in plasma, only about 0.3% of the Cu(II) binding site on HSA is occupied (Peters, 1996). The plasma concentration of Ni(II) is less than 3×10^{-8} M; thus, only a minimal fraction of HSA molecules is involved in Ni(II) transport (Peters, 1996). It should be noticed, however, that HSA circulating in the human bloodstream exhibits a distinct heterogeneity at its N-terminus, about 25% of HSA molecules having their first two amino-acid residues, Asp1-Ala2, cleaved off. This results in the under representation of NTS in HSA derived from human blood. This defect was not observed in other mammalian SAs (Chan et al., 1995; Sokołowska et al., 2009).

Co(II) binds to NTS in an octahedral environment, with the $\beta\text{-COO}^-$ group of Asp1 and the $\epsilon\text{-NH}_2$ group of Lys4 axially contributing to the metal ion coordination sphere. Recently, a new rapid clinical assay for myocardial ischemia has been proposed, based on an interaction of Co(II) ions with HSA derived from plasma, followed by dithiothreitol (DTT) treatment (Bar-Or et al., 2001a) (see Section 8.2.3). This test is based on the assumption that the clipping of two first residues of HSA, Asp1-Ala2, is related to ischemia, and on the assumption that NTS is the primary binding site for Co(II) ions (Bar-Or et al., 2001a). Nevertheless, further spectroscopic studies demonstrated that NTS is not the primary binding site for Co(II) (Mothes and Faller, 2007). A complete thermodynamic investigation of Co(II) binding to HSA has now elucidated the occurrence of three distinct binding sites corresponding to MTS-B, MTS-A and NTS, with dissociation equilibrium constants of 1×10^{-5} M, 9×10^{-5} M and 1×10^{-4} M, respectively (Sokołowska et al., 2009).

4.4.2. The Cys34 binding site

Cys34 is the only thiol group of HSA that does not take part to intramolecular disulfide bridges. About 40% of the circulating HSA has Cys34 not coupled to low molecular weight thiols, such as cysteine and glutathione (see Section 3.2.1). The Cys34 side-chain is located at the bottom of a crevice, which results in a limited accessibility and elevated specificity in metal ion interactions (Sokołowska et al., 2009). The peculiar topology of the Cys34 site accounts for its unambiguous involvement in Au(I) (from anti-arthritis drugs) and Pt(II) (from cisplatin) complexes (Shaw, 1989; Ivanov et al., 1998; Talib et al., 2006; Sokołowska et al., 2009). Ag(I) is also expected to bind with high affinity to Cys34 ($K_d = 1 \times 10^{-5}$ M) on the basis of the observation of typical Ag-S bands in the Raman spectrum of Ag(I)-HSA. Additionally, a secondary site for Ag(I) binding has been postulated (Shen et al., 2003).

Hg(II) added to plasma, either as inorganic salts or as organomercurials, becomes 90% bound to HSA, primarily at the thiol group of Cys34. However, multiple stoichiometries and multiple binding sites have been proposed; among them, two sites display higher affinity, the dissociation equilibrium constants being 1×10^{-7} and 1×10^{-6} M (Li et al., 2007).

The binding of Cd(II) and Zn(II) ions to Cys34 was excluded on the basis of nuclear magnetic resonance (NMR) and site-directed mutagenesis (Stewart et al., 2003).

4.4.3. The primary multimetal binding site or Cadmium site A

MBS-A is located at the I/II interdomain contact region, surrounded by FA1, FA2, and FA7 (see Section 2.7). This site was characterized by metal displacement studies using circular dichroism (CD), NMR and site-directed mutagenesis (Sadler and Viles, 1996; Stewart et al., 2003; Sokołowska et al., 2009). Cu(II), Ni(II), and Zn(II) ions bind at MBS-A with dissociation equilibrium constants in the micromolar range, while Cd(II) ions bind with a dissociation equilibrium constant larger by an order of magnitude (Sadler and Viles, 1996; Bal et al., 1998). Due to its high affinity for Zn(II), HSA is the major plasma carrier of Zn(II) (concentration in blood about 2×10^{-5} M); consequently, more than 98% of Zn(II) is bound to HSA (Giroux and Henkin, 1973; Peters, 1996; Blindauer et al., 2009).

Structural details on MBS-A have been obtained by means of Zn K-edge extended X-ray absorption fine structure (EXAFS) spectroscopy, complemented by modeling studies (Blindauer et al., 2009). The structural model obtained shows that the major Zn(II) site on HSA (i.e., MBS-A) is a five-coordinate site with average Zn-O/N distances of 1.98 Å and a weak sixth O/N bond of 2.48 Å, consistent with coordination to His67 and Asn99 from domain I, His247 and Asp249 from domain II, as already postulated for Cd(II) binding (Stewart et al., 2003; Lu et al., 2008), plus a water ligand. These residues are conserved in all sequenced mammalian SAs, and no HSA variants with mutations of any of these residues are known, so far. The coordination sphere of Zn(II) in MBS-A is a trigonal bi-pyramid or a distorted octahedron if the backbone carbonyl oxygen of His247 is considered (Blindauer et al., 2009). The metal ion is coordinated from N-atom ligands from His67 and His247 and O-atom ligands from Asn99 and Asp249. The His N-atoms are in the axial positions of a distorted trigonal bi-pyramid: His67 coordinates to Zn(II) via the $N\epsilon$ atom, and His247 coordinates via the $N\delta$ atom. Asn99Asp and Asn99His mutations enhance Zn(II) binding but alter protein stability, whereas the His67Ala mutation removes an interdomain hydrogen-bond and weakens

Zn(II) binding. Remarkably, the same set of ligands coordinating Zn(II) in HSA has been found in Zn(II)-enzymes (Blindauer et al., 2009).

The location of MBS-A at the domain I/II interface accounts for its role in affecting the HSA conformational transition(s) and consequently allosteric properties (see Section 7). Zn(II) binding induces cooperative allosteric effects related to those of the pH- and ligand-dependent N-to-B transition. Therefore, metal ions binding is dramatically affected by FAs loading of HSA. FAs binding to FA2 trigger a spring-lock mechanism, which disengages the upper (His67 and Asn99) and lower (His247 and Asp249) halves of the Zn(II) binding site. This provides a possible mechanism whereby FAs (and perhaps other small molecules) could influence the transport and delivery of metal ions (e.g., Zn(II)) in blood (Stewart et al., 2003; Blindauer et al., 2009). The allosteric regulation of HSA operated by Zn(II) ions is in keeping with the observation of receptor-mediated vesicular co-transport of HSA-Zn(II) complexes across the endothelium and Zn(II) uptake by erythrocytes, where it binds to glutathione and Hb, thus increasing the O₂ affinity (Peters, 1996; Stewart et al., 2003; Blindauer et al., 2009).

Also Cd(II) binds primarily to MBS-A, the fifth coordination position being weakly occupied by a chloride ion. The value of the dissociation equilibrium constant for Cd(II) binding to HSA is 5.0×10^{-6} M (Mothes and Faller, 2007).

MBS-A acts as a secondary Cu(II) (and possibly Ni(II)) binding site (the primary one being the NTS; see Section 4.4.1), where Cu(II) is coordinated in a tetragonal geometry as inferred from CD and electron paramagnetic resonance (EPR) spectroscopy (Bal et al., 1998; Sokolowska et al., 2002).

4.4.4. The secondary multimetal binding site or Cadmium site B

The ¹¹¹Cd and ¹¹³Cd NMR experiments on HSA demonstrated the existence of two Cd(II) binding sites, one of them only shows competition with Zn(II) (Stewart et al., 2003; Lu et al., 2008). While the site affected by Zn(II) corresponds to MBS-A, the second site still has to be identified and is generically labeled as the secondary multimetal binding site or Cadmium site B (MBS-B). Despite of similar affinity for Cd(II), MBS-B is expected to have a different coordination topology, with one His donor and several carboxylates (Sadler and Viles, 1996; Stewart et al., 2003). MBS-B has been recently suggested to be the main Co(II) binding site (see Section 4.4.1).

4.4.5. Other metal binding sites

HSA is an important carrier for Ca(II) ions in blood. Indeed, about 45% of 2.4×10^{-3} M circulating Ca(II) is free, 45% bound to plasma proteins, chiefly HSA, and the remaining 10% is complexed to small anions such as citrate and phosphate (Vorum et al., 1995; Peters, 1996). Nevertheless, a distinct binding site has not been identified and no typical calcium binding motifs are present on the HSA surface. Instead, the addition of excess of Ca(II) ions perturbed the ¹¹³Cd NMR spectra (see Section 4.4.4). The binding of several Ca(II) ions with dissociation constants higher than millimolar were reported (Vorum et al., 1995). Altogether, Ca(II) ions are currently thought to interact with HSA rather unspecifically via multiple carboxylates present on the protein surface. Long-chain FAs, particularly oleic acid and linoleic acid, increase Ca(II) binding to HSA. The mechanism is not established, and could be due either to direct binding of Ca(II) ions by the COO⁻ group of FAs or by conformational shift(s) caused by FAs binding. The effect could be clinically significant in lowering Ca(II) levels in plasma incident to lipid infusions or heparin administration or adrenalin stimulation (Peters, 1996).

The affinity of Mg(II) for HSA is closely similar to that of Ca(II). The concentration of Mg(II) in plasma is about half of that of Ca(II), and again about 45% is bound by proteins, so less than one site on HSA is usually occupied by Mg(II) (Peters, 1996).

Although Zn(II) binds strongly to MBS-A (see Section 4.4.3), a secondary site for Zn(II) was suggested (André and Guillaume, 2004; Bal et al., 1998). The possibility that MBS-B could act as a secondary binding site for Zn(II), as observed for Cd(II), was ruled out by the evidence that Zn(II) cannot compete with Cd(II) for MBS-B (Sadler and Viles, 1996; Stewart et al., 2003).

Fe(III) binding to HSA is considered to be an important determinant of the plasma anti-oxidant capacity (Loban et al., 1997). In healthy subjects about 10^{-5} M circulating Fe(III) is bound to small chelating molecules (i.e., citrate) or to proteins other than transferrin. HSA is usually reported as a low-affinity Fe(III)-binding protein. Nevertheless, values for HSA-Fe(III) dissociation equilibrium constant(s) have not been reported, although a single site with $K_d = 3.5 \times 10^{-8}$ M was reported for the Fe(III)-bovine serum albumin complex (BSA-Fe(III)) (Xu et al., 2008). Interestingly, Fe(III) does not compete with Ca(II) for its HSA binding site (Silva and Hider, 2009).

Minor findings have been reported for Al(III) and Mn(II) binding to HSA. Indeed, transferrin, rather than HSA, represents the major carrier for Al(III) and Mn(II). Two Al(III) binding sites have been identified on HSA. The binding properties of these two distinct sites appear to undergo reciprocal influences, suggesting a possible interaction between the corresponding protein moieties (Zatta et al., 2003).

4.5. Less frequent binding sites

Ligand binding sites other than those described above have been reported for selected molecules. In particular, a 10th FA binding site has been observed to host a capric acid molecule at the surface of HSA close to the FA6 cleft (Bhattacharya et al., 2000a). Moreover, iophenoxic acid has been reported to bind not only to FA1, FA3–FA4, and FA7, but also to an additional pocket located at the interface between subdomains IIIA and IIIB (Ryan et al., 2011b).

4.6. Molecular evolution of albumin binding properties

Despite the wealth of information available on ligand binding to SA, little has been reported so far in terms of molecular evolution of the ligand binding clefts. SA occurs as far down the evolutionary scale as lampreys. SA sequences longer than 200 residues available in the NCBI protein database are reported in Table 4 (Fanali et al., 2012).

The multivalent function of SA mirrors the taxonomic classification of vertebrates. Although it is not common to infer phylogenetic relationships from sequence analysis of a single protein, the cladogram shown in Fig. 6 reveals that the molecular evolution of SA orthologs, available in the NCBI protein database, allows to cluster operational taxonomic units (OTU) into classes and orders. Interestingly, reptiles are split into two OTU groups, with snakes having more ancient SA sequences compared to lizards and even to amphibia (both frogs and salamanders, that are clustered in the same OTU outgroup). Similar considerations were drawn for partial sequence stretches from fish SA (Metcalf et al., 2003). As the analysis was restricted to sequences longer than 200 residues, important intermediate OTU such as elasmobranchs (sharks and rays) and lungfishes were not considered (Fanali et al., 2012). Indeed, SA sequences display a well-characterized repetition of three conserved domains of about 180 residues each, with a high sequence variability among domains (Fanali et al., 2012).

Table 4

SA sequences longer than 200 residues available in the NCBI protein database.

Organism	NCBI protein gi	Description	Length	Identities with human SA ^a
<i>Ailuropoda melanoleuca</i>	281342393	Hypothetical protein panda_018508	564	209/561 (37%)
<i>Ambystoma maculatum</i>	18032210	Serum albumin precursor	626	248/618 (40%)
<i>Ambystoma texanum</i>	18032208	Serum albumin precursor	624	225/612 (37%)
<i>Anas platyrhynchos</i>	132353350	Preproalbumin	226	101/224 (45%)
<i>Anolis carolinensis</i>	327279666	Predicted: serum albumin-like	573	216/564 (38%)
<i>Bombina maxima</i>	62421448	Albumin 2	607	241/606 (40%)
<i>Bombina maxima</i>	62421446	Albumin 1	607	241/602 (40%)
<i>Bos indicus</i>	76445989	Serum albumin	476	341/477 (71%)
<i>Bos taurus</i>	30794280	Serum albumin	607	464/608 (76%)
<i>Canis lupus familiaris</i>	55742764	serum albumin precursor	608	485/607 (80%)
<i>Capra hircus</i>	193085052	Albumin precursor	583	435/584 (74%)
<i>Cavia porcellus</i>	290563384	Serum albumin	608	444/608 (73%)
<i>Elephas maximus</i>	50953792	Serum albumin precursor	583	428/581 (74%)
<i>Equus asinus</i>	76363596	Serum albumin	607	467/608 (77%)
<i>Equus caballus</i>	126723507	Serum albumin precursor	607	466/608 (77%)
<i>Felis catus</i>	57977283	Serum albumin precursor	608	493/600 (82%)
<i>Gallus gallus</i>	45383974	Serum albumin precursor	615	292/613 (48%)
<i>Homo sapiens</i>	28592	Serum albumin	609	609/609 (100%)
<i>Hoplostactylus maculatus</i>	21309935	Serum albumin	406	170/406 (42%)
<i>Lethenteron japonicum</i>	126143340	Plasma albumin	1401	142/596 (24%)
<i>Macaca mulatta</i>	2492797	Serum albumin	608	568/608 (93%)
<i>Meleagris gallopavo</i>	326918904	Predicted: serum albumin-like	615	295/610 (48%)
<i>Meriones unguiculatus</i>	3121749	Serum albumin	609	448/609 (74%)
<i>Mesocricetus auratus</i>	298351527	Serum albumin	608	461/608 (76%)
<i>Microtus fortis calamorum</i>	58585562	Albumin	608	460/608 (76%)
<i>Microtus fortis fortis</i>	58585560	Albumin	608	458/608 (75%)
<i>Monodelphis domestica</i>	126330670	Predicted: similar to albumin	608	404/608 (66%)
<i>Mus musculus</i>	163310765	serum albumin precursor	576	414/576 (72%)
<i>Naja kaouthia</i>	2134234	serum albumin precursor	614	201/610 (33%)
<i>Nomascus leucogenys</i>	332233172	Predicted: serum albumin isoform 1	609	591/609 (97%)
<i>Nomascus leucogenys</i>	332233174	Predicted: serum albumin isoform 2	609	591/609 (97%)
<i>Nomascus leucogenys</i>	332233176	Predicted: serum albumin isoform 3	523	442/456 (97%)
<i>Oncorhynchus mykiss</i>	295419235	serum albumin 1 protein	281	71/278 (26%)
<i>Ornithorhynchus anatinus</i>	149466340	Predicted: similar to serum albumin	602	204/608 (34%)
<i>Oryctolagus cuniculus</i>	126723746	serum albumin precursor	608	457/608 (75%)
<i>Ovis aries</i>	57164373	serum albumin precursor	607	459/608 (75%)
<i>Pan troglodytes</i>	332819547	Predicted: serum albumin	609	601/609 (99%)
<i>Petromyzon marinus</i>	3023295	Serum albumin SDS-1	1423	137/604 (23%)
<i>Rana catesbeiana</i>	113579	Serum albumin	382	141/383 (37%)
<i>Rana shqiperica</i>	4096806	Serum albumin precursor	603	198/606 (33%)
<i>Rattus norvegicus</i>	158138568	Serum albumin precursor	608	445/608 (73%)
<i>Salmo salar</i>	185132509	Serum albumin 1 precursor	608	159/586 (27%)
<i>Salmo salar</i>	185133567	Serum albumin 2 precursor	608	158/586 (27%)
<i>Sphenodon punctatus</i>	21309933	Serum albumin	527	240/528 (45%)
<i>Sus scrofa</i>	52353352	Serum albumin precursor	605	460/606 (76%)
<i>Taeniopygia guttata</i>	224049069	Predicted: albumin	629	254/566 (45%)
<i>Trimeresurus flavoviridis</i>	56790036	Serum albumin	614	198/610 (32%)
<i>Xenopus laevis</i>	148237263	Serum albumin A precursor	606	233/609 (38%)
<i>Xenopus laevis</i>	148236611	Serum albumin B precursor	607	239/609 (39%)

^a Identical/aligned residues (identity %).

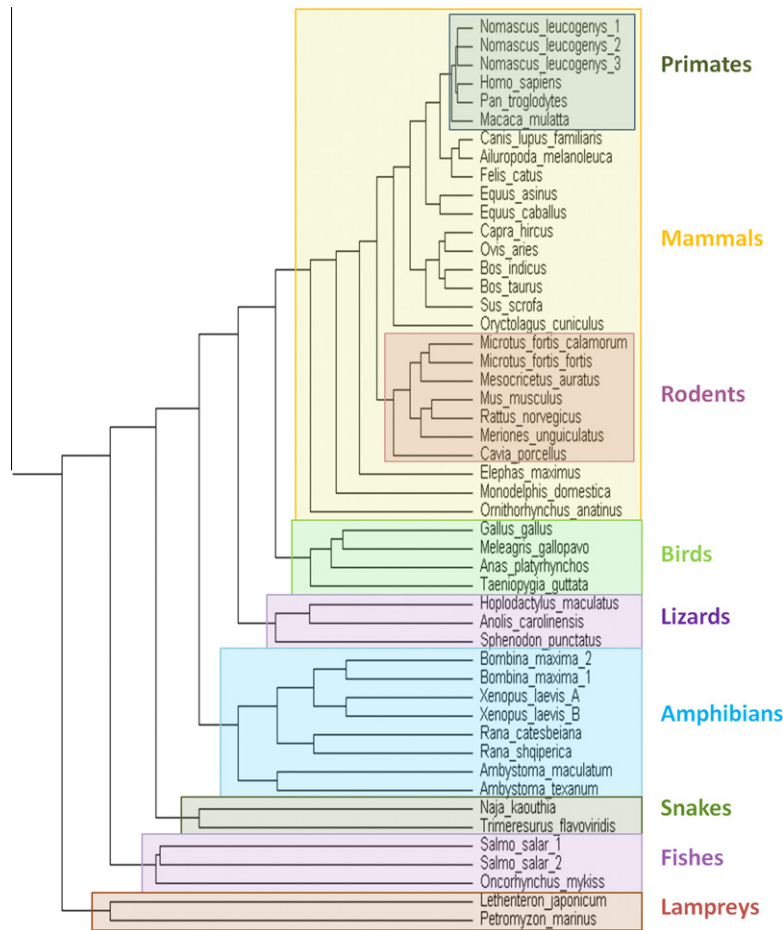


Fig. 6. Cladogram tree of the 50 SA sequences available in protein sequence databases. SA fragments less than 200 residues in length have been removed. For details, see Section 4.6.

Sequence similarity of a domain is greater between species than it is intramolecularly (McLachlan and Walker, 1977). Thus, HSA domain III is 40% identical with rat SA domain III and 28% identical with salmon SA domain III, but only 20% identical with HSA domain I. Salmon SA domain III is only 15% and 18% identical with its own domains I and II, respectively (Peters, 1996).

All sequences reported in Table 4, with the exclusion of the two lampreys (*Lethenteron japonicum* and *Petromyzon marinus*), show a conserved three-domain architecture similar to that of HSA (Fig. 2). As far as lampreys are concerned, a seven-fold repetition of the *Albumin* (NCBI CDD entry cd00015) conserved domain is observed (Gray and Doolittle, 1992; Metcalf et al., 2003; Fanali et al., 2012). Usually, the three-domain SA structure has been associated to a gene triplication event starting from an ancestral monomeric SA (Sargent et al., 1981). The analysis by Gray and Doolittle (1992) also suggested that the seven domains of lamprey SA could have originated from a single-domain ancestor before the gene replication events occurred to form the familiar three-domain structure. The basis for this choice was that the seven-domain lamprey SA show little relation among each other, and can be considered to have changed markedly during the long developmental period. In fact, SA is one of the most rapidly changing proteins, having substituted 70–80% of its residues in 500 My. Thus, evolution of the triple-domain structure was suggested to occur shortly (evolutionally speaking) after the cyclostome radiation by relationships among domains (Gray and Doolittle, 1992; Peters, 1996). On the other hand, multiple sequence analysis of lower vertebrate sequences allowed to localize the boundaries of the aligned stretches (Fanali et al., 2012). Interestingly, the three-domain SA could be the result of a contraction of a larger, seven domains of ancestral SA which is to date present in cyclostomes. The ancestor protein, similar to that of actual lampreys, could have been endowed with different transport functions that, in higher vertebrates, are carried out by different paralog albuminoids (Fasano et al., 2007). In particular, the functional adaptation could be limited to domain III, that appears to be evolved from different domains of the ancestral SA. In this view, domains I and II could represent the allosteric core of SA (see Section 2.7), domain III having evolved as a ligand sink for the removal of FA excess so that only the third equivalent of FA binds to the allosteric effector site (Fanali et al., 2012).

Table 5

Values of maximum absorption and extinction coefficient of HSA–heme–Fe derivatives in the absence and presence of drugs, acting as allosteric effectors.^a

HSA derivative	$\lambda_{\max}$ (nm)	ε (M ⁻¹ cm ⁻¹)	Allosteric effector
HSA–heme–Fe(III)	403	1.1×10^5	–
HSA–heme–Fe(III)	375	8.2×10^4	Ibuprofen, rifampicin
HSA–heme–Fe(III)–NO	368	5.4×10^4	–
HSA–heme–Fe(III)–NO	421	1.5×10^5	Abacavir
HSA–heme–Fe(II)	418	1.1×10^5	–
HSA–heme–Fe(II)	422	1.2×10^5	Ibuprofen, warfarin
HSA–heme–Fe(II)–NO	389	6.4×10^4	–
HSA–heme–Fe(II)–NO	419	1.3×10^5	Abacavir, rifampicin
HSA–heme–Fe(II)–CO	415	1.2×10^5	–
HSA–heme–Fe(II)–CO	414	1.6×10^5	Rifampicin
HSA–heme–Fe(II)–CO	421	1.4×10^5	Ibuprofen

^a From Ascenzi and Fasano (2007), Nicoletti et al. (2008), Ascenzi et al. (2010b, 2011c,d), and Cao et al. (2012).

FA1 (i.e., the heme binding site) appears to be shared by all mammals, with a progressively reduced conservation in rodents and lower mammals. This finding is consistent with an active role of SA in heme–Fe transport and delivery. The Lys190 residue, providing a salt bridge with one of the heme–Fe propionates, is present only in primates and in birds (Arg in snakes), therefore it seems not to be a critical residue for heme–Fe docking. The Phe149–Tyr150 dyad, that seems to play a fundamental role in the allosteric modulation of ligand binding to HSA (Ascenzi and Fasano, 2010), is conserved in mammals only. This suggests that heme–Fe binding could induce the conformational switch that allows the receptor-mediated heme–Fe reuptake only in mammals. As already highlighted for FA binding, heme–Fe recognition by SA could serve to sequester free heme–Fe and/or to carry it to target tissues. Nevertheless, receptor-mediated endocytosis of the SA-bound cargo is functionally linked to the allosteric modulation of the SA conformational transition (Fanali et al., 2012).

On the basis of structural and functional considerations on human albuminoids, HSA and AFP have been shown to have a high affinity for the heme–Fe (Zizkowsky et al., 1983; Fasano et al., 2001; Wardell et al., 2002; Zunszain et al., 2003; Fanali et al., 2005). It has been hypothesized that AFM binds the heme–Fe with low affinity, while DBP seems to be unable to bind the heme–Fe at all (Fasano et al., 2007). Consequently, the heme–Fe binding specificity should not be present in the precursor DBP, gradually appearing in more recent members of the albuminoid superfamily, and evolving from the ancestor DBP through the “phylogenetic intermediate” AFM (Fasano et al., 2007). Altogether, the molecular evolution of the heme cleft in human albuminoids resumes the corresponding one in SA orthologs (Fanali et al., 2012).

FA2 could have different ligand affinity in different species, in turn modulating the effective ligand concentration needed to activate the conformational switch (see Section 4.1.2). In fact, only Arg257 within residues stabilizing the FA carboxylate is conserved over the whole set of sequences (excluded birds) (Fanali et al., 2012).

The large cavity composed of FA3 and FA4 sites (i.e., Sudlow's site II in HSA) appears to be well conserved, with relevant differences in fishes, amphibians, and snakes. In particular, the Arg410–Tyr411 dyad is present in all species under investigation (Arg is replaced by Lys in snakes and Tyr in fishes), thus suggesting that the accommodation of large hydrophobic anions is allowed throughout the vertebrates. The Arg348 and Arg485 residues present in HSA display a large variability in lower vertebrates. Interestingly, these residues confer to the FA3 site a high affinity for FAs that is fundamental to sequester low levels of FAs without triggering the conformational change that leads to FAs delivery through receptor-mediated endocytosis (van der Vusse, 2009; Ascenzi and Fasano, 2010). This evidence is in keeping with the appearance of the allosteric regulation at the mammalian radiation. All the other aliphatic side-chains, conferring hydrophobic properties to the FA3–FA4 cleft, are well conserved (Fanali et al., 2012).

FA5 shows an uncommon abundance of aromatic side-chains widely conserved across the species, suggesting an active role of this site as a carrier of aromatic ligands. However, the basic Lys402 residue, that stabilizes FAs by polar interaction, is present in primates only. On the basis of this finding and of the analysis of the cladogram obtained from the hierarchical clustering of sequences delimiting FA5 (Fanali et al., 2012), the recent evolution of this site, partially overlapping with Tr-3 and Tr-4 sites (see Section 4.2), may be postulated to account for the specialization of the thyroid function in mammals and for differences in T₃/T₄ signaling across species (Richardson, 2008). Indeed, different vertebrates display a different expression pattern of thyroid hormone distributor proteins including SA (Gross and Manzon, 2011). The role of SA as thyroid hormone distributor protein could have changed along evolution from lampreys to primates. Indeed, the occurrence of aromatic residues in FA5 is increased in mammals, where SA is no longer the principal thyroid hormone distributor protein expressed by the organism (Yamauchi and Ishihara, 2009).

All the aliphatic residues and the polar side-chains Arg209 and Glu354, that secure the ligand by salt bridging, are conserved in FA6. Due to its low complexity, this ligand binding site appears to have been slightly modified along the evolution of vertebrates (Fanali et al., 2012).

FA7 (i.e., Sudlow's site I in HSA), binding preferentially bulky heterocyclic anions, is characterized by the presence of several Arg residues (see Section 4.1.6) (Curry, 2009; Ascenzi and Fasano, 2010). Actually, Arg257 bridges FA2 with FA7,

endowing these sites with linked functions that appear to be present in all species excluding birds. Polar residues not involved in FA binding, such as Arg218, Arg222, and His242, and the small Ala291 side-chain, altogether delimiting the large distal cavity of FA7 are poorly conserved, indicating a recent evolution of the ligand recognition specificity of this site (Fanali et al., 2012).

As far as FA8 and FA9 sites are concerned, their low complexity in terms of structural definition did not allow investigators to draw any conclusion on the molecular evolution of their binding properties (Fanali et al., 2012).

As a whole, the molecular evolution of FA1–FA7 sites seem to reflect three fundamental aspects: (i) the modulation of FA affinity, (ii) the selectivity for endogenous ligands other than FA (e.g., heme–Fe and T₄), and (iii) the appearance of allosteric properties that seem to be restricted at the mammalian radiation (Fanali et al., 2012).

5. Human serum heme–albumin

HSA is crucial for heme–Fe scavenging, providing protection against free heme–Fe oxidative damage, limiting the access of pathogens to heme–Fe, and contributing to iron homeostasis by recycling the heme–Fe-atom. In fact, during the first seconds after heme–Fe appearance in plasma, more than 80% of this powerful oxidizer binds to HDL and LDL, and only the remaining 20% binds to HSA and hemopexin (HPX). Then, HSA and HPX remove most of the heme–Fe from HDL and LDL. Afterwards, heme–Fe transits from HSA to HPX, that releases it into hepatic parenchymal cells after internalization of the HPX–heme–Fe complex by CD91 receptor-mediated endocytosis. HDL and LDL, the most oxidatively intolerant plasma components, bind the heme–Fe with high affinity, however kinetics of heme–Fe transfer from HDL and LDL to HSA and HPX is faster than the heme–Fe-induced lipoprotein oxidation (Smith and Hunt, 1990; Peters, 1996; Conrad et al., 1999; Miller and Shaklai, 1999; Conrad and Umbreit, 2000, 2002; Delanghe and Langlois, 2001; Goswami et al., 2002; Tolosano and Altruda, 2002; Ascenzi et al., 2005b; Hvidberg et al., 2005; Tolosano et al., 2010).

Under physiological conditions, the HSA–heme–Fe(III) plasmatic level is about 1×10^{-6} M. However, in patients affected with a variety of severe hematologic diseases characterized by excessive intravascular hemolysis, the HSA–heme–Fe(III) plasmatic level increases to a maximum of about 4×10^{-5} M (Muller-Eberhard et al., 1968; Miller and Shaklai, 1999). Thus, HSA acts as the main heme–Fe(III) plasma depot (Miller and Shaklai, 1999; Ascenzi et al., 2005b). Indeed, upon increasing heme–Fe plasma level, hemopexin, whose plasma concentration ($\sim 1.5 \times 10^{-5}$ M) (Miller and Shaklai, 1999; Ascenzi et al., 2005b) is about two orders of magnitude lower than that of HSA ($\sim 7 \times 10^{-4}$ M) (Peters, 1996), undergoes heme–Fe saturation, highlighting the role of HSA as a heme–Fe scavenger (Morgan et al., 1976; Pasternack et al., 1983, 1985; Miller and Shaklai, 1999; Ascenzi et al., 2005b).

Remarkably, heme–Fe binding confers to HSA globin-like spectroscopic and reactivity properties, thus providing a suitable tool to investigate allosteric and competitive properties (Antonini and Brunori, 1971; Bolognesi et al., 1997; Carter et al., 1999; Grinberg et al., 1999; Yamasaki et al., 1999; Komatsu et al., 2000; Mattu et al., 2001; Monzani et al., 2001, 2002; Fasano et al., 2002, 2005; Kamal and Behere, 2002; Komatsu et al., 2004b, 2005a; Ascenzi and Fasano, 2007, 2009, 2010; Ascenzi et al., 2008, 2009a,b, 2010a, 2010c, 2011a,b,c; Tsuchida et al., 2009; di Masi et al., 2011; Cao et al., 2011). The effects arising from heme–Fe binding to HSA might have some role in the regulation of biological functions. Since these actions are dependent on the transient interaction of a ligand (e.g., heme–Fe) with a carrier (e.g., HSA), they have been called “chronosteric effects” (Antonini et al., 1983; Fasano et al., 2008).

5.1. Determination of human serum heme–albumin concentration

HSA–heme–Fe can be determined spectrophotometrically (Ascenzi et al., 2008, 2009a,b, 2011a,b,c; Nicoletti et al., 2008; Cao et al., 2012) and by ¹H NMR relaxometry (Fasano et al., 2003) at concentrations lower than the physiological level, i.e., in the micromolar concentration range.

The concentration of HSA–heme–Fe derivatives can be easily determined using extinction coefficients reported in Table 5. However, the presence in the plasma of colored endogenous and exogenous compounds (e.g., bilirubin) affects significantly the spectrophotometric determination of HSA–heme–Fe (III) (Peters, 1996).

The determination of the HSA–heme–Fe(III) concentration by ¹H NMR relaxometry is based on the relaxometric properties of HSA–heme–Fe(III). Actually, the binding of the paramagnetic heme–Fe(III) to FA1 determines a strong paramagnetic enhancement of the water ¹H NMR relaxation rate. Although a linear relationship may be seen by operating at 20 MHz on conventional electromagnets (millimolar relaxivity $3.9 \text{ mM}^{-1} \text{ s}^{-1}$), the sensibility of the ¹H NMR relaxometric method is improved by working at 0.02 MHz on a field-cycling instrument (millimolar relaxivity $13.9 \text{ mM}^{-1} \text{ s}^{-1}$). This method does not suffer from the presence in plasma of endogenous and exogenous colored compounds. Moreover, the paramagnetic ferric Hb contribution can be selectively quenched by cyanide binding (Fasano et al., 2003).

The use of both spectrophotometric and ¹H NMR relaxometric methods need some cautions if HSA–heme–Fe-bound ligands are concerned. In fact, endogenous and exogenous ligands binding to HSA–heme–Fe sites different from FA1 (i.e., the heme–Fe binding site) could affect allosterically the spectroscopic properties of the HSA–heme–Fe derivatives (see Section 7.5) (Ascenzi and Fasano, 2009, 2010). Moreover, heme–Fe-bound ligands affect HSA–heme–Fe spectroscopic properties (see Table 5), as reported for globins (Antonini and Brunori, 1971; Bolognesi et al., 1997).

5.2. Ligand binding properties of human serum heme-albumin

Under conditions where the HSA:heme-Fe and HSA:Fe(II)P stoichiometry is 1:1, ligand association to and dissociation from the heme-Fe-atom often follows multistep mechanisms reflecting the presence of multiple coordination states of the heme-Fe-atom (*i.e.*, tetra-, penta-, and hexa-coordinated species) interconverting each other very slowly (Ascenzi et al., 2008; Nicoletti et al., 2008; Ascenzi et al., 2009b; Tsuchida et al., 2009; Ascenzi et al., 2011c; Cao et al., 2012). Also ligand binding to HSA-Fe(II)P species showing the 1:2–1:8 HSA:Fe(II)P stoichiometry follows multistep mechanisms (Komatsu et al., 2000, 2005a; Nakagawa et al., 2006; Tsuchida et al., 2009). The occurrence of multiple conformations may have great impact on the function of HSA-heme-Fe as a metabolite and drug transporter since the heme-Fe-ligation and the redox state of HSA-heme-Fe could affect its capability of interacting with molecules circulating in the bloodstream (Tsuchida et al., 2009; Ascenzi and Fasano, 2009, 2010).

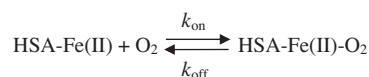
5.2.1. O₂ binding to ferrous human serum heme-albumin

Substantial efforts have been directed to prepare artificial O₂ carriers involving a synthetic heme(s) under physiological conditions, which may represent a red blood cell substitute (Mometeau and Reed, 1994; Komatsu et al., 2002; Collman et al., 2004; Cabrales et al., 2005). In this respect, HSA-heme-Fe(II) has been proposed not only as O₂ carrier for red blood cell substitute, but also as O₂-therapeutic agent (Carter et al., 1999; Komatsu et al., 2000, 2001, 2002, 2004a, 2005a,b, 2009b; Huang et al., 2005; Nakagawa et al., 2006; Tsuchida et al., 2009). However, even if HSA-heme-Fe(III) is reduced to HSA-heme-Fe(II), the latter is immediately re-oxidized by O₂. Indeed, HSA lacks the proximal His residue that enables the heme-Fe(II)-atom to bind O₂ reversibly (Marden et al., 1989; Wardell et al., 2002; Zunszain et al., 2003; Tsuchida et al., 2009).

Two different HSA-heme-Fe-based strategies have been developed to reproduce the O₂-binding ability of Hb and myoglobin (Mb). First, artificial HSA-heme-Fe(II) has been built complexing heme-Fe(II) with HSA mutants bearing a proximal His residue (Komatsu et al., 2004b, 2005b, 2007, 2009b; Tsuchida et al., 2009). Second, HSA-Fe(II)P complexes, bearing a covalently linked proximal imidazole, have been prepared (Komatsu et al., 2000, 2005a; Nakagawa et al., 2006; Tsuchida et al., 2009).

Artificial HSA-heme-Fe(II) has been obtained complexing heme-Fe(II) with HSA mutants. Principal modifications to the heme pocket, necessary to confer reversible O₂ binding, are: (i) the replacement of Tyr161 by a hydrophobic amino acid (Leu or Phe), (ii) the introduction of His as a proximal base at position 42, and (iii) the modification of the distal amino acids Leu185 and Arg186. However, the single HSA(Ile142His)-heme-Fe(II) mutant is unable to bind O₂ since the polar Tyr161 residue is likely to promote the oxidation of the heme-Fe(II)-atom. Remarkably, the HSA(Ile142His,Tyr161Leu/Phe)-heme-Fe(II)-O₂ complexes are very stable. Lastly, mutations of the distal amino acid residues (*i.e.*, Leu185Asn/Gln/His and Arg186Leu/Phe) have a considerable effect on the modulation of O₂ binding (Tsuchida et al., 2009).

Multiphasic kinetics have been reported for O₂ binding to HSA-heme-Fe(II) and HSA-Fe(II)P. Each independent phase may be accounted for a bimolecular oxygenation process according to the reaction mechanism depicted by Scheme 3 (Komatsu et al., 2000, 2004b, 2005b, 2007, 2009b; Nakagawa et al., 2006; Tsuchida et al., 2009):



Scheme 3. Minimum reaction mechanism for dioxygen binding to HSA-heme-Fe(II).

where HSA-Fe(II) indicates HSA-heme-Fe(II) or HSA-Fe(II)P, k_{on} is the second order rate constant for HSA-heme-Fe(II) and HSA-Fe(II)P oxygenation, k_{off} is the first order rate constant for the dissociation of the HSA-heme-Fe(II)-O₂ and HSA-Fe(II)P-O₂ complexes, and $K_d (= k_{\text{off}}/k_{\text{on}})$ is the dissociation equilibrium constant.

The affinity of O₂ for double and triple mutants of HSA-heme-Fe(II) (P_{50} ranging between 1 Torr and 2×10^2 Torr; *i.e.*, $1 \times 10^{-6} \text{ M} < K_d < 3 \times 10^{-4} \text{ M}$) is lower than that of ferrous sperm whale Mb, but similar to that of ferrous human Hb. Values of the second order rate constant for the oxygenation of HSA-heme-Fe(II) species (k_{on}) range between $7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, and values of the first order rate constant for deoxygenation of HSA-heme-Fe(II)-O₂ species (k_{off}) range between $1 \times 10^2 \text{ s}^{-1}$ and $9 \times 10^3 \text{ s}^{-1}$. Biphasic O₂ dissociation kinetics from HSA-heme-Fe(II)-O₂ mutants has been postulated to reflect two different geometries of the axial His142 and His185 residues arising from the two orientations of the porphyrin plane in the heme pocket (Komatsu et al., 2004b, 2005b, 2007, 2009a; Tsuchida et al., 2009).

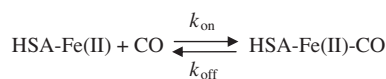
Monomeric, dimeric, trimeric, tetrameric, and octameric HSA-Fe(II)P species bind O₂ reversibly. Actually, HSA-Fe(II)P-O₂ having the His-Gly tail coordinated to the Fe(II)P atom is the most stable HSA-Fe(II)P-O₂ complex investigated. The affinity of O₂ for HSA-Fe(II)P species ranges between 0.1 Torr and 30 Torr (*i.e.*, $1 \times 10^{-7} \text{ M} < K_d < 4 \times 10^{-5} \text{ M}$), being similar to that of ferrous sperm whale Mb(II) and ferrous human Hb(II). Although the O₂ binding equilibrium curve to HSA-Fe(II)P species shows no cooperativity, the O₂ transporting efficiency of HSA-Fe(II)P-O₂ species between the lungs ($P_{\text{O}_2} \sim 110$ Torr) and muscle tissue ($P_{\text{O}_2} \sim 40$ Torr) is 22%, which is identical to that of red blood cells. Values of the second order rate constant

for the oxygenation of HSA–Fe(II)P species (k_{on}) range between $1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, and values of the first order rate constant for deoxygenation of the HSA–Fe(II)P–O₂ species (k_{off}) range between 2 s^{-1} and $1 \times 10^3 \text{ s}^{-1}$. Multi-exponential kinetics for oxygenation and deoxygenation of HSA–Fe(II)P(–O₂) complexes reflect the different protein environment of Fe(II)P bound to multiple HSA clefts and possibly different geometries of Fe(II)P at the same binding site(s) (Komatsu et al., 2000, 2005a; Nakagawa et al., 2006; Tsuchida et al., 2009).

A defect of HSA–Fe(II)P is that Fe(II)P slowly dissociates when the HSA–Fe(II)P complex is infused into animals, because Fe(II)P is non-covalently-bound to HSA. Two strategies have been developed to avoid Fe(II)P release. First, a Fe(II)P derivative showing a succinimidyl(glutamyl) group that can react covalently with Lys residues of HSA has been synthesized. The O₂ binding properties of the covalent HSA–Fe(II)P complex are almost identical to those of the non-covalent HSA–Fe(II)P species. Second, the surface of non-covalent HSA–Fe(II)P complexes has been modified by PEGylation, that delays the oxidation of the heme–Fe-atom of HSA–Fe(II)P–O₂ and prolongs the circulation life-time of HSA–Fe(II)P *in vivo* (i.e., prevents the rapid clearance of HSA–Fe(II)P). Notably, the O₂ affinity of PEG-modified HSA–Fe(II)P is almost identical to that of the original complex (Tsuchida et al., 2009).

5.2.2. CO binding to ferrous human serum heme-albumin

Multiphasic kinetics have been reported for CO binding to HSA–heme–Fe(II) and HSA–Fe(II)P. Each independent phase may be accounted for by a bimolecular carbonylation process according to the reaction mechanism depicted by Scheme 4 (Marden et al., 1989; Komatsu et al., 2005b, 2009a; Tsuchida et al., 2009; Cao et al., 2012):



Scheme 4. Minimum reaction mechanism for carbon monoxide binding to HSA–heme–Fe(II).

where HSA–Fe(II) indicates HSA–heme–Fe(II) or HSA–Fe(II)P, k_{on} is the second order rate constant for HSA–heme–Fe(II) and HSA–Fe(II)P carbonylation, k_{off} is the first order rate constant for the dissociation of the HSA–heme–Fe(II)–CO and HSA–Fe(II)P–CO complexes, and K_{d} ($=k_{\text{off}}/k_{\text{on}}$) is the dissociation equilibrium constant.

Values of the second order rate constant for the HSA–heme–Fe(II) carbonylation (k_{on}) range between $4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, and values of the first order rate constant for HSA–Fe(II)P–CO decarbonylation (k_{off}) range between $3 \times 10^{-3} \text{ s}^{-1}$ and $1 \times 10^2 \text{ s}^{-1}$. Moreover, carbonylation of hexa-coordinated HSA–heme–Fe(II) is limited by dissociation of the sixth endogenous ligand of the heme–Fe-atom (k_{L} values ranging between 1 s^{-1} and $2 \times 10^1 \text{ s}^{-1}$), preceding the formation of the carbonylated adduct (Cao et al., 2012). A similar behavior has been reported for bovine SA–heme–Fe(II) carbonylation (Marden et al., 1989).

The affinity of CO for HSA–heme–Fe(II) mutants is grossly similar to that of ferrous sperm whale Mb and ferrous human Hb, P_{50} values ranging between $1 \times 10^{-3} \text{ Torr}$ and $3 \times 10^{-1} \text{ Torr}$ (i.e., $1 \times 10^{-9} \text{ M} < K_{\text{d}} < 4 \times 10^{-7} \text{ M}$). Values of the second order rate constant for the carbonylation of HSA–heme–Fe(II) mutants (k_{on}) range between $3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, and values of the first order rate constant for the decarbonylation of HSA–heme–Fe(II)–CO mutants (k_{off}) range between $8 \times 10^{-3} \text{ s}^{-1}$ and $2 \times 10^{-1} \text{ s}^{-1}$ (Komatsu et al., 2005b, 2009a; Tsuchida et al., 2009).

Values of the second order rate constant for the carbonylation of HSA–Fe(II)P species (k_{on}) range between $7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Komatsu et al., 2000; Tsuchida et al., 2009).

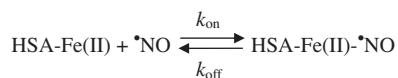
Multiphasic (de)carbonylation kinetics may reflect the different orientation of heme–Fe(II) in HSA, the presence of multiple coordination states of the heme–Fe-atom in HSA–heme–Fe(II), and the different protein environment of Fe(II)P bound to multiple HSA clefts (Komatsu et al., 2000; Tsuchida et al., 2009; Cao et al., 2012). Lastly, CO binding to human and bovine SA–heme–Fe(II) is modulated by pH between 5 and 10 (Marden et al., 1989; Cao et al., 2012), mirroring the N-to-B reversible conformational transition (Ascenzi and Fasano, 2010; Cao et al., 2012) (see Section 7.1).

5.2.3. NO binding to ferrous human serum heme-albumin

NO binds to HSA(–heme–Fe) sites FA1 and FA7; moreover, Cys34 undergoes NO-mediated S-nitrosylation (see Section 3.4). Therefore, NO can affect HSA(–heme–Fe) structure and function(s) by different mechanisms (Peters, 1996; Kharitonov et al., 1997; Komatsu et al., 2001; Fasano et al., 2005; Ascenzi et al., 2006a, 2008, 2009b, 2010a,c, 2011a,b,c; Yamakura and Ikeda, 2006; di Masi et al., 2011).

HSA–Fe(II)P forms a hexa-coordinated nitrosyl complex. Indeed, the heme proximal imidazole does not dissociate from the heme–Fe(II)-atom when NO binds to the *trans* side (Komatsu et al., 2001; Tsuchida et al., 2009). In contrast, the HSA–heme–Fe(II)–NO complex is penta-coordinated, the hexa-coordination of the nitrosylated heme–Fe(II)-atom being induced by allosteric effectors (e.g., drugs) (Baroni et al., 2001; Mattu et al., 2001; Fasano et al., 2002; Nicoletti et al., 2008) (see Section 7.6).

NO binds reversibly to HSA–heme–Fe(II) and HSA–Fe(II)P according to the minimum reaction mechanism depicted by Scheme 5 (Kharitonov et al., 1997; Komatsu et al., 2001; Ascenzi et al., 2008, 2009b, 2011c; di Masi et al., 2011):



Scheme 5. Minimum reaction mechanism for nitrogen monoxide binding to HSA-heme-Fe(II).

where HSA-Fe(II) indicates HSA-heme-Fe(II) or HSA-Fe(II)P, k_{on} is the second order rate constant for HSA-heme-Fe(II) and HSA-Fe(II)P nitrosylation, k_{off} is the first order rate constant for the dissociation of the HSA-heme-Fe(II)-NO and HSA-Fe(II)P-NO complexes, and $K_{\text{d}} (=k_{\text{off}}/k_{\text{on}})$ is the dissociation equilibrium constant.

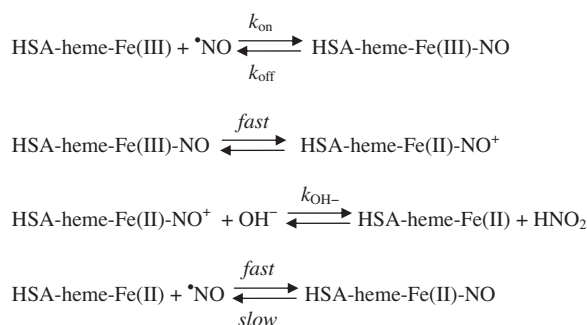
Values of k_{on} , k_{off} , and K_{d} for HSA-heme-Fe(II) and HSA-Fe(II)P nitrosylation are $\sim 1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, $\sim 1 \times 10^{-4} \text{ s}^{-1}$, and $\sim 1 \times 10^{-11} \text{ M}$, respectively. However, nitrosylation of hexa-coordinated HSA-heme-Fe(II) is limited by dissociation of the sixth endogenous ligand of the heme-Fe-atom ($k_{\text{L}} \sim 1 \times 10^{-3} \text{ s}^{-1}$), preceeding the formation of the nitrosylated adduct. (Kharitonov et al., 1997; Komatsu et al., 2001; Ascenzi et al., 2008, 2009b, 2011b, 2011c; di Masi et al., 2011). Interestingly, O_2 -mediated oxidation of HSA-heme-Fe(II)-NO is limited by $\cdot\text{NO}$ dissociation (Ascenzi et al., 2011c).

Drug (e.g., ibuprofen and warfarin) binding to FA2, FA3-FA4, FA6, and/or FA7 impairs HSA-heme-Fe(II) nitrosylation, the value of k_{on} decreasing from $6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, in the absence of heterotropic effectors, to $4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, in the presence of drugs; in parallel, the value of k_{off} increases from 1×10^{-4} to $1 \times 10^{-2} \text{ s}^{-1}$ (Ascenzi et al., 2008, 2009b, 2011b,c; di Masi et al., 2011).

Kinetic and thermodynamic parameters for $\cdot\text{NO}$ binding to HSA-heme-Fe(II) and HSA-Fe(II)P are similar to those reported for ferrous sperm whale Mb and ferrous human Hb (de)nitrosylation (Moore and Gibson, 1976; Olson et al., 1987; Kharitonov et al., 1997; Brucker et al., 1998; Komatsu et al., 2001; Draghi et al., 2002; Ascenzi et al., 2008, 2009b, 2011c; di Masi et al., 2011). Unexpectedly, the k_{off} value for $\cdot\text{NO}$ dissociation from penta-coordinated HSA-heme-Fe(II)-NO is closely similar to those for denitrosylation of hexa-coordinated HSA-Fe(II)P, ferrous sperm whale Mb-NO and of the R-state of ferrous human Hb-NO. This may reflect different protein structure-dependent “cage effects”; following $\cdot\text{NO}$ dissociation, there is a significant likelihood that $\cdot\text{NO}$ undergoes different geminate recombination with the heme-Fe(II)-atom before it escapes to the surrounding solvent (Kharitonov et al., 1997).

5.2.4. $\cdot\text{NO}$ binding to ferric human serum heme-albumin

HSA-heme-Fe(III) binds reversibly $\cdot\text{NO}$ at pH 5.5, leading to the formation of HSA-heme-Fe(III)-NO, k_{on} , k_{off} , and $K_{\text{d}} (=k_{\text{off}}/k_{\text{on}})$ values being $1.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $2.0 \times 10^{-1} \text{ s}^{-1}$, and $1.5 \times 10^{-5} \text{ M}$, respectively (see Scheme 6). By contrast, at pH ≥ 6.5 , the addition of NO to HSA-heme-Fe(III) leads to HSA-heme-Fe(II)-NO, according to the minimum reaction mechanism depicted by Scheme 6 (Ascenzi et al., 2010c):

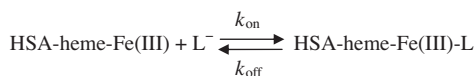


Scheme 6. Minimum reaction mechanism for reductive nitrosylation of HSA-heme-Fe(III).

The rate-limiting step for $\cdot\text{NO}$ -mediated conversion of HSA-heme-Fe(III) to HSA-heme-Fe(II)-NO is represented by the OH^- -mediated reduction of HSA-heme-Fe(II)-NO⁺ to HSA-heme-Fe(II), with a k_{OH^-} value equal to $4.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (Ascenzi et al., 2010c). Reductive nitrosylation of HSA-heme-Fe(III) is in agreement with the role of HSA-heme-Fe in scavenging (pseudo-)enzymatically RNS and ROS (see Section 5.3.1) (Ascenzi and Fasano 2007; Ascenzi et al., 2007, 2009a, 2010a, 2011a).

5.2.5. Azide, cyanide, fluoride, and imidazole binding to ferric human serum heme-albumin

Anionic ligands bind reversibly to HSA-heme-Fe(III) according to the minimum reaction mechanism depicted by Scheme 7 (Fasano et al., 2001; Monzani et al., 2001):



Scheme 7. Minimum reaction mechanism for anionic ligand binding to HSA-heme-Fe(III).

where L^- is the anionic ligand, k_{on} is the second order rate constant for the formation of HSA-heme-Fe(III)-L complexes, k_{off} is the first order rate constant for the dissociation of the HSA-heme-Fe(III)-L adducts, and $K_d (=k_{\text{off}}/k_{\text{on}})$ is the dissociation equilibrium constant.

Values of the dissociation equilibrium constant for azide, fluoride, and imidazole binding to HSA-heme-Fe(III) (i.e., 7.6×10^{-1} M, 1.4×10^{-1} M, and 8.7×10^{-2} M, respectively) are higher than those reported for the binding of anionic ligands to ferric sperm whale Mb and ferric human Hb (Antonini and Brunori, 1971; Bolognesi et al., 1997; Fasano et al., 2001; Monzani et al., 2001). The low affinity of anionic ligands for HSA-heme-Fe(III) may reflect steric clash of the heme-Fe(III)-bound anionic ligand with the heme distal residues and/or the scarce stabilization of the HSA-heme-Fe(III)-ligand complex by the protein matrix (Monzani et al., 2001).

5.3. (Pseudo-)enzymatic properties of human serum heme-albumin

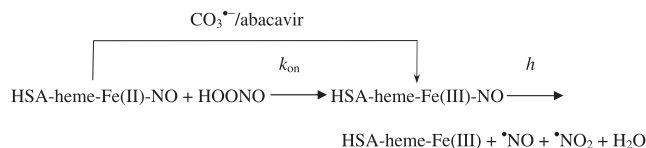
The (pseudo-)enzymatic activity of HSA-heme-Fe may have biological relevance in maintenance of anti-oxidative homeostasis in extracellular fluids that contain little or no anti-oxidant enzymes (Halliwell and Gutteridge, 1990; Peters, 1996; Monzani et al., 2001; Fasano et al., 2005; Ascenzi and Fasano, 2007, 2010). Interestingly, transient heme-based (pseudo-)enzymatic properties of HSA-heme-Fe (Cha and Kim, 1996; Monzani et al., 2001; Ascenzi and Fasano, 2007; di Masi et al., 2011) represent a case for “chronosteric effects”, i.e., time-dependent properties reflecting the life-time of the HSA-heme-Fe complex (Ascenzi and Fasano, 2009, 2010).

5.3.1. Scavenging of reactive nitrogen and oxygen species by human serum heme-albumin

Heme-proteins (e.g., human Hb and sperm whale Mb) facilitate scavenging of RNS and ROS by heme-based reactions (Gow et al., 1999; Brunori, 2001; Flögel et al., 2001; Frauenfelder et al., 2003; Wu et al., 2003; Frey and Kallio, 2005; Herold and Fago, 2005; Poole, 2005; Ascenzi and Visca, 2008; Bonamore and Boffi, 2008; di Masi et al., 2011). Remarkably, values of (pseudo-)enzymatic parameters for scavenging of RNS and ROS by HSA-heme-Fe are similar to those reported for globins (Ascenzi and Fasano 2007; Ascenzi et al., 2007; di Masi et al., 2011). Moreover, HSA-heme-Fe-based actions are modulated allosterically by drugs, outlining the role of heterotropic ligands in modulating HSA-heme-Fe functions (Ascenzi and Fasano, 2009, 2010). This behavior could be relevant for the drug-dependent protective role of HSA-heme-Fe *in vivo* (Ascenzi and Fasano, 2007; Ascenzi et al., 2007; di Masi et al., 2011). However, HSA-heme-Fe(III) displays weak catalase and peroxidase activities (Cha and Kim, 1996; Monzani et al., 2001; Kamal and Behere, 2002) as reported for sperm whale Mb and human Hb (Antonini and Brunori, 1971; Alayash et al., 2001).

Drug-dependent $\cdot\text{NO}$ and peroxynitrite scavenging by HSA-heme-Fe may occur in patients affected with a variety of severe hematologic diseases characterized by excessive intravascular hemolysis and displaying high HSA-heme-Fe plasmatic levels (Ascenzi and Fasano, 2007; Ascenzi et al., 2009a, 2010a).

5.3.1.1. Peroxynitrite scavenging by ferrous nitrosylated human serum heme-albumin. Peroxynitrite scavenging by HSA-heme-Fe(II)-NO is modulated by CO_2 and abacavir (Ascenzi and Fasano, 2007). Mixing of HSA-heme-Fe(II)-NO and peroxynitrite solutions leads to HSA-heme-Fe(III) by way of HSA-heme-Fe(III)-NO according to the minimum reaction mechanism depicted by Scheme 8 (Ascenzi and Fasano, 2007):



Scheme 8. Minimum reaction mechanism for peroxynitrite scavenging by HSA-heme-Fe(II)-NO.

where k_{on} is the second order rate constant for the formation of the HSA-heme-Fe(III)-NO complex and h is the first order rate constant for the dissociation of the HSA-heme-Fe(III)-NO adduct.

The first step of kinetics for peroxynitrite-mediated oxidation of HSA-heme-Fe(II)-NO (indicated by k_{on} in Scheme 8) is a bimolecular process, as observed under pseudo-first order conditions. Values of k_{on} are $6.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ in the absence of CO_2 and abacavir, $1.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of CO_2 , $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of abacavir, and

$3.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of both abacavir and CO_2 . The second step (indicated by h in Scheme 8) follows a monomolecular behavior independent of the peroxynitrite concentration, and the value of h ($=1.8 \times 10^{-1} \text{ s}^{-1}$) is CO_2 - and abacavir-independent (Ascenzi and Fasano, 2007). Remarkably, CO_2 enhances the rate of peroxynitrite scavenging by heme-Fe(II)-NO proteins (i.e., k_{on}) via rapid formation of the transient reactive species $\text{CO}_3^{\cdot-}$ and $\cdot\text{NO}_2$; notably, $\text{CO}_3^{\cdot-}$ is a stronger oxidant than $\cdot\text{NO}_2$ and peroxynitrite (Goldstein et al., 2005; Papina and Koppenol, 2006; Ascenzi and Visca, 2008; Goldstein and Merényi, 2008). On the other hand, abacavir facilitates peroxynitrite scavenging by HSA-heme-Fe(II)-NO inducing allosterically the penta-to-hexa-coordination transition of the heme-Fe(II)-NO-atom by binding to FA7 (i.e., Sudlow's site I) (see Section 7.5) (Ascenzi and Fasano, 2007).

5.3.1.2. Peroxynitrite scavenging by ferric human serum heme-albumin. Peroxynitrite scavenging by HSA-heme-Fe(III) is modulated by CO_2 and drugs (e.g., ibuprofen and warfarin) (Ascenzi et al., 2009a, 2010a). Mixing of HSA-heme-Fe(III) and peroxynitrite solutions leads to HSA-heme-Fe(III) and NO_3^- by way of HSA-heme-Fe(III)-OONO, in the absence and presence of CO_2 , according to the reaction mechanism depicted by Scheme 9 (Ascenzi et al., 2007, 2009a, 2010a, 2011a; di Masi et al., 2011):



Scheme 9. Minimum reaction mechanism for peroxynitrite isomerization by HSA-heme-Fe(III).

where k_{on} is the second order rate constant for the formation of the HSA-heme-Fe(III)-OONO complex.

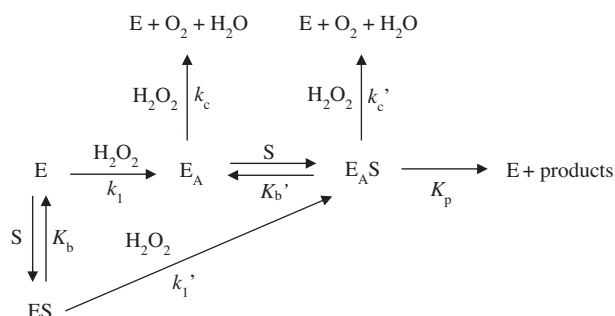
The first step of kinetics for peroxynitrite isomerization by HSA-heme-Fe(III) (indicated by k_{on} in Scheme 9) is a bimolecular process as observed under pseudo-first order conditions; the value of k_{on} is $5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. The formation of the HSA-heme-Fe(III)-OONO adduct represents the rate limiting step, the second step leading to the release of NO_3^- (indicated by *fast* in Scheme 9) being very fast (Ascenzi et al., 2007; Ascenzi et al., 2009a, 2010a; di Masi et al., 2011).

HSA-heme-Fe(III) prevents peroxynitrite-mediated nitration of free added L-Tyr. In the absence and presence of CO_2 , drugs impair dose-dependently peroxynitrite isomerization by HSA-heme-Fe(III) and facilitates the nitration of free added L-Tyr. Indeed, values of k_{on} decrease on increasing the drug concentration, however values of k_{on} are CO_2 -independent. The HSA-heme-Fe(III)-catalyzed isomerization of peroxynitrite has been ascribed to the reactive penta-coordinated heme-Fe(III)-atom. Drugs binding to FA7 and FA3-FA4 (i.e., Sudlow's sites I and II, respectively) and FA2 impair allosterically peroxynitrite isomerization by HSA-heme-Fe(III) inducing the hexa-coordination of the unreactive heme-Fe(III) atom (Ascenzi et al., 2007, 2009a, 2010a; 2011a; di Masi et al., 2011). Similarly, cardiolipin modulates allosterically peroxynitrite detoxification by monomeric horse heart cytochrome c (Ascenzi et al., 2011d).

5.3.2. Catalase and peroxidase activity of human serum heme-albumin

HSA participates in the prevention of the toxic effects of plasma heme-Fe by trapping the macrocycle (Peters, 1996; Monzani et al., 2001; Ascenzi et al., 2005b; Fasano et al., 2005; Ascenzi and Fasano, 2010). This impairs the activation of the heme-Fe-atom by H_2O_2 , the common step for both peroxidase- and catalase-like activities of the heme-Fe proteins (Grinberg et al., 1999).

HSA-heme-Fe(III) exhibits weak catalase and peroxidase activity in the oxidation of phenolic compounds related to Tyr (i.e., *p*-cresol, 3-(*p*-hydroxyphenyl)propionic acid, tyramine, and tyrosine) (Monzani et al., 2001), and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (Kamal and Behere, 2002). The weak catalase and peroxidase activity of HSA-heme-Fe(III) has been postulated to follow the reaction mechanism depicted in Scheme 10 (Monzani et al., 2001):



Scheme 10. Minimum reaction mechanism for catalase and peroxidase activity of HSA-heme-Fe(III).

where E is HSA–heme–Fe(III), E_A is active HSA–heme–Fe(III), S the substrate, ES is the HSA–heme–Fe(III)–S adduct, E_{AS} is the active HSA–heme–Fe(III)–S adduct, k_1 is the active species formation constant, k_1' the kinetic constant for the transformation of ES into E_{AS} , k_p the kinetic constant for product formation, k_c and k_c' are the kinetic constants for decomposition of peroxide by EA and E_{AS} , respectively, and K_b and K_b' are the substrate affinity constants for E and E_A , respectively. In this simplified mechanism the binding processes were considered fast with respect to the reaction processes. Values of k_1 and of the k_p/k_c' ratio, that rule the competition between catalase and peroxidase activity, range between $4.1 \times 10^1 \text{ M}^{-1} \text{ s}^{-1}$ and $1.6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$, and between $5.3 \times 10^{-3} \text{ M}$ and $6.3 \times 10^{-2} \text{ M}$, respectively, depending on the substrate.

The catalytic mechanism of HSA–heme–Fe(III) appears to be different from that of peroxidases. In fact, the rate of formation of the active HSA–heme–Fe(III) species is lower than its reaction rate with either the substrate (in a peroxidase reaction), or the peroxide (in a catalase reaction), or the heme–Fe(III) itself (Monzani et al., 2001). The main factors affecting the catalase and peroxidase activity of HSA–heme–Fe(III) are the reduced accessibility of the heme–Fe(III) center, and the lack of an Arg residue in the HSA–heme–Fe(III) pocket that in peroxidases assists the cleavage of bound peroxide and accelerates the formation of the active species (Monzani et al., 2001).

6. Enzymatic properties of human serum albumin

The (pseudo-)enzymatic activity of SA was first noted in 1951 and investigated extensively for decades; remarkably, it appears to be modulated allosterically and inhibited competitively. Moreover, the enzymatic properties of SA can be stereospecific as reported for the ligand binding properties (see Sections 4 and 9) (Kurono et al., 1992; Dubois-Presle et al., 1995; Peters, 1996; Salvi et al., 1997; Watanabe et al., 2000; Kragh-Hansen et al., 2002; Sakurai et al., 2004; Ahmed et al., 2005; Jurkowski et al., 2009).

6.1. Esterase activity of human serum albumin

The human plasma esterase activity towards aspirin (acetylsalicylic acid) is mainly due to HSA and butyrylcholinesterase (see Liyasova et al., 2010). However, the esterase activity of HSA in aspirin deacetylation is a pseudo-enzymatic activity, since aspirin stably acetylates HSA with the concomitant release of salicylate (Liyasova et al., 2010). Remarkably, aspirin-based acetylation of HSA affects the protein conformation (Peters, 1996; Kragh-Hansen et al., 2002; Gresner et al., 2006; Yang et al., 2007). In turn, FAs, such as myristic acid, alter both the affinity of aspirin for bovine and human SA and the stability of the anti-coagulant–SA complexes (Bojko et al., 2008).

Although several HSA sites have been reported to display esterase activity (Lockridge et al., 2008), Lys residues appear to be the main targets for acetylation (see Kurono et al., 1992; Peters, 1996; Watanabe et al., 2000; Kragh-Hansen et al., 2002; Yang et al., 2007; Liyasova et al., 2010). In fact, Lys199, Lys402, Lys519, and Lys545 residues become acetylated upon treatment with $3.0 \times 10^{-4} \text{ M}$ aspirin. However, the treatment with $2.0 \times 10^{-2} \text{ M}$ aspirin results in the acetylation of 26 Lys residues (Liyasova et al., 2010). Aspirin binds to subdomain IIA, the acetoxyl group being nucleophilically attacked by the Lys199 residue (Yang et al., 2007).

The low resolution structure (4.0 Å) of the HSA–aspirin complex was mentioned by He and Carter (1992), but no details were given. More recently, the detailed crystallographic analysis of HSA complexes with aspirin and salicylate at a resolution of 2.7 Å and 2.9 Å, respectively, has been reported (Yang et al., 2007). Both aspirin and salicylate bind to myristate–HSA at similar location (*i.e.*, at the FA7 site) but in different orientations, reflecting the acetylation of Lys199 (Yang et al., 2007).

The overall structure of the FA–HSA–aspirin complex (*i.e.*, of the myristate–acetyl-Lys199–HSA–salicylate adduct) has the known heart shape (see Section 2.7). Five myristate molecules are distributed asymmetrically in FA1, FA2, FA3, FA4, and FA5 sites. In the presence of myristate, only one salicylate molecule was observed in the FA7 site (Yang et al., 2007). However, two aspirin molecules bind to FA3–FA4 and FA7 sites, in the absence of myristate (He and Carter, 1992).

The phenyl group of salicylate in the FA7 site is located in the binding pocket that is formed by acetyl-Lys199, Leu219, Arg222, Phe223, Leu238, Ile260, Ile264, Ile290, and Ala291 making mostly hydrophobic contacts. The carboxylate group of salicylate is surrounded by a hydrophobic side of the pocket, which is delimited by Leu238, Ile260, Ser287, and Ile290. Although the hydroxyl group of salicylate is surrounded by acetyl-Lys199, His242, and Leu238, no HSA–ligand hydrogen bonds occur. Acetyl-Lys199 is close to Trp214, Arg218, Leu238, and His242; the acetyl group of acetyl-Lys199 forms a hydrogen bond with Arg218. Compared with Lys199 in myristate–HSA, the acetyl-Lys199 side-chain in the myristate–HSA–aspirin complex stretches out to position the amine group of Lys199 toward the hydroxyl group of salicylate. Although there are some conformational changes in side-chains located in the aspirin binding site, the acetylation of Lys199 does not cause overall changes in the secondary structures of HSA (Yang et al., 2007). On the other hand, the location of salicylate in the myristate–HSA–aspirin complex is different from the location of salicylate in the myristate–HSA–salicylate adduct. Indeed, the salicylate of the myristate–HSA–aspirin adduct is closer to the entry area of the FA7 site of HSA and away from the hydrophobic lining of the binding pocket defined by residues Leu238, Ile260, Ile264, Ile290, and Ala291. This difference might result in a weaker binding affinity of the hydrolysis product(s) of aspirin on acetyl-Lys199 HSA compared with aspirin (Yang et al., 2007).

Interestingly, the esterase activity of HSA is inhibited by salicylate. This effect has been ascribed to the stronger binding affinity of salicylate toward HSA compared with that of aspirin (Honma et al. (1991)). Notably, salicylate has more and/or closer contacts with HSA than with acetyl-Lys199 HSA (Yang et al., 2007).

The esterase activity of HSA should be taken into account from the pharmaceutical and clinical points of view to activate pro-drugs. Indeed, the esterase activity of HSA could be used to convert an acetylated pro-drug into a functional drug. However, the biological consequences of HSA acetylation *in vivo* are not fully understood. Notably, the aspirin resistance syndrome, characterized by asthma, rhinitis, and nasal polyps, is induced by aspirin but not by other salicylates (Kragh-Hansen et al., 2002; Yang et al., 2007).

6.2. RNA-hydrolyzing activity of human and bovine serum albumin

Human and bovine SA catalyze the hydrolysis of RNA molecules between the U and A bases (Takahashi et al., 2006; Gerasimova et al., 2008a). Remarkably, acetylation, glycation, *N*-homocysteinylation, and *N*-phosphorylation of Lys residues (see Section 3) inhibit the hydrolysis of RNA molecules catalyzed by human and bovine SA (Takahashi et al., 2006; Gerasimova et al., 2008a, b). These findings suggest that the degradation of mRNAs containing an AU-rich element(s) might be regulated by positive charges of polypeptides and proteins (Takahashi et al., 2006).

6.3. Enolase activity of mammalian serum albumin

SA possesses enolase activity towards dihydrotestosterone, converting it from the 3-keto to the 3-enol form. SA of all mammalian species investigated displays enolase activity, the specific activity however is very low in the plasma of dogs. The enolase activity depends linearly on the SA concentration, the optimum pH being 9.2. The enolase activity of SA is inhibited by Ni(II) and Cu(II) ions, oleic acid, cholesterol, and surface-active agents. The administration of dihydrotestosterone to mice does not influence the SA or enolase levels in their plasma. The plasma enolase activity was found to be a feature of intracellular SA also. Plasma and intracellular SA exhibit the same specific activity and affinity for dihydrotestosterone (Drmanovic et al., 1999; Kragh-Hansen et al., 2002).

Remarkably, benign and malignant tumors of the human mammary gland tissue differ substantially with respect to their percentage of HSA (see Section 8.2.1). Significant differences occur also in the enolase activity. Remarkably, polymeric HSA present in the malignant tissue exhibits a decreased enzymatic activity, compared to its monomeric form, exclusively encountered in benign breast specimens. This offers a possibility of reliable differentiation between benign and malignant breast tumors (Drmanovic et al., 1999; Kragh-Hansen et al., 2002).

6.4. Glucuronidase activity of human serum albumin

During the phase II metabolic pathway, the conjugation of drugs containing carboxylic acid functionalities with D-glucuronic acid by uridine diphosphoglucuronyltransferases operates as a detoxication pathway by enhancing the hydrophilicity of the parent drug, thus allowing its final elimination into urine or bile (di Masi et al., 2009). However, glucuronide conjugates are intrinsically reactive molecules (both *in vivo* and *in vitro*) that can undergo a number of reactions; by far, hydrolysis back to the parent aglycon is the predominating process (Knadler and Hall, 1991; Dubois-Presle et al., 1995; Bailey and Dickinson, 2003; di Masi et al., 2009). Interestingly, SA has been reported: (i) to facilitate the isomerization and the stereoselective hydrolysis of glucuronide conjugates, and (ii) to act as a scavenger of glucuronide conjugates decreasing their plasma levels by reversible and/or irreversible binding (Dubois-Presle et al., 1995; Bedford, 1998; Mizuma et al., 1998, 1999; Bueno et al., 2009).

6.5. Lipid peroxidase activity of human serum albumin

HSA displays a thioredoxin- (Trx)-dependent lipid peroxidase activity in the presence of palmitoyl-CoA. Indeed, palmitoyl-CoA binding induces a conformational change(s) of HSA, promoting the enzymatic activity. The redox-active disulfide Cys392–Cys438, which can be specifically reduced by Trx and is responsible for the Trx-dependent lipid peroxidase activity, is located in the Pro299–Leu585 region. The chemical modification of Cys392 and Cys438 residues with the thiol-specific agent *N*-ethylmaleimide results in the loss of the peroxidase activity. However, carboxyl group-modified HSA shows a 10-fold stronger lipid peroxidase activity than that of native HSA. Interestingly, these chemical modifications, reflecting the redox state of HSA, could modulate the lipid peroxidase activity *in vivo* (Lee and Kim, 2001; Cha and Kim, 2006).

6.6. Aldolase activity of human serum albumin

HSA catalyzes the aldol reaction of aromatic aldehydes and acetone, with saturation kinetics and moderate and opposite enantioselectivity. The aldolase activity of HSA occurs in the FA7 site, where Lys199 shows a low pK_a value. Indeed, a common feature of aldolases is the presence of an amino acid residue with a low pK_a value (*i.e.*, a nucleophilic Lys) in the catalytic site. This residue is surrounded by a hydrophobic environment, which catalyzes the aldol/retroaldol reaction by an enamine/

Schiff base mechanism. The Lys199 residue in HSA is involved in the covalent binding of substrates, and is responsible for the HSA ability to behave as an enzyme-like catalyst in β -eliminations, in the decomposition of Meisenheimer adducts, and in the Kemp elimination (Benedetti et al., 2011).

6.7. Glutathione-linked thiol peroxidase activity of human serum albumin

HSA displays thiol-specific anti-oxidant properties preventing the inactivation of glutamine synthetase activity and the peroxidation of lipid by a metal-catalyzed oxidation system. These anti-oxidant activities are supported by a thiol-reducing equivalent such as dithiothreitol and reduced glutathione. HSA shows a significant capability to reduce H_2O_2 in the presence of reduced glutathione, resulting in the production of oxidized glutathione. The thiol-specific anti-oxidant activity of HSA is greatly activated by halide ions, especially by the chloride ion, and completely abolished by the reaction with *N*-ethylmaleimide and iodoacetate. This indicates that HSA acts as a major and predominant anti-oxidant exerting a glutathione-linked thiol peroxidase activity which removes ROS, such as H_2O_2 , within blood plasma (Cha and Kim, 1996).

6.8. Anti-oxidant activity of human serum albumin

The anti-oxidant activity of HSA results from the redox properties of the Cys34 residue (see Section 3.5.1) and from its metal-binding properties (see Section 4.4). Indeed, Cu(I/II) and Fe(II/III) (either free or as heme) are very efficient to generate ROS after reaction with O_2 . Free Cu(I) and Fe(II) ions can react with H_2O_2 leading to the formation of the deleterious hydroxyl radical via the Fenton reaction. Cu(I) and Fe(II) binding to HSA usually promotes their oxidation to Cu(II) and Fe(III), thus limiting their ability to participate in the Fenton reaction. As thoroughly discussed in Sections 4.4.5 and 5, HSA traps Fe(III) and heme-Fe(III), playing a pivotal anti-oxidative role in Fe(III)- and heme-Fe(III)-overload diseases (see Ascenzi et al., 2006b, 2009b; Walter et al., 2006). The (Asp-Ala-His)-Cu(II) coordination (i.e., Cu(II) bound to NCS, see Section 4.4.1) shows a superoxide dismutase-like activity that significantly prevents ROS formation. By trapping Cu(II), HSA prevents LDL lipid peroxidation (Bar-Or et al., 2001b). Moreover, HSA and the Asp-Ala-His-Lys tetrapeptide prevent neuronal death in murine cortical cell cultures exposed to oxidative stress generated by the H_2O_2 /Cu(I)/ascorbic acid reagent (Gum et al., 2004).

HSA displays an indirect anti-oxidant activity by binding several compounds, including oxysterols, bilirubin, and homocysteine (Roche et al., 2008). Cholesterol undergoes oxidation *in vitro* and *in vivo*, forming biologically active derivatives known as oxysterols (van Reyk et al., 2006). Remarkably, HSA releases oxysterols less rapidly than cholesterol to cells limiting their detrimental effects (Roche et al., 2008). Bilirubin binding to HSA endows the tetrapyrrole to act as an inhibitor of lipid peroxidation, to protect α -tocopherol from damage mediated by peroxy radicals, and to prolong the survival of human ventricular myocytes against *in situ*-generated oxidative stress (Wu et al., 1991; Neuzil and Stocker, 1993, 1994; Roche et al., 2008). Furthermore, homocysteine trapping by HSA protects from atherosclerosis, elevated plasma homocysteine levels being a well-known risk factor (Papatheodorou and Weiss, 2007).

During its long life-time (28–36 days), a HSA molecule makes about 15000 passes through the circulation (Peters, 1996) incurring some damages that affect its ligand binding and anti-oxidant properties (Roche et al., 2008).

In particular, HSA glycation in patients affected by diabetes mellitus (Cohen, 2003) impairs both ligand binding and anti-oxidant properties (Bourdon et al., 1999; Sakata et al., 2002; Van Campenhout et al., 2006; Barzegar et al., 2007; Faure et al., 2008a). Since the Cu(II) binding capacity of glycated HSA is lower than that of the non-glycated protein (Sakata et al., 2002), glycated HSA exacerbates Cu(II)-induced LDL oxidation, probably by the generation of superoxide (Bourdon et al., 1999; Sakata et al., 2002). Moreover, the Fe(III)-binding anti-oxidant capacity of HSA is markedly reduced in diabetes (Van Campenhout et al., 2006). Lastly, binding and transport of Trp is reduced in glycated HSA (Barzegar et al., 2007).

Several receptors for advanced glycation end products initiate intracellular signaling and enhance ROS formation in cells through recognition and binding of glycated (macro)molecules including HSA (Goldin et al., 2006; Mera et al., 2007). Remarkably, HOCl-mediated carbonylation of Lys residues of glycated HSA represents a major antigenic advanced glycation end product in hyperglycemia and in inflammation (Mera et al., 2007). Then, advanced glycation end products including glycated SA impair vascular endothelial NO synthase activity *in vivo* in rabbit aortas (Xu et al., 2003).

Glycated HSA displays a toxic effect on microglial cells associated with impairments in cellular proteolytic systems, possibly reflecting the role of advanced glycation end products in neurodegeneration (Vitek et al., 1994; Cohen, 2003; de Arriba et al., 2006; Stolzing et al., 2006).

Several patients with diabetes (30–40%) develop nephropathies that require hemodialysis treatment. Both di-Tyr and carbonyl contents were found increased in HSA isolated from patients on hemodialysis. Such damaged HSA has impaired ligand-binding capacity and anti-oxidant properties (Lim et al., 2007).

The occurrence of oxidative damages in patients with acute respiratory distress syndrome has been reported. Remarkably, a beneficial effect of HSA administration was evidenced by the resulting enhancement in plasma thiol-dependent anti-oxidant status and in the reduction in protein oxidative damage (Quinlan et al., 2004).

Very recently, alteration in the anti-oxidant properties of HSA has been observed in patients affected by the obstructive sleep apnea syndrome. This reflects the impaired anti-oxidant HSA activity, which is associated with the enhanced glycation level of HSA in patients affected by this sleep disorder (Faure et al., 2008b).

7. Human serum albumin: a monomeric allosteric protein

HSA can bind different classes of ligands at multiple sites (see Section 4 and Table 3). The multidomain structural organization of this monomeric protein is at the root of its allosteric properties, that recall those of multimeric proteins. The comprehension of allosteric regulation in HSA has been essentially achieved, at least for what is best known to date, by joining the evidence of multiple conformations, based on crystallographic investigations of HSA in the absence and presence of ligands (see Table 3), with solution studies describing linked functions dependent on binding of homotropic and heterotropic effectors (see Ascenzi et al., 2006a; Ascenzi and Fasano, 2009, 2010; Varshney et al., 2010).

The conformational adaptability of HSA involves more than the immediate vicinity of the binding site(s), affecting both the structure and the ligand binding properties of the whole molecule. HSA conformational transitions are not only associated to pH changes (see Section 2.7), but also to endogenous and exogenous ligand (e.g., FAs, heme, and drugs) binding. Indeed, the pH-induced N-to-B transition (occurring in the 6–9 pH range) replicates that induced by FAs (van der Vusse, 2009). Remarkably, crystallographic and solution studies indicate that the HSA conformational transitions are not determined by the highest affinity binding events, but take place when HSA is half-saturated (van der Vusse, 2009; Ascenzi and Fasano, 2010).

Remarkably, allosteric regulation of ligand binding to HSA is relevant not only under physiological conditions but also in the pharmacological therapy management. Indeed, drug binding to plasma proteins is an important determinant for their biological efficacy, in turn drug availability to the intended target is modulated by binding to and dissociation from blood components. As an example, the increase of plasma levels of the heme-Fe under pathological conditions (e.g., severe hemolytic anemia, crash syndrome, and post-ischemic reperfusion) may induce a release of HSA-bound drugs with the concomitant intoxication of the patient. As expected, the plasma heme-Fe concentration may increase in patients after drug administration (Ascenzi et al., 2006a; Ascenzi and Fasano, 2010).

7.1. pH-dependent allosteric transition(s) in human serum albumin

Among the four reversible pH-dependent conformational states of HSA (i.e., E, F, N, and B), only the N-B transition gained experimental support. A detailed description of structural changes linked to pH changes is given in Section 2.7. Remarkably, the HSA N and B forms display different affinity for some ligands, thus suggesting that the pH-dependent allosteric transition(s) should overlap those induced by endogenous and exogenous ligands (Peters, 1996; van der Vusse, 2009; Ascenzi and Fasano, 2010).

7.2. Fatty acid-induced allosteric transition(s) in human serum albumin

Two medium-affinity sites (i.e., FA2 and FA3) are located at the interfaces between subdomains IA and IIA and between subdomains IIB and IIIA, respectively. For their peculiar interdomain location, these sites drive the conformational transition(s) observed upon FA binding. In fact, the methyl end of the hydrophobic tail in domain I and the polar head of FAs are located in the two separate sub-sites that constitute the FA2 site. In FA3, the interaction between the carboxylate group of FAs and Arg348 causes a local distortion of subdomain IIIA by displacing Glu450. As a result, the rotation of Asp451 toward domain I gives the formation of a salt bridge with Lys195 and produces the rigid body turning of domain I and III with respect to domain II. It should be taken into account that the FA-loaded conformational state of HSA corresponds to the B state of the protein. This conformational transition probably has a physiological meaning as it triggers a mechanism of receptor-mediated endocytosis to deliver FAs to hepatocytes (Fasano et al., 2007; van der Vusse, 2009; Ascenzi and Fasano, 2010). Actually, it has been suggested that the conformational transition induced by FA binding allows FA-loaded HSA to be recognized by a putative HSA receptor (Fasano et al., 2007; van der Vusse, 2009). Since the conformational transition occurs when the third FA equivalent binds to HSA, the FA cargo would be delivered only when a critical FA concentration is exceeded. Eventually, a decrease of pH in the endocytotic vesicle would shift the conformational equilibrium towards the FA-unloaded species, the cargo being released and the receptor being recycled (Fanali et al., 2005; Ascenzi and Fasano, 2010).

7.3. FA1 and FA7 sites of human serum albumin are allosterically coupled

The first evidence of functionally-linked sites refers to FA1 (i.e., the heme binding site) and FA7 (i.e., Sudlow's site I). In fact, the affinity of heme-Fe(III) for HSA decreases by about one order of magnitude upon FA7 ligand (e.g., warfarin) binding. According to linked functions (Wyman 1964; Di Cera, 1994), heme-Fe(III) binding to HSA decreases the FA7 ligand affinity by the same extent (Ascenzi et al., 2005c, 2006a; Bocedi et al., 2005; Ascenzi and Fasano, 2009, 2010).

More in general, FAs are effective in the regulation of heme-Fe(III) binding to HSA, both by direct competition and allosteric mechanisms. FAs cooperate with exogenous ligands (e.g., drugs) in modulating heme-Fe(III) binding to HSA. In fact, FA7 ligands (e.g., warfarin) have a higher affinity for the FA-free conformation, whereas FA1 ligands (e.g., heme-Fe(III)) have a higher affinity for the FA-bound conformation. As a whole, heme-Fe(III) inhibits ligand binding to FA7 by stabilizing the B form of HSA, whereas ligand association to FA7 impairs HSA-heme-Fe(III) formation by stabilizing the N state of HSA. The

structural mechanism that functionally links FA1 and FA7 sites has been ascribed to the tilt of the interdomain h10–h1 helix (see Section 7.6) (Ascenzi et al., 2006a; Simard et al., 2006; Fanali et al., 2007, 2009; Ascenzi and Fasano, 2009, 2010).

Modulation of heme–Fe(III) binding to HSA by drugs and metabolites might be relevant in pharmacological therapy management. Indeed, the increase of the heme–Fe(III) plasma level under pathological conditions, such as severe hemolytic anemia, crash syndrome, and post-ischemic reperfusion, (reaching $\sim 5 \times 10^{-5}$ M) (Muller-Eberhard et al., 1968; Miller and Shakhai, 1999) may induce the release of HSA-bound endogenous and exogenous ligands (e.g., metabolites and drugs) increasing their bio-availability and leading to the potential intoxication of the patient. In turn, the toxic plasma heme–Fe(III) concentration may increase in patients after drug administration (Bocedi et al., 2004, 2005; Ascenzi et al., 2005c; Fasano et al., 2005).

7.4. FA1 and FA6 sites of human serum albumin are allosterically coupled

Structural and solution studies of ibuprofen binding to HSA have shown that the ibuprofen primary binding site (i.e., Sudlow's site II formed by FA3 and FA4 clefts) may not be allosterically-linked to the heme binding site (i.e., FA1) (Ascenzi et al., 2009a,b; di Masi et al., 2011). Anyway, a link between the secondary ibuprofen binding site (i.e., FA6) and FA1 (i.e., heme binding site) occurs. It has been hypothesized that ligand binding to FA6 would have the same effect as binding to FA7, i.e., ligands would stabilize the HSA N state and in turn reduce the heme–Fe(III) affinity (Fanali et al., 2009). Accordingly, ibuprofen binding to FA6 reduces the heme–Fe(III) affinity for FA1 by one order of magnitude in the truncated rHSA (Asp1–Glu382) lacking the primary ibuprofen binding site (Fanali et al., 2009). Also in this case, the tilt of the interdomain h10–h1 helix affects the conformation of the FA6 cleft, thus establishing a functional link between FA1 and FA6 (see Section 7.6).

7.5. Heme-based reactivity of human serum heme-albumin is allosterically modulated

The heme-based reactivity of HSA–heme–Fe(II) and HSA–heme–Fe(III) is modulated allosterically by drug binding to multiple FA sites. In turn, the oxidation state of the heme–Fe-atom and its ligated or unligated form modulate drug binding to HSA–heme–Fe (Ascenzi et al., 2005c, 2006a, 2009a; Cao et al., 2012). Remarkably, drug binding to FA7 (e.g., warfarin) modulates peroxynitrite isomerization to NO_3^- by HSA–heme–Fe(III), HSA–heme–Fe(II)(–NO) nitrosylation and denitrosylation, peroxynitrite-mediated oxidation of HSA–heme–Fe(II)–NO, and O_2 -mediated HSA–heme–Fe(II)–NO oxidation (Ascenzi and Fasano, 2007; Ascenzi et al., 2008, 2010a, 2011a,b,c). Drug binding to FA2 and FA6 also modulate HSA–heme–Fe(II)(–NO) nitrosylation and denitrosylation as well as HSA–heme–Fe(III)-mediated peroxynitrite isomerization both in full-length and in truncated rHSA (Asp1–Glu382) (Ascenzi et al., 2009a,b; di Masi et al., 2011). However, ibuprofen modulates allosterically NO dissociation from HSA–heme–Fe(II)–NO by binding to not only to FA2 and FA6, but also to FA3–FA4 (Ascenzi et al., 2009b).

7.6. Ligand-dependent activation of the conformational transition

It is a currently-accepted view that ligand binding to FA2 drives the HSA conformational transition toward the B form (Ascenzi and Fasano, 2010). Thus, ligand binding to FA2 forces the 16° rotation of domain I with respect to domain II. Actually, binding of myristate to FA2 attracts Tyr150 and Arg252, two key residues positioned in the center of the apolar chamber of the FA3–FA4 site, towards the carboxylate moiety of the FA. On the other hand, the reorientation of Tyr150 drives the interaction of Phe149 with the heme, thus making a stabilizing π – π interaction and accounting for the allosteric modulation observed in solution studies. This effect is not observed for short FAs (e.g., octanoate) that preferably bind to the FA3–FA4 site without inducing HSA allosteric rearrangement(s) (Ascenzi and Fasano, 2010). On this ground, FA2 ligands such as nevirapine, Δ^9 -tetrahydrocannabinol, and ibuprofen (binding to FA2 as the third, low-affinity site) consistently improve the heme–Fe(III) affinity for HSA by one order of magnitude and affect heme–Fe-based spectroscopic and reactivity properties (Ascenzi and Fasano, 2010; Fanali et al., 2011).

Ligand binding to the FA2 site also affects the neighbouring FA1 cleft. In order to account for marked changes in the spectroscopic properties of the heme–Fe chromophore upon the occupancy of FA2 by high ibuprofen concentration ($>10^{-3}$ M), the role of His146 has been proposed. Indeed, His146 could change its interaction from the heme–Fe(III) propionate to the heme–Fe-atom, thus providing the sixth donor atom for the metal coordination (Nicoletti et al., 2008; Ascenzi et al., 2009a,b, 2011c; di Masi et al., 2011; Fanali et al., 2011; Kaneko et al., 2011; Cao et al., 2012).

8. Biomedical aspects

HSA is widely used clinically to treat serious burn injuries, hemorrhagic shock, hypoproteinemia, fetal erythroblastosis, and ascites caused by cirrhosis of the liver (Alexander et al., 1979; Hastings and Wolf, 1992). HSA is also used as an excipient for vaccines or therapeutic protein drugs and as a cell culture medium supplement in the production of vaccines and pharmaceuticals (Marth and Kleinhappl, 2001). Moreover, HSA could act as a carrier of O_2 (see Sections 2.6.2.4 and 5.2.1) (Tsuchida et al., 2009), drugs (see Section 8.5) (Fasano et al., 2005; Cai et al., 2006; Curry, 2009), and fusion peptides (see Section 9.5) (Subramanian et al., 2007; Leung, 2008a,b,c; Yazaki et al., 2008; Furukawa et al., 2011).

The market demand for HSA is estimated at more than 500 tons per year worldwide. Currently, commercial production of HSA is primarily based on collected human plasma, which has a high clinical demand, but is limited in supply. Remarkably, pdHSA may be of potential risk for transmission of blood-derived infectious pathogens (Chamberland et al., 2001). To eliminate the potential risk of viral contamination, regulatory agencies have encouraged pharmaceutical companies to use non-animal-derived sources for pharmaceutical production (Merten, 2002). Thus, the development of a low-cost method for the production of rHSA is essential as a safer and potentially unlimited alternative to pdHSA (see Section 2.5).

8.1. Human serum albumin metabolism

HSA ($\sim 7 \times 10^{-4}$ M) makes up 50% of the protein present in the plasma of normal healthy individuals, is the main determinant (80%) of plasma oncotic pressure, and plays a pivotal role in modulating the distribution of fluids between body compartments. The plasma concentration of HSA is a function of the biosynthesis and degradation rates and of its distribution between intravascular and extravascular compartments. HSA is predominantly an interstitial protein with concentration of about 3×10^{-4} M and a total mass of approximately 160 g. HSA is partially tissue-bound and, therefore, is unavailable for circulation. The HSA concentration in the plasma is about 7×10^{-4} M and the intravascular mass is about 120 g. HSA circulates from the blood across the capillary wall into the interstitial compartments, including cerebrospinal fluid, and returns to the blood through the lymphatic system with a circulation half-life of approximately 16 hours. The movement of HSA across the capillary wall is defined as the transcapillary escape rate (5% per hour), which indicates the percentage of intravascular HSA leaving the intravascular compartment per hour (Peters, 1996; Margaron and Soni, 1998; Evans, 2002; Mendez et al., 2005a; Prajapati et al., 2011).

In the 28–36 days between its birth and death, one HSA molecule makes about 15000 passes through the circulation, shuttling cargo of various kinds, incurring in some damage, and accumulating ligands that resist offloading (Doweiko and Nompleggi, 1991; Peters, 1996; Margaron and Soni, 1998; Mendez et al., 2005a).

The liver is the chief source of HSA. Although minute quantities of mRNA, detected in kidney and pancreas, there is no evidence that the mRNA in these extra-hepatic tissues is translated, in agreement with studies on the lack of circulating SA in the liverless dog or rat. In addition, whether HSA found in milk is produced by the mammary gland or derived from the circulation has not yet been resolved (Peters, 1996). On the other hand, chick skeletal muscle synthesizes small amount of protein identical to circulating HSA, but it is never secreted from the muscle cell. Recently, convincing evidences of HSA synthesis in bone tissues, as well as microglial cells in brain, have been provided (Yamaguchi et al., 2003; Ahn et al., 2008).

In healthy young adults, about 12–25 g of HSA per day are synthesized in polysomes bound to the endoplasmic reticulum of hepatocytes. As a member of secretory proteins, the nascent HSA quickly passes into the endoplasmic reticulum cisternae where it folds to its native configuration and forms its 17 serial disulfide bonds (see Section 2.7). HSA is not stored hepatically and there is therefore no reserve for release on demand (Evans, 2002). Under physiological circumstances only 20–30% of hepatocytes produce HSA and its synthesis can therefore be increased on demand by a factor of 200–300% (Peters, 1996).

HSA is synthesised only in a suitable nutritional, hormonal, and osmotic environment. The colloid osmotic pressure and the osmolality of the interstitial liquid around the hepatocytes is the most important regulator of HSA biosynthesis. In the acute-phase reaction, i.e., the generalized response of the body to a stressful insult or trauma, burn, or acute infection, the concentration of the hepatic HSA mRNA is depressed. The regulatory agents appear to be hepatic cytokines (e.g., interleukin-6 and tumor necrosis factor α). In addition, hormones control HSA mRNA, insulin being required for adequate HSA synthesis. Diabetic subjects have a decreased HSA synthetic rate that improves with insulin infusion. Estrogens do not affect HSA transcription, but act by modifying the stability of the HSA mRNA. Remarkably, an increase of HSA synthesis with combination of corticosteroids, insulin, and amino acids has been observed. HSA synthesis can be rate limited by amino acid deficiencies, but these are rarely seen clinically, except in states of extreme malnutrition. Whether or not synthesis can be enhanced by amino acid supplementation is unclear (Nicholson et al., 2000; Evans, 2002).

The catabolism of HSA takes place in several organs at a rate of about 14 g per day in a 70 kg healthy adult, or 4% of whole-body protein turnover (Nicholson et al., 2000; Friedman and Fadem, 2010). The rate of HSA degradation depends on its concentration; protein and calories deprivation accelerates HSA catabolism. The plasma HSA level may fall during periods of stress, trauma or sepsis despite its long half-life. The drop may result from accelerated redistribution from the intravascular space, decreased synthesis, and increased catabolism. Injury and infection result in the decrease of the HSA level of approximately 2×10^{-4} M within about five days (Peters, 1996; Margaron and Soni, 1998; Mendez et al., 2005a). The mechanism of HSA breakdown involves protein uptake into endocytotic vesicles which fuse with lysosomes of endothelial cells (Peters, 1996).

Circulating HSA is also lost into the intestinal tract (about 1 g each day), where digestion releases amino acid and peptides which are re-absorbed. There is minimal urinary loss of HSA in healthy subjects. Indeed, of the 70 kg of HSA that passes through the kidneys each day, only few milligrams are secreted from kidney tubules (Peters, 1996).

8.2. Human serum albumin functions

Clinicians and medical laboratories are aware that a decline in the circulating HSA level is a common feature in disease, and that a normal HSA concentration is one of the most frequently tested indicators of health. The HSA/globulin ratio,

although being a crude measurement, still has widespread application. The major physiological functions of HSA, recognized for over 70 years, are the regulation of both colloid osmotic pressure and capillary membrane permeability, ligand binding and transport, free radical scavenging, as well as anti-oxidant and circulatory protective properties (Peters, 1996; Evans, 2002; Mendez et al., 2005a).

8.2.1. Colloid osmotic pressure effect

HSA is responsible for 80% of the oncotic pressure of plasma (25–33 mm Hg). Indeed, changes in the oncotic pressure are the prime factors stimulating the synthesis of HSA. About two thirds of this pressure is represented by the simple osmotic pressure, to which HSA contributes disproportionately because its molecular mass of 67 kDa is lower than that of the average of the plasma globulins, about 170 kDa (Peters, 1996). The other third arises from the Donnan effect essentially due to HSA and its low isoelectric point, which gives to the protein a global negative charge at physiological pH (Figge et al., 1991; Peters, 1996). However, HSA is also the predominant protein in the interstitium, contributing to the interstitial colloid osmotic pressure. Notably, the colloid osmotic pressure gradient across the capillary membrane is of greater importance in determining fluid shift into the interstitium, rather than the absolute plasma value (Margarson and Soni, 1998).

8.2.2. Binding and transport

The HSA tertiary structure allows it to bind and transport quite diverse molecules, including metabolites (e.g., cholesterol, FAs, cations, and anions), gas (e.g., NO), and exogenous substances (e.g., drugs and diet-derived compounds) (see Section 4) (Margarson and Soni, 1998; Evans, 2002; Bolli et al., 2010). HSA functions as a reservoir for short-lived free NO, bound to the free thiol of Cys34; NO is released upon exposition to low pH (see Section 3.5.2). In case of tissue hypoxia, HSA undergoes structural transitions and releases NO, thus maintaining the vascular tone (Minamiyama et al., 1996).

Notably, HSA is involved in pharmacokinetics of many drugs (Peters, 1996; Kratz, 2008) (see Section 8.5) and acts as a toxic waste handler (Peters, 1996; Abe et al., 2001) (see Sections 4.1.1.3 and 4.3).

Does the delivery mechanism of HSA require a specific receptor? Although until now no evidence for the presence of any HSA receptor has been reported, a receptor-mediated endocytosis mechanism of the HSA-bound cargo has been postulated (Fasano et al., 2007; Van der Vusse, 2009).

8.2.3. Anti-oxidant effect

HSA is known to exert anti-oxidant activities in plasma, a body compartment exposed to continuous oxidative stress. Indeed, HSA is the major extracellular source of reduced sulfhydryl groups, which act as scavengers of ROS and RNS (see Section 5.3.1). HSA can also limit the production of these reactive species by binding free Cu(II) (see Section 4.4), a metal important in accelerating the production of free radicals. It appears that administered HSA acts as a donor of thiol groups and rejuvenates other intravascular proteins by donating electrons (Margarson and Soni, 1998). The redox state of the protein becomes important for covalent binding. Other main binding sites could be mentioned for their involvement in the anti-oxidative properties of HSA. For instance, the more exposed Met residues are particularly susceptible to oxidation, and could serve as a scavenger of ROS (see Section 5.3.1) to protect proteins from extensive alterations (Gutteridge, 1986; Roche et al., 2008; Rondeau and Bourdon, 2011). Recent studies have clearly documented that HSA oxido-redox potential changes upon oxidation by different oxidants thus becoming a deputy biomarker of this process. Upon stable oxidation, HSA properties are altered: the protein becomes more susceptible to trypsin digestion and is degraded faster compared to the non-oxidized counterpart (see Section 3.5) (Candiano et al., 2009).

8.2.4. Circulatory effect

Further but poorly understood properties of HSA are the anti-coagulant and anti-thrombotic functions. These functions may in part be mediated by the HSA capacity to bind NO forming S-nitrosothiols, thereby inhibiting the rapid inactivation of NO and allowing prolongation of its anti-aggregatory effects on platelets (Evans, 2002).

Because of its long half-life time, compared to other proteins, and of its high concentration, HSA is a plasmatic protein highly sensitive to glycation (see Section 3.3). However, other circulatory proteins, including Hb, insulin, and immunoglobulins are also affected by glycation. An initial study in this area has suggested that HSA may protect other proteins from glycation in the initial stages of diabetes (Bhonsle et al., 2008). Glycation-induced modifications, which are to a certain extent age-dependent, have a determinant impact on HSA conformation and, thus, functional properties of HSA result impaired, including anti-oxidant capacity and drug, FA and metabolites binding and delivery (see Section 3.3). Elevated levels of glycated HSA induce irreversible damages associated with the metabolic disorders observed in diabetes mellitus, such as retinopathy, nephropathy, neuropathy, and coronary artery disease (see Section 8.2.6) (Evans, 2002; Rondeau and Bourdon, 2011).

8.2.5. Capillary membrane permeability

HSA is postulated to influence membrane permeability. In fact, it has been suggested that HSA may bind within the subendothelium and the interstitial matrix and alter the permeability of these layers to macromolecules and other solutes. However, these data, which come from *in vitro* studies, have not yet been confirmed in cell monolayer studies (Margarson and Soni, 1998).

8.2.6. Neuroprotective effect

HSA has been implicated in many neurological diseases owing to its ability to regulate hemodynamic properties of the brain circulation as well as the direct neuroprotective actions on neuronal and glial cells. In the experimental ischemic stroke, exogenous HSA administration has been found to be neuroprotective *via* reducing brain swelling, prevention of post-ischemic thrombosis, anti-oxidant activity, hemodilution, and increasing the perfusion to the ischemic tissue. In the experimental models of Alzheimer's disease, HSA has been implicated in neuroprotection by inhibiting polymerisation and enhancing the clearance of β -amyloid. These neuroprotective effects of HSA are partly attributed to its anti-oxidant properties and to the modulation of intracellular signaling of neuronal or glial cells (Prajapati et al., 2011).

8.2.7. Sex differences in human serum albumin functions

There are marked differences between men and women in the incidence and expression of many major disease entities. These sex-based differences in the patho-physiology of disease imply, in turn, that there are important underlying differences in functions. Despite the importance of this topic, sex differences in physiology are not systematically addressed. In line with this, also information about differences in HSA levels and activities are very scarce. Studies *in vitro* with hepatocytes or liver microsomes isolated from male and female rats showed an increase in SA synthesis with the age only in male rats. However, an increase in total SA elimination and urinary protein excretion and urinary SA excretion was observed in females with age. A proportional increase in the contribution of SA to the urinary protein excretion was also observed (Horbach et al., 1988). In human beings (*i.e.*, in females relative to males), there is a lower absorbing force (low plasma oncotic pressure) in the exchange vessels. The effects of sex and age on HSA plasma level are openly debated. Indeed, before age 14, plasma protein levels are sex-independent. The greatest sex difference, 0.3 g/dL, occurs between 18 and 24 years; after which HSA levels in both sexes decline until they again are sex-independent for 55–74 years old. Because HSA functions are not limited to colloid osmotic pressure regulation, sex differences in HSA levels likely influences the anti-oxidant and transport properties of a wide variety of substances (Huxley and Wang, 2010).

During pregnancy, the concentration of HSA decreases along with other plasma proteins (Haram et al., 1983; Maher et al., 1993) although an increase of endogenous ligands, such as FAs, occurs (Hill and Abramson, 1988; Notarianni, 1990). Moreover, the HSA binding capacity may be reduced by chemical modifications (Perucca and Crema, 1982).

8.3. Human serum albumin and diseases

HSA allosteric properties and multiple activities suggest that mutations localized in critical residues for specific ligand binding could significantly compromise tissue and organ physiology, with consequences on human health.

8.3.1. Analbuminemia

Analbuminemia is a very rare recessive disorder in which HSA is either absent or drastically reduced (HSA levels ranging from 1×10^{-8} M to 1×10^{-4} M) (Peters, 1996; <http://www.albumin.org>). Some of the higher values might be influenced by administration of intravenous HSA before the nature of the condition was realized. An arbitrary upper limit of 1×10^{-5} M in untreated subjects has been proposed for classifying future cases as analbuminemia (Peters, 1996).

Analbuminemia was first reported in 1954 in a 31-years-old woman in Germany, who came to attention due to an elevated erythrocyte sedimentation rate, pre-menstrual ankle edema, and fatigue, but otherwise not ill (Bennhold et al., 1954). To date, 50 cases of analbuminemia have been identified in 41 families worldwide, which yields an estimated frequency of ~ 1 million for most populations (Watkins et al., 1994a; Peters, 1996; <http://www.albumin.org>).

The diagnosis of analbuminemia is hypothesized when persistent unexplained hypoproteinemia is observed, and confirmed by the absence of a HSA band in the plasma protein electrophoresis pattern (Lyon et al., 1998). The average age at detection is about 24 years and the main functional sign is some degree of edema and fatigue. A constant feature in analbuminemic patients is also gross hyperlipemia, which is considered secondary to the severe HSA level reduction, and consists of lipoprotein and total cholesterol values well above the upper limits (Baldo et al., 1983); however, atherosclerosis and arterial disease do not appear to be more frequent than in the general population (Peters, 1996). Such mild symptoms are probably attributable to a compensatory increase in the hepatic biosynthesis of other plasma proteins, of which lipoproteins assume many of the transport functions of HSA. Indeed, the body compensates with lowered blood pressure, lowered colloid oncotic pressure, and elevated plasma globulin concentrations (except for IgG and IgA) (Peters, 1996). Longevity is unaffected, indeed only 7 of the 50 subjects are believed to have died, at an average age of 61 years (<http://www.albumin.org>).

The low frequency of analbuminemia in the fetal state or during early infancy indicates that most cases probably do not survive gestation and that the disorder may be more severe during childhood (Watkins et al., 1994a). Therefore, the rarity of analbuminemia in humans may reflect the fact that the lack of HSA has a deleterious effect on fetal development, and therefore many, if not most, cases of analbuminemia do not survive gestation (Watkins et al., 1994a; Campagnoli et al., 2002).

Analbuminemia is acquired by the inheritance of abnormal *ALB* alleles from both parents, who have been found to be consanguineous in all reported cases in which genealogic data are available (Peters, 1996; <http://www.albumin.org>). In particular, analbuminemia occurs in homozygous and compound heterozygous individuals, where minute amounts of circulating

HSA (spanning the range from 2×10^{-7} M to 2×10^{-5} M) have been reported (Tárnoky, 1980). In the heterozygous state, a single normal *ALB* allele is sufficient to produce more than half the normal amount of HSA (Peters, 1996).

Seventeen mutations of the *ALB* gene have been identified in patients affected by analbuminemia; these mutations are localized within either the coding regions or the intron-exon junctions of *ALB* (Peters, 1996; Minchiotti et al., 2008; <http://www.albumin.org>) (see Table 1). Among the 17 mutations, 7 are nonsense mutations creating a premature stop codon (Papi et al., 1983; Watkins et al., 1994a; Watkins et al., 1994b; Campagna et al., 2005; Campagnoli et al., 2005a,b; Caridi et al., 2009; Ruhoff et al., 2010), 7 are frameshift mutations leading to premature stop codon (Watkins et al., 1994a; Galiano et al., 2002; Campagna et al., 2005; Dolcini et al., 2007; Davis et al., 2008; Dagnino et al., 2010a, 2010b), and 3 are undetected splicing variants (Ruffner and Dugaiczky, 1988; Campagnoli et al., 2002; Caridi et al., 2008) (see Table 1). The protein products codified by the 17 HSA variants are predicted to range in length from 19 to 532 amino acid residues, although no evidence was found for the presence in plasma of a truncated HSA. The production of small amounts of HSA is believed to be due to “leakage” in the splicing process which allows formation of a small amount of complete mRNA (Treisman et al., 1983).

8.3.2. Familial dysalbuminemic hyperthyroxinemia and familial dysalbuminemic hypertriiodothyroninemia syndromes

The familial dysalbuminemic hyperthyroxinemia and the familial dysalbuminemic hypertriiodothyroninemia (FDH) are genetic diseases transmitted in an autosomal dominant manner (Henneman et al., 1979; Lee et al., 1979; Yabu et al., 1987). FDH is the most common inherited cause of increase in plasma total T_4 (TT_4) in the Caucasian population (Croxson et al., 1985), with the highest prevalence in communities of Portuguese or Hispanic origin (De Cosimo et al., 1987; Sunthornthepvarakul et al., 1994; Petersen et al., 1996; Pannain et al., 2000). Indeed, FDH is characterized by an approximate doubling of TT_4 and, to a lesser extent, total reverse T_3 (TrT_3) (Weiss et al., 1995). Although subjects with FDH are euthyroid, falsely elevated free T_4 (FT_4) values, as measured by standard clinical laboratory techniques (Stockigt et al., 1983), have often led to the erroneous diagnosis of hyperthyroidism, resulting in inappropriate thyroid gland ablative or drug therapy (Croxson et al., 1985; Fleming et al., 1987; Wood et al., 1987; Weiss et al., 1995).

Although earlier studies based on electrophoretic, chemical, and immunological properties have associated the TT_4 abnormality to a HSA variant (Lee et al., 1979; Stockigt et al., 1981; Silverberg and Premachandra, 1982; Laloz et al., 1983; Weiss et al., 1995), the precise defect was identified only in 1994 (Petersen et al., 1994; Sunthornthepvarakul et al., 1994). In particular, the missense mutation Arg218His produces a HSA variant (named FDH-2) with 10- to 15-fold higher affinity for T_4 than the wild-type molecule, and a 5-fold increase in affinity for T_3 (Sunthornthepvarakul et al., 1994; Petersen et al., 1996; Petitpas et al., 2003).

The first report of FDH in an Asian family was published in 1997 (Wada et al., 1997). The Authors reported a Japanese family with dysalbuminemic hyperthyroxinemia, in whom plasma T_4 levels were extremely high (1.76–2.74 μ M, the normal range being 66–165 nM) as compared to Caucasian FDH patients with plasma T_4 levels of 2- to 3-fold the normal upper limit. This phenotype was due to the HSA variant, named FDH-3, carrying the missense mutation Arg218Pro, characterized by an 83-fold increase of the T_4 affinity (Wada et al., 1997). The presence of a further abnormal HSA variant (named FDH-2; Arg218His) with predominantly increased affinity for T_3 was observed in a Japanese patient with Graves' disease, who had undergone subtotal thyroidectomy (Sunthornthepvarakul et al., 1994). Both HSA variants have a lesser increase in affinity for T_3 , although only subjects with the Arg218Pro mutation have consistently high plasma total T_3 (TT_3) levels (Weiss et al., 1995; Wada et al., 1997).

The structural analysis of Arg218His and Arg218Pro HSA mutants showed that the increased affinity of TT_4 arises because the substitution of the Arg218 residue, which contacts the hormone bound in subdomain IIA, produces localized conformational changes to relax steric restrictions limiting T_4 binding (Petitpas et al., 2003). Moreover, although FA binding competes with T_4 at all four sites, it induces conformational changes that create a fifth hormone-binding site in the cleft between domains I and III, at least 9 Å from Arg218 (see Section 4.2 and Fig. 5). These structural observations are consistent with binding data showing that HSA retains a high-affinity site for T_4 in the presence of an excess in FA that is insensitive to FDH mutations (Petitpas et al., 2003). These data have provided important structural insights into the factors modulating T_4 binding to HSA, the effects of FA binding, and the impact of FDH mutations. Moreover, they play a pivotal role in the development of drugs based on T_4 analogues (Klabunde et al., 2000; Baxter et al., 2002; Petitpas et al., 2003).

The third mutation (i.e., Leu66Pro) in the *ALB* gene, causing alterations in plasma iodothyronine concentrations, was identified in 1998 in a Thai kindred (Sunthornthepvarakul et al., 1998). This HSA variant (named FDH-1) differed from the other two HSA variants (i.e., Arg218His and Arg218Pro) in that the molecule has a higher affinity for T_3 than for T_4 . The 40-fold higher affinity constant for T_3 , but only 1.5-fold increase in the binding affinity for T_4 , are responsible for the predominant increase in TT_3 plasma concentration (Sunthornthepvarakul et al., 1998).

8.3.3. Familial hyperzincemia

HSA is the major transport protein for Zn(II) in blood (see Section 4.4), a metal ion required for physiological processes and recruited by various drugs and toxins. Zn(II) is an essential element in the body, being critical for the development and function of all cells, for the immune system, and for transmission of genetic information. Notably, Zn(II) has catalytic or

structural roles in a wide range of proteins (Lipscomb and Sträter, 1996). Moreover, Zn(II) recruitment is used as a drug design strategy for enhancing the potency of organic serine protease inhibitors (Janc et al., 2000). The concentration of Zn(II) in blood plasma is $\sim 2 \times 10^{-5}$ M, most of which is bound to HSA; indeed, the dissociation equilibrium constant for Zn(II) binding to HSA (K_d) is $\sim 3 \times 10^{-8}$ M (Masuoka et al., 1993). HSA modulates Zn(II) uptake by endothelial cells (Bobilya et al., 1993). Moreover, receptor-mediated vesicular co-transport across the endothelium has been demonstrated for Zn(II)–HSA complexes *in vitro* (Tibaduiza and Bobilya, 1996). HSA also facilitates the uptake of Zn(II) by erythrocytes (Gálvez et al., 2001), where it binds glutathione (Rabenstein and Isab, 1980), and Hb (Simons, 1991), increasing the O₂ affinity (Oelshlegel et al., 1974).

Familial hyperzincemia can be defined a “nondisease” caused by the enhanced binding of Zn(II) by HSA. However, the biochemical basis for this abnormal binding is still unknown, and it has been hypothesized that a HSA genetic variant may be responsible for this phenotype (Krebs et al., 1985). The first case of heritable hyperzincemia with no apparent clinical symptoms or abnormalities, and associated to extremely high concentration of Zn(II) bound to HSA, was found in five out of seven members of a family and in two out of three second generation individuals (Smith et al., 1976). In 1982, the analysis of plasmas from three brothers with chronic hyperzincemia indicated that in normal plasma Zn(II) was bound to HSA, and the additional complement of Zn(II) in familial hyperzincemic plasma was associated to HSA (Failla et al., 1982). Research data suggest that sustained hyperzincemia can predispose to increased coagulability, and hypozincemia to blood clotting time increase, both disturbances of platelet aggregation and source of abnormal bleeding times (Tubek et al., 2008).

8.3.4. Albumin oxidation and human health

HSA oxidation, with different agents at different sites (see Section 3.5), has various consequences on its physiological functions. Diabetes mellitus, liver diseases, and nephropathy are just a few examples of disorders in which oxidative stress is involved and alters HSA functions. The oxidative modifications of HSA, including glycation, disulfide bond formation, and carbonylation, may alter its binding properties decreasing or increasing ligand affinity (see Sections 3.3–3.5). The redox state of HSA can affect the ligand binding properties allosterically, inducing short- and long-range conformational changes, and competitively. The levels of oxidatively-modified HSA vary over a wide range and are crucial in assessing the clinical relevance of altered ligand binding properties of a given oxidized HSA species in various diseases (Narazaki and Otagiri, 1997; Kawakami et al., 2006; Oettl and Stauber, 2007).

The oxidative stress is believed to play an important role in the pathogenesis of liver failure, the HSA level having been related to prognosis in advanced liver disease. Progressive oxidative modification of HSA parallels with increasing severity of liver failure, indicated by an increased content of carbonyl groups and oxidation of Cys34, with a preferred binding of bilirubin to the fully reduced form of HSA in patients with acute-on-chronic liver failure (Oettl et al., 2008).

The oxidative stress is enhanced in correlation with the level of renal dysfunction among patients with chronic renal failure (Terawaki et al., 2004). HSA has been found to undergo massive oxidation in primary nephrotic syndrome (Musante et al., 2006). Oxidized HSA (a reliable biomarker of oxidative stress in hemodialysis patients) is the major plasma protein target of oxidant stress in chronic renal failure and hemodialysis patients (Himmelfarb and McMonagle, 2001). The increase in plasma protein carbonyl levels in hemodialysis patients is largely due to an increase in oxidized HSA. In hemodialysis patients, HSA has been found to have a low anti-oxidant activity, and to trigger the oxidative burst of neutrophils. Uremia plays an important role in the progression of oxidative stress in hemodialysis patients because uremic toxins may possibly play a role in mediating free radical-initiated protein damage (Himmelfarb and McMonagle, 2001; Wratten et al., 2001). Diabetic complications exacerbate the oxidative stress by further increasing the amount of oxidized HSA (Mera et al., 2005a,b).

The end-stage renal disease is associated with enhanced oxidative stress and may contribute to substantial cardiovascular complications in hemodialysis patients. The HSA molecule in hemodialysis patients appears to be altered and therefore its physiological role is impaired. Intravenous Fe(III) administration, which is effective for correcting anemia in hemodialysis patients, enhances the generation of hydroxyl radicals and thus the oxidation of HSA (Anraku et al., 2004, 2008). Oxidative alterations of HSA might adversely affect the vasculoprotective effects in hemodialysis patients. Hence, anti-oxidant strategies should become part of treatment for pre-dialysis renal failure (Lim et al., 2007).

The increase of the carbonyl content of plasma proteins (*i.e.*, fibrinogen and HSA) is due to several diseases such as liver failure, diabetes mellitus, sepsis, Alzheimer's disease, and rheumatoid arthritis. Oxidative modifications reduce the affinity of bilirubin and progesterone for HSA (Oettl and Stauber, 2007). Moreover, the occurrence of oxidative damages in patients with acute respiratory distress syndrome has been reported (Quinlan et al., 1994). Remarkably, a beneficial effect of HSA administration was evidenced by the resulting enhancement in plasma thiol-dependent anti-oxidant status and in the reduction of protein oxidative damage (Quinlan et al., 2004). HSA carbonylation has been observed in the cerebrospinal fluid of ischemic stroke patients, as well as in animal models of ischemic occlusion. In the attempt to identify biomarkers for S₂O₈²⁻ exposure, oxidized HSA peptides in nasal lavage fluid samples from subjects challenged with S₂O₈²⁻ were detected. The oxidation degree before and after challenge remained constant for HSA peptides containing MetSO, whereas the oxidation degree increased after exposure for HSA peptides containing the oxidized Trp residue. Some of these oxidized peptides may then be suitable as biomarkers for exposure to oxidants (Mörtstedt et al., 2011).

8.4. Human serum albumin as a biomarker of diseases

HSA represents a valuable biomarker for several pathological conditions including cancer, rheumatoid arthritis, ischemia, post-menopausal obesity, severe acute graft-versus-host disease, and diseases that need monitoring of the glycemic control.

Potential macromolecular biomarkers have been observed in the HSA-enriched fraction and have been found to be bound to HSA. This makes the HSA-enriched fraction and the albuminome an important tool in biomarker discovery pursuits. Therefore, it is predicted that the albuminome changes with disease and that change could be a potential biomarker (Gundry et al., 2007).

8.4.1. Human serum albumin and solid tumors

The first evidence demonstrating that tumors are able to trap plasma proteins and utilize their degradation products for proliferation appeared in the middle of the 20th century (Babson and Winnick, 1954). The enhanced uptake of macromolecules in tumor tissue cannot be solely explained by the enhanced permeability of the vascular system, since it would affect smaller molecules in a similar manner, but it is also due to a reduced clearance from the tumor when the molecular weight exceeds 40 kDa. Whereas smaller molecules have been shown to be rapidly cleared from the tumor interstitium, large molecules are retained, thus showing high intratumor concentrations (Yuan et al., 1995). This enhanced retention of macromolecules in tumor tissues is primarily caused by a lack of lymphatic drainage due to an impaired or absent lymphatic system (Kratz, 2008).

The determination of HSA levels in blood represents one of the several methods of assessing in cancer patients the nutritional status, the severity of the disease, the disease progression, and the prognosis. HSA has also been described as an independent prognosticator of survival in various cancers, like lung (Lam et al., 2007), pancreatic (Siddiqui et al., 2007), gastric (Onate-Ocana et al., 2007), colorectal (Heys et al., 1998; Boonpipattanapong and Chewatanakornkul, 2006; Cengiz et al., 2006) and breast (Lis et al., 2003) cancer. Low HSA level has also been shown to be an independent indicator for prognosis in cancer patients with unknown primaries (Seve et al., 2006).

In recent years, the role of malnutrition as a predictor of survival in cancer has received considerable attention. In cancer patients, the inverse correlation between the body weight index and the HSA synthesis supports the possibility of a compensatory enhanced HSA synthesis (Gupta and Lis, 2010). Several studies, in which HSA was either radio-labeled or conjugated with dyes, showed that between 3% and 25% of the applied dose was found in the tumor (Kratz and Beyer, 1998; Kratz, 2008). The high HSA turnover in tumors has been explained by the fact that HSA represents the main energy and nutrition source for the tumor growth, with liver having an active metabolic role (Stehle et al., 1997; Kratz, 2008). This latter aspect represents an important factor for the genesis of cachexia, a condition associated with loss of weight and muscle mass caused by several diseases, such as cancer (Kratz, 2008). However, in the later stages of disease, malnutrition and inflammation suppress HSA synthesis (Ballmer et al., 1994; Yeun and Kaysen, 1998). The reduced HSA concentration in blood seems to be due to the production of cytokines, such as interleukin-6, which modulate the production of HSA by hepatocytes (Barber et al., 1999). Alternatively, the tumor necrosis factor α may increase the permeability of the microvasculature, thus allowing an increased transcapillary passage of HSA. The presence of micrometastatic tumor cells in the liver may indicate that the Kupfer cells produce a variety of cytokines, which may modulate HSA synthesis by hepatocytes (Barber et al., 1999; McMillan et al., 2001). As a consequence, there is slight or no hypoalbuminemia in the early stages of cancer, but as the disease progresses HSA levels drop significantly, serving as a good indicator of cancer prognosis (Barber et al., 1999; McMillan et al., 2001; Dixon et al., 2003; Sun et al., 2009; Gupta and Lis, 2010).

The advantages and disadvantages of HSA level as an indicator of the nutritional status of cancer patients deserve some considerations. Indeed, HSA level is not only a window into the patient nutritional status, but also a useful factor for predicting patient prognosis (Lien et al., 2004). The determination of the HSA level as a pretreatment prognostic factor in cancer patients is potentially advantageous being inexpensive, reproducible, and powerful (Sun et al., 2009). Moreover, HSA may be used as an independent indicator of the need for aggressive nutrition intervention because its low levels are associated with poor outcome in cancer patients (Lis et al., 2003). Among the main disadvantages, the interpretation of HSA levels is often difficult because non-nutritional factors, such as the hydration state and the disease progress, can obscure the effects of actual nutrient deprivation (Detsky et al., 1984). Furthermore, HSA has a relatively long half-life, thus, assessing changes in the nutritional status over a short period of time is challenging (Gupta and Lis, 2010).

8.4.2. Human serum albumin and rheumatoid arthritis

Patients with active rheumatoid arthritis frequently develop hypoalbuminemia, primarily caused by high HSA uptake at sites of inflammation (Wilkinson et al., 1965; Ballantyne et al., 1971; Niwa et al., 1990; Kratz, 2008). The metabolism of synovial cells is highly up-regulated, and the HSA uptake is probably a relevant source covering their high demand for nitrogen and energy. The permeability of the blood-joint barrier for HSA in rheumatoid arthritis patients is markedly increased (Kratz, 2008). Remarkably, using preclinical models it has been demonstrated that SA accumulates in the arthritic paws of mice suffering from collagen-induced arthritis (Wunder et al., 2003). Since the anti-rheumatic drug methotrexate bound to SA has shown promising activity in the collagen-induced murine arthritis model, HSA may represent an attractive drug carrier to target drugs to inflamed joints of patients with rheumatoid arthritis (Kratz, 2008).

8.4.3. Human serum albumin and ischemia

Low HSA levels are associated with an increased risk of mortality as well as an increased incidence of the coronary heart disease (Chan et al., 1995). This relationship has been attributed primarily to the ability of HSA to bind various ligands, including metals (Chan et al., 1995; Bar-Or et al., 2001a). Remarkably, the exposure to ischemic tissue modifies the HSA N-terminus (representing the primary binding site for metals; see Section 4.4) decreasing its binding capacity for metals and resulting in the formation of ischemia-modified HSA (Bar-Or et al., 2001a). The presence of ischemia-modified HSA has been observed in patients affected by hypoxia, acidosis, superoxide-radical injury, energy-dependent membrane disruption, and exposure to free iron and copper (Bar-Or et al., 2001b; Roy et al., 2006). The generation of ischemia-modified HSA depends strongly on the high oxidative stress state in different ischemia-reperfusion models, affecting not only myocardium but also other organs (Sbarouni et al., 2011).

The HSA cobalt binding test is a clinical chemistry assay that indirectly detects ischemia-modified HSA measuring the decreased binding capacity of HSA for cobalt. When a pre-determined amount of Co(II) ions are added to patient sera, they bind to normal HSA but not to ischemia-modified HSA. Thus, the increase of ischemia-modified HSA is related proportionally to unbound Co(II), which can be measured spectrophotometrically following the addition of colorizing reagent (*i.e.*, dithiothreitol) (see Section 4.4.1) (Wu et al., 2001).

However, the ischemia-modified HSA does not appear to be a tissue or clinically-specific marker of ischemia. Indeed, the increase of ischemia-modified HSA has been observed also in cerebrovascular-ischemic stroke, subarachnoid and intracranial hemorrhage, peripheral vascular disease, end-stage renal disease, advanced liver cirrhosis, acute infections, malignancies, systemic sclerosis, intrauterine disorders, and prostatic diseases. In addition, the ischemia-modified HSA does not appear to increase in immune system disorders, in gastrointestinal disorders, and in non-ischemic cardiac diseases (Sbarouni et al., 2011).

Low levels of ischemia-modified HSA exclude the presence of ischemia in a population with a low prevalence of coronary artery disease. Moreover, ischemia-modified HSA is currently the most reliable biomarker for the detection of myocardial ischemia without necrosis. In recent reports, the diagnostic power of sensitive cardiac troponin assays for the detection of unstable angina was low to moderate, suggesting that these assays may be of limited value in the diagnosis of unstable angina (Reichlin et al., 2009). The significance of ischemia-modified HSA as an indicator of outcome is gradually being revealed, and its prognostic performance has not yet been studied in patients with unstable angina and with normal troponin levels (Sbarouni et al., 2011).

Little is known about the use of ischemia-modified HSA in primary prevention and about its ability to detect asymptomatic ischemia or hypoxic stress in high-risk cardiovascular patients. Many questions remain unanswered regarding the ischemia-modified HSA mechanism of action, including HSA cardio-specificity, HSA kinetics during the first hours after acute coronary syndromes, and HSA optimal cut-point for clinical validation. Additional clinical evidence will be necessary in order to support the intended claims before routine clinical use of ischemia-modified HSA as a biomarker (Sbarouni et al., 2011).

8.4.3.1. Ischemia-modified human serum albumin and ischemia reperfusion. Ischemia-modified HSA has performed reasonably well in clinical models of transient myocardial ischemia triggered by a primary reduction in coronary blood flow (supply ischemia), as seen in patients undergoing percutaneous coronary intervention (Garrido et al., 2004; Sbarouni et al., 2011). Ischemia-modified HSA seems to act as a “sacrificial” anti-oxidant to reduce injury during reperfusion; its production decreases in patients with collateral vessels, implying a protective effect of collateral circulation against percutaneous coronary intervention-induced myocardial ischemia-reperfusion injury (Garrido et al., 2004). Ischemia-modified HSA release may depend on reperfusion-induced events rather than on ischemia *per se* (Hjortshøj et al., 2009). Moreover, ischemia-modified HSA levels during balloon angioplasty are related to the number, pressure, and duration of inflation. This suggests that ischemia-modified HSA reflects the magnitude and duration of ischemia during percutaneous coronary intervention, and may not be just a simple marker of free radical damage (Quiles et al., 2003). The ischemia-modified HSA has also been suggested to be a biomarker for transient myocardial ischemia induced by coronary vasospasm during the intracoronary ergonovine spasm provocation test (Cho et al., 2007).

8.4.3.2. Ischemia-modified human serum albumin and acute coronary syndrome. The ischemia-modified HSA measurements demonstrate high sensitivity and high negative prognostic value for predicting troponin-negative or troponin-positive findings from 3 to 24 hours after onset of symptoms, for both low- and high-risk acute coronary syndrome patients. Remarkably, ischemia-modified HSA has poor predictive power for discriminating between a final diagnosis of acute myocardial infarction, whereas it showed good discrimination for patients with unstable angina (Sbarouni et al., 2011).

Remarkably, the ischemia-modified HSA adds diagnostic value to standard biomarkers of myonecrosis (Anwaruddin et al., 2005; Peacock et al., 2006; Lee et al., 2007; Collinson and Gaze, 2008; Liyan et al., 2009). The use of the calculated albumin-adjusted ischemia-modified HSA value increases the sensitivity of the diagnosis of acute coronary syndrome compared with the conventional ischemia-modified HSA value (98.4% versus 93%) (Lee et al., 2007). Further, the addition of ischemia-modified HSA to traditional biomarkers increases sensitivity. Although problems with ischemia-modified HSA need to be resolved, it remains the only assay for ischemia that has reached the clinical validation stage (Sbarouni et al., 2011).

8.4.3.3. Ischemia-modified human serum albumin and myocardial injuries. The ischemia-modified HSA may be a sensitive marker for excluding pulmonary embolism, known to be associated with right ventricular dysfunction and myocardial ischemia

(Turedi et al., 2007). The ischemia-modified HSA has also been investigated in patients with stable dilated cardiomyopathy, and although troponin may be increased in chronic heart failure, ischemia-modified HSA has not been found to differ compared to normal controls (Sbarouni et al., 2009). In addition, ischemia-modified HSA may be of prognostic value in patients with cardiac arrest following cardiopulmonary resuscitation (Turedi et al., 2009).

8.4.3.4. Ischemia-modified human serum albumin and atherosclerotic plaque burden. Ischemia-modified HSA appears to be involved in the initial stage of development of atheromatous plaques, and may be associated with early assessment of overall patient risk (Piwowar et al., 2008; Duarte et al., 2009; Kazanis et al., 2009). Ischemia-modified HSA has also been described to be elevated in patients with hypercholesterolemia and to correlate with total cholesterol, LDL cholesterol, oxidized LDL antibodies and high-sensitivity C reactive protein (Duarte et al., 2009). In patients with documented and stable coronary artery disease, ischemia-modified HSA is higher compared to controls (Kazanis et al., 2009).

8.4.3.5. Ischemia-modified human serum albumin and post-menopausal obesity. Cardiovascular disease represents one of the leading causes of death in post-menopausal women (van der Schouw, 2009). Menopause is associated with the increase of the visceral adipose tissue (Deibert et al., 2007), the increase of the oxidative stress (Kassi et al., 2009), and alterations in the metabolic and endocrine status that could be related to the development of the metabolic syndrome, atherosclerosis, and cardiovascular disease (Lobo, 2008; Gaspard, 2009). The existence of biomarkers upstream from markers of myocardial necrosis that could provide an earlier assessment of overall patient risk would prevent a number of cardiovascular death (Apple et al., 2005).

High circulating ischemia-modified HSA levels have been associated with post-menopausal obesity, possibly due to obesity-associated oxidative stress. However, further larger clinical studies are required to better understand the role of ischemia-modified HSA as a potential marker of oxidative stress in obesity, and to explore whether ischemia-modified HSA measurement provides, alone or together with other plasma biomarkers, an earlier assessment of atherosclerotic burden and cardiovascular risk (Kazanis et al., 2011).

8.4.3.6. Ischemia-modified human serum albumin and perioperative ischemia. The ischemia-modified HSA increases immediately following coronary bypass surgery, with an immediate post-operative peak (Montagnana et al., 2006; Sbarouni et al., 2009). However, ischemia-modified HSA decreases significantly over the next four days and remains considerably higher compared to baseline values, implying different kinetics than in cases of percutaneous coronary intervention (Sbarouni et al., 2009). Therefore, ischemia-modified HSA could represent a useful tool for the early recognition of sub-clinical cardiac involvement, and for clinical risk stratification and management following non-cardiac surgery (Sbarouni et al., 2011).

8.4.4. Human serum albumin and severe acute graft-versus-host disease

The graft-versus-host disease is a common complication of allogeneic bone marrow transplantation in which functional immune cells in the transplanted marrow recognize the recipient as “foreign” and mount an immunologic attack. Notably, the graft-versus-host disease can also take place in a blood transfusion under certain circumstances. Biomarkers capable of predicting the onset and severity of the acute graft-versus-host disease after allogeneic hematopoietic cell transplantation would enable preemptive and risk-stratified therapy. Severe acute graft-versus-host disease leads to gastrointestinal protein loss, resulting in hypalbuminemia. It has been demonstrated that the reduction of HSA levels from baseline to initiation of the acute graft-versus-host disease treatment represents an inexpensive, readily available, and predictive biomarker of graft-versus-host disease treatment severity and mortality after allogeneic hematopoietic cell transplantation (Rezvani et al., 2011).

8.4.5. Levels of glycated human serum albumin as a glycemic control biomarker

It is known that protein glycation is increased in diabetic patients compared with non-diabetic subjects (Taniguchi, 1997). Some glycated proteins are suggested to be involved in the development and progression of chronic diabetic complications (Cohen, 1988). Remarkably, glycated Hb (HbA_{1c}) is commonly used as the biomarker of glycemic control in the clinical setting (Koenig et al., 1976; Bunn et al., 1978). However, HbA_{1c} does not accurately reflect the status of the glycemic control in some conditions where plasma glucose changes during short term, and in patients affected by diseases such as anemia and Hb variants (Takahashi et al., 2007; Koga and Kasayama, 2010). Indeed, glycated HSA increases prior than HbA_{1c} when the glycemic control status worsens during short term, thus allowing the detection of the worsened status at an earlier stage. Furthermore, due to the increase of plasma glucose during very short term, the extent of elevation of HbA_{1c} is suggested to be larger than that of HbA_{1c} at the diagnosis of fulminant type 1 diabetes mellitus (Koga and Kasayama, 2010). As a result, the glycated HSA/HbA_{1c} ratio is significantly higher in patients with fulminant type 1 diabetes mellitus, thus representing a helpful method for the diagnosis of this pathology (Koga and Kasayama, 2010). Moreover, glycated HSA is not affected by changes in the survival time of erythrocytes in the case of type 2 diabetes (Kosecki et al., 2005) and is not influenced by HSA concentration since it calculates the ratio to total HSA (Kouzuma, 2004).

In the circulation, HSA becomes nonenzymatically glycated by reducing sugars, and the reference range in normal humans is 6–10% of glycated HSA (see Section 3.3). However, this proportion typically increases to 20%–30% in hyperglycemic patients (Bourdon et al., 1999). As a result, glycated HSA has been proposed as a useful test for short-term control

(2–4 weeks) of diabetics (Peters, 1996). Although glycated HSA is not influenced by anemia and Hb variants, it is influenced in patients affected by disorders of HSA metabolism (Schleicher et al., 1993). Glycated HSA shows lower values in relation to glycemia in patients with nephrotic syndrome, hyperthyroidism, and glucocorticoid administration in which HSA metabolism increases (Koga et al., 2009a). On the contrary, glycated HSA presents higher values relative to plasma glucose levels in patients with liver cirrhosis (Koga et al., 2008; Bando et al., 2009) and hypothyroidism (Koga et al., 2009a) in which HSA metabolism decreases. Moreover, in obese subjects glycated HSA levels were found to set lower in relation to glycemia (Koga et al., 2006, 2007; Nishimura et al., 2006). Furthermore, it has been suggested that chronic micro-inflammation increases HSA catabolism in obese subjects (Bulló et al., 2003; Don and Kaysen, 2004), and glycated HSA decreases relative to plasma glucose levels as a result of its shortened half-life (Koga et al., 2007). Indeed, it has been shown that glycated HSA was set lower in relation to plasma glucose levels in smokers, hyperuricemic and hypertriglyceridemic patients as well as patients with non-alcoholic fatty liver disease with high alanine aminotransferase levels in whom chronic inflammation is evoked (Koga et al., 2009b, 2010a).

8.4.5.1. Glycated human serum albumin and anemia. HbA_{1c} presents lower values in relation to glycemia in patients with hemolytic anemia, because the lifespan of erythrocytes is shortened in these patients (Panzer et al., 1982). Meanwhile, patients with iron deficiency anemia (the most frequently described anemia) conversely present higher HbA_{1c} values relative to plasma glucose levels (Coban et al., 2004; Kim et al., 2010b). Since approximately one half of pre-menopausal women are in iron deficient status, a great number of pre-menopausal women present higher HbA_{1c} values relative to plasma glucose levels (Kim et al., 2010b; Koga et al., 2010b). On the other hand, when patients with iron deficiency anemia are treated with iron supplements, HbA_{1c} transiently decreases because the lifespan of erythrocytes shortens (Gram-Hansen et al., 1990; Koga et al., 2009c). In contrast, glycated HSA is not influenced by these conditions, and thus glycated HSA is a preferable biomarker of glycemic control in pre-menopausal women who frequently suffer from iron deficiency anemia (Koga et al., 2009c, 2010b).

8.4.5.2. Glycated human serum albumin and pregnancy. In pregnant women displaying either diabetes mellitus or gestational diabetes, intensive glycemic control during pregnancy is needed to lower the risk of intrauterine fetal death, fetal growth disorders, and maternal complications (Evers et al., 2002; Lauenborg et al., 2003). One of the reasons why HbA_{1c} decreases from the first trimester to the second trimester of pregnancy is considered to be the decrease in plasma glucose levels, although the reason why HbA_{1c} increases again from the second trimester to the third trimester is unknown. These findings suggest that HbA_{1c} is not an appropriate biomarker of the glycemic control during pregnancy. Meanwhile, glycated HSA is not affected by iron deficiency and its level does not change much during this period (Sanaka, 2006). Thus, during pregnancy, glycated HSA reflects the short-term status of the glycemic control, and thus it is considered to be a preferable biomarker of the glycemic control (Koga and Kasayama, 2010).

8.4.5.3. Glycated human serum albumin and chronic liver diseases. Since liver is a pivotal organ regulating plasma glucose levels, glucose metabolic abnormalities occur frequently in patients with chronic liver diseases, such as chronic hepatitis and liver cirrhosis. In patients with chronic liver diseases, about 70% to 90% are diagnosed as impaired glucose tolerance and 30% to 60% of them as diabetes mellitus (Kingston et al., 1984). It is important to maintain a good glycemic control status because chronic liver disease patients with poor glycemic control have been shown to offer poor prognosis (Bianchi et al., 1994). HbA_{1c} was shown to be useful as a biomarker of glycemic control in chronic liver diseases patients (Koga et al., 2008). Since HbA_{1c} shows lower and glycated HSA shows higher values relative to glycemia in chronic liver disease patients, the glycated HSA/HbA_{1c} ratio is set higher in these patients (Koga and Kasayama, 2010). Therefore, the glycated HSA/HbA_{1c} ratio reflects the hepatic function independently of plasma glucose levels (Bando et al., 2009; Koga and Kasayama, 2010).

8.4.5.4. Glycated human serum albumin and diabetic nephropathy. In patients with chronic renal failure, HbA_{1c} shows lower values in relation to glycemia due to renal anemia. Furthermore, when erythropoietin is administered to patients with renal anemia, HbA_{1c} shows even lower values because the lifespan of erythrocytes is shortened (Inaba et al., 2007; Peacock et al., 2008). Meanwhile, glycated HSA is a useful biomarker of glycemic control in hemodialysis patients with diabetes because glycated HSA is not affected by renal anemia (Chujo et al., 2006; Inaba et al., 2007; Peacock et al., 2008). Additionally, in the examination of patients with diabetes mellitus receiving peritoneal dialysis, glycated HSA reflects properly the status of glycemic control whereas HbA_{1c} does not (Freedman et al., 2010).

8.5. Human serum albumin therapy in clinical practice

HSA infusions have been used for more than 60 years in the management of a diverse range of medical and surgical problems, including hypovolemia, shock, burns, surgical blood loss, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, hemodialysis, acute liver failure, chronic liver disease, nutrition support, resuscitation, and hypoalbuminemia (Tullis, 1977a,b; Alexander et al., 1982; Erstad et al., 1991; Hastings and Wolf, 1992; Haynes et al., 2003; Alderson et al., 2004; Liberati et al., 2006). Moreover, HSA infusions have multiple effects, such as volume expansion, increase of the colloid osmotic pressure, and hemodilution. Although the use of HSA therapy in different clinical situations

and its influence in morbidity and mortality remains controversial, advantages of HSA therapy in the management of ascites and the use of HSA in volume expansion have been reported (De Gaudio, 1995; Peters, 1996; Wilkes and Navickis, 2001; Mendez et al., 2005a; Myburgh and Finfer, 2009; Niemi et al., 2010; Bunn et al., 2011).

Commercially available HSA derives from pooled human plasma and its use is not without potential side effects. Complications associated with HSA administration include fluid overload, coagulation defects, hemolysis, and myocardial depression (perhaps related to the binding of calcium ions). Allergic reactions are rare but may occur, usually to contaminants or to polymers that form during processing. Viral transmission is highly unlikely due to prolonged heat treatment during preparation. Complaints of nausea, fever, or chills usually resolve with a reduction in the rate of transmission or discontinuation of therapy. The risk of Al(III) toxicity may be increased by the use of HSA in patients with renal failure (Al(III) is introduced into commercially available HSA via materials used in manufacturing process). Lastly, HSA administration may decrease endogenous synthesis and increase the degradation rate (Margarson and Soni, 1998; Boldt, 2000; Nicholson et al., 2000; Mendez et al., 2005a).

8.5.1. Parenteral uses of human serum albumin

The efficacy of fluid resuscitation after hemorrhagic shock can be assessed by the recovery of cardiovascular stability, restoration of organ perfusion, and long-term hemodynamic effects. Outcome is significantly affected by the nature of the plasma expander used to restore blood volume, an issue for which there is no generalized agreement on the availability of a material with the ideal properties. A principal criterion for rating the efficacy of plasma expanders is the ability to maintain circulatory volume, which is determined by its intravascular retention time. However, prolonged volume expansion produces hemodilution, a condition that is not accompanied in all circumstances by the maintenance of microvascular perfusion. In this context, volume restitution without maintenance of microvascular function can lead to tissue damage resulting from the lack of perfusion and eventually multi-organ failure (Cabrales et al., 2008). Therefore, an optimal plasma expander should provide efficient recovery of intravascular volume and sufficient restoration of blood that insures microvascular perfusion, tissue oxygen delivery, and metabolic balance (Dubick and Atkins, 2003).

HSA is considered to be a near optimal resuscitation fluid, because it is naturally occurring in plasma. Remarkably, 4% of hexa-polyethyleneglycolylated-HSA (PEG-HSA) and 10% HSA provide an early similar recovery of systemic and microvascular conditions. Moreover, restoration of vascular homeostasis during hemorrhagic shock is improved by increasing the molecular size of HSA. These hyper-oncotic solutions provide rapid restoration of blood pressure and blood gas parameters. A potential future improvement of the PEG-HSA configuration will be to increase its viscosity to the level shown to be even more beneficial for resuscitation (Wettstein et al., 2004). Thus, future development of the technology of “PEGylation” may lead to enhanced plasma viscosity, while maintaining its oncotic characteristics at moderate protein concentrations.

Colloids including HSA are widely used as plasma substitutes for short-term replacement of fluid volume in critically ill patients efficiently expanding the circulating blood volume (Peters, 1996; Myburgh and Finfer, 2009; Niemi et al., 2010; Bunn et al., 2011). Up to about 25% of administered HSA is for the treatment of acute hypovolemia following surgical blood loss, trauma, and hemorrhage. However, the optimal composition of fluid for volume resuscitation is openly debated. Moreover, no markedly evidence that HSA solution administration reduces mortality and morbidity was found (Peters, 1996; Mendez et al., 2005a; Myburgh and Finfer, 2009; Niemi et al., 2010; Bunn et al., 2011).

Actually, there is no evidence that HSA is more effective or safe than any other colloid and crystalloid solution and may be detrimental in trauma patients (Mendez et al., 2005a; Heier et al., 2006). Thus, ready availability, lower cost, and lesser risk of infectious complications may favor the use of isotonic crystalloids than colloids (Roberts and Bratton, 1998; Waikar and Chertow, 2000; Bunn et al., 2003; Rhee et al., 2003; Boldt, 2004; Mendez et al., 2005a; Niermeyer, 2006; Perel and Roberts, 2007; Myburgh and Finfer, 2009; Niemi et al., 2010; Schrier, 2010; Bunn et al., 2011).

Although HSA and saline are clinically equivalent treatments for intravascular volume resuscitation in a heterogeneous population of critically ill patients (Alderson et al., 2004; Fan and Stewart, 2004; Myburgh and Finfer, 2009), 4.5% HSA is approximately four-times as effective in expanding the plasma volume as sodium-containing crystalloids. Therefore, colloids (e.g., HSA) have been proposed to be combined with crystalloids to obviate administration of large crystalloid volumes (Groeneveld, 2000). Moreover, the administration of colloids (e.g., HSA) restores the intravascular volume with minimal risk of tissue edema in comparison with crystalloid solutions alone (Boldt, 2000; Horsey, 2002; Mendez et al., 2005a; Jacob et al., 2008; Ertmer et al., 2009; Niemi et al., 2010). In contrast to crystalloids, HSA solution should remain primarily within the intravascular space and provide an oncotic gradient favoring the entry of water from the interstitial space. However, this does not seem to be the case in septic shock due to damage of the vascular endothelium and transcapillary leak (Waikar and Chertow, 2000; Mendez et al., 2005a). Moreover, colloid (e.g., HSA) solution administration in critically ill patients could induce potential deleterious effects of fluid overload on lung, heart, and kidney function that could contribute to increased mortality (Alderson et al., 2002; Schrier, 2010). The choice of clinical endpoints to guide dosage (infused volume) of colloids (e.g., HSA) may be more important than the choice between individual products (Vercueil et al., 2005).

The use of HSA-containing solutions for the resuscitation of patients with sepsis appears to be associated with lower mortality compared with other fluid resuscitation regimens (Schrier, 2010; Delaney et al., 2011). Moreover, HSA may be useful in patients with hypoalbuminaemia at risk of complications (e.g., sepsis and acute lung injury) (Bagshaw and Bellomo, 2007; Vincent, 2009).

In non-cardiac surgery, fluid requirements as well as pulmonary and intestinal edema were decreased by HSA compared with control fluids (Haynes et al., 2003; Mendez et al., 2005a; Jacob et al., 2008). In cardiac surgery, HSA administration

resulted in lower fluid requirements, higher colloid oncotic pressure, and reduced pulmonary edema than with crystalloid (Haynes et al., 2003; Mendez et al., 2005a).

In patients with traumatic head injury, HSA administration is openly debated. In fact, HSA administration to patients affected by brain and closed head injury has been associated with a significantly higher mortality (Bagshaw and Bellomo, 2007; Myburgh and Finfer, 2009; Hartog et al., 2011). In contrast, disability has been reported to be reduced by therapy with hyperoncotic HSA in brain injury (Jacob et al., 2008).

8.5.2. Human serum albumin administration in chronic liver disease

Colloid (e.g., HSA) solutions are best used in patients with decompensated cirrhosis. HSA may be effective in preventing the hemodynamic derangements associated with total paracentesis or serial large volume paracentesis (Salerno et al., 1987; Luca et al., 1995; Ruiz-del-Arbol et al., 1997; Mendez et al., 2005a) to decrease frequency of renal impairment and mortality in patients with spontaneous bacterial peritonitis (Sort et al., 1999; Mendez et al., 2005a), and, in association with splanchnic vasopressors, in caring for patients with the hepatorenal syndrome (Guevara et al., 1998; Angeli et al., 1999; Duvoux et al., 2002; Reith and Werdan, 2004; Mendez et al., 2005a; Jacob et al., 2008; Vincent, 2009; Polli and Gattinoni, 2010).

The controversy regarding the use of intravenous HSA is intense when it is used after paracentesis, because the preventable circulatory dysfunction is transitory and mortality is not affected. The use of intravenous HSA infusion remains questionable but not unreasonable, considering that the alternative is very close outpatient follow-up with serial laboratory tests, which is also expensive and often impractical or unrealistic (Runyon, 1998; Mendez et al., 2005a). Intravenous infusion using the vasoconstrictor terlipressin has been reported as effective as the intravenous HSA infusion in preventing post-paracentesis circulatory dysfunctions (Moreau et al., 2002; Mendez et al., 2005a). Lastly, the volume expanders Dextran-70 and polygeline are less effective than HSA (Ginès et al., 1996; Mendez et al., 2005a).

The use of intravenous HSA in patients with spontaneous bacterial peritonitis is less controversial. Frequency of renal impairment, the in-hospital mortality rate, and the three-month mortality rate appear to be reduced with the administration of intravenous HSA (Sort et al., 1999; Mendez et al., 2005a).

Intravenous HSA is used as part of regimens to reverse the hepatorenal syndrome. Due to the clear impact in survival and the relative non-invasiveness of the therapeutic regimens, the use of HSA is well justified in this setting. Indeed, the management of renal dysfunction in liver disease has changed drastically over the past few years, and mortality from hepatorenal syndrome has been substantially improved (Ginès et al., 1993; Angeli et al., 1999; Duvoux et al., 2002; Mendez et al., 2005a).

8.5.3. Human serum albumin in nutrition support and hypoalbuminemia

The plasma HSA concentration can decrease rapidly over a few hours after the increased loss of HSA and the altered distribution between the intra- and extra-vascular compartments. The altered distribution of HSA associated with increased capillary permeability is probably the most frequent cause of hypoalbuminemia in critically ill patients. Note that decreased HSA levels following decreased synthesis (e.g., severe liver failure) occur over a period of weeks to months (Uhing, 2004; Mendez et al., 2005a). Because the plasma level of HSA is affected by many factors, HSA correlates very poorly with the assessment of the nutrition status (Downs and Haffejee, 1998; Fuhrman, 2002; Waitzberg and Correia, 2003; Mendez et al., 2005a; Friedman and Fadem, 2010). As an indicator of morbidity and mortality, HSA concentration is a reliable tool (Gibbs et al., 1999; Mendez et al., 2005a). Hypoalbuminemia has been shown to be associated with increased mortality and morbidity in hospitalized patients and community-dwelling elderly persons (Covinsky et al., 2002; Mendez et al., 2005a). In elective surgery, an association between hypoalbuminemia and adverse outcomes has also been recognized for years. Indeed, plasma HSA concentration has been reported to be a better predictor of surgical outcomes than many other pre-operative patient characteristics, with an inverse relation between 30-day morbidity and mortality and pre-operative plasma HSA levels (Gibbs et al., 1999; Mendez et al., 2005a).

The provision of adequate nutrition support may take weeks to months in order to raise the plasma HSA concentration. The administration of HSA as adjunctive therapy in hypoalbuminemic patients who are receiving parenteral nutrition has been assessed; overall, no significant effect of HSA administration on mortality was observed (Mendez et al., 2005a). Moreover, reduced morbidity has been reported by administering exogenous HSA in hypoalbuminemic patients (Rubin et al., 1997; Vincent et al., 2003; Mendez et al., 2005a).

8.5.4. Clinical trials using recombinant human serum albumin

While in pre-clinical tests similarity between pdHSA and rHSA produced using *P. pastoris* was intensively evaluated, in clinical studies emphasis was placed on the safety aspects of rHSA administration. Data obtained in both tests indicated that rHSA appeared to be identical to pdHSA, having the same safety characteristics (Kobayashi, 2006).

8.5.4.1. Phase I clinical trial. To evaluate the safety of rHSA, 25 normal volunteers were subjected to intravenous administration for 3 consecutive days of either 50 ml of rHSA (12.5 g rHSA/body) or progressively increased doses of rHSA in a bolus injection (5–100 ml; 1.25–25 g rHSA/body). No abnormalities relating to the administration of rHSA were observed during the trial in physiological tests (i.e., blood pressure, pulse, body weight, electrocardiogram fundus examination); moreover, subjective and objective symptoms, hematological analysis or urine analysis, and elevation of antibody to rHSA were not observed in 3 months after administration (Kobayashi, 2006).

8.5.4.2. Phase II and III clinical trials. As clinical administration of HSA results in the recovery of colloid osmotic pressure and of circulatory volume, the phase II clinical trials were conducted in patients suffering from hemorrhagic shock and cirrhosis with ascites. High efficacy with little or no adverse reaction was confirmed in both studies (Kobayashi, 2006).

In phase III clinical trials conducted in patients with cirrhosis with ascites, rHSA was compared with pdHSA by randomized multi-center study, and no differences were observed. Repeated administration of rHSA was then tested in relapse cases which had been treated with rHSA in the phase II study in cirrhosis with ascites or in a phase III study in nephrosis with edema. No elevation of antibody against rHSA was observed in either case. In all cases, high efficacy with little or no adverse reaction was confirmed (Kobayashi, 2006).

8.6. Human serum albumin as a drug carrier

Most drugs circulate in the bloodstream (reversibly) bound to plasma proteins. HSA is the major carrier for acidic and neutral drugs; however basic drugs are bound to a lesser extent by HSA, mainly binding to α_1 -acid glycoprotein. Drug binding to other plasma proteins (e.g., LDL and HDL) only occurs at a much smaller extent (Meyer and Guttman, 1968; Vallner, 1977; Peters, 1996; Bertucci and Domenic, 2002; Kragh-Hansen et al., 2002; Fasano et al., 2005; Otagiri, 2005; Ascenzi et al., 2006a; Ascenzi and Fasano, 2010; Varshney et al. 2010).

8.6.1. Human serum albumin influences the pharmacokinetics and pharmacodynamics of drugs

Binding to plasma proteins greatly influences the pharmacokinetics and pharmacodynamics of most commonly prescribed drugs (Meyer and Guttman, 1968; Vallner, 1977; D'Arcy and McElnay, 1982; Rolan, 1994; Grandison and Boudinot, 2000; Schmidt et al. 2010). For instance, binding to plasma proteins results in increased solubility for lipophilic drugs, thus allowing them to reach their site of action. However, since the bound drug cannot readily leave the capillaries, only the unbound drug can be distributed to tissues therefore having pharmacological activity, as well as toxic effects. Thus, depending on the drug and the target, the high affinity of drugs for plasma proteins (e.g., HSA) may be either beneficial or a drawback for efficacy (Smith et al., 2010).

Usually, a drug is considered highly bound to plasma proteins when the fraction bound exceeds about 90% of the total drug concentration (Pellegatti et al., 2011). Examples of highly bound drugs include warfarin and the non-steroidal anti-inflammatory drugs phenylbutazone and ibuprofen (see Sections 4.1.3 and 4.1.6), that show about 99% protein binding in human plasma at normal therapeutic levels. Most or all of these binding processes occur with HSA (Lindup and Orme, 1981; Gly596_Leu609delinsSerLeuCysSerGly Bocedi et al., 2004, 2005; Fasano et al., 2005; Otagiri, 2005; Ascenzi et al., 2006a; Ascenzi and Fasano, 2010; Varshney et al., 2010).

The apparent volume of distribution of a drug (V_d) gives an indication of the extent of drug binding and distribution (Lindup and Orme, 1981; Schmidt et al., 2010). Drugs highly bound to plasma proteins usually have low V_d , because their strong association with plasma proteins confines them to the vascular space (Ariens, 1974). Conversely, drugs that are largely free in plasma are generally available for distribution to tissues. However, the V_d is also affected by the drug affinity for tissue proteins (Jusko and Gretch, 1976; Grandison and Boudinot, 2000; Schmidt et al., 2010). Several drugs, such as amiodarone, digoxin and tricyclic anti-depressants, although highly bound to plasma proteins, are bound with greater affinity to tissue proteins, resulting in a large V_d value (Jusko and Gretch, 1976; Grandison and Boudinot, 2000). Thus, drug distribution is a function of both plasma protein binding and tissue protein binding.

Besides distribution, binding to plasma proteins also affects drug metabolism and elimination, since both hepatic uptake and glomerular filtration are directly proportional to the free drug fraction present in the plasma (Lindup and Orme, 1981; Schmidt et al., 2010). If a drug is highly bound to plasma proteins and has a high extraction ratio from the liver, which involves active transport mechanisms to concentrate the drug in the hepatic cells, then plasma proteins act as delivery systems. Indeed, the rate of metabolism of some highly bound drugs such as propranolol depends on the rate of delivery to the liver via the bloodstream (the so-called flow-dependent hepatic elimination). Alternatively, if a drug has a low extraction ratio by the liver, then metabolism may be decreased by high plasma protein binding. Drugs bound to plasma proteins cannot undergo glomerular filtration, with the result that the half-life of drugs that are neither actively secreted by the renal tubules nor rapidly metabolized by the liver is increased. Conversely, similar to hepatic uptake, if a drug has a high renal extraction ratio due to active tubular secretion, then plasma protein binding will promote drug elimination by transporting the drug to the kidney (Lindup and Orme, 1981; Schmidt et al., 2010).

The extent of protein binding is a function of drug and protein concentrations, reflects the affinity constant for the drug-protein interaction, and depends on the number of protein binding sites per class of plasma proteins (Vallner, 1977; Lindup and Orme, 1981; Schmidt et al., 2010).

Drug binding to plasma proteins is a dynamic process. For most drugs, distribution occurs by passive diffusion of the unbound drug, usually occurring very rapidly; therefore, an equilibrium exists between the unbound drug concentrations in plasma and at the site(s) of action. Any change in either plasma or tissue protein binding may affect the free drug fraction (Vallner, 1977; Lindup and Orme, 1981; Rolan, 1994; Grandison and Boudinot, 2000; Schmidt et al., 2010).

The clinical relevance of changes in protein–drug association is a source of a never-ending debate (D'Arcy and McElnay, 1982; Rolan, 1994; Benet and Hoener, 2002; DeVane, 2002; Smith et al., 2010; Pellegatti et al., 2011). It is nowadays generally accepted that, since the free drug can easily leave the plasma compartment, and since it is the free drug concentration at the therapeutic target that is crucial for *in vivo* activity, and not the free drug fraction, changes in drug binding to plasma

proteins, alone, have usually little effects on the *in vivo* efficacy of drugs (Benet and Hoener, 2002; Schmidt et al., 2010; Smith et al., 2010; Pellegatti et al., 2011).

8.6.2. Factors affecting drug binding to plasma proteins

The interaction of a drug with plasma proteins is an important factor affecting their distribution and elimination, as well as the duration and intensity of their pharmacological actions. Remarkably, the concurrent administration of drugs, pathological conditions, age, and sex are the major factors that can affect drug binding to plasma proteins (Grandison and Boudinot, 2000; Turnheim, 2003, 2004; Ascenzi and Fasano 2009, 2010; Schmidt et al., 2010).

8.6.2.1. Concurrent administration of drugs. The concurrent administration of drugs with high affinity for HSA is one of the major factor that can affect drug-protein association (Vallner, 1977; Lindup and Orme, 1981; Rolan, 1994; Kragh-Hansen et al., 2002; Otagiri, 2005; Schmidt et al., 2010; Smith et al., 2010). Drug binding to HSA can be inhibited by a second drug. The mechanism may be either competitive, when two drugs administered concurrently bind to the same sites, or allosteric, when drug binding causes a conformational change(s) in the HSA molecule that affects the binding of further drug molecules. The ability of one drug to modulate the binding of the other is a function of their relative concentrations, binding affinity, and binding specificity. Drug disposition also depends on binding of endogenous and exogenous ligands to HSA (see Section 4) (Koch-Weser and Sellers, 1976; D'Arcy and McElnay, 1982; Peters, 1996; Bocedi et al., 2005; Fasano et al., 2005; Ascenzi et al., 2006a; Ascenzi and Fasano 2009, 2010).

The most important drug-displacing agents at HSA binding sites are acidic compounds (e.g., FAs) (Sellers and Koch-Weser, 1971; D'Arcy and McElnay, 1982). Drug metabolites may also induce the displacement of HSA-bound drugs: for instance, trichloroacetic acid, a metabolite of chloral hydrate, displaces warfarin from FA7 (Sellers and Koch-Weser, 1970). Moreover, aspirin affects drug binding by acetylating HSA Lys residues (D'Arcy and McElnay, 1982) (see Section 3.1). In particular, aspirin modulates the binding of flufenamic acid and phenylbutazone and possibly other anionic drugs to HSA (Kramer and Routh, 1973; Pinckard et al., 1973; Hultmark et al., 1975).

Over the years, the clinical relevance of drug–drug interactions due to displacement from plasma proteins has been the subject of intense debate (D'Arcy and McElnay, 1982; MacKichan, 1989; Rolan, 1994; Sansom and Evans, 1995; Benet and Hoener, 2002; Bertucci and Domenici, 2002; DeVane, 2002; Fasano et al., 2005; Ascenzi and Fasano, 2010; Smith et al., 2010; Pellegatti et al., 2011). In fact, the importance of plasma binding displacement as a mechanism of drug–drug interaction has been overestimated and overstated, being based mainly on *in vitro* data (Dayton et al., 1973; D'Arcy and McElnay, 1982; McElnay and D'Arcy, 1983; Smith et al., 2010). However, the fact that most studies have been performed *in vitro* limits their clinical significance, for several reasons. In fact, when *in vitro* studies are performed using animal SA, species differences in binding might be expected (Dayton et al., 1973). Moreover, certain *in vitro* assays use cells that overexpress target proteins in order to enhance the assay signal and its sensitivity (Smith et al., 2010). However, the overexpressed *in vitro* target requires a higher drug concentration to inhibit or to activate the larger amount of the target protein(s). As a consequence, the free drug concentration that is efficacious *in vivo* can be lower than the *in vitro* activity, due to the much lower expression of the target *in vivo* (Smith et al., 2010). Another limitation of *in vitro* studies is that they do not take into account that, in the clinical practice, patients are often treated with more than two drugs (Grandison and Boudinot, 2000). Lastly, plasma binding displacement might be not the only phenomenon occurring *in vivo*, since other pharmacokinetic processes (e.g., drug metabolism) may occur (di Masi et al., 2009).

Two clinically significant drug–drug interactions observed in the Sixties and originally attributed to plasma protein binding displacement support this notion (Rolan, 1994). First, a marked increase in prothrombin time was observed in patients treated with phenylbutazone in addition to warfarin (Fox, 1964; Aggeler et al., 1967). Since warfarin binds to HSA and is displaced by phenylbutazone, it was hypothesized that the concurrent administration of warfarin and phenylbutazone would result in an enhanced risk of hemorrhage in patients due to higher concentration of the free anti-coagulant drug. However, further investigation led to a more plausible explanation: indeed, besides displacing warfarin from its HSA binding sites (*i.e.*, FA7), phenylbutazone inhibits the metabolism of both the *S*-isomer of warfarin and of its less potent *R*-isomer (O'Reilly et al., 1980; Banfield et al., 1983). Therefore, both phenomena, *i.e.*, protein binding displacement and altered metabolism, are likely responsible of the pharmacokinetic interaction between the two drugs. Another example of a clinically significant drug interaction originally attributed to plasma protein displacement was the severe hypoglycemia described in patients simultaneously treated with sulfonamides and the hypoglycemic drug tolbutamide (Christensen et al., 1963). Since tolbutamide binds to plasma proteins with high affinity, it was postulated that sulfonamides displace tolbutamide from plasma carriers, thus increasing the free drug fraction in plasma. However, other mechanisms were found to be involved in the tolbutamide-sulfonamides interaction: indeed, sulfonamides inhibit the metabolism of tolbutamide, thus resulting in reduced tolbutamide clearance and sustained increase in its hypoglycemic effect (Hansen and Christensen, 1977; Pond et al., 1977).

The only pharmacological interactions that have been hypothesized to occur through pure displacement from plasma proteins are the warfarin-clofibrate and warfarin-chloral hydrate interactions, due to warfarin displacement by clofibrate and trichloroacetic acid, a metabolite of chloral hydrate (Sellers and Koch-Weser, 1970; Griner et al., 1971; Koch-Weser et al., 1971; Bjornsson et al., 1979a,b; McElnay and D'Arcy, 1983; Palareti and Legnani, 1996). However, absent or minimal clinical consequences of these interactions have been described. This supports the notion that ligand displacement from plasma proteins, alone, is not the major cause of clinically relevant drug interactions, and that further interaction mechanisms might be involved. Indeed, since the displaced drug can normally easily distribute out of the plasma compartment,

the increase of plasma free drug concentrations caused by displacement from plasma proteins is usually transient and too small to affect the total pool of the free drug in the body; therefore, it will not have a significant effect on free drug concentrations in tissues after reaching the equilibrium state (D'Arcy and McElnay, 1982; Rolan, 1994; di Masi et al., 2009; Smith et al., 2010).

Clinically important drug–drug interactions formerly considered to be caused *via* displacement from plasma protein binding sites usually have another interaction mechanism involved, like altered metabolism or changes in renal excretion. However, pharmacokinetic interactions due to displacement from plasma proteins become clinically important in specific situations: (i) for drugs highly bound to plasma proteins that have low clearance, narrow therapeutic index and small V_d , like warfarin, phenytoin, and tolbutamide (Rolan, 1994; Christensen et al., 2006), and (ii) when a displacing drug is co-administered to the patient via rapid intravenous injection (D'Arcy and McElnay, 1982; Rolan, 1994). As a consequence, the concentration of the primary drug in the plasma will quickly rise reaching receptors or entering a compartment into which it would usually reach lower concentrations. Compared to drug–drug interactions due to displacement from plasma proteins, interactions due to displacement from tissue proteins have a greater potential to cause adverse effects in the patient, since the displaced drugs will be forced from extravascular sites back into the plasma (D'Arcy and McElnay, 1982). The resulting increased drug plasma levels will lead to enhanced pharmacological effects and, possibly, toxicity. The displacement of drugs from binding sites simultaneously in both plasma and tissues will increase the free drug concentration in the plasma leading to over-activity of the displaced drug (D'Arcy and McElnay, 1982; McElnay and D'Arcy, 1983).

In conclusion, although displacement from plasma proteins per se is not a major underlying mechanism of clinically relevant drug–drug interactions, the study of protein binding and displacement is important for two reasons. First, to predict the involvement of other interaction mechanisms, like changes in metabolism, renal excretion, and changes in tissue binding. Second, to avoid incorrect recording of pharmacokinetic data of new drugs, which may lead to inappropriate dose recommendations and selection criteria of patient.

8.6.2.2. Pathological conditions. The extent of plasma protein binding can be altered in several pathological conditions: thus, the bound fraction of a certain drug can be decreased in patients suffering of analbuminemia, hypoalbuminemia, uremia, and hyperbilirubinemia, with consequent increase in the free drug concentrations (Vallner, 1977; Lindup and Orme, 1981; Pacifici et al., 1986; Viani et al., 1992; Grandison and Boudinot, 2000; Kragh-Hansen et al., 2002; Palatini et al., 2010; Schmidt et al., 2010). As previously stated, any reduction in drug binding is unlikely to give rise to adverse effects in patients. However, several diseases are likely to affect also metabolism and excretion processes. Altogether, changes in drug–protein binding, hepatic metabolism, and excretion may alter the activity of a certain drug from the therapeutic to the toxic range. For instance, the anti-epileptic drug phenytoin extensively binds to HSA, and increased adverse reactions have been reported in hypoalbuminemic patients (Vallner, 1977). Similarly, adverse reactions to diazepam (ranging from 2.9% in normal subject to 9.3% in patients suffering of hypoalbuminaemia) have been reported (Greenblatt and Koch-Weser, 1974). Remarkably, while hyperalbuminemia is a rare condition, hypoalbuminemia can occur more frequently, e.g., in severe liver or renal diseases (particularly in the nephrotic syndrome) (Grandison and Boudinot, 2000). In patients with renal insufficiency, the decreased binding of acidic and neutral drugs to HSA has been related to competition, to accumulation of endogenous metabolites and to conformational changes of HSA (Shoeman et al., 1973; Vallner, 1977). Liver dysfunctions may also result in decreased protein binding of certain drugs (Vallner, 1977). For instance, cirrhotic patients show lower HSA concentrations, and this may lead to an increased fraction of unbound drugs. Indeed, protein binding of phenytoin decreases in patients with hepatic disease compared to healthy subjects (Hooper et al., 1974).

Liver diseases are usually associated with altered metabolism processes. The combination of metabolic inhibition and altered plasma protein binding might result in a disproportionate rise in free drug concentration (Palatini et al., 2010). Conversely, pathological conditions resulting in the acute-phase reaction response, such as cancer, arthritis and myocardial infarction, lead to increased levels of α_1 -acid glycoprotein and therefore enhanced binding of basic drugs (Pacifici et al., 1986; Kremer et al., 1988).

8.6.2.3. Age and sex differences. Age is an important physiological factor that may influence HSA binding properties. Remarkably, phenytoin, salicylate, and nafcillin binding to HSA differs in newborns compared to adults, with the unbound fraction of these drugs being greater in neonates than adults. It has been suggested that these variations might be due to changes in FA plasma levels occurring during the neonatal period (Krasner et al., 1973; Vallner, 1977; Verbeeck et al., 1984; Notarianni, 1990; Grandison and Boudinot, 2000).

Unfortunately, very few conclusions have been reported on HSA binding properties occurring during aging and in elderly populations (Wallace and Verbeeck, 1987; Grandison and Boudinot, 2000; Turnheim, 2003, 2004). In fact, age has an effect on HSA binding properties depending not only on drugs, but also on the manner in which the study is conducted. Indeed, several studies in the elderly population involve patients affected by various diseases making difficult to assess the effect of age alone on HSA binding properties (Grandison and Boudinot, 2000; Turnheim, 2003, 2004). For instance, a number of pathological conditions very common in the elderly, including renal disease, hepatic dysfunction, acute myocardial infarction, and arthritis, alter protein levels and therefore drug–protein binding (Grandison and Boudinot, 2000; Mangoni and Jackson, 2004; Corsonello et al., 2010). Thus, HSA levels are approximately 19% lower in the elderly than in young populations because of reduced renal function and altered capacity of the liver to synthesize plasma proteins (Greenblatt, 1979; Corsonello et al., 2010). Furthermore, decreased protein binding might occur in patients subjected to restricted diets or

who are taking multiple medications. Another factor that may lead to changes in drug distribution in the elderly is the different body composition (Grandison and Boudinot, 2000; Mangoni and Jackson, 2004; Corsonello et al., 2010). Indeed, the elderly population usually shows an increased percentage of adipose tissue in the body. The elevation of fat tissues increases the V_d of lipophilic drugs and decreases the V_d of hydrophilic drugs. As a consequence, protein binding of lipophilic compounds decreases and protein binding of hydrophilic compounds increases (Dawling and Crome, 1989; Grandison and Boudinot, 2000).

The clinical outcome of changes in protein binding occurring with age depends also on the pharmacokinetic characteristics of the drug and on its therapeutic index. For most drugs, age-related changes in protein binding, although statistically significant, are not likely to be clinically relevant. The free fraction is changed by more than 50% in the elderly for only a few drugs, e.g., acetazolamide, diflunisal, etomidate, naproxen, salicylate, valproate, and zimeidine (Grandison and Boudinot, 2000).

There are also sex-specific differences in drug–protein binding (Koch-Weser and Sellers, 1976; Verbeeck et al., 1984; Gilmore et al., 1992). For instance, stereospecific age-related changes in plasma protein binding between males and females have been reported. The prototype β -adrenoceptor antagonist propranolol is highly bound to α_1 -acid glycoprotein and is marketed as an equal mixture of two enantiomers. The affinity of the β -adrenoceptor for the *S*-enantiomer of propranolol is at least 100 times higher than that of the *R*-isomer. No age-related difference in α_1 -acid glycoprotein concentration or protein binding of either enantiomer of propranolol has been reported (Gilmore et al., 1992). However, there is a sex-related difference with the females having significantly greater binding of the *S*-enantiomer than the males, and therefore less percentage of unbound *S*-propranolol (Gilmore et al., 1992). This sex effect might lead to an apparent resistance of females because higher concentrations of drug would be necessary to produce the same concentration of the active *S*-enantiomer in females. However, the clinical relevance of this finding needs to be further addressed (Mehvar and Brocks, 2001).

The effect of age and sex on plasma protein binding of both acidic (e.g., salicylate, phenytoin) and basic (e.g., chlorpromazine, meperidine, propranolol, and desipramine) drugs has been investigated in detail. Only the correlation of the free plasma fraction of salicylate with age was statistically different between males and females. However, the difference in the free plasma fraction of salicylate between sexes was too small to be clinically significant (Verbeeck et al., 1984). Moreover, no significant difference in the plasma protein binding of salicylate between males and females ranging in age from 23 to 34 years was found (Miaskiewicz et al., 1982).

The effect of age and sex on the protein binding of different benzodiazepines was reported. Remarkably, the plasma protein binding of these compounds is more affected by age than by sex (Greenblatt et al., 1980, 1981; Divoll et al., 1981). Lastly, physiological changes in pregnancy include significant changes in plasma composition. As a result, the plasma protein binding of many drugs, including propranolol, salicylate, diazepam, valproic acid, and sulfafurazole, is reduced during pregnancy (Perucca et al., 1981; Perucca and Crema, 1982; Nau et al., 1984; Bardy et al., 1990; Notarianni, 1990; Loebstein et al., 1997; Grandison and Boudinot, 2000).

9. Biotechnological aspects

SA is a multi-functional protein having several biotechnological and biopharmaceutical applications, including O_2 transport and delivery (Tsuchida et al., 2009), nanodelivery of drugs (Qu and Komatsu, 2010; Komatsu et al., 2011a, 2011b), and fusion of peptides (Furukawa et al., 2011). Moreover, HSA appears to play a role in the development of implantable biomaterials, surgical adhesives, and surgical sealants due to its ability to coagulate (Subramanian et al., 2004; Spotnitz and Burks, 2008). Remarkably, the use of rHSA provides the well recognized benefits of using pdHSA and avoids the risk of transmitting pathogenic contaminants (e.g., virus and prion) (Otagiri and Chuang, 2009).

9.1. Serum albumin as an adjuvant of cell growth and productivity

Mammalian cell culture has played an important part in the advancement of the life sciences over many decades and more recently has been a key factor in the application of biomedical research to the development of new therapeutic strategies. In the last decade the benefits of mammalian cell culture have become increasingly important for the production of new protein biopharmaceuticals, including monoclonal antibodies and cytokines, as well as providing new strategies for vaccine production. Tissue engineering and therapies arising from advances in stem cell science are broadening the applications for cell culture technology (Merten, 2006).

SA is added to cell culture media as a supplement to increase the growth and productivity of cells. Moreover, thanks to SA ability to carry and to deliver important nutrients to cells, it increases overall cell health. SA binds toxins thus avoiding toxic effects (see Section 4.3), binds excessive proteins acting as a buffer, and binds hormones and growth peptides keeping them stable (see Section 4.2). SA also binds free radicals to reduce damage to cells (see Section 5.3). In serum-free media, SA is often used in place of fetal bovine serum in cell culture media, improving the performance of a wide range of cell types, including stem cells and primary cells (Mather, 1998).

9.2. Human serum albumin nanotubes

Organic nanotubes have attracted great interest because of their biotechnological and biomedical applications. Indeed, (i) multi-functionalities can be introduced on the inner and outer surfaces of nanotubes independently, (ii) open-end terminals of nanotubes enable quick loading of target molecules without structural change(s), and (iii) nanotubes can have long circulation persistence in the bloodstream (Sun et al., 2002; Geng et al., 2007; Komatsu et al., 2011a).

Recently, HSA nanotubes trapping either HSA binding molecules or biotin or hepatitis B virus and displaying catalytic activity have been obtained. The nanotubes were prepared using an alternating layer-by-layer assembly of HSA and oppositely charged poly-L-Arg into a nanoporous polycarbonate membrane. The subsequent dissolution of the polycarbonate template yielded (poly-L-Arg/HSA) cylinders (Qu and Komatsu, 2010; Komatsu et al., 2011ab). Although the secondary structure of α -helices in HSA is slightly affected during the adsorption process and the random coils connecting α -helices are strongly altered, HSA retains functional properties (Shen et al., 2008).

The nanotubes, displaying HSA as the inner layer, bind typical HSA ligands such as uranyl ion, 3,3'-diethylthiacarbocyanine iodide, heme-Fe(III), and heme-Zn(II); remarkably, ligands can be released from nanotubes by FA-dependent competitive and allosteric modulation as observed in free HSA. Moreover, the HSA nanotubes showing either avidin or hepatitis B surface polyclonal antibody as the inner layer bind strongly biotin and the hepatitis B virus, respectively. Furthermore, the HSA nanotubes internally decorated with α -glucosidase catalyze the hydrolysis of 4-methyl-umbelliferyl- α -D-glucopyranoside to form α -D-glucose (Qu and Komatsu, 2010; Komatsu et al., 2011a,b). Very recently, HSA nanotubes with esterase activity have been produced; HSA retains its esterase activity and catalyzes *p*-nitrophenyl acetate hydrolysis (Komatsu et al., 2012). Lastly, recombinant HSA, which is currently manufactured on an industrial scale (Kobayashi, 2006), enables to develop the HSA nanotubes in practical use (Komatsu et al., 2011a).

9.3. Human serum albumin biochromatography

(Macro)molecular recognition represents the principal mechanism that controls living processes. Therefore, new drugs discovery involves a detailed analysis of biological activities of a large series of compounds, including binding to specific targets such as HSA (Bertucci and Domenici, 2002).

The affinity chromatography constitutes a powerful and predictive approach to investigate potential drugs binding to target bio-polymers. This technique (*i.e.*, biochromatography) involves: (i) the immobilization of a target bio-polymer (*e.g.*, enzyme, receptor, and transport protein), (ii) the assessment of unchanged protein functions after immobilization, and (iii) the determination of the ligand binding capacity, after the insertion of the immobilized protein in a separation system. The drug action, absorption, distribution, excretion, and receptor activation are dynamic processes in nature and similar to the basic mechanisms involved in chromatographic retention. Thus, the behavior of compounds in both biological and chromatographic environments is determined by the basic intermolecular interactions (*e.g.*, hydrophobic, electrostatic, and hydrogen bonding). The advantages of this approach are: (i) the possibility of revealing differences in binding of solutes in the presence of fast association-dissociation kinetics, due to the establishment of a large number of consecutive equilibria between free and bound ligands, (ii) the increased stability of bio-polymers (several weeks), (iii) the use of small amounts of bio-polymers, (iv), the recovery for re-use, (v) the reproducibility, and (vi) the on-line high-throughput screening for enzyme inhibitors and ligands (Ascoli et al., 1998, 2006; Bertucci and Domenici, 2002; Bertucci et al., 2003, 2010).

The development of several immobilized chromatographic columns is based on carrier proteins such as HSA (*i.e.*, HSA-HPLC), that possesses the highest enantioselectivity among plasma proteins. Immobilized HSA preserves the binding specificity and the conformation of the native form, allowing the detection of stereoselective and allosteric interactions. This method lets to study the drug binding process and to determine the extent of drug binding and the enantioselectivity of the process. Thus, it is possible to determine small differences in drug binding affinity and in its modulation by experimental parameters or by the presence of modulators. The chromatographic process can be a direct probe for ligand binding specificity and stereoselectivity, allows to investigate drug–drug, drug–metabolite, and drug–ion interactions, and evaluates the ligand-induced changes in the HSA structure (Ascoli et al., 1998, 2006; Bertucci and Domenici, 2002; Bertucci et al., 2003, 2010).

9.4. Bilirubin removal by human serum albumin in dialysis

Bilirubin, the principal mammalian bile pigment, is the end product of the heme-Fe catabolism (Ascenzi et al., 2005). Bilirubin is a high affinity endogenous SA ligand, and is considered as a substitute to evaluate the depurative capacity of HSA-bound toxins (*i.e.*, bile acids, aromatic amino acids, indols, and mercaptans) by blood purification modalities (Zunszain et al., 2008). Like many weakly-polar, poorly-soluble compounds, bilirubin is transported in blood tightly bound to HSA, with less than 0.01% of total bilirubin circulating in the unbound form (*i.e.*, free bilirubin). Free bilirubin governs the diffusion of HSA-bound bilirubin into tissues, and therefore it is responsible for both its beneficial and toxic effects. Although bilirubin was long thought to be a non-functional waste product, recent studies have shown that the anti-oxidant effects of mildly elevated plasma bilirubin levels, as well as activation of heme oxygenase, may protect against diseases associated with oxidative stress, such as atherosclerosis. By contrast, markedly elevated plasma levels of bilirubin may cause severe neurological

damage, especially in neonates. Therefore, the regulation of the cellular content of bilirubin, by conjugation, oxidation, and export, is of pivotal importance for cellular health (Vitek and Ostrow, 2009).

Bilirubin accumulates to toxic levels in individuals with impaired or immature liver function. The resulting jaundice is managed with totally artificial or bioartificial extracorporeal systems (Santoro et al., 2007; Saito, 2010). Moreover, phototherapy induces the isomerization of the biosynthetic 4Z,15Z-bilirubin-IX α to more soluble and excretable isomers, such as 4Z,15E-bilirubin (Maisels and McDonagh, 2008). Then, bilirubin and its configurational isomers are transported to the liver by HSA (Ascenzi et al., 2005b).

The prognosis of patients with an acute accumulation of pathogenic or toxic substances in their body fluids can be significantly improved by blood purification techniques including hemodialysis, hemofiltration, hemodiafiltration, continuous hemofiltration, hemadsorption, and plasma exchange. Each modality has different removal capacities and limitations; therefore, it is necessary to thoroughly evaluate the time and the duration of use in the case of different disease conditions. The use of fresh frozen plasma as a replacement fluid in plasma exchange is also important in order to replenish the deficient coagulation factors and essential metabolic factors. Activation of tissue/organ regeneration by the removal of pathogenic factors or by the substitution of factors essential for regeneration might be important in the case of multiple organ dysfunction syndromes. In critically ill patients with composite conditions, the use of more than two blood purification techniques at the same time or at different times during the course of the diseases can improve patient prognosis more than the use of single methods. Lastly, stem cells are however the real great hope for the future of patients with end-stage liver disease (see Santoro et al., 2007; Saito, 2010).

HSA dialysis is widely accepted as a liver-support technique for patients with liver failure and was introduced clinically in 1993. In the Molecular Adsorbent Recirculating System (MARS) and the single-pass HSA dialysis (SPAD), HSA is the exogenous trapper for the toxic substances. The removal of bilirubin by MARS and SPAD is a diffusion process, dependent on the free bilirubin concentration. In contrast, with the Prometheus system (the most recent system developed for a wide Liver Support Systems detoxification), HSA-bound toxins are directly removed in two separate cartridges with different solute affinity, without the need for exogenous HSA; indeed, plasmadsorption is then coupled with a renal dialysis process. MARS is an efficient system in the removal of accumulated toxins in patients with various forms of liver failure and its complications, in the treatment of highly protein-bound drug overdose, cerebral edema, hepatic encephalopathy, high intracranial pressure, and progressive hyperbilirubinemia (see Drexler et al., 2009). It has been demonstrated that removal of HSA ligands by MARS leads to improved HSA binding capacity *in vivo* and that this correlates with improved survival (Klammt et al., 2008). A variation of the MARS method was tested clinically in a small number of cases. Indeed, SPAD uses a standard continuous venovenous hemodialysis (CVVHD) or venovenous hemodiafiltration system without any additional columns or circuits to dialyze the blood against a HSA dialysate. Both methods have the HSA-containing dialysate, the non-HSA leaking membrane in common, but the major difference is the HSA concentration used. MARS is typically carried out with either 100/120 g of HSA (i.e., 600 or 500 mL of 20% HSA solution) to fill the re-circulating HSA circuit. The resulting concentrations in the dialysate are between 1×10^{-3} M and 2×10^{-3} M HSA. Typical HSA concentrations in SPAD range between 3×10^{-4} M and 6×10^{-4} M HSA in the dialysate (Drexler et al., 2009). Even though the MARS is quite expensive (i.e., equipment and a special training of the operator), it is considered the best treatment for the bilirubin blood removal (Uzun and Denizli, 2006; Santoro et al., 2007; Gong et al., 2008; Boonsrirat et al., 2009; Saito, 2010).

9.5. Human serum albumin depletion from human serum

Plasma is a rich source of biochemical products that can be indicators of the physiological or clinical status of the patient. Several methods of proteome analysis are used to find additional clinically informative protein markers, but the high abundance of HSA in plasma can interfere with the resolution and sensitivity of many proteome profiling techniques (Gundry et al., 2007). Indeed, many efforts have been made to remove HSA from plasma by developing an immunoaffinity resin with high specificity and capacity. In particular, to maximize the yield of the process, it has been developed a resin with immobilized monoclonal antibodies against HSA. Remarkably, each antibody reacts with high affinity to unique epitope on the HSA molecule, thus removing both the full-length protein and many of its fragments present in the plasma. Moreover, many dye-based resins optimized for HSA binding are commercially available (e.g., Cibacron Blue cross-linked to agarose gel), and can interact with the protein by: (i) ion-exchange, (ii) hydrophobic interaction, (iii) exclusion-diffusion, and (iv) affinity binding. However, they show a specificity lower than that of the antibody method (Steel et al., 2003).

9.6. Human serum albumin fusion proteins

Biologically active peptides or low molecular weight protein can be fused genetically to HSA, which has a longer plasma half-life, so that the half-life of the short peptide can be extended (Chuang et al., 2002; Chuang and Otagiri, 2007). In comparison to chemical modification methods, HSA fusion method has not been reported to produce an accelerated blood clearance process (Wang et al., 2007).

9.6.1. Human serum albumin–thioredoxin fusion protein

Trx is a small ubiquitous protein that is redox active and is induced in response to various oxidative stress conditions (Arner and Holmgren, 2000). Recently, a number of studies exploiting the biological activities of Trx in treating diseases

caused by oxidative stress have been attempted (Okubo et al., 1997; Shibuki et al., 1998; Takagi et al., 1999; Hoshino et al., 2003; Callister et al., 2006; Nakamura et al., 2007; Hu et al., 2008; Nakamura et al., 2009).

The fusion protein of HSA and Trx (HSA–Trx) has been produced, and the evaluation of both the structural and functional properties has been performed (Furukawa et al., 2011). The HSA–Trx fusion protein exhibits similar pharmacokinetic properties as HSA, and a much improved blood retention property than that of Trx alone. Although fusion to HSA causes about 40% reduction in the Trx biological activity *in vitro*, HSA–Trx fusion protein has proven to be therapeutically effective in the septic shock mouse model (Furukawa et al., 2011). It is noteworthy that HSA–Trx shows higher distribution in the lungs than in other organs, as well as 10 times longer plasma half-life than Trx in normal mice (Ikuta et al., 2010).

9.6.2. Human serum albumin-single chain antibody fusion protein

Radiolabeled monoclonal antibodies have been developed for both the diagnosis and the treatment of tumors (Wu and Senter, 2005; Kenanova and Wu, 2006). Single-chain variable fragments (scFvs) of monoclonal antibodies with a molecular mass of 25 kDa are cleared very rapidly from the circulation, but they exhibit poor tumor retention, due to their lower affinity, than the parent antibody (Wu et al., 1996). On the other hand, bivalent antibody fragments possess more ideal tumor-targeting characteristics, including rapid tissue penetration, high target retention, and rapid blood clearance (Leung, 2008a,b,c). The diabody fragment (a dimer of scFvs) and the minibody fragment (a dimer of scFvs-CH3 with a linker region comprising 18 amino acids) of a murine/human chimeric anti-carcinoembryonic antigen T84.66 antibody, have been evaluated for targeting in several tumor antigen systems with rapid tumor localization and high-contrast imaging (Wu et al., 1996; Viti et al., 1999). The carcinoembryonic antigen (CEA) is a β -glycoprotein expressed primarily on the cell surface; its levels are increased in a variety of carcinomas and in certain inflammatory conditions, such as inflammatory bowel disease (Wahl et al., 1983; Kowalsky and Falen, 2004). Remarkably, its expression has been observed in patients with various carcinomas of the colon, lungs, thyroid, uterus, ovaries, pancreas, and medullary thyroid (Leung, 2008a,b,c).

As already discussed, HSA is known to accumulate in tumors (Wang et al., 1994) and has a long blood half-life (Dennis et al., 2002) (see Section 8.2.1). Therefore, a T84.66 single-chain antibody (scFv) has been joined with HSA using recombinant technology to form the T84.66 scFv-HSA fusion protein which was radiolabeled with either ^{64}Cu , ^{111}In , or ^{125}I , for localization of CEA-positive tumors in mice with single-photon emission computed tomography (Leung, 2008a,b,c; Yazaki et al., 2008).

Biodistribution studies of ^{64}Cu -, ^{111}In -, and ^{125}I -labeled T84.66 scFv-HSA in nude mice, using a LS174T human colon carcinoma subcutaneous xenograft model, have been performed. The positron emission tomography imaging with the ^{64}Cu -labeled T84.66 scFv-HSA was visualized at 4 hours and reached the highest level at 24 hours. There was relatively low radioactivity elsewhere (except the liver) in the mice. On the contrary, the ^{111}In -labeled T84.66 scFv-HSA exhibits a markedly slower blood clearance than the T84.66 minibody and diabody but clears faster than the parent T84.66 antibody. The single-photon emission computed tomography imaging with the ^{111}In -labeled T84.66 scFv-HSA showed high localization to the CEA-positive tumor and relatively low activity elsewhere (except the liver) in the mice. Finally, the ^{125}I -labeled T84.66 scFv-HSA exhibits a markedly slower blood clearance than the T84.66 minibody and diabody, but clears faster than the parent T84.66 antibody. The single-photon emission computed tomography imaging with the ^{125}I -labeled T84.66 scFv-HSA showed high localization to the CEA-positive tumor and relatively low activity elsewhere (except the thyroid) in the mice (Leung, 2008a,b,c; Yazaki et al., 2008). Overall, radiolabeled HSA-single chain antibody fusion proteins may represent invaluable tools in anti-tumor therapy (Leung, 2008a,b,c; Yazaki et al., 2008).

10. Conclusion and perspectives

HSA, the most abundant circulating protein in the blood, represents the main determinant of plasma oncotic pressure and the main modulator of fluid distribution between body compartments. Although monomeric, HSA displays extraordinary ligand binding properties which are reminiscent of those of multimeric proteins. Indeed, endogenous and exogenous ligand binding to HSA is modulated not only by ligand-ligand competition for the same site but also by intramolecular communication(s) within multiple clefts. Remarkably, ligand binding to HSA is a transient event(s); therefore, the HSA–ligand complexes may display time-dependent specific functional properties. These transient ligand-dependent events have been called “chronosteric effects”.

HSA is widely used clinically to treat serious burn injuries, hemorrhagic shock, hypoproteinemia, fetal erythroblastosis, and ascites caused by liver cirrhosis. Moreover, HSA represents a valuable biomarker of many diseases, including cancer, ischemia, severe acute graft-versus-host disease, and diseases that need monitoring of the glycemic control.

HSA natural and artificial genetic mutants can provide valuable informations concerning structural and reactivity properties. Moreover, they are relevant in biotechnological and biopharmaceutical applications, including O_2 transport and delivery, nanodelivery of drugs, fusion peptides, implantable biomaterials, surgical adhesives, and surgical sealants. HSA is also used as an excipient for vaccines or therapeutic protein drugs, and as a cell culture medium supplement. Remarkably, the use of rHSA provides the well recognized benefits of using pdHSA and avoids the risk of transmitting pathogenic contaminants (e.g., virus and prion). rHSA will thus open new doors in its applications and contribute to self-sufficiency in blood and blood products.

Lastly, although HSA is one of the most investigated proteins, some questions are in order. Is HSA essential? Indeed, humans almost totally lacking HSA safely survive. Does occur a specific HSA receptor in humans? Interestingly, the albuminoid

protein AFP is recognized by a specific receptor. Answers to these and other questions could unveil several still unrecognized physiological roles of HSA.

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