Claims

1. A mutein of mature human tear lipocalin, wherein the mutein comprises:

(a) a mutated amino acid residue at any one or more of the sequence positions 26-34, 56-58, 80, 83, 104-106, and 108 of the linear polypeptide sequence of the mature human tear lipocalin set forth in SEQ ID NO: 1, and

(b) a mutated amino acid residue at any one or more of the sequence positions 61, 101, 111, 114, and 153 of the linear polypeptide sequence of the mature human tear lipocalin set forth in SEQ ID NO 1,

wherein the mutein contains at least one of the following amino acid substitutions in comparison to the mature human tear lipocalin:

Arg 26 → Ser, Phe, Trp, His or Thr;

Glu 27 → Ser, Gln, Thr, Phe, Lys, Ala or Arg;

Phe 28 → Cys, Arg, Lys, Trp, Asp, Gly, His, Leu or Asn;

Pro 29 → Gly, Asp, Asn, Ile, Leu or Met;

Glu 30 → Arg, Asp, Thr, Ser, Gly, Ala or Asn;

Met 31 → Ala, Gly, His, Pro, Ser Aps, Glu or Gln;

Asn 32 → Ile, Leu, Tyr, Met or Trp;

Leu 33 → Trp, Tyr, Phe, Pro or Ala;

Glu 34 → Asn, Thr, Arg or Gly;

Leu 56 → Met, Ser, Gln, Phe, His or Asn;

Ile 57 → Tyr, Trp, His, Gln, Thr or Arg;

Ser 58 → Lys, Ala, Arg, Trp or Pro;

Ser 61 → Trp or Phe;

Asp 80 → Ser, Met, Pro, Ile, Gln, Tyr, Ser, Val or Thr;

Lys 83 → Arg, Ser, Gln, Thr or Glu;

Glu 104 → Leu, Pro, Ser, Ala, Asn, Thr, Lys or Asp;

Leu 105 → Cys, Tyr, Trp, Glu, Arg, Ser, His, Ala, Val, Asp, Pro, Gly or Lys;

His 106 → Pro, Gln, Gly, Arg, Val, Thr, Asn or Leu;

Lys 108 → Gln, Ala, Trp, Tyr, Arg, Asp, Asn, Ser, Glu or Thr; and wherein the mutein binds specifically to proprotein convertase subtilisin/kexin type 9 (PCSK9).

2. The lipocalin mutein of claim 1, wherein the lipocalin mutein is an antagonist of PCSK9.

3. The lipocalin mutein of claim 1, wherein the lipocalin mutein is competitive for binding of LDL-R to PCSK9.

4. The lipocalin mutein of claim 1, wherein the lipocalin mutein is competitive for binding of a monoclonal antibody comprising SEQ ID NO: 29 and SEQ ID NO: 33 to PCSK9.

5. The lipocalin mutein of claim 1, wherein the lipocalin mutein is able to fully or partially inhibit PCSK9-mediated downregulation of LDL-R.

6. The lipocalin mutein of claim 1, wherein the lipocalin mutein is able to restore LDL uptake in the presence of PCSK9.

7. The lipocalin mutein according to any one of the claims 1-6, wherein the mutein binds to a non-human primate PCSK9 or an immunogenic fragment thereof with a dissociation constant (K_D) equal to or less than 10 nM.

8. The lipocalin mutein according to any one of the claims 1-6, wherein the mutein binds to mouse PCSK9 or an immunogenic fragment thereof with a dissociation constant (K_D) equal to or less than 10 nM.

9. The lipocalin mutein according to any one of the claims 1-6, wherein the mutein binds to human PCSK9 or a fragment thereof with a dissociation constant (K_D) equal to or less than 10 nM.

10. The lipocalin mutein according to any one of the claims 1-6, wherein the mutein binds to human PCSK9 or a fragment thereof with a dissociation constant (K_D) equal to or less than 1 nM.

11. The lipocalin mutein according to any one of the claims 1-6, wherein the mutein binds to human PCSK9 or a fragment thereof with a dissociation constant (K_D) equal to or less than 0.1 nM.

12. The lipocalin mutein according to any one of the claims 1-6, wherein mutein binds to human PCSK9 or a fragment thereof with a dissociation constant (K_D) equal to or less than 1 pM.

13. The mutein according to any one of claims 1-12, comprising one of the following sets of amino acid substitutions in comparison to mature human tear lipocalin:

(a) Arg 26 → Phe; Asn 32 → Ile; Glu 34 → Thr; Leu 56 → Met; Ser 58 → Ala and Lys 83 → Ser,

(b) Arg 26 → Trp; Asn 32 → Leu; Glu 34 → Thr; Leu 56 → Ser and Ser 58 → Ala,

(c) Arg 26 → His; Asn 32 → Tyr; Glu 34 → Thr; Leu 56 → Ser; Ser 58 → Arg and Lys 83 → Gln;

(d) Arg 26 → Phe; Asn 32 → Met; Glu 34 → Thr; Leu 56 → Gln; Ser 58 → Ala and Lys 83 → Thr;

(e) Asn 32 → Trp; Glu 34 → Arg; Leu 56 → Asn; Ser 58 → Trp and Lys 83 → Ser,

(f) Arg 26 → Phe; Asn 32 → Leu; Glu 34 → Thr; Leu 56 → Phe; Ser 58 → Ala and Lys 83 → Arg,

(g) Arg 26 → Thr; Asn 32 → Trp; Glu 34 → Asn; Leu 56 → His; Ser 58 → Pro and Lys 83 → Ser,

(h) Asn 32 → Trp; Glu 34 → Asn; Leu 56 → Phe; Ser 58 → Arg and Lys 83 → Glu,

(i) Arg 26 → Trp; Asn 32 → Leu; Glu 34 → Thr; Leu 56 → Met; Ser 58 → Ala and Lys 83 → Ser, or

(j) Asn 32 → Trp; Glu 34 → Gly; Leu 56 → Gln; Ser 58 → Ala and Lys 83 → Gln.

14. The mutein of according to any one of claims 1-12, comprising one of the following sets of amino acid substitutions in comparison to mature human tear lipocalin:

(a) Glu 27 → Ser; Phe 28 → Arg; Pro 29 → Gly; Glu 30 → Asp; Met 31 → Ala; Leu 33 → Trp; Ile 57 → Tyr; Asp 80 → Met; Glu 104 → Pro; Leu 105 → Tyr; His 106 → Gln; Lys 108 → Ala,

(b) Glu 27 → Gln; Phe 28 → Cys; Pro 29 → Asp; Glu 30 → Thr; Met 31 → Gly; Leu 33 → Trp; Ile 57 → Tyr; Leu 105 → Cys; His 106 → Gly; Lys 108 → Trp,

(c) Glu 27 → Glu; Phe 28 → Trp; Pro 29 → Asn; Glu 30 → Gly; Met 31 → His; Leu 33 → Tyr; Ile 57 → Tyr; Asp 80 → Pro; Glu 104 → Ser; Leu 105 → Trp; His 106 → Pro; Lys 108 → Tyr,

(d) Glu 27 → Thr; Phe 28 → Asp; Pro 29 → Asn; Glu 30 → Ser; Met 31 → Pro; Leu 33 → Phe; Ile 57 → Tyr; Asp 80 → Ile; Glu 104 → Ala; Leu 105 → Glu; His 106 → Arg; Lys 108 → Arg,

(e) Glu 27 → Phe; Phe 28 → Lys; Pro 29 → Ile; Glu 30 → Ala; Met 31 → Ser; Leu 33 → Pro; Ile 57 → Trp; Asp 80 → Gln; Glu 104 → Asn; Leu 105 → Arg; His 106 → Gln; Lys 108 → Asp,

(f) Glu 27 → Lys; Phe 28 → Gly; Pro 29 → Pro; Glu 30 → Thr; Met 31 → Pro; Leu 33 → Trp; Ile 57 → His; Asp 80 → Tyr; Glu 104 → Ala; Leu 105 → Ser; His 106 → Val; Lys 108 → Asn,

(g) Glu 27 → Glu; Phe 28 → His; Pro 29 → Leu; Glu 30 → Ala; Met 31 → Asp; Leu 33 → Ala; Ile 57 → Gln; Asp 80 → Ile; Glu 104 → Ala; Leu 105 → Tyr; His 106 → Pro; Lys 108 → Ser,

(h) Glu 27 → Ala; Phe 28 → Asp; Pro 29 → Met; Glu 30 → Gly; Met 31 → Asp; Leu 33 → Pro; Ile 57 → Thr; Asp 80 → Thr; Glu 104 → Thr; His 106 → Thr; Lys 108 → Arg,

(i) Glu 27 → Arg; Phe 28 → Leu; Pro 29 → Asp; Glu 30 → Asn; Met 31 → Glu; Leu 33 → Trp; Ile 57 → Tyr; Asp 80 → Gln; Glu 104 → Pro; Leu 105 → Arg; His 106 → Asn; Lys 108 → Ala,

(j) Glu 27 → Lys; Phe 28 → Asn; Pro 29 → Met; Glu 30 → Gly; Met 31 → Gln; Leu 33 → Pro; Ile 57 → Arg; Asp 80 → Ile; Glu 104 → Asp; Leu 105 → Arg; His 106 → Leu; Lys 108 → Thr, or

(k) Glu 27 → Ser; Phe 28 → Arg; Pro 29 → Gly; Glu 30 → Asp; Met 31 → Ala; Leu 33 → Trp; Ile 57 → Tyr; Asp 80 → Met; Glu 104 → Pro; Leu 105 → Gly; His 106 → Gln; Lys 108 → Ala.

15. The mutein according to any one of claims 1-12, comprising the following combination of amino acid substitutions: Arg 26 → Phe, Glu 27 → Ser, Phe 28 → Arg, Pro 29 → Gly, Glu 30 → Asp, Met 31 → Ala, Asn 32 → Ile, Leu 33 → Trp, Glu 34 → Thr, Leu 56 → Met, Ile 57 → Tyr, Ser 58 → Ala, Lys 83 → Ser, Glu 104 → Pro, and Lys 108 → Thr, in comparison to mature human tear lipocalin.

16. The mutein according to claim 15, further includes one or more of the following amino acid substitutions in comparison to mature human tear lipocalin: Thr 43 → Ile or Ala, Glu 45 → Gly, Asn 48 → Gly, Glu 63 → Gly, Ala 66 → Val, Glu 69 → Val, Lys 70 → Arg, Ala 79 → Thr, Met or Val, Asp 80 → Met or Ser, Gly 82 → Ser, His 84 → Gln, Val 85

→ Gly, Tyr 87 → Ser, Ile 89 → Thr or Leu, His 92 → Pro, Leu 105 → His, Gly, or Tyr, and His 106 → Gln or Arg.

17. The mutein according to any one of claims 1-12, comprising the following combination of amino acid substitutions: Glu 27 → Phe, Phe 28 → Lys, Pro 29 → Ile, Asn 32 → Trp, Leu 33 → Pro, Glu 34 → Arg, Leu 56 → Asn, Ile 57 → Trp, His 106 → Gln, and Lys 108 → Glu, in comparison to mature human tear lipocalin.

18. The mutein according to claim 17, further includes one or more of the following amino acid substitutions in comparison to mature human tear lipocalin: Glu 45 → Gly, Ser 58 → Trp or Arg, Glu 63 → Asp, Glu 69 → Gly, Lys 70 → Arg, Asp 80 → Gln, Val or Thr, Gly 82 → Asp, Lys 83 → Ser or Arg, Ala 86 → Glu or Ser, Phe 99 → Leu, Glu 102 → Lys or Val, Glu 104 → Asn or Lys, and Pro 106 → Thr.

19. The mutein according to any one of claims 1 to 18, wherein the mutein comprises an amino acid substitution of a native amino acid by a cysteine residue at positions 28 or 105 with respect to the amino acid sequence of mature human tear lipocalin.

20. The mutein according to one of claims 1 to 19, wherein the mutein has at least 75% identity to the sequence of mature human tear lipocalin.

21. The mutein of any one of claims 1 to 12, wherein the mutein has an amino acid sequence as set forth in any one of SEQ ID NOs: 3-28, 62-71, and 82 or of a fragment thereof.

22. The mutein of any one of claims 1 to 12, wherein the mutein has an amino acid sequence as set forth in SEQ ID NO: 23 or of a fragment thereof.

23. The mutein of any one of claims 1 to 12, wherein the mutein has an amino acid sequence as set forth in SEQ ID NO: 13 or of a fragment thereof.

24. The mutein of any one of claims 1 to 12, wherein the mutein has an amino acid sequence as set forth in SEQ ID NO: 20 or of a fragment thereof.

25. The mutein of any one of claims 1 to 12, wherein the mutein has an amino acid sequence as set forth in SEQ ID NO: 22 or of a fragment thereof.

26. The mutein of any one of claims 1-25, wherein the mutein is conjugated to a label moiety.

27. The mutein of any one of claims 1-25, wherein the mutein is conjugated to a moiety that can target a specific body region, organism, tissue, organ, or cell within a subject.

28. The mutein of any one of claims 1-25, wherein the mutein is conjugated to a moiety that can extend the serum half-life of the mutein.

29. The mutein of any one of claims 1-25, wherein the mutein is conjugated to a polyalkylene glycol molecule.

30. The conjugated mutein of claim 29, wherein the conjugated mutein comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 30-32.

31. The mutein of any one of claims 1-25, wherein the mutein is conjugated to an albumin binding protein.

32. The mutein of any one of claims 1-25, wherein the mutein is fused to a moiety, wherein the moiety is selected from the group consisting of alkaline phosphatase, horseradish peroxidase, glutathione-S-transferase, the albumin-binding domain of protein G, protein A, an antibody fragment, an oligomerization domain, a lipocalin mutein of same or different binding specificity, and a toxin.

33. A method of generating one or more muteins of mature human tear lipocalin set forth in SEQ ID NO: 1, wherein the one or more mutein(s) bind to proprotein convertase subtilisin/kexin type 9 (PCSK9), comprising:

(a) subjecting a nucleic acid molecule encoding mature human tear lipocalin to mutagenesis at:

(i) any one or more of the amino acid sequence positions 26-34, 56-58, 80, 83, 104-106, and 108 of the linear polypeptide sequence of mature human tear lipocalin**,** and

(ii) any one or more of the amino acid sequence positions 61, 101, 111, 114, and 153 of the linear polypeptide sequence of the mature human tear lipocalin,

thereby obtaining one or more nucleic acids molecule(s) encoding one or more mutein(s) of mature human tear lipocalin,

(b) expressing the one or more nucleic acid molecule(s) obtained in (a) in an expression system, thereby obtaining one or more mutein(s) of mature human tear lipocalin, and

(c) further selecting the one or more mutein(s) obtained in (b),

wherein the one or more generated muteins contain at least one of the following amino

acid substitutions in comparison to the mature human tear lipocalin:

Arg 26 → Ser, Phe, Trp, His or Thr;

Glu 27 → Ser, Gln, Thr, Phe, Lys, Ala or Arg;

Phe 28 → Cys, Arg, Lys, Trp, Asp, Gly, His, Leu or Asn;

Pro 29 → Gly, Asp, Asn, Ile, Leu or Met;

Glu 30 → Arg, Asp, Thr, Ser, Gly, Ala or Asn;

Met 31 → Ala, Gly, His, Pro, Ser Aps, Glu or Gln;

Asn 32 → Ile, Leu, Tyr, Met or Trp;

Leu 33 → Trp, Tyr, Phe, Pro or Ala;

Glu 34 → Asn, Thr, Arg or Gly;

Leu 56 → Met, Ser, Gln, Phe, His or Asn;

Ile 57 → Tyr, Trp, His, Gln, Thr or Arg;

Ser 58 → Lys, Ala, Arg, Trp or Pro;

Ser 61 → Trp or Phe;

Asp 80 → Ser, Met, Pro, Ile, Gln, Tyr, Ser, Val or Thr;

Lys 83 → Arg, Ser, Gln, Thr or Glu;

Glu 104 → Leu, Pro, Ser, Ala, Asn, Thr, Lys or Asp;

Leu 105 → Cys, Tyr, Trp, Glu, Arg, Ser, His, Ala, Val, Asp, Pro, Gly or Lys;

His 106 → Pro, Gln, Gly, Arg, Val, Thr, Asn or Leu;

Lys 108 → Gln, Ala, Trp, Tyr, Arg, Asp, Asn, Ser, Glu or Thr.

34. The method according to claim 33, wherein step (c) further comprises:

(ci) providing PCSK9 or an immunogenic fragment thereof,

(cii) contacting the one or more mutein(s) obtained through selection with the PCSK9 or the immunogenic fragment thereof, thereby allowing the formation of a complex between the PCSK9 or the immunogenic fragment thereof and the mutein having binding affinity for the same, and

(ciii) removing one or more mutein(s) having no or no substantial binding affinity.

35. The method of claims 33 or 34, wherein the selection in step (c) is carried out under competitive conditions.

36. The method of any one of claims 33-35, wherein step (a) further comprises: (a)(iii) subjecting the nucleic acid molecule encoding mature human tear lipocalin to

mutagenesis at any one or more of the amino acid sequence positions 79, 92, and 105 of the linear polypeptide sequence of mature human tear lipocalin**.**

37. A nucleic acid molecule comprising a nucleotide sequence encoding a mutein of any one of claims 1-32.

38. A nucleic acid molecule as set forth in any one of SEQ ID NOs: 36-61, 72-81, and 86-87.

39. A host cell containing a nucleic acid molecule of claim 37 or 38.

40. The use of a mutein according to any one of claims 1-32 or of a composition comprising such mutein for the manufacture of a medicament for lowering low density lipoprotein cholesterol (LDL-C) levels.

41. The use of a mutein according to any one of claims 1-32 for the detection of PCSK9, comprising:

    (a) contacting the mutein with a test sample suspected to contain PCSK9 under suitable conditions, thereby allowing the formation of a complex between the mutein and PCSK9 or a domain or fragment thereof, and

    (b) detecting the complex by a suitable signal.

42. The use of a mutein according to any one of claims 1-32 for the separation of PCSK9, comprising:

    (a) contacting the mutein with a sample supposed to contain PCSK9 under suitable conditions, thereby allowing formation of a complex between the mutein and PCSK9 or a domain or fragment thereof, and

    (b) separating the complex from the sample.

43. A kit comprising a mutein according to any one of claims 1-32.

44. The kit of claim 43, wherein the kit is a diagnostic kit.