



**ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES**

**Assessment of avidity of IgG antibodies against meningococcal
serogroup A polysaccharide and its correlation with pre-existing data
on bactericidal activity in meningitis patients and controls from
Ethiopia.**

A Thesis Submitted to the School of Graduate Studies of Addis Ababa
University in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Applied Genetics

BY
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DECLARATION

I the undersigned, declare that this thesis is my original work and has not been presented for a degree at this or any other university and that all source materials used for the thesis have been duly acknowledged.

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LIST OF ABBREVIATIONS

AHRI- Armauer Hansen Research Institute

AI- avidity index

ANOVA - analysis of variance

CDC - The Centers for Disease Control and Prevention

CFU- colony forming unit

CI - confidence interval

ELISA - enzyme linked immunosorbent assay

FCS - fetal calf serum

GMAI - geometric mean avidity index

GMC- geometric mean concentrations

GMT - geometric mean titer

Hib - *Haemophilus influenza* type B

hSBA- serum bactericidal activity using human sera as a complement source

IgG - immunoglobulin G

Log - logarithm

LOS - lipooligosaccharide

menA - meningitis caused by serogroup A *N. meningitidis*

menAPS - serogroup A *N. meningitidis* capsular polysaccharide

MLST - multi-locus sequence typing

NIBSC - National Institute for Biological standards and Controls

NIPH - Norwegian Institute of Public health

OD - optical density

OMP - outer membrane protein

OMV - outer membrane vesicle

PBS - phosphate buffered saline

PCR - polymerase chain reaction

r - Pearson's correlation coefficient

rSBA- serum bactericidal activity using baby rabbit sera as a complement source

SBA- serum bactericidal activity

SNNPR- South Nations and Nationalities People Region

ST - sequence type

WHO - World Health Organization

ABSTRACT

Studies in the “meningitis belt” countries such as Burkina Faso, Gambia, Ethiopia and Sudan showed the presence of high antibody levels against serogroup A capsular polysaccharide (APS) of *Neisseria meningitidis*. The antibody concentrations usually exceeded the suggested protective antibody level ($\geq 2\mu\text{g/ml}$ of total Ig against APS). In spite of this, the populations in this area are affected by recurring epidemics of meningococcal meningitis. Therefore, to determine the level of anti-APS IgG that correlates with protection in the “meningitis belt” area, it may be appropriate to consider the quality of the antibodies along with quantity. Though quality of antibodies might be affected by several factors, this study considered the avidity of antibodies against APS, which is believed to better correlate with serum bactericidal activity (SBA). Two modified ELISA methods using the chaotropic agent ammonium thiocyanate (NH_4SCN) that disrupts weak antibody binding were employed to measure avidity indices (AI) of anti-APS IgG. In one of the methods a constant concentration of SCN^- and varying serum dilutions, and in the other, a single serum dilution with varying SCN^- concentrations were used. 114 acute and convalescent sera from Ethiopian meningitis patients and controls were analysed. High correlation was observed between the avidity indices calculated from the two methods ($r=0.8$). When assaying patient sera, anti-APS IgG concentrations correlated with SBA using baby rabbit sera as a complement source (rSBA) both before and after elution with 120mM SCN^- ($r=0.64$ and $r=0.65$, $p<0.0001$). Similar results were observed with SBA by employing human sera as a complement source (hSBA) ($r = 0.46$ and 0.41 $p<0.0001$). However, correlation between AI and SBA was only seen in the late convalescent sera. The anti-APS IgG rose in early convalescent but declined in late convalescent sera though the AI remained high in the late convalescent sera indicating affinity maturation. Age dependent increase in IgG concentration was seen in controls and late convalescent sera, and lower levels of avidity indices were seen in sera of the test groups aged 0.5-6 years. Further studies to standardize avidity measurement and other factors that might affect the quality of antibody responses (e.g. IgG subclasses) will be necessary to obtain a correct picture of protective immunity against serogroup A *N. meningitidis* infections in the “meningitis belt” of Africa.

Key words – Avidity index, Capsular polysaccharide A, ELISA, “Meningitis belt”, Serum bactericidal activity, *Neisseria meningitidis* A

1. INTRODUCTION

Under the family Neisseriaceae and genus *Neisseria*, the species *Neisseria meningitidis* is an obligate commensal of humans (Caugant and Maiden, 2009). However, certain strains of this species can cause a variety of diseases including septicemia, meningococcal pneumonia and mostly meningitis (inflammation of the meninges). *N. meningitidis* are gram-negative, encapsulated diplococci with their adjacent sides flattened, giving them a bean like shape. Size could vary from 0.6 to 1.0 μm in diameter. The bacteria's outer surface is triple layered, where the inner cytoplasmic membrane is surrounded by a peptidoglycan cell wall, which is enclosed by the outer membrane. The outer membrane is composed of lipids, lipooligosaccharides (LOS or endotoxin), outer membrane proteins (OMPs), and surface appendages, called pili or fimbriae (Poolman, *et al.*, 1982; Pollard and Levin, 2000). The most external layer is the polysaccharide capsule (Baron, 2000) (Figure 1).

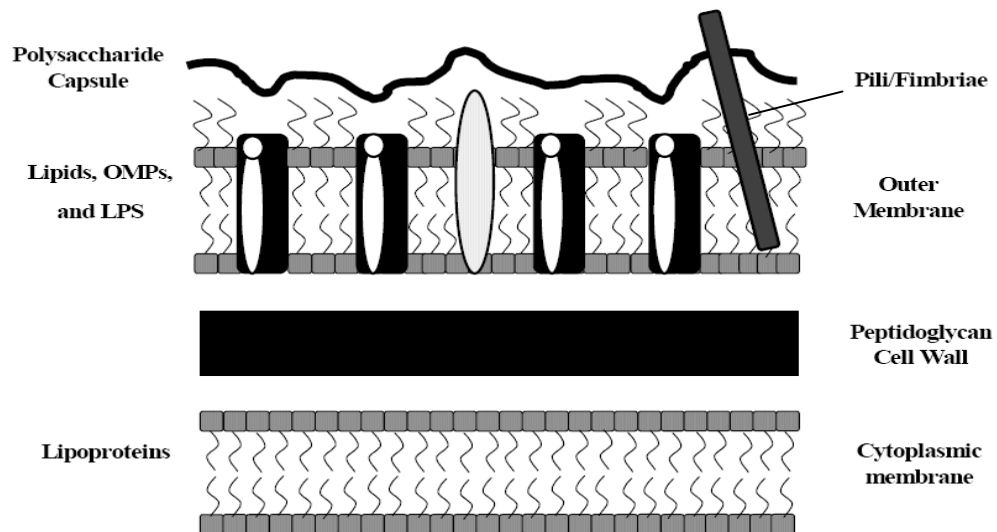


Figure 1. The membrane structure of *N. meningitidis*
(Source: Prinz, 2004)

Though meningococcal strains are diverse, majority of the cases are caused by a minority of antigenic types and genotypes. This asserts the importance of detailed and accurate isolate characterization of strains which is vital to handle cases efficiently and perform studies including meningococcal epidemiology, population biology and evolution (Maiden *et al.*, 1998).

The survival and proliferation of *N. meningitidis* in the host is by the virtue of several bacterial virulence factors (Table 1). The main virulence factor is the polysaccharide capsule that protects the organism from environmental insults and is anti-phagocytic. The biochemical composition of the capsule has been mainly used as a basis for classifying strains into serogroups. 13 serogroups A, B, C, D, H, I, K, L, X, Y, Z, 29E, and W135) have been identified. The polysaccharide capsule is an important virulence factor of the organism (Kroll and Moxon, 1990). It is known to have antiphagocytic nature and down regulates the alternative pathway of complement activation (Estabrook, *et al.*, 1992 and Finne *et al.*, 1983; Jarvis and Vedros, 1987).

Numerous surface antigens and OMPs have been described and shown to attribute to the virulence of the meningococcus. Many of the surface antigens are not conserved between serogroups and are highly variable. These proteins enable the organism to adhere to host cells, confer serum resistance, act as transporter proteins, and aid in pathogenesis of disease (Prinz, *et al.*, 1999; Vogel and Frosch, 1999).

Meningococci also demonstrate a high degree of “blebbing” that consist of surface antigens such as LOS, periplasmic proteins, OMPs and phospholipid (Devoe and Gilchrist 1973; Andersen *et al.*, 1979; Zhou, *et al.*, 1998). It has been suggested that blebbing may be caused by peptidoglycan turnover or cell autolysis (Pollard and Levin, 2000). Blebbing contributes to the pathogenicity of the meningococcus by drawing attention away from the organisms as the immune system targets the blebs.

Based on the OMPs of *N. meningitidis*, strains are further classified into serotypes and serosubtypes. Serotyping is based on variants of the PorB OMP and serosubtyping is based on variants of the PorA OMP (Frasch *et al.*, 1985, Wedege *et al.*, 1990).

The LOS is known to reduce C3 and C9 deposition and down regulates the alternative complement pathway activation (Jarvis and Vedros, 1987; Hammerschmidt, 1994; Esabrook, *et al.*, 1992; Griffiss, 1991; Mandrell, 1988). Another classification based on the antigenic diversity of meningococcal LOS is immunotyping. The LOS types (L1-L11) are independent of the protein serotypes (Mandrell and Zollinger, 1977).

Along with OMPs (Opa and Opc), the pili predominantly influence bacterial adhesion to host cells. Pili and Opa proteins also determine host and tissue specificity while Opa and Opc facilitate efficient cellular invasion by these pathogens (Stephens and McGee, 1981; Virji, 1996; Carbonnelle *et al.*, 2009).

Table 1. Some functions of major surface structures of *N. meningitidis*

Structure	Function	Vaccine potential
Polysaccharide capsule	Anti-phagocytic, reduced immunogenicity, down regulates alternative complement pathway activation, antigenic mimicry	Serogroup A, C, Y and W135 plain polysaccharide vaccines in routine use. Protein-polysaccharide conjugate C vaccine introduced in the UK in 1999. Conjugate A vaccine is safe and immunogenic. Conjugate Y and W135 vaccines under development
Lipopolysaccharide (LPS)	Antigenic mimicry, Reduces C3 and C9 deposition and downregulates alternative complement pathway activation	Induces antibody response following immunization. Detoxified LPS under consideration as a vaccine antigen
Pili	Adhesion	Anti-pilin antibodies might block adherence and could reduce colonization
PorA (class 1)	Cation porin	Major component of Dutch and Norwegian OMV vaccines. Immunogenic but shows some antigenic heterogeneity
PorB (class 2:3)	Anion porin	Immunogenic protein, with vaccine potential but shows some antigenic heterogeneity

Class 4, reduction modifiable protein (Rmp)	Unknown	May induce blocking antibodies making it undesirable as a vaccine component
Opa (class 5)	Adhesion, invasion	Antigenic variability, but antibodies raised have the potential to block
Opc (class 5c)	Adhesion or invasion	Included in Norwegian OMV vaccine. Induces bactericidal antibody following vaccination
Transferrin binding protein 1 (Tbp1)	Transferrin binding	Immunogenic and shows limited antigenic heterogeneity
Transferrin binding protein 2 (Tbp2)	Transferrin binding	Immunogenic and shows limited antigenic heterogeneity
Ferric enterobactin A (FetA)	Ferric-enterobactin receptor	Potential vaccine component. Antigenic heterogeneity
Ferric binding protein (FbpA)	Iron-binding	Potential vaccine component
Neisserial surface protein A (NspA)	Unknown	Immunogenic and protective in animal

(Source: Pollard and Frasch, 2001)

Different methods of strain typing have been developed but each has its own advantage and disadvantage. One of the major drawbacks of some of the commonly used serotyping methods is poor discrimination and reproducibility within and between laboratories. And the lack of accurately identifying infectious agents could have negative impact on epidemiological surveillances and public health decisions (Maiden *et al.*, 1998).

Molecular typing techniques are advantageous over the serology based classifications. The methods include polymerase chain reaction (PCR) with repetitive element or arbitrary primers, pulse-field gel electrophoresis (PFGE), multi-locus enzyme electrophoresis (MLEE) and multi-locus sequence typing (MLST), which is being widely used. MLST is a molecular characterization method that indexes the variation present in nucleotide sequencing of 400-500 base pair internal fragments from housekeeping genes. Seven loci are used to identify meningococcal clonal complexes. MLST is nowadays the

method most used for characterization of bacterial pathogens for global surveillance in that it could generate reproducibility, reliability, cost effectiveness and throughput (Maiden *et al.*, 1998).

1.1 Global epidemiology

According to WHO's weekly epidemiological record of 2010, meningococcal meningitis is a significant global health problem causing 1.2 million cases and 350,000 deaths per year. It is still the leading cause of bacterial meningitis and septicemia in children under the age of 2 yrs, with a secondary peak incidence in adolescent years (Moore 1992; Kaczmarek 1997; Rosenstein *et al.*, 1999). The incidence of meningococcal disease varies from 1-3 cases per 100,000 individuals in developed countries to 10-25 cases per 100,000 in developing countries (Schlech *et al.*, 1985; Harrison and Broome 1987; Wenger *et al.*, 1990).

As described by Harrison *et al.*, (2009), *N. meningitidis* has a fluid epidemiology, characterized by substantial cyclical fluctuations of meningococcal disease incidence and the occurrence of outbreaks and epidemics. Furthermore, meningococcal incidence and serogroup distribution are highly regional (Figure 2).

Although 13 serogroups have been described, serogroups A, B, C, Y and W-135 account for more than 90% of all cases of meningococcal disease (Harrison, 2006). The majority of epidemics are the result of serogroup A, whereas sporadic disease is usually caused by serogroups B, C, Y and W-135 (Peltola, 1998). Epidemic can be defined as $\geq 100/100,000$ individuals in a 1-year period (Riedo *et al.*, 1995).

Serogroups B, C, Y and W-135 commonly cause sporadic meningococcal disease in developed countries. Serogroup B mainly occurs in Europe, South America, and in the United States, accounting for approximately 46% of the cases in these countries (Riedo, *et al.*, 1995; Lepow, *et al.*, 1999). Serogroup C causes endemic meningococcal disease in the United States and sporadic disease in Canada, Australia, and Europe (Pearce *et al.*, 1995; De Wals and Erickson 2002; Healy, *et al.*, 2002). This serogroup accounts for 45%

of cases seen annually, with one-third occurring in the United States alone (Racoosin *et al.*, 1999; Stephens, 1999). Lastly, serogroups Y and W-135 account for the remaining cases of sporadic disease in the United States and Europe (approximately 5%) (Rosenstein *et al.*, 1999; Aguilera *et al.*, 2002)

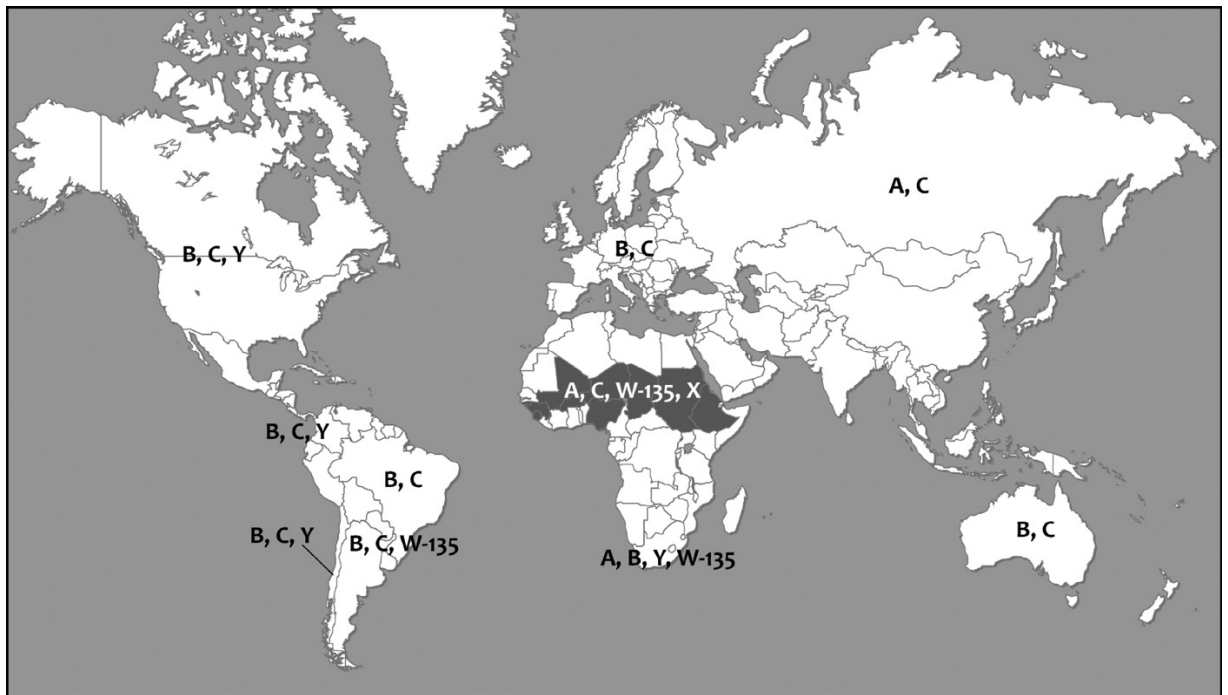


Figure 2. Worldwide distribution of major meningococcal serogroups. The darker region that extends from Senegal in the west to Ethiopia in the east is known as the “meningitis belt” (Source: Harrison *et al.*, 2009)

1.2 Serogroup A *Neisseria meningitidis* in Sub-Saharan Africa

N. meningitidis is responsible for recurrent bacterial meningitis epidemics in Sub-Saharan Africa known as the “meningitis belt” (Lapeyssonnie, 1963). The “meningitis belt” stretches from Senegal in the west to Ethiopia in the east (Figure 2), including 21 countries and a population of approximately 400 million people. This area is characterized by hyperendemicity, seasonal epidemics which usually start in the dry season (December-June) and end in the rainy season, and occur in 8-12 year cycles (Greenwood *et al.*, 1987). Annual incidence during epidemics can reach 10% and

between the years 2003 and 2009, meningitis epidemics have resulted in approximately 270,000 cases and 25,000 deaths in the “meningitis belt” alone (WHO, 2010).

Colomboni *et al.*, (2008) described epidemic meningitis in sub Saharan countries as one cause of “societal chaos and an important precipitating cause of poverty in affected families”. And the available data of over the past four decades show the importance of serogroup A *N. meningitidis* as the dominant etiologic agent (LaForce *et al.*, 2009; WHO, 2010).

Serological studies confirmed the importance of serogroup A *N. meningitidis* as the major cause of epidemics in the “meningitis belt”. Records in three countries of the “meningitis belt” (Burkina Faso, Mali and Niger) have clearly shown the dominance of this serogroup in causing meningitis epidemics (LaForce *et al.*, 2009) (Table 2).

Multilocus enzyme electrophoresis (MLEE), a genetic method for strain characterization, has shown three clonal complexes of serogroup A to be the major causes of bacterial meningitis in the past 40 years (Achtman, 1991). A more recent molecular technique, MLST, also confirmed the above result (Caugant and Nicolas, 2008).

Table 2. Percent distribution of *N. meningitidis* groups in districts reaching epidemic status (>10 cases/100,000/week) in the “meningitis belt”: 2003–2007.

Year	2003	2004	2005	2006	2007	Total
No. of epidemic districts	44	22	19	74	96	257
<u>Percent distribution by <i>N. meningitidis</i> group</u>						
Group A	70.5%	95.5%	89.5%	85.1%	95.8%	87.8%
Group W135	29.5%	4.5%	10.5%	9.5%	3.1%	10.2%
Group X	0	0	0	4.1%	1.0%	1.6%
Group X&A	0	0	0	1.4%	0	0.4

Source: LaForce *et al.*, 2009

Ethiopia has been affected by both endemic and epidemic forms of meningococcal meningitis for over a century (Norheim *et al.*, 2006). Outbreaks and epidemics have been reported in 1935, the 1940s, the 1950s, 1964, 1976 to 1977, 1981 to 1983, and 1988 to 1989 (Habte-Gabr *et al.*, 1984; Haimanot *et al.*, 1990). Prior to 1988, the majority of epidemic cases occurred in the north, the northwest, and parts of the central regions of Ethiopia, which lie within the eastern end of the meningitis belt (Lapeyssonnie, 1963). After the devastating epidemic of 1988 to 1989, however, this pattern changed, and the whole country has been affected by outbreaks (Tedla, 1993), although increased awareness could also contribute to this observation. Epidemics were also reported in Ethiopia in the years 2001 to 2003. While the epidemics in 1981 and 1988 to 1989 struck with the magnitude of 40,000 to 50,000 cases these recent epidemics were much smaller; most cases occurred in the Amhara region and the Southern Nations, Nationalities, and Peoples' Region (SNNPR), respectively (Haimanot *et al.*, 1990, Tedla, 1993). The strain ST-5 of the serogroup A was dominant and is replaced by the ST-7 of the serogroup A between 1995 and 2000 (Norheim *et al.*, 2006).

1.3 Pathogenesis of Meningococcal Disease

Neisseria meningitidis inhabits the upper respiratory tract in humans. Meningococci are spread by intimate contact with the oral secretions or respiratory droplets of an infected individual (Peltola, 1983). *N. meningitidis* can cause meningitis, meningococcemia, and septicemia, but more commonly results in asymptomatic nasopharyngeal carriage. Approximately, 10% of adults and up to 30% of adolescents carry the meningococcus in their nasopharynx at any one time (Riedo, *et al.*, 1995; Morley and Pollard 2001).

There are different stages involved in the establishment of meningococcal infection (van Deuren, *et al.*, 2000). First, those meningococci expressing type IV pili, attach to specific receptor sites such as CD46 and other proteins expressed on epithelial cells in the oropharynx. The meningococci start to multiply locally and influence the physical appearance of the epithelium cells during the first 24 hours (Johansson, *et al.*, 2003, Stephens and Farley, 1991, Read and Goodwin, 2001).

After proliferation, the meningococci initiate a parasite-directed endocytosis, passing through the epithelial cells. In adenoid tissue covered with intact epithelium, meningococci are gradually engulfed by the epithelial cells. The sparsely ciliated epithelium which overlies the tonsils and the adenoids may facilitate adherence of meningococci (Stephens and Farley, 1991, Read and Goodwin, 2001).

After adherence, adaptation and initial proliferation, the meningococci gradually move through the cytoplasm to the basal membrane. Virulent meningococci expressing capsular polysaccharide are carried in vacuoles, whereas uncapsulated variants are transported through the cytoplasm without vacuoles. The attachment of meningococci to epithelial cells induces specific structural changes as a consequence of altered gene expression in the cells. In a way not clearly understood, the meningococci pass through the basal membrane then divide within macrophages in submucosa. How they gain access to the circulation is not exactly known but meningococci located in the extracellular space may penetrate between or through capillary endothelial cells into the lumen. It is speculated that neutrophils and monocytes may carry them as into the bloodstream without killing them (Stephens and Farley, 1991, Read and Goodwin, 2001, Plant *et al.*, 2004)

IgA1 protease, which is secreted by pathogenic meningococci may neutralize secretory IgA on the mucosal surface and thereby thwart a first-line defense mechanism, facilitating the attachment of meningococci to the mucosal epithelial cells. Although high levels of IgA1 protease may induce the production of proinflammatory cytokines *in vitro*, the cytokine response in patients also appears primarily to be triggered by LPS or endotoxin (Parsons *et al.*, 2004).

A prerequisite for causing clinical disease is the ability of *N. meningitidis* to resist the bactericidal capacity of the blood caused by the combined action of antibodies and complement and to circumvent rapid phagocytosis by stationary and circulating phagocytes. Subsequently, meningococci are spread in specific areas of the body,

primarily the skin and the meninges. The early symptoms of meningococemia, including general malaise, fever and sometimes muscle ache are recognizable as the number of multiplying bacteria in the blood surpasses a certain threshold (Dinarello, 2004).

Diagnosis of meningococcal infection is determined by blood and cerebrospinal fluid cultures and specimens of skin lesions and other infected areas (Lepow, *et al.* 1999). The first clinical classification of meningococcal disease is “bacteremia without sepsis” which is defined as the presence of bacteria in the blood regardless of clinical symptoms. The second classification is “meningococemia without sepsis” which is associated with leukocytosis, headache, muscle aches, rash, and seizures that occur in approximately 20% of infected individuals (Lepow, *et al.* 1999; van Deuren, *et al.* 2000). The third classification of meningococcal disease is “meningitis with or without meningococemia.” Meningeal signs in this classification are associated with <100 white blood cells/mm³ of cerebrospinal fluid. The infected individual presents with headache, fever, with either a full alert or depressed mental status. The last classification of meningococcal infection is “meningoencephalitic manifestation” defined as ≥ 100 white blood cells/mm³ of cerebrospinal fluid, with an increased concentration of endotoxin and cytokines in the plasma (van Deuren, *et al.* 2000). This is the most severe form of disease resulting in shock, multiple organ failure, skin and limb necrosis and cerebral edema. Other symptomatic problems as a result to meningococcal infection include myocarditis, endocarditis, pericarditis, and purulent arthritis (Mason, *et al.* 1979). Although there are 4 outlined clinical classifications of meningococcal disease, individuals who are infected with the bacteria present with various manifestations that may not fall into one specific category.

The progression from nasopharyngeal carriage to invasive disease remains unclear but is influenced by a variety of factors associated with both the host and the infecting bacteria. These predisposing factors include rate of bacterial propagation, climatological and social conditions, and the immune status of the infected individual. Influences by climatic conditions have been documented in the Sudan where the incidence of disease varies seasonally (Peltola, 1983). Overcrowding and poor living conditions are predisposing

conditions to infection. This has been shown in the crowded living conditions associated with military camps and more recently with an increase of incidence in university dormitory settings (Peltola, 1983; Neal *et al.*, 1999; Brundage, *et al.*, 2002; Williams *et al.*, 2003). Close contact in living conditions and households with an infected individual increase the risk of colonization. Smoking is another risk factor associated with meningococcal disease due to the physical damage sustained to the nasopharyngeal mucosa resulting in increased risk of carriage (Stanwell-Smith *et al.*, 1994). Current infections, such as acute respiratory disease or those caused by immunosuppressive viruses, alter the integrity of the mucosal surface and increase the risk of disease by transiently suppressing the immune system (Haneberg *et al.*, 1983; Rouse and Horohov, 1986; Cartwright *et al.*, 1991).

Immunodeficient individuals, such as asplenic persons or those with complement or IgM/IgG2 deficiencies, have a greater risk of succumbing to invasive meningococcal disease (Francke and Neu 1981; Bass *et al.*, 1983; Ross and Densen 1984; Hobbs 1986). Additional risk factors also include chronic alcoholism, low socioeconomic status, and an excess of anti-polysaccharide IgA antibodies that may inhibit the binding of the bacteria with anti-polysaccharide IgM or IgG antibodies (Hedrich 1952; Wenzel *et al.*, 1973; Griffiss 1975, 1982; Salmi *et al.*, 1976).

1.4 Immunity against *N. meningitidis*

As reviewed by Pollard and Frasch (2001), early scientists such as Matsunami and Kolmer have in general shown the importance of blood borne immunologic factors for immunity to meningococci. They also presented the first evidences of age dependent immunity. Heist then showed the existence of variation in immune response between individuals as he noticed that some individuals, including young children were more susceptible than others. Sadly, he died of meningitis before his important work was published.

Later, the classic studies of Goldschneider *et al.*, (1969, 1972, and 1973) and Gotschlich *et al.*, (1969) gave improved insight into the immune response against this disease. They scrutinized the relationship between functional anti-meningococcal antibodies and level of susceptibility to infection. Their subsequent studies also showed the importance of complement-dependent bactericidal activity of sera as a major host defence mechanism against this disease.

The importance of serum bactericidal activity can be seen in the rarity of this disease in newborns. Most newborns have anti-meningococcal antibodies transferred to them through placenta from their mother. But after six months, their immunity is likely to wane. This time is when young children are at high risk of infection. These were more elaborated by Shahid *et al.*, (2002) as they showed immunized pregnant women gave birth to babies with higher antibody levels. However, as people get older, they start to develop bactericidal antibodies either due to cross reaction or carriage (Goldschneider *et al.*, 1969) (Figure 3).

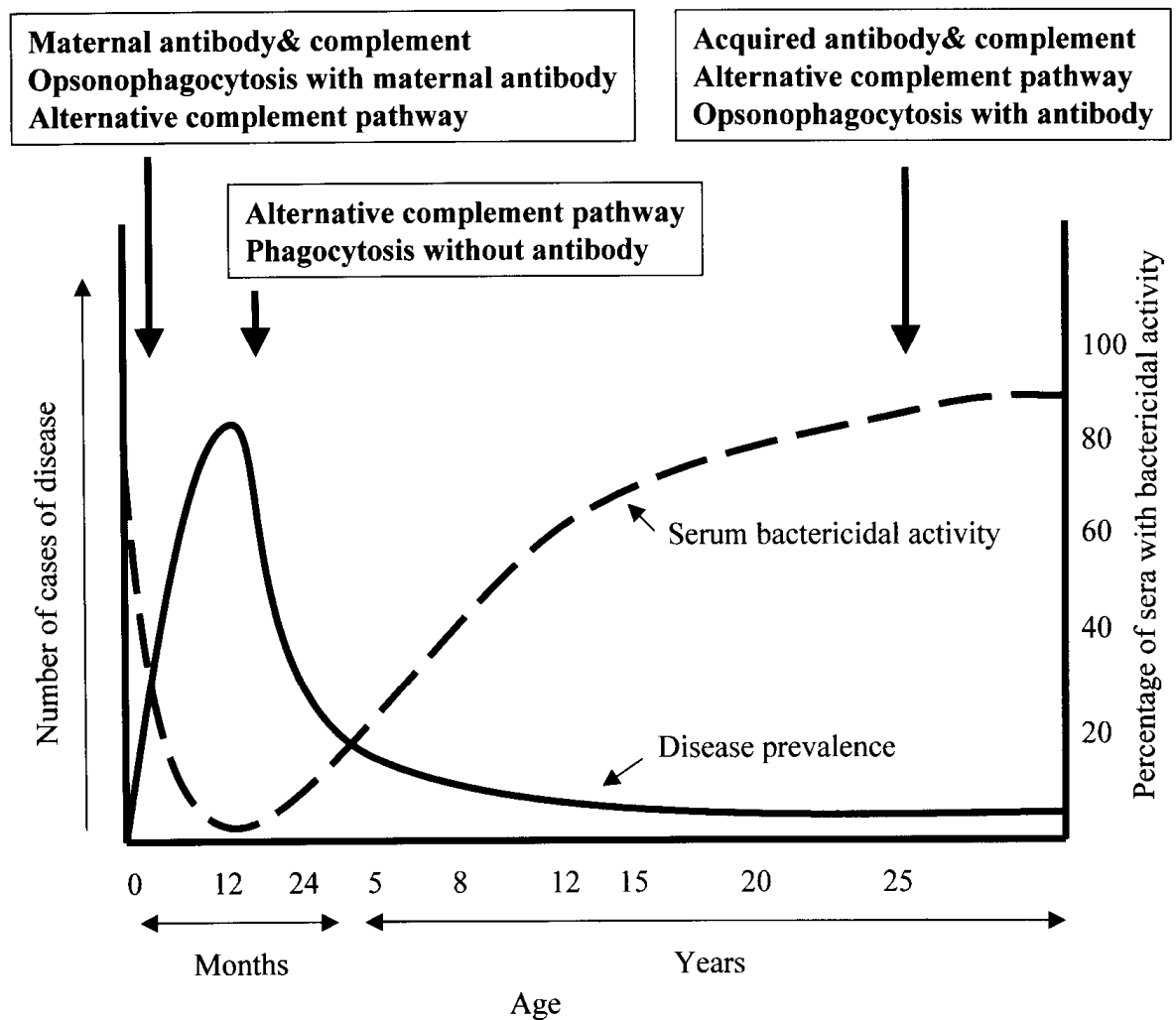


Figure 3. The age dependent prevalence of meningococcal disease in relation to population immunity measured by serum bactericidal activity
(Source: Pollard and Frasch, 2001)

Studies in immunodeficient individuals have unraveled the importance of antibody in playing the central roles of body's defence mechanisms against these pathogens. Salit, (1981) showed the importance of antibody in immunity against meningococci as individuals with hypogammaglobulinemia* have a greatly increased risk of invasive meningococcal infection.

*Deficient levels of IgG, IgM, and IgA serum immunoglobulins that may be attributable to either decreased synthesis or increased loss. Hypogammaglobulinemia can be physiologic in neonates.

Anti-meningococcal specific IgA antibody is produced following infection and may block complement-mediated lysis of meningococci by IgG or IgM and may increase susceptibility to meningococcal disease. The blocking IgA is probably directed against the meningococcal capsule and may arise as a result of enteric exposure to other bacteria expressing cross-reacting antigens. Elevated levels of specific IgA are associated with epidemic meningococcal disease (Griffis 1975, 1982).

Besides early *in vitro* studies (Goldschnieder *et al.*, 1969) that confirmed the importance of complement for bactericidal activity of antibodies directed against meningococci, studies in individuals with certain complement deficiencies have clearly shown the central role played by complement in defence against these bacteria. For instance the study of Nicholson and Lepow (1979) on human sera that are deficient in complement component 8 (C8-D) has shown the importance of terminal components of complement for extraphagocytic bactericidal activity of host's sera in defence against pathogenic *N. meningitidis*.

The importance of complement in fighting off these bacteria is summarized by Prinz, (2004) as (1) lysis by anti-meningococcal antibodies followed by activation of the classical complement pathway, (2) lysis and activation of complement via the alternative complement pathway, (3) opsonization by complement or phagocytosis by other opsonins such as mannose binding lectin via its receptor, and (4) opsonization by anti-meningococcal antibodies and/or complement followed by phagocytosis of the bacteria.

1.5 Importance of antibody avidity for immunity against *N. meningitidis*

Both concentration and affinity are important determining factors of antibody titer elicited against certain antigen. Affinity is defined as the strength of interaction between a monovalent antigen and a single antibody binding region (Harris *et al.*, 2007). It can be explained as the equilibrium association constant K , defined by the law of mass action and calculated by $K = [AgAb] / [Ag][Ab]$ where $[AgAb]$ represents concentration of antibody-antigen complex, $[Ag]$ represents concentration of free antigen and, $[Ab]$ the concentration of free antibody.

Exact measurement of affinity requires ideal conditions like homogenous solution with pure antibodies and antigens. However, in studying heterogeneous antibody population such as in the human sera, estimating affinity is difficult. Thus, it is important to consider an overall affinity measurement, antibody avidity, which has also been termed as 'functional affinity' by Karush (1978) (Goldblatt, 2001).

Antibody avidity can be defined as the total strength of the multivalent interactions between antibody and antigen. It has previously been shown (Schlesinger and Granoff, 1992; Vermont *et al.*, 2002) that antibodies with higher avidity are more active in important immune responses such as complement activation and opsonisation. The measurement of antibody avidity indices was also used as surrogate marker for the development of immunological memory and has been used to study conjugate vaccines (Antilla, *et al.*, 1998; Goldblatt and Miller, 1998; Joseph *et al.*, 2001; Richmond *et al.*, 1999a,b and 2001).

Modified ELISAs for measuring avidity of antibodies have been described by several researchers. One of them is a competition inhibition assay, where increasing concentrations of free antigens are used to competitively inhibit the binding of antibodies of decreasing affinity (Anderson, 1970). The other is elution assay. The principle is that elution of low avidity antibodies from antigens may take place at a lower concentration of chaotrope or denaturant when compared to antibodies of high avidity. The drawback of such solid phase ELISAs is that the analysis is done on antigen- antibody complexes that have been bound to plates. This might alter the antigen conformation, which in turn might

affect binding of antibodies to the antigens. However, these methods have been very useful as they permit to correctly rank avidity of antibodies against a particular antigen (Goldblatt, 2001).

Among the commonly used elution methods is the use of chaotropic salts such as ammonium or sodium thiocyanate (Macdonald *et al.*, 1988; Anttila *et al.*, 1999; Goldblatt *et al.*, 2002; Borrow *et al.*, 2003). The use of these salts is based on their ability to dissociate antibody-antigen complexes of low avidity while complexes of high avidity remain intact. Goldblatt *et al.*, (2001) explain that chaotropic ions dissociate antibody-antigen complexes through breaking hydrophobic bonds and through electrostatic shielding of charged groups which decreases the strength of ionic binding. Anttila *et al.*, (1998) also refer to this method as the simplest.

The mechanism of action of the chaotropic ions on macromolecules generally, and upon antigen-antibody complexes in particular, probably includes several types of effects. (1) The addition of ions may be expected to exert effects by electrostatic shielding, thus weakening the interaction between charged groups. (2) There is a classical salting-out effect at the high ionic strengths employed, which should tend to promote folding-up of the protein molecule and to decrease its solubility (Dandliker *et al.*, 1967).

1.6 Vaccines against *N. meningitidis*

Since the introduction of meningococcal serogroup A and C capsular polysaccharide vaccines by Gotschlich *et al.*, (1969), such vaccines are still used in response to epidemics in the “meningitis belt”. The capsular polysaccharide vaccines have proven to be effective in adults but are poorly immunogenic in children less than 2 years of age due to the T cell independent nature of the capsular polysaccharides (Gold *et al.*, 1979). On the other hand, the serogroup A polysaccharide has been shown to elicit anti-APS antibodies that are persistent in children as young as 3-9 months of age. The levels of anti-APS antibodies are usually short-lived though, and are not comparable to adult antibody levels until 4-5 years of age (Kayhty, *et al.*, 1980; Reingold, *et al.*, 1985).

Later attempts of conjugating the meningococcal polysaccharide to proteins helped overcome the T independence (Jennings and Lugowski, 1981). Several studies with meningococcal conjugate vaccines in infants, children and adults have demonstrated that the vaccines are safe, well tolerated, and immunogenic (Anderson, *et al.*, 1994; Richmond, *et al.*, 1999b; Campbell, *et al.*, 2002). It has been described that despite low titers of antibodies, the conjugate will provide long term protection via immunologic memory as shown for the Hib conjugate. Immunization studies with meningococcal conjugate vaccines in adults has been limited, but current studies have demonstrated a slight increase in levels of anti-polysaccharide antibodies induced by the conjugate, but not significantly different from those induced by the plain polysaccharide vaccine. Conjugated polysaccharide vaccines were shown to have higher avidity antibodies than the plain polysaccharide vaccines (Joseph *et al.*, 2001). Though polyvalent conjugate vaccines are very expensive for developing countries, a cheaper monovalent men A conjugate vaccine is developed with funding from Bill and Melinda Gates foundation (Jodar *et al.*, 2003). The vaccine has been shown to be safe and to generate a sustained immunologic response with functional antibody 20 times higher than that seen with polysaccharide vaccine in a phase II clinical trials (LaForce *et al.*, 2009).

Alternative meningococcal vaccine candidates consist of non-capsular surface antigens such as serogroup/serotype specific epitopes on outer membrane proteins (OMPs). These vaccine candidates are targeted predominantly for serogroup B meningococci as the capsule of this serogroup is poorly immunogenic (Rosenqvist *et al.*, 1995, Holst *et al.*, 2009). This is due to the fact that the capsular polysaccharide of serogroup B structurally mimics polysialated glycoproteins that are expressed in various human tissues, especially in the fetal brain on the neural cell adhesion molecule. Therefore, efforts to develop effective vaccines against serogroup B *N. meningitidis* focus on sub-capsular components such as OMPs, lipopolysaccharides or OMVs (Finne, *et al.*, 1983; Holst, 2007, Holst *et al.*, 2009).

1. RATIONALE OF THE STUDY

Meningococcal meningitis is a significant global health challenge, especially for sub-Saharan countries (Rosenstein *et al.*, 2001). In the “meningitis belt”, serogroup A *N. meningitidis* is responsible for the large number of epidemics that have been recorded (WHO, 2001). Occasional outbreaks of other serogroups (e.g W135 and X) have also been reported (Traore *et al.*, 2006; Boiser *et al.*, 2007). In this part of Africa, the previously common ST5 strain of serogroup A *N. meningitidis* have been replaced by ST7 between 1995 and 2000 (Nicolas *et al.*, 2005; Norheim *et al.*, 2006). As part of the “meningitis belt”, Ethiopia also suffers from recurring epidemics of this disease.

Antibody responses in patients with meningococcal disease caused by serogroup A, ST7 strains from the epidemics of 2002 and 2003 in Ethiopia, was the subject of study by Norheim *et al.*, (2007). The results demonstrated high proportion of both acute patient and control sera with putatively protective titers in serum bactericidal activity (SBA) assays. It was also found that the disease induces high amount of IgG in nearly all of the patients. In general, this work confirmed the presence of significant background immunity against serogroup A *N. meningitidis* in the Ethiopian populations.

Other studies in the “meningitis belt” countries such as Sudan (Amir *et al.*, 2005), Burkina Faso (Mueller *et al.*, 2006) and Gambia (Greenwood *et al.*, 1987) also showed that these populations have higher antibody levels than the antibody level which is assumed to confer protection ($\geq 2\mu\text{g/ml}$ of total Ig against APS from studies in Finland (Makela *et al.*, 1975; Peltola *et al.*, 1977)).

However, the populations in the “meningitis belt” are affected by recurring epidemics of this disease. Therefore, in order to estimate the level of anti-APS antibodies correlating with protection for this area, it may be important to consider quality of the antibodies along with quantity (Norheim *et al.*, 2007). The quality of antibodies might be affected by different factors including Ig isotype, IgG subclass and avidity. This study undertook determination of avidity of anti- APS antibodies, since avidity is assumed to correlate with SBA.

Another reason for choosing avidity study is its potential use as an indicator of immunological memory. Avidity ELISA has been very useful in assessing vaccines efficacy and memory response (Joseph *et al.*, 2001; Richmond *et al.*, 1999 and 2001). Vaccines efficacy trials are time consuming and very expensive, thus laboratory immunogenicity studies that assess vaccines' efficacy can provide additional immunogenicity information. Thus, avidity measurement could be handy in evaluating the existing plain polysaccharide vaccines and the new conjugate vaccine which is hoped to reduce the burden of meningococcal meningitis in the "meningitis belt" (Jodar *et al.*, 2003). Therefore, standardizing such serological assays to obtain comparable results in different laboratories is crucial to infer useful information from laboratory data which in turn could be very useful for public health recommendations.

Complement-mediated bactericidal antibody has been considered as surrogate of protection against meningococcal meningitis and was used to assess efficacies of plain meningococcal A polysaccharide vaccines and serogroup C conjugate vaccines. In the 1999 WHO's report, several points were raised and discussed as the relationship between the antibody titres measured by these assays and protection against disease showed some disagreement. Limitations of the existing assays were described and recommendations were put forward. Finally the importance of more experiments was asserted in order to validate serological assays for serogroup A/C *N. meningitidis* vaccines. Though the report is now more than ten years old, only few studies (Joseph *et al.*, 2001; Norheim *et al.*, 2007; Bårnes *et al.*, personal communication) has been done to evaluate the correlation between protection and immune responses against APS. This paper is the first to assess the avidity of IgG antibodies elicited against APS in meningitis patients caused by serogroup A *N. meningitidis* to the best of the authors' knowledge.

In general, this study addresses the avidity of IgG antibodies against APS; a vaccine relevant antigen. This will hopefully provide improved insight into the quality of immune responses after meningococcal disease. This may also provide some explanation to the apparent lack of protection in populations affected by recurring epidemics. Overall, the results may also provide additional information for development of improved vaccine that is more effective.

2. HYPOTHESIS

The avidity of total Anti-APS IgG antibody correlates with SBA titers and increases with time in convalescing meningitis patients.

3. OBJECTIVES

4.1 General objective

To assess the avidity of total IgG antibody APS in Ethiopian sera and study its correlation with SBA.

4.2 Specific objectives

- * Characterize the avidity of total IgG antibody against the serogroup A polysaccharide, APS, in Ethiopian patients following serogroup A meningococcal disease.
- * Estimate the correlation of anti-APS IgG with SBA in a selection of sera from Ethiopian serogroup A meningococcal disease patients and controls.
- * Determine if avidity increases with time after infection.
- * Compare two methods of avidity index calculation.

4. MATERIALS AND METHOD

5.1 Serum samples

Blood samples have been collected from meningitis patients and controls in the years 2002 and 2003, when the last major meningitis epidemics occurred in Ethiopia. The samples were collected from Sidama and Gedeo Zones in the Southern Nations Nationality Peoples Region (SNNPR) and in the Northern Gondar Zone in the Amhara region of Ethiopia. Molecular and serological analysis of part of the sera was done at Norwegian Institute of Public health (NIPH), Norway and the results were published (Norheim *et al.*, 2006, 2007 and 2008). Further analysis of the aliquots of the sera, kept at Armauer Hansen Research Institute (AHRI), was done at NIPH in collaboration with AHRI.

The inclusion criteria were clinical diagnosis of meningitis, presentation of a turbid cerebrospinal fluid, and patients being of age ≥ 6 months. Patient sera included in the study were confirmed to have serogroup A *N. meningitidis* using the standard strain identification methods (Norheim *et al.*, 2007). Blood samples were obtained in the acute phase (0 to 7 days after the onset of disease), the early convalescent phase (8 days to 6 weeks), and the late convalescent phase (≥ 72 days after the onset of disease). The date of onset of disease was defined as the date of the first severe symptom related to the meningitis disease episode reported by the patient. The sera were stored in aliquots at 20°C freezing and transporting of sera was done on dry ice.

Controls were recruited from hospitals or health centres in Gondar in 2003 and in SNNPR in 2002 and 2003 and blood samples were collected from individuals of age ≥ 6 months.

Sera with anti-APS IgG antibody concentration greater than 1 $\mu\text{g/ml}$ were considered for statistical analysis since AIs were not evaluable with sufficient accuracy for values less than this (Richmond *et al.*, 1999). Thus, from the 125 sera tested, avidity indices were calculated for 114 (Table 3).

Table 3. Number of sera for which avidity indices were calculated for the study population from Sidama and Gedeo Zones in the SNNPR and in the Northern Gondar Zone in the Amhara region of Ethiopia during the 2002/2003 epidemics.

Age in years*	Number of controls	Number of patients			Total no. of patients
		Acute Phase sera (0-7 days)	Early convalescent phase sera (8-32 days)	Late convalescent phase sera (≥ 72 days)	
≥ 0.5 to < 7	11	6	6	2	14
≥ 7 to < 16	7	11	10	11	32
≥ 16	10	16	14	8	38
Total=114	28	33	32*	21	86

*age of 2 patients was unregistered

5.2 Avidity ELISA

Although the standard ELISA method for measuring serum antibody concentration to meningococcal A or C polysaccharides is both sensitive and reproducible (Carlone *et al.*, 1992; Gheesling *et al.*, 1994), its correlation with SBA was previously shown to be non-significant, especially while dealing with heterogeneous antibody populations (Granoff *et al.*, 1998; Norheim *et al.*, 2007). Therefore, attempts have been made to develop modified ELISA protocols that better correlate with serum bactericidal activity (Granoff *et al.*, 1998) and other protection indicators such as opsonic activity (Anttila *et al.*, 1999).

The superiority of high avidity antibodies in bactericidal activity has been repeatedly mentioned (Schlesinger *et al.*, 1992; Lucas *et al.*, 1995 and Usinger *et al.*, 1999). A study

on serum antibodies to pneumococcal serotypes 6B and 23F indicated the importance of avidity in antibody effector functions and protection (Anttila *et al.*, 1998). The importance of avidity for priming immunological memory was in addition shown by Joseph *et al.*, (2001 and 2003)

In another project at NIPH, chaotropic ELISA has been established and used to measure IgG antibody concentrations and avidities in Ugandan sera, after vaccination with ACYW135 polysaccharide vaccine (Guerin *et al.*, 2008; Bårnes, *et al.*, personal communication). In this study, the IgG antibody avidity against serogroup A polysaccharide in meningococcal patient sera was measured using a modification of the methods employed by Bårnes *et al.*, (personal communication) and Romero-Steiner *et al.*, (2005).

Coating ELISA plates - MaxiSorp 96 well microtiter plates from Nunc (Roskilde, Denmark) were used for the assays. The use of such special high-binding plates has been recommended by Rosenqvist *et al.*, (2001), as antigens like meningococcal capsular polysaccharides bind poorly to the commonly used polystyrene plates. The plates were coated with meningococcal A polysaccharide (APS) complexed with methylated human serum albumin (mHSA) at a final concentration of 5 µg/ml. A coating buffer prepared from distilled water with 2% phosphate buffered saline (PBS), pH 7.0, and 0.02% sodium-azide was employed. The plates were incubated overnight at +4°C and kept up to 3 weeks until use.

The plates were washed five times in PBS with 0.1% Brij and 0.02% sodium-azide, and blocked for 1 hour at room temperature with blocking buffer (3% fetal calf serum (FCS), F3885, Sigma-Aldrich, St.Louis, USA, in PBS, pH 7.0, with 0.02% sodium-azide).

Reference and control sera - The Centers for Disease Control and Prevention (CDC 1992) reference serum from National Institute for Biological Standards and Controls (NIBSC), (Hertfordshire, UK) with an assigned IgG value of 91.8 µg/ml against serogroup A polysaccharide was used (Holder *et al.*, 1995). A serum from one Ethiopian vaccinee (AC polysaccharide) was also used as a positive control serum on every plate.

Serum dilution – Test, positive control and reference (CDC 1992) sera were two-fold diluted with dilution buffer containing PBS, pH 7.0, with 3% FCS, 0.1% Brij and 0.02% sodium azide. 100 µl of test, positive control and reference sera were added to the wells. The test and reference sera were run in duplicate on each plate. The microtiter plates were then incubated overnight (16-20 h) at +4°C.

Elution with chaotrope - The following day, the plates were washed five times. From the 4M stock solution of the ammonium thiocyanate, different molar solutions (0mM, 60mM, 120mM, 180mM and 240mM) were prepared fresh for every assay. These solutions were prepared by diluting the salt (molecular weight = 304.48 gram) in coating buffer containing PBS with 0.02% sodium azide. The solutions were added to the plate wells according to the template (Figure 4) and incubated at room temperature for 15 minutes.

Preliminary experiments were performed to see the effect of temperature and time on incubation of sera with thiocyanate solutions. Three plates were run with similar sera and sera dilutions. The first plate was incubated at room temperature for 15 minutes, the second at room temperature for 30 minutes and the third at 37°C for 30 minutes. The results showed that the first and the second plates gave similar result, while the third showed some deviations. Thus the 15 minute incubation at room temperature was chosen as also used in *Streptococcus pneumoniae* (Antilla *et al.*, 1998), *Haemophilus influenzae* (Romero-Steiner *et al.*, 2005) and *N. meningitidis* (Richmond *et al.*, 2001, Joseph *et al.*, 2003) ELISAs.

Reference Sera (CDC 1992)		NH ₄ SCN concentrations in mM									
		0mM		60mM		120mM		180mM		240mM	
1:1600	1:1600	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1
1:3200	1:3200	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1	serum no.1
1:6400	1:6400	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2
1:12800	1:12800	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2	serum no.2
1:25600	1:25600	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3
blank	1:1600	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3	serum no.3
blank	1:3200	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4
blank	1:6400	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4	serum no.4

Positive control serum

Figure 4. ELISA plate template

Conjugate and substrate - After incubation with the chaotrope and washing the plates five times, secondary antibody (Anti-Human IgG (γ -chain specific) conjugated to Alkaline Phosphatase produced in goat (A3187, Sigma-Aldrich, St.Louis, USA), was added and the plates were further incubated at +37°C for 2 h. After washing five times, the plates were developed using p-nitrophenyl phosphatase substrate tablets (S0942, Sigma-Aldrich, St.Louis, USA) prepared as 10% in diethanolamine buffer, pH 9.8.

Plate reading - Optical densities (OD) were read using the SOFTmax software (version 2.35 Dale Quantz, Molecular Devices Corp.) when the test sera with no thiocyanate reached an OD of approximately between 0.75 and 1.25 at wavelength 405 nm. OD values for wells incubated without sera were subtracted as background.

IgG antibody concentrations were calculated using a 4-parameter logistic curve-fitting analysis against the standard reference curve. Those data points obtained from dilutions

that yielded OD values in the linear portion of the curve were averaged and IgG geometric mean concentrations (GMC) were calculated. Finally, geometric mean concentrations (GMCs) of APS IgG and geometric mean avidity indices (GMAIs) were estimated for each group at 95% confidence interval.

5.2.1 Avidity indices (AI) - were calculated in two ways:

Method 1. Avidity determination using elution with a single concentration of the chaotrope (Antilla *et al.*, 1998; Romeiro-Steiner *et al.*, 2005, Bårnes *et al.*, personal communication) - from the series of thiocyanate solutions used in the assays, a single concentration was chosen to calculate avidity indices. As mentioned by Romero-Steiner *et al.*, (2005), three groups of sera were chosen for this method. Sera in each group involve those which show low, medium and high OD reduction after being eluted by the increasing concentration of the chaotrope solutions (Table 4). Average of percent reduction in OD₄₅₀ of 3 sera for each group was plotted against the series of different concentrations of NH₄SCN (Figure 5). The thiocyanate concentration which shows the highest discrimination between the three panels of sera was chosen. The chosen salt concentration was 120mM, which was also used by Bårnes *et al.*, (personal communication). Then the avidity indices were calculated as the percent IgG antibody concentration after being eluted by the 120mM thiocyanate salt solution ($AI-1 = \text{IgG concentration of the serum sample with NH}_4\text{SCN treatment} / \text{IgG concentration of the sample without NH}_4\text{SCN treatment} * 100\%$). The avidity indices are referred to as AI-1 for convenience.

Table 4. The average % OD reduction in 9 randomly selected patient sera. 3 sera are included in each group and their average % OD reduction is recorded. Sera in each group show relatively low, medium and high OD reductions after being eluted by the increasing concentration of the chaotrope solutions (60mM, 120mM, 180mM and 240mM).

NH ₄ SCN conc. (mM)	Average % OD reduction		
	Group 1	Group2	Group 3
60	21	26	47
120	37	49	76
180	48	63	87
240	55	69	90

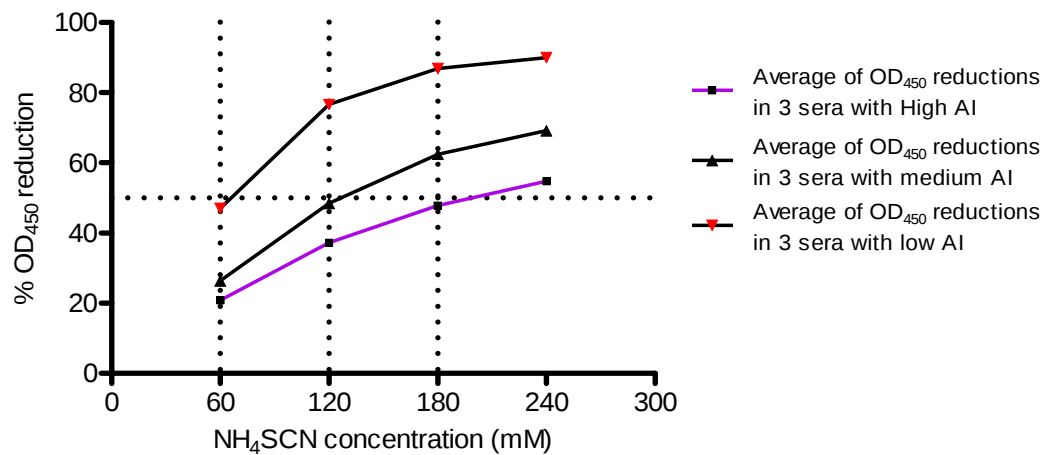


Figure 5. Average percent reductions in OD₄₅₀ of 9 sera, 3 in each group, plotted against concentration of NH₄SCN in millimolar (mM). These patient sera are randomly selected from sera collected from Sidama and Gedeo Zones in the SNNPR and in the Northern Gondar Zone in the Amhara region of Ethiopia during the 2002/2003 epidemics. Sera in each group show relatively low, medium and high OD reductions or have high AI after being eluted by the increasing concentration of the chaotrope.

Method 2. Avidity determination using elution with increasing concentrations of the chaotrope (Richmond *et al.*, 1999; Romeiro-Steiner *et al.*, 2005) – the OD values of the sera after elution with 0mM , 60mM, 120mM, 180mM and 240mM salt were used to interpolate the exact thiocyanate concentration that resulted in 50% reduction of the OD readings of each serum. This was performed using cubic spline curve fitting. This is a simple curve fitting method for calculating unknowns from known values using GraphPad Prism, version 5.0 (GraphPad Software, Inc. San Diego, USA). Then the resulting values were considered as Avidity Indices which are put in milli-molars (mM) and referred to as AI-2.

5.3 Serum bactericidal activity assays

Serum bactericidal activity assays (SBA) were previously performed on these sera using the tilt method with some modifications (Norheim *et al.*, 2007). Since this is a retrospective study, data from the previous study was used for analytical purpose.

5.4 Ethical clearance

The main project titled “Characterization of epidemic serogroup A meningococcal strains from Ethiopia and antibody responses in patients following disease” has obtained ethical clearance in 2002 from AHRI/ALERT Ethics Review Committee, the National Ethical Review Committee (Ethiopian Science and Technology Commission) and the Norwegian Regional Committee for Medical Research Ethics in Western Norway. Informed written consent was obtained from patients (above 18 years of age) or their parents or guardians (for those patients below 18 years of age or with lack of consciousness) before enrollment in the study, and information of study participants has been kept confidential.

Since this study employed stored samples which were collected for the main project, ethical clearance for the continuation of the study was obtained from AHRI/ALERT Ethics Review Committee with the running title “Importance of antibodies against vaccine relevant antigens from serogroup A meningococci in protection against meningococcal disease”. A new material transfer agreement (MTA) was also signed between AHRI and NIPH before sending the sera to NIPH.

5.5 Statistical analyses

IgG concentrations and IgG avidity indices with 95% confidence intervals were calculated for the control sera, acute (0 to 7 days after the onset of disease) sera, early-convalescent (8 to 32 days) and late-convalescent phase (>72 days after the onset of disease) patient sera. The geometric means for different test groups were calculated at 95% confidence interval. Since the data obtained was non-normally distributed, the IgG concentrations and the avidity indices were compared using the Mann-Whitney and the Kruskal-Wallis test. For correlation analyses, Pearson correlation test, after log transforming the data, was used. All data were analyzed using SPSS statistics version 17.0 and GraphPad Prism version 5.0.

6. RESULTS

In this study, avidity of antibodies elicited against serogroup A *N. meningitidis* capsular antigen were measured using modified chaotropic ELISAs. Correlations of these results with SBA, using both baby rabbit and human complement (Norheim *et al.*, 2007), were also scrutinized.

While looking at the avidity indices calculated from the elution ELISA using a single ammonium thiocyanate concentration, the acute and early convalescent sera showed almost similar geometric mean avidity indices (GMAI-1=34.1, GMAI-1=33.3 respectively), but the value rose to GMAI-1=40.9 in late convalescing patients (Table 4). The avidity indices in the controls were significantly higher than the acute sera ($P=0.031$) and early convalescent sera ($P=0.027$) and combining data of all patients ($P=0.025$). However, the control and the late convalescent sera avidity indices did not vary significantly ($P=0.74$) (Figure 6). Except for the acute sera, the patients aged 0.5-6, had lower avidity indices than the older patient groups. This was also true for controls.

The geometric mean avidity indices showed increment with time while considering avidity indices calculated from the second elution method, where different concentrations of thiocyanate were used. The geometric mean avidity indices (GMAI-2) of acute sera, 75.3mM, increased to 89.6mM in early convalescent sera and further rose to 96.94mM in late convalescing patients. The avidity indices of controls were significantly higher than the acute sea ($P = 0.003$) though not significantly different from early ($P=0.32$) and late convalescent sera ($P=0.11$). The controls avidity indices were significantly higher than patients when all disease phases were combined ($P = 0.018$).

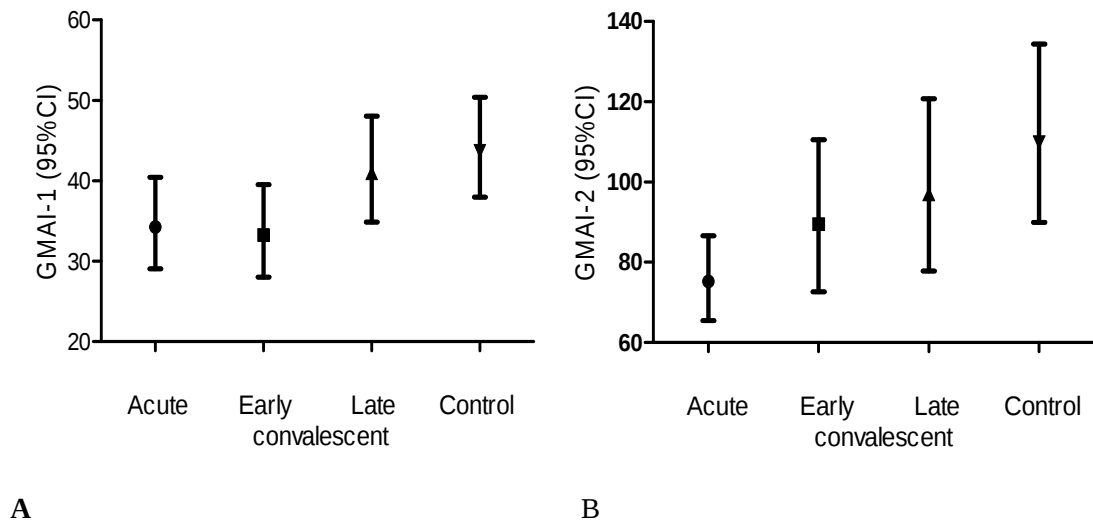


Figure 6. Time dependent increase of avidity indices as the patients progressed through different disease phases, and avidity indices of the controls. A. Data points are geometric mean avidity indices (GMAI-1) of patients and controls as calculated by the elution ELISA method that employed a single thiocyanate concentration. B. Data points are geometric mean avidity indices (GMAI-2) of patients and controls as calculated by the elution ELISA method that employed a series of thiocyanate concentrations.

Table 5. Geometric mean avidity indices (GMAI-1 and GMAI-2) of IgG antibodies elicited against APS calculated using the two elution ELISA methods employed.

Patient group			n	GMAI-1 (95%CI)	GMAI-2 (95%CI)
Controls	0.5 - 6 yrs		11	42.6 (31.14-58.40)	108.1 (70.96-164.70)
	7 -15 yrs		7	44.1 (36.24-53.57)	114.3 (80.13-163)
	≥ 16 yrs		10	44.8 (34.84-57.47)	109.0 (75.27-157.90)
	All		28	43.7 (37.99-50.37)	109.9 (89.94-134.40)
Patients	Acute phase	0.5-6 yrs	6	34.6 (22.15-54.06)	76.4 (57.41-101.70)
		7-15 yrs	11	34.8 (24.61-49.25)	75.8 (56.17-102.20)
		≥ 16 yrs	16	33.8 (26.24-43.53)	74.6 (59.66-93.24)
		All	33	34.1 (29.06-40.45)	75.3 (65.46-86.63)
	Early convalescent phase	0.5-6 yrs	6	26.3 (18.34-37.73)	69.7 (48.67-99.78)
		7-15 yrs	10	36.8 (24.94-54.26)	100.1 (71.00-141.20)
		≥ 16 yrs	14	35.8 (26.98-47.63)	100.3 (66.92-150.20)
		All	32*	33.3 (28.01-39.56)	89.6 (72.63-110.5)
	Late convalescent phase	0.5-6 yrs	2	38.9 (8.34-180.9)	80.5 (12.21-531.10)
		7-15 yrs	11	40.5 (30.56-53.56)	102.7 (73.74-143.00)
		≥ 16 yrs	8	42.2 (32.5-54.69)	93.8 (59.56-147.80)
		All	21	40.9 (34.09-48.04)	96.9 (77.83-120.70)

*age of 2 early convalescing patients was unregistered

Except in the acute sera, where the avidity indices are similar in all age groups, the age group 0.5-6 have the lowest avidity indices. The older age groups (7-15 yrs and ≥ 16 yrs) showed similar avidity indices (Table 5).

The first method of avidity measurement, which employed a single thiocyanate concentration, requires calculating antibody concentration. Thus the geometric mean anti-APS IgG concentrations were calculated before and after elution with 120mM NH_4SCN (Table 5). The table shows that all patients and controls have anti-APS IgG concentrations that exceed $2\mu\text{g/ml}$, both before and after elution with the thiocyanate.

Table 6. Geometric mean concentrations (GMC) of IgG elicited against APS with no and 120mM ammonium thiocyanate.

Patient group			n	GMC anti-APS IgG with no NH ₄ SCN (95% CI)	GMC anti-APS IgG with 120mM NH ₄ SCN (95% CI)
Controls	0.5 - 6 yrs		11	6.1 (2.6-14.3)	2.6 (1.4-5.0)
	7 -15 yrs		7	9.5 (2.8-32.7)	4.2 (1.1-16.5)
	≥ 16 yrs		10	30.3 (10.4-88.3)	13.7 (4.4-42.5)
	all		28	12.1 (6.7-21.6)	5.3 (3.0-9.5)
Patients	Acute phase	0.5-6 yrs	6	40.3 (6.5-248.8)	13.0 (2.5-68.5)
		7-15 yrs	11	17.2 (6.9-42.9)	5.7 (2.6-12.7)
		≥ 16 yrs	16	3.6 (2.3-5.8)	1.2 (0.8-1.9)
		all	33	9.4 (5.5-16.4)	3.1 (1.9-5.3)
	Early convalescent phase	0.5-6 yrs	6	89.1 (33.4-237.4)	32.3 (7.9-133.2)
		7-15 yrs	10	52.3 (27.1-101.0)	25.1 (11.2-56.5)
		≥ 16 yrs	14	21.4 (9.1-50.4)	8.8 (3.7-20.7)
		all	32*	39.7 (25.1-62.9)	9.1 (5.4-15.4)
	Late convalescent phase	0.5-6 yrs	2	7.5	2.9
		7-15 yrs	11	14.3 (4.5-45.4)	8.9 (3.3-24.2)
		≥ 16 yrs	8	17.3 (5.2-56.8)	3.5 (2.1-5.9)
		all	21	14.5 (7.4-28.5)	7.4 (3.8-14.4)

Unlike the avidity indices, the anti-APS IgG increased from 9.4 $\mu\text{g/ml}$ in acute phase sera to 39.7 $\mu\text{g/ml}$ in early convalescent sera and declined in late convalescent sera to 14.5 $\mu\text{g/ml}$ (Figure 7). The early convalescent sera anti-APS IgG is significantly higher than the controls ($P=0.005$), the acute sera ($P=0.0006$) and the late convalescent sera ($P=0.02$). However, the controls, the acute and early convalescent sera anti-APS IgG are not statistically different ($P=0.5$) (Table 6).

After being eluted by 120mM SCN, the geometric mean anti-APS IgG of acute phase sera (3.14 $\mu\text{g/ml}$) increased to 9.1 $\mu\text{g/ml}$ and then declined to 7.4 $\mu\text{g/ml}$. The acute phase anti-APS IgG increased significantly ($P = 0.006$) in early convalescent sera and declined in late convalescent sera. However, the decline is not statistically significant ($P = 0.5$). The early convalescent sera anti-APS IgG is not also significantly different from that of the controls ($P=0.14$) (Table 6).

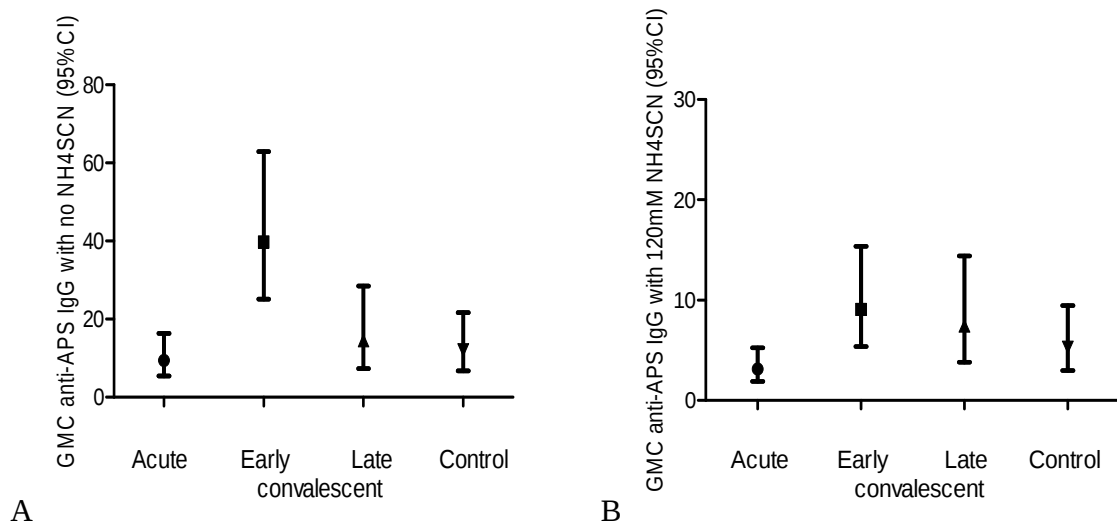


Figure 7. The time dependent variation of anti-APS IgG in the different disease phases, and of the controls (A) Data points are geometric mean anti-APS IgG concentrations of patients and controls with no thiocyanate (B) Data points are geometric mean anti-APS IgG concentrations of patients and controls with 120 mM thiocyanate.

Age dependent increase in IgG concentration is seen in control and in the late convalescent patient sera. However statistically significant increase is seen in control sera after being eluted with 120mM ammonium thiocyanate ($p=0.046$) (Figure 8).

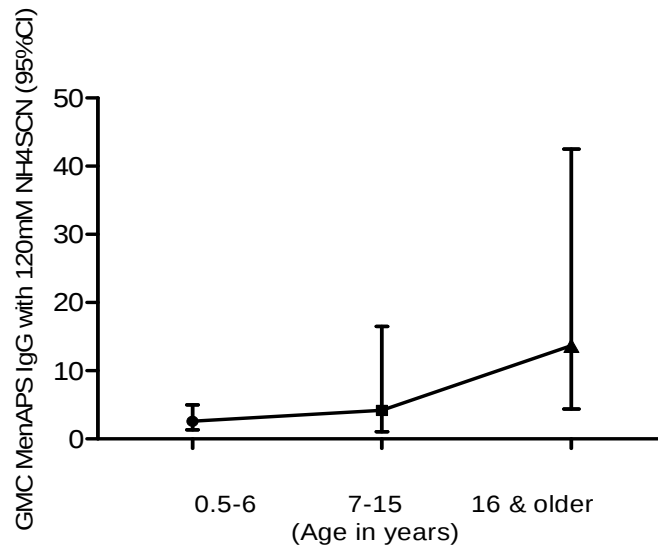


Figure 8. The age dependent increase of geometric mean anti-APS IgG concentration in control sera after elution with 120mM ammonium thiocyanate.

The avidity indices calculated from the two elution methods correlate significantly for all patients ($r = 0.80$, $P<0.0001$) and controls($r = 0.93$, $P<0.0001$) (Figure 9).

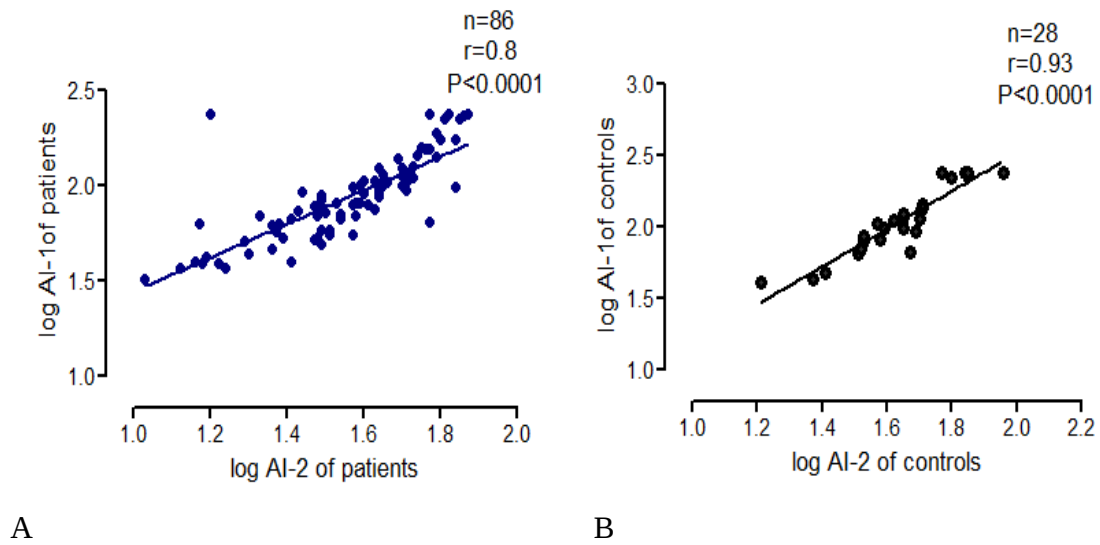


Figure 9. Correlations between avidity indices calculated by the two elution methods.

A) All patient sera B) control sera

Correlation between all patients sera rSBA and avidity indices (AI-1 and AI-2) were not significant ($P = 0.33$ and $P = 0.055$ respectively), however the correlations were significant with anti-APS IgG before ($r = 0.64$, $P < 0.0001$) and after elution with 120mM thiocyanate ($r = 0.65$, $P < 0.0001$). While considering hSBA results, the correlations were significant with AI-1 though negative ($r = -0.26$, $P = 0.028$) and no significant correlation with AI-2 ($P = 0.28$). The correlations between hSBA and anti-APS IgG concentrations before and after thiocyanate elution were significant ($r = 0.41$, $P = 0.0001$ and $r = 0.46$, $P = 0.0006$ respectively) (Figure 10).

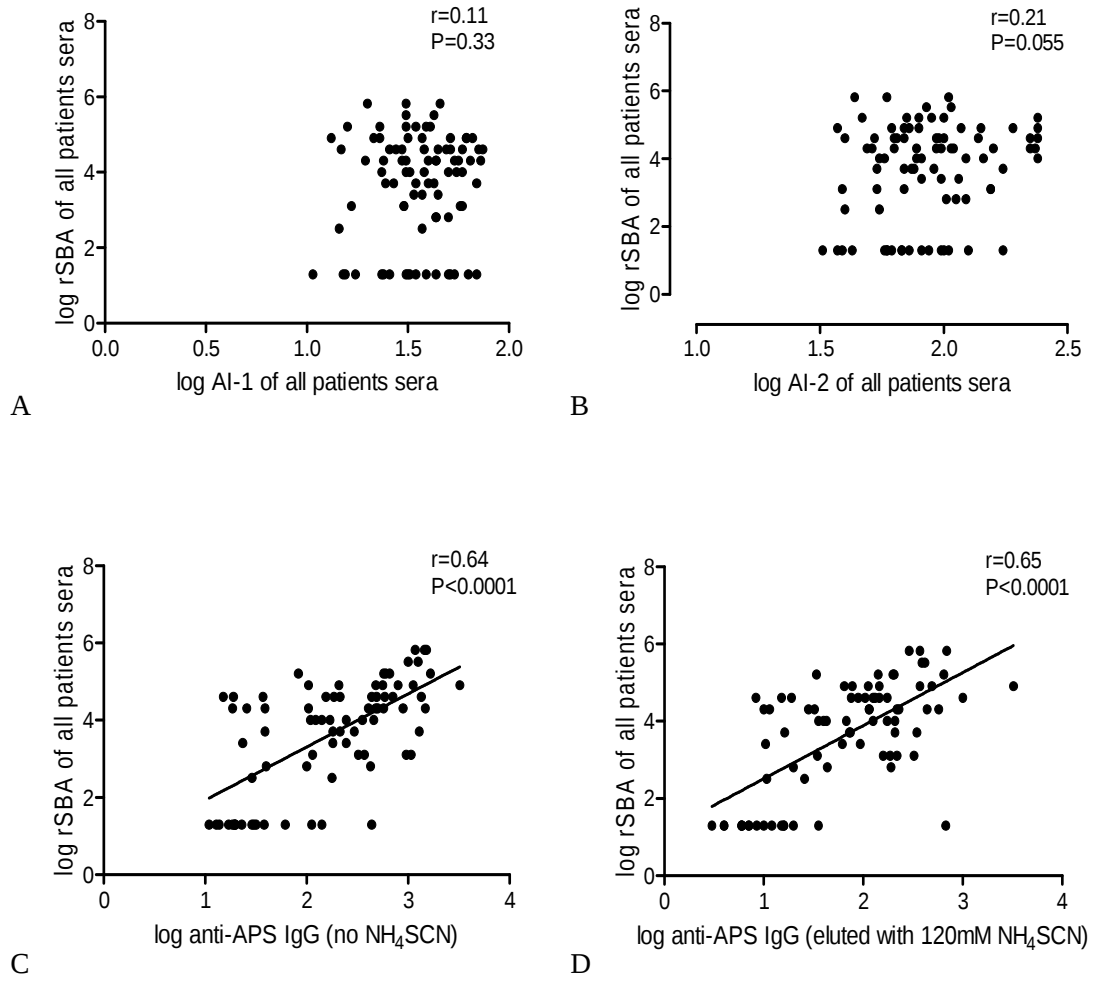


Figure 10. Correlations between SBA and Avidity indices, and anti-APS IgG in all patients. A) correlation between rSBA and AI-1. B) correlation between rSBA and AI-2. C) correlation between rSBA and IgG (no NH_4SCN). D) correlation between rSBA and IgG (after elution with 120 mM NH_4SCN). E) correlation between hSBA and AI-1. F) correlation between hSBA and AI-2. G) correlation between hSBA and IgG (no NH_4SCN). H) correlation between hSBA and IgG (after elution with 120 mM NH_4SCN) (Continued on next page)

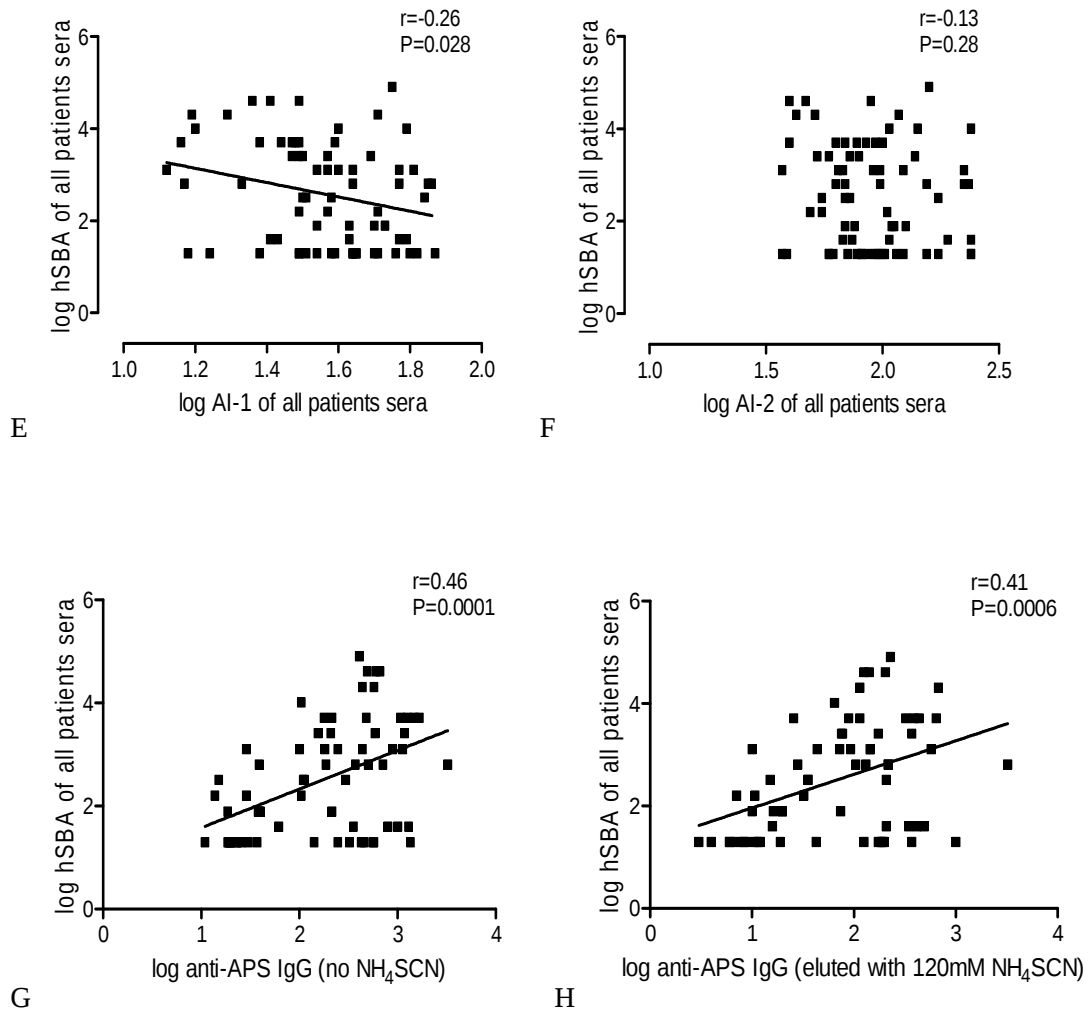


Figure 10. Continued

Considering the 0.5-6 age patients, significant correlation was observed between rSBA and the anti-APS IgG concentrations before and after elution ($r = 0.58$ $P = 0.03$, $r = 0.61$ $P = 0.02$, respectively). Though no significant correlation was observed with the avidity indices. No correlation was observed with hSBA. For those aged 7-15, significant correlation was seen with the anti-APS IgG before and after elution ($r = 0.49$ $P = 0.008$, $r = 0.42$ $P = 0.03$ respectively) and no correlations with hSBA. In those aged 16 and above, significant correlations were seen between rSBA and AI-2 ($r = 0.34$, $P = 0.04$) and anti-APS IgG before and after elution with thiocyanate ($r = 0.7$ $P < 0.0001$, $r = 0.76$, $P < 0.0001$ respectively), though only IgG correlated with hSBA.

Looking at the acute patients sera, the correlation between rSBA and anti-APS IgG concentrations before and after elution were significant ($r = 0.75$, $r = 0.79$ respectively, $P < 0.0001$). However the correlations were not significant with both avidity indices ($P = 0.81$). The results were similar with the hSBA, where the correlations with anti-APS IgG concentrations before and after elution were significant ($r = 0.8$, $r = 0.84$ respectively, $P < 0.0001$), though not significant with the avidity indices ($P = 0.088$, $P = 0.67$ for AI-1 and AI-2 respectively) (Figure 11).

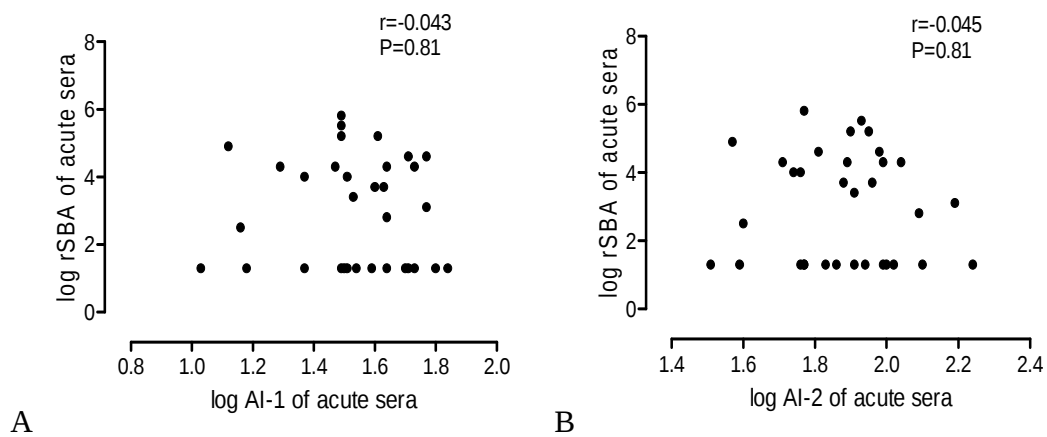


Figure 11. Correlations between SBA and Avidity indices, and anti-APS IgG in acute patients. A) correlation between rSBA and AI-1. B) correlation between rSBA and AI-2. C) correlation between rSBA and IgG (no NH_4SCN). D) correlation between rSBA and IgG (after elution with 120 mM NH_4SCN). E) correlation between hSBA and AI-1. F) correlation between hSBA and AI-2. G) correlation between hSBA and IgG (no NH_4SCN). H) correlation between hSBA and IgG (after elution with 120 mM NH_4SCN) (Continued on next page)

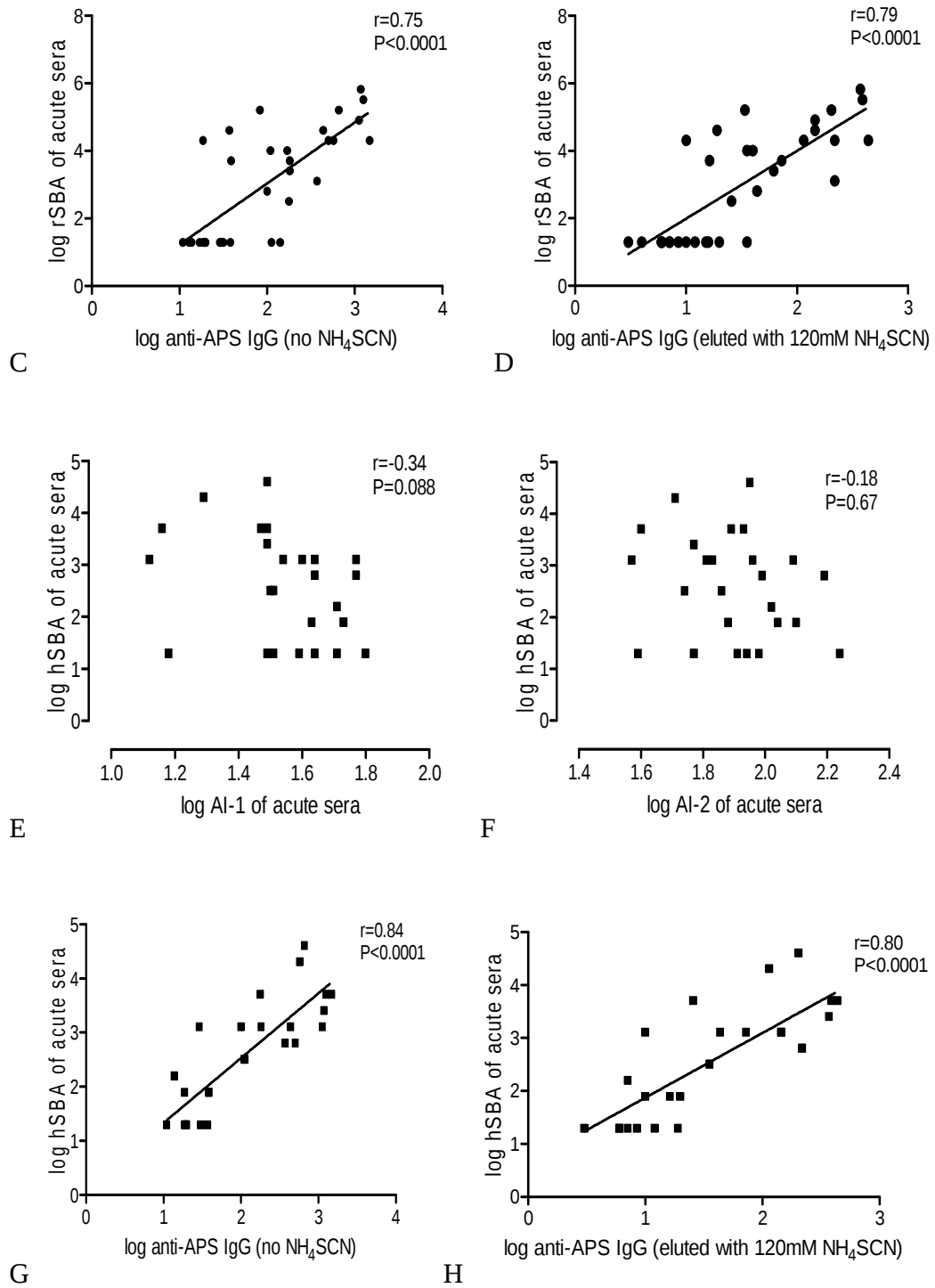


Figure 11. Continued

For the early convalescent sera, the only significant correlation seen was between the anti-APS IgG with no thiocyanate and rSBA. For the late convalescent sera, significant correlation was observed between rSBA and both avidity indices ($r = 0.58$, $P = 0.006$ for AI-1 and $r = 0.48$, $P = 0.03$ for AI-2 respectively). However, no correlations were observed with the IgG concentrations (Figure 12).

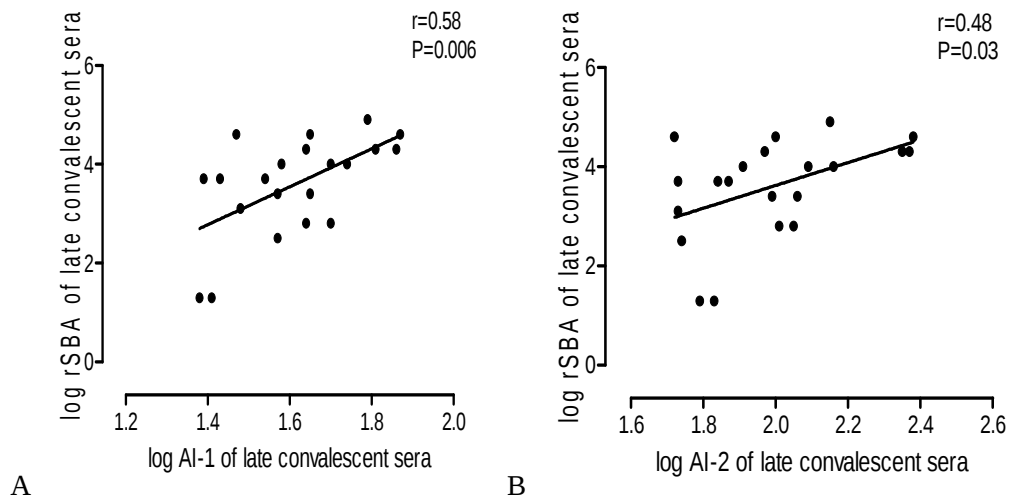


Figure 12. Correlations between SBA and Avidity indices in late convalescent patients. A) correlation between rSBA and AI-1. B) correlation between rSBA and AI-2.

For the controls, significant correlations were seen between the rSBA and anti-APS IgG concentration before and after elution ($r = 0.61$, $P = 0.0006$ and $r = 0.51$, $P = 0.002$ respectively). But the correlations were insignificant with the avidity indices ($P = 0.19$ and $P = 0.35$ for AI-1 and AI-2 respectively). No significant correlation was observed in the different age groups (Figure 13).

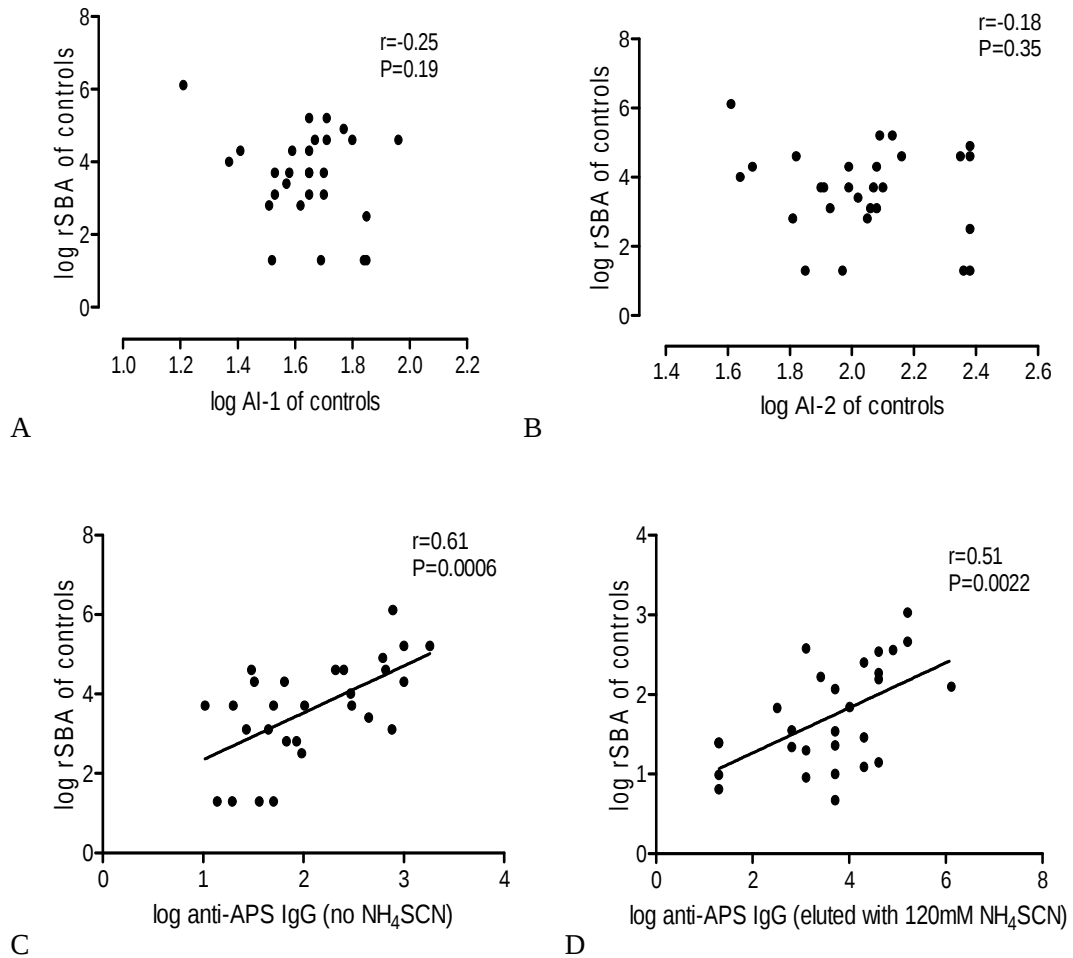


Figure 13. Correlations between SBA and Avidity indices, and anti-APS IgG in controls. A) correlation between rSBA and AI-1. B) correlation between rSBA and AI-2. C) correlation between rSBA and IgG (no NH_4SCN). D) correlation between rSBA and IgG (after elution with 120 mM NH_4SCN).

In patients sera considered to be protected based on rSBA titer (that means $\geq 1:128$), significant correlation between rSBA and anti-APS IgG before and after elution was observed ($r = 0.3$, $P = 0.02$) (figure 14). Sera considered to be protected based on hSBA results (that means $\geq 1:4$) result, correlation was significant with the anti-APS IgG before ($r = 0.38$, $P = 0.007$) and after elution ($r = 0.33$, $P = 0.02$), and negative correlation with AI-1 ($r = -0.32$, $P = 0.02$) (Figure 15). For patient sera considered to be protected according to both rSBA and hSBA titers, there was no correlations observed.

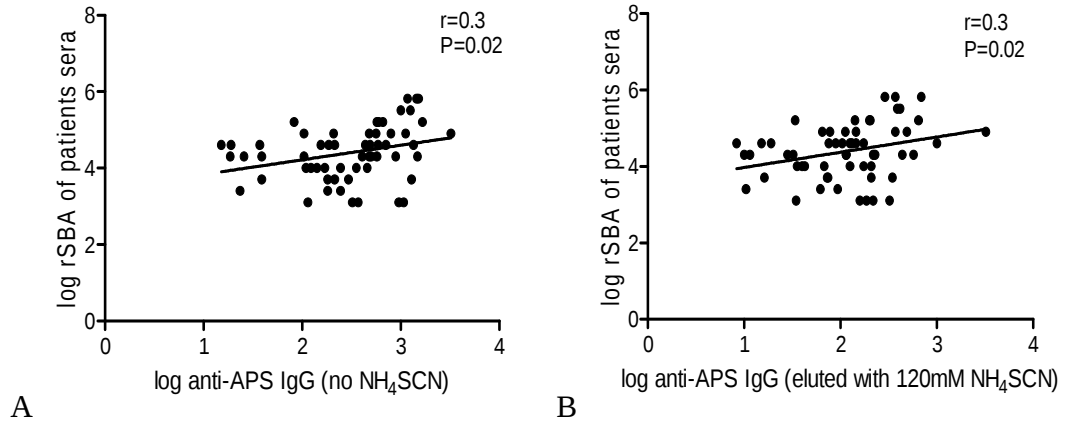
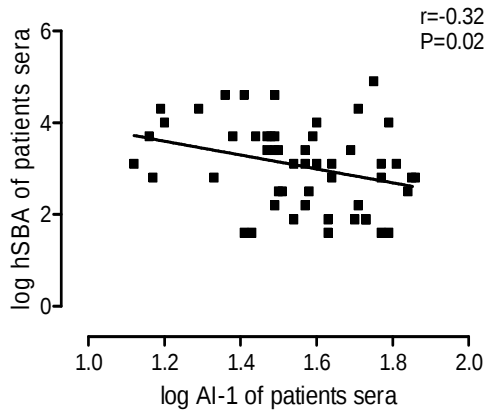
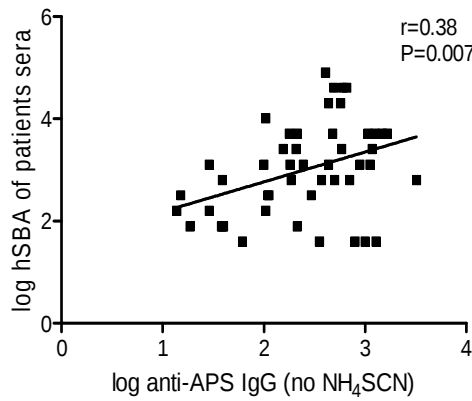


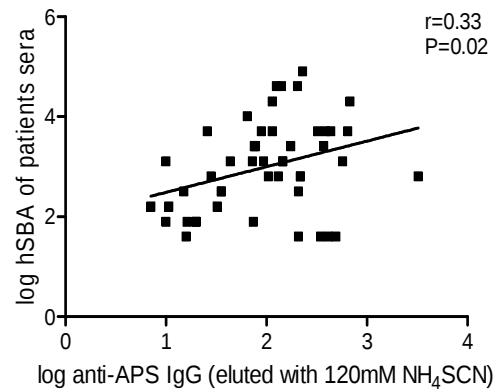
Figure 14. Correlations between rSBA and Avidity indices, and anti-APS IgG in all patients considered to be protected according to rSBA titer ($\geq 1:128$) (A) Correlation between rSBA and IgG (no NH_4SCN). (B) Correlation between rSBA and IgG (after elution with 120 mM NH_4SCN).



A



B



C

Figure 15. Correlations between hSBA and Avidity indices, and anti-APS IgG in all patients considered to be protected according to hSBA titer ($\geq 1:4$) (A) Correlation between hSBA and AI-1 (B) Correlation between hSBA and IgG (no NH_4SCN) (C) Correlation between hSBA and IgG (after elution with 120 mM NH_4SCN).

The geometric mean anti-APS IgG concentrations before and after elution in sera assumed to be protected based on their SBA titer ($\text{rSBA} < 1:128$ and $\text{hSBA} < 1:4$) showed that those assumed to have protective titer have higher values ($p < 0.0001$). The geometric mean avidity indices in sera assumed to have protective rSBA titer and hSBA is higher than those assumed to have not protective titers, however the values are not statistically significant (Table 7).

Table 7. Geometric mean avidity indices and anti-APS IgG concentrations before and after elution in sera assumed to be protected based on their SBA titer (rSBA $\geq$ 1:128 and hSBA $\geq$ 1:4)

	GMAI-1 (95% CI)	GMAI-2 (95% CI)	GMC anti-APS IgG with no NH ₄ SCN (95% CI)	GMC anti-APS IgG with 120mM NH ₄ SCN (95% CI)
Sera with rSBA $\geq$ 1:128	38.0 (34.8-41.6)	94.4 (84.7-105.3)	25.56 (18.8-34.9)	10.2 (7.5-13.8)
Sera with rSBA < 1:128	35.3 (29.2-42.8)	81.75 (66.9-100.0)	4.2 (2.9-6.2)	1.6 (1.0-2.5)
Sera with hSBA $\geq$ 1:4	35.4 (31.3-39.9)	87.8 (76.5-100.7)	24.4 (16.5-36.1)	9.6 (6.5-14.0)
Sera with hSBA < 1:4	39.7 (33.3-47.2)	94.6 (75.6-118.4)	7.9 (4.1-15.3)	3.2 (1.5-6.6)
Sera with rSBA $\geq$ 1:128 and hSBA $\geq$ 1:4	35.8 (31.2-41.0)	91.0 (77.5-106.8)	36.0 (24.2-53.6)	13.8 (9.6-20)
Sera with rSBA < 1:128 and hSBA < 1:4	34.0 (23.7-48.9)	78.7 (51.3-120.8)	2.9 (1.3-6.7)	1.0 (0.4-2.7)

7. DISCUSSION

The results of this study showed correlations between the avidity indices and bactericidal activity of the IgG antibodies only in the late convalescent and patients sera greater than or equal to 16 years of age using baby rabbit sera as a complement source. The correlations were however not significant while using human sera as a complement source. Bårnes *et al.*, (personal communication) also observed no correlation between rSBA and avidity indices in Ugandan vaccinees. However, these results were inconsistent with other studies that showed significant correlations between SBA and avidity of antibodies against related strains (Granoff *et al.*, 1998, Richmond *et al.*, 1999b, Joseph *et al.*, 2001 and 2003).

The poor correlation of avidity indices and serum bactericidal activity, which was observed in the present study, might be due to the fact that correlation was attempted to be shown between the serum's bactericidal activity as a whole i.e. directed against both capsular and non capsular antigens, and IgG antibodies elicited against a specific capsular antigen, APS. This idea is further supported by Amir *et al.*, (2005) as they showed the relative importance of antibodies for bactericidal activity against specific antigen could vary. Their study illustrated that among bactericidal Sudanese sera, 58% showed inhibition of bactericidal activity by APS, and the value was lower for Ugandan and USA sera.

Although not statistically significant, it was seen that in sera considered to be protective based on the rSBA titers, the avidity indices were higher than those assumed not protective. This supported the findings of Granoff *et al.*, (1998) and Romero-Steiner *et al.*, (1999) where they associated protective SBA titers with high avidity and reduced functional activity of antibodies with low avidity.

The correlations of the IgG antibodies elicited against the capsular antigen before and after elution by ammonium thiocyanate and rSBA were significant for the controls and all patient sera with the highest correlation in patients aged ≥ 16 years. The correlations with hSBA were however not significant except in the acute sera where the highest value was observed between the IgG elicited against the capsular antigen before and after elution by

ammonium thiocyanate and hSBA. The fact that correlation was seen in rSBA and not hSBA might be because the rSBA titers might have been exaggerated due to the employment of heterologous species sera as a source of complement as reported by Zollinger and Mandrell (1983). Or the strain used in rSBA may not be representative of the epidemic strain under consideration (Norheim *et al.*, 2007). The ideal exogenous complement is human serum from patients with agammaglobulinaemia and normal complement activity, since with that source the risk of introducing antibodies that can affect the measurement of bactericidal activity in the test sera is negligible. However, WHO (1999) recommended the use of infant rabbit complement since it is not easy to get human complement source that fulfils such criteria.

The age dependent increase in antibody levels seen in controls and late convalescent sera resembled the classic studies of Goldschneider *et al.*, (1969). This might be due to the fact that exposure to the antigen from asymptomatic carriage or previous infection is likely to increase with age as Caugant and Maiden (2009) showed age to be one of the important factors influencing meningococcal carriage rates. Increase in avidity index based on age was also seen in all test groups except for the acute sera where similar avidity indices were seen in all age groups. Goldblatt *et al.*, (2002) showed that antibody induced by polysaccharide antigens in adults is of relatively high avidity than that of children. However other factors such as poor socio economic status in Africa might influence the age dependent distribution of carriage rates as other respiratory tract infections and closeness of social contacts is very high (Caugant and Maiden, 2009).

Grannof *et al.*, (1998) had recommended a modified ELISA for measuring IgG antibody responses to meningococcal C polysaccharide that ensure specificity and favor detection primarily of high-avidity antibodies. They reported that solid phase meningococcal C polysaccharide mixed with methylated human albumin could result in high absorbance values when serum samples from some unvaccinated children or adults were assayed. And, for the modified ELISA, they used adipic acid-derivitized polysaccharide as the solid-phase antigen and reported a better correlation with SBA. In the present study, a meningococcal A polysaccharide mixed with methylated human serum albumin was used as a solid phase antigen. Therefore, the results of the present study might have been

exaggerated by the solid phase antigen preparation method used. However, the method recommended by Grannof *et al.*, (1998) has not been compared between different laboratories and also not tested with serogroup A capsular polysaccharide.

Although both rabbit and human complements were used in SBA assays (Norheim *et al.*, 2007), correlations of these assays with the two avidity ELISA indices were low. This could in part be due to the limitations of the methodologies employed since every protocol has its own advantage and disadvantage. Goldblatt (2001) mentions several shortcomings of the solid phase ELISA methods for measuring antibody avidity of which are antigen density and steric hindrance which may cause possible complications that relate to conformational changes in antigens during binding to solid phase, which in turn might affect antibody binding. Gray and Shaw (1993) also mentioned points that could result in artifacts while using thiocyanate elution ELISA for estimating relative antibody avidity. These include low thiocyanate concentrations and the choice of secondary antibody. They mentioned that though these methods are simple and mostly accurate, they might not be applicable to all antibody/antigen systems. However their results were based on IgM elicited against pneumococcal polysaccharide which might have differences in the dynamics of antibody antigen reaction from the current study.

Comparison of the two elution methods employed showed the avidity indices calculated from the two methods to correlate highly although the correlation with the SBA results were not statistically significant for most test groups. Furthermore, Romero-Steiner *et al.*, (2005) mentioned the limitations of different methods of avidity determination and the difficulty of getting comparable results using such methods. They compared 3 methods and advised the employment of elution with a single chaotrope concentration in measuring antibody avidity. In another study by Harris *et al.*, (2007), inhibition and elution ELISAs with a single concentration of chaotrope were compared. They mentioned several shortcomings of the chaotropic ELISA and finally described a modified inhibition ELISA that could provide better results. However, this method is more complex and not widely used.

The study populations in the present study were highly varied in antibody concentration, which made it difficult to obtain OD readings of approximately 1 that was also used by Romero-Steiner *et al.*, (2005). Diluting serum to a certain OD reading is difficult due to the variation in IgG concentrations and polyclonal nature of human serum. This also has a limitation of failing to gain parallelism with standards and accurate measurement of antibody concentration.

To lessen the effect of highly varied antibody population, a series of chaotrope concentrations with a series of serum dilutions were employed and a wider range of ODs, approximately between 0.75 and 1.25 was tolerated as used by Joseph *et al.*, (2003). This allowed the choice of OD readings which fairly fulfill parallelism with standard, though it made it hard to primarily choose OD readings that are approximately 1. A single chaotrope concentration with serially diluted sera was chosen, which gave this study an advantage of comparing avidity indices calculated in two ways and see their correlation with SBA assay results.

Varying salt concentrations have been employed by different researchers. Each has chosen a protocol depending on the objective of the study and the antigen tested in particular. In another study at NIPH (Bårnes, *et al.*, personal communication), avidity of serogroup A meningococcal IgG antibodies in Ugandan vaccinees was studied using the chaotropic ELISA method. The optimum chaotrope concentration to dissociate the strongly and weakly bound antibodies was chosen to be 120mM, the same salt concentration considered optimum for this study. 0.5M was considered optimum for anti-pneumococcal polysaccharide (Anttila *et al.*, 1998) and 0.75M for serogroup C meningococcal polysaccharide (Granoff *et al.*, 1998). However, Romero-Steiner *et al.*, (2005) mentioned some artifact OD readings in some adult sera with a relatively lower concentration (0.15M). Furthermore, Gray and Shaw (1993) also mention false results that might appear with low chaotrope concentrations. The chaotrope concentration used as optimum for this study (0.12M), which is relatively lower than concentrations used by other studies may have to be validated by comparing it with other liquid phase avidity measurements. Further studies on the capsular polysaccharides of different strains that have different properties will also be necessary for a more conclusive determination.

A recent study attempting to validate measurements of avidity by using monoclonal antibodies compared values obtained by using two different methods_ thiocyanate elution and the liquid phase radioantigen binding assay (RABA) and correlating these values with complement-dependent antibody bactericidal activity (Reykjalín *et al*, 2010). The results showed that both methods gave significant correlations though in some cases, the avidity indexes calculated after thiocyanate elution did not accurately reflect antibody functional activity. Their speculation for this phenomenon is that chaotropic disruption of antigen binding may in few cases reflect unique combining site chemistries that are not directly related to avidity determination and the exact reason for such exceptions is not known.

The presence of high antibody level in the Ethiopian population studied is not different from that in other countries of the “meningitis belt”. Studies in this area had shown significantly high antibody concentrations in these populations (Greenwood *et al.*, 1987; Mueller *et al.*, 2003 and Amir *et al.*, 2005). Amir *et al.*, (2005) showed that 100% of Sudanese sera tested have higher IgG antibody concentrations than 2µg/ml, previously assumed to be the protective level (Makela *et al.*, 1975 and Peltola *et al.*, 1977). However, the antibody levels were lower for sera from Uganda, which is a bordering country of the “meningitis belt”, and even lower in the USA population. The reason that Amir *et al.*, (2005) considered important is the nasopharyngeal colonization by this pathogen, which is very common in epidemic areas. Makela *et al.*, (1975) also discussed the relation between immunity and carriage where they state that close contact with patients or asymptomatic carriers highly increase carriage rate and antibody concentrations in individuals. Thus considering the epidemic season as time of sample collection and the fact that controls were also recruited from hospitals where overcrowding is typical, the presence of high antibody levels in both patients and controls from Ethiopia is to be expected.

The finding that avidity indices of controls were significantly higher than that of the acute and early convalescent patient sera might be due to previous vaccination status or asymptomatic carriage of the controls. Though vaccination is not routine in Ethiopia, it is common during epidemic seasons. Asymptomatic carriage of similar serogroup strains

was also previously shown to result in bactericidal antibodies by a study in Gambia (Brieske *et al.*, 1999). Though the carriage rate varies, in some cases it could reach up to 100% and at one time in life any individual could be an asymptomatic carrier (Caugant and Maiden, 2009).

Compared to 2-3 year old controls in UK (Joseph *et al.*, 2003), the avidity indices in the Ethiopian study populations were lower although the antibody levels were higher. In Ugandan population, where it has been previously shown by Amir *et al.*, (2005) to have lower antibody concentrations than in Sudan, the avidity indices of Ugandan vaccinees (Bårnes *et al.*, personal communication) were similar to the Ethiopian patients and controls. This might indicate that protective antibody levels might depend on the exposure of individuals to the organisms and this might vary geographically as the rate of flow and strain type varies temporally and spatially.

8. CONCLUSION

In the present study, it was attempted to consider antibody avidity, which was anticipated to provide some explanation for the presence of high level of IgG antibodies against APS, though not conferring protection against *N. meningitidis* in the Ethiopian populations. However, the results did not show correlation between avidity of anti-APS IgG and the primary correlate of protection, SBA, as previously assumed. But there are still questions that need to be addressed before ruling out avidity measurement as another correlate of protection which WHO (1999) recommended the use of such serological assay for evaluating efficacy of serogroup A/C vaccines.

Although the results of this study did not clearly show correlation between protection against *N. meningitidis* and avidity of IgG antibodies to APS, a vaccine relevant antigen, important observations were made from the results. These include:

- Exposure to *N. meningitidis* resulted in affinity maturation as the avidity indices increased in convalescing patients, though the antibody levels declined with time.
- It was also seen that protection could relate with quality of immune response, as sera with high SBA titers tended to have higher avidity indices.
- Youngest study participants (2-6 yr olds) showed low antibody concentration and avidity indices and this substantiates the fact that immunity of individuals against serogroup A *N. meningitidis* develops after exposure to antigens which could be due to asymptomatic carriage, disease or vaccination.
- The two methods of calculating avidity indices employed gave comparable results. The first method, which employs a single thiocyanate concentration and a series of serum dilution, can be used if a standard reference serum is available, and if not the other method which uses different thiocyanate concentrations and does not require calculating antibody concentration can be employed to measure avidity of IgG antibodies elicited against APS.

9. RECOMMENDATIONS

Avidity studies have proven useful as correlates of protection in studies with related strains: serogroup C *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. Thus it might be important to re-examine the existing methods for determining antibody avidity so that standard protocols are developed for serogroup A *N. meningitidis* which is responsible for most of the meningitis epidemics that occurred in the “meningitis belt”.

Quality of the immune response might be affected by several factors, thus it might be necessary to consider other factors that might affect antibody quality, such as IgG subclasses. It could also be interesting to study other protective indicators such as antibody dependent opsonisation.

It might also be crucial to consider subcapsular antigens of serogroup A *N. meningitidis* that were previously shown to induce strong immune responses. Thus, to get a better picture of immune protection against *N. meningitidis*, it might be important to consider the SBA that is elicited against the particular antigen which could be seen by absorbing sera with the purified antigens (capsular and subcapsular) and analyzing them in SBA before and after absorption.

It is likely that detailed studies of antibody responses mounted after disease may contribute to the understanding of meningitis epidemics in sub-Saharan Africa, and these are important to recommend appropriate correlates of protection against serogroup A *N. meningitidis* in the “meningitis belt”.

10. LIMITATIONS OF THE STUDY

The vaccination status of the study participants was not available. This might have provided additional explanation for the presence of high anti-APS IgG avidity in controls.

In addition to the total IgG antibody avidity addressed in this study, looking at avidity of IgG subclasses could have provided more information on the correlation with protection.

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