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# **Apolipoprotein H, an Acute Phase Protein, a Performing Tool for Ultra-Sensitive Detection and Isolation of Microorganisms from Different Origins**

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## **1. Introduction**

Apolipoprotein H (ApoH), also known as beta2-glycoprotein I ( $\beta$ 2-GPI), is a plasma glycoprotein of 50 kDa. ApoH is present in human plasma at a concentration of between 150 and 300 mg/ml (Bouma et al., 1999). In blood, ApoH circulate in free conformations or bound to lipoproteins: chylomicrons, very low-density lipoprotein (VLDL), low density lipoprotein (LDL) and high-density lipoprotein (HDL). In addition, ApoH has a high affinity for triglyceride-rich lipoproteins. The amount of ApoH associated with plasma lipoproteins in healthy individuals varies according to the authors from 4 to 13% (Gambino et al., 1999a) up to about 40% (Polz & Kostner, 1979). ApoH is able to activate lipoprotein lipases (Lee et al., 1983). ApoH was isolated from the fraction of plasma lipoproteins, and described for the first time in 1961 by H. Schultze E (Schultze, 1961). In a lesser extent, ApoH is also associated to  $\beta$ 2-globulin fraction.

ApoH is expressed in human liver, in intestinal cells and tissues (Averna et al., 1997). In rats, other sites of synthesis in low concentrations were identified as the kidney, small intestine, brain, cardiomyocytes of the heart, and at even lower in the spleen, stomach and prostate (Ragusa et al., 2006).

ApoH is an acute phase protein and because when activated, ApoH bind, with a relative high affinity, to pathogens or their proteins, ApoH is also considered as an element of the host innate immune response, particularly during the acute phase. It is difficult to classified as positive or negative acute phase protein. This property is used as a mean to drastically improve diagnostic of pathogens from different origins, including human, animal or environmental and nature, including enveloped or non-enveloped viruses, parasites, and Gram+ or Gram - bacteria. Indeed, activated ApoH coupled to solid supports is used to concentrate and “clean” pathogens (from inhibitor of detection methods) to detect

pathogens with molecular or immunological methods and thus avoiding false negative diagnostics.

## 2. ApoH expression

### 2.1 Structure and localization of the ApoH gene

The gene encoding ApoH was localized by fluorescent *in situ* hybridization, on chromosome 17, locus q23-24, and extends over 18 kilobases (kb). It consists of eight exons (~ 1.2 kb) separated by large introns (~ 16.2 kb) encoding a protein of 345 amino acids (aa). This protein is subsequently cleaved at a signal peptide of 19 aa located at the N-terminal position (N-ter) to generate the matured protein (Mehdi et al., 1991; Steinkasserer et al., 1991).

Eight exons will form the matured protein constituted of five domains. Exon 1 encodes the 5'-untranslated region (5'-UTR) and the signal peptide. Exons 3 and 4 encode the domain II and exons 2, 5, 6 and 7 respectively encode domains I, III, IV and V of the protein. Exon 8 encodes the C-terminal (C-ter), the stop codon and 3'-UTR region (Fig. 1) (Okkels et al., 1999).

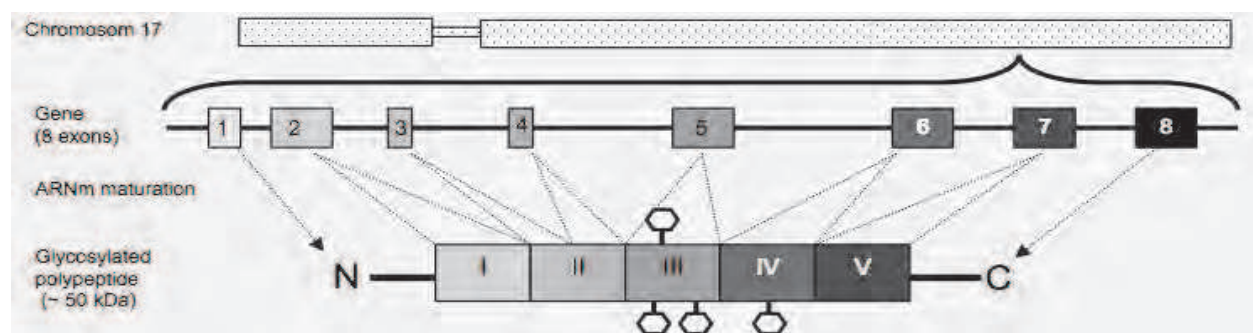


Fig. 1. ApoH gene organization (Sodin-Semrl & Rozman, 2007)

### 2.2 Promoter of the ApoH gene

Using the HepG2 liver cell line, deletion analyses in the 5' region of ApoH gene evidenced that its promoter is located between positions -197 and 7 (Wang & Chiang, 2004). The ApoH gene expression is governed by several transcription factors including a liver-specific atypical TATA box (TATTA) located between positions -97 and -92 and the transcription factor HNF-1 (Hepatic nuclear factor-1) located upstream of the TATTA box. The soluble transactivation factor HNF-1 $\alpha$  interacts with HNF-1 leading to transactivation of the ApoH promoter. The gene expression increasing of endogenous ApoH is correlated with an overproduction HNF-1 $\alpha$ .

Several transcription factors are able to bind multiple ApoH regulating sites. In liver cells, these sites are located near the promoter (up-670pb), as shown in Fig. 2. These transcription factors are:

- STAT Signal Transducer and Activator of Transcription.
- Leucine zipper domains: CREB (cAMP Response Element Binding) and C/EBP $\beta$  (CCAAT-Enhancer-Binding Proteins).
- AP-1 (Activating Protein 1) also known as TPA-responsive element (TRE)
- HNF-3 $\beta$  (Hepatocyte Nuclear Factor 3 $\beta$ ) involved in embryo development

- HNF1 (Hepatocyte Nuclear Factor 1), a transcription factor that regulates the expression of several hepatic proteins, including serum albumin or  $\alpha$ 1-antitrypsin.
- The protein NFAT (nuclear factor of activated T-cells) activated in the cytoplasm by calcium-dependent phosphatase. The signal may interact with transcription factors AP-1 and NF-AT.

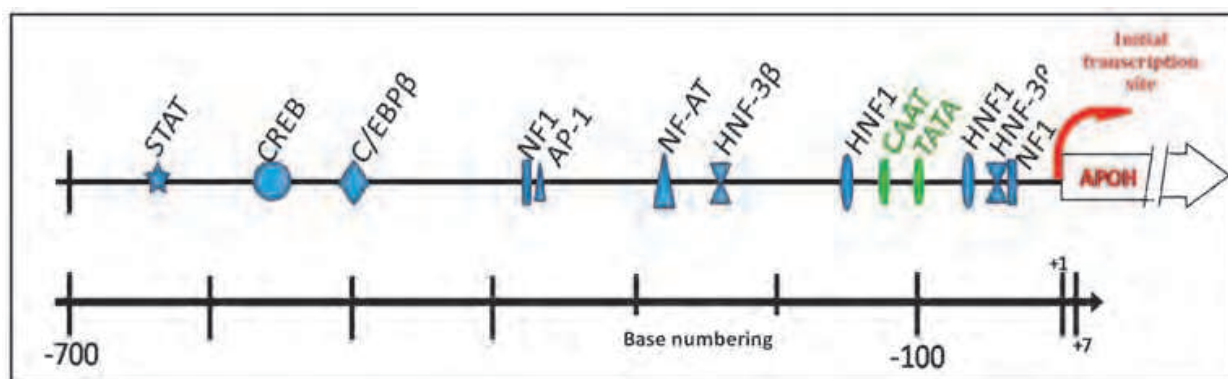


Fig. 2. Promoter region of the ApoH gene and transcription factors (Wang & Chiang, 2004). The transcription sites in the promoter (TATA and CAAT boxes) are located near the initiation of ApoH transcription site (+1). Regulatory sites (blue), CAAT & TATA boxes (green), initiation site of transcription (red).

## 2.3 Protein structure of ApoH

### *Primary structure and domain concepts*

The peptide sequence of ApoH was identified in 1984. Its primary structure consists in a single polypeptide chain of 326 amino acids. With the exception of collagen and other related molecules, ApoH appears as one of the richest eukaryotic structures containing prolines, exhibiting 31 proline residues per molecule. ApoH is also rich in lysine (30 residues), glycine (23 residues) and cysteine (22 residues) (Lozier et al., 1984) amino-acids.

The peptide sequence can be divided into four continuous domains of about 60 amino acids each, called SCR (short consensus repeat) and a fifth domain in the C-terminal region (Lozier et al., 1984; Steinkasserer et al., 1991). SCR domains are also known under the term of Sushi domain, or CCP (Complement Control Protein) that are motifs often found in proteins of the complement system, but also in some proteins, such as interleukin-2 receptor. These SCR confer to the ApoH sequence exhibit a homology with several proteins such as VCP (vaccinia virus protein), CD46 (binding protein of measles virus) and HI, a Drosophila protein that belongs to the family of immunoglobulins and complement proteins. As shown in Fig. 3, each SCR contains four cysteines associated by pairs, Cys-1 to Cys-3 and Cys-2 to Cys-4 linked by disulfide (S-S) bridges (Okkels et al., 1999) that confers a high structural stability to ApoH.

In contrast, the domain V of ApoH exhibits a very different structure. A sequence of six amino-acids (KNKEKK) is inserted within a highly positive charged region, an extension of 19 residues at the C-terminal and the presence of an additional S-S bridge (Steinkasserer et al., 1991). A highly conserved tryptophan within the domain V will contribute to hydrophobic interactions with the ApoH partners.

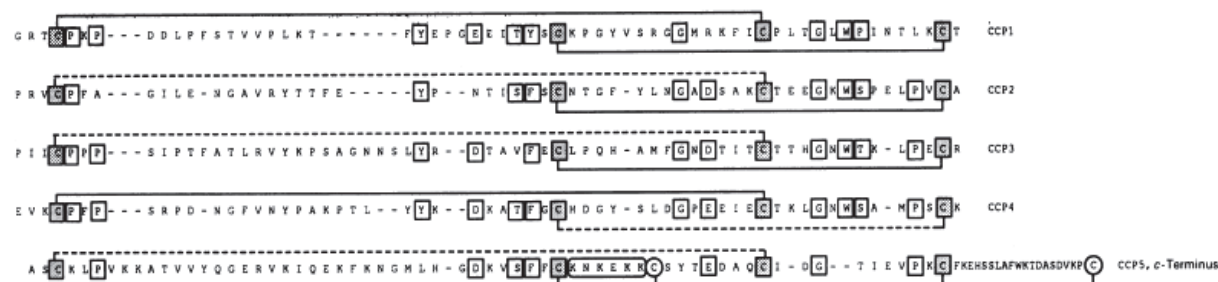


Fig. 3. Alignment and organization of the disulfide bonds of ApoH (Steinkasserer et al., 1991).

Secondary structure of ApoH

ApoH contains four N-glycosylation sites and one site of O-glycosylation linked to domain III and IV of the protein (Fig.4A). The glycosylated chains of ApoH are composed of galactose, mannose, N-acetylglucosamin, fructose, N-acetylneuraminic acid and sialic acid (Kamboh et al., 1988). They are linked to the peptide sequence *via* a nitrogen or oxygen atom (Gambino et al., 1999b).

Secondary structures of each domain are composed of several anti-parallel beta-sheets, shorts, and coiled around a hydrophobic core (Fig. 4B). Two S-S bridges, respectively located in N-ter and C-ter stabilize the structure. A long central  $\beta$ -sheet is framed along of the longitudinal axis by short beta-sheets parallels anti-parallel located near the C-ter and N-ter. The fifth domain, folds into a central  $\beta$ -sheet composed of four anti-parallel  $\beta$ -sheets and two short  $\alpha$ -helices and this domain is stabilized by three S-S bridges (Fig. 4C) (Bouma et al., 1999).

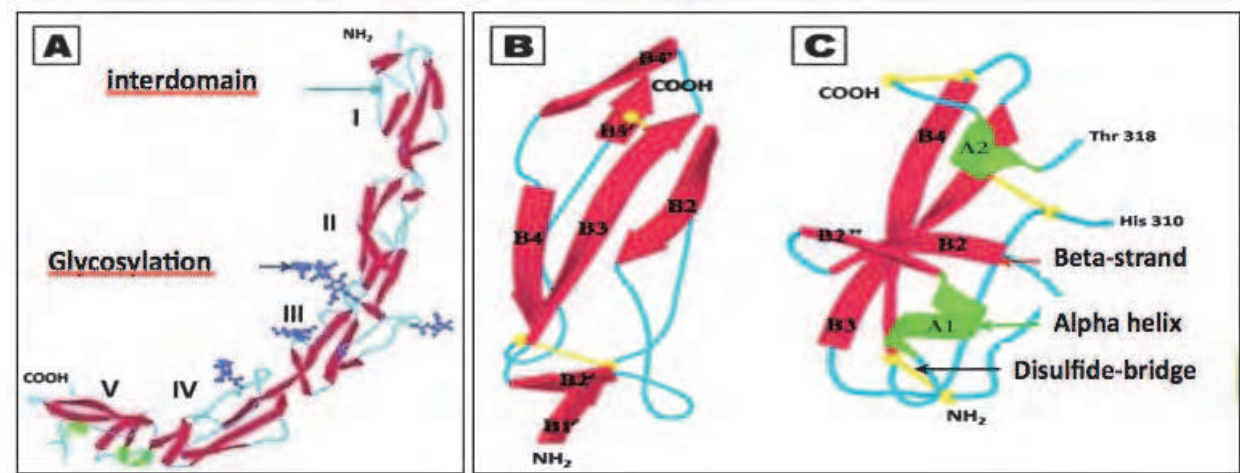


Fig. 4. The different structures of glycosylated chains of ApoH (Gambino et al., 1997)

Composition and organization of sugar chains

There are three possible structures for the glycosilated chains (Fig. 5) (Gambino et al., 1997): Complex, hybrid and Mannose-rich. These structures have a common motif consisting in two N-acetylglucosamins and three mannoses, differing in the antennas composition, and their number varies from two to five.



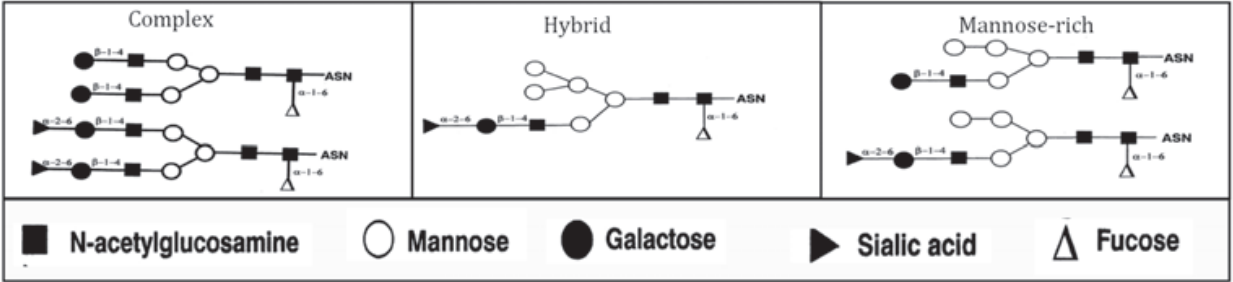


Fig. 5. Structural representation ApoH and domains (Bouma et al., 1999). (A) Secondary structure ApoH; (B) Secondary structure of domain III of ApoH; (C) Secondary structure of domain V of ApoH.

*The ApoH inter-domain regions*

Different ApoH domains are linked each other by sequences respectively composed of three amino-acids, between domains IV and V, and four amino-acids for other domains. Between domains II-III and III-IV residues form chains of  $\beta$ -sheets connecting the N-ter-B5 B4' sheet at the C-ter-sheet B1' B2'. The inter-domain represents 10-15% of the molecular surface. The inter-domain orientation varies in its inclination angle, ranging from 128 to 160° and a rotation angle ranging from 41 to 137°. Within the conserved domains, all are obtuse angles ranging from 120 to 162°, while the rotation angles vary widely between 22 and 180 ° (Bouma et al., 1999).

*Tertiary structure*

The analysis of crystallized structure of ApoH revealed a spatial organization to form a "J" or fishhook-like shape (Bouma et al., 1999; Schwarzenbacher et al., 1999). The four SCRs are arranged along a single axis with a slight spiral to the right to join the domain V. This very compact form suggests a certain rigidity of the protein. The X-ray analysis shows that in solution, the protein is folded, bulkier and exhibiting an "S-shape" (Fig. 6) (Hammel et al., 2002).

These differences may be explained by the absence of sugar chains in the crystallized protein and by changing the orientation of different domains due to the use of ions to form the crystal.

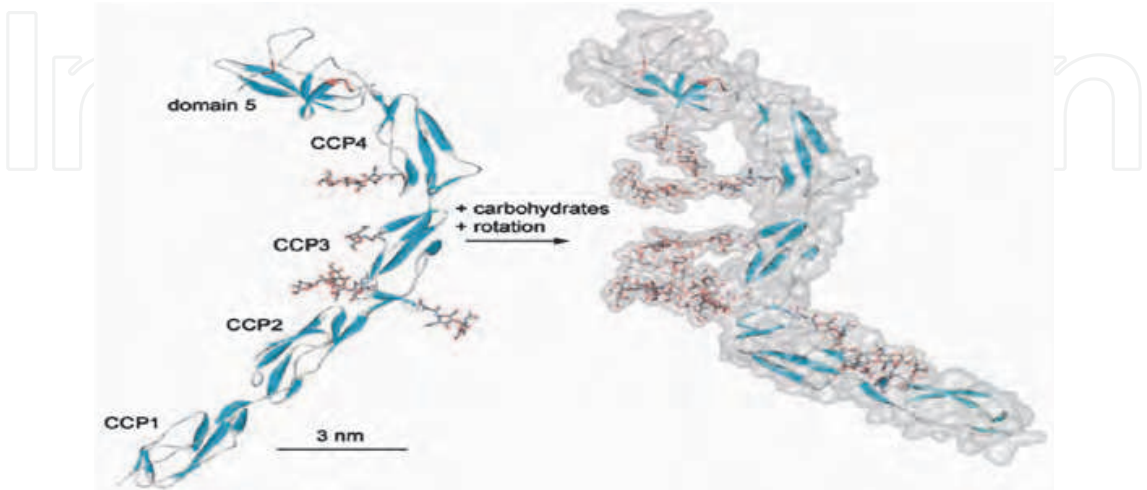


Fig. 6. The ApoH structure (Hammel et al., 2002). Crystal form (left) compared with the schematic atomic structure model in solution (right).

However, recent studies on electronic microscopy has evidenced the presence of inactive ring forms of ApoH as well as the fishhook-like active ApoH able to catch LPS present in sepsis cases (Agar et al., 2011).

### 3. ApoH and physiology

The role of ApoH *in vivo* remains only partially elucidated. Studies conducted *in vitro* have demonstrated that ApoH has the particularity to bind, not only to negatively charged molecules and structures, including anionic phospholipids (PL), but also binds platelets, apoptotic cells (d'Angeac et al., 2005), mitochondria, DNA as well as bile acids (Schousboe, 1983). Apart from the PL, ApoH also has the ability to bind cellular proteins such as the kidney calmodulin (Rojkjaer et al., 1997), megalin, an endocytic receptor of the renal epithelium (Moestrup et al., 1998), Annexin II (Ma et al., 2000) as well as other binding proteins or receptors including phosphatidylserine PS, LPS (Agar et al., 2011) and TLR2 (Alard et al., 2010).

Expression studies in HepG2 cells demonstrate that ApoH mRNA is regulated in a cell cycle-dependent manner, with very low expression in low cycling conditions and increasing levels in proliferating cells. p21 WAF-dependent growth arrest, induced by butyrate treatment, down-regulate ApoH mRNA levels. Immunolocalization in normal rat liver shows a non-homogeneous pattern, being mainly present in the centrolobular area; post-hepatectomy regenerating rat liver is uniformly immuno-stained and mitotic elements show the highest protein expression. Albumin gene expression, studies as control liver specific product, was not affected by sodium butyrate induced growth arrest. As previously reported for endothelial cells, ApoH behaves as survival factor for HepG2 cells: when increasing amounts of the protein (10–50 µg) have been added to serum deficient cultured liver cells a progressive reduced cell loss was observed (Averna et al., 2004).

In some autoimmune diseases, such as anti-phospholipid syndrome (APS), anti-ApoH antibodies and the complex ApoH-PL has been reported. These antibodies impact the inhibition of coagulation. They are associated with clinical manifestations of thrombotic venous or arterial type and recurrent fetal loss (Asherson & Cervera, 1993; Kandiah et al., 1998). Thus, APS may be associated with various clinical manifestations: thrombocytopenia, coronary or valve damage, neurological disorders, and autoimmune hemolytic anemia. The presence of these antibodies may also occur in infectious diseases (viral, bacterial or parasitic) and neoplasia (solid tumors, lympho-proliferative disorders) (Arnoux & Boutière, 2006; Harel et al., 2005).

ApoH is also involved in the whole process of hemostasis regulation. ApoH inhibits both the factor Xa genesis, in the presence of platelets and the activation of factor XI (Shi et al., 2005; Shi et al., 2004), as well as fibrinolysis, by preventing the generation of plasmin (Yasuda et al., 2004). ApoH also inhibits activated protein C that is an inhibitor of hemostasis (Keeling et al., 1993). ApoH therefore, exhibits both procoagulant and anticoagulant activities (McNeil et al., 1990).

The presence of antibodies to form an immune complex ApoH consisting in ApoH-oxidized LDL:anti-ApoH antibodies, complex that is phagocytized by macrophages. Thus, there would be presentation of ApoH epitopes, by major the histocompatibility complex type II (MHC II), at the macrophages surface, leading CD4<sup>+</sup> T cells activation. Moreover, it follows an excessive of macrophage lipids burden, and these macrophages are transformed into foam cells, the early stage of plaque formation.

Thus, ApoH is highly present in atherosclerotic plaques (George et al., 1999). ApoH allow binding and internalization of LDL in macrophages because of its ability to bind to LDL and oxidized LDL (Kochl et al., 1997). Moreover, ApoH is involved in apoptosis by binding to phosphatidylserine (PS), present at the surface of cells undergoing apoptosis (d'Angeac et al., 2005). Altogether, these data contribute to characterize ApoH as a scavenger-like protein. Finally, ApoH is also necessary to placental homeostasis and is involved in activation of endothelial cells and apoptotic mechanisms (Miyakis et al., 2004). However, despite available data showing pleiotropic functions of ApoH, many precise physiological roles of ApoH remain to be elucidated.

The high correlation between ApoH and CRP expression during systemic inflammation indicates that ApoH is a part of the group of acute phase proteins (Sellar et al., 1993). However, the expression of the acute phase protein, ApoH, sometimes is upregulated and sometimes is down regulated, reflecting its pleiotropic functions. Then, it become difficult to classify this APP as positive or negative. Indeed, divers data are sometime contradictory such as the observation of ApoH reduction in CSF during cerebral malaria (Agar et al., 2011) and the increase of ApoH in several other infectious diseases (Gast et al., 2006; Myles et al., 2003; van Hemert et al., 2006).

Due to different results presented here, ApoH can be considered as one of the major elements of the first line of the innate immune response regulating homeostasis.

#### 4. Interactions between ApoH and phospholipids

ApoH has a strong ability to bind anionic phospholipids (PL), such as cardiolipin or phosphatidylserine, rather than the neutral PL, such as phosphatidylcholin (Wang et al., 1998; Willems et al., 1996; Wurm, 1984). This interaction is ionic strength-dependent.

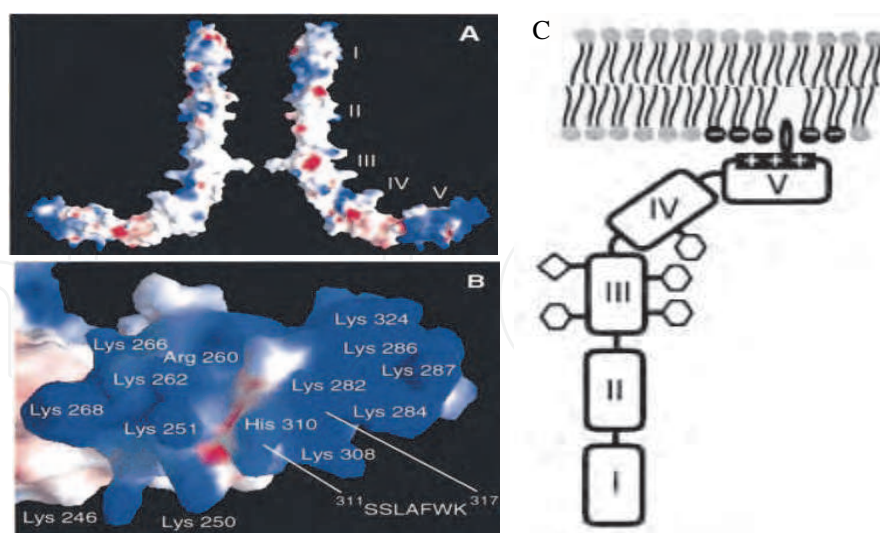


Fig. 7. Binding between ApoH and membranes (Bouma et al., 1999). (A) Representation of the electrostatic potential ApoH surface, with a view rotated 180°. ApoH domains are numbered from I to V. negative charges (red); positive charges (blue); (B) Prominent representation of half of the domain V. The positive charges are mainly due to the presence of lysine; negative charges (red), positive charges (blue); (C) scheme of the binding between ApoH (including the Tryptophan from domain V) and phospholipids bilayer.



Mechanisms of interaction between the anionic PL and ApoH could involve two types of bonds: (i) ionic and (ii) hydrophobic, this latter involving the V domain of ApoH (Hammel et al., 2001; Wang et al., 1998). Initially, the sequence C-C-KNKEKK 281-288, highly positively charged (Steinkasserer et al., 1991), and most likely exposed on the surface of the protein would be responsible for the initiation of ionic bonds with anionic heads responsible for PL. A second step, would involve the insertion of a mobile hydrophobic loop of the protein in the PL layer (Fig. 8). The authors report a sort of anchor protein to the interface head polar/non-polar tail of PL *via* the hydrophobic (tryptophan) residue of the mobile loop region located in the position 311-SSLAFWK-317 (Hong et al., 2001; Mehdi et al., 2000).

## 5. ApoH-viruses binding

### *Enveloped and non-enveloped viruses*

Human and animal viruses such as influenza have a lipid bilayer coat covering their capsid, which is a protein box protecting the nucleic acid (short or large RNA or DNA). These envelopes are derived from portions of the host cell membranes including phospholipids together with specific viral glycoproteins that frequently mature during virus development. These glycoproteins play a pivotal role in the cell entry of these viruses. The cell entry of the non-enveloped viruses is less well known. However, for some viruses, such as for rotaviruses A, trypsin plays a role in preparing the capsid to find a good “conformation” of some peptides to fuse with the cell membrane to enter cells.

## 6. The use of ApoH for drastic improvement of viral detection

An interesting feature of ApoH, is its ability to bind both enveloped and non-enveloped viruses. Thus, it was shown that the protein bind hepatitis B virus (HBV), and more particularly the surface antigen HBsAg (Mehdi et al., 1994). HBsAg exists in three molecular forms: S for small, small protein, M for middle, moderate protein and L for Large protein (Fig. 9). The viral anionic PL are also involved in the interaction between ApoH and HBsAg (Neurath & Strick, 1994; Stefas et al., 2001). These results are reinforced by the fact that the recombinant HBsAg protein and anionic PL share the domain V of ApoH as binding domain (Mehdi et al., 2008). When viral PL becomes oxidized, the binding affinity ApoH-HBsAg increases. It has been shown that ApoH has an affinity for infectious Dane particles

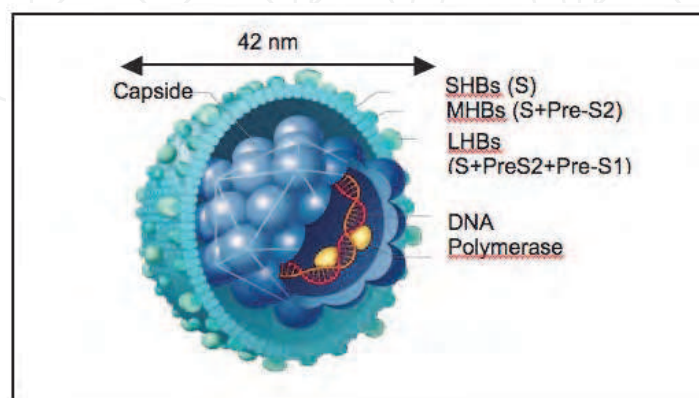


Fig. 8. Structure of hepatitis B virus ([http://dicos.ens-lyon.fr/vie/viro/image/V05\\_2H1\\_Hepatitis\\_8HBV.jpg](http://dicos.ens-lyon.fr/vie/viro/image/V05_2H1_Hepatitis_8HBV.jpg))

that are characterized by the presence of a myristyl group on the pre-S1 domain of protein L. The presence of the myristyl group enhances its binding with ApoH (Stefas et al., 2001). Among experiments carried out by us, four HBV/HBsAg and HBV-DNA-positive patients' sera were preincubated for 1 hr at 37°C in the presence or absence of 100 µg/mL of polymyxin B or 2 mmol/L of iron or a mixture of both. The ability of HBV+ from patient sera (diluted at 1/100) to bind to ApoH-coated ELISA wells was assessed in the following conditions: sera HBV+ alone, in the presence of either polymyxin B (PB) or in the presence of Fe<sup>3+</sup>. ApoH-coated wells preincubated with PB or Fe<sup>3+</sup> and washed were used as controls (Fig.9).

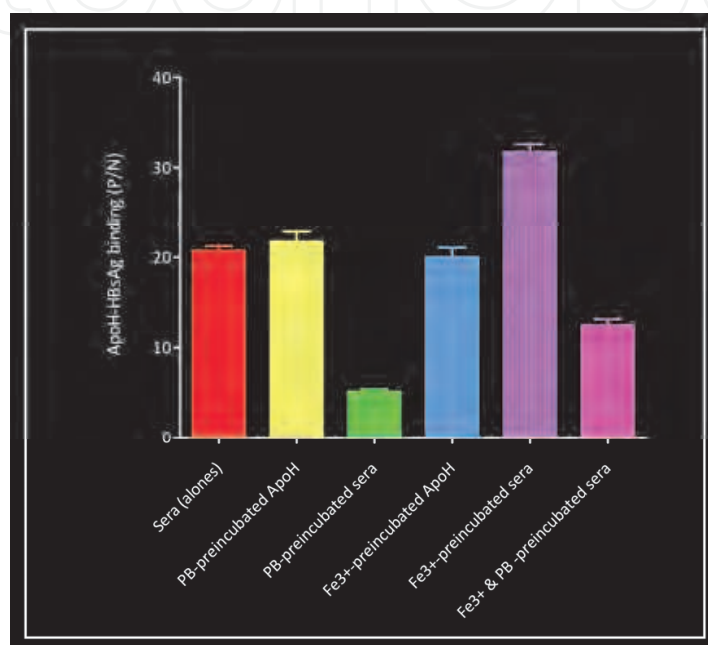


Fig. 9. Effects of polymyxin B and/or metal ions on the ApoH-HBsAg binding.

The preincubation of HBV+ sera with PB significantly reduced HBV/HBsAg binding to ApoH and this interaction was dose-dependent (data not shown). In contrast, the preincubation of HBV sera with Fe<sup>3+</sup> increased HBV/HBsAg binding. No effect was observed when ApoH-coated wells were preincubated with polymyxin and washed, before the addition of serum. These data strongly suggest that viral phospholipids are involved in the ApoH-HBV/HBsAg interaction.

It has been also shown that ApoH interact with antigens of human immunodeficiency virus (HIV) types I and II, particularly the Gag proteins of HIV 1 and 2 of p18 and p26 of HIV-1 HIV-2 or of gp160 of HIV-1 (Stefas et al., 1997), suggesting that the interaction of ApoH with HIV proteins may result in part from the ApoH affinity for PL. According to Stefas et al. (1997), experimental conditions, such as acidic pH and/or the presence of detergent, could lead to conformational changes of ApoH or viral proteins thereby inducing the binding between ApoH and HIV-1.

More recently, within the European USDEP project ([www.usdep.eu](http://www.usdep.eu)), it has been shown that ApoH capture viruses including Hantavirus in both serum and urine from infected patients (Godoy et al., 2009), and very interestingly avoiding false negative generated by conventional and standard PCR methods (Fig. 10) and from stools rotavirus-infected patients permitting a very highly immune detection as sensitive as quantitative PCR

(Adlhoch et al., 2011). Within the same project, many other viruses have been shown strongly bind to ApoH, including poxvirus virus, hepatitis C virus, pseudorabies virus, vesicular stomatitis virus, and Altogether these data clearly demonstrate that ApoH binds enveloped or non-enveloped DNA or RNA virus with high affinity permitting their ultrasensitive detection.

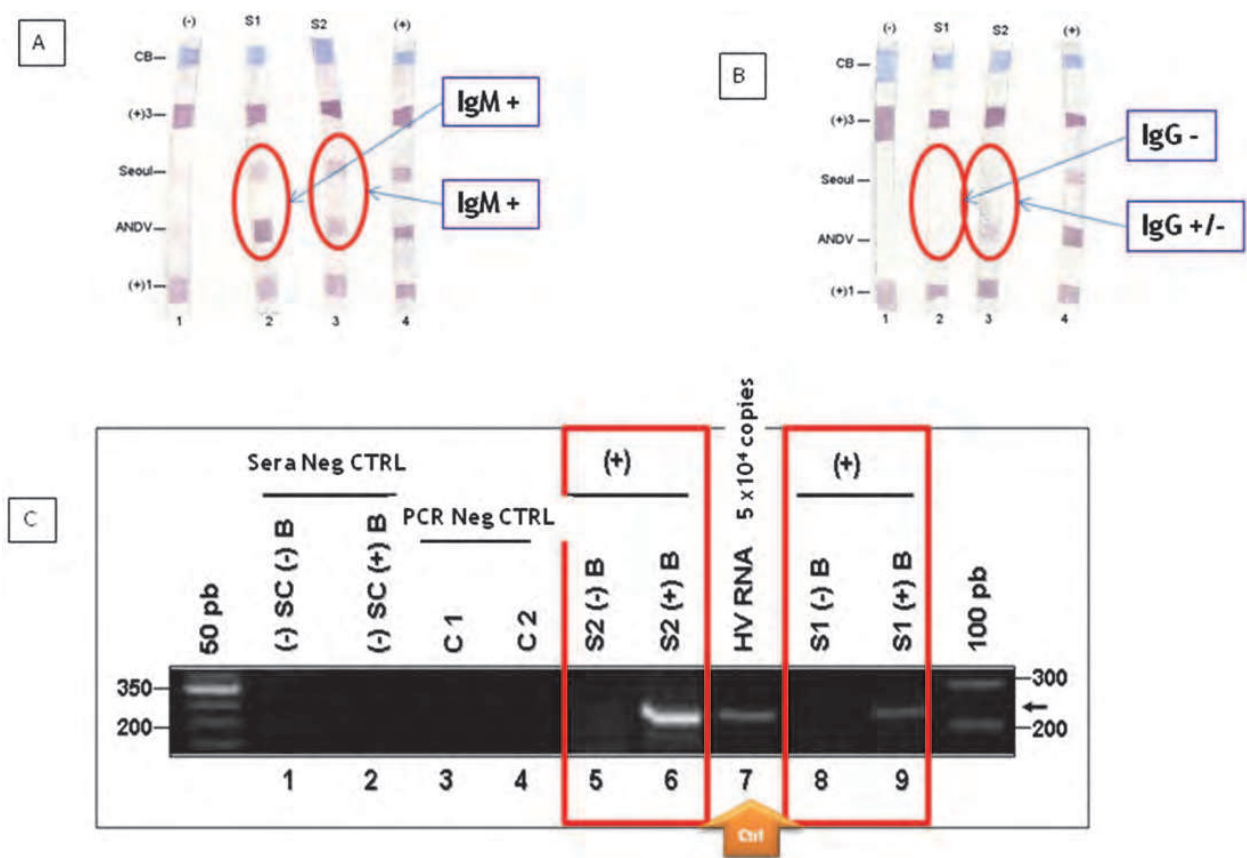


Fig. 10. ANDV binds serum-purified human ApoH (Godoy et al., 2009). Serum from patients S1 and S2 were tested for the presence of anti-hantavirus IgM (A) and IgG (B) antibodies by SIA using affinity-purified recombinant ANDV and Seoul virus N antigen. As internal controls, two levels (+1 and +3) of human IgG were applied to the blot. Serum from a negative donor (-) and of from a known HCPS-positive patient (+) were used as controls. Coomassie blue (CB) was used to determine the orientation of the strip. (C) Serum samples from patients S1 and S2 were divided in two. RNA was directly extracted from 300  $\mu$ L of sample one without the addition of ApoH beads [(-) B; lanes 5 and 8]. In parallel, the second sample of each patient was submitted to an additional step of ANDV capture by ApoH-coated magnetic beads prior to RNA extraction [(+) B; lanes 6 and 9]. Extracted RNA was used as the template in a RT-PCR/heminested PCR assay. As an additional control, total RNA extracted from sera of healthy negative donors with [(-) SC (+) B; lane 2] or without [(-) SC (-) B; lane 1] a step of virus capture and concentration by ApoH-coated magnetic beads was used as the negative control. Water controls for the RT-PCR (C1; lane 3) and heminested PCR (C2; lane 4) were included. In vitro-transcribed RNA corresponding to the ANDV S segment was used as a positive control for RT-PCR (HV RNA; lane 7). The arrow indicates the expected amplicon.

7. ApoH and bacteria relationship

Circulating anti-ApoH antibodies in APS and infections

In addition to circulating anti-ApoH antibodies in serum of patients exhibiting APS, an increased amount of anti-ApoH antibodies is also associated with several bacterial infections including: (i) urinary tract bacterial infections (Stojanovic et al., 2004); (ii) presence of *Helicobacter pylori* associated to ulcers (Pellicano et al., 1999); (iii) bacteria-associated rheumatic fever (Blank et al., 2004) and some other infections summarized in Table 1.

Infection agents or pathology	Frequency (%)
Typhus	20
<i>Mycobacterium leprae</i>	33 – 67
<i>Mycobacterium tuberculosis</i>	27 – 53
Bacterial endocarditis	5 – 44
<i>Helicobacter pylori</i>	ND
<i>Mycoplasma pneumoniae</i>	20 – 53
<i>Staphylococcus aureus</i>	43
<i>Streptococcus epidermidis</i>	80
<i>Staphylococcus pyogenes</i>	0 – 80
<i>Salmonella typhi</i>	60
<i>Escherichia coli</i>	67
<i>Coxiella burnetti</i>	42 – 84
<i>Leptospirosis</i>	50
<i>Borrelia burgdorferi</i>	14 – 41
<i>Sacharomyces cerevisiae</i>	ND
<i>Plasmodium falciparum</i>	30

Table 1. Prevalence of anti-ApoH in infection associated diseases (Blank et al., 2004). \*ND= undetermined

Moreover, it was shown that epitopes recognized by the anti-ApoH antibodies, exhibited similarities with peptides associated to several infectious diseases (Blank et al., 2002; Gharavi et al., 2002). The ApoH-like epitope expressed by microorganisms, might use by these latter to escape the immune response *via* molecular mimicry mechanisms. Thus, pathogen ApoH-like epitope expression could induce autoimmune response. The involvement of molecular mimicry in the induction of APS was demonstrated in a mouse experimental model. Balb/c mice immunized with pathogens having sequence homology with the peptide TLRVYK, a peptide recognized by anti-ApoH. The mouse anti-TLRVYK Ab were purified and injected into new BALB/c, which have subsequently developed an



experimental APS (Blank & Shoenfeld, 2004). Synthetic peptides corresponding to the ApoH-phospholipid binding site and having a high sequence homology with cytomegalovirus, adenovirus and *Bacillus subtilis* were used to induce an APS (Gharavi et al., 2002; Gharavi et al., 1999).

Changes of ApoH amounts have been reported in some bacterial infections. In a rare clinical case showing that a *purpura fulminans* phenomenon following an infection with *Haemophilus influenzae* in an adult was associated to a drastic elevation of the ApoH plasma concentration (Gast et al., 2006). A gene expression study performed on *Helicobacter hepaticus*-infected mice showed an increased expression of the gene of ApoH three months after infection, once a chronic inflammation was established (Myles et al., 2003). The infection of chickens with *Salmonella* leads to high ApoH gene over-expression approximately 40-fold excess as compared with uninfected controls (van Hemert et al., 2006). In contrast, it has been reported that *Legionella pneumophila* produces enzymes able to cleave ApoH (Muller, 1980). Altogether these reports suggest that increased gene expression of ApoH or even its "degradation" by enzymes induced by microorganisms may be in favor of active recognition of microorganisms by ApoH.

Some ApoH-derived peptides have shown antibacterial effects against both Gram-positive (*Staphylococcus pyogenes* and *S. aureus*) and Gram-negative bacteria (*Escherichia coli*), while the intact entire ApoH protein did not exhibit any antibacterial effects. At the infection site, polymorphonuclear neutrophils (PMN) active secrete proteolytic enzymes. These enzymes generate antibacterial peptides derived from ApoH that can be found in high concentrations at the infected sites. These peptides bind to released bacterial cell wall and induce bacterial lysis (Fig. 11A). Thus, ApoH contributes as a precursor to the human innate immunity.

However, it has been hypothesized that some bacteria, including *S. pyogenes*, may have developed defense mechanisms against the ApoH-derived peptides (Nilsson et al., 2008).

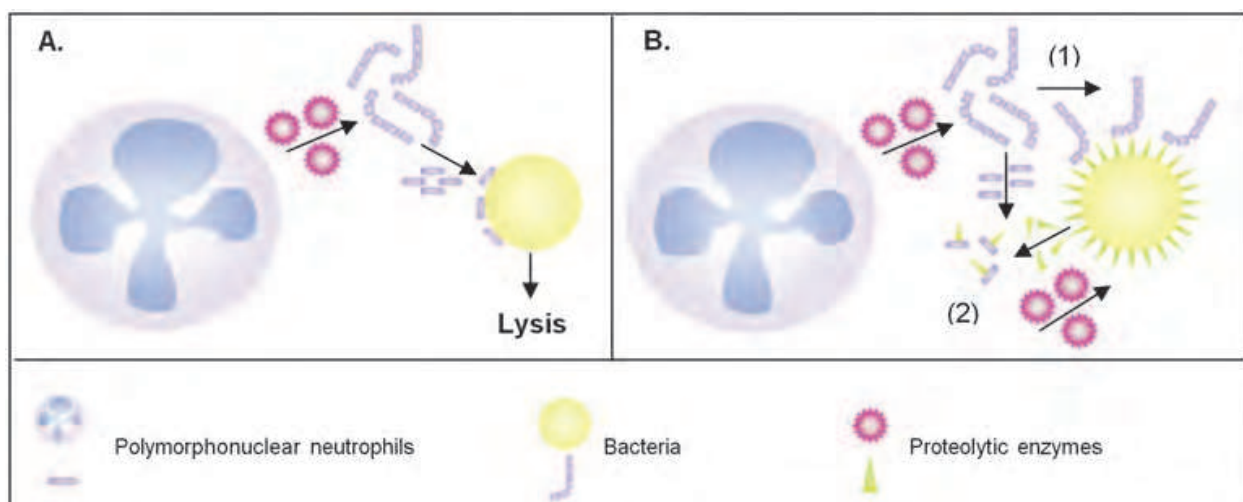


Fig. 11. Mechanisms used by *Streptococcus pyogenes* AP1 to neutralize the effect of anti-bacterial peptides derived from ApoH (Nilsson et al., 2008). (A) At the site of infection, activated neutrophils secrete proteolytic enzymes generating lytic antibacterial ApoH-derived peptides. (B) Mechanisms proposed to be used by AP1 bacteria to prevent the antibacterial activity of ApoH: (i) The binding of ApoH to *S. pyogenes* surface proteins M1 and H prevents the cleavage of the ApoH; (ii) soluble proteins M1 and H interact with ApoH-derived peptides to neutralize their anti-bacterial effects.

The first mechanism is the link between the bacterium and the ApoH. Once ApoH is bound to bacteria, proteinases from PMN cannot cleave ApoH and therefore the production of antibacterial ApoH-derived peptides is reduced (Fig. 11B1). The second mechanism circumventing host immune defenses is the binding of soluble proteins M1 and H with peptides derived from ApoH inducing the inactivation of these latter. The proteins M1 and H became soluble due to proteases from the PMN that cleaved proteins M and H of the bacterium surface (Fig. 11B2).

Thus ApoH, a precursor of antibacterial peptides, would be a mechanism of host protection against infectious agents. It is possible to speculate that certain bacteria are capable to neutralize ApoH *via* their binding mechanisms and thereby escaping the host immune responses.

#### *General structure of bacteria*

Bacteria belong to one independent branch of the tree main phyla of life (Woese, 1987). Bacteria have characteristics that are not found in other living prokaryotes (Archaea) or eukaryotes. These are bacterial surface structures, which have the most unique features, not shared with other living beings. Among these structures, the bacterial wall, the exopolysaccharides, protein structures as external S layer, pili, flagella. Most of these surface structures are involved in bacteria-host interactions through their adhesion molecules at their surfaces with host's cells. In addition, these interactions play a pivotal role in bacterial pathogenicity.

Despite their simple morphology and unicellular organization, bacteria present both large organic and structural diversities. This structural diversity is primarily expressed in surface structures. Structure and function properties of the bacteria envelope structures of are a direct consequence of their adaptive strategies.

#### *Bacterial cell wall*

Bacteria cells are particularly robust because of their cell wall, which allow them to withstand particularly high variations in osmotic pressure. The wall structure is a base for the determination of major bacterial groups mainly including: Gram-positive bacteria, Gram-negative and mycobacteria.

The common component of bacterial cell wall or murein is a peptidoglycane. Murein is a polymer of two sugar-derivatives (N-acetylglucosamin and N-acetylmuraminic) and several amino-acids and/or lipids that largely differ in a specie-depending manner.

#### *Gram-positive's cell wall*

The wall of Gram-positive bacteria is a homogeneous structure with thickness variation from 10 to 80 nm. It consists mostly of a thick layer of multimolecular murein associated with lesser amounts of other dispersed polymers, especially the teichoic and teichuronic acids).

There are a wide variety of these compounds being highly antigenic. The nature of their carbohydrate skeleton, and position of many substitutions are submitted to considerable variations.

Numerous other components are also present in some species, such as proteins or polysaccharides, and they can play an important role in the antigenic properties or pathogenicity. For example, protein A, found in over 98% of strains of *Staphylococcus aureus*, is a receptor for the Fc fragment of immunoglobulin G that when present on bacteria surface will inhibits opsonization. In addition, bacterial mutants lacking protein A present a lower virulence in mouse experimental infections.

### *Gram-negative's cell wall*

The wall of Gram-negative bacteria is more complex. We distinguish an inner membrane and outer membrane, which defines a periplasmic space containing a thin layer of peptidoglycan. The wall is much thinner than those of Gram-positive bacteria, of about 10 nm. Murein of gram-negative bacteria does not contain teichoic acid.

Periplasmic space contains the Braun lipoproteins, which connects the outer membrane to peptidoglycan involved in the cohesion of the wall.

The outer membrane has a distinct chemical composition. Its structure is a phospholipid bilayer with the inner layer has a composition similar to that of the cytoplasmic membrane. Its outer layer, by cons, has a particular constituent embodiment in the PL: lipopolysaccharide (LPS) is a complex molecule consisting of three components: (i) The lipid A, which anchors the LPS in the outer leaflet of the outer membrane. This lipid A is also called endotoxin because of these properties highly toxic for the host. Endotoxin is responsible for septic shock in systemic infections with Gram-negative bacteria; (ii) short series of sugar in the center including: desoxyoctonoic keto-acid and heptose; (iii) long carbohydrate chain whose length is 40 sugars that constitutes the O antigen, covering the surface of the bacterium highly antigenic.

The outer membrane also contains a large number of proteins such as porins involved in the permeability of small hydrophilic molecules (<600 Da) and proteins involved in bacterial virulence (<http://www.bacterio.cict.fr/bacdico/bacteriogene/structure.html>).

## **7.1 Bacteria and ApoH binding**

*In vitro* direct interaction has been shown between *S. aureus* and ApoH via the Sbi protein (Zhang et al., 1999). Furthermore, a study from a DNA library of *S. aureus* phages and on human blood proteins that adhere to implanted materials, such as catheters, showed the presence of ApoH on these biomaterials. This study attest to the connection on the *ex vivo* biomaterial ApoH *S. aureus* (Bjerketorp et al., 2004). More recently (Agar et al., 2011), it has been shown that upon interaction of LPS with domain V of ApoH, a conformational change occurs in ApoH binds LPS from the ring or close form to a "J or fishhook-like form". Thus, the 'active' fishhook-like conformation of ApoH in complex with LPS is then able to bind to the LPR receptor after which the ApoH-LPS complex is cleared (4). The scavenging of LPS by ApoH leads to a decreased binding of LPS to the TLR-4 receptor resulting in a decreased expression of the inflammatory markers TNF $\alpha$ , IL-6 and IL-8.

ApoH also interacts with *Streptococcus pyogenes*. At least two streptococcal proteins are involved in this interaction, M1 protein and H factor, which are surface proteins. M1 protein is a virulence factor, allowing the bacteria to resist phagocytosis. The M1 protein would interact with plasma proteins (fibrinogen, C4b binding protein, H factor) to avoid bacterial lysis (Nilsson et al., 2008).

## **7.2 The use of ApoH to drastic improvement of bacterial detection**

As different bacteria are able to interact with ApoH, we used these fundamental property to propose that ApoH can be a useful tool to capture, from different complex biological media, different kinds of bacteria (Gram- or Gram+) and proceed to their detection and/or diagnostic (Fig. 12) by using different methods including ATP detection by luminometry, cultivation in an appropriated media or PCR.

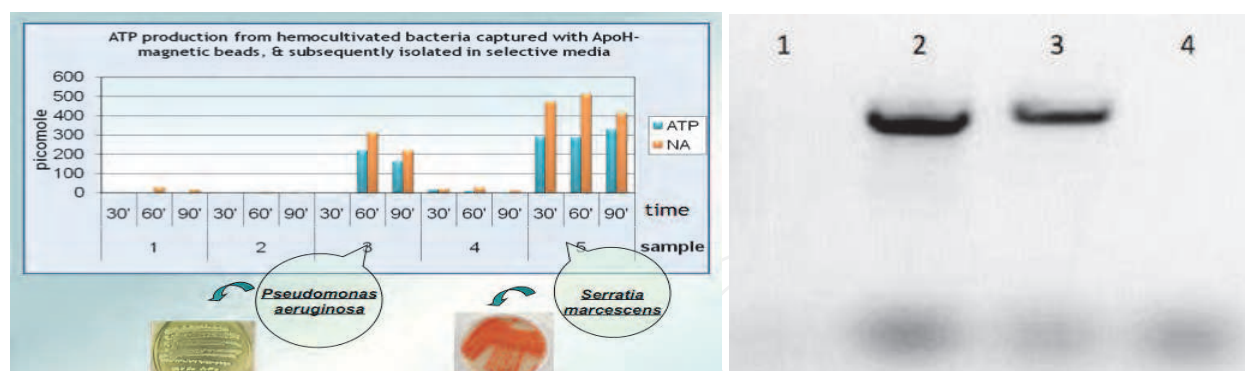


Fig. 12. Bacterial detection. (left) ATP detection and specific cultivation of *Pseudomonas aeruginosa* and *Serratia marcescens* from clinical hemocultivation; (right) 16S RNA PCR. Bacterial Luria broth (LB) Medium (1), ApoH-captured *Escherichia coli* from LB medium (2), positive PCR control, *E. coli* DNA (3), H<sub>2</sub>O (4)

## 8. Conclusion

Taking into account literature data and our own data, it is possible to mention that, probably one of the main roles for the acute phase protein ApoH is to be an important actor of host innate immune response through its capacity to bind with high affinity to a large panel of pathogens and or antigens from different origins. This ApoH property can be applied to face a tremendous public health problem that is the existence of (re)-emerging pathogens and their difficulties to be detected.

Indeed, according to the Center for Disease Control and Prevention in Atlanta (CDC), 70% of emerging infectious diseases in humans, are zoonotic pathogens. Most of these human diseases are caused by the introduction of existing pathogens into human populations from other species or dissemination from endemic areas into larger populations. This process depends upon a complex interaction of factors such as ecological, environmental and/or climatic changes, the basic biology of pathogens, host and cell tropism, the route of transmission, the natural host reservoir and the vector, social behavior, international travel and commerce, political instability, breakdown in public health measures, etc.

Nucleic acid amplification is widely used for the detection and identification of pathogens. Sequencing and comparison to known microorganisms may allow identification of unidentified pathogens. Extraction and careful purification of DNA and/or RNA is an essential step for sensitivity and reliability of these diagnostic techniques, but often incompatible within high throughput screening in routine diagnostics eg. in blood transfusion centres where large series of samples have to be tested. Several major diagnostic companies have been involved in the development of universal viral nucleic acid purification systems from plasma and serum, compatible with IVD (In Vitro Diagnostics) assays. However, despite a gain in sensitivity brought by these methods, the EQA studies revealed that molecular diagnostics still showed a poor overall test proficiency compared to serological diagnostics. One of the main problems for pathogen detection in clinical but also in environmental samples is that they generate false negative results. This problem is mainly due to 3 reasons:

- Presence of inhibitors such as heme, anticoagulants like EDTA and heparin, high concentrations of leukocyte DNA, immunoglobulins and other unknown inhibitors of nucleic acid amplification in the plasma or serum (Al-Soud et al., 2000; Al-Soud &



Radstrom, 2001; Wilson, 1997). Such an inhibition was described at a frequency of 0.34 to 2.1% of tests for patients infected with human immunodeficiency virus type 1 or hepatitis C virus, respectively (Drosten et al., 2001; Nolte et al., 2001). In the case of patients with severe viral hemorrhagic fever false-negative results are also likely to occur, especially in the acute phase of the disease where a rapid confirmation is required for the introduction of countermeasures (Drosten et al., 2002). In this scenario it is extremely important to develop methods for pathogen concentration allowing their separation from these reaction inhibitors. Such a technology is critical for the development of novel methodologies of sample preparation which would result in the reliable detection of pathogens present within complex biological samples such as blood.

- ii. Absence of a universal extraction method that efficiently lyses the pathogens and allow the separation of nucleic acids (RNA or DNA) from other components of the microorganisms or cellular materials that might interfere with downstream processes. Different nucleic acid extraction techniques have shown to result in variable diagnostic outcome for viruses detected by the polymerase chain reaction (PCR). The choice of appropriate RNA and DNA extraction methods, thus, is a critical step for the successful and valid use of RT-PCR and PCR based exams on clinical samples (Fredricks et al., 2005; Labayru et al., 2005; Scansen et al., 2005),
- iii. Lack of a rapid and reliable pathogen concentration methodology. Often pathogens present in clinical samples and particularly in environmental samples are diluted in large sample volumes poorly amenable for molecular methods (Brassard et al., 2005; Dreier et al., 2004; Fung et al., 2000); in this case, working with a small fraction of the sample may lead to false-negative results due to the specific sensitivity of the used detection method. Yet, simple and inexpensive methods for the concentration of pathogens, present within the samples, that could be easily introduced into the normal working scheme used by most clinical microbiology laboratories involved in the routine screening of large numbers of human samples are currently not at hand.

One of the main concerns for pathogen detection in clinical, but also in environmental samples, is that they generate false negative results that are mainly due to: (i) presence of inhibitors, (ii) absence of suitable molecular detection methods (e.g. PCR) and (iii) lack of a rapid and reliable pathogen concentration methodology.

The above-mentioned disadvantages have been compensated by the use of a matrix-bound ApoH to concentrate pathogens from biologically complex fluids (blood, feces, tissues, etc). After capture, washing of the ligand-pathogen(s) complex would allow removal of the inhibitors of the subsequent diagnostic steps. Thus, in this novel diagnostic schema the concentrated pathogen(s) would be highly accessible to any subsequent method of extraction, thus increasing the expected efficiency and sensitivity of nucleic acid detection.

Thus, ApoH-coated solid matrix is used as a generic capture method for a wide range of pathogens in order to concentrate them from large sample volumes of complex biological mixtures, otherwise poorly amenable for molecular methods and thus to improve their detection threshold (Fig. 13).

Tools and protocols have been established to use this simple, fast and broad target method adapted to different types of specimen, saliva, plasma, serum, urine and feces as well as various environmental samples. Thus, the pre-analytical sample processing by ApoH allows an efficient and ultrasensitive detection of very divers pathogens from their originated media using simple or sophisticated methods related to immunological (ELISA) or molecular (real-time RT-PCR) detection techniques, therefore drastically diminishing false negative diagnostics generated by classical standardized method.

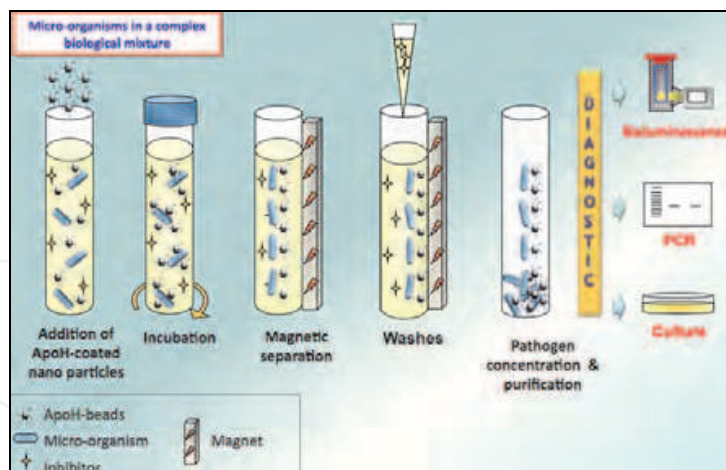


Fig. 13. A schematic representation of different steps to capture and concentrate microorganisms from complex biological fluids using ApoH-coated nano-magnetic beads to drastically improve their ultra-sensitive detection.

## 9. Acknowledgement

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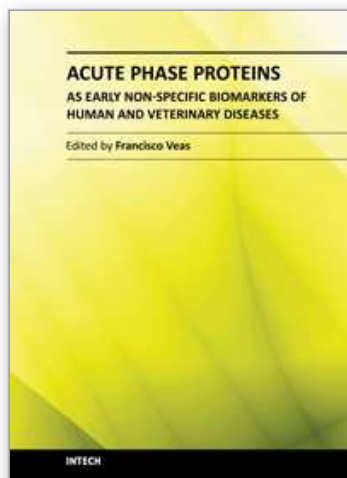
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## **Acute Phase Proteins as Early Non-Specific Biomarkers of Human and Veterinary Diseases**

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The two volumes of Acute Phase Proteins book consist of chapters that give a large panel of fundamental and applied knowledge on one of the major elements of the inflammatory process during the acute phase response, i.e., the acute phase proteins expression and functions that regulate homeostasis. We have organized this book in two volumes - the first volume, mainly containing chapters on structure, biology and functions of APP, the second volume discussing different uses of APP as diagnostic tools in human and veterinary medicine.

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