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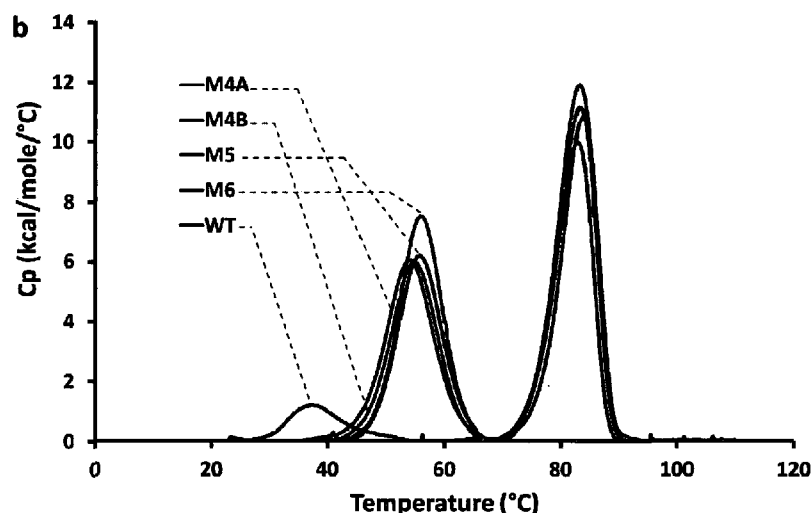
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Figure 5B



(57) Abstract: The invention relates to a modified V2 fHbp having increased stability over a wild type V2 fHbp; a modified V2 fHbp having an amino acid sequence with at least 85% identity to the sequence of Seq ID No: 1, wherein both Ser¹³⁷ and Gly¹³⁸ are mutated or both Val¹¹² and Leu¹¹⁴ are mutated; immunogenic, pharmaceutical and vaccine compositions; nucleic acid and a host cell; and methods of use of such compositions.

NEISSERIA MENINGITIDIS FHBP VARIANT AND ITS USE FOR VACCINATION

The present invention relates to a factor H binding protein with improved stability, and in particular to the use of such a protein in immunogenic compositions for eliciting an immune response to pathogenic organisms. In particular the invention relates to immunogenic compositions for *Neisseria meningitidis*.

Neisseria meningitidis (meningococcus) is an encapsulated Gram-negative diplococcal bacterium that inhabits the nasopharynx/upper respiratory tract of humans and is an important cause of sepsis and meningitis. Carriage rates in the general population are usually around 10%. There are approximately 500,000 cases of meningococcal disease each year with around 50,000 deaths. In developed countries, the bacterium is a leading infectious cause of mortality among children, has important public health impacts during outbreaks in schools and universities, and can cause profound disability in survivors. Mortality for meningococcal septicaemia remains around 10%, and patients can develop neurological deficits and/or loss of limbs.

20

The *Neisseria meningitidis* organism is sensitive to several front line antibiotics. However, despite this, a significant number of patients diagnosed with meningococcal infection die of overwhelming disease or suffer serious complications. Mortality rates vary from 2-3% in cases of uncomplicated meningitis to 50% or more in cases of septic shock (Cartwright, K. A. and D. A. Ala'Aldeen (1997) J Infect 34:15-19).

25

The extremely rapid progression of meningococcal disease and non-specific early symptoms mean that vaccination is the best way to protect populations from the devastating consequences of meningococcal disease.

30

Current vaccines only cover subsets of strains. Vaccines based on the polysaccharide capsule (which define the serogroup classification) are only effective against certain serogroups. This approach cannot be used against serogroup B disease which accounts for over 95% of clinical isolates in England and Wales, and predominates in developed countries. This is because the B capsule is identical to a modification on human NCAM-1. Outer membrane vesicle (OMVs) vaccines have been used successfully to prevent epidemics (most recently in New Zealand) but only confer protection against strains expressing the same variant of PorA (an outer membrane porin) as in the OMV. There is still no serogroup B vaccine that is suitable for routine immunisation schedules in Europe and the USA.

Another potential vaccine candidate is the factor H binding protein (fHbp) found on the surface of *Neisseria meningitidis*.

In healthy individuals, the activation of complement is precisely controlled through membrane-bound and soluble plasma-regulatory proteins including factor H (fH). Factor H is a 155 kDa protein composed of twenty domains (termed complement control protein repeats, or CCPs). Several pathogens have adapted to avoid complement-mediated killing by sequestering fH to their surface.

fHbp on *Neisseria meningitidis* is a 27 kDa lipoprotein that consists of two beta barrels (an N terminal barrel and a C terminal barrel) joined by a short amino acid linker. While charged carbohydrates on the surface of the vascular endothelium engage fH, charged amino acids in the fHbp bind fH at nanomolar affinities at the same site of this complement regulator.

Based on differences in the nucleotide and predicted amino acid sequences, fHbps from different *Neisseria meningitidis* strains have been categorised using several schemes. These include two subfamilies (A and B) (Murphy E, et al. (2009) The Journal of Infectious Diseases 200: 379-389) or three variant groups (V1, V2, and V3) (Masignani V, et al. (2003) The Journal of Experimental Medicine 197: 789-799), with subfamily A corresponding to V2 and V3, and subfamily B to V1 (which is the most abundant). Of note, fHbps belonging to the same variant group share over 85% amino acid similarity, and only 60-70% similarity between the three variant groups. fHbp is also an antigen that elicits serum bactericidal antibody responses in immunised individuals and is a key component of investigational vaccines for the prevention of meningococcal, in particular serogroup B, disease that are currently being evaluated in clinical trials. However immunisation with a protein belonging to one variant fHbp family generates a variant specific response, with no cross-reactivity to other variant groups. So a single fHbp will not provide universal protection against meningococcal disease, so immunisation with representatives from each of the three variants, V1, V2 and V3, is necessary for a broad based vaccine.

20

To date, no vaccine studies have included V2 fHbp even though strains expressing this variant account for around 20-30% of all isolates.

It is therefore an aim of the present invention to provide a novel V2 fHbp, and in particular a novel V2 fHbp that can be used in an immunogenic composition, wherein the novel protein may then be used to elicit an immune response against *Neisseria meningitidis*. The immune response elicited against *Neisseria meningitidis* may be protective.

30 According to a first aspect, the present invention provides a modified V2 fHbp having increased stability over a wild type (WT) V2 fHbp.

A modified V2 fHbp with increased stability may be defined as a protein in which the maximum value of the left hand peak in a DSC analysis profile (corresponding to the N terminal beta barrel) is increased by at least about 5°C compared with a wild-type fHbp protein.

The DSC profile for a WT V2 fHbp shows the independent unfolding of the two barrels, the N terminal barrel and the C terminal barrel. In a WT fHbp the peak representing unfolding of the C-terminal barrel shows melting at temperatures above 80°C. In contrast, in WT V2 fHbp the N-terminal barrel exhibits a much reduced melting point, between 30°C and 40°C, indicating that this barrel readily unfolds. This unfolding of the N-terminal barrel in WT V2 fHbp may explain the increased susceptibility of the protein to proteolytic cleavage, as protease recognition sites in an unstable protein are accessible to the enzyme. In a modified V2 fHbp the maximum melting point of the N terminal barrel may be increased to between about 40°C and about 60°C.

According to another aspect, the present invention provides a modified V2 fHbp having increased immunogenicity over a wild type (WT) V2 fHbp.

The sequence of six WT V2 fHbps is given in Figure 3.

Reference herein to a modified V2 fHbp refers to a protein with an amino acid sequence that is not normally found in nature and instead is artificially produced and/or modified.

In one embodiment the modified V2 fHbp has one or more amino acid substitutions in the N terminal beta barrel. The amino acids which are

mutated may be internally positioned in the beta barrel when the protein is folded. Not more than four consecutive amino acids may be mutated.

The amino acid substitution may be at one or more of the amino acids at position 35, 36, 42, 43, 46, 107, 112, 114, 137 and 138 in a protein of Seq ID No: 1 or a protein with at least 85%, 90%, 95% or more identity to Seq ID No: 1.

The substitution may be at one or more of Ser35, Leu36, Asp42, Glu43, Arg46, Asp107, Val112, Leu114, Ser137, Gly138.

The substitution may be at one or more of Ser35, Leu36, Asp42, Gln43, Arg46, Asp107, Val112, Leu114, Ser137, Gly138. The substitution may be at Ser35.

The modified V2 fHbp may have a mutation at one or both of Ser¹³⁷ and Gly¹³⁸. The modified V2 fHbp may have a mutation at both of Ser¹³⁷ and Gly¹³⁸. The modified V2 fHbp may have a mutation at Ser¹³⁷.

The modified V2 fHbp may have a mutation at Ser35, Asp107, Ser137 and Gly138. The modified V2 fHbp may have a mutation at Ser35, Leu114, Ser137 and Gly138. The modified V2 fHbp may have a mutation at Ser35, Asp107, Leu114, Ser137 and Gly138. The modified V2 fHbp may have a mutation at Ser35, Asp107, Val112, Leu114, Ser137 and Gly138. The modified V2 fHbp may have a mutation at both of Val112 and Leu114.

The modified V2 fHbp may have one or both of the following mutations, Ser¹³⁷Gly and Gly¹³⁸Asp. The modified V2 fHbp may have both of the following mutations, Ser¹³⁷Gly and Gly¹³⁸Asp. The modified V2 fHbp may have the following mutations, Ser³⁵Gly, Asp¹⁰⁷Ser, Ser¹³⁷Gly and

Gly¹³⁸Asp. The modified V2 fHbp may have the following mutations, Ser³⁵Gly, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp. The modified V2 fHbp may have the following mutations, Ser³⁵Gly, Asp¹⁰⁷Ser, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp. The modified V2 fHbp may have the following mutations, Ser³⁵Gly, Asp¹⁰⁷Ser, Val¹¹²Thr, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp. The modified V2 fHbp may have the following mutations, Val¹¹²Thr and Leu¹¹⁴Phe. The modified V2 fHbp may have the following mutation, Ser¹³⁷Gly.

- 10 Alternatively, or additionally, one or more amino acid substitutions, insertions or deletion may be made in the linker region connecting the N and C terminal barrels. One or more amino acid substitutions, insertions or deletion may be made in amino acids 141 to 153 in a protein of Seq ID No: 1 or a protein with at least 85%, 90%, 95% or more identity to Seq ID No: 1.

Reference herein to a fHbp may refer to the factor H binding protein from *Neisseria meningitidis*.

- 20 The modified V2 fHbp may not be a chimeric protein. The modified V2 fHbp may not have beta barrels from different fHbp variants.

- In this invention at least one modified V2 fHbp may be used as an antigen. The fHbp may be further modified to prevent, or reduce, binding of fH to the fHbp. The binding of fH to the fHbp following administration of the fHbp in a vaccine formulation to a subject may impair the success of the vaccine. First, the presence of fH on the fHbp may limit recognition of critical epitopes on the factor H binding protein by the host immune system. Antibodies generated against these epitopes may be both bactericidal and inhibit fH binding to bacteria rendering them sensitive to

complement mediated lysis. Second, immune responses against the fHbp may also elicit responses against bound fH, in a manner similar to a hapten. This may lead to a potential, unwanted, autoimmune response. Finally, complement activation is involved in immune responses as
5 complement proteins have adjuvant activity. Reduction of complement activation at the site of immunisation by recruitment of fH could impair the level of immunity obtained following vaccination.

According to another aspect the invention provides a modified V2 fHbp
10 wherein both Ser¹³⁷ and Gly¹³⁸ are mutated, the mutations may comprise Ser¹³⁷Gly and Gly¹³⁸Asp.

According to another aspect the invention provides a modified V2 fHbp wherein Ser35, Asp107, Ser137 and Gly138 are mutated, or wherein
15 Ser35, Leu114, Ser137 and Gly138 are mutated, or wherein Ser35, Asp107, Leu114, Ser137 and Gly138 are mutated, or wherein Ser35, Asp107, Val112, Leu114, Ser137 and Gly138 are mutated, or wherein Val112 and Leu114 are mutated, or wherein Ser35 is mutated.

20 The mutations may comprise Ser¹³⁷Gly and/or Gly¹³⁸Asp; or Ser¹³⁷Gly and Gly¹³⁸Asp; or Ser³⁵Gly, Asp¹⁰⁷Ser, Ser¹³⁷Gly and Gly¹³⁸Asp; or Ser³⁵Gly, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp; or Ser³⁵Gly, Asp¹⁰⁷Ser, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp; or Ser³⁵Gly, Asp¹⁰⁷Ser, Val¹¹²Thr, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp; or Val¹¹²Thr and Leu¹¹⁴Phe; or Ser¹³⁷Gly.

25

The mutations may be in the N terminal beta barrel of the folded protein.

A V2 fHbp comprising both of the mutations Ser¹³⁷Gly and Gly¹³⁸Asp may be more stable than a V2 variant which does not.

30

The modified fHbp may have at least 80%, 85%, 90%, 95% or more sequence identity with a sequence of Figure 3, such as the sequence of Seq ID No: 1, but is modified in at least one position which increases the protein stability. The modification may be a different amino acid.

5

Percentage sequence identity is defined as the percentage of amino acids in a sequence that are identical with the amino acids in a provided sequence after aligning the sequences and introducing gaps if necessary to achieve the maximum percent sequence identity. Alignment for the purpose of determining percent sequence identity can be achieved in many ways that are well known to the man skilled in the art, and include, for example, using BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool).

10

15 Variations in percent identity may be due, for example, to amino acid substitutions, insertions or deletions. Amino acid substitutions may be conservative in nature, in that the substituted amino acid has similar structural and/or chemical properties, for example the substitution of leucine with isoleucine is a conservative substitution.

20

According to a further aspect, the invention provides an immunogenic composition comprising a modified V2 fHbp according to any aspect of the invention.

25 An immunogenic composition is a composition that is capable of eliciting an immune response to the modified V2 fHbp when the composition is administered to a subject. The subject may be a human or non-human animal. The subject may be a mammal.

30 The immune response elicited by the composition of the invention may affect the ability of *N. meningitidis* to infect an immunised human. The

ability of *N. meningitidis* to infect a human immunised with the composition of the invention may be impeded or prevented. This may be achieved in a number of ways. The immune response elicited may recognise and destroy *N. meningitidis*. Alternatively, or additionally, the immune response elicited may impede or prevent replication of *N. meningitidis*. Alternatively, or additionally, the immune response elicited may impede or prevent *N. meningitidis* causing disease in the human or non-human animal. The composition may elicit a bactericidal antibody response effective against bacterial strains expressing the V2 fHbp. The immune response elicited by the composition of the invention may be equivalent or greater than an immune response elicited by wild type V2 fHbp.

The modified fHbp may be recombinantly produced (e.g. from a genetically-engineered expression system) or be a synthetic product, for example produced by *in vitro* peptide synthesis or *in vitro* translation.

The composition of the invention may also comprise a further one or more antigens, in addition to the modified V2 fHbp. The further antigens may also be derived from *N. meningitidis* and may be capable of eliciting an immune response directed to *N. meningitidis*.

The composition may be used to elicit/produce a protective immune response when administered to a subject. The protective immune response may cause *N. meningitidis* to be killed upon infecting the subject, or it may prevent or inhibit *N. meningitidis* from replicating and/or from causing disease.

The composition may be used as a prophylactic or a therapeutic vaccine directed to *N. meningitidis*.

According to a further aspect, the invention provides a pharmaceutical composition comprising a modified V2 fHbp according to the invention and a pharmaceutically acceptable carrier or excipient.

- 5 The pharmaceutical composition may be capable of producing a protective immune response to *N. meningitidis*.

The phrase “producing a protective immune response” as used herein means that the composition is capable of generating a protective response
10 in a host organism, such as a human or a non-human mammal, to whom it is administered. A protective immune response may protect against subsequent infection by *N. meningitidis*. The protective immune response may eliminate or reduce the level of infection by reducing replication of *N. meningitidis* or by affecting the mode of action of *N. meningitidis* to
15 reduce disease.

Suitable acceptable excipients and carriers will be well known to those skilled in the art. These may include solid or liquid carriers. Suitable liquid carriers include water and saline. The proteins of the composition
20 may be formulated into an emulsion or they may be formulated into biodegradable microspheres or liposomes.

The composition may further comprise an adjuvant. Suitable adjuvants will be well known to those skilled in the art, and may include Freund's
25 Incomplete Adjuvant (for use in animals), and metal salts, such as aluminium or calcium salts.

The composition may also comprise polymers or other agents to control the consistency of the composition, and/or to control the release of the
30 antigen/secreted protein from the composition.

The composition may also comprise other agents such as diluents, which may include water, saline, glycerol or other suitable alcohols etc; wetting or emulsifying agents; buffering agents; thickening agents for example cellulose or cellulose derivatives; preservatives; detergents, antimicrobial
5 agents; and the like.

The active ingredients in the composition may be greater than 50% pure, usually greater than 80% pure, often greater than 90% pure. The active ingredients may be greater than 95%, 98% or 99% pure. With active
10 ingredients approaching 100% pure, for example about 99.5% pure or about 99.9% pure, being used most often.

The composition of the present invention may be used as vaccine against infections caused by *N. meningitidis*. The composition may be used as a
15 vaccine directed to meningitis or other invasive meningococcal diseases including septicaemia or septic shock. The vaccine may be administered prophylactically to those at risk of exposure to *N. meningitidis*, and/or therapeutically to persons who have already been exposed to *N. meningitidis*.

20

If the composition is used as a vaccine, the composition may comprise an immunologically effective amount of antigen wherein the composition comprises at least one modified V2 fHbp. An “immunologically effective amount” of an antigen is an amount that when administered to an
25 individual, either in a single dose or in a series of doses, is effective for treatment or prevention of infection by *N. meningitidis*. This amount will vary depending upon the health and physical condition of the individual to be treated and on the antigen. Determination of an effective amount of an immunogenic or vaccine composition for administration to an organism is
30 well within the capabilities of those skilled in the art.

A composition according to the invention may be for oral, systemic, parenteral, topical, mucosal, intramuscular, intravenous, intraperitoneal, intradermal, subcutaneous, intranasal, intravaginal, intrarectal, transdermal, sublingual, inhalation or aerosol administration.

5

The composition may be arranged to be administered as a single dose or as part of a multiple dose schedule. Multiple doses may be administered as a primary immunisation followed by one or more booster immunisations. Suitable timings between priming and boosting
10 immunisations can be routinely determined.

A composition according to the invention may be used in isolation, or it may be combined with one or more other immunogenic or vaccine compositions, and/or with one or more other therapeutic regimes.

15

Compositions of the invention may be able to induce a serum bactericidal antibody responses and elicit antibodies which mediate opsonphagocytosis after being administered to a subject. These responses can be conveniently measured in mice and the results are a standard indicator of
20 vaccine efficacy.

The compositions of the invention may also, or alternatively, be able to elicit an immune response which neutralises bacterial proteins or other molecules, thereby preventing them from having their normal function
25 and preventing or reducing disease progression without necessarily destroying the pathogenic organism/bacteria, in this case to *N. meningitidis*.

A composition according to the invention may also comprise a further one
30 or more antigens, in addition to the modified V2 fHbp. The further antigens may also be derived from *N. meningitidis* and may be capable of

eliciting an immune response directed to *N. meningitidis*. The further antigens may include one or more of a V1 fHbp and a V3 fHbp.

According to a further aspect, the present invention provides the use of
5 one or more modified V2 fHBps in the preparation of a medicament for eliciting an immune response. The medicament may be used for the prophylactic or therapeutic vaccination of subjects against *N. meningitidis*. The medicament may be a prophylactic or a therapeutic vaccine. The vaccine may be for meningitis, septicaemia and/or septic
10 shock caused by *N. meningitidis*.

According to a yet further aspect, the invention provides a composition comprising one or more modified V2 fHbp for use in generating an immune response to *N. meningitidis*. The immune response may be
15 prophylactic or therapeutic. The composition may be for use as a vaccine.

According a still further aspect, the present invention provides a method of protecting a human or non-human animal from the effects of infection by *N. meningitidis* comprising administering to the human or non-human
20 animal a composition according to any aspect of the invention or a modified occurring V2 fHbp according to any aspect of the invention. The composition may be a vaccine.

According to another aspect, the invention provides a method for raising
25 an immune response in a human or non-human animal comprising administering a pharmaceutical composition according to the invention to the human or non-human animal. The immune response is may be protective. The method may raise a booster response in a patient that has already been primed. The immune response may be prophylactic or
30 therapeutic.

One way to check the efficacy of a therapeutic treatment comprising administration of a composition according to the invention involves monitoring for *N. meningitidis* infection after administration of the composition. One way to check the efficacy of a prophylactic treatment
5 comprising administration of a composition according to the invention involves monitoring immune responses to *Neisseria meningitidis* after administration of the composition.

According to another aspect, the invention provides the use of one or
10 more modified V2 fHbps in the preparation of a medicament for use in the immunisation of human or non-human mammals against infection by *N. meningitidis*.

According to a further aspect the invention provides a kit for use in
15 inducing an immune response in an organism, comprising an immunogenic or vaccine composition according to the invention and instructions relating to administration.

In addition to their potential use as vaccines, compositions according to
20 the invention may be useful as diagnostic reagents and as a measure of the immune competence of a vaccine.

According to another aspect the invention provides a nucleic acid
encoding a modified V2 fHbp according to the invention.

25

According to a still further aspect the invention provides a host cell containing the above nucleic acid.

The skilled man will appreciate that any of the optional features discussed
30 above can be applied to any of the aspects or embodiments of the invention.

Embodiments of the present invention will now be described, merely by way of example, with reference to the following figures and examples.

5 **Figure 1A** - DSC of a wild-type V2 protein (green/dark trace) and the modified fHbp V2MUT (red/light trace, with the modifications Ser¹³⁷Gly and Gly¹³⁸Asp) which contains two amino acid changes in the N terminal barrel. The two peaks for each protein represent unfolding of the N terminal barrel (left hand peak, at lower
10 temperatures) and C terminal barrel (right hand peak). Modification of the V2 protein increases the peak associated with the C terminal barrel and shifts the peak to higher temperatures.
15 **Figure 1B** - DSC of V1 (red line), V2 (blue line) and V3 (green line) fHbp showing unfolding of the C-terminal barrel at around 80oC for all variants, and the N-terminal barrel at lower temperatures.

20 **Figure 2** - Sensitivity of the wild-type (V2WT) and stabilised (V2MUT) V2 protein to proteolytic cleavage. Proteins were incubated in the presence of the protease for increasing concentrations of tyrpsin (right to left) and analysed by SDS:PAGE.

25 **Figure 3** - shows sequences of some WT V2 fHbps, identical amino acids are shown with asterisks. FHbp_V2_peptide_22 is referred to herein as Seq ID No: 1. FHbp_V2_peptide_23 is referred to herein as Seq ID No: 2. FHbp_V2_peptide_25 is referred to herein as Seq ID No: 3. FHbp_V2_peptide_21 is referred to herein as Seq ID No: 4. FHbp_V2_peptide_16 is
30 referred to herein as Seq ID No: 5. FHbp_V2_peptide_19 is referred to herein as Seq ID No: 6. In all sequence the N terminal

beta barrel spans amino acids numbers 20 to 141, and amino acids residues 141 to 153 represent a linked between the N and C terminal beta barrels.

5 **Figure 4** - shows a cartoon representation of V1 fHbp structure with the six V2 fHbp stabilising amino acid changes mapped on it. Figure created using PyMol based on pdb entry 2W80.

10 **Figure 5** - illustrates the effect of stabilising mutations in V2 fHbp. The heat capacity (Cp) of V2 fHbp mutants was measured by DSC. **Figure 5A** - V2 fHbp with single and double amino acid substitutions. **Figure 5B** - Four combinations of mutations that showed the greatest increase in stability. **Figure 5C** - M6 compared to WT V1, V2 and V3 fHbp.

15 **Figure 6** - shows the susceptibility of stabilised V2 fHbps to protease digestion. SDS:PAGE stained with Coomassie blue of V2 fHbp following protease treatment in serial dilutions (lanes 3-10).

20 **Figure 7** - shows SBA titres of mice. Animals received different doses of the wild-type and M6 V2 fHbp (shown) on three occasions, and SBA was determined for pooled sera against a clinical isolate expressing V2 fHbp. Error bars: SD of assays performed in triplicate.

25 The data presented herein demonstrates the inherent instability of the WT V2 fHbp, in particular the instability of the N terminal beta barrel. This instability demonstrates its unsuitability for vaccine development, both for reliable antigen product and because several bactericidal antibodies are
30 known to require conformational epitopes involving both the N and C terminal beta barrels.

Further more, the data also demonstrates that by mutating the amino acid sequence of the N terminal beta barrel the stability of the V2 fHbp can be significantly increased. The stabilised protein can then be used as an immunogen.

Stable Modified V2 fHbp

WT V2 fHbp with the sequence of Figure 3, Seq ID No: 1, was mutated using PCR to change the amino acid at position 137 from serine to glycine and the amino acid a position 138 from glycine to aspartic acid. Both of these mutations are in the N terminal beta barrel of the protein. The modified protein produced is referred to herein as V2MUT.

The stability of the mutant produced was assessed by differential scanning calorimetry (DSC, Figure 1) and by susceptibility to protease cleavage (Figure 2).

Figure 1 clearly demonstrates that the V2MUT protein has dramatically increase stability compared to the wild type protein. The N terminal barrel has a mean melting temperature of around 50°C, compared to about 35°C for the wild type protein. No significant change in stability was seen in the C terminal barrel of the V2MUT protein.

The improved stability of the mutant V2 fHbp is further supported by the results of proteolytic cleavage study (Figure 2). More cleavage product (lower band,) is generated when the wild-type protein is incubated with protease than when the V2MUT protein is treated under identical conditions. This demonstrates that V2MUT is less susceptible to digestion, due to increased stability of the protein.

Efficacy of V2MUT as an Immunogen

Female six to eight-week-old BALB/c mice (Charles Rivers) are immunised with antigens (20 μ g of WT V2 fHbp or V2MUT fHbp) with
5 aluminium hydroxide adsorbed by spinning the mixture for one hour at room temperature. Immunogens are given intraperitoneally (transgenic mice) on days 0, 21 and 35; sera are collected on day 49. In immunisation studies with C57Bl/6 transgenic mice, antigens are given intraperitoneally to twelve to sixteen-weeks-old mice on days 0, 21 and
10 35, and whole blood collected by terminal anaesthesia and cardiac puncture from the mice on day 49. All procedures are conducted in accordance with Home Office guidelines.

Wells of ELISA plates (Nunc) are coated with mutant V2 (V2MUT) fHbp
15 (100 ng) overnight at 4°C, washed, blocked for one hour with 3% normal goat serum diluted in PBS-T, then sera added at a range of dilutions. Binding is detected using goat anti-mouse HRP-conjugated IgG (Dako, 1 in 1, 000) and incubated for one hour at room temperature. The substrate (ONPG, Sigma) is added to wells, the reaction is stopped with 3N HCl,
20 and the A492 read with a Multiskan photometer (Thermo Scientific).

For serum bactericidal assays, *N. meningitidis* MC58 is re-suspended in SBA assay buffer (0.1% glucose in PBS) to a final concentration of 5×10^4 CFU/ml and mixed with an equal volume of human complement.
25 Control wells are also prepared containing bacteria without serum or without complement. Sera are pooled from groups of non-transgenic mice ($n > 8$), and immunisations repeated on two or three occasions for each antigen; for transgenic mice, sera are tested from individual animals. Following incubation, 10 μ l from each well is plated onto solid media,
30 and the number of surviving bacteria is determined after overnight

growth. The bactericidal activity is expressed as the reciprocal of the highest dilution of sera required to kill more than 50% of bacteria.

- Mice immunised as described above with the WT and mutant (V2MUT)
- 5 V2 fHbp will demonstrate that the stabilised modified occurring V2 fHbp (V2MUT) is significantly more immunogenic than the WT fHbp.

Further studies

- 10 V2 fHbp is not included in either of the two vaccines currently undergoing clinical trials. This could be because its inherent instability prevents it from being a good vaccine candidate (Johnson et al, 2012). Vaccine coverage could be improved by including V2 fHbps as 20-30% of *N. meningitidis* isolates express V2 fHbps. In order to do this V2 fHbp
- 15 would need to be stabilised.

Previously, we found that a region of V2 fHbp is involved in its susceptibility to trypsin digestion. This region was used as the site for a large scale screen to identify mutations that increase V2 fHbp's stability.

- 20 Amino acids in this region were mutated to their equivalent residue in V1 Six fHbp. mutations (S35G, D107S, V112T, L114F, S137G, G138D) were identified that enhance the stability of V2 fHbp (Fig. 4). These six mutations are all in the N terminal barrel of fHbp.

- 25 To identify the most effective combination of changes for stabilising V2 fHbp, 13 V2 fHbps were expressed (Table 1) with different combinations of these six amino acid changes.

Name of mutant	Amino acid changes
M4A	S35G/D107S/S137G/G138D

M4B	S35G/L114F/S137G/G138D
M5	S35G/D107S/L114F/S137G/G138D
M6	S35G/D107S/V112T/L114F/S137G/G138D
V112T	V112T
D107S	D107S
L114F	L114F
S35G	S35G
S137G/G138D	S137G/G138D
V112T/L114F	V112T/L114F
D107S/L114F	D107S/L114F
S35G/D107S	S35G/D107S
S35G/D107S/L114F	S35G/D107S/L114F

Table 1: Stabilised V2 fHbps. The stability of these V2 fHbp mutants was analysed. The table contains the name of the mutant and the amino acid changes made in WT V2 fHbp.

- 5 The stability of the V2 fHbps was assessed by Differential Scanning Calorimetry (DSC) to determine the melting temperatures of the two α barrels. The N terminal α barrel of WT fHbp melts first (T_{m1}) at 37.30°C, while the C terminal α barrel melts (T_{m2}) at 83.06°C. This indicates that the N terminal barrel is less stable than the C terminal
- 10 barrel. The DSC profiles were determined for all thirteen mutants. The DSC profiles of four mutants, that contain the six mutations in single or double combinations, are shown in fig. 5A. The increased T_{m1} value relative to WT V2 fHbp indicates an increase in stability of the N terminal barrel. There was no change in the T_{m2} values between the WT
- 15 and the mutants, showing there is no change in the stability of the C terminal barrel. The DSC profiles of all thirteen mutants (not shown) showed that the four mutants that had the greatest increase in stability were: M4A, M4B, M5 and M6 (Fig.5B). Their T_{m1} values (Table 2) had the largest increase, indicating greater stability of their N terminal barrel.

From DSC M6 was more stable than either M4A, M4B or M5 with a T_{m1} of 55.96°C for M6 compared with 54.03, 54.79, 55.46°C for M4A, M4B and M5 respectively. The T_{m1} value of M6 is lower than the T_{m1} values of WT V1 and V3 fHbp (Fig. 5C), indicating that its N terminal barrel is less stable.

mutant	T_{m1} (°C)	$\Delta H1$ (kJ)	T_{m2} (°C)	$\Delta H2$ (kJ)
WT	37.30	24.6	83.06	101.7
L114F	36.06	32.7	83.06	114.6
D107S/L114F	39.35	44.3	82.95	96.8
V112T	39.95	22.7	83.00	107.6
D107S	40.23	48.4	83.14	109.5
S35G	42.14	49.2	83.20	111.2
S35G/D107S/L114F	42.50	50.5	82.90	101.8
V112T/L114F	43.81	44.6	83.08	101.2
S35G/D107S	47.26	66.2	83.05	115.7
S137G/G138D	49.56	58.1	82.54	111.5
M4A	54.03	70.6	82.56	104.6
M4B	54.79	70.4	82.57	105.0
M5	55.46	76.2	82.40	100.9
M6	55.96	79.6	82.56	108.7

Table 2: Melting temperature of the N and C terminal α -barrels (T_{m1} and T_{m2} , respectively) and the enthalpy of melting of the N and C terminal α -barrels ($\Delta H1$ and $\Delta H2$, respectively) of stabilised V2 fHbp mutants in comparison with WT V2 fHbp.

Selecting the most stable V2 fHbp

To further define the stability of the four most stable V2 fHbp mutants, protease digestion assays were performed. Five different proteases were

used: trypsin, proteinase K, collagenase, dispase and papain. All four mutants showed enhanced resistance to protease digestion compared to WT V2 fHbp. M6 was the least susceptible to protease digestion with collagenase, proteinase K, dispase, and papain (Fig. 6). All four mutants (M4A, M4B, M5 and M6) showed enhanced resistance to trypsin digestion in comparison with WT V2 fHbp, but there was no clear difference between them with this protease. For proteinase K, dispase and papain, M6 showed the most resistance to protease digestion, followed by M5, consistent with V2 fHbp mutants containing more mutations being more stable. M4A and M4B show intermediate levels of susceptibility. In summary both DSC and protease digestion indicate that the V2 fHbp mutant with six stabilising amino acid changes (M6) is the most stable.

fH binding to stable V2 fHbps

To determine the effect of stabilising V2 fHbp on fH binding, SPR was carried out using the four most stable V2 fHbp mutants immobilised on the sensor chip and flowing fH₆₇ over them. K_d values for fH₆₇ binding to the stable V2 fHbps were calculated from the sensogram (fH₆₇ binding to WT V2 fHbp shown in Appendix 3a) and compared to fH₆₇ binding to WT V2 fHbp (Table 3). The K_d values for M4A, M4B and M5 were between two and four fold higher (indicating a lower binding affinity) than for WT V2 fHbp, whilst the K_d for M6 is almost twenty fold higher. The decrease in fH₆₇ binding of M6 is potentially beneficial as this will help to produce a stable V2 fHbp vaccine candidate that does not bind fH.

Protein	Average K_d (nM)	Relative K_d
WT	3.18 ± 0.11	1.00

M4A	7.60 ± 0.17	2.39
M4B	12.50 ± 0.21	3.92
M5	12.20 ± 0.71	3.84
M6	61.30 ± 6.86	19.29

Table 3: fH binding to V2 fHbps: table of K_d values and relative K_d values for the V2 fHbps in comparison with WT V2 fHbp. Mean ± standard deviation of two determinations.

5 Immunogenicity of M6 in comparison with WT V2 fHbp

The immunogenicity of M6 was compared with WT V2 fHbp. Non transgenic mice were immunised with three different amounts of both WT and M6 V2 fHbp in order to establish a dose response. To assess the immune response, the serum bactericidal activity (SBA) of sera from immunised mice was determined. The SBA is a correlate of protection against meningococcal disease. The results show that M6 V2 fHbp has equivalent or better immunogenicity than the wild-type protein because SBA is still detected in mice vaccinated with the lowest dose of M6 V2 fHbp, while mice receiving the same amount of the wild-type protein fail to elicit SBA (Fig. 7).

MATERIALS AND METHODS

Bacterial growth and Western analysis

N. meningitidis was grown in 5% CO₂ on Brain Heart Infusion (BHI) agar plates with Levanthal's supplement, and *Escherichia coli* propagated in LB liquid medium with shaking at 200 r.p.m. or on LB agar plates (1.5% agar wt/vol). Whole cell lysates were prepared of *N. meningitidis* grown overnight on solid media then re-suspended in PBS. The concentration of bacteria was determined by measuring the optical density at 260 nm of bacterial lysates in 1% SDS/0.1M NaOH [15] and adjusted to 1 x 10⁹ CFU/ml, and re-suspended with an equal volume of 2x SDS-PAGE loading buffer (100 mM Tris-HCl pH 6.8, 20 μM β-mercaptoethanol, 4%

SDS, 0.2% bromophenol blue, 20% glycerol), and boiled for 10 minutes; polyacrylamide gels which were either stained with Coomassie blue or proteins were transferred to nitrocellulose membranes in a Mini Trans-Blot Cell. Membranes were incubated with primary then secondary
5 antibodies diluted in PBS-T and 1% skimmed milk (PBS-TM), which were detected using Amersham ECL Western blot detection method (GE Healthcare). To detect fH binding, blots were incubated in either normal human serum (NHS 1:100), purified (5 μ g/ml) or recombinant fH diluted in PBS-TM for two hours, washed then incubated with goat anti-fH pAb
10 (Quidel, 1 in 2,000); membranes were then incubated with murine anti-goat HRP-conjugated IgG (Sigma, 1 in 10,000).

Modification of fHbp and fH

Point mutations in fHbp were introduced by site directed PCR
15 mutagenesis with Roche Expand High Fidelity enzyme or using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer's protocols, and the primers

Stijn-2.P22-S137G/G138D-fwd:

CGCTCCTTCCTTGTCGGCGATTGTTGGGTGGAGAACAT

20 Stijn-2.P22-S137G/G138D-rev:

ATGTTCTCCACCCAAATCGCCGACAAGGAAGGAGCG

His-tagged proteins were expressed in E. coli B834 (DE3) cells and isolated using Ni-NTA Magnetic Agarose Beads (Qiagen) following the manufacturer's protocols and dialysed against 50 mM Sodium acetate,
25 pH 4.5.

DSC analysis

DSC experiments were carried out using a VP Capillary DSC (GEHealthcare) using a heating rate of 1 °C/min from 30 to 110°C. The

V2 sample was repeated from 10 to 110°C when its lower melting event was identified at around 35°C to ensure that this transition was flanked by sufficient baseline to allow analysis. Samples contained 20uM of each variant in 25mM Tris pH7.5, 150mM NaCl. Samples and buffer were
5 degassed by stirring under vacuum before running. Data analysis was done with the software supplied with the instrument by the manufacturers (Origin version 7.0) with buffer reference subtracted from the sample data and baseline correction.

10 **Proteolytic cleavage**

The sensitivity of WT and mutant (V2MUT) V2 fHbp to proteolytic cleavage was investigated by trypsin digestion. Five µg of fHbp V2 protein was incubated with trypsin ranging from 50 ng to 0.2 ng for 1 h at 37°C and cleavage was visualised by SDS:PAGE.

15

Reference

Design and evaluation of meningococcal vaccines through structure-based modification of host and pathogen molecules. Johnson S, Tan L, van der Veen S, Caesar J, Goicoechea De Jorge E, Harding RJ, Bai X, Exley
20 RM, Ward PN, Ruivo N, Trivedi K, Cumber E, Jones R, Newham L, Staunton D, Ufret-Vincenty R, Borrow R, Pickering MC, Lea SM, Tang CM.

PLoS Pathog. 2012;8(10):e1002981.

CLAIMS

1. A modified V2 fHbp having increased stability over a wild type V2 fHbp.
2. The modified V2 fHbp of claim 1 wherein the maximum value of the left hand peak in a DSC analysis profile of the protein is increased by at least about 5°C compared with a wild-type fHbp.
3. The modified V2 fHbp of any of claims 1 or 2 comprising one or more amino acid substitutions in the N terminal beta barrel.
4. The modified V2 fHbp of any preceding claim comprising one or more amino acid substitutions at position 35, 36, 42, 43, 46, 107, 112, 114, 137 and 138 in a protein of Seq ID No: 1 or a protein with at least 85%, 90%, 95% or more identity to Seq ID No: 1.
5. The modified V2 fHbp of claim 4 comprising a mutation at one or both of Ser¹³⁷ and Gly¹³⁸.
6. The modified V2 fHbp of claim 5 comprising both of the mutations Ser¹³⁷Gly and Gly¹³⁸Asp.
7. The modified V2 fHbp of claim 4 comprising a mutation at Ser35, Asp107, Ser137 and Gly138; and optionally wherein the mutations comprise Ser³⁵Gly, Asp¹⁰⁷Ser, Ser¹³⁷Gly and Gly¹³⁸Asp.
8. The modified V2 fHbp of claim 4 comprising a mutation at Ser35, Leu114, Ser137 and Gly138; and optionally wherein the mutations comprise Ser³⁵Gly, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp.

9. The modified V2 fHbp of claim 4 comprising a mutation at Ser³⁵, Asp¹⁰⁷, Leu¹¹⁴, Ser¹³⁷ and Gly¹³⁸; and optionally wherein the mutations comprise Ser³⁵Gly, Asp¹⁰⁷Ser, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp.

10. The modified V2 fHbp of claim 4 comprising a mutation at Ser³⁵, Asp¹⁰⁷, Val¹¹², Leu¹¹⁴, Ser¹³⁷ and Gly¹³⁸; and optionally wherein the mutations comprise Ser³⁵Gly, Asp¹⁰⁷Ser, Val¹¹²Thr, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp.

11. The modified V2 fHbp of claim 4 comprising a mutation at Val¹¹² and Leu¹¹⁴; and optionally wherein the mutations comprise Val¹¹²Thr and Leu¹¹⁴Phe.

12. A modified V2 fHbp having an amino acid sequence with at least 85% identity to the sequence of Seq ID No: 1, wherein both Ser¹³⁷ and Gly¹³⁸ are mutated.

13. The modified V2 fHbp of claim 12 wherein the mutations are Ser¹³⁷Gly and Gly¹³⁸Asp.

14. The modified V2 fHbp of claim 12 wherein Ser³⁵, Asp¹⁰⁷, Ser¹³⁷ and Gly¹³⁸ are mutated; and optionally wherein the mutations are Ser³⁵Gly, Asp¹⁰⁷Ser, Ser¹³⁷Gly and Gly¹³⁸Asp.

15. The modified V2 fHbp of claim 12 wherein Ser³⁵, Leu¹¹⁴, Ser¹³⁷ and Gly¹³⁸ are mutated; and optionally wherein the mutations are Ser³⁵Gly, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp.

16. The modified V2 fHbp of claim 12 wherein Ser³⁵, Asp¹⁰⁷, Leu¹¹⁴, Ser¹³⁷ and Gly¹³⁸ are mutated; and optionally wherein the mutations are Ser³⁵Gly, Asp¹⁰⁷Ser, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp.

17. The modified V2 fHbp of claim 12 wherein Ser³⁵, Asp¹⁰⁷, Val¹¹², Leu¹¹⁴, Ser¹³⁷ and Gly¹³⁸ are mutated; and optionally wherein the mutations are Ser³⁵Gly, Asp¹⁰⁷Ser, Val¹¹²Thr, Leu¹¹⁴Phe, Ser¹³⁷Gly and Gly¹³⁸Asp.

18. A modified V2 fHbp having an amino acid sequence with at least 85% identity to the sequence of Seq ID No: 1, wherein both Val¹¹² and Leu¹¹⁴ are mutated; and optionally wherein the mutations are Val¹¹²Thr and Leu¹¹⁴Phe.

19. An immunogenic composition comprising a modified V2 fHbp according to any preceding claim.

20. The immunogenic composition of claim 19, wherein the composition that is capable of eliciting a bactericidal antibody response to *N. meningitidis*.

21. The immunogenic composition of claim 19 or 20 comprising a further one or more antigens in addition to the modified V2 fHbp.

22. A prophylactic or a therapeutic vaccine directed to *N. meningitidis* comprising a modified V2 fHbp according to any of claims 1 to 18.

23. A pharmaceutical composition comprising a modified V2 fHbp according to any of claims 1 to 18 and a pharmaceutically acceptable carrier or excipient.

24. A pharmaceutical composition according to claim 23 which is capable of producing a protective immune response to *N. meningitidis*.

25. The use of one or more modified V2 fHbps according to any of claims 1 to 18 in the preparation of a medicament for eliciting an immune response.

26. The use of claim 25 wherein the immune response elicited is to *N. meningitidis*.

27. A composition comprising one or more modified V2 fHbp according to any of claims 1 to 18 for use in generating an immune response to *N. meningitidis*.

28. A method of protecting a human or non-human animal from the effects of infection by *N. meningitidis* comprising administering to the human or non-human animal a modified V2 fHbp according to any of claims 1 to 18.

29. The use of one or more modified V2 fHbps according to any of claims 1 to 18 in the preparation of a medicament for use in the immunisation of human or non-human mammals against infection by *N. meningitidis*.

30. A nucleic acid encoding a modified V2 fHbp according to any of claims 1 to 18.

31. A host cell containing a nucleic acid according to claim 30.

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Figure 1A

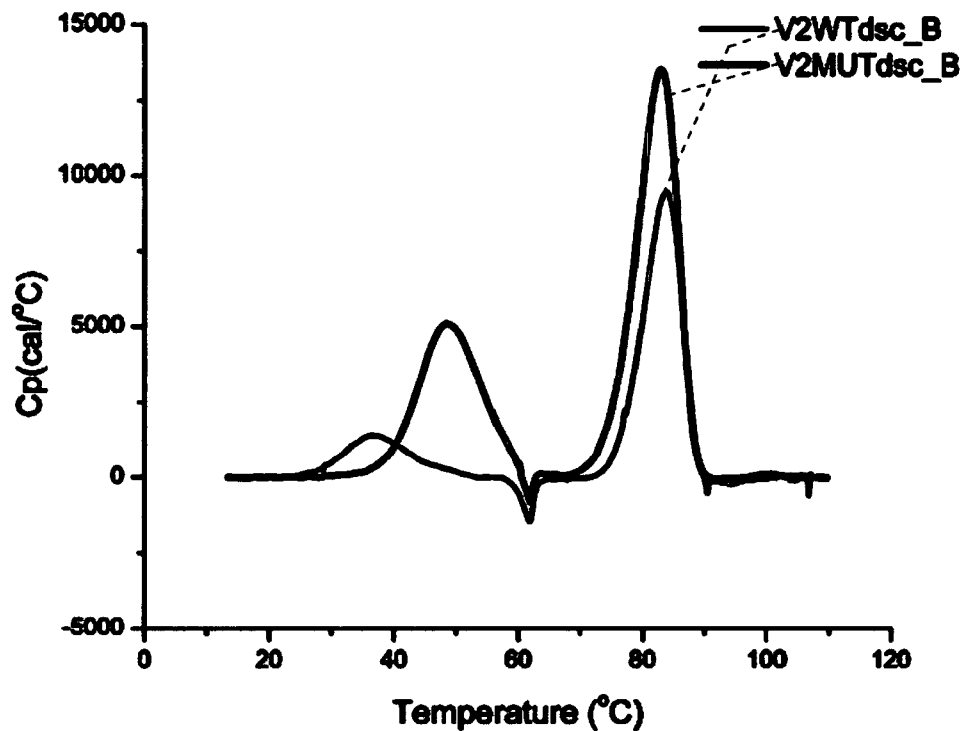
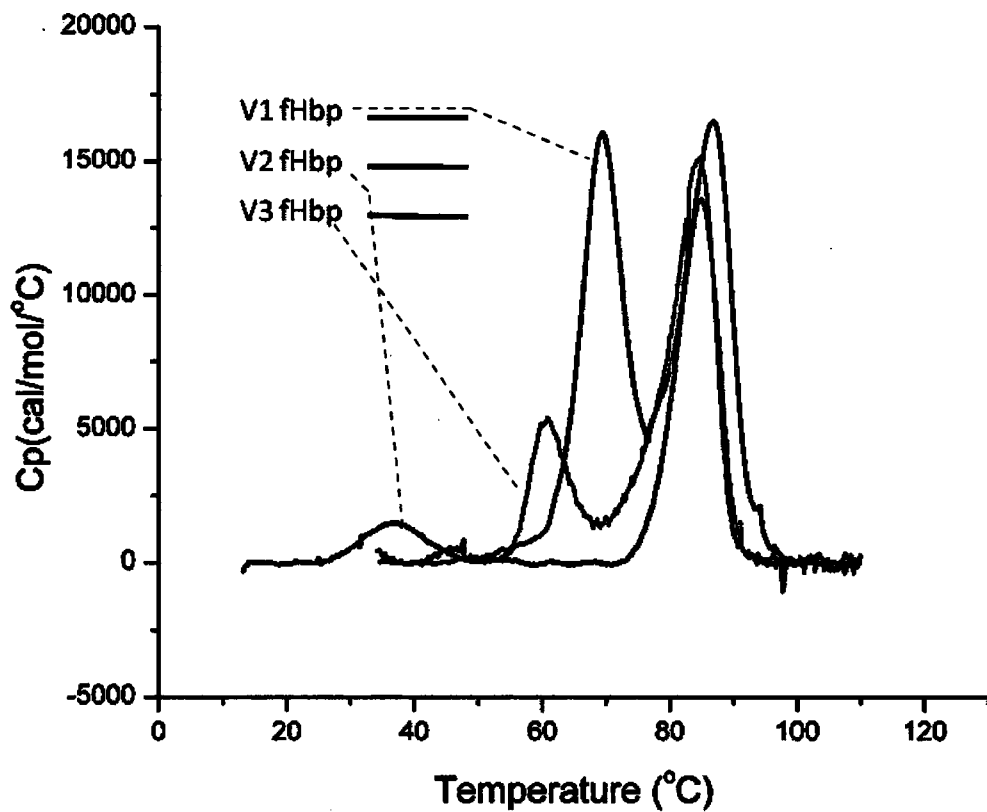


Figure 1B



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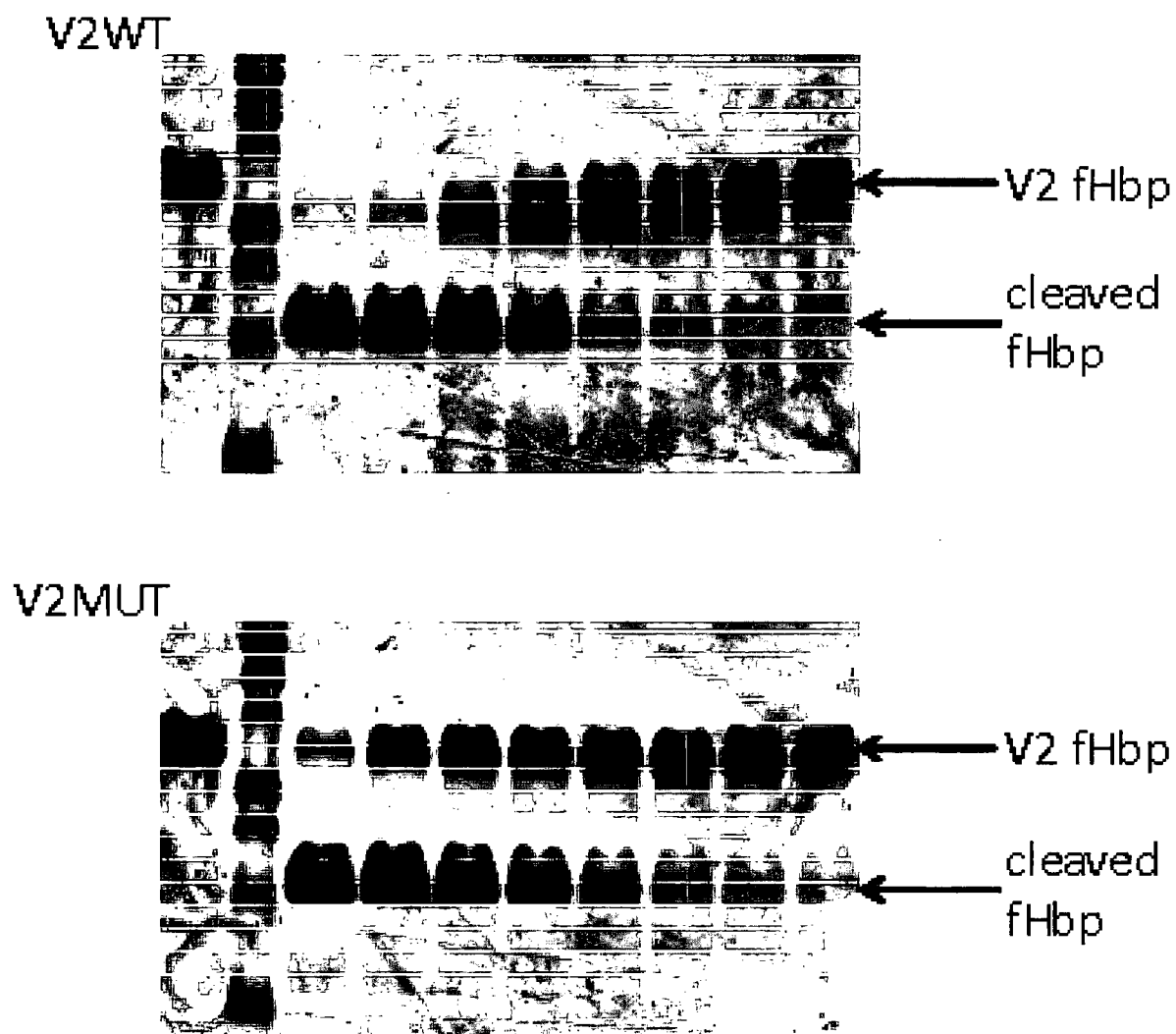


Figure 2

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FHbp_V2 2.P22 5 CSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAQAQGAEKTYGNG 65
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FHbp_V2 2.P25 5 CSSGGGGVAADIGAGLADALTTPLDHKDKSLQSLTLDQSVRKNEKLKLAQAQGAEKTYGNG 65
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*****:*****.*****

FHbp_V2 2.P22 66 DSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKI 125
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***** *** *****

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Figure 3

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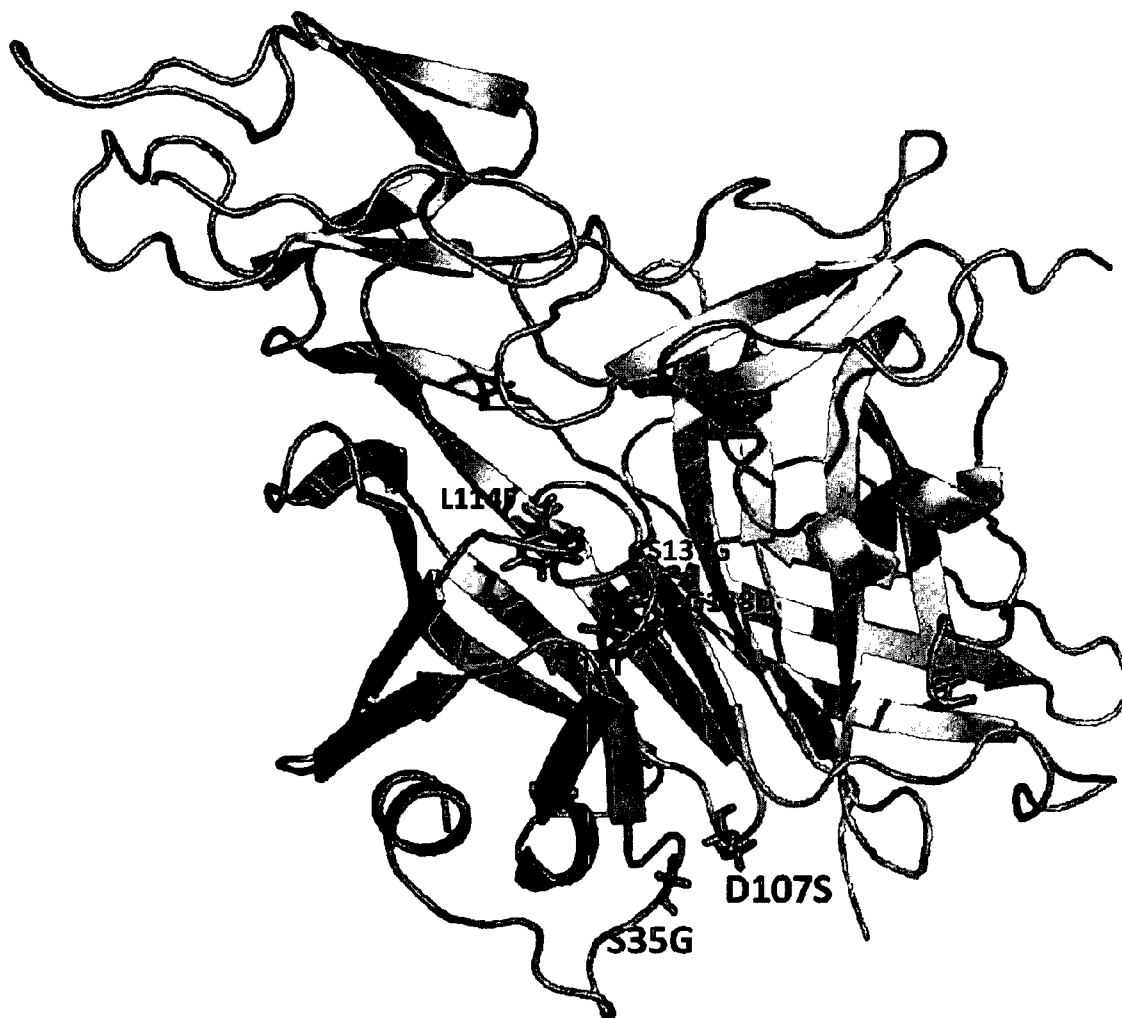


Figure 4

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Figure 5A

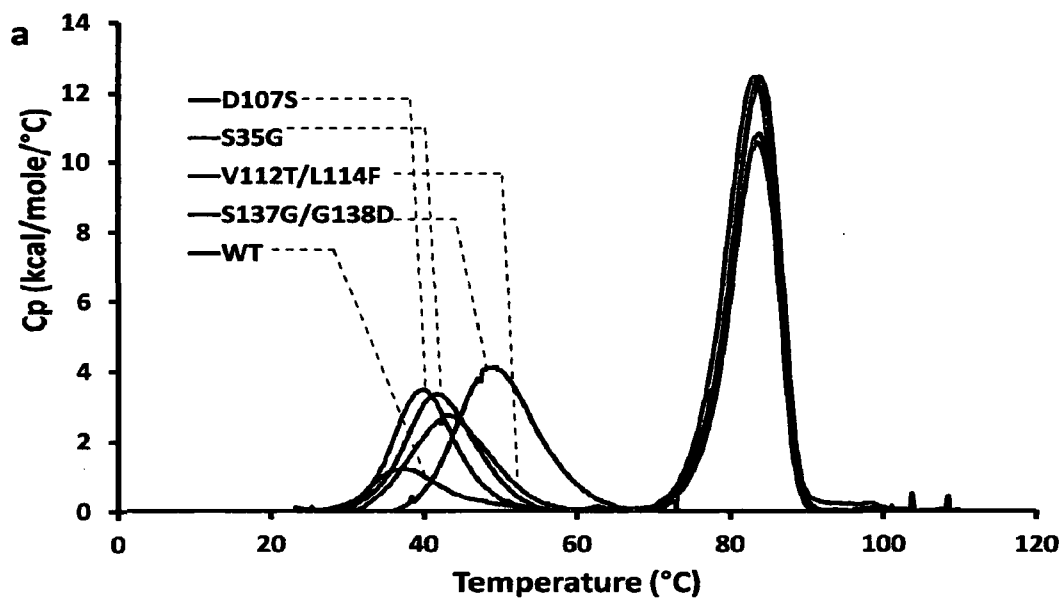
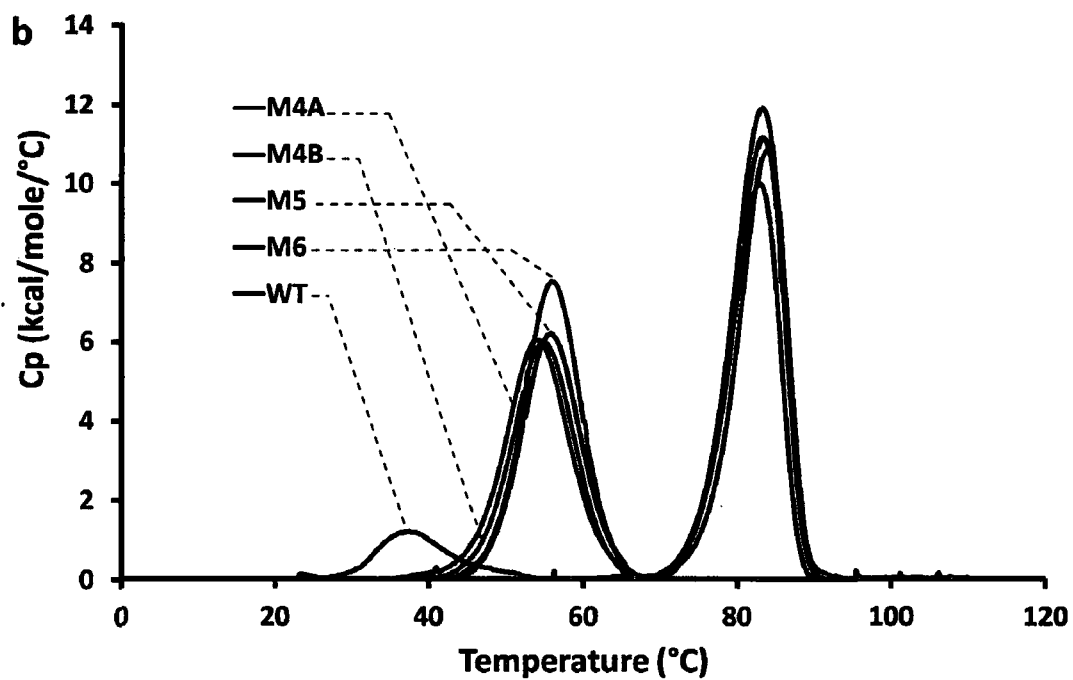
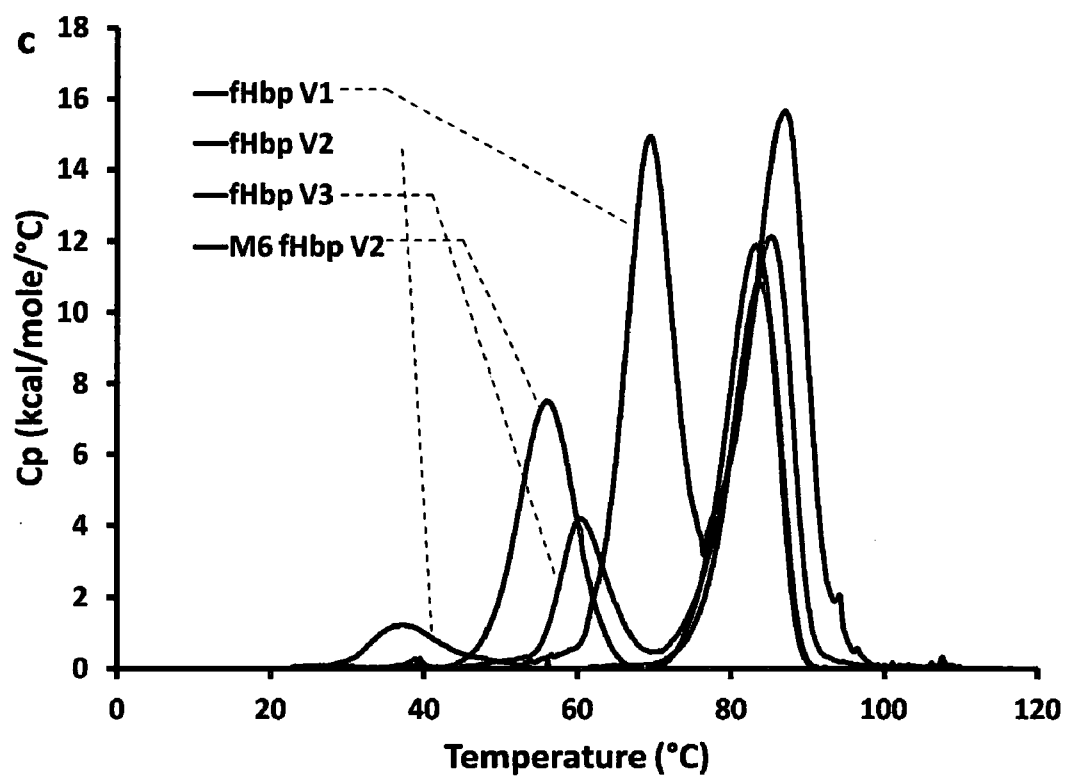


Figure 5B

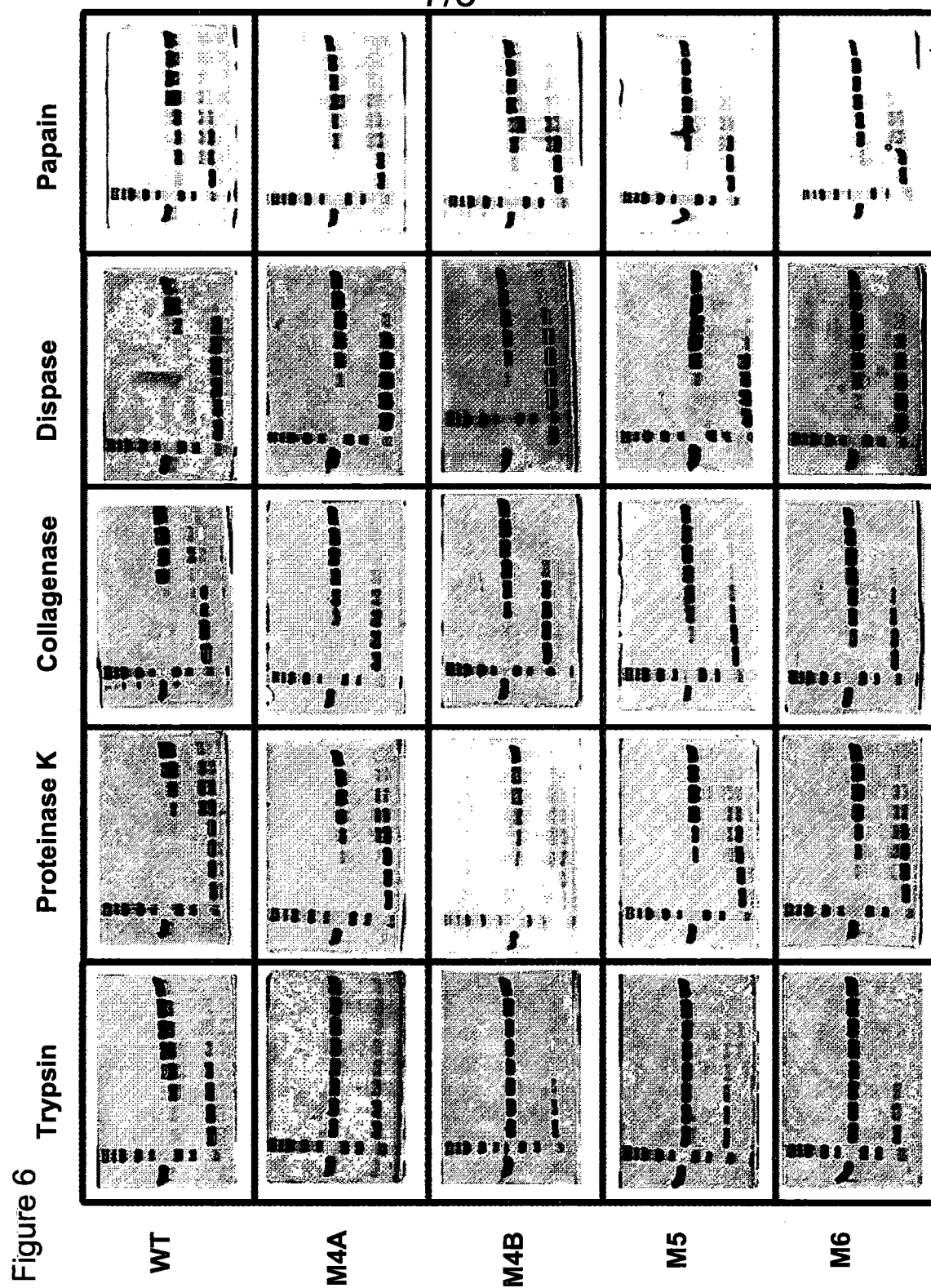


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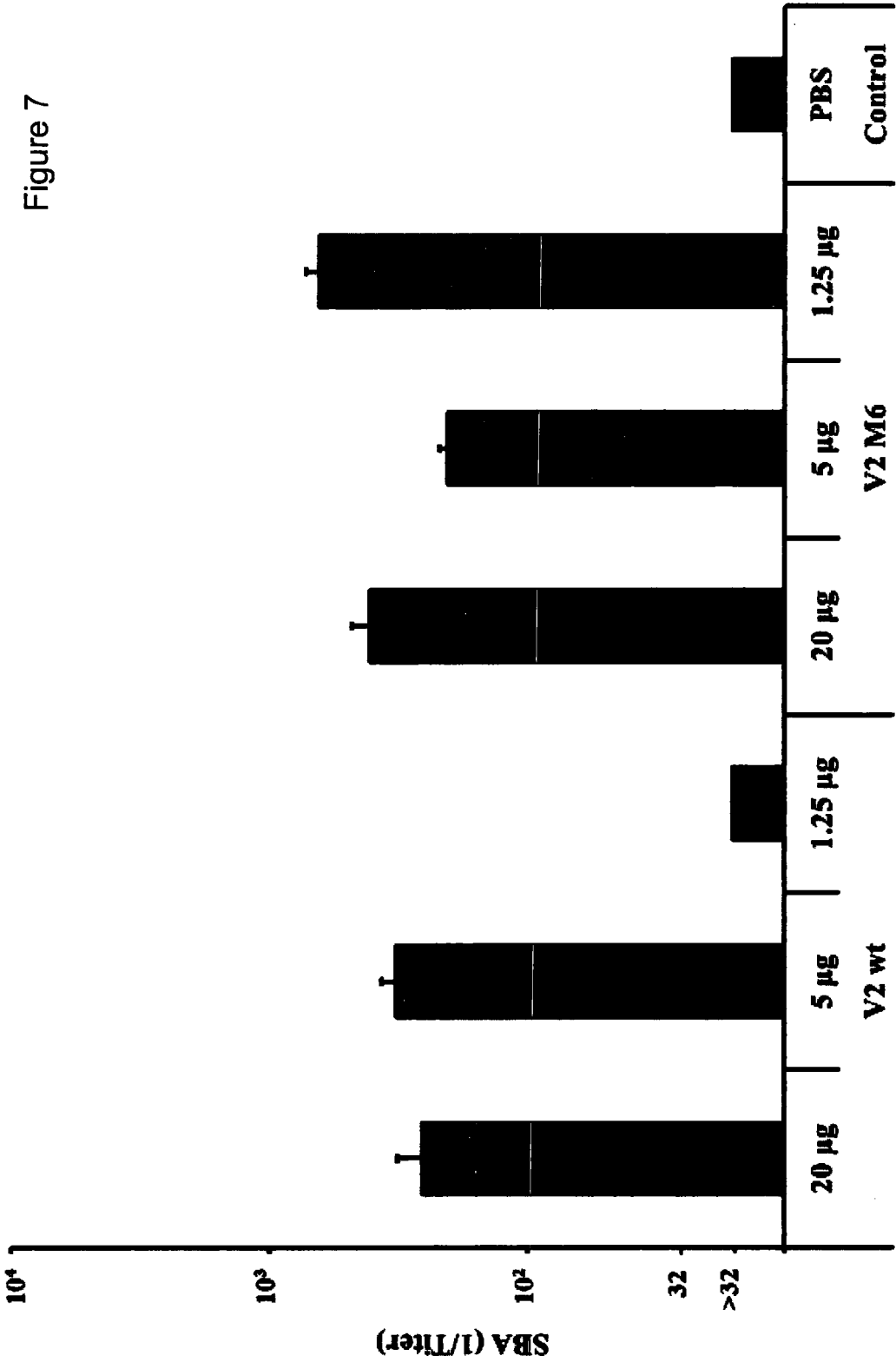
Figure 5C



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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/052215

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/095 C12N15/00 C07K14/22
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, Sequence Search, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MURPHY ELLEN ET AL: "Sequence diversity of the factor H binding protein vaccine candidate in epidemiologically relevant strains of serogroup B Neisseria meningitidis", JOURNAL OF INFECTIOUS DISEASES.JID, UNIVERSITY OF CHICAGO PRESS, CHICAGO, IL, vol. 200, no. 3, 1 August 2009 (2009-08-01), pages 379-389, XP002617504, ISSN: 0022-1899, DOI: 10.1086/600141 sequence -/--	1-5, 19-31



Further documents are listed in the continuation of Box C.



See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 January 2014

Date of mailing of the international search report

23/01/2014

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Voigt-Ritzer, Heike

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2013/052215

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& DATABASE UniProt [Online] 5 May 2009 (2009-05-05), "SubName: Full=Factor H binding protein variant A42_001; Flags: Fragment;", XP002718688, retrieved from EBI accession no. UNIPROT:C0JFP2 Database accession no. C0JFP2 sequence -----	
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