PCSK9 vaccine

FIELD OF THE INVENTION

The present invention relates to the provision of novel immunogens comprising an antigenic PCSK9 peptide preferably linked to an immunogenic carrier for the prevention, treatment or alleviation of PCSK9-related disorders. The invention further relates to methods for production of these medicaments, immunogenic compositions and pharmaceutical composition thereof and their use in medicine.

BACKGROUND

Proprotein convertase subtilisin-kexin type 9 (hereinafter called "PCSK9"), also known as neural apoptosis- regulated convertase 1 ("NARC-I"), is a proteinase K-like subtilase identified as the 9th member of the mammalian PCSK family; see Seidah et al, 2003 PNAS 100:928-933. The gene for PCSK9 localizes to human chromosome 1p33-p34.3. PCSK9 is expressed in cells capable of proliferation and differentiation including, for example, hepatocytes, kidney mesenchymal cells, intestinal ileum, and colon epithelia as well as embryonic brain telencephalon neurons.

Original synthesis of PCSK9 is in the form of an inactive enzyme precursor, or zymogen, of ~72-kDa which undergoes autocatalytic, intramolecular processing in the endoplasmic reticulum ("ER") to activate its functionality. The gene sequence for human PCSK9, which is ~22-kb long with 12 exons encoding a 692 amino acid protein, can be found, for example, at Deposit No. NP_777596.2. Human, mouse and rat PCSK9 nucleic acid sequences have been deposited; see, e.g., GenBank Accession Nos.: AX127530 (also AX207686), AX207688, and AX207690, respectively.

Human PCSK9 is a secreted protein expressed primarily in the kidneys, liver and intestines. It has three domains: an inhibitory pro-domain (amino acids 1-152; including a signal sequence at amino acids 1-30), a catalytic domain (amino acids 153-448), and a C-terminal domain 210 residues in length (amino acids 449-692), which is rich in cysteine residues. PCSK9 is synthesized as a zymogen that undergoes autocatalytic cleavage between the pro- domain and catalytic domain in the endoplasmic reticulum. The pro-domain remains bound to the mature protein after cleavage, and the complex is secreted. The cysteine-rich domain may play a role analogous to the P-(processing) domains of other Furin/Kexin/Subtilisin-like serine proteases, which appear to be essential for folding and regulation of the activated protease. Mutations in PCSK9 are associated with abnormal levels of low density lipoprotein cholesterol (LDL-c) in the blood plasma (Horton et al., 2006 Trends. Biochem. Sci. 32(2):71-77).

PCSK9 has been ascribed a role in the differentiation of hepatic and neuronal cells (Seidah et al, supra), is highly expressed in embryonic liver, and has been strongly implicated in cholesterol homeostasis.

The identification of compounds and/or agents effective in the treatment of cardiovascular affliction is highly desirable. Reductions in LDL cholesterol levels have already demonstrated in clinical trials to be directly related to the rate of coronary events; Law et al, 2003 BMJ 326: 1423-1427. More, recently moderate lifelong reduction in plasma LDL cholesterol levels has been shown to be substantially correlated with a substantial reduction in the incidence of coronary events; Cohen et al, supra. This was found to be the case even in populations with a high prevalence of non-lipid-related cardiovascular risk factors; supra.

Accordingly, it is of great importance to indentify therapeutic agent permiting the control of LDL cholesterol levels.

Accordingly, it would be of great importance to produce a medicament that inhibits or antagonizes the activity of PCSK9 and the corresponding role PCSK9 plays in various therapeutic conditions.

Expression or upregulation of PCSK9 is associated with increased plasma levels of LDL cholesterol, and inhibition or the lack of expression of PCSK9 is associated with low LDL cholesterol plasma levels. Significantly, lower levels of LDL cholesterol associated with sequence variations in PCSK9 have conferred protection against coronary heart disease; Cohen, 2006 N. Engl. J. Med. 354: 1264-1272.

SUMMARY OF THE INVENTION

The present invention relates to an immunogen comprising an antigenic PCSK9 peptide and optionally an immunogenic carrier.

The invention also relates to methods for producing such antigenic PCSK9 peptide optionally linked to an immunogenic carrier.

The invention also relates to immunogenic compositions comprising such antigenic PCSK9 peptide optionally linked to an immunogenic carrier, optionally comprising one or several adjuvants, preferably one or two adjuvants.

Another aspect of the invention relates to pharmaceutical compositions comprising an antigenic PCSK9 peptide according to the invention, or an immunogenic composition thereof, as well as to medical uses of such compositions.

In particular, the invention relates to an antigenic PCSK9 peptide of the invention, or an immunogenic or pharmaceutical composition thereof, for use as a medicament, preferably in treatment, alleviation or prophylaxis of PCSK9-related disorders.

In particular, the invention relates to an antigenic PCSK9 peptide of the invention, or an immunogenic or pharmaceutical composition thereof, for use as a medicament preferably in treatment, alleviation or prophylaxis of diseases associated with an elevated level of cholesterol.

The antigenic PCSK9 peptides of the invention are particularly suitable for treating human patients having, or at risk for, elevated LDL-cholesterol or a condition associated with elevated LDL-cholesterol, e.g., a lipid disorder (e.g., hyperlipidemia, type I, type II, type III, type IV, or type V hyperlipidemia, secondary hypertriglyceridemia, hypercholesterolemia, familial hypercholesterolemia, xanthomatosis, cholesterol acetyltransferase deficiency). Antigenic PCSK9 peptide of the invention are also suitable for treating human patients having arteriosclerotic conditions (e.g., atherosclerosis), coronary artery disease, cardiovascular disease, and patients at risk for these disorders, e.g., due to the presence of one or more risk factors (e.g., hypertension, cigarette smoking, diabetes, obesity, or hyperhomocysteinemia).

In yet another aspect, the present invention provides the use of an antigenic PCSK9 peptide of the invention or of an immunogenic composition or a pharmaceutical composition thereof, in the manufacture of a medicament for the treatment of Alzheimer's disease.

In one embodiment, the antigenic PCSK9 peptide or an immunogenic composition or a pharmaceutical composition thereof is administered together with another agent.

BRIEF DESCRIPTION OF THE DRAWING:

Figure 1: The PDB structure of human PCSK9 bound to the EGF-A domain of the LDL-R (3BPS) showing 5 peptide sequences in PCSK9 (peptide 1-5) chosen as being involved in the interaction between these two proteins.

Figure 2: Mice were immunized with peptides VR_9.1 to VR_9.9 conjugated to VLPs, using Alum plus CpG as adjuvant and antibody responses to full-length recombinant human PCSK9 were measured by titrating sera in an ELISA assay. Results are shown as reciprocal titres for each of 6 mice per group, with the reciprocal titre measured as the dilution of serum giving an optical density reading of 0.5.

Figure 3: Antibody responses to full-length recombinant mouse PCSK9 protein as described in Figure 2.

Figure 4: Plasma cholesterol levels measured in the sera of vaccinated mice (same samples as used for antibody assays in Figures 2 and 3)

Figure 5: Plasma samples used in Figs 2 to 4 were tested at different dilutions for their ability to inhibit the interaction between recombinant PCSK9 and the extracellular domain of the LDL receptor as measured by FRET assay.

Figure 6: Dilutions of plasma samples from peptides VR_9.5 and VR_9.6 vaccinations in FRET assay, showing dose-responsive inhibition of interaction between PCSK9 and the LDL receptor.

Figure 7: Complex of PCSK9 (ribbons) and EGF-A (space fill) from PDB:3BPS. Potential regions of PCSK9 that may interact with domains of LDLR other than EGF-A are indicated by the elipse.

Figure 8: Complex of PCSK9 (ribbons) and EGF-A (space fill), with the amino acids corresponding to peptides VR_13/14 (A) and VR_15/16 (B) and VR_9.5 (C) is displayed.

Figure 9 and 10: Plasma antibody responses of mice vaccinated with peptides VR_9.5 and VR_9.10 to VR_9.16. Antibodies to mouse PCSK9 were measured by ELISA assay of serial plasma dilutions using full-length mouse PCSK9 protein. Individual titration curves are shown for 8 mice per group, with ELISA responses of plasma from mice immunized with unconjugated VLP shown as a control.

Figure 11: Serum antibody responses to full-length human PCSK9 protein induced in BALB/c and C57BL/6 mice vaccinated with either peptide VR_9.5 or VR_9.10 conjugated to VLPs (using Alum +/- CpG as adjuvant) or CRM197 (using TiterMax as adjuvant). Antibodies to human PCSK9 were measured by titrating sera in an ELISA assay. Results are shown as log reciprocal titres determined at an optical density of 1.0 for each of 8 mice per group.

Figure 12: Serum antibody responses to full-length mouse PCSK9 protein induced in BALB/c and C57BL/6 mice vaccinated as described for Figure 11. Results are shown as log reciprocal titres determined at an optical density of 0.5 for each of 8 mice per group.

Figure 13: Total cholesterol levels measured in the serum samples from BALB/c vaccinated mice (same samples used for antibody assays in Figures 11 and 12).

Figure 14: Total cholesterol levels measured in the serum samples from C57BL/6 vaccinated mice (same samples used for antibody assays in Figures 11 and 12).

Figure 15: Antibody responses to full-length human PCSK9 induced in BALB/c mice immunized with peptides VR_9.5 or VR_9.17 to VR_9.35 conjugated to VLPs using Alum plus CpG as adjuvant. Antibodies to human PCSK9 were measured by titrating sera in an ELISA assay. Results are shown as log reciprocal titres determined at an optical density of 1.0 for each of 8 mice per group.

Figure 16: Antibody responses to full-length mouse PCSK9 induced in BALB/c mice immunized as described for Figure 15. Antibodies to mouse PCSK9 were measured by titrating sera in an ELISA assay. Results are shown as log reciprocal titres determined at an optical density of 0.5 for each of 8 mice per group.

Figure 17: Total cholesterol levels measured in the serum samples from BALB/c vaccinated mice (same samples used for antibody assays in Figures 15 and 16).

Figure 18: Complex of PCSK9 (ribbons) and EGF-A (space fill) with regions of PCSK9 containing amino acids sequences linked to gain- or loss-of-function mutations and/or protein surface exposed epitopes indicated by elipses.

DETAILED DESCRIPTION OF THE INVENTION

Antigenic PCSK9 peptide of the invention

The present invention relates to an immunogen comprising an antigenic PCSK9 peptide optionally linked to an immunogenic carrier.

In one embodiment, the antigenic PCSK9 peptide is a portion of PCSK9 comprising between 4 to 20 amino acids and, when administered to a subject, is able to lower the LDL-cholesterol level in blood of said subject. Preferably, said subject is a mammal, preferably a human. Preferably, said antigenic PCSK9 peptide is able to lower the LDL-cholesterol level by at least 2%, 5%, 10%, 20%, 30% or 50%.

In one embodiment, the antigenic PCSK9 peptide is a portion of PCSK9 which participates in the interaction of PCSK9 with the LDL receptor.

In one embodiment, the antigenic PCSK9 peptide is a portion of PCSK9 which participates in the interaction of PCSK9 with the LDL receptor, comprising between 4 and 20 amino acids and, when administered to a subject is able to lower the LDL-cholesterol level in blood of said subject. Preferably,

said subject is a mammal, preferably a human. Preferably, said antigenic PCSK9 peptide is able to lower the LDL-cholesterol level by at least 2%, 5%, 10%, 20%, 30% or 50%.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397,398, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587 and 588.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 1 to 312, 330 to 398, 421, 423, 424, 426 and 428 to 588.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195,

196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397 and 398.

In one embodiment, the antigenic PCSK9 peptide is a portion of PCSK9 which may participate in the interaction with the domain EGF-A of the LDL receptor. Examples of such portions are represented on figure 1.

In one embodiment, the antigenic PCSK9 peptide is a portion of PCSK9 which may participate in the interaction with the domain EGF-A of the LDL receptor, comprising between 4 and 20 amino acids and, when administered to a subject, is able to lower the LDL-cholesterol level in blood of said subject. Preferably, said subject is a mammal, preferably a human. Preferably, said antigenic PCSK9 peptide is able to lower the LDL-cholesterol level by at least 2%, 5%, 10%, 20%, 30% or 50%.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 13, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 1.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44 and 45.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 15, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 46.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 and 101.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 14, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 102.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 102,103,104,105,106,107,108,109,110,111,112,113,114,115,116,117,118,119,120,121,122,123,124,125, 126,127,128,129,130,131,132,133,134,135,136,137,138,139,140,141,142,143,144,145 and 146.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 13, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 147.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180 and 181.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 13, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 182.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225 and 226.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 13, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 330.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358 and 359.

In a preferred embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 19, 56, 63, 109, 153, 165, 184, 186, 187, 188, 332 and 424.

In a preferred embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 19, 56, 63, 109, 153 and 184.

In a preferred embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 56, 184, 186, 187, 188 and 332.

In a most preferred embodiment, the antigenic PCSK9 peptide is a peptide of sequence SEQ ID No 56.

In a more prefered embodiment, the antigenic PCSK9 peptide is a peptide of sequence SEQ ID No 184 or 187.

In a most preferred embodiment, the antigenic PCSK9 peptide is a peptide of sequence SEQ ID No 184.

In a most preferred embodiment, the antigenic PCSK9 peptide is a peptide of sequence SEQ ID No 332.

In one embodiment, the antigenic PCSK9 peptide is selected in a region of PCSK9 which may participate in the interaction with a region of the LDL receptor other than the EGF-A domain. Examples of such portions are represented on figures 7 and 8.

In one embodiment, the antigenic PCSK9 peptide is a portion of PCSK9 which may participate in the interaction with a region of the LDL receptor other than the EGF-A domain, comprising between 4 and 20

amino acids and, when administered to a subject, is able to lower the LDL-cholesterol level in blood of said subject. Preferably, said subject is a mammal, preferably a human. Preferably, said antigenic PCSK9 peptide is able to lower the LDL-cholesterol level by at least 2%, 5%, 10%, 20%, 30% or 50%.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 12, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 227.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261 and 262.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 13, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 263.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306 and 307.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 13, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 360.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397 and 398.

In an embodiment, the antigenic PCSK9 peptide is selected in a region of PCSK9 pro-domain (SEQ ID No 329).

In one embodiment, the antigenic PCSK9 peptide is a portion of PCSK9 pro-domain, comprising between 4 and 20 amino acids and, when administered to a subject, is able to lower the LDL-cholesterol level in blood of said subject. Preferably, said subject is a mammal, preferably a human. Preferably, said antigenic PCSK9 peptide is able to lower the LDL-cholesterol level by at least 2%, 5%, 10%, 20%, 30% or 50%.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 308, 309, 310, 311 and 312.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 12, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 309.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 309, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462 and 463.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 12, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 508.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542 and 543.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 13, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 310.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 310, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506 and 507.

In one embodiment, the antigenic PCSK9 peptide is a peptide comprising 5 to 13, preferably 6 to 8, consecutive amino acids of the PCSK9 fragment of SEQ ID No 544.

In one embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, and 588.

In a preferred embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 312, 421, 422, 423, 426, 427, 428, 445, 482, 525, and 563.

In a more preferred embodiment, the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 445, 482, 525, and 563.

In a most preferred embodiment, the antigenic PCSK9 peptide is a peptide of sequence SEQ ID No 445.

Such antigenic PCSK9 peptides may be used alone or in combination, preferably when conjugated to an immunogenic carrier, to induce auto anti-PCSK9 antibodies in a subject in order to treat, prevent or ameliorate PCSK9-related disorders.

It will be apparent to the man skilled in the art which techniques may be used to confirm whether a specific construct falls within the scope of the present invention. Such techniques include, but are not

restricted to, the techniques described in the Example section of the present application, and also to the following.

The ability of the antigenic PCSK9 peptide of the invention to induce auto anti-PCSK9 antibodies may be measured in mice, using the test disclosed in example 3 of the present application. The ability of auto-antibodies induced by the antigenic PCSK9 peptide of the invention to decrease the level of circulating plasma cholesterol may be measured in mice, using the test disclosed in example 3. The ability of auto-antibodies induced by the antigenic PCSK9 peptide of the invention to inhibit the interaction between PCSK9 and LDL receptors may be measured directly using the test disclosed in example 3 (FRET assay) or indirectly by measuring the upregulation of cell surface LDL receptors which is a consequence of blocking PCSK9-mediated down-regulation (as well described in the relevant literature, either using cell lines in vitro or by measuring LDL receptor levels in liver biopsies of antibody expressing animals (e.g.by Western blotting)).

The term "antigenic PCSK9 peptide biological activity", when used herein, refers to the ability of the antigenic PCSK9 peptides of the invention to induce auto anti-PCSK9 antibodies in a patient.

Preferably said antigenic PCSK9 peptide, when administered to a subject, is able to lower the LDL-cholesterol level in blood of said subject. Preferably, said subject is a mammal, preferably a human. Preferably, said antigenic PCSK9 peptide is able to lower the LDL-cholesterol level by at least 2%, 5%, 10%, 20%, 30% or 50%.

In an embodiment the antigenic PCSK9 peptides of the present invention are of a size such that they mimic a region selected from the whole PCSK9 domain in which the native epitope is found. In a particular embodiment, the antigenic PCSK9 peptides of the invention are less than 100 amino acids in length, preferably shorter than 75 amino acids, more preferably less than 50 amino acids, even more preferably less than 40 amino acids. The antigenic PCSK9 peptides of the invention are typically 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acids in length, preferably from 4 to 20 amino acids, for example 6 to 12, 6 to 8 or 9 to 12 amino acids. Specific examples of antigenic PCSK9 peptides of the invention are provided in the sequence listing and include peptides ranging from 5 to 17 amino acids in length.

The antigenic peptides of the invention include an amino acid sequence derived from a portion of a mammalian PCSK9, preferably a human PCSK9 (SEQ ID No 399) or mouse PCSK9 (SEQ ID Nos 400), more preferably human PCSK9, such derived portion of PCSK9 either corresponding to the amino acid sequence of naturally occurring PCSK9 or corresponding to variant PCSK9, i.e. the amino acid sequence of naturally occurring PCSK9 in which a small number of amino acids have been substituted, added or deleted but which retains essentially the same immunological properties. In addition, such derived PCSK9 portion can be further modified by amino acids, especially at the N- and C-terminal ends to allow the antigenic PCSK9 peptide to be conformationally constrained and/or to allow coupling of the antigenic PCSK9 peptide to an immunogenic carrier after appropriate chemistry has been carried out.

The antigenic PCSK9 peptides disclosed herein encompass functionally active variant peptides derived from the amino acid sequence of PCSK9 in which amino acids have been deleted, inserted or substituted without essentially detracting from the immunological properties thereof, i.e. such functionally active variant peptides retain a substantial antigenic PCSK9 peptide biological activity. Typically, such functionally variant peptides have an amino acid sequence homologous, preferably highly homologous, to an amino acid sequence selected from the group consisting of SEQ ID Nos: 1 to 312, 330 to 398 and 420 to 588.

In one embodiment, such functionally active variant peptides exhibit at least 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% identity to an amino acid sequence selected from the group consisting of SEQ ID Nos: 1 to 312, 330 to 398 and 420 to 588.

Sequence similarity for polypeptides, which is also referred to as sequence identity, is typically measured using sequence analysis software. Protein analysis software matches similar sequences using measures of similarity assigned to various substitutions, deletions and other modifications, including conservative amino acid substitutions. For instance, GCG contains programs such as "Gap" and "Bestfit" which can be used with default parameters to determine sequence homology or sequence identity between closely related polypeptides, such as homologous polypeptides from different species of organisms or between a wild type protein and a mutein thereof. See, e.g., GCG Version 6.1. Polypeptide sequences also can be compared using FASTA using default or recommended parameters, a program in GCG Version 6.1. FASTA (e.g., FASTA2 and FASTA3) provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences (Pearson, *Methods Enzymol.* **183**:63-98 (1990); Pearson, *Methods Mol. Biol.* **132**:185-219 (2000)). An alternative algorithm when comparing a sequence of the invention to a database containing a large number of sequences from different organisms is the computer program BLAST, especially blastp or tblastn, using default parameters. *See, e.g.*, Altschul et al., *J. Mol. Biol.* **215**:403-410 (1990); Altschul et al., *Nucleic Acids Res.* **25**:3389-402 (1997).

Functionally active variants comprise naturally occurring functionally active variants such as allelic variants and species variants and non-naturally occurring functionally active variants that can be produced by, for example, mutagenesis techniques or by direct synthesis.

A functionally active variant differs by about, for example, 1, 2, 3, 4 or 5 amino acid residues from any of the peptide shown at SEQ ID Nos: 1 to 312, 330 to 398 and 420 to 588, and yet retain an antigenic PCSK9 biological activity. Where this comparison requires alignment the sequences are aligned for maximum homology. The site of variation can occur anywhere in the peptide, as long as the biological activity is substantially similar to a peptide shown in SEQ ID Nos: 1 to 312, 330 to 398 and 420 to 588.

Guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie et al., Science, 247: 1306-1310 (1990), which teaches that there are two main strategies for studying the tolerance of an amino acid sequence to change.

The first strategy exploits the tolerance of amino acid substitutions by natural selection during the process of evolution. By comparing amino acid sequences in different species, the amino acid positions which have been conserved between species can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions in which substitutions have been tolerated by natural selection indicate positions which are not critical for protein function. Thus, positions

tolerating amino acid substitution can be modified while still maintaining specific immunogenic activity of the modified peptide.

The second strategy uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene to identify regions critical for protein function. For example, site-directed mutagenesis or alanine-scanning mutagenesis can be used (Cunningham et al., Science, 244: 1081-1085 (1989)). The resulting variant peptides can then be tested for specific antigenic PCSK9 biological activity.

According to Bowie et al., these two strategies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at certain amino acid positions in the protein. For example, the most buried or interior (within the tertiary structure of the protein) amino acid residues require nonpolar side chains, whereas few features of surface or exterior side chains are generally conserved.

Methods of introducing a mutation into amino acids of a protein is well known to those skilled in the art. See, e. g., Ausubel (ed.), Current Protocols in Molecular Biology, John Wiley and Sons, Inc. (1994); T. Maniatis, E. F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor laboratory, Cold Spring Harbor, N. Y. (1989)).

Mutations can also be introduced using commercially available kits such as "QuikChangeTM Site-Directed Mutagenesis Kit" (Stratagene) or directly by peptide synthesis. The generation of a functionally active variant to an antigenic PCSK9 peptide by replacing an amino acid which does not significantly influence the function of said antigenic PCSK9 peptide can be accomplished by one skilled in the art.

A type of amino acid substitution that may be made in one of the peptides according to the invention is a conservative amino acid substitution. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain R group) with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity or degree of similarity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well-known to those of skill in the art. See e.g. Pearson, *Methods Mol. Biol.* 243:307-31 (1994).

Examples of groups of amino acids that have side chains with similar chemical properties include 1) aliphatic side chains: glycine, alanine, valine, leucine, and isoleucine; 2) aliphatic-hydroxyl side chains: serine and threonine; 3) amide-containing side chains: asparagine and glutamine; 4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; 5) basic side chains: lysine, arginine, and histidine; 6) acidic side chains: aspartic acid and glutamic acid; and 7) sulfur-containing side chains: cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine.

Alternatively, a conservative replacement is any change having a positive value in the PAM250 log-likelihood matrix disclosed in Gonnet et al., *Science* 256:1443-45 (1992). A "moderately conservative" replacement is any change having a nonnegative value in the PAM250 log-likelihood matrix.

A functionally active variant peptide can also be isolated using a hybridization technique. Briefly, DNA having a high homology to the whole or part of a nucleic acid sequence encoding the peptide of interest, e.g. SEQ ID Nos: 1 to 312, 330 to 398 and 420 to 588, is used to prepare a functionally active peptide. Therefore, an antigenic PCSK9 peptide of the invention also includes peptides which are functionally

equivalent to one or more of the peptide of SEQ ID Nos: 1 to 312, 330 to 398, and 420 to 588 and which are encoded by a nucleic acid molecule which hybridizes with a nucleic acid encoding any one of SEQ ID Nos: 1 to 312, 330 to 398 and 420 to 588, or a complement thereof. One of skill in the art can easily determine nucleic acid sequences that encode peptides of the invention using readily available codon tables. As such, these nucleic acid sequences are not presented herein.

The stringency of hybridization for a nucleic acid encoding a peptide, polypeptide or protein that is a functionally active variant is, for example, 10% formamide, 5 x SSPE, 1 x Denhart's solution, and 1 x salmon sperm DNA (low stringency conditions). More preferable conditions are, 25% formamide, 5 x SSPE, 1 x Denhart's solution, and 1 x salmon sperm DNA (moderate stringency conditions), and even more preferable conditions are, 50% formamide, 5 x SSPE, 1 x Denhart's solution, and 1 x salmon sperm DNA (high stringency conditions). However, several factors influence the stringency of hybridization other than the above-described formamide concentration, and one skilled in the art can suitably select these factors to accomplish a similar stringency.

Nucleic acid molecules encoding a functionally active variant can also be isolated by a gene amplification method such as PCR using a portion of a nucleic acid molecule DNA encoding a peptide, polypeptide or protein of interest, e.g. any one of the peptides shown SEQ ID Nos: 1 to 312, 330 to 398 and 420 to 588, as the probe.

In one embodiment of the invention, a peptide of the invention is derived from a natural source and isolated from a mammal, such as a human, a primate, a cat, a dog, a horse, a mouse, or a rat, preferably from a human source. A peptide of the invention can thus be isolated from cells or tissue sources using standard protein purification techniques.

Alternatively, peptides of the invention can be synthesized chemically or produced using recombinant DNA techniques.

For example, a peptide of the invention can be synthesized by solid phase procedures well known in the art. Suitable syntheses may be performed by utilising "T-boc" or "F-moc" procedures. Cyclic peptides can be synthesised by the solid phase procedure employing the well-known "F-moc"procedure and polyamide resin in the fully automated apparatus. Alternatively, those skilled in the art will know the necessary laboratory procedures to perform the process manually. Techniques and procedures for solid phase synthesis are described in 'Solid Phase Peptide Synthesis: A Practical Approach' by E. Atherton and R. C. Sheppard, published by IRL at Oxford University Press (1989) and 'Methods in Molecular Biology, Vol. 35: Peptide Synthesis Protocols (ed. M. W.Pennington and B. M. Dunn), chapter 7, pp91-171 by D. Andreau et al.

Alternatively, a polynucleotide encoding a peptide of the invention can be introduced into an expression vector that can be expressed in a suitable expression system using techniques well known in the art, followed by isolation or purification of the expressed peptide, polypeptide, or protein of interest. A variety of bacterial, yeast, plant, mammalian, and insect expression systems are available in the art and any such expression system can be used. Optionally, a polynucleotide encoding a peptide of the invention can be translated in a cell-free translation system.

Antigenic PCSK9 peptides of the invention can also comprise those that arise as a result of the existence of multiple genes, alternative transcription events, alternative RNA splicing events, and alternative translational and postranslational events. A peptide can be expressed in systems, e.g. cultured cells, which result in substantially the same postranslational modifications present as when the peptide is expressed in a native cell, or in systems that result in the alteration or omission of postranslational modifications, e.g. glycosylation or cleavage, present when expressed in a native cell.

An antigenic PCSK9 peptide of the invention can be produced as a fusion protein that contains other non-PCSK9 or non- PCSK9-derived amino acid sequences, such as amino acid linkers or signal sequences or immunogenic carriers as defined herein, as well as ligands useful in protein purification, such as glutathione-S-transferase, histidine tag, and staphylococcal protein A. More than one antigenic PCSK9 peptide of the invention can be present in a fusion protein. The heterologous polypeptide can be fused, for example, to the N- terminus or C-terminus of the peptide of the invention. A peptide of the invention can also be produced as fusion proteins comprising homologous amino acid sequences, i. e., other PCSK9 or PCSK9-derived sequences.

The antigenic PCSK9 peptides of the invention might be linear or conformationally constrained. As used herein in reference to a peptide, the term "conformationally constrained" means a peptide, in which the three-dimensional structure is maintained substantially in one spatial arrangement over time. Conformationally constrained molecules can have improved properties such as increased affinity, metabolic stability, membrane permeability or solubility.

In addition, such conformationally constrained peptides are expected to present the antigenic PCSK9 epitope in a conformation similar to their native loop conformation, thereby inducing anti-PCSK9 antibodies more susceptible to recognize intact, native self PCSK9 molecules or with an increased affinity to recognize self PCSK9 molecules. Methods of conformational constraint are well known in the art and include, without limitation, bridging and cyclization.

There are several approaches known in the prior art to introduce conformational constraints into a linear peptide. For example, bridging between two neighbouring amino acids in a peptide leads to a local conformational modification, the flexibility of which is limited in comparison with that of regular peptides. Some possibilities for forming such bridges include incorporation of lactams and piperazinones (for review see Giannis and. Kolter, Angew. Chem. Int. Ed., 1993,32: 1244).

As used herein in reference to a peptide, the term “cyclic” refers to a structure including an intramolecular bond between two non-adjacent amino acids or amino acid analogs. The cyclization can be effected through a covalent or non-covalent bond. Intramolecular bonds include, but are not limited to, backbone to backbone, side-chain to backbone, side-chain to side-chain, side chain to end-group, end-to-end bonds. Methods of cyclization include, without limitation, formation of an amide bond between the N-term residue and the C-term residue of a peptide, formation of a disulfide bond between the side-chains of non-adjacent amino acids or amino acid analogs; formation of an amide bond between the side-chains of Lys and Asp/Glu residues; formation of an ester bond between serine residues and Asp/Glu residues; formation of a lactam bond, for example, between a side-chain group of one amino acid or analog thereof to the N-terminal amine of the amino-terminal residue; and formation of lysinonorleucine and dityrosine bonds. Carbon versions of a disulfide linkage, for example an ethenyl or ethyl linkage, could also be used (*J. Peptide Sc.*, 2008, 14, 898-902) as well as alkylation reactions with an appropriately polysubstituted

electrophilic reagent such as a di-, tri- or tetrahaloalkane (*PNAS*, 2008, 105(40), 15293-15298; *ChemBioChem*, 2005, 6, 821-824). Various modified proline analogs can also be used to incorporate conformational constraints into peptides (Zhang et al., J. Med Chem., 1996,39: 2738-2744; Pfeifer and Robinson, Chem. Comm., 1998,1977-1978). Chemistries that may be used to cyclise peptides of the invention result in peptides cyclised with a bond including, but not limiting to the following: lactam, hydrazone, oxime, thiazolidine, thioether or sulfonium bonds.

Yet another approach in the design of conformationally constrained peptides, which is described in US10/114918, is to attach a short amino acid sequence of interest to a template, to generate a cyclic constrained peptide. Such cyclic peptides are not only structurally stabilized by their templates, and thereby offer three-dimensional conformations that may imitate conformational epitopes on native proteins such as on viruses and parasites or on self proteins (autologous mammalian proteins such as PCSK9), but they are also more resistant than linear peptides to proteolytic degradation in serum. US 10/114918 further discloses the synthesis of conformationally constrained cross-linked peptides by preparation of synthetic amino acids for backbone coupling to appropriately positioned amino acids in order to stabilize the supersecondary structure of peptides. Cross-linking can be achieved by amide coupling of the primary amino group of an orthogonally protected (2S, 3R) -3-aminoproline residue to a suitably positioned side chain carboxyl group of glutamate. This approach has been followed in the preparation of conformationally constrained tetrapeptide repeats of the CS protein wherein at least one proline has been replaced by2S, 3R) -3-aminoproline and, in order to introduce a side chain carboxyl group, glutamate has been incorporated as a replacement for alanine.

Cross-linking strategies also include the application of the Grubbs ring-closing metathesis reaction to form 'stapled' peptides designed to mimic alpha-helical conformations (*Angew. Int. Ed. Engl.*, 1998, 37, 3281 ; *JACS*, 2000, 122, 5891); use of poly-functionalised saccharides ; use of a tryptathionine linkage (*Chemistry Eu. J.*, 2008, 24, 3404-3409); use of 'click' reaction of azides and alkynes which could be incorporated as either a side chain amino acid residues or located within the backbone of the peptide sequence (*Drug Disc. Today*, 2003, 8(24), 1128-1137). It is also known in the literature that metal ions can stabilise constrained conformations of linear peptides through sequestering specific residues e.g. histidine, which co-ordinate to metal cations (*Angew. Int. Ed. Engl.*, 2003, 42, 421). Similarly, functionalising a linear peptide sequence with non-natural acid and amine functionality, or polyamine and polyacid functionality can be used to allow access to cyclised structures following activation and amide bond formation.

According to one embodiment, the antigenic PCSK9 peptide is conformationally constrained by intramolecular covalent bonding of two non-adjacent amino acids of the antigenic PCSK9 peptide to each other, e.g. the N- and C- terminal amino acids. According to another embodiment, the antigenic PCSK9 peptide of the invention is conformationally constrained by covalent binding to a scaffold molecule. According to a further embodiment, the antigenic PCSK9 peptide is simply constrained, i.e. coupled either at one end, (C or N terminus) or through another amino acid not located at either end, to the scaffold molecule. According to another embodiment, the antigenic PCSK9 peptide is doubly constrained, i.e. coupled at both C and N termini to the scaffold molecule. According to another embodiment, the antigenic peptide is constrained by cyclising via the templating effect of a heterochiral Diproline unit (D-Pro-L-Pro) (Spath et al, 1998, Helvetica Chimica Acta 81, p1726-1738).

The scaffold (also called 'platform') can be any molecule which is capable of reducing, through covalent bonding, the number of conformations which the antigenic PCSK9 peptide can assume. Examples of conformation-constraining scaffolds include proteins and peptides, for example lipocalin-related molecules such as beta-barrel containing thioredoxin and thioredoxin-like proteins, nucleases (e.g. RNaseA), proteases (e.g. trypsin), protease inhibitors (e.g. eglin C), antibodies or structurally-rigid fragments thereof, fluorescent proteins such as GFP or YFP, conotoxins, loop regions of fibronectin type III domain, CTL-A4, and virus-like particles (VLPs).

Other suitable platform molecules include carbohydrates such as sepharose. The platform may be a linear or circular molecule, for example, closed to form a loop. The platform is generally heterologous with respect to the antigenic PCSK9 peptide. Such conformationally constrained peptides linked to a platform are thought to be more resistant to proteolytic degradation than linear peptide.

According to a preferred embodiment, the scaffold is an immunogenic carrier as defined in the present application. In a further embodiment, the antigenic PCSK9 peptide is simply constrained onto the immunogenic carrier. In another further embodiment, the antigenic PCSK9 peptide is doubly constrained onto the immunogenic carrier. In this manner, the antigenic PCSK9 peptide forms a conformationally constrained loop structure which has proven to be a particularly suitable structure as an intracellular recognition molecule.

The antigenic PCSK9 peptides of the invention may be modified for the ease of conjugation to a platform, for example by the addition of a terminal cysteine at one or both ends and/or by the addition of a linker sequence, such a double glycine head or tail plus a terminal cysteine, a linker terminating with a lysine residue or any other linker known to those skilled in the art to perform such function. Details of such linkers are disclosed hereafter. Bioorthogonal chemistry (such as the click reaction described above) to couple the full peptide sequence to the carrier, thus avoiding any regiochemical and chemoselectivity issues, might also be used. Rigidified linkers such as the one described in Jones *et al.* Angew. Chem. Int. Ed. 2002, 41:4241-4244 are known to elicit an improved immunological response and might also be used.

In a further embodiment, the antigenic PCSK9 peptide is attached to a multivalent template, which itself is coupled to the carrier, thus increasing the density of the antigen (see below). The multivalent template could be an appropriately functionalised polymer or oligomer such as (but not limited to) oligoglutamate or oligochitosan (see figure 19).

Immunogenic carrier of the invention

In an embodiment of the present invention, the antigenic PCSK9 peptide of the invention is linked to an immunogenic carrier molecule to form immunogens for vaccination protocols, preferably wherein the carrier molecule is not related to the native PCSK9 molecule.

The term "immunogenic carrier" herein includes those materials which have the property of independently eliciting an immunogenic response in a host animal and which can be covalently coupled to a peptide, polypeptide or protein either directly via formation of peptide or ester bonds between free carboxyl, amino or hydroxyl groups in the peptide, polypeptide or protein and corresponding groups on the immunogenic carrier material, or alternatively by bonding through a conventional bifunctional linking group, or as a fusion protein.

The types of carriers used in the immunogens of the present invention will be readily known to the person skilled in the art. Examples of such immunogenic carriers are: serum albumins such as bovine serum

albumin (BSA); globulins; thyroglobulins; hemoglobins; hemocyanins (particularly Keyhole Limpet Hemocyanin [KLH]); polylysin; polyglutamic acid; lysine-glutamic acid copolymers; copolymers containing lysine or ornithine; liposome carriers; the purified protein derivative of tuberculin (PPD); inactivated bacterial toxins or toxoids such as tetanus or diptheria toxins (TT and DT) or fragment C of TT, CRM197 (a nontoxic but antigenically identical variant of diphtheria toxin) other DT point mutants, such as CRM176, CRM228, CRM 45 (Uchida et al J. Biol. Chem. 218; 3838-3844, 1973); CRM 9, CRM 45, CRM102, CRM 103 and CRM107 and other mutations described by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc, 1992; deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala 158 to Gly and other mutations disclosed in US 4709017 or US 4950740; mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in US 5917017 or US 6455673; or fragment disclosed in US 5843711 , pneumococcal pneumolysin (Kuo et al (1995) Infect Immun 63; 2706-13) including ply detoxified in some fashion for example dPLY-GMBS (WO 04081515, PCT/EP2005/010258) or dPLY-formol, PhtX, including PhtA, PhtB, PhtD, PhtE (sequences of PhtA, PhtB, PhtD or PhtE are disclosed in WO 00/37105 or WO 00/39299) and fusions of Pht proteins for example PhtDE fusions, PhtBE fusions, Pht A-E (WO 01/98334, WO 03/54007, WO2009/000826), OMPC (meningococcal outer membrane protein - usually extracted from N. meningitidis serogroup B - EP0372501), PorB (from N. meningitidis), PD (Haemophilus influenzae protein D - see, e.g., EP 0 594 610 B), or immunologically functional equivalents thereof, synthetic peptides (EP0378881 , EP0427347), heat shock proteins (WO 93/17712, WO 94/03208), pertussis proteins (WO 98/58668, EP0471 177), cytokines, lymphokines, growth factors or hormones (WO 91/01146), artificial proteins comprising multiple human CD4+ T cell epitopes from various pathogen derived antigens (Falugi et al (2001) Eur J Immunol 31 ; 3816-3824) such as N19 protein (Baraldoi et al (2004) Infect Immun 72; 4884-7) pneumococcal surface protein PspA (WO 02/091998), iron uptake proteins (WO 01/72337), toxin A or B of C. difficile (WO 00/61761).

In a preferred embodiment, the immunogenic carrier of the invention is CRM197.

In another embodiment, the immunogenic carrier is a virus-like particle (VLPs), preferably a recombinant virus-like particle.

As used herein, the term "virus-like particle" refers to a structure resembling a virus particle but which has been demonstrated to be non pathogenic. In general, virus-like particles lack at least part of the viral genome. Also, virus-like particles can often be produced in large quantities by heterologous expression and can be easily purified. A virus-like particle in accordance with the invention may contain nucleic acid distinct from their genome. A typical and preferred embodiment of a virus-like particle in accordance with the present invention is a viral capsid such as the viral capsid of the corresponding virus, bacteriophage, or RNA-phage.

As used herein, the term "virus- like particle of a bacteriophage" refers to a virus-like particle resembling the structure of a bacteriophage, being non replicative and noninfectious, and lacking at least the gene or genes encoding for the replication machinery of the bacteriophage, and typically also lacking the gene or genes encoding the protein or proteins responsible for viral attachment to or entry into the host. This definition should, however, also encompass virus-like particles of bacteriophages, in which the

aforementioned gene or genes are still present but inactive, and, therefore, also leading to non-replicative and noninfectious virus-like particles of a bacteriophage.

The capsid structure formed from the self-assembly of 180 subunits of RNA phage coat protein and optionally containing host RNA is herein referred to as a "VLP of RNA phage coat protein". Specific examples are the VLP of Qbeta, MS2, PP7 or AP205 coat proteins. In the specific case of Qbeta coat protein, for example, the VLP may either be assembled exclusively from Qbeta CP subunits (generated by expression of a Qbeta CP gene containing, for example, a TAA stop codon precluding any expression of the longer A1 protein through suppression, see Kozlovska, T. M., et al., Intervirology 39: 9-15 (1996)), or additionally contain A1 protein subunits in the capsid assembly. Generally, the percentage of Qbeta A1 protein relative to Qbeta CP in the capsid assembly will be limited, in order to ensure capsid formation. Examples of VLPs suitable as immunogenic carriers in the context of the present invention include, but are not limited to, VLPs of Qbeta, MS2, PP7, AP205 and other bacteriophage coat proteins, the capsid and core proteins of Hepatitis B virus (Ulrich, et al., Virus Res. 50: 141-182 (1998)), measles virus (Warnes, et al., Gene 160: 173-178 (1995)), Sindbis virus, rotavirus (U. S. Patent Nos. 5,071, 651 and 5,374, 426), foot-and-mouth-disease virus (Twomey, et al., Vaccine 13: 1603-1610, (1995)), Norwalk virus (Jiang, X., et al., Science 250: 1580-1583 (1990); Matsui, S. M., et al., J Clin. Invest. 87: 1456-1461 (1991)), the retroviral GAG protein (PCT Patent Appl. No. WO 96/30523), the retrotransposon Ty protein pl, the surface protein of Hepatitis B virus (WO 92/11291), human papilloma virus (WO 98/15631), human polyoma virus (Sasnauskas K., et al., Biol. Chem. 380 (3): 381-386 (1999); Sasnauskas K., et al., Generation of recombinant virus-like particles of different polyomaviruses in yeast. 3rd Interational Workshop"Virus-like particles as vaccines. "Berlin, September 26-29 (2001)), RNA phages, Ty, frphage, GA-phage, AP 205-phage and, in particular, Qbeta-phage, Cowpea chlorotic mottle virus, cowpea mosaic virus, human papilloma viruses (HPV), bovine papilloma viruses, porcine parvovirus, parvoviruses such as B19, porcine (PPV) and canine (CPV) parvovirues, caliciviruses (e.g. Norwalk virus,rabbit hemorrhagic disease virus [RHDV]), animal hepadnavirus core Antigen VLPs, filamentous/rod-shaped plant viruses, including but not limited *to* Tobacco Mosaic Virus (TMV), Potato Virus X (PVX), Papaya Mosaic Virus (PapMV), Alfalfa Mosaic Virus (AlMV), and Johnson Grass Mosaic Virus (JGMV), insect viruses *such as* flock house virus (FHV) and tetraviruses, polyomaviruses such as Murine Polyomavirus (MPyV), Murine Pneumotropic Virus (MPtV), BK virus (BKV), and JC virus (JCV).

As will be readily apparent to those skilled in the art, the VLP to be used as an immunogenic carrier of the invention is not limited to any specific form. The particle can be synthesized chemically or through a biological process, which can be natural or nonnatural. By way of example, this type of embodiment includes a virus-like particle or a recombinant form thereof. In a more specific embodiment, the VLP can comprise, or alternatively consist of, recombinant polypeptides of any of the virus known to form a VLP. The virus-like particle can further comprise, or alternatively consist of, one or more fragments of such polypeptides, as well as variants of such polypeptides. Variants of polypeptides can share, for example, at least 80%, 85%, 90%, 95%, 97%, or 99% identity at the amino acid level with their wild-type counterparts. Variant VLPs suitable for use in the present invention can be derived from any organism so long as they are able to form a “virus-like particle” and can be used as an “immunogenic carrier” as defined herein.

Preferred VLPs according to the invention include the capsid protein or surface antigen of HBV (HBcAg and HBsAg respectively) or recombinant proteins or fragments thereof, and the coat proteins of RNA-

phages or recombinant proteins or fragments thereof, more preferably the coat protein of Qbeta or recombinant proteins or fragments thereof.

In one embodiment, the immunogic carrier used in combination with an antigenic PCSK9 peptide of the invention is an HBcAg protein. Examples of HBcAg proteins that can be used in the context of the present invention can be readily determined by one skilled in the art. Examples include, but are limited to, HBV core proteins described in Yuan et al., (J. Virol. 73: 10122-10128 (1999)), and in WO00/198333, WO 00/177158, WO 00/214478, WO WO00/32227, WO01/85208, WO02/056905, WO03/024480, and WO03/024481. HBcAgs suitable for use in the present invention can be derived from any organism so long as they are able to form a "virus-like particle" and can be used as an "immunogenic carrier" as defined herein.

HBcAg variants of particular interest that could be used in the context of the present invention are those variants in which one or more naturally resident cysteine residues have been either deleted or substituted. It is well known in the art that free cysteine residues can be involved in a number of chemical side reactions including disulfide exchanges, reaction with chemical substances or metabolites that are, for example, injected or formed in a combination therapy with other substances, or direct oxidation and reaction with nucleotides upon exposure to UV light. Toxic adducts could thus be generated, especially considering the fact that HBcAgs have a strong tendency to bind nucleic acids. The toxic adducts would thus be distributed between a multiplicity of species, which individually may each be present at low concentration, but reach toxic levels when together. In view of the above, one advantage to the use of HBcAgs in vaccine compositions which have been modified to remove naturally resident cysteine residues is that sites to which toxic species can bind when antigens or antigenic determinants are attached would be reduced in number or eliminated altogether.

In addition, the processed form of HBcAg lacking the N-terminal leader sequence of the Hepatitis B core antigen precursor protein can also be used in the context of the invention, especially when HBcAg is produced under conditions where processing will not occur (e.g. expression in bacterial systems).

Other HBcAg variants according to the invention include i) polypeptide sequence having at least 80%, 85%, 90%, 95%, 97% or 99% identical to one of the wild-type HBcAg amino acid sequences, or a subportion thereof, using conventionally using known computer programs, ii) C-terminal truncation mutants including mutants where 1, 5, 10, 15, 20, 25, 30, 34 or 35, amino acids have been removed from the C-terminus, ii) N-terminal truncation mutants including mutants where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus, iii) mutants truncated in both N-terminal and C-terminal include HBcAgs where 1, 2 ,5, 7, 9, 10, 12, 14, 15 or 17 amino acids have been removed from the N-terminus and 1, 5, 10, 15, 20, 25, 30, 34 or 35 amino acids have been removed from the C-terminus.

Still other HBcAg variant proteins within the scope of the invention are those variants modified in order to enhance immunogenic presentation of a foreign epitope wherein one or more of the four arginine repeats has been deleted, but in which the C-terminal cysteine is retained (see e.g. WO01/98333), and chimeric C-terminally truncated HBcAg such as those described in WO02/14478, WO03/102165 and WO04/053091.

In another embodiment, the immunogic carrier used in combination with an antigenic PCSK9 peptide of the invention is an HBsAg protein. HBsAg proteins that could be used in the context of the present invention can be readily determined by one skilled in the art. Examples include, but are limited to, HBV

surface proteins described in US5792463, WO02/10416, and WO08/020331. HBsAgs suitable for use in the present invention can be derived from any organism so long as they are able to form a "virus-like particle" and can be used as an "immunogenic carrier" as defined herein.

In still another embodiment, the immunogic carrier used in combination with an antigenic PCSK9 peptide or polypeptide of the invention is a Qbeta coat protein.

Qbeta coat protein was found to self-assemble into capsids when expressed in E. coli (Kozlovska TM. et al., GENE 137: 133-137 (1993)). The obtained capsids or virus-like particles showed an icosahedral phage-like capsid structure with a diameter of 25 nm and T=3 quasi symmetry. Further, the crystal structure of phage Qss has been solved. The capsid contains 180 copies of the coat protein, which are linked in covalent pentamers and hexamers by disulfide bridges (Golmohammadi, R. et al., Structure 4: 5435554 (1996)) leading to a remarkable stability of the capsid of Qbeta coat protein. Qbeta capsid protein also shows unusual resistance to organic solvents and denaturing agents. The high stability of the capsid of Qbeta coat protein is an advantageous feature, in particular, for its use in immunization and vaccination of mammals and humans in accordance of the present invention.

Examples of Qbeta coat proteins that can be used in the context of the present invention can be readily determined by one skilled in the art. Examples have been extensively described in WO02/056905, WO03/024480, WO03/024481 (incorporated by reference in their entirety) and include, but are not limited to, amino acid sequences disclosed in PIR database, accession No. VCBPQbeta referring to Qbeta CP; Accession No. AAA16663 referring to Qbeta Al protein; and variants thereof including variants proteins in which the N-terminal methionine is cleaved; C-terminal truncated forms of Qbeta A1 missing as much as 100, 150 or 180 amino acids; variant proteins which have been modified by the removal of a lysine residue by deletion or substitution or by the addition of a lysine residue by substitution or insertion (see for example Qbeta-240, Qbeta-243, Qbeta-250, Qbeta-251 and Qbeta-259 disclosed in WO03/024481, incorporated by reference in its entirety), and variants exhibiting at least 80%, 85%, 90%, 95%, 97%, or 99% identity to any of the Qbeta core proteins described above. Variant Qbeta coat proteins suitable for use in the present invention can be derived from any organism so long as they are able to form a "virus-like particle" and can be used as "immunogenic carriers" as defined herein.

The antigenic PCSK9 peptides of the invention may be coupled to immunogenic carriers via chemical conjugation or by expression of genetically engineered fusion partners. The coupling does not necessarily need to be direct, but can occur through linker sequences. More generally, in the case that antigenic peptides either fused, conjugated or otherwise attached to an immunogenic carrier, spacer or linker sequences are typically added at one or both ends of the antigenic peptides. Such linker sequences generally comprise sequences recognized by the proteasome, proteases of the endosomes or other vesicular compartment of the cell.

In one embodiment, the peptides of the present invention are expressed as fusion proteins with the immunogenic carrier. Fusion of the peptide can be effected by insertion into the immunogenic carrier primary sequence, or by fusion to either the N-or C-terminus of the immunogenic carrier. Hereinafter, when referring to fusion proteins of a peptide to an immunogenic carrier, the fusion to either ends of the subunit sequence or internal insertion of the peptide within the carrier sequence are encompassed. Fusion, as referred to hereinafter, may be effected by insertion of the antigenic peptide into the sequence

of carrier, by substitution of part of the sequence of the carrier with the antigenic peptide, or by a combination of deletion, substitution or insertions.

When the immunogenic carrier is a VLP, the chimeric antigenic peptide-VLP subunit will be in general capable of self-assembly into a VLP. VLP displaying epitopes fused to their subunits are also herein referred to as chimeric VLPs. For example, EP 0 421 635 B describes the use of chimaeric hepadnavirus core antigen particles to present foreign peptide sequences in a virus-like particle.

Flanking amino acid residues may be added to either end of the sequence of the antigenic peptide to be fused to either end of the sequence of the subunit of a VLP, or for internal insertion of such peptidic sequence into the sequence of the subunit of a VLP. Glycine and serine residues are particularly favored amino acids to be used in the flanking sequences added to the peptide to be fused. Glycine residues confer additional flexibility, which may diminish the potentially destabilizing effect of fusing a foreign sequence into the sequence of a VLP subunit.

In a specific embodiment of the invention, the immunogenic carrier is a HBcAg VLP. Fusion proteins of the antigenic peptide to either the N-terminus of a HBcAg (Neyrinck, S. et al., Nature Med. 5: 11571163 (1999)) or insertions in the so called major immunodominant region (MIR) have been described (Pumpens, P. and Grens, E., Intervirology 44: 98114 (2001)), WO 01/98333), and are specific embodiments of the invention. Naturally occurring variants of HBcAg with deletions in the MIR have also been described (Pumpens, P. and Grens, E., Intervirology 44: 98-114 (2001)), and fusions to the N-or C-terminus, as well as insertions at the position of the MIR corresponding to the site of deletion as compared to a wt HBcAg are further embodiments of the invention. Fusions to the C-terminus have also been described (Pumpens, P. and Grens, E., Intervirology 44: 98-114 (2001)). One skilled in the art will easily find guidance on how to construct fusion proteins using classical molecular biology techniques. Vectors and plasmids encoding HBcAg and HBcAg fusion proteins and useful for the expression of a HBcAg and HBcAg fusion proteins have been described (Pumpens, P. and #38; Grens, E. Intervirology 44: 98-114 (2001), Neyrinck, S. et al., Nature Med. 5: 1157-1163 (1999)) and can be used in the practice of the invention. An important factor for the optimization of the efficiency of self-assembly and of the display of the epitope to be inserted in the MIR of HBcAg is the choice of the insertion site, as well as the number of amino acids to be deleted from the HBcAg sequence within the MIR (Pumpens, P. and Grens, E., Intervirology 44: 98-114 (2001); EP 0 421 635; U. S. Patent No. 6,231, 864) upon insertion, or in other words, which amino acids form HBcAg are to be substituted with the new epitope. For example, substitution of HBcAg amino acids 76-80, 79-81, 79-80, 75-85 or 80-81 with foreign epitopes has been described (Pumpens, P. and Grens, E., Intervirology 44: 98-114 (2001); EP0421635; US 6,231, 864, WO00/26385). HBcAg contains a long arginine tail (Pumpens, P. and Grens, E., Intervirology 44: 98-114 (2001)) which is dispensable for capsid assembly and capable of binding nucleic acids (Pumpens, P. and Grens, E., Intervirology 44: 98-114 (2001)). HBcAg either comprising or lacking this arginine tail are both embodiments of the invention.

In another specific embodiment of the invention, the immunogenic carrier is a VLP of a RNA phage, preferably Qbeta. The major coat proteins of RNA phages spontaneously assemble into VLPs upon expression in bacteria, and in particular in E. coli. Fusion protein constructs wherein antigenic peptides have been fused to the C-terminus of a truncated form of the A1 protein of Qbeta, or inserted within the A1 protein have been described (Kozlovska, T. M., et al., Intervirology, 39: 9-15 (1996)). The A1 protein is

generated by suppression at the UGA stop codon and has a length of 329 aa, or 328 aa, if the cleavage of the N-terminal methionine is taken into account. Cleavage of the N-terminal methionine before an alanine (the second amino acid encoded by the Qbeta CP gene) usually takes place in E. coli, and such is the case for N-termini of the Qbeta coat proteins. The part of the A1 gene, 3' of the UGA amber codon encodes the CP extension, which has a length of 195 amino acids. Insertion of the antigenic peptide between position 72 and 73 of the CP extension leads to further embodiments of the invention (Kozlovska, T. M., et al., Intervirology 39: 9-15 (1996)). Fusion of an antigenic peptide at the C-terminus of a C-terminally truncated Qbeta A1 protein leads to further preferred embodiments of the invention. For example, Kozlovska et al., (Intervirology, 39: 9-15 (1996)) describe Qbeta A1 protein fusions where the epitope is fused at the C-terminus of the Qbeta CP extension truncated at position 19.

As described by Kozlovska et al. (Intervirology, 39: 9-15 (1996)), assembly of the particles displaying the fused epitopes typically requires the presence of both the Al protein-antigen fusion and the wt CP to form a mosaic particle. However, embodiments comprising virus-like particles, and hereby in particular the VLPs of the RNA phage Qbeta coat protein, which are exclusively composed of VLP subunits having an antigenic peptide fused thereto, are also within the scope of the present invention.

The production of mosaic particles may be effected in a number of ways. Kozlovska et al., Intervirology, 39: 9-15 (1996), describe three methods, which all can be used in the practice of the invention. In the first approach, efficient display of the fused epitope on the VLPs is mediated by the expression of the plasmid encoding the Qbeta A1l protein fusion having a UGA stop codon between CP and CP extension in a E. coli strain harboring a plasmid encoding a cloned UGA suppressor tRNA which leads to translation of the UGA codon into Trp (pISM3001 plasmid (Smiley B. K., et al., Gene 134: 33-40 (1993))). In another approach, the CP gene stop codon is modified into UAA, and a second plasmid expressing the A1 protein-antigen fusion is cotransformed. The second plasmid encodes a different antibiotic resistance and the origin of replication is compatible with the first plasmid. In a third approach, CP and the A1 protein-antigen fusion are encoded in a bicistronic manner, operatively linked to a promoter such as the Trp promoter, as described in Figure 1 of Kozlovska et al., Intervirology, 39: 9-15 (1996).

Further VLPs suitable for fusion of antigens or antigenic determinants are described in WO03/024481 and include bacteriophage fr, RNA phase MS-2, capsid proteine of papillomavirus, retrotransposon Ty, yeast and also Retrovirus-like-particles, HIV2 Gag, Cowpea Mosaic Virus, parvovirus VP2 VLP, HBsAg (US 4,722, 840, EP0020416B1). Examples of chimeric VLPs suitable for the practice of the invention are also those described in Intervirology 39: 1 (1996). Further examples of VLPs contemplated for use in the invention are: HPV-1, HPV-6, HPV-11, HPV-16, HPV-18, HPV-33, HPV-45, CRPV, COPV, HIV GAG, Tobacco Mosaic Virus. Virus-like particles of SV-40, Polyomavirus, Adenovirus, Herpes Simplex Virus, Rotavirus and Norwalk virus.

For any recombinantly expressed antigenic PCSK9 peptide according to the invention coupled or not to an immunogenic carrier, the nucleic acid which encodes said peptide or protein also forms an aspect of the present invention, as does an expression vector comprising the nucleic acid, and a host cell containing the expression vector (autonomously or chromosomally inserted). A method of recombinantly producing the peptide or protein by expressing it in the above host cell and isolating the immunogen therefrom is a further aspect of the invention. The full-length native PCSK9 molecule or the full-length native DNA sequence encoding it are not covered by the present invention.

In another embodiment, the peptide of the invention is chemically coupled to an immunogenic carrier, using techniques well known in the art. Conjugation can occur to allow free movement of peptides via single point conjugation (e.g. either N-terminal or C-terminal point) or as locked down structure where both ends of peptides are conjugated to either a immunogenic carrier protein or to a scaffold structure such as a VLP. Conjugation occurs via conjugation chemistry known to those skilled in the art such as via cysteine residues, lysine residues or other carboxy moiety's commonly known as conjugation points such as glutamic acid or aspartic acid. Thus, for example, for direct covalent coupling it is possible to utilise a carbodiimide, glutaraldehyde or (N- [y- malcimidobutyryloxy] succinimide ester, utilising common commercially available heterobifunctional linkers such as CDAP and SPDP (using manufacturers instructions). Examples of conjugation of peptides, particularly cyclised peptides, to a protein carrier via acylhydrazine peptide derivatives are described in WO03/092714. After the coupling reaction, the immunogen can easily be isolated and purified by means of a dialysis method, a gel filtration method, a fractionation method etc. Peptides terminating with a cysteine residue (preferably with a linker outside the cyclised region) may be conveniently conjugated to a carrier protein via maleimide chemistry.

When the immunogenic carrier is a VLP, several antigenic peptide, either having an identical amino acid sequence or a different amino acid sequence, may be coupled to a single VLP molecule, leading preferably to a repetitive and ordered structure presenting several antigenic determinants in an oriented manner as described in WO00/32227, WO03/024481, WO02/056905 and WO04/007538.

In a preferred embodiment, the antigenic PCSK9 peptide is bound to the VLP by way of chemical cross-linking, typically and preferably by using a heterobifunctional cross-linker. Several hetero-bifunctional cross-linkers are known to the art. In some embodiments, the hetero-bifunctional crosslinker contains a functional group which can react with first attachment sites, i. e. with the side-chain amino group of lysine residues of the VLP or VLP subunit, and a further functional group which can react with a preferred second attachment site, i. e. a cysteine residue fused to the antigenic peptide and optionally also made available for reaction by reduction. The first step of the procedure, typically called the derivatization, is the reaction of the VLP with the cross-linker. The product of this reaction is an activated VLP, also called activated carrier. In the second step, unreacted cross-linker is removed using usual methods such as gel filtration or dialysis. In the third step, the antigenic peptide is reacted with the activated VLP, and this step is typically called the coupling step. Unreacted antigenic peptide may be optionally removed in a fourth step, for example by dialysis. Several hetero-bifunctional crosslinkers are known to the art. These include the preferred cross-linkers SMPH (Pierce), Sulfo-MBS, Sulfo-EMCS, Sulfo-GMBS, Sulfo-SIAB, Sulfo-SMPB, Sulfo-SMCC, SVSB, SIA and other cross-linkers available for example from the Pierce Chemical Company (Rockford, IL, USA), and having one functional group reactive towards amino groups and one functional group reactive towards cysteine residues. The above mentioned cross-linkers all lead to formation of a thioether linkage.

Another class of cross-linkers suitable in the practice of the invention is characterized by the introduction of a disulfide linkage between the antigenic peptide and the VLP upon coupling. Preferred cross-linkers belonging to this class include for example SPDP and Sulfo-LC-SPDP (Pierce). The extent of derivatization of the VLP with cross-linker can be influenced by varying experimental conditions such as the concentration of each of the reaction partners, the excess of one reagent over the other, the pH, the

temperature and the ionic strength. The degree of coupling, i. e. the amount of antigenic peptide per subunits of the VLP can be adjusted by varying the experimental conditions described above to match the requirements of the vaccine.

Another method of binding of antigenic peptides to the VLP, is the linking of a lysine residue on the surface of the VLP with a cysteine residue on the antigenic peptide. In some embodiments, fusion of an amino acid linker containing a cysteine residue, as a second attachment site or as a part thereof, to the antigenic peptide for coupling to the VLP may be required. In general, flexible amino acid linkers are favored. Examples of the amino acid linker are selected from the group consisting of: (a) CGG; (b) N-terminal gamma 1-linker; (c) N-terminal gamma 3-linker; (d) Ig hinge regions; (e) N-terminal glycine linkers; (f) (G) kC (G) n with n=0-12 and k=0-5; (g) N-terminal glycine-serine linkers; (h) (G) kC (G) m (S) i (GGGGS) n with n=0-3, k=0-5, m=0-10, i=0-2; (i) GGC; (k) GGC-NH2; (1) C-terminal gamma 1-linker; (m) C-terminal gamma 3-linker; (n) C-terminal glycine linkers; (o) (G) nC (G) k with n=0-12 and k=0-5; (p) C-terminal glycine-serine linkers; (q) (G) m (S) t (GGGGS) n (G) oC (G) k with n=0-3, k=0-5, m=0-10, 1=0-2, and o=0-8. Further examples of amino acid linkers are the hinge region of immunoglobulins, glycine serine linkers (GGGGS) n, and glycine linkers (G) n all further containing a cysteine residue as second attachment site and optionally further glycine residues. Typically preferred examples of said amino acid linkers are N-terminal gamma 1: CGDKTHTSPP; C-terminal gamma 1: DKTHTSPPCG; N-terminal gamma 3: CGGPKPSTPPGSSGGAP; C-terminal gamma 3: PKPSTPPGSSGGAPGGCG; N-terminal glycine linker: GCGGGG and C-terminal glycine linker: GGGGCG.

Other amino acid linkers particularly suitable in the practice of the invention, when a hydrophobic antigenic peptide is bound to a VLP, are CGKKGG, or CGDEGG for N-terminal linkers, or GGKKGC and GGEDGC, for the C-terminal linkers. For the C-terminal linkers, the terminal cysteine is optionally C-terminally amidated.

In some embodiments of the present invention, GGCG, GGC or GGC-NH2 ("NH2"stands for amidation) linkers at the C-terminus of the peptide or CGG at its N-terminus are preferred as amino acid linkers. In general, glycine residues will be inserted between bulky amino acids and the cysteine to be used as second attachment site, to avoid potential steric hindrance of the bulkier amino acid in the coupling reaction. In a further embodiment of the invention, the amino acid linker GGC-NH2 is fused to the C-terminus of the antigenic peptide.

The cysteine residue present on the antigenic peptide has to be in its reduced state to react with the hetero-bifunctional cross-linker on the activated VLP, that is a free cysteine or a cysteine residue with a free sulfhydryl group has to be available. In the instance where the cysteine residue to function as binding site is in an oxidized form, for example if it is forming a disulfide bridge, reduction of this disulfide bridge with e. g. DTT, TCEP or p- mercaptoethanol is required. Low concentrations of reducing agent are compatible with coupling as described in WO 02/05690, higher concentrations inhibit the coupling reaction, as a skilled artisan would know, in which case the reductand has to be removed or its concentration decreased prior to coupling, e. g. by dialysis, gel filtration or reverse phase HPLC.

Binding of the antigenic peptide to the VLP by using a hetero-bifunctional cross-linker according to the methods described above, allows coupling of the antigenic peptide to the VLP in an oriented fashion. Other methods of binding the antigenic peptide to the VLP include methods wherein the antigenic peptide is cross-linked to the VLP using the carbodiimide EDC, and NHS.

In other methods, the antigenic peptide is attached to the VLP using a homo-bifunctional cross-linker such as glutaraldehyde, DSGBM [PEO] 4, BS3, (Pierce Chemical Company, Rockford, IL, USA) or other known homo-bifunctional cross-linkers with functional groups reactive towards amine groups or carboxyl groups of the VLP.

Other methods of binding the VLP to an antigenic peptide include methods where the VLP is biotinylated, and the antigenic peptide expressed as a streptavidin-fusion protein, or methods wherein both the antigenic peptide and the VLP are biotinylated, for example as described in WO 00/23955. In this case, the antigenic peptide may be first bound to streptavidin or avidin by adjusting the ratio of antigenic peptide to streptavidin such that free binding sites are still available for binding of the VLP, which is added in the next step. Alternatively, all components may be mixed in a "one pot" reaction. Other ligand-receptor pairs, where a soluble form of the receptor and of the ligand is available, and are capable of being cross-linked to the VLP or the antigenic peptide, may be used as binding agents for binding antigenic peptide to the VLP. Alternatively, either the ligand or the receptor may be fused to the antigenic peptide, and so mediate binding to the VLP chemically bound or fused either to the receptor, or the ligand respectively. Fusion may also be effected by insertion or substitution.

One or several antigen molecules can be attached to one subunit of the capsid or VLP of RNA phages coat proteins, preferably through the exposed lysine residues of the VLP of RNA phages, if sterically allowable. A specific feature of the VLP of the coat protein of RNA phages and in particular of the QP coat protein VLP is thus the possibility to couple several antigens per subunit. This allows for the generation of a dense antigen array.

VLPs or capsids of Q coat protein display a defined number of lysine residues on their surface, with a defined topology with three lysine residues pointing towards the interior of the capsid and interacting with the RNA, and four other lysine residues exposed to the exterior of the capsid. These defined properties favor the attachment of antigens to the exterior of the particle, rather than to the interior of the particle where the lysine residues interact with RNA. VLPs of other RNA phage coat proteins also have a defined number of lysine residues on their surface and a defined topology of these lysine residues.

In a further embodiment of the present invention, the first attachment site is a lysine residue and/or the second attachment comprises sulfhydryl group or a cysteine residue. In an even further embodiment of the present invention, the first attachment site is a lysine residue and the second attachment is a cysteine residue. In further embodiments of the invention, the antigen or antigenic determinant is bound via a cysteine residue, to lysine residues of the VLP of RNA phage coat protein, and in particular to the VLP of Qbeta coat protein.

Another advantage of the VLPs derived from RNA phages is their high expression yield in bacteria that allows production of large quantities of material at affordable cost. Moreover, the use of the VLPs as carriers allow the formation of robust antigen arrays and conjugates, respectively, with variable antigen density. In particular, the use of VLPs of RNA phages, and hereby in particular the use of the VLP of RNA phage Qbeta coat protein allows a very high epitope density to be achieved.

According to an embodiment of the present invention the antigenic PCSK9 peptide disclosed herein are linked, preferably chemically cross linked, to CRM197, either directly or *via* one of the peptide linker disclosed herein, to generate an immunogen. In an embodiment, the antigenic PCSK9 peptide disclosed

herein is linked to CRM197, by way of chemical cross-linking as described herein and preferably by using a heterobifunctional cross-linker, as disclosed above.

Preferred heterobifunctional cross-linkers for use with CRM197 are BAANS (bromoacetic acid N-hydroxysuccinimide ester), SMPH (Succinimidyl-6-[ß-maleimidopropionamido]hexanoate), Sulfo-MBS, Sulfo-EMCS, Sulfo-GMBS, Sulfo-SIAB, Sulfo-SMPB, Sulfo-SMCC, SVSB, SIA and other cross-linkers available for example from the Pierce Chemical Company (Rockford, IL, USA). In a preferred embodiment of the present invention, the hetero-bifunctional crosslinker is BAANS or SMPH.

Alternatively, cross-linkers suitable allowing the introduction of a disulfide linkage between the antigenic peptide and CRM197 could also be used in the context of the invention. Preferred cross-linkers belonging to this class include for example SPDP and Sulfo-LC-SPDP (Pierce).

In a particular embodiment, when the sequence of the antigenic PCSK9 peptide disclosed herein comprises a cysteine, said antigenic PCSK9 peptide may be covalently linked to CRM197 directly via said cysteine.

In some embodiments of the invention, immunogenic compositions of the invention may comprise mixtures of immunogenic conjugates, i.e. immunogenic carriers coupled to one or several antigenic PCSK9 peptides of the invention. Thus, these immunogenic compositions may be composed of immunogenic carriers which differ in amino acid sequence. For example, vaccine compositions could be prepared comprising a "wild-type" VLP and a modified VLP protein in which one or more amino acid residues have been altered (e. g., deleted, inserted or substituted). Alternatively, the same immunogenic carrier might be used but coupled to antigenic PCSK9 peptides of different amino acid sequences.

The invention therefore also relates to method for producing an immunogen according to the invention comprising i) providing an antigenic PCSK9 peptide according to the invention, ii) providing an immunogenic carrier according to the invention, preferably a VLP, and iii) combining said antigenic PCSK9 peptide and said immunogenic carrier. In one embodiment, said combining step occurs through chemical cross-linking, preferably through an heterobifunctional cross-linker.

In an embodiment of the present invention, the antigenic PCSK9 peptide disclosed herein is linked to an immunogenic carrier molecule. In an embodiment said immunogenic carrier is selected from the group consisting of any of the immunogenic carrier described herein. In another embodiment said immunogenic carrier is selected from the group consisting of: serum albumins such as bovine serum albumin (BSA); globulins; thyroglobulins; hemoglobins; hemocyanins (particularly Keyhole Limpet Hemocyanin [KLH]) and virus-like particle (VLPs). In a preferred embodiment said immunogenic carrier is Diphtheria Toxoid, CRM197 mutant of diphtheria toxin, Tetanus Toxoid, Keyhole Limpet Hemocyanin or virus-like particle (VLPs). In an even preferred embodiment, said immunogenic carrier is DT, CRM197 or a VLP selected from the group consisting of HBcAg VLP, HBsAg VLP, Qbeta VLP, PP7 VLP, PPV VLP, Norwalk Virus VLP or any variant disclosed herein. In an even preferred embodiment, said immunogenic carrier is a bacteriophage VLP such as Qbeta VLP selected from the group consisting of Qbeta CP; Qbeta A1, Qbeta-240, Qbeta-243, Qbeta-250, Qbeta-251 and Qbeta-259 (disclosed in WO03/024481) or PP7.

In another preferred embodiment, said immunogenic carrier is CRM197.

In an embodiment, said immunogenic carrier is covalently linked to the antigenic PCSK9 peptide disclosed herein either directly or via a linker. In an embodiment, said immunogenic carrier is linked to the antigenic PCSK9 peptide disclosed herein by expression of a fusion protein as described herein. In another embodiment, the antigenic PCSK9 peptide disclosed herein is linked to the immunogenic carrier, preferably a VLP, by way of chemical cross-linking as described herein and preferably by using a heterobifunctional cross-linker. Several hetero-bifunctional cross-linkers are known to the art. In some embodiments, the hetero-bifunctional crosslinker contains a functional group which can react with first attachment sites, i.e. with the side-chain amino group of lysine residues of the VLP or VLP subunit, and a further functional group which can react with a preferred second attachment site, i.e. a cysteine residue fused to the antigenic peptide made available for reaction by reduction.

Antigenic PCSK9 peptide of the invention comprising a linker

In an embodiment of the present invention the antigenic PCSK9 peptide disclosed herein further comprise either at its N-terminus, or at its C-terminus or at both the N-terminus and C-terminus a linker which is able to react with an attachment site of the immunogenic carrier in a chemical cross-linking reaction. In an embodiment, the antigenic PCSK9 peptide disclosed herein further comprise at its C-terminus a linker having the formula $(G)_nC$, $(G)_nSC$ or $(G)_nK$, preferably $(G)_nC$ wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, preferably in the group consisting of 0, 1, 2, 3, 4 and 5, more preferably in the groups consisting of 0, 1, 2 and 3, most preferably n is 0 or 1 (where n is equal to 0 said formula represents a cysteine). Preferably the antigenic PCSK9 peptide disclosed herein further comprise at its C-terminus a linker having the formula GGGC, GGC, GC or C.

In another embodiment of the present invention the antigenic PCSK9 peptide disclosed herein further comprise at its N-terminus a linker having the formula $C(G)_n$, $CS(G)_n$ or $K(G)_n$, preferably $C(G)_n$ wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, preferably in the group consisting of 0, 1, 2, 3, 4 and 5, more preferably in the groups consisting of 0, 1, 2 and 3, most preferably n is 0 or 1 (where n is equal to 0, the formula represents a cysteine). Preferably the antigenic PCSK9 peptide disclosed herein further comprise at its N-terminus a linker having the formula CGGG, CGG, CG or C.

In another embodiment the antigenic PCSK9 peptide disclosed herein further comprise at its C-terminus a linker having the formula $(G)_nC$, $(G)_nSC$ or $(G)_nK$, preferably $(G)_nC$ wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, preferably in the group consisting of 0, 1, 2, 3, 4 and 5, more preferably in the groups consisting of 0, 1, 2 and 3, most preferably n 0 or 1 (where n is equal to 0 said formula represents a cysteine) and at its N-terminus a linker having the formula $C(G)_n$, $CS(G)_n$ or $K(G)_n$, preferably $C(G)_n$ wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, preferably in the group consisting of 0, 1, 2, 3, 4 and 5, more preferably in the groups consisting of 0, 1, 2 and 3, most preferably n is 0 or 1 (where n is equal to 0, the formula represents a cysteine). Preferably the antigenic PCSK9 peptide disclosed herein further comprise at its N-terminus a linker having the formula CGGG, CGG, CG or C and at its C-terminus a linker having the formula GGGC, GGC, GC or C. Preferably, the antigenic PCSK9 peptide disclosed herein further comprises at its N-terminus a cysteine and at its C-terminus a cysteine.

Representative of said antigenic PCSK9 peptides further comprising such a linker are disclosed at SEQ ID NO 313, 314, 315, 316, 317, 322, 323, 324, 325, 326, 327, 328, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418 and 419.

Representative of said antigenic PCSK9 peptides further comprising such a linker are disclosed at SEQ ID NO 313, 314, 315, 316, 317, 322, 323, 324, 325, 326, 327 and 328.

Preferred antigenic PCSK9 peptides comprising a linker are disclosed at SEQ ID Nos 317, 322, 323, 324, 401, 402, 403, 413, 414, 415 and 416.

Preferred antigenic PCSK9 peptides comprising a linker are disclosed at SEQ ID Nos 317, 322, 323 and 324.

Most preferred antigenic PCSK9 peptides comprising a linker are disclosed at SEQ ID Nos 317, 322, 402 and 413.

In one embodiment, the antigenic PCSK9 peptide is cyclised. In one embodiment, the cyclised antigenic PCSK9 peptide is attached to an immunogenic carrier. In one embodiment, said cyclised antigenic PCSK9 peptide is attached to an immunogenic carrier by covalent binding. In one embodiment, said cyclised antigenic PCSK9 peptide is attached to an immunogenic carrier by covalent binding of one of the side chain of its amino acids to the carrier. In one embodiment, a cysteine, a GC or a CC fragment comprising a variable number of glycine residues and one cysteine residue is added to the cyclised PCSK9 peptides to enable the covalent binding to the immunogenic carrier through the added cysteine.

In one embodiment, the antigenic PCSK9 peptide is cyclised and comprises a a cysteine, a $(G)_nC$ or a $C(G)_n$ fragment wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, preferably in the group consisting of 0, 1, 2, 3, 4 and 5, more preferably in the groups consisting of 0, 1, 2 and 3, most preferably n is 0 or 1 (where n is equal to 0, the formula represents a cysteine).

Non limitative examples of such conformationally constrained antigenic PCSK9 peptide are the peptides of SEQ ID Nos 318, 319, 320 and 321. A preferred cyclised peptide is the peptide of SEQ ID Nos 318.

Examples of conjugations of antigenic PCSK9 peptides with carrier or scaffolds described above, all within the scope of the present invention and constituting various embodiments, using various linkers are provided below:

Peptide – GGGGGC – scaffold, peptide – GGGGC – scaffold, peptide – GGGC – scaffold, peptide – GGC – scaffold, peptide – GC – scaffold, peptide – C – scaffold, peptide – GGGGGK – scaffold, peptide – GGGGK – scaffold, Peptide – GGGK – scaffold, Peptide – GGK – scaffold, Peptide – GK – scaffold, Peptide – K– scaffold, Peptide – GGGGSC – scaffold, Peptide – GGGSC – scaffold, Peptide – GGSC – scaffold, Peptide – GSC – scaffold, Peptide – SC – scaffold, Scaffold – CSGGGG – Peptide, Scaffold – CSGGG– Peptide, Scaffold – CSGG– Peptide, Scaffold – CSG– Peptide, Scaffold – CS – Peptide, Scaffold – KGGGG – Peptide, Scaffold – KGGG– Peptide, Scaffold – KGG– Peptide, Scaffold – KG– Peptide, Scaffold – K – Peptide.

In an embodiment, the peptide consists of any of the antigenic PCSK9 peptide disclosed herein and the scaffold consists of any of the immunogenic carrier disclosed herein, preferably a VLP.

Exemplary combinations of conjugations using various linkers and doubly constrained peptides are provided below, where the carrier can be the identical monomer of a carrier or a differential monomer of a

carrier. (In the example below, the GC linker can be substituted by any of the GK linker or GSC linker exemplified above or any other known to those skilled in the art):

Carrier – CGGGGG – Peptide – GGGGGC – carrier, Carrier – CGGGG – Peptide – GGGGC – carrier, Carrier – CGGGG – Peptide – GGGGC – carrier, Carrier – CGGG – Peptide – GGGC – carrier, Carrier – CG – Peptide – GC – carrier, Carrier – CG – Peptide – C – carrier, Carrier – C – Peptide – C – carrier.

In an embodiment, the peptide consists of any of the antigenic PCSK9 peptide disclosed herein and the carrier consists of any of the immunogenic carrier disclosed herein, preferably a VLP.

In an embodiment, the invention relates to an immunogen comprising an antigenic PCSK9 peptide consisting of, or consisting essentially of, an amino acid sequence selected from the group consisting of SEQ ID Nos: 1 to 312, 330 to 398 and 420 to 588, wherein said antigenic antigenic PCSK9 peptide further comprises at its C-terminus or at its N-terminus a cysteine which is chemically cross linked to an immunogenic carrier via a thioether linkage. In a preferred embodiment, said immunogenic carrier is selected from the group consisting of DT (Diphtheria toxin), TT (tetanus toxid) or fragment C of TT, PD (Haemophilus influenzae protein D), CRM197, other DT point mutants, such as CRM176, CRM228, CRM 45, CRM 9, CRM102, CRM 103 and CRM107. Preferably said immunogenic carrier is CRM197.

In an embodiment, the invention relates to an immunogen comprising an antigenic PCSK9 peptide consisting of, or consisting essentially of, an amino acid sequence selected from the group consisting of 1 to 312, 330 to 398 and 420 to 588, wherein said antigenic antigenic PCSK9 peptide further comprises at its C-terminus or at its N-terminus a cysteine which is chemically cross linked to an immunogenic carrier *via* a thioether linkage using SMPH (Succinimidyl-6-[ß-maleimidopropionamido]hexanoate) or BAANS (bromoacetic acid N-hydroxysuccinimide ester) as cross linker. In a preferred embodiment, said immunogenic carrier is selected from the group consisting of DT (Diphtheria toxin), TT (tetanus toxid) or fragment C of TT, PD (Haemophilus influenzae protein D, CRM197, other DT point mutants, such as CRM176, CRM228, CRM 45, CRM 9, CRM102, CRM 103 and CRM107. Preferably said immunogenic carrier is CRM197.

In an embodiment, the invention relates to an immunogen comprising an antigenic PCSK9 peptide consisting of, or consisting essentially of, an amino acid sequence selected from the group consisting of SEQ ID Nos: 1 to 312, 330 to 398 and 420 to 588, wherein said antigenic antigenic PCSK9 peptide further comprises at its C-terminus a cysteine which is chemically cross linked to an immunogenic carrier *via* a thioether linkage using SMPH (Succinimidyl-6-[ß-maleimidopropionamido]hexanoate) or BAANS (bromoacetic acid N-hydroxysuccinimide ester) as cross linker, said linkage being between a lysine residue of CRM197 and the cysteine residue of said antigenic peptide.

<u>Compositions comprising an antigenic PCSK9 peptide of the invention</u>

The present invention further relates to compositions, particularly immunogenic compositions also referred to as “subject immunogenic compositions”, comprising an antigenic PCSK9 peptide of the invention, preferably linked to an immunogenic carrier, and optionally at least one adjuvant. Such immunogenic compositions, particularly when formulated as pharmaceutical compositions, are deemed useful to prevent, treat or alleviate PCSK9-related disorders.

In some embodiments, a subject immunogenic composition according to the invention comprises an antigenic PCSK9 peptide, optionally comprising a linker, comprising an amino acid sequence selected

from SEQ ID Nos 1 to 328, 330 to 398, and 401 to 588 and functionally active variants thereof. In some embodiment, said antigenic PCSK9 peptide is linked to an immunogenic carrier, preferably a DT, CRM197 or a VLP, more preferably to a HBcAg, HBsAg, Qbeta, PP7, PPV or Norwalk Virus VLP.

In a preferred embodiment, a subject immunogenic composition according to the invention comprises an antigenic PCSK9 peptide, optionally comprising a linker, comprising an amino acid sequence selected from SEQ ID Nos 1 to 328, 330 to 398 and 401 to 588, and functionally active variants thereof linked to a VLP, preferably a Qbeta VLP.

In a preferred embodiment, a subject immunogenic composition according to the invention comprises an antigenic PCSK9 peptide optionally comprising a linker, comprising an amino acid sequence selected from SEQ ID Nos 1 to 328, 330 to 398, and 401 to 588 and functionally active variants thereof linked to CRM197.

A subject immunogenic composition comprising an antigenic PCSK9 peptide according to the invention can be formulated in a number of ways, as described in more detail below.

In some embodiments, a subject immunogenic composition comprises single species of antigenic PCSK9 peptide, e.g., the immunogenic composition comprises a population of antigenic PCSK9 peptides, substantially all of which have the same amino acid sequence. In other embodiments, a subject immunogenic composition comprises two or more different antigenic PCSK9 peptides, e.g., the immunogenic composition comprises a population of antigenic PCSK9 peptides, the members of which population can differ in amino acid sequence. A subject immunogenic composition can comprise from two to about 20 different antigenic PCSK9 peptides, e.g., a subject immunogenic composition can comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11-15, or 15-20 different antigenic PCSK9 peptides, each having an amino acid sequence that differs from the amino acid sequences of the other antigenic PCSK9 peptides.

In other embodiments, a subject immunogenic composition comprises a multimerized antigenic PCSK9 polypeptide, as described above. As used herein, the terms “immunogenic composition comprising an antigenic PCSK9 peptide” or “immunogenic composition of the invention” or “subject immunogenic composition” refers to an immunogenic composition comprising either single species (multimerized or not) or multiple species of antigenic PCSK9 peptide(s) coupled or not to an immunogenic carrier. Where two or more peptides are used coupled to a carrier, the peptide may be coupled to the same carrier molecule or individually coupled to carrier molecules and then combined to produce an immunogenic composition.

Another aspect of the invention relates to methods for producing an immunogen according to the invention, said method comprising coupling an antigenic PCSK9 peptide to an immunogenic carrier. In one embodiment, said coupling is chemical.

Adjuvants

In some embodiments, a subject immunogenic composition comprises at least one adjuvant. Suitable adjuvants include those suitable for use in mammals, preferably in humans. Examples of known suitable adjuvants that can be used in humans include, but are not necessarily limited to, alum, aluminum phosphate, aluminum hydroxide, MF59 (4.3% w/v squalene, 0.5% w/v polysorbate 80 (Tween 80), 0.5% w/v sorbitan trioleate (Span 85)), CpG-containing nucleic acid (where the cytosine is unmethylated),

QS21 (saponin adjuvant), MPL (Monophosphoryl Lipid A), 3DMPL (3-O-deacylated MPL), extracts from Aquilla, ISCOMS (see, e.g., Sjölander et al. (1998) *J. Leukocyte Biol.* 64:713; WO90/03184, WO96/11711, WO 00/48630, WO98/36772, WO00/41720, WO06/134423 and WO07/026190), LT/CT mutants, poly(D,L-lactide-co-glycolide) (PLG) microparticles, Quil A, TiterMax classic, TiterMax Gold, interleukins, and the like. For veterinary applications including but not limited to animal experimentation, one can use Freund's adjuvant, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion.

Further exemplary adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59™ (WO90/14837; Chapter 10 in *Vaccine design: the subunit and adjuvant approach,* eds. Powell & Newman, Plenum Press 1995), containing 5% Squalene, 0.5% Tween 80 (polyoxyethylene sorbitan mono-oleate), and 0.5% Span 85 (sorbitan trioleate) (optionally containing muramyl tri-peptide covalently linked to dipalmitoyl phosphatidylethanolamine (MTP-PE)) formulated into submicron particles using a microfluidizer, (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) RIBI™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components such as monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (DETOX™); (2) saponin adjuvants, such as QS21, STIMULON™ (Cambridge Bioscience, Worcester, MA), Abisco® (Isconova, Sweden), or Iscomatrix® (Commonwealth Serum Laboratories, Australia), may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes), which ISCOMS may be devoid of additional detergent *e.g.* WO00/07621; (3) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (4) cytokines, such as interleukins *(e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 (WO99/44636), *etc.),* interferons *(e.g.* gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), *etc.;* (5) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) *e.g.* GB-2220221, EP-A-0689454, optionally in the substantial absence of alum when used with pneumococcal saccharides *e.g.* WO00/56358; (6) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions *e.g.* EP-A-0835318, EP-A-0735898, EP-A-0761231; (7) oligonucleotides comprising CpG motifs [Krieg *Vaccine* 2000, 19, 618-622; Krieg *Curr opin Mol Ther2001* 3:15-24; Roman *et al., Nat. Med.,* 1997, 3, 849-854; Weiner *et al., PNAS USA,* 1997, 94, 10833-10837; Davis *et al, J. Immunol,* 1998, 160, 870-876; Chu *et al., J. Exp.Med,* 1997, 186, 1623-1631; Lipford *et al, Ear. J. Immunol.,* 1997, 27, 2340-2344; Moldoveami *et al., Vaccine,* 1988, 16, 1216-1224, Krieg *etal., Nature,* 1995, 374, 546-549; Klinman *et al., PNAS USA,* 1996, 93, 2879-2883; Ballas *et al, J. Immunol,* 1996, 157, 1840-1845; Cowdery *et al, J. Immunol,* 1996, 156, 4570-4575; Halpern *et al, Cell Immunol,* 1996, 167, 72-78; Yamamoto *et al, Jpn. J. Cancer Res.,* 1988, 79, 866-873; Stacey *et al, J. Immunol.,* 1996, 157,2116-2122; Messina *et al, J. Immunol,* 1991, 147, 1759-1764; Yi *et al, J. Immunol,* 1996, 157,4918-4925; Yi *et al, J. Immunol,* 1996, 157, 5394-5402; Yi *et al, J. Immunol,* 1998, 160, 4755-4761; and Yi *et*

al, J. Immunol, 1998, 160, 5898-5906; International patent applications WO96/02555, WO98/16247, WO98/18810, WO98/40100, WO98/55495, WO98/37919 and WO98/52581] *i.e.* containing at least one CG dinucleotide, where the cytosine is unmethylated; (8) a polyoxyethylene ether or a polyoxyethylene ester *e.g.* WO99/52549; (9) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol (WO01/21207) or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol (WO01/21152); (10) a saponin and an immunostimulatory oligonucleotide *(e.g.* a CpG oligonucleotide) (WO00/62800); (11) an immunostimulant and a particle of metal salt *e.g.* WO00/23105; (12) a saponin and an oil-in-water emulsion *e.g.* WO99/11241; (13) a saponin *(e.g.* QS21) + 3dMPL + IM2 (optionally + a sterol) *e.g.* WO98/57659; (14) other substances that act as immunostimulating agents to enhance the efficacy of the composition, such as Muramyl peptides include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-25 acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutarninyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE), (15) ligands for toll-like receptors (TLR), natural or synthesized (e.g. as described in Kanzler et al 2007, Nature Medicine 13, p1552-9), including TLR3 ligands such as polyI:C and similar compounds such as Hiltonol and Ampligen.

In a particular embodiment, said adjuvant is an immunostimulatory oligonucleotide and more preferably a CpG oligonucleotide. A CpG oligonucleotide as used herein refers to an immunostimulatory CpG oligodeoxynucleotide (CpG ODN), and accordingly these terms are used interchangeably unless otherwise indicated. Immunostimulatory CpG oligodeoxynucleotides contain one or more immunostimulatory CpG motifs that are unmethylated cytosine-guanine dinucleotides, optionally within certain preferred base contexts. The methylation status of the CpG immunostimulatory motif generally refers to the cytosine residue in the dinucleotide. An immunostimulatory oligonucleotide containing at least one unmethylated CpG dinucleotide is an oligonucleotide which contains a 5' unmethylated cytosine linked by a phosphate bond to a 3' guanine, and which activates the immune system through binding to Toll-like receptor 9 (TLR-9). In another embodiment the immunostimulatory oligonucleotide may contain one or more methylated CpG dinucleotides, which will activate the immune system through TLR9 but not as strongly as if the CpG motif(s) was/were unmethylated. CpG immunostimulatory oligonucleotides may comprise one or more palindromes that in turn may encompass the CpG dinucleotide. CpG oligonucleotides have been described in a number of issued patents, published patent applications, and other publications, including U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068.

Different classes of CpG immunostimulatory oligonucleotides have been identified. These are referred to as A, B, C and P class, and are described in greater detail below. Methods of the invention embrace the use of these different classes of CpG immunostimulatory oligonucleotides.

Any of the classes may be subjugated to an E modification which enhances its potency. An E modification may be a halogen substitution for the 5' terminal nucleotide; examples of such substitutions include but are not limited to bromo-uridine or iodo-uridine substitutions. An E modification can also include an ethyl-uridine substituation for the 5' terminal nucleotide.

The "A class" CpG immunostimulatory oligonucleotides are characterized functionally by the ability to induce high levels of interferon-alpha (IFN-α) from plasmacytoid dendritic cells (pDC) and inducing NK cell activation while having minimal effects on B cell activation. Structurally, this class typically has

stabilized poly-G sequences at 5' and 3' ends. It also has a palindromic phosphodiester CpG dinucleotide-containing sequence of at least 6 nucleotides, for example but not necessarily, it contains one of the following hexamer palindromes: GACGTC, AGCGCT, or AACGTT described by Yamamoto and colleagues. Yamamoto S et al. J. Immunol 148:4072-6 (1992). A class CpG immunostimulatory oligonucleotides and exemplary sequences of this class have been described in U.S. Non-Provisional Patent Application Serial No. 09/672,126 and published PCT application PCT/USOO/26527 (WO 01/22990), both filed on September 27, 2000.

In an embodiment, the "A class" CpG oligonucleotide of the invention has the following nucleic acid sequence: 5' GGGGACGACGTCGTGGGGGGG 3'

Some non-limiting examples of A-Class oligonucleotides include:

5' G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G 3' ; wherein * refers to a phosphorothioate bond and _ refers to a phosphodiester bond.

The B class CpG oligonucleotide sequences of the invention are those broadly described above as well as disclosed in published PCT Patent Applications PCT/US95/01570 and PCT/US97/19791, and in USPs 6,194,388, 6,207,646, 6,214,806, 6,218,371, 6,239,116 and 6,339,068. Exemplary sequences include but are not limited to those disclosed in these latter applications and patents.

In an embodiment, the "B class" CpG oligonucleotide of the invention has the following nucleic acid sequence:

5' TCGTCGTTTTTCGGTGCTTTT 3' (SEQ ID No 589), or

5' TCGTCGTTTTTCGGTCGTTTT 3'(SEQ ID No 590)or

5' TCGTCGTTTTGTCGTTTTGTCGTT 3' (SEQ ID No 591) or

5' TCGTCGTTTCGTCGTTTTGTCGTT 3' (SEQ ID No 592), or

5' TCGTCGTTTTGTCGTTTTTTTCGA 3' (SEQ ID No 593).

In any of these sequences, all of the linkages may be all phosphorothioate bonds. In another embodiment, in any of these sequences, one or more of the linkages may be phosphodiester, preferably between the "C" and the "G" of the CpG motif making a semi-soft CpG oligonucleotide. In any of these sequences, an ethyl-uridine or a halogen may substitute for the 5' T; examples of halogen substitutions include but are not limited to bromo-uridine or iodo-uridine substitutions.

Some non-limiting examples of B-Class oligonucleotides include:

5' T*C*G*T*C*G*T*T*T*T*T*C*G*G*T*G*C*T*T*T*T 3', or

5' T*C*G*T*C*G*T*T*T*T*T*T*C*G*G*T*C*G*T*T*T*T 3' or

5' T*C*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T 3', or

5' T*C*G*T*C*G*T*T*T*T*C*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T 3' , or

5' T*C*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T*T*T*T*T*T*T*C*G*A 3'.

wherein * refers to a phosphorothioate bond.

The "C class" of CpG immunostimulatory oligonucleotides is characterized functionally by the ability to activate B cells and NK cells and induce IFN-α. Structurally, this class typically includes a region with one or more B class-type immunostimulatory CpG motifs, and a GC -rich palindrome or near-palindrome region that allows the molecules to form secondary (e.g., stem-loop) or tertiary (e.g., dimer) type structures. Some of these oligonucleotides have both a traditional "stimulatory" CpG sequence and a

"GC-rich" or "B-cell neutralizing" motif. These combination motif oligonucleotides have immune stimulating effects that fall somewhere between the effects associated with traditional B class CpG oligonucleotides (i.e., strong induction of B cell activation and dendritic cell (DC) activation), and the effects associated with A class CpG ODN (i.e., strong induction of IFN-α and NK cell activation but relatively poor induction of B cell and DC activation). Krieg AM et al. (1995) Nature 374:546-9; Ballas ZK et al. (1996) J Immunol 157:1840-5; Yamamoto S et al. (1992) J Immunol 148:4072-6.

The C class of combination motif immune stimulatory oligonucleotides may have either completely stabilized, (e.g., all phosphorothioate), chimeric (phosphodiester central region), or semi-soft (e.g., phosphodiester within CpG motif) backbones. This class has been described in U.S. patent application US 10/224,523 filed on August 19, 2002.

One stimulatory domain or motif of the C class CpG oligonucleotide is defined by the formula: 5' X_1DCGHX_2 3'. D is a nucleotide other than C. C is cytosine. G is guanine. H is a nucleotide other than G. X_1 and X_2 are any nucleic acid sequence 0 to 10 nucleotides long. X_1 may include a CG, in which case there is preferably a T immediately preceding this CG. In some embodiments, DCG is TCG. X_1 is preferably from 0 to 6 nucleotides in length. In some embodiments, X_2 does not contain any poly G or poly A motifs. In other embodiments, the immunostimulatory oligonucleotide has a poly-T sequence at the 5' end or at the 3' end. As used herein, "poly- A" or "poly-T" shall refer to a stretch of four or more consecutive A's or T's respectively, e.g., 5' AAAA 3' or 5' TTTT 3'. As used herein, "poly-G end" shall refer to a stretch of four or more consecutive G's, e.g., 5' GGGG 3', occurring at the 5' end or the 3' end of a nucleic acid. As used herein, "poly-G oligonucleotide" shall refer to an oligonucleotide having the formula 5' X_1X_2GGGX_3X_4 3' wherein X_1, X_2, X_3, and X_4 are nucleotides and preferably at least one of X_3 and X_4 is a G. Some preferred designs for the B cell stimulatory domain under this formula comprise TTTTTCG, TCG, TTCG, TTTCG, TTTTCG, TCGT, TTCGT, TTTCGT, TCGTCGT.

The second motif of the C class CpG oligonucleotide is referred to as either P or N and is positioned immediately 5' to X_1 or immediately 3' to X_2.

N is a B cell neutralizing sequence that begins with a CGG trinucleotide and is at least 10 nucleotides long. A B cell neutralizing motif includes at least one CpG sequence in which the CG is preceded by a C or followed by a G (Krieg AM et al. (1998) Proc Natl Acad Sd USA 95:12631-12636) or is a CG containing DNA sequence in which the C of the CG is methylated. Neutralizing motifs or sequences have some degree of immunostimulatory capability when present in an otherwise non- stimulatory motif, but when present in the context of other immunostimulatory motifs serve to reduce the immunostimulatory potential of the other motifs.

P is a GC-rich palindrome containing sequence at least 10 nucleotides long.

As used herein, "palindrome" and equivalently "palindromic sequence" shall refer to an inverted repeat, i.e., a sequence such as ABCDEE'D'C'B'A' in which A and A', B and B', etc., are bases capable of forming the usual Watson-Crick base pairs.

As used herein, "GC-rich palindrome" shall refer to a palindrome having a base composition of at least two-thirds G's and Cs. In some embodiments the GC- rich domain is preferably 3' to the "B cell stimulatory domain". In the case of a 10- base long GC-rich palindrome, the palindrome thus contains at least 8 G's and Cs. In the case of a 12-base long GC-rich palindrome, the palindrome also contains at least 8 G's and Cs. In the case of a 14-mer GC-rich palindrome, at least ten bases of the palindrome are G's and Cs. In some embodiments the GC-rich palindrome is made up exclusively of G's and Cs.

In some embodiments the GC-rich palindrome has a base composition of at least 81 % G's and Cs. In the case of such a 10-base long GC-rich palindrome, the palindrome thus is made exclusively of G's and Cs. In the case of such a 12-base long GC-rich palindrome, it is preferred that at least ten bases (83 %) of the palindrome are G's and Cs. In some preferred embodiments, a 12-base long GC-rich palindrome is made exclusively of G's and Cs. In the case of a 14-mer GC-rich palindrome, at least twelve bases (86 %) of the palindrome are G's and Cs. In some preferred embodiments, a 14-base long GC-rich palindrome is made exclusively of G's and Cs. The Cs of a GC-rich palindrome can be unmethylated or they can be methylated.

In general this domain has at least 3 Cs and Gs, more preferably 4 of each, and most preferably 5 or more of each. The number of Cs and Gs in this domain need not be identical. It is preferred that the Cs and Gs are arranged so that they are able to form a self-complementary duplex, or palindrome, such as CCGCGCGG. This may be interrupted by As or Ts, but it is preferred that the self-complementarity is at least partially preserved as for example in the motifs CGACGTTCGTCG or CGGCGCCGTGCCG. When complementarity is not preserved, it is preferred that the non-complementary base pairs be TG. In a preferred embodiment there are no more than 3 consecutive bases that are not part of the palindrome, preferably no more than 2, and most preferably only 1. In some embodiments, the GC-rich palindrome includes at least one CGG trimer, at least one CCG trimer, or at least one CGCG tetramer. In other embodiments, the GC-rich palindrome is not CCCCCCGGGGGG or GGGGGGCCCCCC, CCCCCGGGGG or GGGGGCCCCC.

At least one of the G's of the GC rich region may be substituted with an inosine (I). In some embodiments, P includes more than one I.

In certain embodiments, the immunostimulatory oligonucleotide has one of the following formulas 5' NX_1DCGHX_2 3', 5' X_1DCGHX_2N 3', 5' PX_1DCGHX_2 3', 5' X_1DCGHX_2P 3', 5' $X_1DCGHX_2PX_3$ 3', 5' $X_1DCGHPX_3$ 3', 5' $DCGHX_2PX_3$ 3', 5' TCGHX2PX3 3', 5' $DCGHPX_3$ 3' or 5'DCGHP 3'.

The invention provides other immune stimulatory oligonucleotides defined by a formula 5' N_1PyGN_2P 3'. N_1 is any sequence 1 to 6 nucleotides long. Py is a pyrimidine. G is guanine. N_2 is any sequence 0 to 30 nucleotides long. P is a GC- rich palindrome containing a sequence at least 10 nucleotides long.

N_1 and N_2 may contain more than 50% pyrimidines, and more preferably more than 50% T. N_1 may include a CG, in which case there is preferably a T immediately preceding this CG. hi some embodiments, N1PyG is TCG, and most preferably a $TCGN_2$, where N_2 is not G.

N_1PyGN_2P may include one or more inosine (I) nucleotides. Either the C or the G in N_1 may be replaced by inosine, but the CpI is preferred to the IpG. For inosine substitutions such as IpG, the optimal activity may be achieved with the use of a "semi-soft" or chimeric backbone, where the linkage between the IG or the CI is phosphodiester. N1 may include at least one CI, TCI, IG or TIG motif.

In certain embodiments N_1PyGN_2 is a sequence selected from the group consisting of TTTTTCG, TCG, TTCG , TTTCG, TTTTCG, TCGT, TTCGT, TTTCGT, and TCGTCGT.

In an embodiment, the "C class" CpG oligonucleotide of the invention has the following nucleic acid sequence:

5' TCGCGTCGTTCGGCGCGCGCCG 3' (SEQ ID No 594), or

5' TCGTCGACGTTCGGCGCGCGCCG 3' (SEQ ID No 595), or

5' TCGGACGTTCGGCGCGCGCCG 3' (SEQ ID No 596), or

5' TCGGACGTTCGGCGCGCCG 3' (SEQ ID No 597), or
5' TCGCGTCGTTCGGCGCGCCG 3' (SEQ ID No 598), or
5' TCGACGTTCGGCGCGCGCCG 3' (SEQ ID No 599), or
5' TCGACGTTCGGCGCGCCG 3' (SEQ ID No 600), or
5' TCGCGTCGTTCGGCGCCG 3' (SEQ ID No 601), or
5' TCGCGACGTTCGGCGCGCGCCG 3' (SEQ ID No 602), or
5' TCGTCGTTTTCGGCGCGCGCCG 3' (SEQ ID No 603), or
5' TCGTCGTTTTCGGCGGCCGCCG 3' (SEQ ID No 604), or
5' TCGTCGTTTTACGGCGCCGTGCCG 3' (SEQ ID No 605), or
5' TCGTCGTTTTCGGCGCGCGCCGT 3' (SEQ ID No 606).

In any of these sequences, all of the linkages may be all phosphorothioate bonds. In another embodiment, in any of these sequences, one or more of the linkages may be phosphodiester, preferably between the "C" and the "G" of the CpG motif making a semi-soft CpG oligonucleotide.

Some non-limiting examples of C-Class oligonucleotides include:
5' T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' , or
5' T*C_G*T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' , or
5' T*C_G*G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' , or
5' T*C_G*G*A*C_G*T*T*C_G*G*C*G*C*G*C*C*G 3' , or
5' T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C*G*C*C*G 3' , or
5' T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' , or
5' T*C_G*A*C_G*T*T*C_G*G*C*G*C*G*C*C*G 3' , or
5' T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C*C*G 3' , or
5' T*C_G*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' , or
5' T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G 3' , or
5' T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*G*C*C*G*C*C*G 3' , or
5' T*C*G*T*C_G*T*T*T*T*A*C_G*G*C*G*C*C_G*T*G*C*C*G 3' , or
5' T*C_G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G*T 3'
wherein * refers to a phosphorothioate bond and _ refers to a phosphodiester bond.
In any of these sequences, an ethyl-uridine or a halogen may substitute for the 5' T; examples of halogen substitutions include but are not limited to bromo-uridine or iodo-uridine substitutions.

The "P class" CpG immunostimulatory oligonucleotides have been described in WO2007/095316 and are characterized by the fact that they contain duplex forming regions such as, for example, perfect or imperfect palindromes at or near both the 5' and 3' ends, giving them the potential to form higher ordered structures such as concatamers. These oligonucleotides referred to as P-Class oligonucleotides have the ability in some instances to induce much high levels of IFN-α secretion than the C-Class. The P-Class oligonucleotides have the ability to spontaneously self-assemble into concatamers either in vitro and/or in vivo. Without being bound by any particular theory for the method of action of these molecules, one potential hypothesis is that this property endows the P-Class oligonucleotides with the ability to more highly crosslink TLR9 inside certain immune cells, inducing a distinct pattern of immune activation compared to the previously described classes of CpG oligonucleotides.

In an embodiment, the CpG oligonucleotide for use in the present invention is a P class CpG oligonucleotide containing a 5' TLR activation domain and at least two palindromic regions, one palindromic region being a 5' palindromic region of at least 6 nucleotides in length and connected to a 3' palindromic region of at least 8 nucleotides in length either directly or through a spacer, wherein the oligonucleotide includes at least one YpR dinucleotide. In an embodiment, said oligoonucleotide is not T*C_G*T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*C*G. In one embodiment the P class CpG oligonucleotide includes at least one unmethylated CpG dinucleotide. In another embodiment the TLR activation domain is TCG, TTCG, TTTCG, TYpR, TTYpR, TTTYpR, UCG, UUCG, UUUCG, TTT, or TTTT. In yet another embodiment the TLR activation domain is within the 5' palindromic region. In another embodiment the TLR activation domain is immediately 5' to the 5' palindromic region. In still another embodiment the 5' palindromic region is at least 8 nucleotides in length. In another embodiment the 3' palindromic region is at least 10 nucleotides in length. In another embodiment the 5' palindromic region is at least 10 nucleotides in length. In yet another embodiment the 3' palindromic region includes an unmethylated CpG dinucleotide. In another embodiment the 3' palindromic region includes two unmethylated CpG dinucleotides. In another embodiment the 5' palindromic region includes an unmethylated CpG dinucleotide. In yet another embodiment the 5' palindromic region includes two unmethylated CpG dinucleotides. In another embodiment the 5' and 3' palindromic regions have a duplex stability value of at least 25. In another embodiment the 5' and 3' palindromic regions have a duplex stability value of at least 30. In another embodiment the 5' and 3' palindromic regions have a duplex stability value of at least 35. In another embodiment the 5' and 3' palindromic regions have a duplex stability value of at least 40. In another embodiment the 5' and 3' palindromic regions have a duplex stability value of at least 45. In another embodiment the 5' and 3' palindromic regions have a duplex stability value of at least 50. In another embodiment the 5' and 3' palindromic regions have a duplex stability value of at least 55. In another embodiment the 5' and 3' palindromic regions have a duplex stability value of at least 60. In another embodiment the 5' and 3' palindromic regions have a duplex stability value of at least 65.

In one embodiment the two palindromic regions are connected directly. In another embodiment the two palindromic regions are connected via a 3 '-3' linkage. In another embodiment the two palindromic regions overlap by one nucleotide. In yet another embodiment the two palindromic regions overlap by two nucleotides. In another embodiment the two palindromic regions do not overlap. In another embodiment the two palindromic regions are connected by a spacer. In one embodiment the spacer is a nucleic acid having a length of 1-50 nucleotides. In another embodiment the spacer is a nucleic acid having a length of 1 nucleotide. In another embodiment the spacer is a non-nucleotide spacer. In one embodiment the non-nucleotide spacer is a D-spacer. In another embodiment the non-nucleotide spacer is a linker. In one embodiment the oligonucleotide has the formula 5' XP_1SP_2T 3', wherein X is the TLR activation domain, P_1 is a palindrome, S is a spacer, P_2 is a palindrome, and T is a 3' tail of 0-100 nucleotides in length. In one embodiment X is TCG, TTCG, or TTTCG. In another embodiment T is 5-50 nucleotides in length. In yet another embodiment T is 5-10 nucleotides in length. In one embodiment S is a nucleic acid having a length of 1-50 nucleotides. In another embodiment S is a nucleic acid having a length of 1 nucleotide. In another embodiment S is a non-nucleotide spacer. In one embodiment the non-nucleotide spacer is a D-spacer. In another embodiment the non-nucleotide spacer is a linker. In another embodiment the oligonucleotide is not an antisense oligonucleotide or a ribozyme. In one embodiment P_1 is A and T rich.

In another embodiment P_1 includes at least 4 Ts. In another embodiment P_2 is a perfect palindrome. In another embodiment P2 is G-C rich. In still another embodiment P_2 is $CGGCGCX_1GCGCCG$, where X_1 is T or nothing.

In one embodiment the oligonucleotide includes at least one phosphorothioate linkage. In another embodiment all internucleotide linkages of the oligonucleotide are phosphorothioate linkages. In another embodiment the oligonucleotide includes at least one phosphodiester-like linkage. In another embodiment the phosphodiester-like linkage is a phosphodiester linkage. In another embodiment a lipophilic group is conjugated to the oligonucleotide. In one embodiment the lipophilic group is cholesterol.

In an embodiment, the TLR-9 agonist for use in the present invention is a P class CpG oligonucleotide with a 5' TLR activation domain and at least two complementarity-containing regions, a 5' and a 3' complementarity-containing region, each complementarity-containing region being at least 8 nucleotides in length and connected to one another either directly or through a spacer, wherein the oligonucleotide includes at least one pyrimidine-purine (YpR) dinucleotide, and wherein at least one of the complementarity-containing regions is not a perfect palindrome. In one embodiment the oligonucleotide includes at least one unmethylated CpG dinucleotide. In another embodiment the TLR activation domain is TCG, TTCG, TTTCG, TYpR, TTYpR, TTTYpR, UCG, UUCG, UUUCG, TTT, or TTTT. In another embodiment the TLR activation domain is within the 5' complementarity-containing region. In another embodiment the TLR activation domain is immediately 5' to the 5' complementarity- containing region. In another embodiment the 3' complementarity-containing region is at least 10 nucleotides in length. In yet another embodiment the 5' complementarity-containing region is at least 10 nucleotides in length. In one embodiment the 3' complementarity- containing region includes an unmethylated CpG dinucleotide. In another embodiment the 3' complementarity-containing region includes two unmethylated CpG dinucleotides. In yet another embodiment the 5' complementarity-containing region includes an unmethylated CpG dinucleotide. In another embodiment the 5' complementarity-containing region includes two unmethylated CpG dinucleotides. In another embodiment the complementarity- containing regions include at least one nucleotide analog. In another embodiment the complementarity-containing regions form an intramolecular duplex. In one embodiment the intramolecular duplex includes at least one non- Watson Crick base pair. In another embodiment the non- Watson Crick base pair is G-T, G-A, G-G, or C-A. In one embodiment the complementarity-containing regions form intermolecular duplexes. In another embodiment at least one of the intermolecular duplexes includes at least one non- Watson Crick base pair. In another embodiment the non- Watson Crick base pair is G-T, G- A, G-G, or C-A. In yet another embodiment the complementarity-containing regions contain a mismatch. In still another embodiment the complementarity-containing regions contain two mismatches. In another embodiment the complementarity-containing regions contain an intervening nucleotide. In another embodiment the complementarity-containing regions contain two intervening nucleotides.

In one embodiment the 5' and 3' complementarity-containing regions have a duplex stability value of at least 25. In another embodiment the 5' and 3' complementarity- containing regions have a duplex stability value of at least 30. In another embodiment the 5' and 3' complementarity-containing regions have a duplex stability value of at least 35. In another embodiment the complementarity-containing regions have a duplex stability value of at least 40. In another embodiment the complementarity-containing regions have a duplex stability value of at least 45. In another embodiment the complementarity-containing regions have a duplex stability value of at least 50. In another embodiment the complementarity-

containing regions have a duplex stability value of at least 55. In another embodiment the complementarity-containing regions have a duplex stability value of at least 60. In another embodiment the complementarity-containing regions have a duplex stability value of at least 65.

In another embodiment the two complementarity-containing regions are connected directly. In another embodiment the two palindromic regions are connected via a 3 '-3' linkage. In yet another embodiment the two complementarity-containing regions overlap by one nucleotide. In another embodiment the two complementarity-containing regions overlap by two nucleotides. In another embodiment the two complementarity-containing regions do not overlap. In another embodiment the two complementarity-containing regions are connected by a spacer. In another embodiment the spacer is a nucleic acid having a length of 1 -50 nucleotides. In another embodiment the spacer is a nucleic acid having a length of 1 nucleotide. In one embodiment the spacer is a non-nucleotide spacer. In another embodiment the non-nucleotide spacer is a D-spacer. In yet another embodiment the non- nucleotide spacer is a linker.

In one embodiment the P-class oligonucleotide has the formula 5' XNSPT 3', wherein X is the TLR activation domain, N is a non-perfect palindrome, P is a palindrome, S is a spacer, and T is a 3' tail of 0-100 nucleotides in length. In another embodiment X is TCG, TTCG, or TTTCG. In another embodiment T is 5-50 nucleotides in length. In another embodiment T is 5-10 nucleotides in length. In another embodiment S is a nucleic acid having a length of 1-50 nucleotides. In another embodiment S is a nucleic acid having a length of 1 nucleotide. In another embodiment S is a non-nucleotide spacer. In another embodiment the non-nucleotide spacer is a D-spacer. In another embodiment the non-nucleotide spacer is a linker. In another embodiment the oligonucleotide is not an antisense oligonucleotide or a ribozyme. In another embodiment N is A and T rich. In another embodiment N is includes at least 4 Ts. In another embodiment P is a perfect palindrome. In another embodiment P is G-C rich. In another embodiment P is $CGGCGCX_1GCGCCG$, wherein X_1 is T or nothing. In another embodiment the oligonucleotide includes at least one phosphorothioate linkage. In another embodiment all interaucleotide linkages of the oligonucleotide are phosphorothioate linkages. In another embodiment the oligonucleotide includes at least one phosphodiester-like linkage. In another embodiment the phosphodiester-like linkage is a phosphodiester linkage. In another embodiment a lipophilic group is conjugated to the oligonucleotide. In one embodiment the lipophilic group is cholesterol.

In an embodiment, the "P class" CpG oligonucleotides of the invention has the following nucleic acid sequence: 5' TCGTCGACGATCGGCGCGCGCCG 3' (SEQ ID No 607).

In said sequences, all of the linkages may be all phosphorothioate bonds. In another embodiment, one or more of the linkages may be phosphodiester, preferably between the “C” and the “G” of the CpG motif making a semi-soft CpG oligonucleotide. In any of these sequences, an ethyl-uridine or a halogen may substitute for the 5' T; examples of halogen substitutions include but are not limited to bromo-uridine or iodo-uridine substitutions.

A non-limiting example of P-Class oligonucleotides include:

5' T*C_G*T*C_G*A*C_G*A*T*C_G*G*C*G*C_G*C*G*C*C*G 3'

wherein * refers to a phosphorothioate bond and _ refers to a phosphodiester bond.

In an embodiment, all the internucleotide linkage of the CpG oligonucleotides disclosed herein are phosphodiester bonds (“soft” oligonucleotides, as described in the PCT application WO2007/026190). In

another embodiment, CpG oligonucleotides of the invention are rendered resistant to degradation (e.g., are stabilized). A "stabilized oligonucleotide" refers to an oligonucleotide that is relatively resistant to *in vivo* degradation (e.g. via an exo- or endo-nuclease). Nucleic acid stabilization can be accomplished via backbone modifications. Oligonucleotides having phosphorothioate linkages provide maximal activity and protect the oligonucleotide from degradation by intracellular exo- and endo-nucleases.

The immunostimulatory oligonucleotides may have a chimeric backbone, which have combinations of phosphodiester and phosphorothioate linkages. For purposes of the instant invention, a chimeric backbone refers to a partially stabilized backbone, wherein at least one internucleotide linkage is phosphodiester or phosphodiester-like, and wherein at least one other internucleotide linkage is a stabilized internucleotide linkage, wherein the at least one phosphodiester or phosphodiester-like linkage and the at least one stabilized linkage are different. When the phosphodiester linkage is preferentially located within the CpG motif such molecules are called “semi-soft” as described in the PCT application WO2007/026190.

Other modified oligonucleotides include combinations of phosphodiester, phosphorothioate, methylphosphonate, methylphosphorothioate, phosphorodithioate, and/or p-ethoxy linkages.

Since boranophosphonate linkages have been reported to be stabilized relative to phosphodiester linkages, for purposes of the chimeric nature of the backbone, boranophosphonate linkages can be classified either as phosphodiester-like or as stabilized, depending on the context. For example, a chimeric backbone according to the instant invention could, in some embodiments, includes at least one phosphodiester (phosphodiester or phosphodiester-like) linkage and at least one boranophosphonate (stabilized) linkage. In other embodiments, a chimeric backbone according to the instant invention could include boranophosphonate (phosphodiester or phosphodiester-like) and phosphorothioate (stabilized) linkages. A "stabilized internucleotide linkage" shall mean an internucleotide linkage that is relatively resistant to in vivo degradation (e.g., via an exo- or endo-nuclease), compared to a phosphodiester internucleotide linkage. Preferred stabilized internucleotide linkages include, without limitation, phosphorothioate, phosphorodithioate, methylphosphonate, and methylphosphorothioate. Other stabilized internucleotide linkages include, without limitation, peptide, alkyl, dephospho, and others as described above.

Modified backbones such as phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made, e.g., as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described. Uhlmann E et al. (1990) Chem Rev 90:544; Goodchild J (1990) Bioconjugate Chem 1:165. Methods for preparing chimeric oligonucleotides are also known. For instance patents issued to Uhlmann et al have described such techniques.

Mixed backbone modified ODN may be synthesized as described in the PCT application WO2007/026190.

The oligonucleotides of the invention can also include other modifications. These include nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is

alkylated. Nucleic acids which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

The size of the CpG oligonucleotide (i.e., the number of nucleotide residues along the length of the oligonucleotide) also may contribute to the stimulatory activity of the oligonucleotide. For facilitating uptake into cells, CpG oligonucleotide of the invention preferably have a minimum length of 6 nucleotide residues. Oligonucleotides of any size greater than 6 nucleotides (even many kb long) are capable of inducing an immune response if sufficient immunostimulatory motifs are present, because larger oligonucleotides are degraded inside cells. In certain embodiments, the CpG oligonucleotides are 6 to 100 nucleotides long, preferentially 8 to 30 nucleotides long. In important embodiments, nucleic acids and oligonucleotides of the invention are not plasmids or expression vectors.
In an embodiment, the CpG oligonucleotide disclosed herein comprise substitutions or modifications, such as in the bases and/or sugars as described at paragraph 134 to 147 of WO2007/026190.

In an embodiment, the CpG oligonucleotide of the present invention is chemically modified. Examples of chemical modifications are known to the skilled person and are described, for example in Uhlmann E. et al. (1990), Chem. Rev. 90:543, S. Agrawal, Ed., Humana Press, Totowa, USA 1993; Crooke, S.T. et al. (1996) Annu. Rev. Pharmacol. Toxicol. 36:107-129; and Hunziker J. et al., (1995), Mod. Synth. Methods 7:331-417. An oligonucleotide according to the invention may have one or more modifications, wherein each modification is located at a particular phosphodiester internucleoside bridge and/or at a particular β-D-ribose unit and/or at a particular natural nucleoside base position in comparison to an oligonucleotide of the same sequence which is composed of natural DNA or RNA.
In some embodiments of the invention, CpG-containing nucleic acids might be simply mixed with immunogenic carriers according to methods known to those skilled in the art (see, e.g. WO03/024480).

In a particular embodiment of the present invention, any of the vaccine disclosed herein comprises from 20µg to 20mg of CpG oligonucleotide, preferably from 0.1mg to 10 mg CpG oligonucleotide, preferably from 0.2 mg to 5 mg CpG oligonucleotide, preferably from 0.3 mg to 3 mg CpG oligonucleotide, even preferably from 0.4 to 2 mg CpG oligonucleotide, even preferably from 0.5 to 1.5 mg CpG oligonucleotide. In a preferred embodiement, any of the vaccine disclosed herein comprises approximately 0.5 to 1mg CpG oligonucleotide.

Preferred adjuvants for use in the present invention are alum, QS21, CpG ODN, alum in combination with CpG ODN, Iscomatrix and Iscomatrix in combination with CpG ODN.

Pharmaceutical compositions of the invention

The invention also provides pharmaceutical compositions comprising an antigenic PCSK9 peptide of the invention or an immunogenic composition thereof, in a formulation in association with one or more pharmaceutically acceptable excipient(s) and optionally combined with one or more adjuvants (as adjuvant described above). The term 'excipient' is used herein to describe any ingredient other than the active ingredient, i.e. the antigenic PCSK9 peptide of the invention eventually coupled to an immunogenic carrier and optionally combined with one or more adjuvants. The choice of excipient(s) will to a large extent depend on factors such as the particular mode of administration, the effect of the

excipient on solubility and stability, and the nature of the dosage form. As used herein, "pharmaceutically acceptable excipient" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Some examples of pharmaceutically acceptable excipients are water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Additional examples of pharmaceutically acceptable substances are wetting agents or minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the active ingredient.

Pharmaceutical compositions of the present invention and methods for their preparation will be readily apparent to those skilled in the art. Such compositions and methods for their preparation may be found, for example, in *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company, 1995). Pharmaceutical compositions are preferably manufactured under GMP conditions.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

Any method for administering peptides, or proteins accepted in the art may suitably be employed for the peptides or proteins of the invention.

The pharmaceutical compositions of the invention are typically suitable for parenteral administration. As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue, thus generally resulting in the direct administration into the blood stream, into muscle, or into an internal organ. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal, intramuscular, intrasternal, intravenous, intraarterial, intrathecal, intraventricular, intraurethral, intracranial, intrasynovial injection or infusions; and kidney dialytic infusion techniques. Preferred embodiments include the intravenous, subcutaneous, intradermal and intramuscular routes, even more preferred embodiments are the intramuscular or the subcutaneous routes.

Formulations of a pharmaceutical composition suitable for parenteral administration typically generally comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampoules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and the like. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents.

In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i.e. powder or granular) form for reconstitution with a suitable vehicle (e.g. sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition. Parenteral formulations also include aqueous solutions which may contain excipients such as salts, carbohydrates and buffering agents (preferably to a pH of from 3 to 9), but, for some applications, they may be more suitably formulated as a sterile non-aqueous solution or as a dried form to be used in conjunction with a suitable vehicle such as sterile, pyrogen-free water. Exemplary parenteral administration forms include solutions or suspensions in sterile aqueous solutions, for example, aqueous propylene glycol or dextrose solutions. Such dosage forms can be suitably buffered, if desired. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, microparticles, or in a liposomal preparation. Formulations for parenteral administration may be formulated to be immediate and/or modified release. Modified release formulations include delayed-, sustained-, pulsed-, controlled-, targeted and programmed release.

For example, in one aspect, sterile injectable solutions can be prepared by incorporating the anti-PCSK9 peptide, preferably coupled to an immunogenic carrier, optionally in combination with one or more adjuvants, in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

An exemplary, non-limiting pharmaceutical composition of the invention is a formulation as a sterile aqueous solution having a pH that ranges from about 5.0 to about 6.5 and comprising from about 0.1 mg/mL to about 20 mg/mL of a peptide of the invention, from about 1 millimolar to about 100 millimolar of histidine buffer, from about 0.01 mg/mL to about 10 mg/mL of polysorbate 80, from about 100 millimolar to about 400 millimolar of trehalose, and from about 0.01 millimolar to about 1.0 millimolar of disodium EDTA dihydrate.

The antigenic PCSK9 peptides of the invention can also be administered intranasally or by inhalation, typically in the form of a dry powder (either alone, as a mixture, or as a mixed component particle, for example, mixed with a suitable pharmaceutically acceptable excipient) from a dry powder inhaler, as an aerosol spray from a pressurised container, pump, spray, atomiser (preferably an atomiser using electrohydrodynamics to produce a fine mist), or nebuliser, with or without the use of a suitable propellant, or as nasal drops.

The pressurised container, pump, spray, atomizer, or nebuliser generally contains a solution or suspension of an antibody of the invention comprising, for example, a suitable agent for dispersing, solubilising, or extending release of the active, a propellant(s) as solvent.

Prior to use in a dry powder or suspension formulation, the drug product is generally micronised to a size suitable for delivery by inhalation (typically less than 5 microns). This may be achieved by any appropriate comminuting method, such as spiral jet milling, fluid bed jet milling, supercritical fluid processing to form nanoparticles, high pressure homogenisation, or spray drying.

Capsules, blisters and cartridges for use in an inhaler or insufflator may be formulated to contain a powder mix of the compound of the invention, a suitable powder base and a performance modifier.

A suitable solution formulation for use in an atomiser using electrohydrodynamics to produce a fine mist may contain a suitable dose of the antigenic PCSK9 peptide of the invention per actuation and the actuation volume may for example vary from 1µL to 100µL.

Suitable flavours, such as menthol and levomenthol, or sweeteners, such as saccharin or saccharin sodium, may be added to those formulations of the invention intended for inhaled/intranasal administration.

Formulations for inhaled/intranasal administration may be formulated to be immediate and/or modified release. Modified release formulations include delayed-, sustained-, pulsed-, controlled-, targeted and programmed release.

In the case of dry powder inhalers and aerosols, the dosage unit is determined by means of a valve which delivers a metered amount. Units in accordance with the invention are typically arranged to administer a metered dose or "puff" of an antibody of the invention. The overall daily dose will typically be administered in a single dose or, more usually, as divided doses throughout the day.

A pharmaceutical composition comprising an antigenic PCSK9 peptide may also be formulated for an oral route administration. Oral administration may involve swallowing, so that the compound enters the gastrointestinal tract, and/or buccal, lingual, or sublingual administration by which the compound enters the blood stream directly from the mouth.

Formulations suitable for oral administration include solid, semi-solid and liquid systems such as tablets; soft or hard capsules containing multi- or nano-particulates, liquids, or powders; lozenges (including liquid-filled); chews; gels; fast dispersing dosage forms; films; ovules; sprays; and buccal/mucoadhesive patches.

Liquid formulations include suspensions, solutions, syrups and elixirs. Such formulations may be employed as fillers in soft or hard capsules (made, for example, from gelatin or hydroxypropylmethylcellulose) and typically comprise a carrier, for example, water, ethanol, polyethylene glycol, propylene glycol, methylcellulose, or a suitable oil, and one or more emulsifying agents and/or suspending agents. Liquid formulations may also be prepared by the reconstitution of a solid, for example, from a sachet.

The compositions of the invention can be used to treat, alleviate or prevent PCSK9-mediated disorders or symptoms in a subject at risk or suffering from such disorder or symptom by stimulating an immune response in said subject by immunotherapy. Immunotherapy can comprise an initial immunization followed by additional, e. g. one, two, three, or more boosters.

An "immunologically effective amount" of an antigenic PCSK9 peptide of the invention, or composition thereof, is an amount that is delivered to a mammalian subject, either in a single dose or as part of a series, which is effective for inducing an immune response against PCSK9 in said subject. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic

group of individual to be treated, the capacity of the individual's immune system to synthesize antibodies, the formulation of the vaccine, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

A "pharmaceutically effective dose" or "therapeutically effective dose" is that dose required to treat or prevent, or alleviate one or more PCSK9-related disorder or symptom in a subject. The pharmaceutically effective dose depends on *inter alia* the specific compound to administer, the severity of the symptoms, the susceptibility of the subject to side effects, the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration such as health and physical condition, concurrent medication, the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, and other factors that those skilled in the medical arts will recognize. For prophylaxis purposes, the amount of peptide in each dose is selected as an amount which induces an immunoprotective response without significant adverse side effects in typical vaccinees. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

It is understood that the specific dose level for any particular patient depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

For example, antigenic PCSK9 peptides or pharmaceutical composition of the invention can be administered to a subject at a dose of about 0.1 µg to about 5 mg, e.g., from about 0.1 µg to about 5 µg, from about 5 µg to about 10 µg, from about 10 µg to about 25 µg, from about 25 µg to about 50 µg, from about 50 µg to about 100 µg, from about 100 µg to about 500 µg, from about 500 µg to about 1 mg, from about 1 mg to about 2 mg, with optional boosters given at, for example, 1 week, 2 weeks, 3 weeks, 4 weeks, two months, three months, 6 months and/or a year later.

In some embodiments, a single dose of an antigenic PCSK9 peptide or pharmaceutical composition according to the invention is administered. In other embodiments, multiple doses of an antigenic PCSK9 peptide or pharmaceutical composition according to the invention are administered. The frequency of administration can vary depending on any of a variety of factors, e.g., severity of the symptoms, degree of immunoprotection desired, whether the composition is used for prophylactic or curative purposes, etc. For example, in some embodiments, an antigenic PCSK9 peptide or pharmaceutical composition according to the invention is administered once per month, twice per month, three times per month, every other week (qow), once per week (qw), twice per week (biw), three times per week (tiw), four times per week, five times per week, six times per week, every other day (qod), daily (qd), twice a day (qid), or three times a day (tid). When the composition of the invention is used for prophylaxis purposes, they will be generally administered for both priming and boosting doses. It is expected that the boosting doses will be adequately spaced, or preferably given yearly or at such times where the levels of circulating antibody fall below a desired level. Boosting doses may consist of the antigenic PCSK9 peptide in the absence of the original immunogenic carrier molecule. Such booster constructs may comprise an alternative immunogenic carrier or may be in the absence of any carrier. Such booster compositions may be formulated either with or without adjuvant.

The duration of administration of an antigenic PCSK9 peptide according to the invention, e.g., the period of time over which an antigenic PCSK9 peptide is administered, can vary, depending on any of a variety

of factors, e.g., patient response, etc. For example, an antigenic PCSK9 peptide can be administered over a period of time ranging from about one day to about one week, from about two weeks to about four weeks, from about one month to about two months, from about two months to about four months, from about four months to about six months, from about six months to about eight months, from about eight months to about 1 year, from about 1 year to about 2 years, or from about 2 years to about 4 years, or more.

A variety of treatment methods are also contemplated by the present disclosure, which methods comprise administering an antigenic PCSK9 peptide according to the invention. Subject treatment methods include methods of inducing an immune response in an individual to self-PCSK9, and methods of preventing, alleviating or treating a PCSK9-related disorder or symptom in an individual.

In one aspect, the present invention provides a method for treating, preventing or alleviating a PCSK9-related disorder or symptom in a subject, comprising administering a therapeutically effective amount of an antigenic PCSK9 peptide of the invention, or immunogenic or pharmaceutical composition thereof, to said subject.

In another aspect, the present invention provides a method for inducing an immune response against self-PCSK9 in a subject, comprising administering a therapeutically or immunogenically effective amount of an antigenic PCSK9 peptide of the invention, or immunogenic or pharmaceutical composition thereof, to said subject.

A PCSK9 related disease or a PCSK9 mediated disease is, for example, a disease where the inhibition of PCSK9 activity or the inhibition of the interaction of PCSK9 with the LDL receptor could be beneficial.

"Treat", "treating" and "treatment" refer to a method of alleviating or abrogating a biological disorder and/or at least one of its attendant symptoms. As used herein, to "alleviate" a disease, disorder or condition means reducing the severity and/or occurrence frequency of the symptoms of the disease, disorder, or condition. Further, references herein to “treatment” include references to curative, palliative and prophylactic treatment. Said subject is preferably human, and may be either male or female, of any age.

Other aspects of the invention relate to an antigenic PCSK9 peptide according to the invention or of an immunogenic composition or a pharmaceutical composition thereof, for use as a medicament, preferably in treatment, alleviation or prophylaxis of PCSK9-related disorders.

In yet another aspect, the present invention provides the use of an antigenic PCSK9 peptide of the invention or of an immunogenic composition or a pharmaceutical composition thereof, in the manufacture of a medicament, preferably for treating a PCSK9-related disorder.

In particular, the invention relates to an antigenic PCSK9 peptide of the invention, or an immunogenic or pharmaceutical composition thereof, for use as a medicament preferably in treatment, alleviation or prophylaxis of diseases associated with an elevated level of cholesterol.

In yet another aspect, the present invention provides the use of an antigenic PCSK9 peptide of the invention or of an immunogenic composition or a pharmaceutical composition thereof, in the manufacture of a medicament, preferably for lowering the LDL-cholesterol level in blood in a subject in need thereof.

In some aspects of the uses or methods of the invention, said PCSK9-related disorder is selected from the group consisting of elevated cholesterol, a condition associated with elevated LDL-cholesterol, e.g., a lipid disorder (e.g., hyperlipidemia, type I, type II, type III, type IV, or type V hyperlipidemia, secondary

hypertriglyceridemia, hypercholesterolemia, familial hypercholesterolemia, xanthomatosis, cholesterol acetyltransferase deficiency), arteriosclerotic conditions (e.g., atherosclerosis), coronary artery disease, and cardiovascular disease.

In yet another aspect, the present invention provides the use of an antigenic PCSK9 peptide of the invention or of an immunogenic composition or a pharmaceutical composition thereof, in the manufacture of a medicament for treating or alleviating diseases where an up-regulation of the LDL receptor or an inhibition of the interaction between PCSK9 and the LDL receptor is beneficial.

In yet another aspect, the present invention provides the use of an antigenic PCSK9 peptide of the invention or of an immunogenic composition or a pharmaceutical composition thereof, in the manufacture of a medicament for the treatment of Alzheimer's disease.

In other aspects of the uses or methods of the invention, said subject is a mammal, preferably a human subject.

In still other aspects of the uses or methods of the invention, said subject suffers from said PSCK9-related disorder. Alternatively, said subject is at risk of suffering from said PCSK9-related disorder, e.g., due to the presence of one or more risk factors (e.g., hypertension, cigarette smoking, diabetes, obesity, or hyperhomocysteinemia).

The antigenic PCSK9 peptide of the invention or an immunogenic composition or a pharmaceutical composition thereof are useful for subjects who are intolerant to therapy with another cholesterol-reducing agent, or for whom therapy with another cholesterol-reducing agent has produced inadequate results (e.g., subjects who experience insufficient LDL-c reduction on statin therapy). The antigenic PCSK9 peptide of the invention described herein can be administered to a subject with elevated LDL-cholesterol.

Preferably a subject with elevated cholesterol is a human subject with total plasma cholesterol levels of 200 mg/dl or greater. Preferably a subject with elevated cholesterol is a human subject with LDL-cholesterol levels of 160 mg/dl or greater.

Total plasma cholesterol levels and LDL-cholesterol levels are measured using standard methods on blood samples obtained after an appropriate fast. Protocols to measure total plasma cholesterol levels and LDL-cholesterol levels are well-known to the man skilled in the art.

In one embodiment the antigenic PCSK9 peptide or an immunogenic composition or a pharmaceutical composition thereof is administered together with another agent, the two can be administered sequentially in either order or simultaneously. In some embodiments, an antigenic PCSK9 peptide or an immunogenic composition or a pharmaceutical composition thereof is administered to a subject who is also receiving therapy with a second agent (e.g., a second cholesterol-reducing agent). Cholesterol reducing agents include statins, bile acid sequestrants, niacin, fibric acid derivatives, and long chain alpha, omego-dicarboxylic acids. Statins inhibit cholesterol synthesis by blocking HMGCoA, a key enzyme in cholesterol biosynthesis. Examples of statins are lovastatin, pravastatin, atorvastatin, cerivastatin, fluvastatin, and simvastatin. Bile acid sequestrants interrupt the recycling of bile acids from the intestine to the liver. Examples of these agents are cholestyramine and colestipol hydrochloride. Examples of fibric acid derivatives are clofibrate and gemfibrozil. Long chain alpha, omego-dicarboxylic

acids are described, e.g., by Bisgaier et al., 1998, J. Lipid Res. 39:17-30; WO 98/30530; U.S. Pat. No. 4,689,344; WO 99/001 16; U.S. Pat. No. 5,756,344; U.S. Pat. No. 3,773,946; U.S. Pat. No. 4,689,344; U.S. Pat. No. 4,689,344; U.S. Pat. No. 4,689,344; and U.S. Pat. No. 3,930,024); ethers (see, e.g., U.S. Pat. No. 4,711,896; U.S. Pat. No. 5,756,544; U.S. Pat. No. 6,506,799). Phosphates of dolichol (U.S. Pat. No. 4,613,593), and azolidinedione derivatives (U.S. Pat. No. 4,287,200) can also be used to reduce cholesterol levels. A combination therapy regimen may be additive, or it may produce synergistic results (e.g., reductions in cholesterol greater than expected for the combined use of the two agents). In some embodiments, combination therapy with an antigenic PCSK9 peptide or an immunogenic composition or a pharmaceutical composition thereof and a statin produces synergistic results (e.g., synergistic reductions in cholesterol). In some subjects, this can allow reduction in statin dosage to achieve the desired cholesterol levels.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1. Selection of antigenic PCSK9 peptides at the PCSK9-EGF-A domain of the LDL receptor interface

The structure of human PCSK9 binding to the EGF-A domain of the LDL receptor has been solved and published (Kwon et al, PNAS 105, 1820-1825, 2008). This structural information (PDB: 3BPS) was used together with information from the structure of free PCSK9, PDB: 2P4E (Cunningham et al, Nature Structural & Molecular Biology, 14, 413-419, 2007) to design the following peptides which would correspond to areas of importance for the PCSK9-LDL receptor interaction (see figure 1).

Peptide 1. ASSDCSTCFV (SEQ ID No: 19)
Peptide 2. GTRFHRQASK (SEQ ID No: 63)
Peptide 3. IQSDHREIEGRV (SEQ ID No: 109)
Peptide 4. SGRDAGVAKGA (SEQ ID No: 153)
Peptide 5. SIPWNLERITP (SEQ ID No: 184)

Since peptides 1-4 represent loops in the PCSK9 structure, the respective sequences (SEQ ID Nos 19, 63, 109 and 153) were made with added Cys, Cys-Gly or Lys linkers to allow coupling via both ends to the VLP carrier to provide a conformational mimetic of the natural loop structure (VR_9.1 to VR_9.4 in Table 1). In addition, cyclised versions of peptides 2-4 were also made (VR_9.6 to VR_9.9 in Table 1)

which provided a Cys residue for coupling to VLPs. Peptide 1 was made with a Lys-Gly-Gly N-terminal linker for coupling purposes so that the two Cys residues were free to disulphide bond as they do in the native PCSK9 structure. Peptide 5 represents the N-terminal of the mature processed form of human PCSK9 and was coupled via a C-terminally added cysteine residue to allow the N-terminus to be free for antibody recognition (VR_9.5 in Table 1). The following table (Table 1) describes 9 peptides generated for evaluation as vaccine candidates.

Table 1. Peptide Sequences

Peptide	Sequence	SEQ ID No
VR_9.1	KGGASSDCSTCFV	313
VR_9. 2	CGGTRFHRQASKC	314
VR_9.3	CGIQSDHREIEGRVC	315
VR_9.4	CSGRDAGVAKGAC	316
VR_9.5	SIPWNLERITPC	317
VR_9.6	ASK-Cys(H)-GDGTRFHRQ	318
VR_9.7	AG-Cys-(H)-GTRFHRQ	319
VR_9.8	GRV-Cys(H)-IQSDHREIE	320
VR_9.9	AGVAKGAG-Cys(H)-SGRD	321

Underscore indicates cysteine residues assed for conjugation purposes and double underscore indicates a GC or KGG linker.

Example 2 - Preparation of Peptide-VLP conjugates for evaluation as vaccine candidates

The peptides were synthesised using a standard Fmoc protocol on CLEAR amide resin. The amino acid coupling reactions were carried out using 5 fold excess of Fmoc-protected amino acid activated with 1 eq of HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) in the presence of HOBt (hydroxybenzotriazole) and NMM (N-methylmorpholine). The deprotection of Fmoc group was achieved with 20% piperidine/DMF. Resin-bound peptide was then cleaved and side chain protecting groups removed simultaneously with Reagent D (TFA/H2O/DODT: 89/3/8). The peptide was made with a free N-terminus and amidated C-terminus. The crude peptide was purified to homogeneity by HPLC using a BEH 130 C18 column and a water/acetonitrile gradient in the presence of 0.1% TFA. The purified peptide was vacuum-dried using a lyophilizer. The peptide was analyzed using mass-spectrometry (LC-MS) and gave satisfactory data.

The Qβ VLP used in this study was produced by bacterial *E.Coli* fermentation in a BL21 (DE3) strain incorporating a pET28 plasmid encoding the 14kD monomer protein: MAKLETVTLGNIGKDGKQTLVLNPRGVNPTNGVASLSQAGAVPALEKRVTVSVSQPSRNRKNYKVQVKI QNPTACTANGSCDPSVTRQAYADVTFSFTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY (Genbank ID: M99039). The fermentation is induced at an OD600 of 0.8 with IPTG and allowed to proceed overnight in terrific broth (TB) with kanamycin. The VLP, which self-assembles in the host cell, was then purified from the fermentation cell pellet using the method described in the patent application EP1736538 with the following differences: after cell disruption, the clarified homogenate was treated with ammonium sulphate at 50% saturation and the cell pellet recovered by centrifugation. Then, the pellet was

redissolved in HEPES buffer and dialysed against HEPES buffer before proceeding to the first column step in the published method. After the ion-exchange column and hydroxylapatite column steps, the material was purified using a further anion-exchange column step and sterile filtered to make the final VLP bulk material, which was analysed by size-exclusion chromatography, SDS-PAGE and electron microscopy with acceptable results.

Conjugation of peptides through cysteine residues:

The Qβ VLP was activated using either N-gamma-maleimido-butyryloxy-succinimide ester (GMBS) or the longer Succinimidyl-6-[ß-maleimidopropionamido]hexanoate linking reagent. The procedure for the usage of both these reagents was similar: Solid reagent was dissolved in dimethyl sulphoxide (DMSO) and added to the VLP solution at ≥10 -fold molar excess. The activation reaction was allowed to proceed for ≥90 minutes and the solution was then desalted using a NAP-25 desalting column into Dulbeccos Phosphate Buffered Saline (DPBS) with 5 mM EDTA or Dulbeccos Phosphate Buffered Saline (DPBS) that had been modified by the addition of solid NaCl (14.6g / L). If necessary, the protein solution was concentrated slightly using 10kD spin microconcentrators prior to the next conjugation reaction.

Prior to the conjugation reaction, the peptides were dissolved in an aliquot of pH 7.4 DPBS, with 5mM EDTA as an additive. The concentration of the peptide in solution was 10 mg/ml. The solubilised peptide was added to an aliquot of TCEP immobilised reducing agent (Pierce Chemical) which had been washed in DPBS containing 5mM EDTA. The aliquot of peptides was incubated with mixing in the presence of the TCEP gel for approximately 1 hour, after which time the aliquot was spun down in a microfuge and the solid pellet discarded. The reduced peptide-containing supernatant was added directly to the activated VLP which had been prepared earlier. One alternative to this procedure however is the addition of solid peptide directly to the sample of activated Qβ VLP. Both methods work equally well for the generation of peptide-VLP conjugates.

The reaction between the VLPs and the reduced peptides was allowed to proceed for at least thirty minutes with very gentle mixing. At the end of the reaction time each sample was desalted into Dulbeccos PBS (DPBS) using NAP-10 or NAP-25 desalting columns (GE Healthcare). The desalted conjugated peptides were analysed for protein content using the Bradford (Coomassie Brilliant Blue, Pierce Chemical Co) assay or BCA protein assay (bicinchoninic acid) (Pierce Chemical Co) as well as by SDS-PAGE and size-exclusion chromatography. The conjugate products were sterile filtered using a 0.22 µm filter and stored at 2-8 ºC until use. Careful attention was paid to these samples during storage to prevent freezing or exposure to extremes in temperature.

The conjugation of the PCSK9 peptide to CRM197 was performed by using BAANS (Bromoacetic acid N-hydroxysuccinimide ester, Sigma B8271). CRM197 was first activated by reacting with BAANS in 0.1 M sodium carbonate pH 8.3 with a molar ratio of 100 in a cold room for 90 minutes. The reaction mixture was passed through a Zeba desalting column, and the flow-through was collected. The PCSK9 peptide at 10 mg/ml was incubated with an equal volume of immobilized TCEP reducing gel (Thermo Scientific) at room temperature for 1 hour, and collected after centrifugation through a 0.2 µm filter. The activated CRM197 was then mixed with the treated peptide in the cold room overnight, followed by an extensive

dialysis in PBS buffer. The conjugate was recovered, concentrated, and sterilized through a 0.22 μm filter. The protein concentration was determined using Coomassie blue assay (Thermo Scientific).

Conjugation of peptides via amines

For the conjugation of peptides to Qβ via amine residues, specifically peptide VR_9.1, the following procedure was followed. Qβ was initially derivatised by the addition of solid succinic anhydride at a ≥10 -fold molar excess relative to the VLP monomer. The succinic anhydride was allowed to dissolve and the derivatisation reaction was allowed proceed for at least 30mins. After this time, the sample was then desalted using a NAP-25 desalting column into Dulbeccos Phosphate Buffered Saline (DPBS) with 5 mM EDTA. Then, the following reagents were added in the order listed at a ≥10 -fold molar excess relative to the VLP monomer: Solid peptide, *N*-hydroxysulfosuccinimide and finally 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide. Following the addition of reagents in the order listed above, the sample was incubated at room temperature and the reaction was allowed proceed for at least 30mins, after which time the VLP-peptide conjugate was desalted using NAP-25 desalting columns into Dulbeccos Phosphate Buffered Saline (DPBS).

The extent of the conjugation for the VLP-peptide samples was measured using SDS-PAGE, and a molecular weight increase was observed for all samples which is consistent with the addition of the peptide to the VLP protein monomer. In addition, samples were tested in the HPLC size-exclusion chromatography assay (using a Tosoh PWXL5000 HPLC column) and found to contain assembled VLP when compared to unconjugated samples of VLP. VLP-peptide conjugation features are summarized in Table 2.

Table 2. VLP-Peptide Conjugates

Peptide	Activated VLP added	Amount of peptide added	Percentage yield of input VLP	Approximate substitution (ug peptide per mg of VLP monomer)
VR_9.1	4 mg	5 mg	40-60%	25 ug
VR_9.2	3 mg	5 mg	40-60%	125 ug
VR_9.3	3 mg	5 mg	40-60%	125 ug
VR_9.4	3 mg	5 mg	40-60%	125 ug
VR_9.5	3 mg	5 mg	40-60%	50 ug
VR_9.6	3 mg	5 mg	40-60%	125 ug
VR_9.7	3 mg	5 mg	40-60%	125 ug
VR_9.8	3 mg	5 mg	40-60%	125 ug
VR_9.9	3 mg	5 mg	40-60%	125 ug
VR_9.10	3 mg	5 mg	40-60%	125 ug
VR_9.11	3 mg	2 mg	40-60%	125 ug
VR_9.12	3 mg	2 mg	40-60%	125 ug
VR_9.13	3 mg	2 mg	40-60%	125 ug
VR_9.14	3 mg	2 mg	40-60%	125 ug
VR_9.15	3 mg	2 mg	40-60%	125 ug
VR_9.16	3 mg	2 mg	40-60%	125 ug
VR_9.17	10 mg	9.5 mg	95%	82 ug

VR_9.18	10 mg	8.7 mg	90%	74 ug
VR_9.19	10 mg	7.6 mg	80%	58 ug
VR_9.20	10 mg	8.7 mg	95%	86 ug
VR_9.21	10 mg	8.8 mg	95%	92 ug
VR_9.22	10 mg	6.6 mg	85%	62 ug
VR_9.23	10 mg	10.0 mg	90%	85 ug
VR_9.24	10 mg	10.5 mg	75%	64 ug
VR_9.25	10 mg	5.1 mg	40%	1 ug
VR_9.26	10 mg	10.0 mg	60%	123 ug
VR_9.27	10 mg	9.6 mg	60%	136 ug
VR_9.28	10 mg	9.4 mg	65%	153 ug
VR_9.29	10 mg	4.2 mg	75%	19 ug
VR_9.30	10 mg	4.4 mg	63%	15 ug
VR_9.31	10 mg	4.4 mg	70%	13 ug
VR_9.32	10 mg	4.4 mg	63%	15 ug
VR_9.33	10 mg	7.4 mg	40%	18 ug
VR_9.34	10 mg	7.3 mg	50%	23 ug
VR_9.35	7.5 mg	4.3 mg	61%	16 ug

*As determined by SDS-PAGE and densitometry calculations

Example 3: PCSK9 peptide immunogenicity

This study aimed to evaluate how efficacious peptides conjugated to a Qbeta VLP (as detailed in Example 2 above) were in inducing an antibody response that can bind to human and mouse PCSK9. Female Balb/c (6-8 weeks) were injected by the intramuscular route (50 microliter volume injected into each *Tibialis anterior* muscle) on days 0, 21 and 42 with VLP-peptide conjugates formulated in Alum with CpG of formula 5' TCGTCGTTTTTCGGTGCTTTT 3'. One group of control mice was immunized with VLP coupled to a control (non-PCSK9) peptide following the same protocol and a second control group was left unimmunised. Necropsy took place on day 49. At necropsy 400 – 600 microliter blood was sampled from euthanised mice by cardiac puncture using an anti-coagulant. Blood was centrifuged to separate the plasma, which was stored frozen until testing.

IgG antibody responses to full length human recombinant PCSK9 protein were measured using a colorimetric ELISA method. Serial dilutions were prepared from sera samples and tested in the assay.

Human PCSK9 ELISA method 1: 384-well high bind assay plates (Corning International Cat#3700) were coated with 25µL/well of human PCSK9 protein stock diluted to 1µg/mL with 0.01M PBS pH 7.4 and incubated on a shaker at RT for 3 hours. After washing x 2 with PBS pH 7.4, plates were blocked using 80µL/well of 0.01M PBS/1% BSA, incubated at RT for 1 hour before a final wash x 3 with 0.01M PBS pH 7.4/0.05% Tween 20. The following day, an 8 point ½ log serial dilution of each sample was prepared starting at 1:25 dilution (PBS/1%BSA diluent), 25µL/well of the serial dilution transferred in duplicate into the human PCSK9 coated plate then incubated shaking at RT for 1.5 hours. After washing x 3 with 0.01M PBS pH 7.4/0.05% Tween 20, 25µL/well of Total IgG detection antibody (Rabbit anti-mu IgG-Fc,

Cat# A90-130A Bethyl Laboratories) at a 1:6000 dilution with 0.01M PBS pH 7.4/1%BSA was added, then incubated shaking at RT for 1 hour. After washing x 5 with 0.01M PBS pH 7.4/0.05% Tween 20, added 25µL/well Bio-Rad kit goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad Cat#172 -1019) 1:3000 with 0.01M PBS pH 7.4/0.05% Tween 20 pH 7.4, then incubated shaking at RT for 1 hour. After washing x 4 with 0.01M PBS pH 7.4/0.05% Tween 20 and then x 1 with 0.01M PBS pH 7.4 only, 25µL/well Mouse Typer HRP Substrate (Bio-Rad Cat#172 -1064) were added and the plates were incubated at RT for 30mins. 25µL/well 2% oxalic acid were added and the absorbance then read at Abs 405nm.

Mouse PCSK9 ELISA method 1: Thermo Immunolon 4HBX 96-well ELISA plates were coated with 100µl of 1µg/ml recombinant mouse PCSK9 in PBS overnight at 4°C. After washing, plates were blocked with 300ml PBS/0.5% BSA (Sigma A7030) for 1 hr, washed 4x with 300µl PBS/0.01% Tween-20 and 100µl of serial dilutions of plasma samples (in PBS/0.5% BSA) added. After incubation with gentle shaking for 1 hour at room temperature, plates were washed 4x with 300µl PBS/0.01% Tween-20 and 100µl of a 1:5000 dilution of goat anti-mouse IgG-HRP (horse radish peroxidase; Pierce 31430) added to each well. The plates were incubated at room temperature with gentle shaking for 45 minutes and then washed x7 with 300µl PBS/0.01% Tween-20. 100µl TMB substrate (Sigma T-4444) was then added, the colorimetric reaction stopped after 4 minutes by addition of 2N sulphuric acid and the absorbance read at 450nm.

Data analysis: Titration curves were plotted for each test sample (sample dilution vs absorbance). The sample titer (subsequently transformed into reciprocal titer) was then taken as the serum dilution achieving a cut-off optical density (0.D.) values of 1.0 or 0.5.

Measurement of plasma/serum cholesterol level

Cholesterol levels in plasma and serum samples were measured using a WAKO Cholesterol E Assay kit (Cat# 439-17501) following the manufacturers' instructions. Dilutions of cholesterol standard or test plasma/serum samples (4µl volume) were added to wells of a 96-well plate and 196µl of prepared cholesterol reagent added. The plate was incubated for 5 minutes at 37°C and the absorbance of the developed colour read at 600nm within 30 minutes.

Measurement of interaction between PCSK9 and the extracellular domain of the LDL receptor

Interruption of LDLR and PCSK9 binding by mouse plasma was determined with TR-FRET assay (time-resolved fluorescence resonance energy transfer assay) using fluorophor-labeled LDLR extracellular domain (LDLR-ECD) and full length wild type PCSK9 protein. LDLR-ECD (R&D system, cat#2148-LD/CF)) was labeled with europium (GE healthcare, Cat#PA99148) according to manufacturer's instruction (at a Eu:LDLR molar ratio 6:1). PCSK9 was biotinylated with Biotin-XX-SSE (Pierce, cat#21237) at a Biotin:PCSK9 molar ratio of 8. The TR-FRET assay was conducted with 5nM LDLR-Eu+3, 30nM PCSK9-biotin and 50nM Alexa Fluor 647 conjugated streptavidin (SA647, Invitrogen, cat# S21374) in 20ul of assay buffer (20mM Hepes, pH7.0, 150mM NaCl, 0.1mM CaCl2 and 0.05%(w/v) BSA) in 384-well black plates (Corning, Cat# 3676). Serial dilution of mouse plasma were pre-incubated with PCSK9-biotin at RT for 30 minutes in humidified chamber, followed by mixing with LDLR and streptavidin-

SA647. After an additional 60 minute incubation at RT in a humidified atmosphere in dark, the plates were read on a Perkin Elmer Victor2 plate reader using a 50µs delay time and a 400µs window. Data are reported as the ratio of the signals at (665nm/615nM) x1000.

Results: as shown in figure 2, all peptides used as immunogens were able to induce antibody responses to the intact full-length human PCSK9 protein, some inducing higher responses than others. In all cases these responses cross-reacted with mouse PCSK9 as shown in Figure 3. Figure 4 shows that peptide VR_9.5 immunization also led to a decrease in plasma cholesterol levels and Figures 5 and 6 show that VR_9.5 and VR_9.6 induced serum antibody responses that could inhibit the interaction between PCSK9 and LDL receptors using a fluorescence resonance energy transfer (FRET) assay.

Example 4 - Design of peptides corresponding to regions distinct from receptor EGF-A domain present in PDB: 3BPS:

The LDL receptor is a multidomain protein the extracellular domains of which consist of an N-terminal ligand binding domain, three EGF like repeats (of which EGF-A is one) a β-propeller domain and an "O linked sugar domain" (Kwon et al, PNAS 105, 1820-1825, 2008). The PDB file 3GCX details the structure of PCSK9 in complex with a soluble form of only the EGF-A domain of the LDL receptor, therefore we postulated that further non-obvious interactions may exist between PCSK9 and the LDL receptor that cannot be deduced from direct analysis of the molecules represented in PDB: 3GCX. Examples of these regions are detailed in figure 7 and specifically two sequences in PCSK9 were identified that could act as additional putative receptor interfaces (NAQDQPVTLGTL and INEAWFPEDQRVL - see figure 8).

SIPWNLERIIP (SEQ ID No 332) (mouse)
NAQDQPVTLGTL (SEQ ID No: 227)
INEAWFPEDQRVL (SEQ ID No: 263)
INMAWFPEDQQVL (SEQ ID No 360) (mouse)

By using murine PCSK9 sequences as found in public databases the murine homologues were also identified (as displayed in table 3). We also postulated that part of the amino acid sequence contained within peptide VR_9.5 (described in Example 1) may also interact with parts of the LDL receptor not visible in the PDB: 3BPS (Figure 8). This concept was probed by refining the sequence VR_9.5 by altering the amino acid linker and the orientation of conjugation. We also identified a peptide corresponding to the mouse homologue of VR_9.5 (peptide VR_9.10). The resultant series of peptide sequences from the approach described above were modified by the addition of amino acids to permit chemical conjugation and were evaluated as vaccine candidates (peptides detailed in table 3).

Table 3 - Peptide sequences

Peptide	Sequence	Species	SEQ ID No
VR_9.10	SIPWNLERIIPC	Mouse	322
VR_9.11	CGGSIPWNLERIIP	Mouse	323
VR_9.12	SIPWNLERIIPGGC	Mouse	324
VR_9.13	CGGNAQDQPVTLGTL	Mouse & Human	325

VR_9.14	NAQDQPVTLGTLGGC	Mouse & Human	326
VR_9.15	CGGINMAWFPEDQQVL	Mouse	327
VR_9.16	INMAWFPEDQQVLGGC	Mouse	328

Residues underlined indicate amino acids added for conjugation purposes.

Example 5

Peptides VR_9.10 to VR_9.16 (as well as VR_9.5 for comparison) were conjugated to Qβ VLP as described in example 2 and used to immunize mice and assess antibody responses to PCSK9 as described in example 3, with an unconjugated VLP used as a control immunogen. As shown in Figure 9 and 10, all peptides induced antibodies that could recognize intact full-length mouse PCSK9 in an ELISA assay.

Example 6

On the basis of our observation of cholesterol lowering being induced by immunization with peptide VR_9.5, representing the N-terminus of the mature processed form of human PCSK9 (SEQ ID No: 184), we hypothesize that, since the cleaved prodomain of immature PCSK9 is known to remain associated with the mature PCSK9 protein, regions of this prodomain (SEQ ID No. 329) are also candidate antibody targets for lowing cholesterol levels.

MGTVSSRRSWWPLPLLLLLLLLLGPAGARAQEDEDGDYEELVLALRSEEDGLAEAPEHGTTATFHRC AKDPWRLPGTYVVVLKEETHLSQSERTARRLQAQAARRGYLTKILHVFHGLLPGFLVKMSGDLLELAL KLPHVDYIEEDSSVFAQ (SEQ ID No: 329)

Specific, non-restrictive, examples of peptides of interest are found within the C-terminal sequence of the prodomain region and surface exposed sequences and loops, including regions containing known loss-of-function or gain-of-function genetic mutations in humans:

VDYIEEDSSVFAQ (SEQ ID No: 308)
RCAKDPWRLPGT (SEQ ID No: 309)
AQAARRGYLTKIL (SEQ ID No: 310)
GDYEELVLALRSEEDG (SEQ ID No: 311)
FLVKMSGDLLELALKLP (SEQ ID No: 312)

The above peptides and truncations thereof are synthesized with N- or C-terminally added linkers (e.g. CGG or GGC), or as cyclised or otherwise conformationally constrained molecules and coupled to Qβ VLPs as described in example 2 and used to immunize mice and assess antibody responses to PCSK9 as described in example 3.

Example 7

Peptides VR_9.5 and 9.10 were conjugated to Qβ VLP or to CRM197 as described in example 2 and used to immunize mice (BALB/c or C57BL/6) using TiterMax Gold, alum or alum-CpG as adjuvant.

Hepatitis B virus derived peptide (amino acids 28-39 (IPQSLDSWWTSL)) was conjugated to Qβ VLP or to CRM197 and used as a control immunogen.

The ELISA method used was slightly different from the one disclosed in example 3: the ELISA method was performed as follows for Human and Mouse PCSK9 ELISA (Method 2): 384-well high bind assay plates (Greiner bio-one 781061) were coated with 25µL/well of human or mouse PCSK9 protein stock diluted to 1µg/mL with 1X PBS pH 7.4 and incubated at 4°C over night. The following day, plates were blocked using 25µL/well of 1X PBS/0.05% Tween-20/1% BSA, incubated on a shaker at RT for 1 hour. A 10 point ½ log serial dilution of each sample was prepared starting at 1:50 or 1:500 dilution (1X PBS/0.05% Tween-20 diluent), 25µL/well of the serial dilution transferred in duplicate into the human or mouse PCSK9 coated plate then incubated shaking at RT for 1 hour. After washing x 3 with 1X PBS pH 7.4/0.05% Tween-20,25µL/well of Total IgG detection antibody (Goat Anti-Mouse IgG (γ), HRP, Invitrogen M30107) at a 1:3000 dilution with 1X PBS pH 7.4/0.05% Tween-20 was added then incubated shaking at RT for 1 hour. After washing x 5 with 1X PBS pH 7.4/0.05% Tween 20, 25µL/well Bio-Rad TMB Peroxidase EIA Substrate Kit (Bio-Rad Cat#172 -1067) were added and the plates incubated for 15 minutes. 12.5µL/well of 1N sulfuric acid were added and the absorbance then read at Abs 450nm.

Results: Peptides VR_9.5 and 9.10 conjugated to Qβ VLP or CRM197 were able to induce antibody responses to the intact full-length human and mouse PCSK9 protein (see figures 11 and 12). Figures 13 and 14 and Table 4 also show that peptide VR_9.5 and 9.10 conjugated to different carriers and in the presence of different adjuvants led to a decrease in serum cholesterol levels.

Table 4 - Total Cholesterol Levels in Immunized Mmice

Mouse Strain	Immunogen	Carrier	Adjuvant(s)	N	Cholesterol (mg/dL)		Stats[a]	Stats[b]
					Mean	SEM		
BALB/c	Untreated	-	-	8	106.5	3.0	-	-
	Control peptide	VLP	Alum + CpG	8	99.0	2.8	P>0.05	-
	VR_9.5	VLP	Alum + CpG	8	77.5	3.6	P<0.001	P<0.001
	VR_9.5	VLP	Alum	8	65.9	2.3	P<0.001	P<0.001
	VR_9.10	VLP	Alum + CpG	8	85.3	2.4	P<0.001	P<0.05
	Control peptide	CRM197	TiterMax	8	96.6	2.3	P>0.05	-
	VR_9.5	CRM197	TiterMax	8	75.0	4.1	P<0.001	P<0.001
	VR_9.10	CRM197	TiterMax	7	70.9	2.8	P<0.001	P<0.001
C57BL/6	Untreated	-	-	8	83.4	5.8	-	-
	Control peptide	VLP	Alum + CpG	8	81.0	3.7	P>0.05	-
	VR_9.5	VLP	Alum + CpG	8	59.7	2.8	P<0.001	P<0.01
	VR_9.5	VLP	Alum	8	59.5	1.5	P<0.001	P<0.01
	VR_9.10	VLP	Alum + CpG	8	64.5	2.7	P<0.01	P<0.05
	Control peptide	CRM197	TiterMax	8	71.5	1.7	P>0.05	-
	VR_9.5	CRM197	TiterMax	8	58.7	4.1	P<0.001	P>0.05
	VR_9.10	CRM197	TiterMax	8	63.8	3.4	P<0.01	P>0.05

Stats[(a)]: 1-way ANOVA statistical comparison with Tukey post-hoc test displaying p values of test groups vs. untreated.

Stats[(b)]: 1-way ANOVA statistical comparison with Tukey post-hoc test displaying p values of test groups vs. matched control peptide-carrier.

Example 8

Additional peptides (SEQ ID No 312, 420, 421, 422, 423, 425, 426, 427, 428, 445, 482, 525 and 563) were chosen from both the prodomain and the C-terminal region of the catalytic domain of PCSK9 for surface exposure and association with gain of function or loss of function mutations identified in humans. The resultant series of peptide sequences from the approach described above were modified by the addition of amino acids to permit chemical conjugation and were evaluated as vaccine candidates (Table 5 peptides 9.23-9.35).

Table 5 - Peptide sequences

Peptide	Sequence	Species	SEQ ID No
VR_9.5	SIPWNLERITPC	Human	317
VR_9.10	SIPWNLERIIPC	Mouse	322
VR_9.17	SIPWNLERIGGC	Human & Mouse	401
VR_9.18	SIPWNLERGGC	Human & Mouse	402
VR_9.19	SIPWNLEGGC	Human & Mouse	403
VR_9.20	CGGSGRDAGVAKGA	Human	404
VR_9.21	CGGSGRDAGVAKGT	Mouse	405
VR_9.22	RDAGVAKGGC	Human	406
VR_9.23	CSRHLAQASQELQ	Human	407
VR_9.24	CRSRPSAKASWVQ	Mouse	408
VR_9.25	CGGDYEELVLALR	Human	409
VR_9.26	CGGDYEELMLALP	Mouse	410
VR_9.27	LVLALRSEEDGGC	Human	411
VR_9.28	LMLALPSQEDGGC	Mouse	412
VR_9.29	AKDPWRLPGGC	Human	413
VR_9.30	SKEAWRLPGGC	Mouse	414
VR_9.31	CGGAARRGYLTK	Human	415
VR_9.32	CGGAARRGYVIK	Mouse	416
VR_9.33	FLVKMSGDLLELALKLPGGC	Human	417
VR_9.34	FLVKMSSDLLGLALKLPGGC	Mouse	418
VR_9.35	CGGEEDSSVFAQ	Human	419

Residues underlined indicate amino acids added for conjugation purposes.

Example 9

Peptides VR_9.17 to VR_9.35 (as well as VR_9.5) were conjugated to Qβ VLP as described in example 2 and used to immunize mice to assess antibody responses to PCSK9 as described in example 3. Hepatitis B virus derived peptide (amino acids 28-39 (IPQSLDSWWTSL)) was conjugated to Qβ VLP and used as a control immunogen. Method No 2 was used for the ELISA.

Result: As shown in figures 15 and 16, most of the the peptides conjugated to Qb VLP were able to induce antibody responses to intact full length PCSK9. No antibody response was detected for peptides 9.31 and 9.32. In certain cases (peptides 9.23, 9.24, 9.27, 9.28, 9.29, 9.30, 9.33, and 9.34) the antibody responses were species specific. Immunization with peptides 9.5, 9.18, and 9.29 also resulted in decreased serum cholesterol, while immunization with peptides 9.17, 9.19, 9.30, 9.31, and 9.32 resulted in a trend to reduced cholesterol (Table 6, figure 17).

Table 6 Total Cholesterol Levels in Immunized Mice

Mouse Strain	Immunogen	Carrier	Adjuvants	N	Cholesterol (mg/dL)		Stats[a]	Stats[b]
					Mean	SEM		
BALB/c	Untreated	-	-	8	77.7	5.5	-	-
	Control peptide	VLP	Alum + CpG	4	78.1	1.2	P>0.05	-
	VR_9.5	VLP	Alum + CpG	8	65.8	1.1	**P<0.05**	P>0.05
	VR_9.17	VLP	Alum + CpG	8	70.4	2.1	P>0.05	P>0.05
	VR_9.18	VLP	Alum + CpG	8	65.9	1.6	**P<0.05**	P>0.05
	VR_9.19	VLP	Alum + CpG	8	71.7	1.7	P>0.05	P>0.05
	VR_9.20	VLP	Alum + CpG	7	85.2	4.0	P>0.05	P>0.05
	VR_9.21	VLP	Alum + CpG	8	77.1	2.0	P>0.05	P>0.05
	VR_9.22	VLP	Alum + CpG	8	84.5	2.6	P>0.05	P>0.05
	VR_9.23	VLP	Alum + CpG	8	79.0	1.8	P>0.05	P>0.05
	VR_9.24	VLP	Alum + CpG	8	76.9	2.1	P>0.05	P>0.05
	VR_9.25	VLP	Alum + CpG	8	77.3	2.5	P>0.05	P>0.05
	VR_9.26	VLP	Alum + CpG	8	79.2	2.2	P>0.05	P>0.05
	VR_9.27	VLP	Alum + CpG	8	71.8	3.4	P>0.05	P>0.05
	VR_9.28	VLP	Alum + CpG	8	76.6	2.2	P>0.05	P>0.05
	VR_9.29	VLP	Alum + CpG	8	67.0	1.6	**P<0.05**	P>0.05
	VR_9.30	VLP	Alum + CpG	8	70.4	2.3	P>0.05	P>0.05
	VR_9.31	VLP	Alum + CpG	8	71.0	1.7	P>0.05	P>0.05
	VR_9.32	VLP	Alum + CpG	8	72.8	1.0	P>0.05	P>0.05
	VR_9.33	VLP	Alum + CpG	8	75.5	2.2	P>0.05	P>0.05
	VR_9.34	VLP	Alum + CpG	8	76.0	2.3	P>0.05	P>0.05
	VR_9.35	VLP	Alum + CpG	8	74.5	2.2	P>0.05	P>0.05

Stats[a]: 1-way ANOVA statistical comparison with Bonferroni post-hoc test displaying p values of test groups vs. untreated group.

Stats[b]: 1-way ANOVA statistical comparison with Bonferroni post-hoc test displaying p values of test groups vs. control peptide group.

Sequence listing:

SEQ ID No 1	IGASSDCSTCFVS	SEQ ID No 53	DGTRFHRQASKCDS
SEQ ID No 2	IGASSDCSTCFV	SEQ ID No 54	DGTRFHRQASKCD
SEQ ID No 3	IGASSDCSTCF	SEQ ID No 55	DGTRFHRQASKC
SEQ ID No 4	IGASSDCSTC	SEQ ID No 56	DGTRFHRQASK
SEQ ID No 5	IGASSDCST	SEQ ID No 57	DGTRFHRQAS
SEQ ID No 6	IGASSDCS	SEQ ID No 58	DGTRFHRQA
SEQ ID No 7	IGASSDC	SEQ ID No 59	DGTRFHRQ
SEQ ID No 8	IGASSD	SEQ ID No 60	GTRFHRQASKCDS
SEQ ID No 9	IGASS	SEQ ID No 61	GTRFHRQASKCD
SEQ ID No 10	GASSDCSTCFVS	SEQ ID No 62	GTRFHRQASKC
SEQ ID No 11	GASSDCSTCFV	SEQ ID No 63	GTRFHRQASK
SEQ ID No 12	GASSDCSTCF	SEQ ID No 64	GTRFHRQAS
SEQ ID No 13	GASSDCSTC	SEQ ID No 65	GTRFHRQA
SEQ ID No 14	GASSDCST	SEQ ID No 66	GTRFHRQ
SEQ ID No 15	GASSDCS	SEQ ID No 67	TRFHRQASKCDS
SEQ ID No 16	GASSDC	SEQ ID No 68	TRFHRQASKCD
SEQ ID No 17	GASSD	SEQ ID No 69	TRFHRQASKC
SEQ ID No 18	ASSDCSTCFVS	SEQ ID No 70	TRFHRQASK
SEQ ID No 19	ASSDCSTCFV	SEQ ID No 71	TRFHRQAS
SEQ ID No 20	ASSDCSTCF	SEQ ID No 72	TRFHRQA
SEQ ID No 21	ASSDCSTC	SEQ ID No 73	TRFHRQ
SEQ ID No 22	ASSDCST	SEQ ID No 74	RFHRQASKCDS
SEQ ID No 23	ASSDCS	SEQ ID No 75	RFHRQASKCD
SEQ ID No 24	ASSDC	SEQ ID No 76	RFHRQASKC
SEQ ID No 25	SSDCSTCFVS	SEQ ID No 77	RFHRQASK
SEQ ID No 26	SSDCSTCFV	SEQ ID No 78	RFHRQAS
SEQ ID No 27	SSDCSTCF	SEQ ID No 79	RFHRQA
SEQ ID No 28	SSDCSTC	SEQ ID No 80	RFHRQ
SEQ ID No 29	SSDCST	SEQ ID No 81	FHRQASKCDS
SEQ ID No 30	SSDCS	SEQ ID No 82	FHRQASKCD
SEQ ID No 31	SDCSTCFVS	SEQ ID No 83	FHRQASKC
SEQ ID No 32	SDCSTCFV	SEQ ID No 84	FHRQASK
SEQ ID No 33	SDCSTCF	SEQ ID No 85	FHRQAS
SEQ ID No 34	SDCSTC	SEQ ID No 86	FHRQA
SEQ ID No 35	SDCST	SEQ ID No 87	HRQASKCDS
SEQ ID No 36	DCSTCFVS	SEQ ID No 88	HRQASKCD
SEQ ID No 37	DCSTCFV	SEQ ID No 89	HRQASKC
SEQ ID No 38	DCSTCF	SEQ ID No 90	HRQASK
SEQ ID No 39	DCSTC	SEQ ID No 91	HRQAS
SEQ ID No 40	CSTCFVS	SEQ ID No 92	RQASKCDS
SEQ ID No 41	CSTCFV	SEQ ID No 93	RQASKCD
SEQ ID No 42	CSTCF	SEQ ID No 94	RQASKC
SEQ ID No 43	STCFVS	SEQ ID No 95	RQASK
SEQ ID No 44	STCFV	SEQ ID No 96	QASKCDS
SEQ ID No 45	TCFVS	SEQ ID No 97	QASKCD
SEQ ID No 46	EDGTRFHRQASKCDS	SEQ ID No 98	QASKC
SEQ ID No 47	EDGTRFHRQASKCD	SEQ ID No 99	ASKCDS
SEQ ID No 48	EDGTRFHRQASKC	SEQ ID No 100	ASKCD
SEQ ID No 49	EDGTRFHRQASK	SEQ ID No 101	SKCDS
SEQ ID No 50	EDGTRFHRQAS	SEQ ID No 102	SIQSDHREIEGRVM
SEQ ID No 51	EDGTRFHRQA	SEQ ID No 103	SIQSDHREIEGRV
SEQ ID No 52	EDGTRFHRQ	SEQ ID No 104	SIQSDHREIEGR

SEQ ID No 105	SIQSDHREIEG	SEQ ID No 156	SGRDAGVA
SEQ ID No 106	SIQSDHREIE	SEQ ID No 157	GRDAGVAKGAS
SEQ ID No 107	SIQSDHREI	SEQ ID No 158	GRDAGVAKGA
SEQ ID No 108	IQSDHREIEGRVM	SEQ ID No 159	GRDAGVAKG
SEQ ID No 109	IQSDHREIEGRV	SEQ ID No 160	GRDAGVAK
SEQ ID No 110	IQSDHREIEGR	SEQ ID No 161	GRDAGVA
SEQ ID No 111	IQSDHREIEG	SEQ ID No 162	RDAGVAKGAS
SEQ ID No 112	IQSDHREIE	SEQ ID No 163	RDAGVAKGA
SEQ ID No 113	IQSDHREI	SEQ ID No 164	RDAGVAKG
SEQ ID No 114	QSDHREIEGRVM	SEQ ID No 165	RDAGVAK
SEQ ID No 115	QSDHREIEGRV	SEQ ID No 166	RDAGVA
SEQ ID No 116	QSDHREIEGR	SEQ ID No 167	DAGVAKGAS
SEQ ID No 117	QSDHREIEG	SEQ ID No 168	DAGVAKGA
SEQ ID No 118	QSDHREIE	SEQ ID No 169	DAGVAKG
SEQ ID No 119	QSDHREI	SEQ ID No 170	DAGVAK
SEQ ID No 120	SDHREIEGRVM	SEQ ID No 171	DAGVA
SEQ ID No 121	SDHREIEGRV	SEQ ID No 172	AGVAKGAS
SEQ ID No 122	SDHREIEGR	SEQ ID No 173	AGVAKGA
SEQ ID No 123	SDHREIEG	SEQ ID No 174	AGVAKG
SEQ ID No 124	SDHREIE	SEQ ID No 175	AGVAK
SEQ ID No 125	SDHREI	SEQ ID No 176	GVAKGAS
SEQ ID No 126	DHREIEGRVM	SEQ ID No 177	GVAKGA
SEQ ID No 127	DHREIEGRV	SEQ ID No 178	GVAKG
SEQ ID No 128	DHREIEGR	SEQ ID No 179	VAKGAS
SEQ ID No 129	DHREIEG	SEQ ID No 180	VAKGA
SEQ ID No 130	DHREIE	SEQ ID No 181	AKGAS
SEQ ID No 131	DHREI	SEQ ID No 182	SIPWNLERITPPR
SEQ ID No 132	HREIEGRVM	SEQ ID No 183	SIPWNLERITPP
SEQ ID No 133	HREIEGRV	SEQ ID No 184	SIPWNLERITP
SEQ ID No 134	HREIEGR	SEQ ID No 185	SIPWNLERIT
SEQ ID No 135	HREIEG	SEQ ID No 186	SIPWNLERI
SEQ ID No 136	HREIE	SEQ ID No 187	SIPWNLER
SEQ ID No 137	REIEGRVM	SEQ ID No 188	SIPWNLE
SEQ ID No 138	REIEGRV	SEQ ID No 189	SIPWNL
SEQ ID No 139	REIEGR	SEQ ID No 190	SIPWN
SEQ ID No 140	REIEG	SEQ ID No 191	IPWNLERITPPR
SEQ ID No 141	EIEGRVM	SEQ ID No 192	IPWNLERITPP
SEQ ID No 142	EIEGRV	SEQ ID No 193	IPWNLERITP
SEQ ID No 143	EIEGR	SEQ ID No 194	IPWNLERIT
SEQ ID No 144	IEGRVM	SEQ ID No 195	IPWNLERI
SEQ ID No 145	IEGRV	SEQ ID No 196	IPWNLER
SEQ ID No 146	EGRVM	SEQ ID No 197	IPWNLE
SEQ ID No 147	VSGRDAGVAKGAS	SEQ ID No 198	IPWNL
SEQ ID No 148	VSGRDAGVAKGA	SEQ ID No 199	PWNLERITPPR
SEQ ID No 149	VSGRDAGVAKG	SEQ ID No 200	PWNLERITPP
SEQ ID No 150	VSGRDAGVAK	SEQ ID No 201	PWNLERITP
SEQ ID No 151	VSGRDAGVA	SEQ ID No 202	PWNLERIT
SEQ ID No 152	SGRDAGVAKGAS	SEQ ID No 203	PWNLERI
SEQ ID No 153	SGRDAGVAKGA	SEQ ID No 204	PWNLER
SEQ ID No 154	SGRDAGVAKG	SEQ ID No 205	PWNLE
SEQ ID No 155	SGRDAGVAK	SEQ ID No 206	WNLERITPPR

SEQ ID No 207	WNLERITPP	SEQ ID No 258	PVTLGT
SEQ ID No 208	WNLERITP	SEQ ID No 259	PVTLG
SEQ ID No 209	WNLERIT	SEQ ID No 260	VTLGTL
SEQ ID No 210	WNLERI	SEQ ID No 261	VTLGT
SEQ ID No 211	WNLER	SEQ ID No 262	TLGTL
SEQ ID No 212	NLERITPPR	SEQ ID No 263	INEAWFPEDQRVL
SEQ ID No 213	NLERITPP	SEQ ID No 264	INEAWFPEDQRV
SEQ ID No 214	NLERITP	SEQ ID No 265	INEAWFPEDQR
SEQ ID No 215	NLERIT	SEQ ID No 266	INEAWFPEDQ
SEQ ID No 216	NLERI	SEQ ID No 267	INEAWFPED
SEQ ID No 217	LERITPPR	SEQ ID No 268	INEAWFPE
SEQ ID No 218	LERITPP	SEQ ID No 269	INEAWFP
SEQ ID No 219	LERITP	SEQ ID No 270	INEAWF
SEQ ID No 220	LERIT	SEQ ID No 271	INEAW
SEQ ID No 221	ERITPPR	SEQ ID No 272	NEAWFPEDQRVL
SEQ ID No 222	ERITPP	SEQ ID No 273	NEAWFPEDQRV
SEQ ID No 223	ERITP	SEQ ID No 274	NEAWFPEDQR
SEQ ID No 224	RITPPR	SEQ ID No 275	NEAWFPEDQ
SEQ ID No 225	RITPP	SEQ ID No 276	NEAWFPED
SEQ ID No 226	ITPPR	SEQ ID No 277	NEAWFPE
SEQ ID No 227	NAQDQPVTLGTL	SEQ ID No 278	NEAWFP
SEQ ID No 228	NAQDQPVTLGT	SEQ ID No 279	NEAWF
SEQ ID No 229	NAQDQPVTLG	SEQ ID No 280	EAWFPEDQRVL
SEQ ID No 230	NAQDQPVTL	SEQ ID No 281	EAWFPEDQRV
SEQ ID No 231	NAQDQPVT	SEQ ID No 282	EAWFPEDQR
SEQ ID No 232	NAQDQPV	SEQ ID No 283	EAWFPEDQ
SEQ ID No 233	NAQDQP	SEQ ID No 284	EAWFPED
SEQ ID No 234	NAQDQ	SEQ ID No 285	EAWFPE
SEQ ID No 235	AQDQPVTLGTL	SEQ ID No 286	EAWFP
SEQ ID No 236	AQDQPVTLGT	SEQ ID No 287	AWFPEDQRVL
SEQ ID No 237	AQDQPVTLG	SEQ ID No 288	AWFPEDQRV
SEQ ID No 238	AQDQPVTL	SEQ ID No 289	AWFPEDQR
SEQ ID No 239	AQDQPVT	SEQ ID No 290	AWFPEDQ
SEQ ID No 240	AQDQPV	SEQ ID No 291	AWFPED
SEQ ID No 241	AQDQP	SEQ ID No 292	AWFPE
SEQ ID No 242	QDQPVTLGTL	SEQ ID No 293	WFPEDQRVL
SEQ ID No 243	QDQPVTLGT	SEQ ID No 294	WFPEDQRV
SEQ ID No 244	QDQPVTLG	SEQ ID No 295	WFPEDQR
SEQ ID No 245	QDQPVTL	SEQ ID No 296	WFPEDQ
SEQ ID No 246	QDQPVT	SEQ ID No 297	WFPED
SEQ ID No 247	QDQPV	SEQ ID No 298	FPEDQRVL
SEQ ID No 248	DQPVTLGTL	SEQ ID No 299	FPEDQRV
SEQ ID No 249	DQPVTLGT	SEQ ID No 300	FPEDQR
SEQ ID No 250	DQPVTLG	SEQ ID No 301	FPEDQ
SEQ ID No 251	DQPVTL	SEQ ID No 302	PEDQRVL
SEQ ID No 252	DQPVT	SEQ ID No 303	PEDQRV
SEQ ID No 253	QPVTLGTL	SEQ ID No 304	PEDQR
SEQ ID No 254	QPVTLGT	SEQ ID No 305	EDQRVL
SEQ ID No 255	QPVTLG	SEQ ID No 306	EDQRV
SEQ ID No 256	QPVTL	SEQ ID No 307	DQRVL
SEQ ID No 257	PVTLGTL	SEQ ID No 308	VDYIEEDSSVFAQ

SEQ ID No 309	RCAKDPWRLPGT	SEQ ID No 319	AGCGTRFHRQ
SEQ ID No 310	AQAARRGYLTKIL	SEQ ID No 320	GRVCIQSDHREIE
SEQ ID No 311	GDYEELVLALRSEEDG	SEQ ID No 321	AGVAKGAGCSGRD
SEQ ID No 312	FLVKMSGDLLELALKLP	SEQ ID No 322	SIPWNLERIIPC
SEQ ID No 313	KGGASSDCSTCFV	SEQ ID No 323	CGGSIPWNLERIIP
SEQ ID No 314	CGGTRFHRQASKC	SEQ ID No 324	SIPWNLERIIPGGC
SEQ ID No 315	CGIQSDHREIEGRVC	SEQ ID No 325	CGGNAQDQPVTLGTL
SEQ ID No 316	CSGRDAGVAKGAC	SEQ ID No 326	NAQDQPVTLGTLGGC
SEQ ID No 317	SIPWNLERITPC	SEQ ID No 327	CGGINMAWFPEDQQVL
SEQ ID No 318	ASKCGDGTRFHRQ	SEQ ID No 328	NMAWFPEDQQVLGGC
SEQ ID No 329	MGTVSSRRSWWPLPLLLLLLLLLGPAGARAQEDEDGDYEELVLALRSEEDGLAEAPEHGTTATFH RCAKDPWRLPGTYVVVLKEETHLSQSERTARRLQAQAARRGYLTKILHVFHGLLPGFLVKMSGDL LELALKLPHVDYIEEDSSVFAQ		
SEQ ID No 330	SIPWNLERIIPAW	SEQ ID No 369	NMAWFPEDQQVL
SEQ ID No 331	SIPWNLERIIPA	SEQ ID No 370	NMAWFPEDQQV
SEQ ID No 332	SIPWNLERIIP	SEQ ID No 371	NMAWFPEDQQ
SEQ ID No 333	SIPWNLERII	SEQ ID No 372	NMAWFPEDQ
SEQ ID No 334	IPWNLERIIPAW	SEQ ID No 373	NMAWFPED
SEQ ID No 335	IPWNLERIIPAW	SEQ ID No 374	NMAWFPE
SEQ ID No 336	IPWNLERIIP	SEQ ID No 375	NMAWFP
SEQ ID No 337	IPWNLERII	SEQ ID No 376	NMAWF
SEQ ID No 338	PWNLERIIPAW	SEQ ID No 377	MAWFPEDQQVL
SEQ ID No 339	PWNLERIIPA	SEQ ID No 378	MAWFPEDQQV
SEQ ID No 340	PWNLERIIP	SEQ ID No 379	MAWFPEDQQ
SEQ ID No 341	PWNLERII	SEQ ID No 380	MAWFPEDQ
SEQ ID No 342	WNLERIIPAW	SEQ ID No 381	MAWFPED
SEQ ID No 343	WNLERIIPA	SEQ ID No 382	MAWFPE
SEQ ID No 344	WNLERIIP	SEQ ID No 383	MAWFP
SEQ ID No 345	WNLERII	SEQ ID No 384	AWFPEDQQVL
SEQ ID No 346	NLERIIPAW	SEQ ID No 385	AWFPEDQQV
SEQ ID No 347	NLERIIPA	SEQ ID No 386	AWFPEDQQ
SEQ ID No 348	NLERIIP	SEQ ID No 387	WFPEDQQVL
SEQ ID No 349	NLERII	SEQ ID No 388	WFPEDQQV
SEQ ID No 350	LERIIPAW	SEQ ID No 389	WFPEDQQ
SEQ ID No 351	LERIIPA	SEQ ID No 390	FPEDQQVL
SEQ ID No 352	LERIIP	SEQ ID No 391	FPEDQQV
SEQ ID No 353	LERII	SEQ ID No 392	FPEDQQ
SEQ ID No 354	ERIIPAW	SEQ ID No 393	PEDQQVL
SEQ ID No 355	ERIIPA	SEQ ID No 394	PEDQQV
SEQ ID No 356	ERIIP	SEQ ID No 395	PEDQQ
SEQ ID No 357	RIIPAW	SEQ ID No 396	EDQQVL
SEQ ID No 358	RIIPA	SEQ ID No 397	EDQQV
SEQ ID No 359	IIPAW	SEQ ID No 398	DQQVL
SEQ ID No 360	INMAWFPEDQQVL		
SEQ ID No 361	INMAWFPEDQQV		
SEQ ID No 362	INMAWFPEDQQ		
SEQ ID No 363	INMAWFPEDQ		
SEQ ID No 364	INMAWFPED		
SEQ ID No 365	INMAWFPE		
SEQ ID No 366	INMAWFP		
SEQ ID No 367	INMAWF		
SEQ ID No 368	INMAW		

SEQ ID No 399	MGTVSSRRSWWPLPLLLLLLLLLGPAGARAQEDEDGDYEELVLALRSEEDGLAEAPEHGTTATFH RCAKDPWRLPGTYVVVLKEETHLSQSERTARRLQAQAARRGYLTKILHVFHGLLPGFLVKMSGDL LELALKLPHVDYIEEDSSVFAQSIPWNLERITPPRYRADEYQPPDGGSLVEVYLLDTSIQSDHRE IEGRVMVTDFENVPEEDGTRFHRQASKCDSHGTHLAGVVSGRDAGVAKGASMRSLRVLNCQGKGT VSGTLIGLEFIRKSQLVQPVGPLVVLLPLAGGYSRVLNAACQRLARAGVVLVTAAGNFRDDACLY SPASAPEVITVGATNAQDQPVTLGTLGTNFGRCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAA HVAGIAAMMLSAEPELTLAELRQRLIHFSAKDVINEAWFPEDQRVLTPNLVAALPPSTHGAGWQL FCRTVWSAHSGPTRMATAVARCAPDEELLSCSSFSRSGKRRGERMEAQGGKLVCRAHNAFGGEGV YAIARCCLLPQANCSVHTAPPAEASMGTRVHCHQQGHVLTGCSSHWEVEDLGTHKPPVLRPRGQP NQCVGHREASIHASCCHAPGLECKVKEHGIPAPQEQVTVACEEGWTLTGCSALPGTSHVLGAYAV DNTCVVRSRDVSTTGSTSEGAVTAVAICCRSRHLAQASQELQ
SEQ ID No 400	MGTHCSAWLRWPLLPLLPPLLLLLLLLCPTGAGAQDEDGDYEELMLALPSQEDGLADEAAHVATA TFRRCSKEAWRLPGTYIVVLMEETQRLQIEQTAHRLQTRAARRGYVIKVLHIFYDLFPGFLVKMS SDLLGLALKLPHVEYIEEDSFVFAQSIPWNLERIIPAWHQTEEDRSPDGSSQVEVYLLDTSIQGA HREIEGRVTITDFNSVPEEDGTRFHRQASKCDSHGTHLAGVVSGRDAGVAKGTSLHSLRVLNCQG KGTVSGTLIGLEFIRKSQLIQPSGPLVVLLPLAGGYSRILNAACRHLARTGVVLVAAAGNFRDDA CLYSPASAPEVITVGATNAQDQPVTLGTLGTNFGRCVDLFAPGKDIIGASSDCSTCFMSQSGTSQ AAAHVAGIVARMLSREPTLTLAELRQRLIHFSTKDVINMAWFPEDQQVLTPNLVATLPPSTHETG GQLLCRTVWSAHSGPTRTATATARCAPEEELLSCSSFSRSGRRRGDWIEAIGGQQVCKALNAFGG EGVYAVARCCLVPRANCSIHNTPAARAGLETHVHCHQKDHVLTGCSFHWEVEDLSVRRQPALRSR RQPGQCVGHQAASVYASCCHAPGLECKIKEHGISGPSEQVTVACEAGWTLTGCNVLPGASLTLGA YSVDNLCVARVHDTARADRTSGEATVAAAICCRSRPSAKASWVQ

SEQ ID No 401	SIPWNLERIGGC	SEQ ID No 434	RCAKDP
SEQ ID No 402	SIPWNLERGGC	SEQ ID No 435	RCAKD
SEQ ID No 403	SIPWNLEGGC	SEQ ID No 436	CAKDPWRLPGT
SEQ ID No 404	CGGSGRDAGVAKGA	SEQ ID No 437	CAKDPWRLPG
SEQ ID No 405	CGGSGRDAGVAKGT	SEQ ID No 438	CAKDPWRLP
SEQ ID No 406	RDAGVAKGGC	SEQ ID No 439	CAKDPWRL
SEQ ID No 407	CSRHLAQASQELQ	SEQ ID No 440	CAKDPWR
SEQ ID No 408	CRSRPSAKASWVQ	SEQ ID No 441	CAKDPW
SEQ ID No 409	CGGDYEELVLALR	SEQ ID No 442	CAKDP
SEQ ID No 410	CGGDYEELMLALP	SEQ ID No 443	AKDPWRLPGT
SEQ ID No 411	LVLALRSEEDGGC	SEQ ID No 444	AKDPWRLPG
SEQ ID No 412	LMLALPSQEDGGC	SEQ ID No 445	AKDPWRLP
SEQ ID No 413	AKDPWRLPGGC	SEQ ID No 446	AKDPWRL
SEQ ID No 414	SKEAWRLPGGC	SEQ ID No 447	AKDPWR
SEQ ID No 415	CGGAARRGYLTK	SEQ ID No 448	AKDPW
SEQ ID No 416	CGGAARRGYVIK	SEQ ID No 449	KDPWRLPGT
SEQ ID No 417	FLVKMSGDLLELALKLPGGC	SEQ ID No 450	KDPWRLPG
SEQ ID No 418	FLVKMSSDLLGLALKLPGGC	SEQ ID No 451	KDPWRLP
SEQ ID No 419	CGGEEDSSVFAQ	SEQ ID No 452	KDPWRL
SEQ ID No 420	SRHLAQASQELQ	SEQ ID No 453	KDPWR
SEQ ID No 421	DYEELVLALR	SEQ ID No 454	DPWRLPGT
SEQ ID No 422	LVLALRSEEDG	SEQ ID No 455	DPWRLPG
SEQ ID No 423	EEDSSVFAQ	SEQ ID No 456	DPWRLP
SEQ ID No 424	SGRDAGVAKGT	SEQ ID No 457	DPWRL
SEQ ID No 425	RSRPSAKASWVQ	SEQ ID No 458	PWRLPGT
SEQ ID No 426	GDYEELMLALP	SEQ ID No 459	PWRLPG
SEQ ID No 427	LMLALPSQED	SEQ ID No 460	PWRLP
SEQ ID No 428	FLVKMSSDLLGLALKLP	SEQ ID No 461	WRLPGT
SEQ ID No 429	RCAKDPWRLPG	SEQ ID No 462	WRLPG
SEQ ID No 430	RCAKDPWRLP	SEQ ID No 464	AQAARRGYLTKI
SEQ ID No 431	RCAKDPWRL	SEQ ID No 465	AQAARRGYLTK
SEQ ID No 432	RCAKDPWR	SEQ ID No 466	AQAARRGYLT
SEQ ID No 433	RCAKDPW	SEQ ID No 467	AQAARRGYL

SEQ ID No 468	AQAARRGY	SEQ ID No 519	CSKEAWRL
SEQ ID No 469	AQAARRG	SEQ ID No 520	CSKEAWR
SEQ ID No 470	AQAARR	SEQ ID No 521	CSKEAW
SEQ ID No 471	AQAAR	SEQ ID No 522	CSKEA
SEQ ID No 472	QAARRGYLTKIL	SEQ ID No 523	SKEAWRLPGT
SEQ ID No 473	QAARRGYLTKI	SEQ ID No 524	SKEAWRLPG
SEQ ID No 474	QAARRGYLTK	SEQ ID No 525	SKEAWRLP
SEQ ID No 475	QAARRGYLT	SEQ ID No 526	SKEAWRL
SEQ ID No 476	QAARRGYL	SEQ ID No 527	SKEAWR
SEQ ID No 477	QAARRGY	SEQ ID No 528	SKEAW
SEQ ID No 478	QAARRG	SEQ ID No 529	KEAWRLPGT
SEQ ID No 479	QAARR	SEQ ID No 530	KEAWRLPG
SEQ ID No 480	AARRGYLTKIL	SEQ ID No 531	KEAWRLP
SEQ ID No 481	AARRGYLTKI	SEQ ID No 532	KEAWRL
SEQ ID No 482	AARRGYLTK	SEQ ID No 533	KEAWR
SEQ ID No 483	AARRGYLT	SEQ ID No 534	EAWRLPGT
SEQ ID No 484	AARRGYL	SEQ ID No 535	EAWRLPG
SEQ ID No 485	AARRGY	SEQ ID No 536	EAWRLP
SEQ ID No 486	AARRG	SEQ ID No 537	EAWRL
SEQ ID No 487	ARRGYLTKIL	SEQ ID No 538	AWRLPGT
SEQ ID No 488	ARRGYLTKI	SEQ ID No 539	AWRLPG
SEQ ID No 489	ARRGYLTK	SEQ ID No 540	AWRLP
SEQ ID No 490	ARRGYLT	SEQ ID No 541	WRLPGT
SEQ ID No 491	ARRGYL	SEQ ID No 542	WRLPG
SEQ ID No 492	ARRGY	SEQ ID No 543	RLPGT
SEQ ID No 493	RRGYLTKIL	SEQ ID No 544	TRAARRGYVIKVL
SEQ ID No 494	RRGYLTKI	SEQ ID No 545	TRAARRGYVIKV
SEQ ID No 495	RRGYLTK	SEQ ID No 546	TRAARRGYVIK
SEQ ID No 496	RRGYLT	SEQ ID No 547	TRAARRGYVI
SEQ ID No 497	RRGYL	SEQ ID No 548	TRAARRGYV
SEQ ID No 498	RGYLTKIL	SEQ ID No 549	TRAARRGY
SEQ ID No 499	RGYLTKI	SEQ ID No 550	TRAARRG
SEQ ID No 500	RGYLTK	SEQ ID No 551	TRAARR
SEQ ID No 501	RGYLT	SEQ ID No 552	TRAAR
SEQ ID No 502	GYLTKIL	SEQ ID No 553	RAARRGYVIKVL
SEQ ID No 503	GYLTKI	SEQ ID No 554	RAARRGYVIKV
SEQ ID No 504	GYLTK	SEQ ID No 555	RAARRGYVIK
SEQ ID No 505	YLTKIL	SEQ ID No 556	RAARRGYVI
SEQ ID No 506	YLTKI	SEQ ID No 557	RAARRGYV
SEQ ID No 507	LTKIL	SEQ ID No 558	RAARRGY
SEQ ID No 508	RCSKEAWRLPGT	SEQ ID No 559	RAARRG
SEQ ID No 509	RCSKEAWRLPG	SEQ ID No 560	RAARR
SEQ ID No 510	RCSKEAWRLP	SEQ ID No 561	AARRGYVIKVL
SEQ ID No 511	RCSKEAWRL	SEQ ID No 562	AARRGYVIKV
SEQ ID No 512	RCSKEAWR	SEQ ID No 563	AARRGYVIK
SEQ ID No 513	RCSKEAW	SEQ ID No 564	AARRGYVI
SEQ ID No 514	RCSKEA	SEQ ID No 565	AARRGYV
SEQ ID No 515	RCSKE	SEQ ID No 566	AARRGY
SEQ ID No 516	CSKEAWRLPGT	SEQ ID No 567	AARRG
SEQ ID No 517	CSKEAWRLPG	SEQ ID No 568	ARRGYVIKVL
SEQ ID No 518	CSKEAWRLP	SEQ ID No 569	ARRGYVIKV

SEQ ID No 570	ARRGYVIK
SEQ ID No 571	ARRGYVI
SEQ ID No 572	ARRGYV
SEQ ID No 573	ARRGY
SEQ ID No 574	RRGYVIKVL
SEQ ID No 575	RRGYVIKV
SEQ ID No 576	RRGYVIK
SEQ ID No 577	RRGYVI
SEQ ID No 578	RRGYV
SEQ ID No 579	RGYVIKVL
SEQ ID No 580	RGYVIKV
SEQ ID No 581	RGYVIK
SEQ ID No 582	RGYVI
SEQ ID No 583	GYVIKVL
SEQ ID No 584	GYVIKV
SEQ ID No 585	GYVIK
SEQ ID No 586	YVIKVL
SEQ ID No 587	YVIKV
SEQ ID No 588	VIKVL
SEQ ID No 589	TCGTCGTTTTTCGGTGCTTTT
SEQ ID No 590	TCGTCGTTTTTCGGTCGTTTT
SEQ ID No 591	TCGTCGTTTTGTCGTTTTGTCGTT
SEQ ID No 592	TCGTCGTTTCGTCGTTTTGTCGTT
SEQ ID No 593	TCGTCGTTTTGTCGTTTTTTTCGA
SEQ ID No 594	TCGCGTCGTTCGGCGCGCGCCG
SEQ ID No 595	TCGTCGACGTTCGGCGCGCGCCG
SEQ ID No 596	TCGGACGTTCGGCGCGCGCCG
SEQ ID No 597	TCGGACGTTCGGCGCGCCG
SEQ ID No 598	TCGCGTCGTTCGGCGCGCCG
SEQ ID No 599	TCGACGTTCGGCGCGCGCCG
SEQ ID No 600	TCGACGTTCGGCGCGCCG
SEQ ID No 601	TCGCGTCGTTCGGCGCCG
SEQ ID No 602	TCGCGACGTTCGGCGCGCGCCG
SEQ ID No 603	TCGTCGTTTTCGGCGCGCGCCG
SEQ ID No 604	TCGTCGTTTTCGGCGGCCGCCG
SEQ ID No 605	TCGTCGTTTTACGGCGCCGTGCCG
SEQ ID No 606	TCGTCGTTTTCGGCGCGCGCCGT
SEQ ID No 607	TCGTCGACGATCGGCGCGCGCCG

CLAIMS

1. An immunogen comprising at least one antigenic PCSK9 peptide, or a functionally active variant thereof, linked to an immunogenic carrier.

2. An immunogen according to claim 1 wherein said immunogenic carrier is selected from Diphtheria Toxoid, CRM197 or a VLP selected from HBcAg, HBsAg, Qbeta, PP7, PPV or Norwalk Virus VLP.

3. An immunogen according to claim 1 wherein said immunogenic carrier is selected from CRM197 or Qbeta VLP.

4. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from a portion of PCSK9 which participates in the interaction of PCSK9 with the LDL receptor.

5. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from a portion of PCSK9 which participates in the interaction of PCSK9 with the EGF-A domain of the LDL receptor.

6. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the prodomain of PCSK9.

7. An immunogen according to any one of claims 1 to 6 wherein said antigenic PCSK9 peptide comprises between 4 and 20 amino acids.

8. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225 and 226.

9. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476,

477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, and 588.

10. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358 and 359.

11. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44 and 45.

12. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 and 101.

13. An immunogen according toany one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145 and 146.

14. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180 and 181.

15. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358 and 359.

16. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 19, 56, 63, 109, 153, 165, 184, 186, 187, 188, 332 and 424.

17. An immunogen according to any one of claims 1 to 3, wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 184, 186, 187, 188 and 332.

18. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is SEQ ID Nos 56.

19. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID Nos 308, 309, 310, 311,312, 421 to 424 and 426 to 588.

20. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID 445, 482, 525 and 563.

21. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID 420 and 425.

22. The immunogen according to any one of claim 1 to 21 wherein said antigenic PCSK9 peptide further comprise either:

- at its C-terminus a linker having the formula $(G)_nC$, $(G)_nSC$ or $(G)_nK$ wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5 ,6 , 7 , 8, 9 and 10,
- at its N-terminus a linker having the formula $C(G)_n$, $CS(G)_n$ or $K(G)_n$ wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5 ,6 , 7 , 8, 9 and 10,

-at its C-terminus a linker having the formula $(G)_nC$, $(G)_nSC$ or $(G)_nK$ wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5 ,6 , 7 , 8, 9 and 10, and at its N-terminus a linker having the formula $C(G)_n$, $CS(G)_n$ or $K(G)_n$ wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5 ,6 , 7 , 8, 9 and 10.

23. The immunogen according to any one of claim 1 to 21 wherein said antigenic PCSK9 peptide further comprise either:

- at its C-terminus a linker having the formula $(G)_nC$, $(G)_nSC$ or $(G)_nK$ wherein n is an integer chosen in the group consisting of 0, 1 or 2,
- at its N-terminus a linker having the formula $C(G)_n$, $CS(G)_n$ or $K(G)_n$ wherein n is an integer chosen in the group consisting of 0, 1 or 2,

-at its C-terminus a linker having the formula $(G)_nC$, $(G)_nSC$ or $(G)_nK$ wherein n is an integer chosen in the group consisting of 0, 1 or 2, and at its N-terminus a linker having the formula $C(G)_n$, $CS(G)_n$ or $K(G)_n$ wherein n is an integer chosen in the group consisting of 0, 1 or 2.

24. The immunogen according to any one of claim 1 to 21 wherein said antigenic PCSK9 peptide further comprise a cysteine at its C-terminus.

25. The immunogen according to claim 24 wherein said antigenic PCSK9 peptide further comprise a CG group or a cysteine at its N-terminus.

26. The immunogen according to any one of claim 1 to 21 wherein said antigenic PCSK9 peptide further comprise a CGG at its N-terminus.

27. The immunogen according to any one of claim 1 to 21 wherein said antigenic PCSK9 peptide further comprise a GGC at its C-terminus.

28. The immunogen according to any one of claim 1 to 21 wherein said antigenic PCSK9 peptide is cyclised and comprises a a cysteine, a $(G)_nC$ or a $C(G)_n$ fragment wherein n is an integer chosen in the group consisting of 0, 1, 2, 3, 4, 5 ,6 , 7 , 8, 9 and 10.

29. The immunogen according to any one of claim 1 to 21 wherein said antigenic PCSK9 peptide is cyclised and comprises a cysteine, a GC or a CG fragment.

30. The immunogen according to any one of claim 1 to 21 wherein said antigenic PCSK9 peptide further comprise a KG or a KGG at its N-terminus.

31. An immunogen according to any one of claims 1 to 3 wherein the antigenic PCSK9 peptide is conformationally constrained.

32. An immunogen according to claim 31 wherein the antigenic PCSK9 peptide is selected from the group consisting of SEQ ID NO 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418 and 419.

33. The immunogen according to any one of claims 1 to 32 wherein said immunogen is able, when administered to a subject, to lower the LDL-cholesterol level in blood of said subject by at least 2%, 5%, 10%, 20%, 30% or 50%.

34. The immunogen according to claim 33 wherein said immunogen is able, when administered to a subject, to lower the LDL-cholesterol level in blood of said subject by at least 10%.

35. A composition comprising at least two immunogens according to any one of claim 1 to 34.

36. An immunogenic composition comprising an immunogen of any one of claims 1 to 33, or a composition of immunogens according to claim 35 further comprising at least one adjuvant.

37. An immunogenic composition according to claim 36 wherein said adjuvant is selected from alum, CpG ODN, QS21 and Iscomatrix.

38. An immunogenic composition according to claim 36 wherein said adjuvant is selected from QS21, alum in combination with CpG ODN or Iscomatrix in combination with CpG ODN.

39. An immunogenic composition according to claim 36 wherein said adjuvant is selected from alum in combination with CpG ODN.

40. An immunogenic composition according to claim 37, 38 or 39 wherein the CpG ODN is selected from
5' TCGTCGTTTTTCGGTGCTTTT 3', or
5' TCGTCGTTTTTCGGTCGTTTT 3', or
5' TCGTCGTTTTGTCGTTTTGTCGTT 3'.

41. A pharmaceutical composition comprising the immunogen of any one of claims 1 to 34, or a composition of immunogens according to claim 35, or the immunogenic composition of any one of claims 36 to 40, and a pharmaceutically acceptable excipient.

42. The immunogen of any one of claims 1 to 34, or a composition of immunogens according to claim 35, or the immunogenic composition of any one of claims 36 to 40 , or the pharmaceutical composition of claim 41 for use as a medicament.

43. The immunogen of any one of claims 1 to 34, or a composition of immunogens according claim 35, or the immunogenic composition of any one of claims 36 to 40, or the pharmaceutical composition of claim 41, for preventing, alleviating or treating a PCSK9-related disorder.

44. The immunogen, immunogenic composition or pharmaceutical composition of claim 43 wherein said PCSK9-related disorder is elevated LDL-cholesterol or a condition associated with elevated LDL-cholesterol.

45. The immunogen, immunogenic composition or pharmaceutical composition of claim 43 wherein said PCSK9-related disorder is selected from a lipid disorder such as hyperlipidemia, type I, type II, type III, type IV, or type V hyperlipidemia, secondary hypertriglyceridemia, hypercholesterolemia, familial hypercholesterolemia, xanthomatosis, cholesterol acetyltransferase deficiency; an ateriosclerotic conditions (e.g., atherosclerosis), a coronary artery disease, and a cardiovascular disease.

46. The immunogen of any one of claims 1 to 34, or a composition of immunogens according to claim 35, or the immunogenic composition of any one of claims 36 to 40 for preventing, alleviating or treating Alzheimer's disease.

47. A method for preventing, alleviating or treating an PCSK9-related disorder in an individual, comprising administering a therapeutically effective amount of the immunogen of any one of claims 1 to 34, or a composition of immunogens according to claim 35, or the immunogenic composition of any one of claims 36 to 40 or the pharmaceutical composition of claim 41.

48. A nucleic acid encoding the immunogen of any one of claims 1 to 34.

49. An expression vector comprising the nucleic acid of claim 48.

50. A host cell comprising the expression vector of claim 49.